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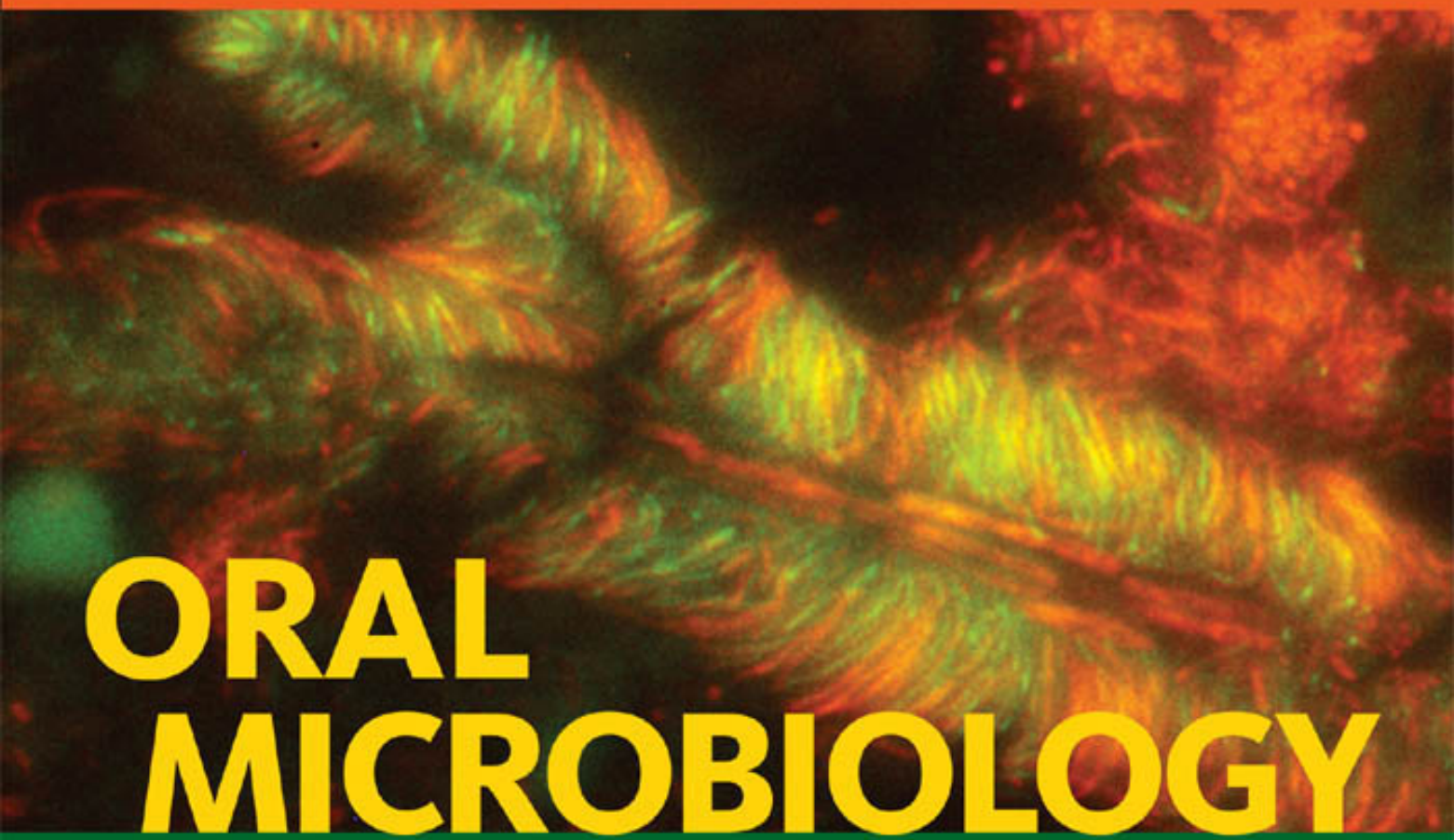
EDITED BY Richard J. Lamont, George N. Hajishengallis, and Howard F. Jenkinson



ORAL MICROBIOLOGY AND IMMUNOLOGY

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Cover image: Longitudinal view of a test-tube brush with a *Lactobacillus* sp. (red rods) as central structures. *Fusobacterium nucleatum* (green) and *Cytophaga-Flavobacterium-Bacteroides* cluster filaments radiate from the central structures. This epifluorescence image is a result of an *in situ* hybridization with fluorescently labeled specific oligonucleotide probes on thin sections of an embedded tooth. Image courtesy of V. Zijnge, M. B. M. van Leeuwen, J. E. Degener, F. Abbas, T. Thurnheer, R. Gmür, and H. J. M. Harmsen. 2010. Oral biofilm architecture on natural teeth. *PLoS ONE* **5**(2)**:**e9321. doi:10.1371/journal.pone.0009321

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Preface

In the seven years since the first edition of this book, the world of microbiology and immunology has seen incredible technological and conceptual advances. It is now almost routine to sequence the genome of a bacterium, and for that matter, a community of bacteria; the catalog of proteins for which the crystal structure is known has increased apace; knockout mice deficient in numerous components of the innate and adaptive immune system are widely available; and the regulatory interplay between the innate and adaptive arms of immunity is now better understood. Development of high resolution and 3D imaging techniques has allowed novel studies of communities growing in biofilms, as well as the more intimate interactions between microbes and host cells. High-throughput techniques and extended computer power have made population biology and epidemiology research more comprehensive. This burgeoning knowledge has changed our understanding of both the etiology of oral diseases and the nature of the pathogenic mechanisms and host responses. These changing perceptions are reflected in the updated and expanded chapters. What has (disappointingly) not improved over the last seven years is the incidence of caries and periodontal disease. It is more important than ever for dental practitioners and the clinical scientists to understand the basic science underlying oral health and disease in order for such knowledge to be translated into future health improvements.

As with the first edition, each chapter is self contained and represents the particular insights and priorities of the authors. Taken separately or together, we hope that the chapters provide the reader with the basic facts as well as with the ecological and biological context.

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About the Editors

Richard J. Lamont received a bachelor of science degree in bacteriology from the University of Edinburgh; he received a doctorate from the University of Aberdeen in 1985. After a postdoctoral fellowship at the University of Pennsylvania focusing on streptococcal adherence mechanisms, he joined the faculty at the University of Washington, in 1989. He is currently the Delta Dental Endowed Professor of oral microbiology at the University of Louisville. His research interests include the molecular mechanisms of polymicrobial synergy and the cellular interactions between oral bacteria and the host epithelium. He has taught microbiology and immunology to dental students and residents for over 25 years.

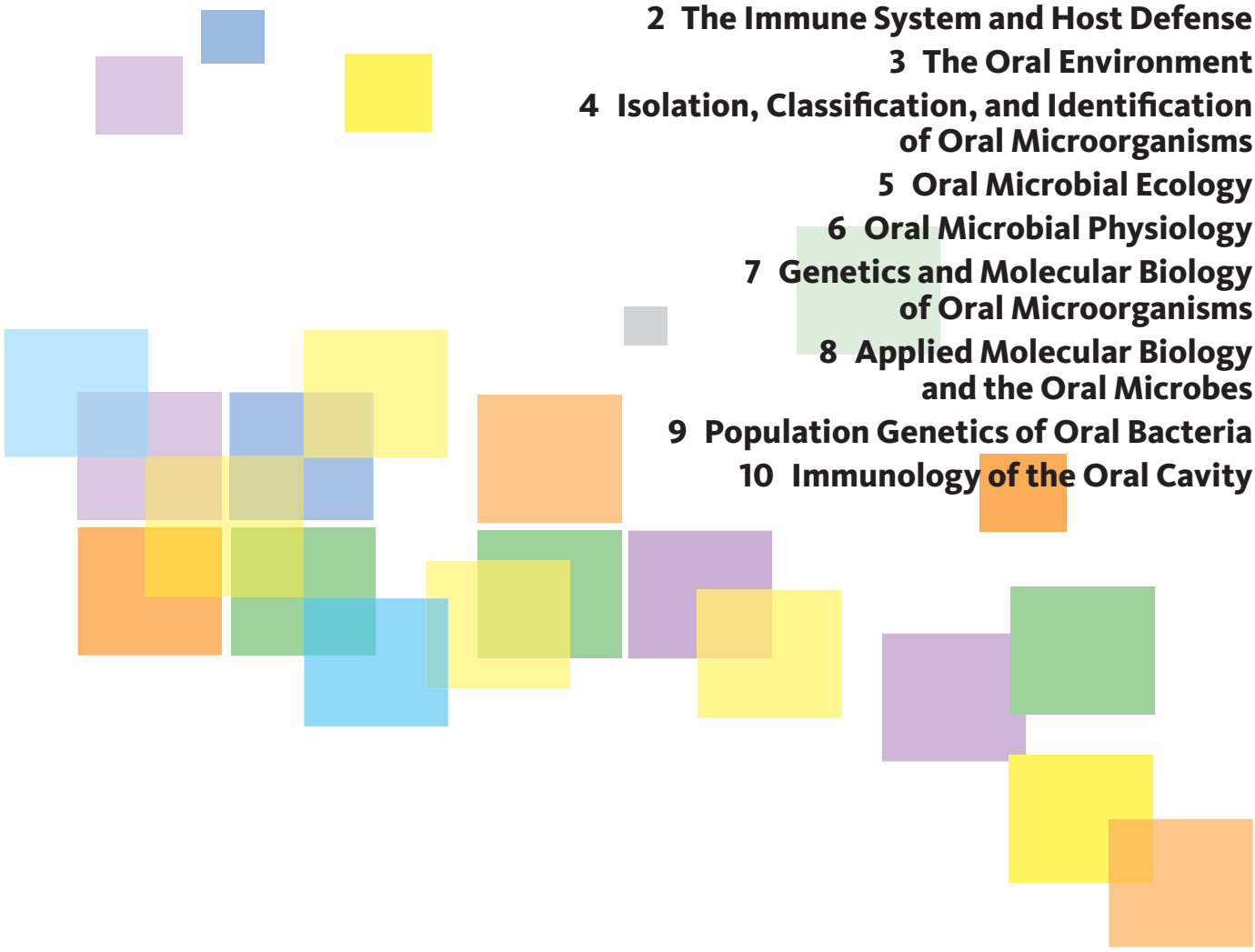
George Hajishengallis was originally trained as a dentist (DDS, 1989, University of Athens, Greece) before pursuing doctoral studies in cellular and molecular biology (PhD, 1994, University of Alabama at Birmingham). His postdoctoral training combined research in mucosal immunology (University of Alabama at Birmingham) and periodontal pathogenesis (State University of New York at Buffalo). He has held faculty appointments at the Louisiana State University, the University of Louisville, and, most recently, the University of Pennsylvania, which he joined in 2012 as a Professor of Microbiology. His field of interest lies at the host-microbe interface focusing on mechanisms of periodontal immunopathogenesis and inflammation. He has taught microbiology and immunology to dental students and residents since 1997.

Howard F. Jenkinson received his bachelor's degree in microbiology and virology from the University of Warwick, England. He completed his PhD training in 1978 at the University of Nottingham. He worked at the University of Oxford for five years as a postdoctoral researcher on the biochemistry and genetics of sporulation in *Bacillus subtilis*. He was appointed Lecturer in Oral Biology at the University of Otago, New Zealand, in 1983 and progressed through the ranks to Professor of Molecular Oral Biology at Otago (1996). He was a visiting Commonwealth

Medical Fellow at the Department of Biochemistry, University of Cambridge (1989–1990), and at the Institute of Molecular Medicine, University of Oxford (1995–1996). In 1997, he moved to the University of Bristol, England, as Professor and Chair of Oral Microbiology. His research interests include the genetics and biochemistry of microbial cell surfaces, principally streptococci and *Candida*, intermicrobial interactions, polymicrobial communities, and infective cardiovascular disease. He has taught molecular microbiology and biochemistry to dental, medical, and basic sciences students since 1983.

SECTION I

GENERAL PRINCIPLES OF ORAL MICROBIOLOGY



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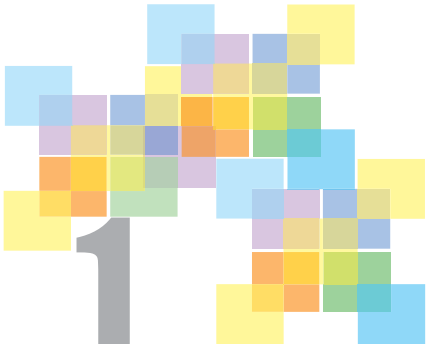

General Microbiology

Howard F. Jenkinson

INTRODUCTION

Antony van Leeuwenhoek was a Dutch scientist who is generally acknowledged as starting the discipline of microbiology. Using simple microscopes that he had fashioned in his workshop, van Leeuwenhoek made the first observations of bacteria and microorganisms, which he named "animalcules." In 1683, van Leeuwenhoek scraped material from his own teeth, describing "a little white matter, which is as thick as if 'twere batter." He continued, "I then most always saw . . . that in the said matter there were many very little living animalcules." When observing a sample from an old man who had not cleaned his teeth, van Leeuwenhoek found "an unbelievably great company of living animalcules, a-swimming more nimbly than any I had ever seen up to this time. Moreover, the other animalcules were in such enormous numbers, that all the water . . . seemed to be alive." These observations of the oral microbiota were among the first recorded sightings of live bacteria. Today, we know that the human oral cavity is a highly dynamic ecosystem that supports the growth of a tremendous number of very diverse organisms. In fact, there are roughly a million microorganisms per milliliter of saliva. The organisms that are present in saliva, mostly bacteria and fungi, are there because they are shed from the hard and soft tissues of the oral cavity and nasopharynx and they multiply in retained pools of saliva. The use of microbiological techniques, coupled with sophisticated and sensitive technologies in molecular biology, has helped us begin to gain an appreciation for the diversity of the oral microbiota. Recent estimates place the number of different species of bacteria in the oral cavity at somewhere near 700. Research into the genetics, physiology, and biochemistry of the oral microbiota has shown that the normal colonizers are a critical component in oral health and has led to an understanding of the importance of oral ecology in the development of diseases.

To fully comprehend how oral microorganisms persist and, under certain circumstances, cause disease, it is necessary to have an understanding of the structure, function, and biological activities of the oral microbiota. Why? Knowledge of the structural components of a microorganism is important because determinants on the cell surface dictate which tissues the organisms can colonize. Likewise, many components that contribute to

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the ability of the organisms to cause disease and damage host tissues are located on the cell surface. It is also important to have an appreciation for the wide variety of biological and biochemical activities that oral microorganisms possess. The metabolic capabilities of the cells—their ability to degrade the substances secreted in saliva and ingested in the diet—are of major importance in oral health and disease. How effectively organisms utilize the available nutrients determines whether an organism will establish and compete effectively at particular sites in the mouth. Moreover, the end products of metabolism of these nutrients, such as organic acids, have harmful effects on the tissues of the mouth. The following sections of this chapter highlight key features of the classification, structure, and functions of bacteria with the goal of providing a foundation for the more detailed descriptions of oral microbes, oral microbial ecology, growth of the oral microbiota, and the virulence mechanisms used by oral pathogens that are presented in the following chapters.

CLASSIFICATION SCHEMES FOR BACTERIA

The system that is commonly used for classification of life on Earth is derived from that developed by Carl Linnaeus in the 18th century. This classification scheme, originally intended for systematics of plants and animals, has been useful in accommodating new forms of life as they were discovered through the centuries. Today, life on Earth is divided into three primary domains: *Eukarya*, which are eukaryotes, and *Bacteria* and *Archaea*, which are prokaryotes, the oldest and most diverse forms of life on the planet (Table 1). Archaea, which are sometimes referred to as archaebacteria, differ genetically and metabolically from true bacteria. In fact, archaea are considered to bridge a major gap in evolution between prokaryotes and eukaryotes. Prokaryotes are distinguished from eukaryotes most notably by lack of a nuclear membrane, which separates the chromosomal DNA of the cell from the cytoplasmic contents. Eukaryotes also possess a variety of organelles and subcellular structures—like mitochondria, the Golgi apparatus, and the endoplasmic reticulum—that are

TABLE 1 General differences between prokaryotes and eukaryotes

	Domain		
Property	**Eukarya**	**Bacteria**	**Archaea**
Nuclear membrane	+	–	–
Chromosomes	>1	1	1
Chromosome organization	Linear	Circular	Circular
Murein in cell wall	–	+	–
Cell membrane lipids	Ester-linked glycerides; unbranched, polyunsaturated	Ester-linked glycerides; unbranched; saturated or monounsaturated	Ether-linked; branched; saturated
Cell membrane sterols	Present	Absent	Absent
Organelles	Present	Absent	Absent
Ribosome size	80S	70S	70S
Transcription/translation coupling	No	Yes	Yes

lacking in prokaryotes. There are a variety of other fundamental differences between these two general classes of life, some of which are summarized in Table 1. Among the more notable differences, the transcription of DNA to mRNA and the translation of RNA to protein occur in separate compartments in eukaryotes but not in prokaryotes.

Members of each of the domains can be found in the oral cavity, although the vast majority of the oral microbiota is composed of bacteria. Through highly sensitive techniques, archaea have been detected in the oral cavity, but current indications are that they appear to represent a small minority of the total organisms present on oral soft or hard tissues. Fungi, which are eukaryotic microorganisms, may also be present in the mouth, but generally, they are there in low numbers. Some of the fungi, e.g., *Candida*, flourish only when there is a restriction of access to saliva or a reduction in immunological competence. Because bacteria comprise the overwhelming majority of oral microorganisms, most of this introductory chapter focuses on bacteria.

BACTERIAL CLASSIFICATION

Most bacteria can be divided into two categories, either gram positive or gram negative, based upon a differential staining technique developed by a Danish bacteriologist, Christian Gram. The Gram stain reveals a major structural difference between the two major groups of bacteria based upon the thickness and degree of cross-linking of the cell wall. Detailed molecular studies have revealed that this relatively simple staining reaction also discloses a major evolutionary split between two major classes of bacteria. Among the bacteria, there are also organisms that cannot appropriately be classified on the basis of Gram staining, such as the agent of tuberculosis, *Mycobacterium tuberculosis*, which has a cell envelope made up of mycolic acids and waxes. Instead of Gram staining, mycobacteria can be stained by the Ziehl-Neelsen staining technique, which is also called acid-fast staining. In contrast, *Mycoplasma* species and closely related organisms are completely devoid of a cell wall, and therefore, these organisms are negative in the Gram reaction—even though genetically they are more closely related to gram-positive bacteria. Gram staining and similar techniques remain useful for bacterial identification, but the phylogenetic relationships, i.e., the evolutionary connections of bacteria, are now based almost exclusively on comparisons of nucleotide and protein sequences of organisms. Chapters 4 and 9 explain in detail many of the techniques used to assign bacteria to species, and they also outline current phylogenetic relationships of the oral microbiota.

One of the most fascinating aspects of studying microorganisms is the tremendous diversity in microbial structure, metabolic capacities, and environments in which these organisms can thrive. In nature, there are bacteria that grow optimally at pH values around 2 (acidophiles), whereas others will only grow well at pH values near 10 (alkalophiles). Some prokaryotes grow very poorly at temperatures above 15°C (psychrophiles), whereas some thrive at 100°C in hydrothermal vents miles below the surface of the ocean (thermophiles). Some microorganisms can grow with jet fuel or kerosene as the primary carbon and energy source, others create tiny internal magnets to use for directed movement, some emit light,

TABLE 2 Microorganisms of importance in the oral cavity

Gram-positive bacteria	Gram-negative bacteria
Streptococcus mutans	*Fusobacterium nucleatum*
S. sanguinis	*F. periodonticum*
S. oralis	*Haemophilus parainfluenzae*
S. mitis	*Porphyromonas gingivalis*
S. gordonii	*P. endodontalis*
S. parasanguinis	*Prevotella intermedia*
S. salivarius	*P. loescheii*
S. anginosus	*P. denticola*
Gemella morbillorum	*P. melaninogenica*
Rothia dentocariosa	*P. nigrescens*
Actinomyces naeslundii	*Tannerella forsythia*
A. gerencseriae	*Bacteroides odontolyticus*
A. odontolyticus	*Neisseria subflava*
A. oris	*Veillonella parvula*
Filifactor alocis	*Aggregatibacter actinomycetemcomitans*
Lactobacillus salivarius	*Capnocytophaga ochracea*
L. fermentum	*C. gingivalis*
L. plantarum	*Campylobacter rectus*
Bifidobacterium dentium	*C. ureolyticus*
Eubacterium nodatum	*Treponema denticola*
Parvimonas micra	*T. socranskii*
Peptostreptococcus anaerobius	*T. vincentii*
Propionibacterium acnes	

and others detoxify mercury in the environment. A variety of bacteria can corrode metals, and many, e.g., *Streptomyces*, synthesize products of significant economic importance, such as antibiotics or complex polysaccharides that are used in foods or pharmaceuticals.

Bacteria in and on the human body outnumber the cells composing the body by about 10 to 1. The number of bacteria that colonize humans is fairly small compared to the total number of known bacteria, and the number that routinely cause disease is substantially smaller still. Interestingly, the oral microbial community is among the most diverse group of organisms colonizing the various environments of a human host. To begin to become familiar with the organisms that comprise the oral microbiota in health and disease, some of the more abundant and significant oral microorganisms are listed in Table 2.

BACTERIAL ARCHITECTURE

Most bacteria are about 1 to 5 μm across the largest dimension of the cell, although there are some interesting exceptions, including a few unusual marine bacteria that are as large as 100 μm in diameter. A bacterial colony of roughly 3 mm in diameter that forms on an agar plate can contain upward of 100 million organisms. Bacteria also come in a wide variety of shapes: coccoid or spherical; bacillary or rod shaped; fusiform or long, thin rods that taper at the ends; helical or corkscrew shaped; curved; irregular; or a combination of shapes. In addition, many bacteria can form complex, multicellular structures or can differentiate into alternative shapes with clearly distinct functions and metabolic potential.

Membranes

As with all living cells, biological membranes separate the contents of the cell from the surroundings. The cytoplasmic membrane of the bacteria separates an amazingly concentrated collection of proteins, nucleic acids, lipids, and other constituents from its surroundings. The protein concentration of a typical bacterial cell is estimated at 350 mg of protein per ml; comparatively, human plasma contains only tens of milligrams of protein per milliliter. Gram-positive bacteria possess a single plasma membrane, or cytoplasmic membrane, whereas gram-negative bacteria are characterized by the presence of two membranes, a cytoplasmic (inner) membrane and an outer membrane (Fig. 1). The region between the inner and outer membranes of gram-negative bacteria is known as the periplasm, which contains the cell wall structure, proteins, and lipids. The cytoplasmic membranes of bacteria are not radically different from those of mammalian or plant cells in the sense that they consist of a phospholipid bilayer. However, unlike eukaryotic membranes, the membranes of bacteria lack sterols, such as cholesterol, and are composed primarily of saturated or monounsaturated fatty acids rather than polyunsaturated fatty acids. Membranes of archaea are also composed of a phospholipid bilayer, but the membrane lipids are attached to the glycerol moiety by ether linkages rather than the ester linkages typical of bacteria and eukaryotes. The actual composition of membranes of a given bacterial species, i.e., the number of carbons in the lipids and whether the lipids are monounsaturated or completely unsaturated, can change depending on growth conditions. However, the lipid composition of a given species remains fairly consistent when the bacteria are grown under similar conditions. On the other hand, the membrane lipid composition of different species of bacteria can vary quite a bit. Consequently, it has been possible to distinguish between even fairly closely related bacteria by comparing the composition of the lipids in the membranes of isolated bacteria grown under defined conditions.

Many biologically important proteins and enzymes are embedded in the membranes of bacteria. The cytoplasmic membrane of bacteria houses the machinery for respiration, sensing environmental signals, and transporting compounds and macromolecules into and out of the cell. Many membrane-integral or -linked proteins contribute to the virulence of the microorganisms. For example, membrane proteins can mediate adherence to the host or can have a biochemical activity that is detrimental to host

FIGURE 1 Schematic diagram illustrating the differences between the surfaces of gram-positive and gram-negative bacteria. See text for details.
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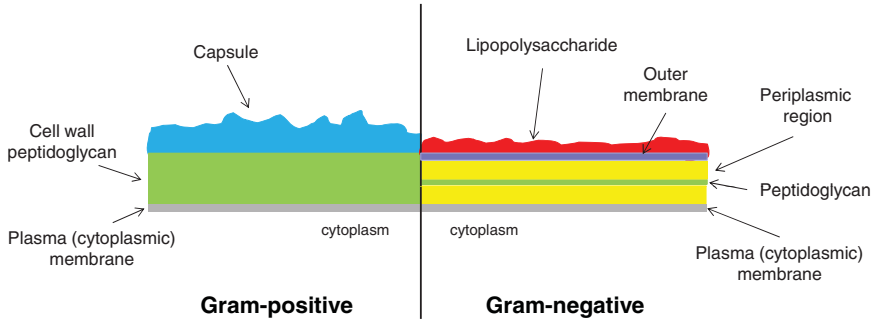

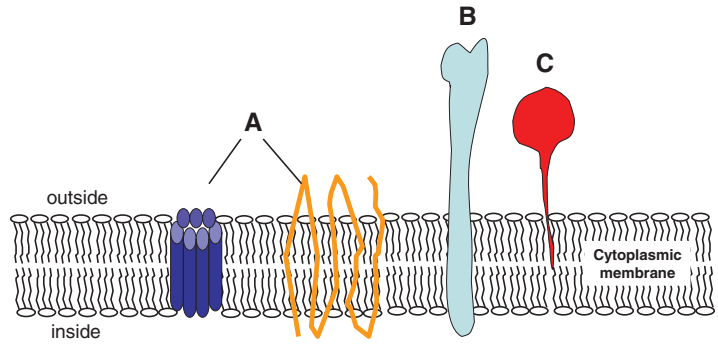

FIGURE 2 Schematic representation of a typical bacterial cytoplasmic membrane with proteins that may be involved in transport of solutes, environmental sensing, adherence, or other critical functions of the cell. See text for descriptions of A, B, and C.
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tissues, such as degradation of host proteins. The association of proteins with membranes occurs by three principal mechanisms. First, a protein can contain multiple hydrophobic domains that are able to weave their way in and out of the membrane, with hydrophilic subdomains of the protein becoming exposed alternately to the cytoplasm and external milieu (Fig. 2, structure A). Such proteins often are capable of forming pores and can be involved in the movement of solutes into or out of the cell, or they can be involved in sensing external stimuli and relaying a signal into the cell. Alternatively, membrane proteins that are in contact with the surroundings can be anchored to the cytoplasmic membrane by a single domain of the protein that is rich in hydrophobic amino acids (Fig. 2, structure B). Finally, membrane proteins can be linked to the membrane by covalent coupling to a lipid moiety, usually via a cysteine residue in the protein (Fig. 2, structure C). These covalently linked lipoproteins have many different functions, including helping bacteria adhere to target tissues.

The outer membranes of gram-negative bacteria also harbor a variety of proteins (outer membrane proteins) that serve many different functions for the organisms, although the distribution, type, and absolute number of outer membrane proteins are highly variable between genera. Porins are a general class of proteins that form pores in the outer membrane that allow nutrients and other small molecules to diffuse into the periplasm, where they can be actively transported across the cytoplasmic membrane. Porins also allow metabolic end products to diffuse out of the periplasm so they do not accumulate to toxic levels or interfere with active transport processes. The outer membrane also contains many other types of individual proteins and complex proteinaceous structures, some of which mediate adhesion to host tissues.

Lipopolysaccharides

Only gram-negative bacteria produce lipopolysaccharide (LPS), a hybrid molecule of lipid and carbohydrate that is abundant in, and adds structural integrity to, the outer membrane of the organisms. LPS consists of three major domains: a lipid A portion of the molecule, which is anchored in the outer membrane, and the core polysaccharide and O side chains, which extend from the cell into the surroundings (Fig. 3). The structure of

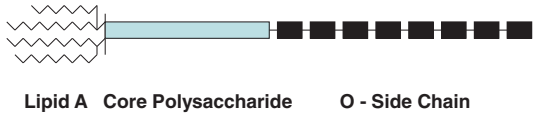

FIGURE 3 Schematic diagram of a typical LPS molecule of gram-negative bacteria.
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LPS varies considerably among gram-negative bacteria, as does the length of the O side chain. Some bacteria have "rough" LPS, which lacks a repeating O side chain, whereas bacteria with "smooth" LPS have an O side chain consisting of a fairly large and variable number of repeating subunits of carbohydrate. The rough and smooth designations do not refer to the LPS molecule directly but rather to the appearance of the colonies on agar media. Strains with long polysaccharide O side chains appear smooth and shiny on agar plates. The classification of bacterial strains by serotyping is frequently based upon the structure and composition of the core polysaccharides and O side chains.

LPS plays major roles in the ability of the organisms to elicit diseases. A listing of some of the biological properties associated with LPS, which is sometimes referred to as endotoxin, is given in Table 3. Among the more important biological effects of LPS are the ability to elicit shock, fever, and apoptosis (programmed cell death) of host cells and the ability to stimulate potent and adverse inflammatory immune reactions through a variety of pathways that ultimately result in tissue damage. Most of the detrimental biologic activities of LPS reside in the lipid A portion of the molecule. Notably, not all bacterial LPS molecules are highly toxic, nor do all elicit the reactions described in Table 3 at biologically meaningful concentrations. Instead, there is a broad spectrum of activity of LPS depending on the organism from which it is isolated. By way of example, *Porphyromonas gingivalis*, which has been implicated in human periodontal diseases, produces an LPS that strongly stimulates bone resorption, a major problem in periodontal diseases, whereas the LPS of some strains of the common intestinal bacterium *Escherichia coli* is comparatively benign in this regard. Some of the mechanisms by which LPS exacerbates periodontal diseases are covered in greater detail in chapter 14.

TABLE 3 Some relevant biological activities of LPS

Lethal toxicity
Stimulation of inflammation
Complement activation
Polymorphonuclear leukocyte activation
Macrophage activation
B-cell mitogen activity
Adjuvant activity
Pyrogenicity
Stimulation of bone resorption
Stimulation of prostaglandin synthesis
Induction of tumor necrosis factor
Hypothermia
Hypotension

Cell Wall Peptidoglycan

With few exceptions, bacteria have cell walls. The material comprising the cell wall is known as peptidoglycan or murein, which is structurally different from the cell walls of plants and fungi. Peptidoglycans consist of a repeating *N*-acetylglucosamine, *N*-acetylmuramic acid carbohydrate backbone linked to a tetrapeptide that generally contains biologically uncommon D-amino acids and diaminopimelic acid (Fig. 4). The peptides are cross-linked to various degrees, depending on the organism and growth conditions, and this cross-linking gives the peptidoglycan a meshwork-like structure that is flexible, yet strong. In gram-negative bacteria, the cell wall lies between the inner and outer membranes and is held in place by covalently bound lipoproteins that anchor the wall to the outer membrane, with the protein portion bound to the wall and the lipid portion buried in the outer membrane (Fig. 5).

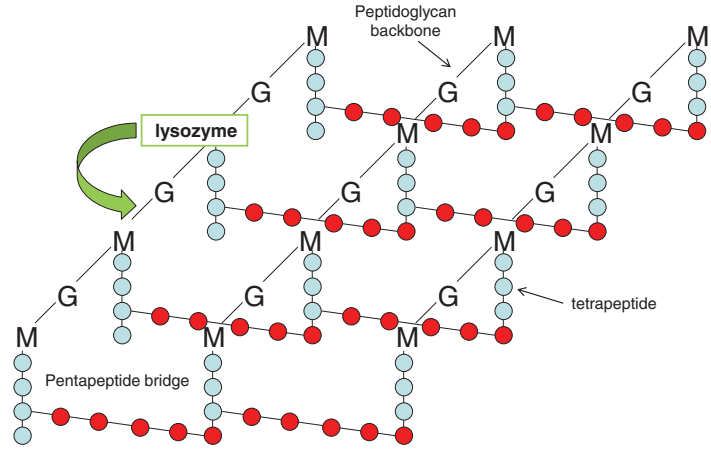
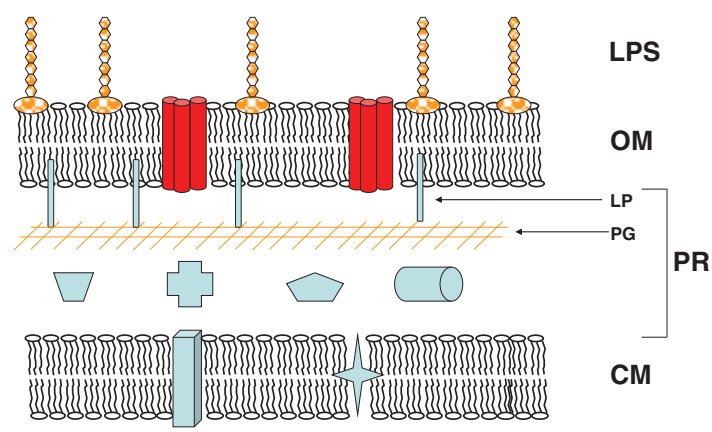


FIGURE 4 Structure of bacterial peptidoglycan, with alternating *N*-acetylmuramic acid (M) and *N*-acetylglucosamine (G). A tetrapeptide is covalently linked to *N*-acetylmuramic acid and is cross-linked by a pentapeptide (red). The peptides often contain unusual D-amino acids and diaminopimelic acid. Lysozyme breaks the β(1, 4) bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine. Many bacteria with capsules are resistant to lysozyme. doi:10.1128/9781555818906.ch1.f4

The cell wall is a comparatively minor architectural structure in gram-negative bacteria, although it does play key roles in the integrity of the cell by helping to prevent bacteria from bursting in hypo-osmotic conditions. The cell walls of gram-positive bacteria are much thicker and more highly cross-linked than those of gram-negative bacteria and are very prominent features of the cellular architecture (Fig. 6). In contrast to that of gram-negative bacteria, the wall of gram-positive bacteria is often the outermost structure of the cell, so it is directly exposed to the environment. Covalently linked to, or sterically anchored in, the cell wall are a diverse group of proteins, enzymes, and polysaccharides, many of

FIGURE 5 Architecture of a gram-negative bacterial surface showing the cytoplasmic membrane (CM) with transport and other proteins embedded (geometric shapes) and the periplasmic region (PR) containing proteins and peptidoglycan (PG) anchored to the outer membrane (OM) by lipoproteins (LP). The outer membrane contains multiple proteins, including porins (red cylinders), as well as lipopolysaccharides (LPS). doi:10.1128/9781555818906.ch1.f5



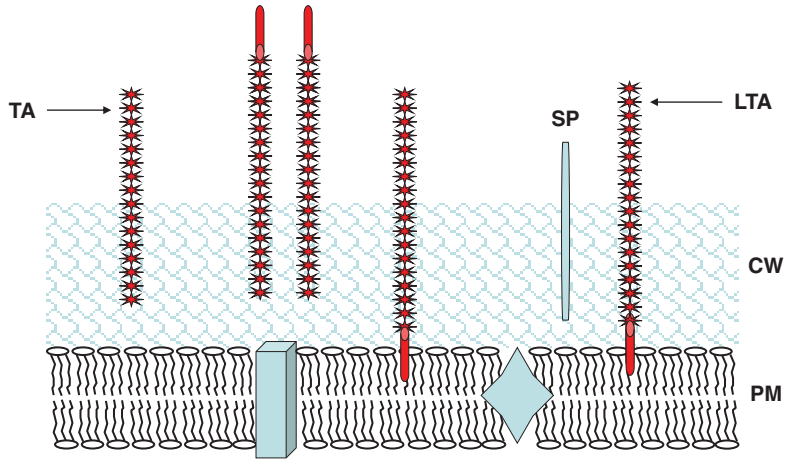


FIGURE 6 Architecture of the exterior of a gram-positive bacterium showing the plasma membrane (PM) with transport proteins embedded. The peptidoglycan meshwork cell wall (CW) is anchored in the membrane. Teichoic acids (TA) and lipoteichoic acids (LTA) with a lipid moiety (in red) are illustrated, along with a surface protein (SP) anchored in the cell wall. doi:10.1128/9781555818906.ch1.f6

which are crucial for persistent colonization of the oral cavity. In many cases, the carbohydrates in the cell wall have been useful for serological discrimination of different strains and species of bacteria, including many streptococci that elicit dental caries.

Lipoteichoic Acids

A major constituent of the outer envelope of gram-positive bacteria is lipoteichoic acid (LTA). LTAs are amphipathic molecules, i.e., they are composed of hydrophilic and hydrophobic constituents. LTAs consist of a lipid moiety that is generally embedded in the cytoplasmic membrane and a teichoic acid moiety, made from repeating units of phosphorylated glycerol or ribitol, which gives the teichoic acid portion of the molecule a strong net negative charge (Fig. 6). Bacteria also contain teichoic acids (TAs), which are deacylated forms, i.e., lacking the lipid moiety, of LTAs. LTAs and TAs appear to contribute to the structural integrity of gram-positive bacteria, but they are also important in pathogenesis and have been implicated in such processes as adhesion to the host and avoidance of immune surveillance. Additionally, both LTAs and TAs can be shed from the cell surface into the surroundings. This can be problematic for the host because, similarly to LPS, LTAs can stimulate a variety of adverse reactions, including apoptosis, although the toxicity of LTAs is generally much less than that of the LPS of pathogenic gram-negative bacteria. Also like LPS, LTAs can be recognized by specific signaling molecules on the surface of host cells called Toll-like receptors, resulting in stimulation of innate and immune defenses (see chapter 2). Finally, it appears as though LTAs can be oriented in the cell wall with the lipid moiety protruding into the environment (Fig. 6). In this case, LTAs are thought to be important in conferring surface hydrophobicity to the bacteria, a trait that is strongly correlated with the ability to adhere to host tissues and saliva-coated teeth.

Some gram-positive bacteria can also produce teichuronic acids. Teichuronic acids are structurally similar to TAs, except the glycerol or ribitol phosphate backbone is replaced by a backbone of a hexuronic acid, such as glucuronic acid, or by other sugars, including *N*-acetylgalactosamine. For organisms capable of producing both TAs and teichuronic acids, growth conditions usually dictate which polymers will dominate, with availability of phosphate in the growth medium having the most profound impact. Not surprisingly, when phosphate is abundant, the formation of TAs is favored, since TAs are rich in phosphate.

Other Important Components Produced by Bacteria

CAPSULES

Capsules, which are produced by a wide variety of bacteria, are extracellular polymers that are loosely attached to the surface of the organisms. Generally, capsules are composed of polysaccharides, although there are a number of examples of nonpolysaccharide capsules, including the poly-D-glutamic acid capsule of the agent of anthrax, *Bacillus anthracis*. Capsules are high-molecular-mass polymers (>10^5 Da) that consist of repeating units of the same building blocks (homopolymers) or of a number of different building blocks (heteropolymers). Capsule synthesis by bacteria usually involves the production within the cell of the core repeating subunit of the capsule using nucleotide sugars, e.g., UDP-glucose. Subsequently, the core unit is translocated across the membrane(s) and polymerized at the surface of the cell. The variety in the structure and composition of bacterial capsules is remarkable. Since microorganisms spend significant amounts of energy to produce capsules, it is not surprising that capsules provide substantial benefits. These include protection against physical insult, serving as a source of food during starvation periods, and conferring substantial resistance to phagocytosis.

Many oral bacteria produce extracellular polysaccharides that are not usually thought of as capsules, yet these polymers have many properties in common with true capsules. In particular, a number of cariogenic oral streptococci, including *Streptococcus mutans* and oral *Actinomyces* species, produce secreted enzymes that convert sucrose to high-molecular-mass (10^5 to 10^8 Da) homopolymers of glucose or fructose, known as glucans (or mutan) and fructans, respectively. Glucans and fructans are often responsible for the "furry" feeling on teeth after consuming foodstuffs containing high concentrations of sucrose. Fructans and glucan polysaccharides produced by oral bacteria represent a large proportion of the dry weight of dental plaques formed in humans fed a diet rich in sucrose. Glucans are produced by the action of glucosyltransferases, which are enzymes that convert sucrose to α-1,3- and α-1,6-linked homopolymers of glucose. Glucans serve mainly as an adhesive scaffolding to support the irreversible binding of a group of cariogenic organisms, known as mutans streptococci, to the teeth. Mutant strains of *S. mutans* that lack the ability to produce glucans are essentially devoid of the ability to cause caries on the smooth surfaces of the teeth (discussed in more detail in chapter 12). Notably, the glucans produced by *S. mutans* cannot be digested by the enzymes of any known oral organisms or by salivary amylase, so once glucans are produced in the mouth, they must be removed by

mechanical forces. Fructans are also rapidly synthesized in the mouth by oral streptococci and certain strains of oral *Actinomyces*, but these polymers are probably not as important for adhesion of bacteria to the teeth. Instead, fructans function as extracellular storage compounds that allow the organisms to accumulate carbohydrates in a form that will not readily diffuse away from plaque. Then the bacteria can degrade these polysaccharides when dietary sources of sugar are exhausted.

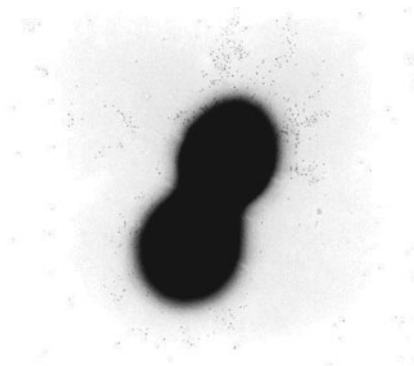


FIGURE 7 Pili of gram-positive bacterium *Streptococcus agalactiae* (Lancefield group B streptococcus). The pilus structures have been revealed by reaction with gold-labeled antibodies that bind to the proteins making up the pilus shaft. Image provided by Angela Nobbs. doi:10.1128/9781555818906.ch1.f7

FIMBRIAE AND PILI

The terms fimbriae and pili are often used interchangeably to refer to filamentous, or hair-like, structures on the surface of bacteria (Fig. 7). A range of oral bacteria produce fimbriae, and these mediate adhesion to salivary proteins; extracellular matrix proteins, e.g., collagen and fibronectin; proteins in the clotting cascade; different types of host cells; and other bacteria. In addition, pili and fimbriae mediate many functions that are detrimental to the host, such as stimulation of bone resorption and facilitation of entry of the bacteria into cells of the host (invasion). Pili can also mediate transfer of DNA from a donor bacterium to suitable recipient bacteria. Many of the fascinating aspects of pilus and fimbria biogenesis and function will be discussed in subsequent chapters.

FIBRILLAR LAYERS

A number of gram-positive bacteria possess a fibrillar layer. Fibrillar layers or "fuzzy coats" are so named because they confer a fuzzy appearance to the cell surface when viewed under the electron microscope. The fibrils, which are shorter and distinct from fimbriae, are often composed of proteins that act as specific adhesins and directly mediate the binding of bacteria to host proteins or host cells. It has been shown that the fibrillar layer helps confer hydrophobicity to the cells, which, as mentioned above, is an important determinant in bacterial adhesion because it appears to stabilize the specific stereochemical interactions governed by cell surface adhesins.

FLAGELLA

Flagella are organelles of locomotion produced by many different species of bacteria, but only a relatively small subset of oral species produce flagella. In conjunction with chemotaxis systems, flagella can facilitate the directed movement of bacteria toward an attractant, such as a nutrient, or away from a repellant, such as a toxic metabolic end product. The distribution of flagella on bacteria is highly variable, but a given species of bacteria generally displays a very specific pattern of flagella. Some bacteria have a single flagellum or have one flagellum located at each end of the cell; these are known as polar flagella. Some have many flagella distributed along the body of the organisms, called peritrichous flagella. In virtually all cases, flagella are in direct contact with the surroundings and spin to propel the bacteria through the media. One notable exception to this rule is the group of organisms known as spirochetes. Some examples of spirochetes include *Treponema pallidum*, the syphilis agent, and *Treponema denticola*, an organism associated with human periodontal diseases. Although the mechanisms of rotation of spirochetal flagella are generally similar to those of other bacteria, the flagella of spirochetes

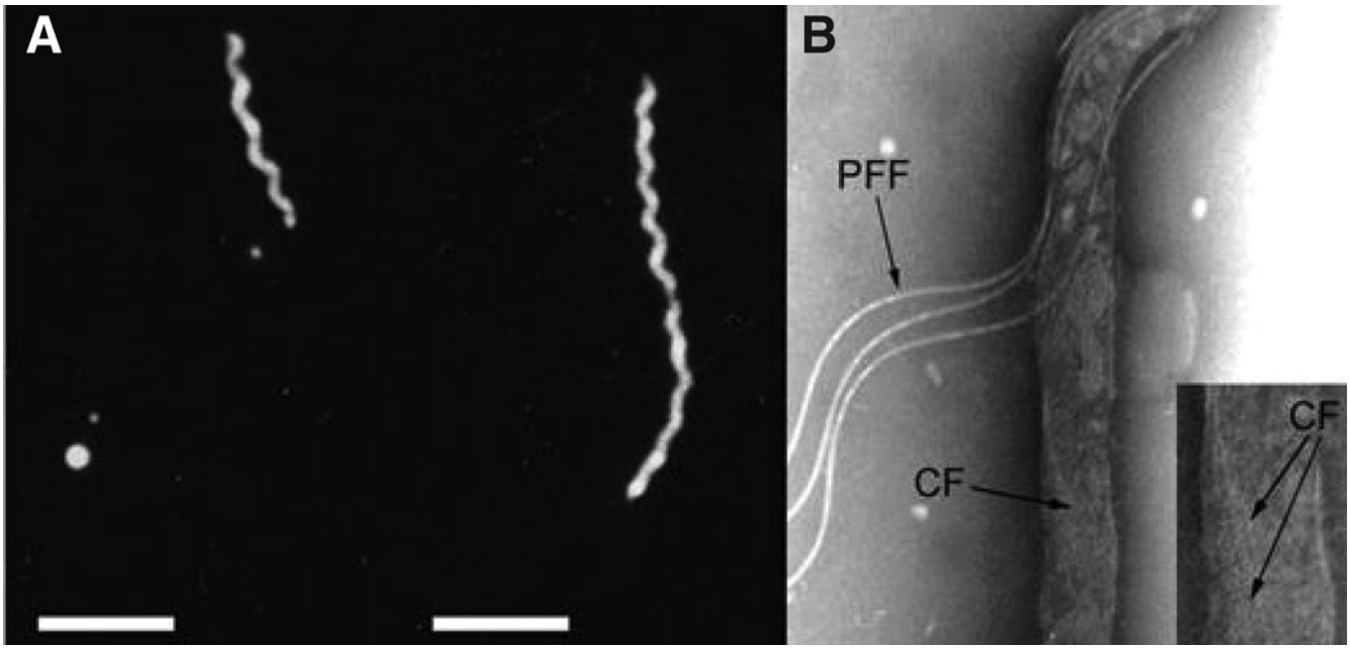


FIGURE 8 (A) Dark-field microscopy of spirochetes showing the characteristic corkscrew shape. (B) Electron micrograph showing periplasmic flagella (PFF) as well as cytoplasmic filaments (CF). Image provided by Jacques Izard.
doi:10.1128/9781555818906.ch1.f8

are located within the periplasm of the organism between the cytoplasmic and outer membranes of the organisms, and thus are known as periplasmic flagella (Fig. 8). In most cases, flagella and motility have not been strongly correlated with colonization or virulence of oral bacteria, but again, an exception is the spirochetes. The corkscrew shape of spirochetes and the periplasmic flagella render these organisms able to penetrate tissues. Interestingly, spirochetes are the bacteria that are most frequently found at the front of a progressing periodontal lesion, which is likely due to their unique motility characteristics.

VESICLES

Vesicle production is a characteristic of many gram-negative bacteria. Vesicles are produced by shedding or "blebbing" of the outer membrane. The small, lipid-rich vesicles that are shed can contain many or all of the components of the outer membrane, including LPS, tissue-damaging enzymes, and other virulence factors. Vesicles are thought to be relevant in oral diseases because they are very small—tens of nanometers in diameter—and can diffuse into host tissues carrying virulence factors and stimulating adverse reactions, such as inflammation and bone resorption.

S-LAYERS

Some oral bacteria can produce a surface layer known as an S-layer, which is a highly ordered proteinaceous coat that covers the surface of the bacterial cell. S-layers are generally composed of a single protein and can be highly effective in protecting the organisms from being phagocytosed and killed by immune cells or from other inimical influences.

ENDOSPORES

Some bacteria have the ability to form endospores. Endospores, or spores as they are more commonly called, are dormant forms of bacteria that are highly resistant to killing by physical or chemical agents—heat, bleach, alcohol, peroxides, and so on. Under favorable conditions, spores can germinate to produce progeny organisms. Sadly, the American public became familiar with bacterial endospores when *B. anthracis* (the causative agent of anthrax) spores were used in assaults on media figures, government officials, and innocent citizens in the fall of 2001. Most oral bacteria are not able to form spores, but spore-forming bacteria are relevant to the practice of dentistry by virtue of their near ubiquity and high level of resistance to antimicrobials, disinfectants, and autoclaving. Characteristics of spores and methods for sterilization and disinfection are covered in chapter 22.

GENETIC ORGANIZATION OF BACTERIA

The Bacterial Chromosome

Bacteria have a single, covalently closed, circular chromosome, although there are some rare exceptions when the chromosomal DNA is linear. The chromosome is double stranded and contains the same bases as human DNA—adenine (A), cytosine (C), guanine (G), and thymine (T). The actual nucleotide composition and size of the chromosome vary widely among species of bacteria. For example, many of the oral streptococci have a chromosome with a combined guanine and cytosine content, or G+C content, of roughly 35%. On the other extreme, *Actinomyces* species, which are gram-positive, non-spore-forming anaerobic bacteria that are abundant colonizers of the teeth and soft tissues, have a G+C content of around 68%. Now that the technology is available to sequence entire genomes, the complete sequence and, thus, the exact sizes of thousands of bacterial chromosomes have been determined, including numerous oral pathogens. For example, the entire chromosome of the dental caries pathogen *S. mutans* is 2,032,327 bp, containing over 2,000 genes. It is noteworthy that the chromosomes of oral bacteria are generally smaller than those of organisms that are able to both exist in the environment and colonize a variety of hosts. The *S. mutans* chromosome (2.03 Mbp) and the chromosome of *P. gingivalis* (2.3 Mbp) are only about a third of the size of the chromosome of *Pseudomonas aeruginosa* (6.2 Mbp), a bacterium that is widely disseminated in water, soil, and plants but that can also cause serious diseases in immunocompromised patients, burn victims, and persons with cystic fibrosis. For additional comparisons, the enteric bacterium and common laboratory pet *E. coli* has a chromosome of about 4.6 Mbp and the chromosome of the sporulating soil bacterium *Bacillus subtilis* is 4.2 Mbp. Thus, it appears as though oral bacteria have lost, or failed to acquire, many of the genes that environmental bacteria need to survive and thrive outside the oral cavity. In spite of the relatively small size of chromosomes of oral bacteria though, these organisms have evolved highly specialized and extremely elegant mechanisms to colonize and persist in the oral cavity. To provide a bit more perspective, the species with the smallest genome of free-living organisms is *Mycoplasma genitalium*, which has a chromosome of 580,074 bp (580 kbp) containing around 520 genes, so oral bacteria have the potential to express many

more genes than may be required simply for growth and replication in a human host. As discussed in subsequent chapters, many of these genes have in fact been shown to be critical for persistence and elicitation of diseases by oral microorganisms.

Chromosome Replication in Bacteria

Replication of the bacterial chromosome begins at a single origin of replication and proceeds bidirectionally to a terminus located asymmetrically on the chromosome. DNA replication in bacteria has been studied extensively, and the machinery required for replication—DNA polymerase, DNA winding and unwinding enzymes, single-stranded DNA-binding proteins, and the use of RNA primers to initiate DNA synthesis—have much in common with that found in mammalian cells. However, the processes to control initiation of replication and the machinery involved in DNA replication are somewhat streamlined in bacteria compared with mammalian cells. Nevertheless, control of DNA replication is a carefully orchestrated process and bacteria face some interesting challenges in coordinating DNA replication with cell division. One of the problems that bacteria face is that replication of the entire chromosome can take substantially longer than it takes for a rapidly growing culture of bacteria to divide. For example, replication of the *E. coli* chromosome is predicted to take a little over 80 min, whereas rapidly growing *E. coli* can divide in less than 20 min. To overcome this problem, bacteria do something substantially different than eukaryotes—they initiate additional rounds of chromosome replication before the initial round is completed. In fact, there are multiple replication events proceeding concurrently in rapidly growing bacteria. Completed chromosomes, and those still replicating, can then be passed on to daughter cells when division takes place. The daughter cells will then have partially replicated chromosomes and can divide faster than could be accomplished if it was necessary to complete one full cycle of DNA replication before division.

Bacteria frequently harbor extrachromosomal elements, i.e., discrete DNA elements that can replicate independent of a direct association with the chromosome. The most common extrachromosomal elements of bacteria are called plasmids. Plasmids usually consist of circular double-stranded DNAs that carry an origin of replication that allows replication of the element by the host machinery. Most plasmids carry additional genetic information, including genes encoding resistance to antibiotics, genes that allow the plasmids to be transferred from one bacterium to another, genes that allow organisms to use certain types of compounds, or in some cases, genes that are crucial to the ability of the organisms to cause disease. Plasmids can be extremely small, less than 2,000 bp, or fairly large, upward of 100 kbp. Other elements that can replicate independent of the chromosome include the genetic material of bacteriophage (bacterial viruses). In these cases, bacteriophage inject their nucleic acid into the host cell, where it can be replicated using the host machinery and, in some cases, with the assistance of bacteriophage-derived enzymes. Extrachromosomal DNA elements have proven to be the essential tools of molecular biology because they allow researchers to work with manageably small DNA fragments and to easily recover engineered DNAs free of the large chromosomes of the organisms (see chapter 7).

Gene Transfer in Bacteria

Genetic exchange between bacteria can enhance the diversity of the population and allows the transfer of genes that can augment the virulence of organisms. Indeed, horizontal gene transfer, i.e., gene transfer between organisms, appears to be the major route for acquisition of resistance to antibiotics. Genetic exchange by bacteria can take place in three general ways: transformation, transduction, or conjugation. These methods are covered in greater detail in chapter 7. Briefly, transformation involves the uptake of DNA from the surroundings, and bacteria that are capable of this are called naturally competent. Work is ongoing to determine whether this type of genetic exchange may be occurring in dental plaque. Transduction involves the transfer of genetic information from one organism to another via bacteriophage. Generalized transducing bacteriophage can package fairly large pieces of DNA from a donor strain and transfer that DNA by infecting a suitable recipient strain. Specialized transducing phage picks up selected segments of DNA from the donor and transfers the DNA, along with some or all of the genetic material of the virus, into a recipient cell. Conjugation involves transfer of genetic material by direct cell-cell contact. In many gram-negative bacteria, DNA transfer is mediated through a sex pilus on the surface of the cell through which DNA can be funneled to the recipient. In some gram-positive bacteria, a sophisticated pheromone system induces clumping between a donor and a compatible recipient and the close cell-cell contact allows DNA to be transferred.

BACTERIAL GROWTH AND NUTRITION

Growth

Bacteria divide by binary fission. Because one bacterium becomes two bacteria, cultures of bacteria exhibit exponential growth: 2 become 4, then 8, 16, 32, and so on. When bacteria that are not already dividing are inoculated into a liquid medium, the organisms must alter their metabolism and gene expression patterns to produce sufficient quantities of the constituents needed to grow as rapidly as is possible in the new environment. During this adjustment period, known as the lag phase (Fig. 9), the organisms begin replicating their chromosomes and synthesizing the machinery to transcribe and translate their genetic material into the building blocks of the cell. The organisms begin to grow and divide during the exponential phase of growth, sometimes called log phase, although exponential phase is the more appropriate term. Cells will continue to divide exponentially until nutrients begin to be exhausted or until inhibitory metabolites start to accumulate in sufficient concentrations to inhibit growth. When cell numbers stop increasing, the organisms are said to have entered the stationary phase. During this period, DNA replication largely is arrested, the number of ribosomes diminishes, and the cell slows production of the enzymes needed to catalyze the synthesis of the building blocks of the cell. Cells also start to synthesize a new subset of proteins that help to enhance survival during nutrient starvation. Stationary-phase cells are more resistant to antibiotics, since most antibiotics target exponentially growing bacteria by acting on cell wall

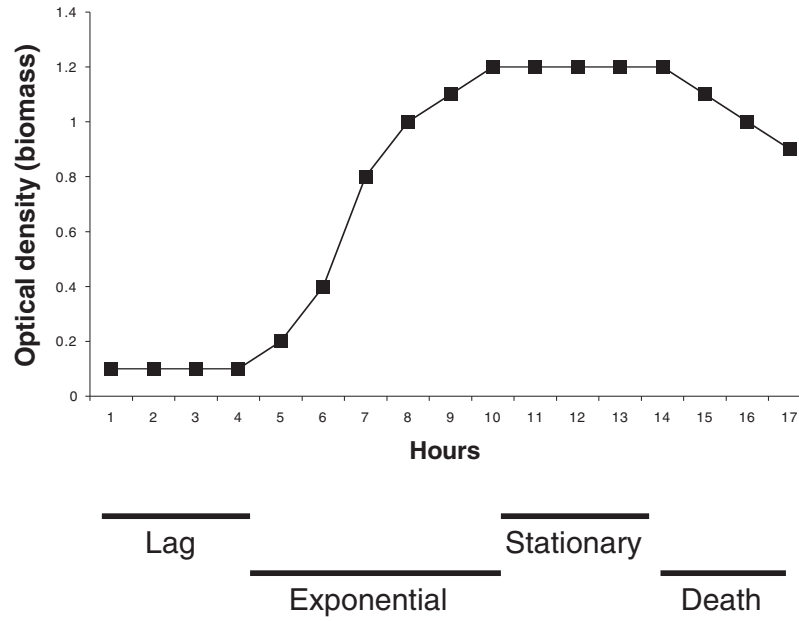


FIGURE 9 Typical growth pattern of bacteria in liquid culture.
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synthesis (penicillins and cephalosporins), DNA replication (nalidixic acid), transcription (rifampin), or translation (tetracyclines and macrolides). Relating the growth phases to bacteria in dental plaque, it is easy to imagine that, during fasting periods, dental plaque bacteria are frequently limited for nutrients, especially carbohydrates. Thus, they may be in a state akin to stationary phase and their phenotypic properties in plaque and their susceptibilities to antimicrobial agents may be different from what we can observe in the laboratory using traditional methods. After some time, cells enter a death phase. This is highly variable, depending on the organisms. Some oral bacteria, like *Streptococcus gordonii*, do not survive for prolonged periods (days) without nutrients, whereas some bacteria can survive for weeks.

Another environmental factor that has a major impact on microbial growth is oxygen, which is present in near atmospheric quantities, about 20%, in the oral cavity. Oxygen itself is not toxic, but single electron reductions of oxygen catalyzed by enzymes of bacteria can lead to the generation of toxic oxygen radicals, including superoxide, peroxides, and hydroxyl radicals, which can damage membranes (lipids), proteins, and DNA. Bacteria vary dramatically in their abilities to utilize oxygen and to cope with oxygen radicals. Some bacteria actually require oxygen for growth because the organisms respire to generate energy and use oxygen as a terminal electron acceptor. These aerobic organisms consume oxygen and have high levels of the enzymes needed to detoxify oxygen radicals. There are relatively few obligately aerobic bacteria in dental plaque. Obligate anaerobes cannot grow in the presence of oxygen, largely because they create toxic oxygen radicals through metabolism but lack sufficient

quantities of, or lack altogether, the enzymes needed to cope with oxygen radicals. Many of the organisms associated with periodontal diseases, *P. gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and oral spirochetes, are obligate anaerobes. These organisms can be abundant in dental plaque, particularly subgingival plaque, and can exist there because they are protected from oxygen metabolites by other plaque microorganisms that can detoxify or consume the oxygen. Facultative or facultatively anaerobic bacteria can grow in the presence of oxygen but also grow well when oxygen is absent. Most of the abundant species in the mouth are facultative organisms, such as oral streptococci. Some bacteria that require but can only tolerate small amounts of oxygen are referred to as microaerophilic. Organisms that have their growth enhanced by carbon dioxide are referred to as capnophilic. Important oral capnophiles include *Aggregatibacter actinomycetemcomitans* and *Capnocytophaga gingivalis*, both of which are associated with human periodontal diseases.

Nutrient Acquisition

For one bacterium to become two bacteria, it is necessary for the organisms to be able to acquire from their surroundings all of the nutrients needed for growth. In many cases, the first step in this process for oral bacteria involves the degradation of complex macromolecules in saliva and crevicular fluids, or in the diet, to compounds that are sufficiently small in size that they can diffuse through, or be transported across, the membranes of the organisms. Some of the types of extracellular enzymes that are produced by oral bacteria that assist in processing nutrients include proteinases (or proteases), which cleave proteins into peptides or individual amino acids; glycohydrolases (or glycosidases), such as amylase, which break down polysaccharides or oligosaccharide side chains on proteins; neuraminidase, which removes sialic acid from glycoproteins; and lipases, which break down lipids.

Once the oral secretions or the diet are processed to constituents that can be assimilated by bacteria, the organisms use many different types of transport proteins, which are found in the cytoplasmic membrane, to internalize the nutrients. These transporters can be divided into several different categories. Most of the transporters are active transporters that use energy in the form of ATP or that exploit the electrochemical gradient (Δp, the proton motive force) to move the desired compounds into the cell. Peptides and some sugars are generally taken up by high-affinity, multisubunit transporters known as ATP-binding cassette transporters. This class of transporters usually contains, at a minimum, a substrate-binding domain, a membrane-spanning domain, and an ATP hydrolysis domain that energizes movement of the compounds into the cells. Another important type of transporter for oral bacteria is a family of proteins that is part of the sugar-phosphotransferase system (PTS) (Fig. 10). The PTS consists of a general PTS protein called enzyme I, which transfers phosphate from phosphoenolpyruvate to a general phosphocarrier protein called HPr. HPr can then donate that phosphate to sugar-specific enzyme II proteins that bind the sugar outside the cell and, with the concomitant transfer of a phosphate group to the sugar, transport the sugar

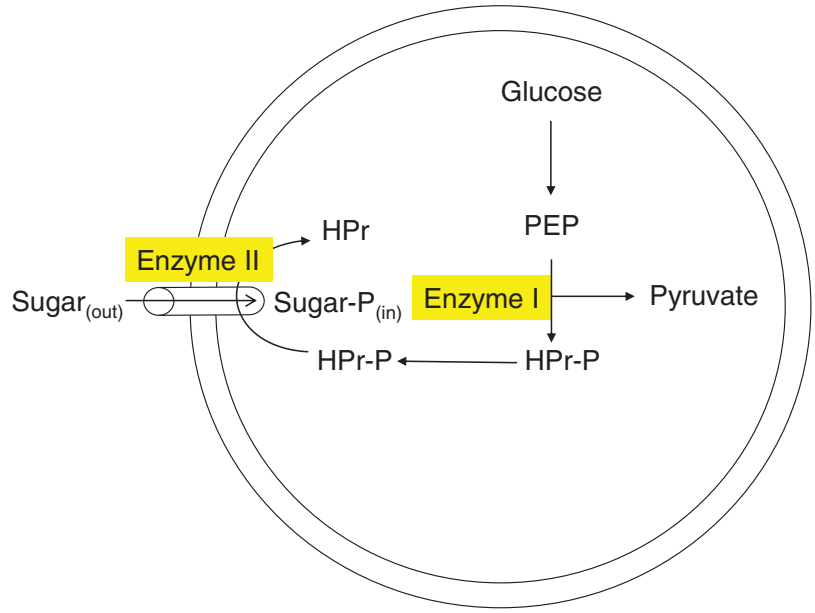

FIGURE 10 The bacterial sugar PTS consists of enzyme I, which transfers a phosphate group from phosphoenolpyruvate (PEP) to the HPr at histidine residue 15. HPr (phosphocarrier protein) can then donate the phosphate to a series of sugar-specific enzyme II proteins that catalyze the transport and concomitant phosphorylation of incoming sugars. doi:10.1128/9781555818906.ch1.f10

phosphate into the cell. For example, an enzyme II specific for glucose binds glucose in the environment and moves it into the cell as glucose-6-phosphate, which can directly enter the glycolytic pathway. The PTS is a very high affinity system, with K_m values (the concentration of substrate at which the system operates at 50% of its maximum rate) for the cognate sugars of approximately 10 μM, which is about the same concentration of glucose that is found in the mouth during fasting periods. The PTS has many important roles in oral bacteria, but it is probably an especially critical pathway when nutrients become limiting because it will allow oral bacteria to scavenge the trace amounts of carbohydrates that are present in the mouth between periods of food consumption.

INTRODUCTION TO FUNDAMENTAL CONCEPTS OF ORAL MICROBIAL ECOLOGY

Microbial Biofilms

Much of what is currently known about the properties and metabolism of oral bacteria has been learned from studying pure cultures of individual species growing in test tubes, primarily because this was the most convenient and reliable way to study the behavior of bacteria. However, growth as suspended populations in liquid medium is not the normal mode of growth for bacteria in the mouth. Oral microorganisms instead grow as compositionally and structurally complex mixtures of species adhering to a surface. These types of populations are generally known as biofilms (Fig. 11). In fact, the simplest definition of a biofilm is "organisms

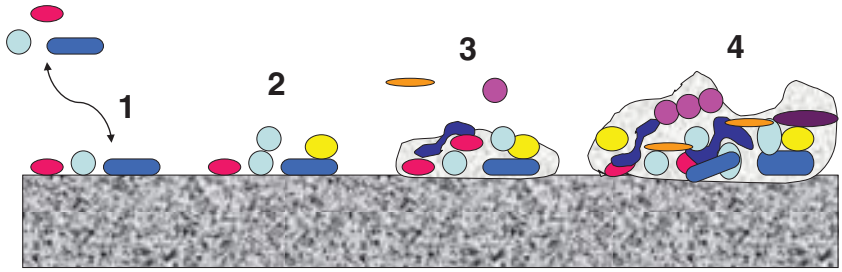

FIGURE 11 General features of biofilm formation. (1) Reversible association of microbes with a surface. (2) Stable adherence of the bacteria. (3) Growth, polymer production, and recruitment of new species to the biofilm through interbacterial interactions. (4) Formation of a mature biofilm, often referred to as a climax community. doi:10.1128/9781555818906.ch1.f11

colonizing a surface and embedded in a polymer-rich matrix," with dental plaque being an archetypical example of a biofilm. Importantly, recognition of the oral microbiota as highly diverse, surface-adherent communities of microorganisms—biofilms—is central to understanding oral microbial ecology and oral disease development.

ADHESIVE AND COOPERATIVE INTERACTIONS HELP TO DICTATE COMMUNITY COMPOSITION AND STABILITY

From multiple studies on dental plaque, it is clear that oral bacteria exist in very close association with one another, and in many cases, oral biofilms appear to have a highly ordered structure. These apparently specific associations among various species of bacteria are believed to be driven, at least in part, by production of surface adhesins that allow groups of species to colonize the same tissues and to adhere to one another with high affinity. Notably, these interbacterial associations may have evolved because of nutritional benefits or other advantages to the two species of growing very close to one another. Such cooperative growth behavior facilitated by adhesive interactions has been seen between *S. mutans*, which produces copious amounts of lactic acid, and another important oral bacterium, *Veillonella parvula*, which consumes lactic acid. Similarly, production of certain fatty acids by *P. gingivalis* can stimulate the growth of the oral spirochete *T. denticola*. Interestingly, strong associations of *S. mutans* and *Veillonella* species with dental caries and of *P. gingivalis* and spirochetes in periodontal diseases are well documented. Thus, there is likely to be a real benefit to bacteria to grow in dense biofilms, and specific associations between certain species may be an essential factor in the persistence of those organisms in plaque.

ANTAGONISTIC INTERACTIONS INFLUENCE COMPOSITION AND PATHOGENIC POTENTIAL OF ORAL BIOFILMS

Antagonistic interactions can also influence the composition and biological activities of the oral biofilms on the teeth and soft tissue. Perhaps the simplest form of antagonism occurs when end products of one organism directly inhibit the growth of other organisms in plaque. One example of this type of antagonism can be seen during the development of dental caries. Consumption by the host of a diet that is rich in carbohydrates leads

to bacterial production of organic acids, such as lactic and acetic acids, that in turn drive the pH of oral biofilms down to values of 4 and below. Acidification of plaque by cariogenic bacteria, such as *S. mutans* and lactobacilli, inhibits the growth of acid-sensitive organisms. Over time, the acid-sensitive organisms can no longer compete effectively, leading to the enrichment in cariogenic biofilms of organisms that are acid tolerant, like *S. mutans* and lactobacilli.

Another example of an antagonistic interaction that may influence plaque ecology is the production of hydrogen peroxide (H_2O_2) by certain streptococci. H_2O_2 is toxic to a number of bacteria that lack, or have low levels of, the enzymes needed to detoxify oxygen radicals. Thus, this simple diffusible compound may kill or inhibit the growth of selected organisms, making the H_2O_2-producing organisms more competitive. Even more sophisticated forms of antagonism exist, such as production of compounds known as bacteriocins by bacteria in plaque. In one well-characterized case, it is known that certain strains of *S. mutans* produce bacteriocins known as mutacins, which are peptide antibiotics that specifically inhibit the growth of closely related species. Through production of bacteriocins, *S. mutans* is able to suppress the growth of other streptococci that would otherwise compete for similar nutrients.

Ecology of the Oral Microbiota and Development of Oral Diseases

The oral environment and ecology of the oral microbiota are covered in detail in chapters 3 and 5. However, it is useful to provide a brief introduction to the current concepts of biofilm homeostasis and the ecological basis of oral diseases. Why? Because the normal microbial constituents are an important contributor to tissue homeostasis in the oral cavity. These usually benign populations that colonize all the surfaces of the mouth establish shortly after birth, persist throughout the individual's lifetime, and generally are compatible with oral health. Unless there is significant perturbation from an adverse environmental stress—such as increased carbohydrates in the diet or reduction of salivary flow, both of which can lead to a sustained lowering of the pH of plaque—there is little or no obvious damage to the colonized tissues. So, the microbes of the oral cavity do not necessarily have an adverse effect on the health of the individual nor is it predetermined that these organisms will eventually cause disease. In fact, there are many reasons that the normal microbial composition of the body is believed to have major health benefits for the host. Not surprisingly then, the concept of stabilizing a microbiota that is compatible with health is more and more frequently being embraced as a way to maintain oral health.

Major challenges facing oral health researchers are how to foster a healthy microbiota and how to prevent the ecologic shifts seen during disease development in the oral cavity, but the benefits to perseverance and development of new preventive and treatment strategies for oral disease will be great. Hopefully, as you make your way through the following chapters, you will gain an appreciation for the complexity of the oral environment, the microbial inhabitants of this environment, and the host responses to disease and you can begin to envision how to exploit this knowledge to improve overall oral health.

KEY POINTS

The three primary domains of life are the *Eukarya*, which encompass all eukaryotes, and the *Bacteria* and *Archaea*, which are the prokaryotes.

Bacteria almost uniformly can be divided into two major classes based on the Gram stain reaction. Gram-positive bacteria have only a single cell membrane and a thick, highly cross-linked cell wall. Gram-negative bacteria have a cytoplasmic and outer membrane and a thin, less highly cross-linked cell wall found in the periplasm between the inner and outer membranes.

A variety of bacterial factors that are relevant to the pathogenic potential of bacteria are located on the surface of the cell, including the LPS of gram-negative organisms, LTAs of gram-positive bacteria, pili, fimbriae, flagella, capsules, and S-layers.

Bacteria divide by binary fission. Replication of the circular chromosomes of bacteria is initiated at a single origin of replication and proceeds bidirectionally to a single terminus. The growth rate of some organisms can be very fast, with cells dividing as rapidly as every 15 min. Many other bacteria grow more slowly, with generation times measured in tens of hours.

The mouth has a variety of habitats, and the bacteria in the mouth exist as adherent biofilms. The biofilm lifestyle facilitates diversity in the population and allows the establishment of metabolic communities, the members of which cooperate to convert the complex constituents of saliva and the diet, such as glycoproteins, into substances that can be used for efficient growth and energy generation. Antagonistic interactions, such as acid production by species that inhibit the growth of acid-sensitive organisms or the overt production of antibiotics by certain species in oral biofilms, also help dictate the composition of dental plaque.

The vast majority of oral diseases arise from a perturbation in the homeostatic mechanisms of oral biofilms, generally driven by environmental changes, such as dietary changes or diminished salivary flow from radiation or hyposalivation-inducing drugs.

ACKNOWLEDGMENT

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The Immune System and Host Defense

P. M. Lydyard and M. F. Cole

INTRODUCTION

The human body has evolved a large variety of mechanisms to protect itself from invasion by pathogenic microorganisms. These host defenses (Fig. 1) include prevention of entry and dealing with the pathogens once they have penetrated the outer defenses of the body. Once a pathogen is inside the body, the immune system must recognize the pathogen as foreign and dispose of it. The overall immune system is divided into two separate but not mutually exclusive systems. The innate system, with which one is born, functions in the same way whether or not one has already encountered the pathogen. The adaptive system adapts to the first encounter with the pathogen (i.e., remembers it) so that subsequent responses are faster, of greater magnitude, and more specific.

The innate and adaptive immune systems work in concert both in recognition and disposal of pathogens. The main differences between the two systems are the speed and fine specificity of the pathogen recognition systems and the memory capacity of the adaptive system, although the boundaries between the two are becoming blurred as we learn more about them.

INNATE IMMUNITY

Microbes penetrating the physical barriers of the body (Fig. 2) pass across epithelial surfaces (mainly mucosal) to enter the tissue. Prior to entry, microbes encounter a hostile environment created by complex resident microbiota that serve to exclude extrinsic microorganisms (including pathogens) and a barrage of secretions from the epithelial and other cells. These include antimicrobial proteins (at most epithelial surfaces), enzymes (mouth, skin, and stomach), low pH (stomach), and mucus. Once in the tissues, a number of cells (Fig. 3) and molecules (Table 1) begin to function in early defense against the invading microbes.

Cells Involved in Early Defense

PHAGOCYTES

There are two main types of phagocytes or "eating" cells. Polymorphonuclear cells (polymorphs, or PMNs) are blood borne (mobile) and make up

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Prevention of entry	Physical barriers Creating an adverse environment	
Once inside the body		
Innate immunity		
First line of defense	Cells	Phagocytes (neutrophils and macrophages) Natural killer cells Mast cells (some link to adaptive immunity)
	Humoral factors	Acute-phase proteins Cytokines Complement proteins Antimicrobial proteins
Adaptive immunity		
Second line of defense	Cells	Lymphocytes and dendritic cells
	Humoral factors	Antibodies Cytokines

FIGURE 1 Summary of host defenses. doi:10.1128/9781555818906.ch2.f1

the majority of the blood granulocytes. In contrast, macrophages derived from circulating monocytes are "tissue-associated" phagocytes and are found in the tissues and organs of the body waiting to encounter invading microbes. On contact with the phagocyte, a susceptible microbe is rapidly ingested and killed inside the cell by antimicrobial proteins, degradative enzymes, and reactive oxygen and nitrogen species within lysosomes. The engulfment process can be enhanced (opsonization) by a number of molecules coating the microbe. Complement and antibodies coating the microbe act as opsonins (see below).

NK CELLS

Natural killer (NK) cells are large lymphocyte-like cells that recognize body cells that are infected with a virus or have become neoplastic. On contact with a virus-infected cell or tumor cell, they release substances that induce the target cell to commit suicide through the process of apoptosis. In this way, the virus particles are retained inside the cell and are killed following phagocytosis of the dying cell.

MAST CELLS

Mast cells are found throughout the body, particularly at epithelial sites and surrounding small blood vessels. Their large cytoplasmic inclusions contain pharmacologically active substances, such as histamine, that play an important role in the acute inflammatory response. They release their granular contents very rapidly on contact with activated complement proteins and through some kinds of antibodies (e.g., immunoglobulin E [IgE]) (see below). Basophils, found in the circulation, are thought to carry out a role similar to that of the tissue-bound mast cells.

There are a number of other cells that play a role in innate immunity. These include epithelial cells, which produce antimicrobial proteins (see

TABLE 1 Molecules of the innate immune system

Antimicrobial proteins
Complement proteins
Acute-phase proteins
Cytokines

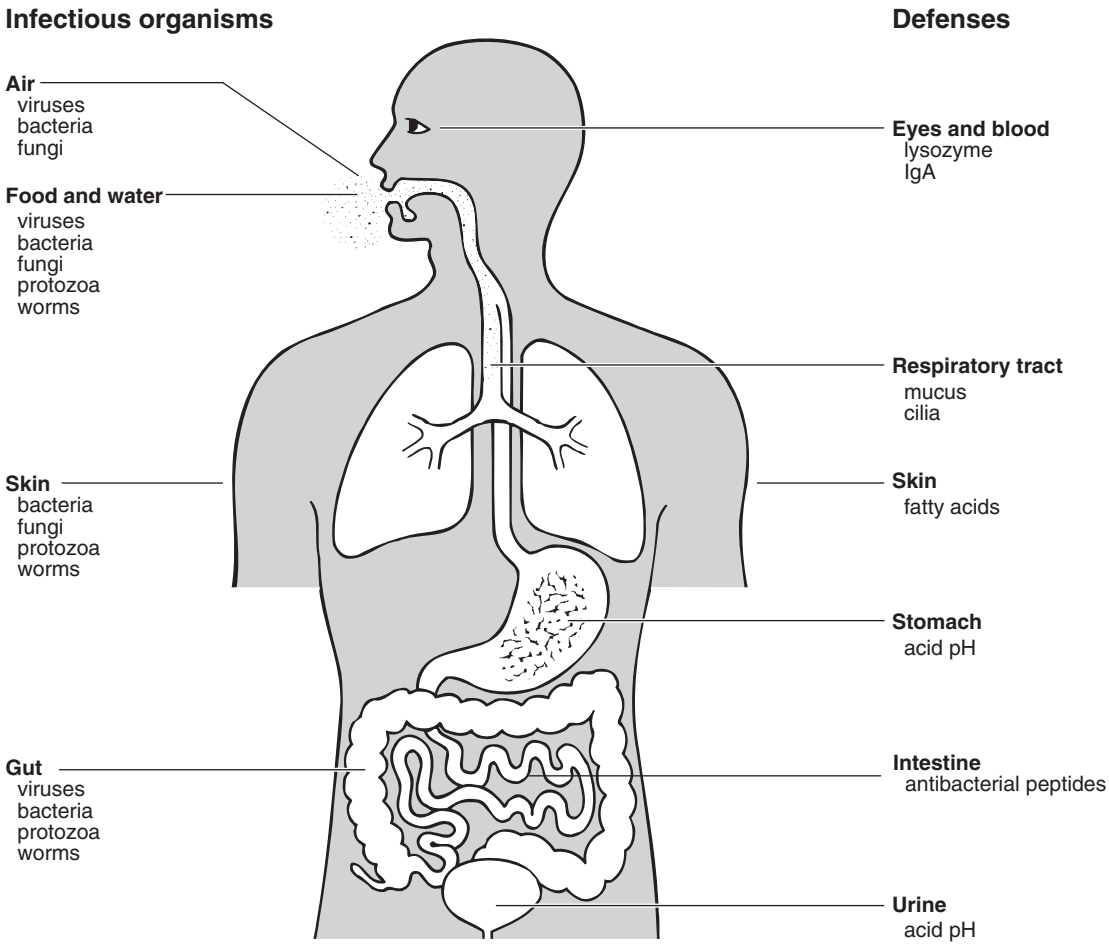

FIGURE 2 External defenses of the body. Humans are exposed daily to large numbers of microbes (mostly bacteria and viruses) through the air that they breathe and the food and drink that they take in. The skin also acts as a physical barrier through its dead external layers. Antimicrobial secretions by the skin and epithelial cells lining the gastrointestinal, genitourinary, and respiratory tracts, gastric acidity, and mucus and cilia in the respiratory tract all help prevent the attachment and entry of microbes. Blood and secretions contain the enzyme lysozyme, which kills many bacteria by attacking their cell walls. IgA is also an important host defense mechanism in secretions that bathe mucous membrane surfaces (see section on antibodies). Reprinted from J. H. L. Playfair and P. M. Lydyard, *Medical Immunology Made Memorable*, 2nd ed. (Churchill Livingston, Edinburgh, United Kingdom, 2000), with permission.
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below); eosinophils, which attack and kill parasites by releasing the cytotoxin, major basic protein; platelets, which on activation, release mediators that activate complement, leading to attraction of leukocytes; and erythrocytes, which bind and remove small immune complexes.

Molecules Involved in Early Defense

AMPS

Antimicrobial proteins (AMPs) are small (less than 60 amino acids) proteins (also called antimicrobial peptides) that have a wide spectrum of antimicrobial activity. They are produced by epithelial cells of the respiratory,

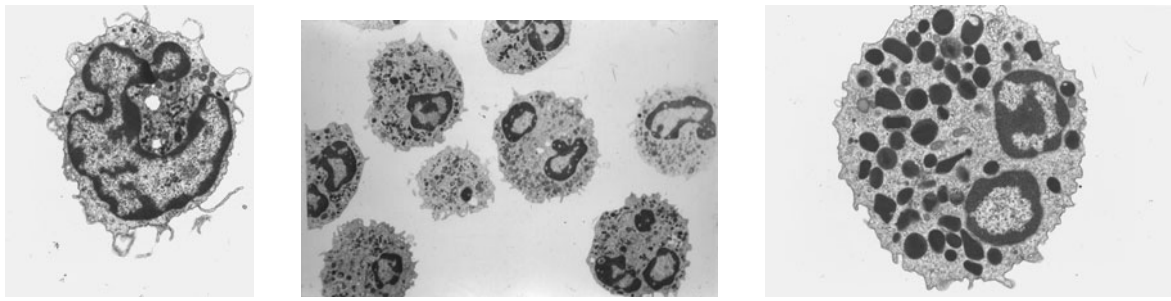


FIGURE 3 Cells involved in innate immune defense. The mobile phagocytic cells called polymorphs or PMNs (left) are present in large numbers in the bloodstream, while macrophages (middle) police the various organs and tissues of the body. Mast cells (right) are found throughout the body but particularly at sites lining mucosal surfaces (lamina propria) and are pivotal cells in the acute inflammatory response.
doi:10.1128/9781555818906.ch2.f3

gastrointestinal, and genitourinary tracts of the body to prevent entry of microbes. Phagocytic cells (both macrophages and neutrophils) also use AMPs to kill microbes that have been ingested following breaching of the epithelial cell barrier. They are active against both gram-positive and -negative bacteria, viruses, and fungi and are induced by microbes through surface pattern recognition receptors or through the effects of cytokines. The defensins and cathelicidins are the main human antimicrobial proteins.

COMPLEMENT

Complement consists of more than 30 different proteins, some of which are regulatory. Components of complement have several important roles in defense, including inflammation, phagocytosis, lysis of susceptible microbes, removal of immune complexes, and activation of B cells. Research in the past decade has also shown that complement cross talks with various other defense systems, including the Toll-like receptors (see below), aiming to orchestrate immune and inflammatory responses. Complement needs to be "activated" for it to function and, like blood clotting, comprises a "cascade" system (Fig. 4). The complement system can be activated directly by microbes (alternative and lectin pathways) and through antibodies (IgM and IgG) bound to the microbes (classical pathway) (see below). The main component of complement is C3, a major serum protein (about 1 g/liter) that is enzymatically split (in all three pathways), or "activated," to give rise to two components, C3a and C3b. When this occurs at the surface of a bacterium, bound C3b acts as an opsonin, resulting in enhanced phagocytosis. The released C3a together with C5a plays an important role in activating mast cells and attracting phagocytic cells (neutrophils—the mobile phagocytes) to the site of the invading bacterium. Later components C5 to C9 form a membrane attack complex that punches a hole in the bacterial surface, causing lysis and the bacterium's demise (Fig. 4).

ACUTE-PHASE PROTEINS

Acute-phase proteins are serum proteins that are generally increased following infection with a microbe. They are important not only in host defense but also in repairing the damage caused by the invaders. Table 2

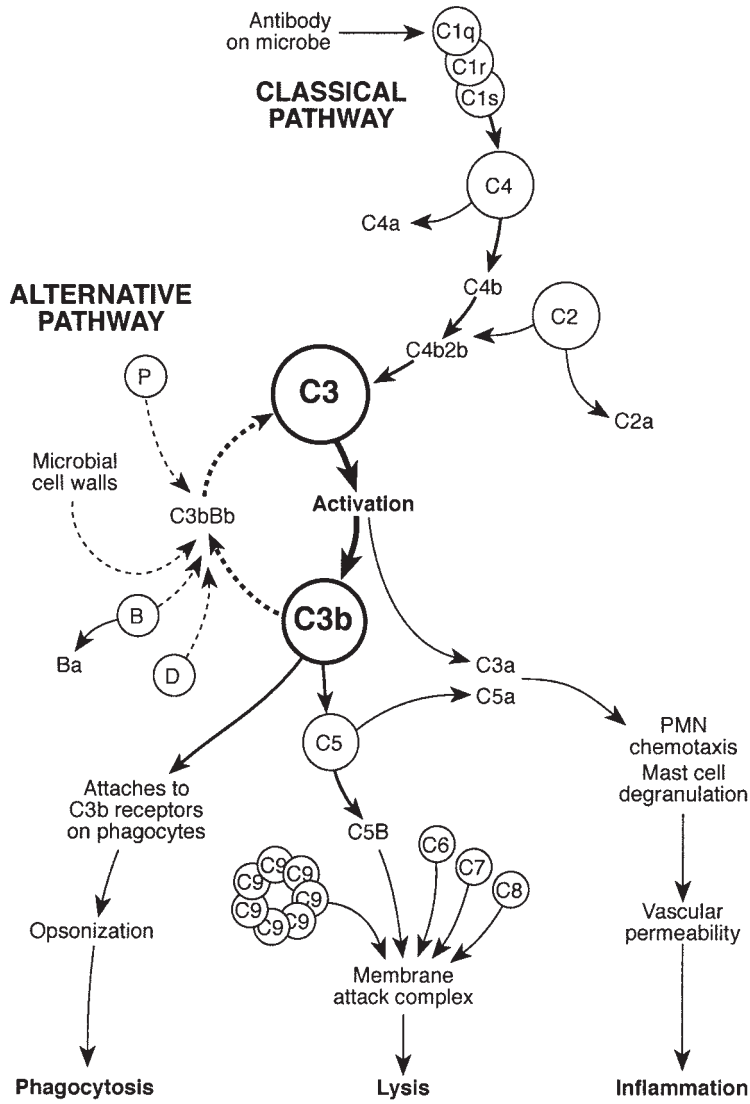


FIGURE 4 The complement system. This figure shows how the pivotal C3 complement component is cleaved into C3a and C3b by enzymes (convertases) through the alternative and classical pathways (activation of complement). The classical pathway involves antibodies while the alternative pathway ticks over slowly and is accelerated when it occurs on surfaces such as those of bacteria. The lectin pathway (not shown) is activated when mannose-binding lectin attaches to mannose residues on the surfaces of microorganisms, and it has its own convertase to cleave C3. Following cleavage of C3, a cascade of events occurs, leading to phagocytosis, lysis, and inflammation, as shown at the bottom of the figure. There are, of course, inhibitory factors that regulate the activation of complement and prevent it from occurring at the surface of the body's own cells. Reprinted from J. H. L. Playfair and P. M. Lydyard, *Medical Immunology Made Memorable*, 2nd ed. (Churchill Livingston, Edinburgh, United Kingdom, 2000), with permission. doi:10.1128/9781555818906.ch2.f4

shows the important acute-phase proteins and their functions. Their production by liver cells is induced mainly through cytokines secreted by macrophages following contact with the microbes, e.g., interleukin-1 (IL-1), tumor necrosis factor (TNF), and IL-6. Certain complement components are also acute-phase proteins.

TABLE 2 Acute-phase proteins

Protein	Function
C-reactive protein	Binds to phosphorylcholine and activates complement (binds to C1q)
Mannose-binding lectin	Binds to arrays of mannose and fucose on the surfaces of bacteria and viruses and activates complement via the lectin pathway; also acts as an opsonin
Complement components	Involved in chemotaxis, opsonization, and lysis (Fig. 4), e.g., C2, C3, C4, C5, C9
Metal-binding proteins	Removal of essential metal ions required for bacterial growth
α_1-Antitrypsin, α_1-antichymotrypsin	Protease inhibitors
Fibrinogen	Coagulation factor

CYTOKINES

Cytokines are small proteins (usually <15 kDa) that have a variety of functions, including producing fever (IL-1) and also enhancing acute inflammatory responses (TNF). These chemical "messengers," or communication molecules, also have important roles in the adaptive immune system (see below). Cytokines important in innate defense against viruses are the interferons (IFNs). This family of proteins, most of which are produced in nucleated cells following viral infection, have the ability to prevent the growth of viruses in other cells local to the infection. IFNs "interfere" with viral protein synthesis through inhibition of RNA translation. IFN-α and IFN-β are produced by most nucleated cells, whereas IFN-γ is made by NK cells and T cells of the adaptive system. A group of cytokines called chemokines functions to attract and activate cells, including lymphocytes, macrophages, and PMNs.

RECOGNITION

The important first step in the innate early defense response is to recognize that the invader is nonself or foreign. Unlike the very fine specific receptors that lymphocytes have to recognize microbial components (see below), many cells of the innate system (especially phagocytes) have receptors that recognize components of microbes that are seen as patterns (called pathogen- or microbe-associated molecular patterns [PAMPS or MAMPS]). The receptors that recognize PAMPS/MAMPS are termed pattern recognition receptors (PRRs). There are several classes of PRRs located either in the cell membrane or in the cytosol. In this way, they are ideally located to monitor what is going on outside the cell as well as inside the cell. The most studied to date are the Toll-like receptor (TLR) family. TLRs are transmembrane proteins and are also found in endosomes. The components of microbes recognized by the different members of the TLR family are shown in Table 3. Another type of transmembrane PRR is the C-type lectin family which comprises proteins that bind sugar motifs. Mannose receptors that recognize mannose molecules on the surface of bacteria (not present on self cells) are members of this family. Scavenger receptors are another group of membrane receptors that bind modified lipoproteins. Cytoplasmic PRR families include the NOD-like receptors (NLRs), RIG-like receptors, and the absence in

TABLE 3 Toll-like receptors: their cellular distribution and specificity[a]

Receptor	Ligands	Microbes recognized	Cellular distribution
TLR1	Lipopeptides	Bacteria	Monocytes/macrophages, DC
TLR2	Lipoteichoic acid, peptidoglycan, zymosan, lipopeptides	Both gram-negative and gram-positive bacteria, some fungi	Monocytes/macrophages, PMN, DC, mast cells, FDC
TLR3[b]	dsRNA	Viruses	Macrophages, DC
TLR4	LPS	Gram-negative bacteria	Macrophages, PMN, DC, mast cells, FDC
TLR5	Flagellin	Bacteria	Monocytes/macrophages, DC
TLR6	Lipopeptides	Bacteria	Monocytes/macrophages, mast cells
TLR7/8[b]	ssRNA	Viruses	Monocytes/macrophages, DC
TLR9[b]	CpG DNA	Bacteria	Monocytes/macrophages, DC
TLR10 (CD290)	Unknown		Plasmacytoid DC

[a]DC, myeloid and plasmacytoid dendritic cells; FDC, follicular dendritic cells; LPS, lipopolysaccharide; PMN, polymorphonuclear cells; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA.

[b]Intracellular TLRs found in endosomes.

melanoma 2 (AIM2) family. NLRs are known to sense gram-positive and gram-negative peptidoglycan fragments, flagellin, and bacterial and viral RNA. RIG-like receptors are mainly involved in the antiviral response, and AIM2 receptors detect intracellular microbial DNA. NLRs are also involved in the inflammasome, which is a caspase-activating complex in granulocytes essential for the processing of immature proinflammatory cytokines IL-1β, IL-18, and IL-33.

Recognition of pathogen-associated molecular patterns is not restricted to cellular receptors, since several soluble PRR-like molecules are able to recognize and bind to microbes, aiding in their demise. These include C-reactive protein, complement components, and others (see below). Complement recognizes some bacteria, for example, those lacking a capsule, and is directly activated (alternative and lectin pathways) via the bacterial surface (Fig. 4).

It is now appreciated that PRRs can also recognize noninfectious material that can cause tissue damage (damage-associated molecular patterns) and, as such, play a role in "sterile inflammation," that is, inflammation caused by, for example, trauma, inhalation of asbestos and silica, gout (uric acid), and amyloid formed in the central nervous system.

ACUTE INFLAMMATION

The archetypal innate immune response is acute inflammation, and it occurs very rapidly following infection. The function of this response is to rapidly bring blood-borne host defenses to bear on the invader. The acute inflammatory response is illustrated in Fig. 5.

THE LYMPHOID SYSTEM

The innate immune system operates rapidly and frequently eliminates the invading organisms. However, usually the second level of defense—the adaptive immune system—is also alerted and may be called into play in case the innate immune system is overrun. The lymphocytes are the main players of the adaptive system and are found in specialized lymphoid tissues and in the bloodstream. The total mass of lymphoid tissues in an adult is about the size of a soccer ball. There are several kinds of lymphocytes

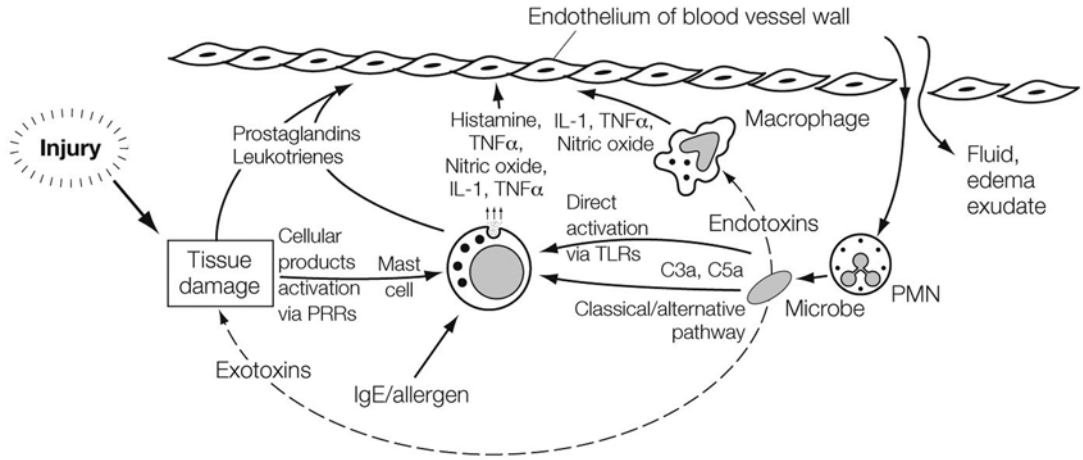


FIGURE 5 The acute inflammatory response. Acute inflammation is a response to cell injury and is a sequence of events that is similar whether the injury is caused by invading microbes or by physical injury. Thus, direct damage by injury or via exotoxins produced by microbes leads to a release of mediators (e.g., prostaglandins and leukotrienes) which, like mast cell mediators (e.g., histamine), increase vascular permeability and vasodilation. The pivotal cell in the acute inflammatory response is the mast cell. Following direct contact with microbes through pattern recognition receptors, microbial activation of complement pathways (alternative, classical, and lectin), and via IgE-allergen complexes, mast cells release an explosive exocytosis of pharmacological mediators such as histamine. Neuropeptides can also stimulate mast cells to release mediators. TNF-α is also released by mast cells, but this is not an immediate response. Microbial endotoxins activate macrophages to produce IL-1 and TNF-α, which have vasodilatory properties. The outcome of the copious local mediator release is loosening of endothelial tight junctions, increased adhesion of PMNs and monocytes, and their migration into the surrounding tissues where they come into contact with and are able to phagocytose the microbes. Fluid containing fibrinogen and antibodies, etc., is released into the area from the bloodstream, and this edema protects the damaged area during repair. Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Bios Scientific, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f5

that are adapted to carry out specific functions. They recirculate around the body and recognize pieces of the foreign microbes (called antigens) through specialized receptors that differ from those of the innate immune system (see below).

Lymphocyte Heterogeneity

The two major lymphocyte populations are T cells, which are produced in the **T**hymus, and B cells, which are produced in the **B**one marrow. In the bloodstream, both B and T lymphocytes are small cells with a thin rim of cytoplasm and cannot morphologically be differentiated from one another. B cells develop into plasma cells, which are specialized to produce antibodies to protect against microbes that live outside body cells (extracellular). On the other hand, the T cells (helper and cytotoxic T cells) have evolved to protect against microbes that live inside cells, such as viruses and certain bacteria (intracellular). The mechanisms by which the lymphocytes carry out these functions are described later. The different lymphocytes can be distinguished from one another by the surface molecules that are important to their function. Further subpopulations of the

TABLE 4 Lymphocyte markers

Cell type	S-Ig	TcR	CD3	CD4	CD8	CD19/20	Cytokine(s) produced
B lymphocytes	+	–	–	–	–	+	IL-1, IL-10
T lymphocytes							
Th1	–	+	+	+	–	–	IL-2, IFN-γ
Th2	–	+	+	+	–	–	IL-4,-5,-13
Th17	–	+	+	+	–	–	IL-17
Tfh	–	+	+	+	–	–	IL-21
Tregs	–	+	+	+	–	–	IL-10, TGF-β[a]
Tc	–	+	+	–	+	–	IFN-γ

[a]TGF-β, transforming growth factor β.

T helper (Th) cells include Th1 and Th2, which are distinguished by the spectrum of cytokines that they produce, and T regulatory cells (Tregs). More recently, another subpopulation was identified, Th17, which produces IL-17 and is important in driving innate immune inflammation and immunity against extracellular pathogens (bacteria and fungi). Moreover, follicular helper T cells (Tfh) are a subset of Th cells that prominently express IL-21 and are engaged in the development and differentiation of B cells to antibody-producing plasma cells. Tregs have an important role in regulation of the immune response. The main "markers" identifying the different lymphocyte populations and subpopulations are shown in Table 4.

Lymphoid Organs and Tissues

There are around 1×10^9 to 2×10^9 lymphocytes per liter of blood (1×10^6 to 2×10^6/ml), which is 5 to 10 times fewer than the innate mobile phagocytes (the PMNs). The lymphocytes are also found in organized lymphoid tissues in the body. These are divided into systemic lymphoid organs and tissues and lymphoid tissues associated with mucosal areas of the body that line the three main tracts (digestive, genitourinary, and respiratory). These tracts are the major sites of entry for microorganisms into the body, so it is not surprising that greater than 50% of the lymphoid tissue is found associated with the mucosal epithelial surfaces. Figure 6 shows the distribution of lymphoid tissues within the body.

Lymph nodes range in size from 1 to 10 mm and are found in superficial and deep locations within the body. T and B lymphocytes are located at different sites within lymphoid tissues and associate with other cells (e.g., dendritic cells) that are important for lymphocyte function. Figure 7 shows the structure of a typical lymph node and its components. The secondary lymphoid follicles found in lymph nodes (and other lymphoid tissues) contain "germinal centers" of B lymphocyte foci proliferating in response to antigens. These are important in the development of memory responses to antigens. As part of the lymphatic system, lymph nodes are mainly localized at the junctions of lymphatic vessels where they act as "filters," trapping any microbes in the lymph and mounting an immune response to them.

The spleen has T and B lymphocytes localized in the white pulp (Fig. 8). The immunological function of the spleen is to filter microbes from the bloodstream and mount an immune response to them.

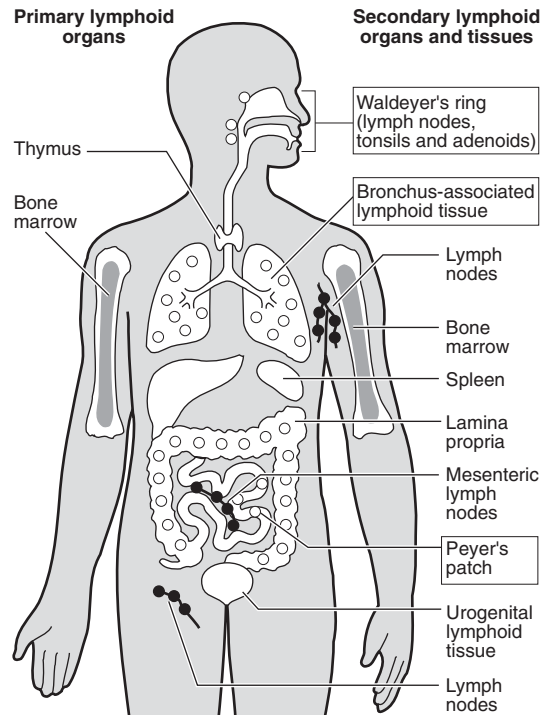


FIGURE 6 The major lymphoid organs and tissues in the body. T and B lymphocytes develop from stem cells in the primary (central) lymphoid organs—thymus and bone marrow—and they migrate to and function in the secondary (peripheral) organs and tissues. These secondary organs and tissues include the extensive mucosa-specific lymphoid tissues (MALT) (shown in boxes). Reprinted from J. H. L. Playfair and P. M. Lydyard, *Medical Immunology Made Memorable*, 2nd ed. (Churchill Livingston, Edinburgh, United Kingdom, 2000), with permission. doi:10.1128/9781555818906.ch2.f6

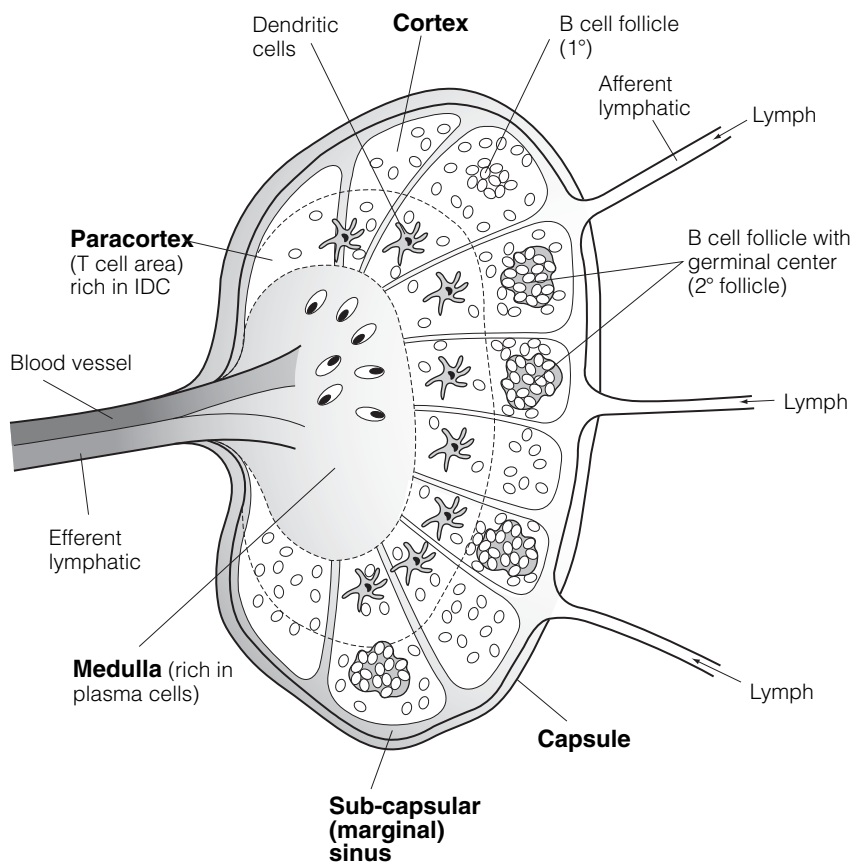


FIGURE 7 Lymph node structure. The outer cortical area of the node contains predominantly B lymphocytes in primary follicles. Some of these contain germinal centers (then called secondary follicles) that are the sites of B-cell proliferation, memory generation, class switching, and antibody affinity maturation. Dendritic antigen-presenting cells are found mainly in the cortical region. The paracortex is dominated by T cells and contains interdigitating dendritic cells (IDC). The medulla is rich in plasma cells. Lymph enters the node from the tissues via the afferent lymphatics and passes across the node into the medulla where it exits via the efferent lymphatic. During passage across the node, any microbe it contains is filtered out by an extensive network of phagocytic macrophages. The efferent lymphatics carry lymph back into the circulation via the thoracic duct. Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Garland Science, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f7

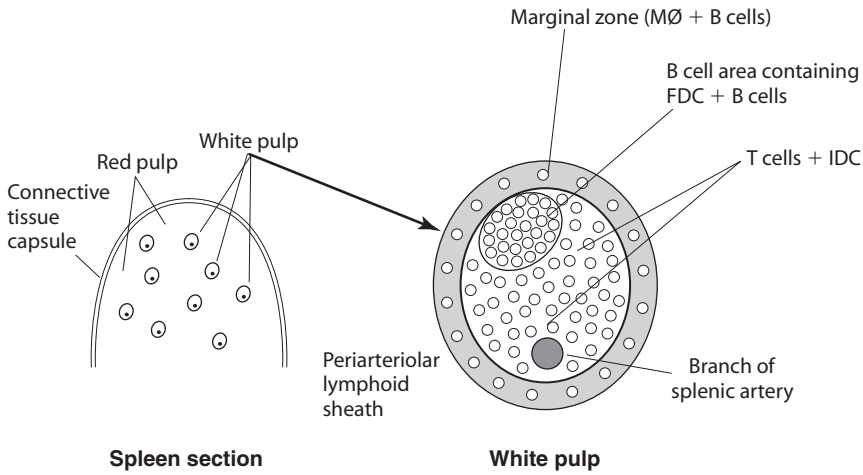


FIGURE 8 The spleen. Lymphocytes are located in the white pulp of the spleen, whereas plasma cells and macrophages are frequent in the red pulp. The white pulp consists of T cells and B cells surrounding a branch of the splenic artery; hence, it is referred to as the periarteriolar lymphoid sheath (PALS). The outer area of the PALS (the marginal zone) contains macrophages (MØ) and B cells. Primary and secondary follicles are also found associated with specialized follicular dendritic cells (FDC), important in the function of B cells. Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Garland Science, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f8

Lymphocytes are not sedentary but recirculate from the bloodstream to the spleen and lymph nodes and back again. Lymphocytes stimulated by antigen in the **M**ucosa-**A**ssociated **L**ymphoid **T**issues (MALT) tend to migrate back to these areas (Fig. 9). Through recirculation, lymphocytes are able to monitor the body for invading organisms through their antigen-specific receptors (see below). They do this through surface adhesion molecules that allow them to bind to specialized endothelial cells of blood vessel walls. On attachment to the endothelial cells, they are then able to migrate between them and gain entry into extravascular spaces. In the lymph node, these specialized endothelial cells (called high endothelial cells because of their tall morphology) are localized in the paracortical region.

ANTIBODIES: SPECIFICITIES, CLASSES, AND FUNCTIONS

Generalized Structure and Specificity

Antibodies have long been known as important molecules that protect against invading microbes. Their basic structure (Fig. 10) is two identical heavy and two identical light polypeptide chains held together with interchain disulfide bonds. Early studies on determining the structure of the molecule recognized two fragments, an $F(ab)_2$ part containing the variable part of the molecule and an Fc crystallizable fragment. The front end (N terminus) is the part that binds to the foreign antigen while the rear end of the two heavy chains is responsible for the secondary function of the molecule (see below). In many cases, the antibodies act as a

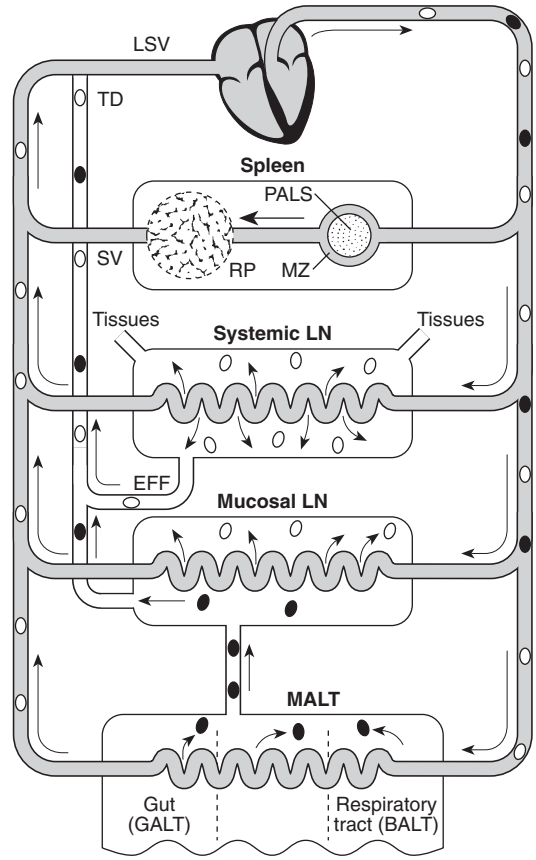
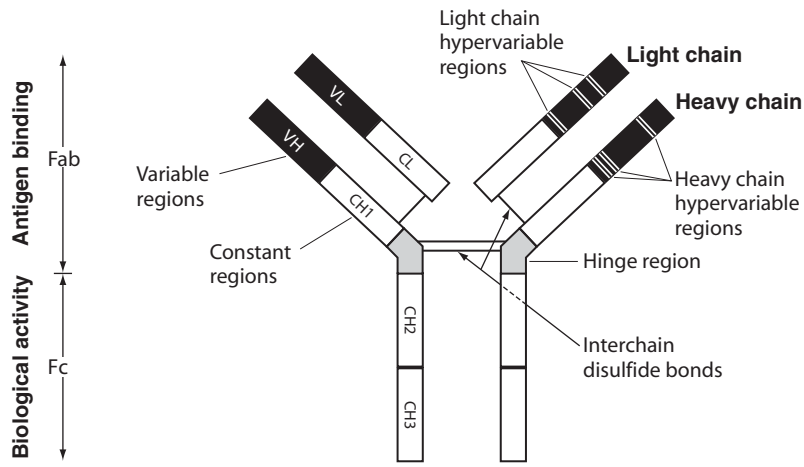


FIGURE 9 Lymphocyte recirculation. Lymphocytes (white ovals) enter the periarteriolar lymphoid sheath (PALS) through the marginal zone (MZ) and leave through the splenic vein (SV) of the red pulp (RP). Lymph node entry of lymphocytes can be from the tissues but is mainly from the bloodstream, facilitated by specialized capillary endothelial cells in the cortico-medullary junction. Lymphocytes leave via the efferent lymphatics (EFF) into the thoracic duct (TD) that empties back into the bloodstream via the left subclavian vein (LSV). Lymph collected in the right upper quadrant of the body empties into the right subclavian vein (not shown). Recirculation through mucosa-associated tissues is unique. Lymphocytes stimulated at one site, e.g., intestine, traffic via the draining lymph nodes into the bloodstream (black ovals) and not only back to the intestine but also to other mucosal surfaces, e.g., bronchus and genitourinary tissues. Reprinted from J. H. L. Playfair and P. M. Lydyard, *Medical Immunology Made Memorable*, 2nd ed. (Churchill Livingston, Edinburgh, United Kingdom, 2000), with permission. doi:10.1128/9781555818906.ch2.f9

FIGURE 10 Antibody structure. The basic antibody unit (here illustrated with the IgG molecule) is composed of two heavy and two light chains held together by disulfide bridges and a hinge region that gives the molecule flexibility when it binds to an antigen. The specific part of the molecule is the $F(ab)_2$ while the biological activity (e.g., binding to Fc receptors on phagocytes) and complement activating activity are located in the Fc region. Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Bios Scientific, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f10



flag to identify the foreign invader to immune cells and other molecules responsible for dealing with them. What makes an antibody specific is the different sequences of amino acids in the hypervariable part of the heavy and light chains. Each chain, heavy and light, has a domain structure defined by intrachain disulfide bonds. Light chains have two domains and heavy chains have four or five, depending on the antibody class (see below). Each of the two N-terminal domains of the heavy and light chains possesses two different sets of amino acids. The other domains of both heavy and light chains are constant for each antibody. The amino acids in the hypervariable region make up the antigen-binding site, each of which attaches to part of the antigen: the antigenic determinant, or epitope (Fig. 11). The reason the amino acids create different specificities is through their varied properties of charge, shape, etc. In fact, the interaction of the binding site with an antigenic determinant is purely physical (Table 5), and the strength of the interaction is called the intrinsic affinity. In practice, the binding of the two binding sites to two antigenic determinants is stronger than the sum of the two separate physical interactions by some thousand-fold. This is the functional affinity of the antibody for the antigen, and it is highest with polymeric antibodies, such as IgM, which has 10 potential antigen-binding sites. Because of the

TABLE 5 Physical forces between antibody molecules and antigenic determinants

Electrostatic forces
Van der Waal forces
Hydrogen bonds
Hydrophobic forces

FIGURE 11 Antibodies recognize antigenic determinants (epitopes). A molecule from a microbe will have several unique structures on it that will elicit an antibody and cellular response. An antigenic determinant (or epitope) is the smallest unit to which an antibody (Ab) or cell can bind. Antibody is shown here binding through its two binding sites to antigenic determinants shared by the same protein molecules on a microbial surface. Antibodies recognize the three-dimensional shape of an antigenic determinant, whereas a linear amino acid sequence is seen by T cells. Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Garland Science, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f11

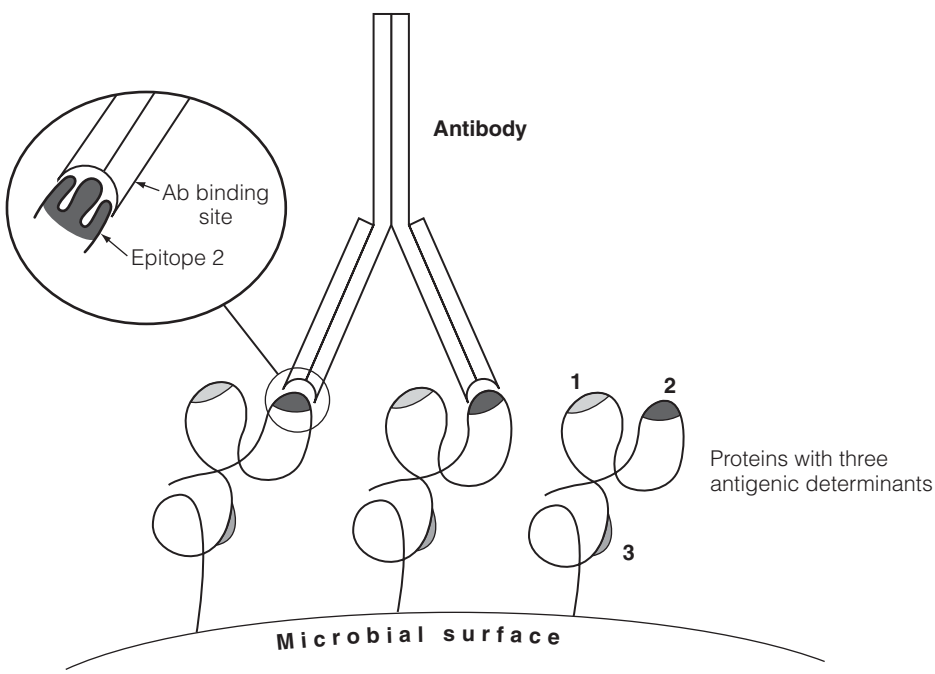


TABLE 6 Antibody classes

Antibody class	Heavy chain	Units	Molecular mass (kDa)	Concn in adult blood (mg/ml)
IgM	μ	5	900	0.4–2
IgG	γ	1	150	8–16
IgD	δ	1	180	0.03
IgA	α	2/1[a]	170–420	1.4–4
IgE	ε	1	190	Nanograms per milliliter

[a]Can exist as dimers or monomers (see Fig. 12).

structure of the binding sites, they are able to bind antigens in solution, giving rise to immune complexes. Immune complexes are usually cleared from the bloodstream following activation of complement (see below). In some situations, these immune complexes can be deposited in blood vessels where they give rise to vasculitis or glomerulonephritis. Immunopathology is the term given to tissue damage resulting from immune mechanisms.

Antibody Classes

Antibodies are called immunoglobulins because they are globular glycoproteins with immune function. There are five major classes of antibodies built on the structure described above. Their differences lie in the types of heavy chains in the molecule. There are 5 different heavy chains that can produce different antibody molecules (Table 6). In addition, different classes of antibodies are of different sizes based on how many of the basic units described above are assembled to form the molecule. For example, IgG exists as a single basic unit (a monomer) (Fig. 10) while IgM is composed of 5 basic units joined together by a small polypeptide chain called J chain (Fig. 12). With regard to the light chain, a given antibody can have one or the other of two kinds of light chain, κ and λ, but not both.

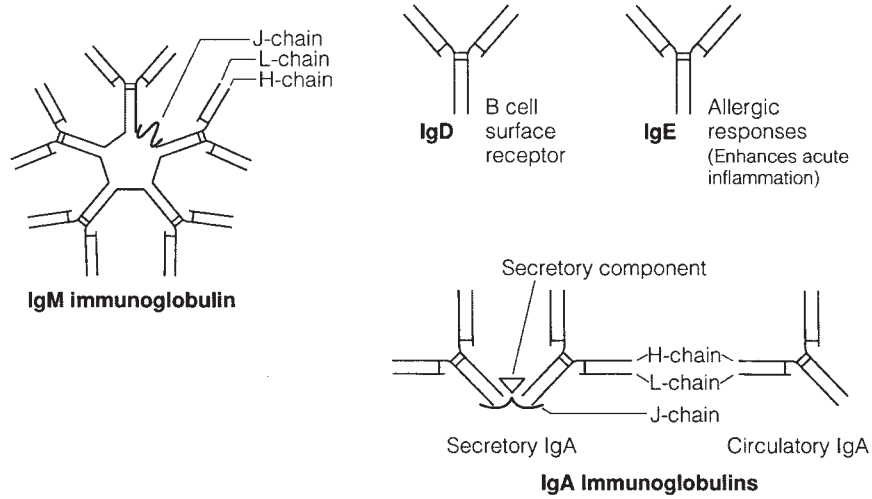


FIGURE 12 Structures of the different antibody classes. Secreted IgM has five antibody units, i.e., it is pentameric, each made up of 2 heavy and 2 light chains held together by a small polypeptide J chain. IgD and IgE (like IgG) are monomeric. Blood-borne IgA exists as a single antibody unit while secretory IgA consists of two or more antibody units, also bound by a J chain. These have a secretory component (polypeptide chain) attached when they are secreted across epithelial surfaces (see Fig. 13). Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Bios Scientific, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f12

Differential Properties of Antibodies

In general, there is a division of labor among the different classes of antibodies. Although there is overlap in their properties, they generally carry out their function at different sites and with different consequences for the microbe.

IgM is the first antibody to be produced in an immune response. It is mainly localized in the bloodstream (Table 6) and, being a large molecule, tends to stay there and protect the body against blood-borne microbes. IgM is very efficient in activating complement through the classical pathway (Fig. 4). It is generally believed that antibodies evolved as a specific way to target encapsulated microbes, as capsules protect microbes against complement activation via the alternative pathways. In a monomeric form, IgM occurs as an antigen receptor on the surface of B cells.

IgG is the predominant antibody class in the bloodstream and, like IgM, can activate complement when two molecules attached to a microbe are close enough together to bind C1q. IgG permeates the tissues of the body and is unique in that during gestation it is transported across the placenta into the bloodstream of the fetus to protect the newborn while its immune system is developing. Motifs on its γ heavy chain allow it to bind to phagocytes (and some other cells) that have an Fc receptor for IgG. There are four IgG subclasses that have slightly different functions from one another.

IgD is mainly restricted to the surface of B cells where, with IgM, it forms an antigen receptor (Fig. 12). Recent evidence suggests that IgD antibodies enhance mucosal immunity and bind to basophils and other innate immune cells where they bring about the release of immune-activating proinflammatory and antimicrobial mediators.

IgA is present as both a monomer and a dimer held together with a J chain. The dimeric form of IgA is transported across mucosal surfaces into the lumen of the major tracts—respiratory, genitourinary, and gastrointestinal—by binding to specialized receptors on epithelial cells at luminal surfaces (Fig. 13). Secretory IgA (S-IgA) is the principal type of antibody that protects mucosal surfaces. There are two IgA subclasses, IgA1 and IgA2, that have slightly different structures and functions. More IgA1 is found within the bloodstream, whereas more IgA2 is found at mucosal surfaces.

IgE is an antibody that has anaphylactic activity. It binds to mast cells through Fc receptors and amplifies the acute inflammatory reaction (Fig. 5). It is thought that IgE evolved to protect the tracts where entry of worms is common. Enhanced entry of blood-borne effector cells and molecules to sites of invading worms results from binding of IgE to specific antigenic determinants on the worm and activation of mast cells.

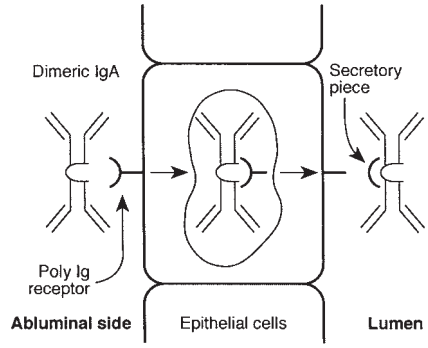


FIGURE 13 IgA is transported across epithelial surfaces. IgA in the lamina propria beneath the epithelium attaches to poly-Ig receptors and is transported via endosomes to the luminal surface. The IgA is released into the lumen with a piece of the poly-Ig receptor (secretory component) attached. This is thought to protect the IgA molecule against the damaging effects of proteolytic enzymes. Reprinted from J. H. L. Playfair and P. M. Lydyard, *Medical Immunology Made Memorable*, 2nd ed. (Churchill Livingston, Edinburgh, United Kingdom, 2000), with permission.
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Antibody-Mediated Protection against Microbes

ANTIBODY ALONE

Prevention of attachment of microbes and toxins to cells

Unless introduced into the tissues by a breach of the surface barriers of the skin and mucosae by trauma and insect bites, etc., pathogenic microorganisms must be able to bind to epithelial cells as a prerequisite for entry into the body. S-IgA antibodies that bathe the epithelial surfaces

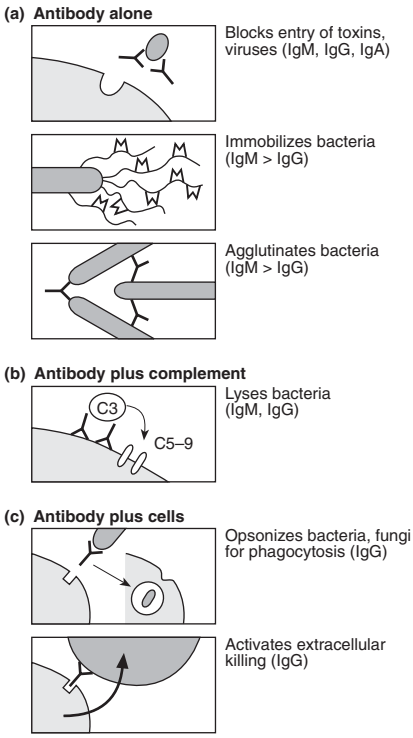

FIGURE 14 Antibody-mediated protection against infection. (a) Antibody alone. IgM and IgG can protect on their own by blocking attachment of viruses, bacteria or toxins. They can also attach to flagellae of bacteria and cause immobilization. In addition, IgM and IgG can bind to antigenic determinants on microbes and through their multiple binding sites (e.g., IgM has 10 binding sites) cause them to stick together, i.e., agglutinate. (b) Antibody with complement. Following complement activation by antibodies, components C5 to C9, the membrane attack complex, cause lysis of bacteria. Note, C3b also acts as an opsonin, to facilitate uptake by phagocytes (not shown here). (c) Antibody plus cells. Antibody, especially IgG, acts as an opsonin through binding to Fc receptors on phagocytes. Binding of IgG attached to large organisms (e.g., worms) through Fc receptors on phagocytes causes extracellular release of enzymes, etc. Reprinted from J. H. L. Playfair and P. M. Lydyard, *Medical Immunology Made Memorable*, 2nd ed. (Churchill Livingston, Edinburgh, United Kingdom, 2000), with permission.
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of the body lining the tracts provide an important protective mechanism by blocking the attachment of microorganisms at these sites (Fig. 14). Viruses that traverse the surface barriers need to attach to specific surface molecules on cells to enter. Antibody (especially IgG and IgM) recognizing the binding site on the virus prevents this (virus neutralization). In the same way, invading bacteria can be prevented from attaching to host cells. Furthermore, IgM and IgG antibodies can prevent pathogenic exotoxins produced by bacteria from binding to body cells.

Immobilization of bacteria by binding to flagella and agglutination

IgM, being pentameric, is very effective at immobilizing bacteria by binding to flagella and agglutination. IgG and IgA can also function in this way.

ANTIBODY ACTING WITH THE COMPLEMENT SYSTEM LEADING TO LYSIS

Complete activation of the complement system via the classical pathway leads to lysis of the bacteria via the membrane attack complex.

ANTIBODY WITH CELLS

Opsonization: making microbes more palatable to phagocytes

IgG antibodies binding to microbial cell surfaces can attach via their Fc part to Fc receptors on phagocytes. This enhances the phagocytic process. Complement component C3b on the surface of microbes, resulting from IgG or IgM antibody-induced activation of the classical pathway, also acts as an opsonin, since the phagocytes also have receptors for this complement component. IgA can also opsonize microbes through binding to Fc receptors but does not activate complement.

Antibody-dependent cellular cytotoxicity

Antibody-dependent cellular cytotoxicity occurs through extracellular release of molecules by effector cells, leading to death of an organism. The best example of this is protection against large worms that cannot be phagocytosed because of their size. Eosinophils attach to antibody (mainly IgG/IgE) coating the tough worm surface through Fc receptors. This causes the release of enzymes that damage the worm surface, leading to its demise. Phagocytes can then deal with broken up pieces of the dead worm.

RECOGNITION OF ANTIGEN BY LYMPHOCYTES

B Lymphocytes

To carry out their function, lymphocytes have to recognize microbial antigens through their specialized receptors. B lymphocytes use antibodies themselves as their antigen receptors. Each B lymphocyte has around 10^5 antibodies in its membrane, and each has the same hypervariable regions in its heavy and light chains, i.e., they have the same specificity. Each B lymphocyte has antibodies with different specificities, and there are probably around 10^8 or so different specific B lymphocytes. This large diversity of specific B lymphocytes is made possible by the random assortment of genes coding for the hypervariable regions during B-cell development.

Since B lymphocytes are specific, only a relatively small number (a few thousand) of B lymphocytes recognize foreign antigens from a given microbe, and only those that can bind the antigen proliferate and mature into plasma cells. This clonal selection by the antigen, also requiring T-helper cells (see below), results in production of a large number of cells derived from the original cell stimulated by antigen (Fig. 15). In addition to maturation into antibody-producing plasma cells, some of the same B-cell clones become memory cells that are important in protection against reinfection by the same microbe. As a result of the primary infection with the microbe, many more B cells (memory cells) with the same specificity will have been produced, resulting in a far larger antibody response to a second infection by the same microbe (Fig. 16). This is the basis of vaccination.

T Lymphocytes

ANTIGEN PROCESSING AND PRESENTATION

Since the function of T cells is to protect against intracellular microbes, their receptors (TCRs) (Fig. 17) allow them to recognize components of the microbe displayed on the surface of an infected cell. If TCRs were able to bind microbial antigens directly, they would be stimulated to respond against soluble antigens, which would be useless to combat intracellular

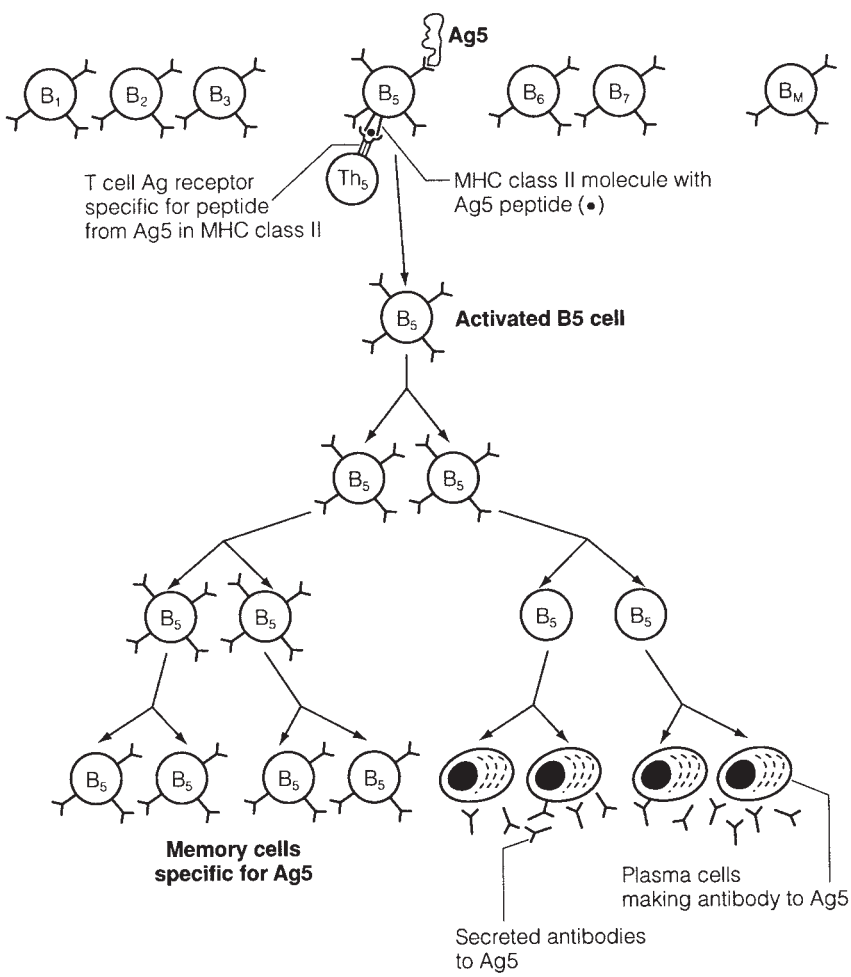


FIGURE 15 Clonal selection, memory cells, and plasma cells. Each B cell is specific for an antigenic determinant. With the help of Th cells that interact with antigenic peptides presented by the B cell in its MHC class II molecules (see below), a particular B cell attaching to its specific antigenic determinant will be activated to divide. Some of the proliferating clonal cells are destined to become plasma cells (antibody factories) with a specificity for the determinant originally recognized by the antigen receptor of the B cell. Another set of proliferating clonal cells become memory cells that are now more frequent than the original B cell and therefore able to provide a quicker response to a second encounter by the microbe. Note that a particular microbe might have a few thousand determinants recognized by different B cells, and shown here is the response of only one specific B cell. Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Garland Science, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f15

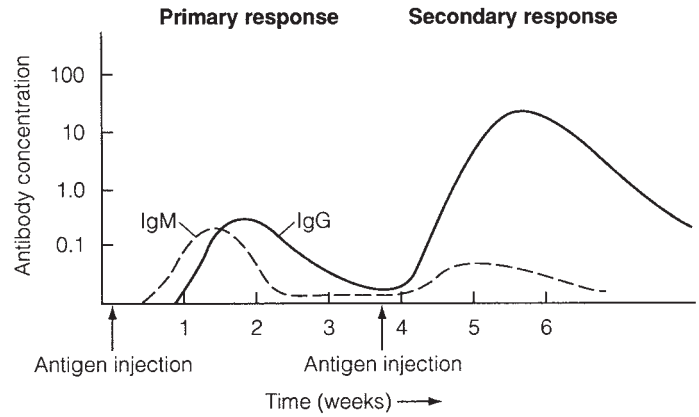
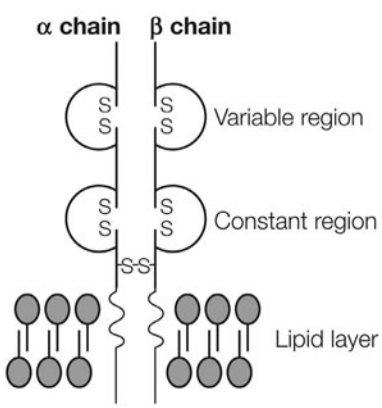


FIGURE 16 Antibody responses. On first encounter with a microbe, many specific B-cell clones will be activated to become plasma cells, first IgM and then IgG (primary antibody response). This takes time, due to the relatively few specific B cells available to recognize the microbe. The antibody concentration rises in the circulation but drops again as the microbe is cleared from the body and the antigenic stimulation of the B cells is removed. On second encounter with the same microbe, memory cells from individual B-cell clones generated in the primary response will be stimulated by their antigenic determinants and produce more antibodies more quickly than in the primary response together with even more clonal memory B cells (secondary response). Note that the primary antibody response is mostly IgM, while the memory response results in the production of IgG and classes other than IgM. Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Garland Science, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f16

FIGURE 17 The T-cell antigen receptor. T cells do not use antibody as their antigen receptor, but instead, most have two polypeptide chains—α and β—held together by covalent bonds and passing across the outer membrane of the T cell. Like antibodies, the N termini of the chains' outer domains have variable amino acid sequences that determine their specificity for antigenic determinants. A smaller number of T cells use two different chains as their receptor—γ and δ. Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Garland Science, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f17



microbes. Thus, TCRs are designed to recognize not only foreign microbial antigens but also self-components that present antigenic pieces of microbe to them (Fig. 18). These antigen-presenting molecules are the major histocompatibility complex (MHC) molecules—called HLA (or human leukocyte antigens for historical reasons) in humans. The HLA molecules are of two major kinds: class I molecules are found on all nucleated cells of the body, whereas class II molecules are normally restricted to dendritic cells, macrophages, and B cells. The distribution is necessarily different so as to direct the T cells, cytotoxic (Tc) and helper (Th) cells, to carry out their appropriate functions. Thus, Tc cells need to be directed to kill cells of the body infected with viruses, and this could be any nucleated cell. They are therefore programmed to recognize pieces of antigen, peptides of about 8 to 10 amino acids, presented with HLA class I molecules. Th cells help macrophages get rid of phagocytosed microbes growing inside them and recognize peptide antigens of 10 to 20 amino acids presented in HLA class II molecules.

For the peptides to assemble with the HLA molecules, they need to be processed by intracellular enzymes. Two different processing pathways determine which of the two HLA molecules display the microbial peptides for T-cell recognition. The pathway used is mainly determined by the mode of entry of the invading microbes. Thus, for viruses that enter cells and are present in the cytosol, the endogenous pathway is used to assemble viral peptides with HLA class I molecules (Fig. 19), whereas for those microbes taken in by phagocytosis, the exogenous pathway is used and the peptides are assembled with HLA class II molecules.

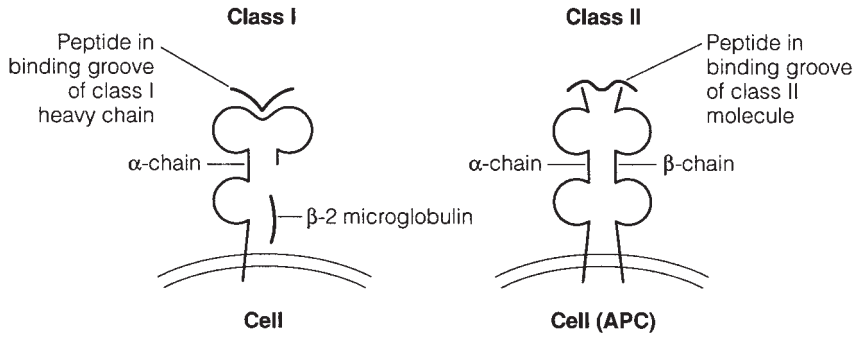


FIGURE 18 T cells recognize antigen presented by MHC class I and II molecules. MHC class I molecules are present on the surface of most nucleated cells of the body, and MHC class II molecules are found on specialized antigen-presenting cells (APC), such as dendritic cells and B cells. They are composed of two polypeptide chains. The α-chain of the MHC class I and both α- and β-chains of MHC class II molecules have amino acid sequences and tertiary structures that allow them to bind (through a binding groove) microbial antigenic peptides within the cell and move to the surface. Here they display the peptides to be recognized by passing T cells. Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Garland Science, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f18

FIGURE 19 Endogenous and exogenous antigen-processing pathways. Endogenous processing pathway: viruses infecting cells enter the cytoplasm where they begin synthesizing their own peptides. Some peptides are cleaved by proteolytic enzymes through proteosomes (not shown) and transported into the endoplasmic reticulum (ER) through specialized transporter molecules (TAP proteins). Viral peptides attach to MHC class I molecules and are transported to the cell surface where they are displayed to T cells. Exogenous processing pathway: microbes entering a cell through endosomes (by phagocytosis or pinocytosis) are digested in part through proteolytic enzymes. Endosomes fuse with vacuoles containing MHC class II molecules that pick up the antigenic peptides and are transported to the cell surface where they display the peptide to T cells. Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 2nd ed. (Garland Science, Taylor and Francis Group, Oxford, United Kingdom, 2004), with permission.
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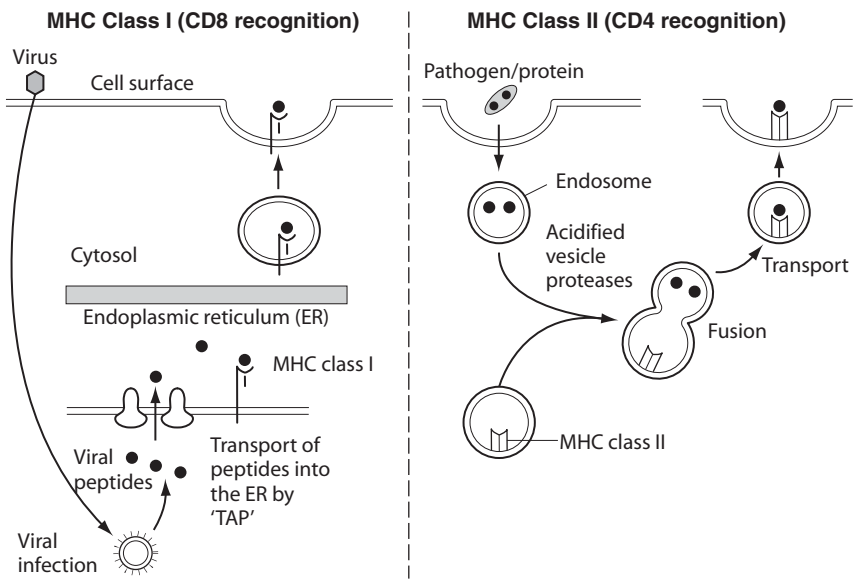


THE ADAPTIVE IMMUNE SYSTEM IN ACTION

Initiation of Adaptive Immune Responses: the Interface between the Innate and Adaptive Systems

Immature dendritic cells, like macrophages, are phagocytic and pick up microbes through some of their PRRs. They mature, and the processed microbial antigen is presented to antigen-specific Th cells (Fig. 20) that recognize peptides via surface HLA class II molecules on the dendritic antigen-presenting cells (Fig. 21). Specific Th cells also recognize peptides on macrophages in the same way. Interaction with antigen-presenting cells activates the T cells to proliferate in a clonal fashion, giving rise to more cells of the same specificity (memory cells), and to produce cytokines—the main function of helper T cells (cf. B-cell clonal activation). Two kinds of helper T cells help different cells in host defense mainly through production of different patterns of cytokines (Fig. 20). They both express CD4 molecules that interact with part of the HLA class II molecules not binding the microbial peptides (i.e., the nonpolymorphic component). Th1 cells are activated by peptides associated with macrophages and dendritic cells (Fig. 21). The production of IL-2 by Th1 cells stimulates proliferation of the T cells (it has autocrine function) while IFN-γ induces (i.e., conditions) the dendritic cells so they are able to present the microbial peptides with HLA class I molecules to cytotoxic $CD8^+$ T-cell precursors. Additional molecules on the surface of the Th1 cells (CD154) interact with their ligand on the surface of the dendritic cells (CD40) to enhance this conditioning process. Different ligands of dendritic cells can also suppress immune responses, for example, to food antigens entering the intestine.

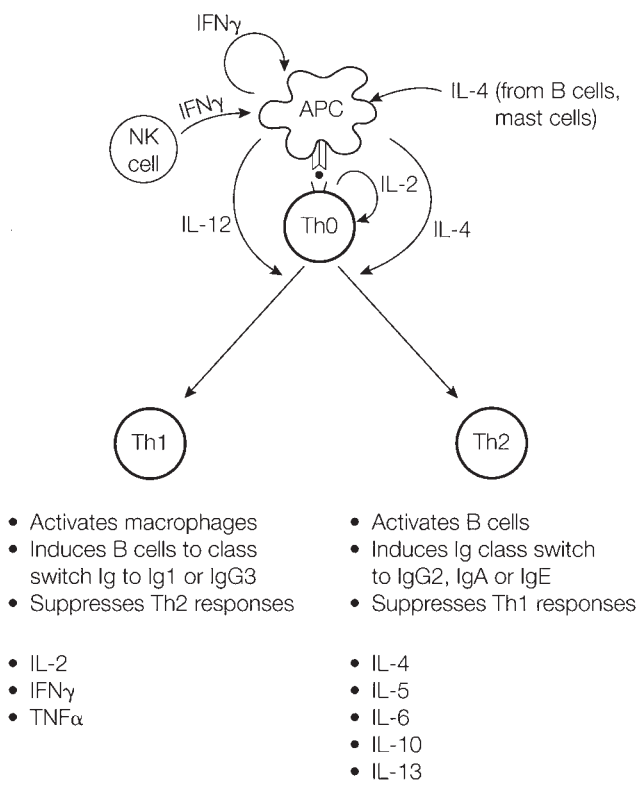


FIGURE 20 Two types of T helper cells. After a microbe has been taken up by a dendritic cell and antigenic pieces displayed in MHC class II molecules, autocrine IFN-γ or that produced by NK cells encourages the antigen presenting cell (APC) to produce IL-12 that induces the Th0 cell to become a Th1 cell. However in the presence of IL-4, i.e., from B cells or mast cells, the T cell is encouraged to become a Th2 cell. The main functions of these two populations and their cytokine profiles are shown in this figure. Whereas Th1 cells are primarily involved in cell-mediated immunity against intracellular microbes, bacteria, and viruses, Th2 cells are mainly involved in helping B cells to divide and class switch to making IgG2 subclass of antibodies and other antibody classes, i.e., IgA and IgE. Th1 cells also help in class switching to IgG1 and IgG3 antibodies. doi:10.1128/9781555818906.ch2.f20

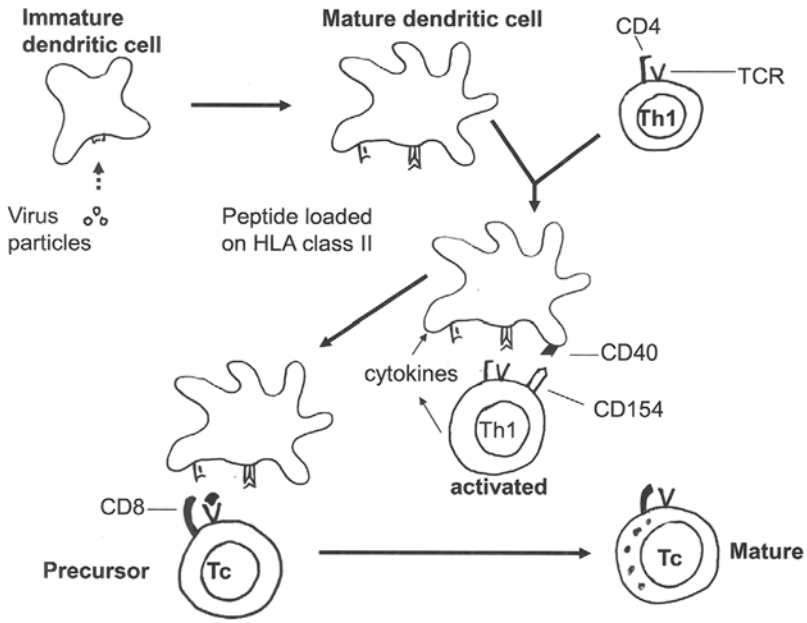


FIGURE 21 Dendritic cells at the interface between innate and adaptive immune responses. Viruses and other microbes are phagocytosed by immature dendritic cells (DC), and as they mature, they are able to present antigenic peptides through MHC class II molecules to Th1 cells. These Th1 cells become activated, and surface CD154 attaches to CD40 on the dendritic cells. This causes release of cytokines that condition the dendritic cell. This conditioning allows the microbial antigens to be processed through the endogenous pathway, and thus, some antigenic peptides are presented through MHC class I molecules to cytotoxic T-cell precursors that have CD8 molecules. These precursor cells, with the aid of other cytokines, e.g., IL-2 provided by the Th1 cells, are able to proliferate and develop into mature cytotoxic cells. Modified from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Garland Science, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f21

Most B Cells Require Help from T Cells

B cells can directly be stimulated to develop into IgM-producing plasma cells by antigens that have repetitive sequences recognized by the specific antigen receptors on B cells. Examples of such antigens are sugars that have a backbone with multiple hexose residues. However, for most antigens, T cells are required. B cells do not phagocytose large microbes but can pick up pieces of microbe or their products that attach to their specific antigen receptors and endocytose them. Whole viruses can also be taken into B cells in this way. Similar to phagocytosis by macrophages, entry of microbes or products into B cells via these receptors triggers the exogenous pathway of antigen processing, and the microbial peptides are assembled in HLA class II molecules on the specific B-cell surface. Th2 cells that have TCRs specific for the microbial peptides assembled with HLA class II molecules will attach to the specific B cells. Cytokines produced by the Th2 cells (IL-4, -5, and -6) help the B cell to proliferate and differentiate into plasma cells (Fig. 22). Interaction of CD154 on the Th2-cell surface with CD40 on the B-cell surface results in antibody class switching so that some of the B cells develop into IgG-producing plasma cells and

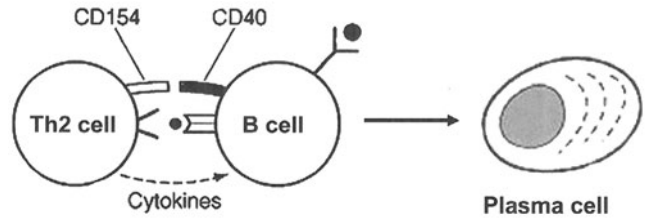


FIGURE 22 T-cell help for B cells. B cells pick up antigen through their specific antigen receptors. Following receptor-mediated endocytosis, the antigen is processed and peptides are displayed on the surface of MHC class II molecules to be recognized by antigen-specific Th cells. Activation of the Th2 cell results in cytokine production required for proliferation and development into plasma cells. In addition, CD154 is produced at the T-cell surface that interacts with B-cell CD40. This is necessary to induce class switching from IgM to the other antibody classes. Modified from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Garland Science, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f22

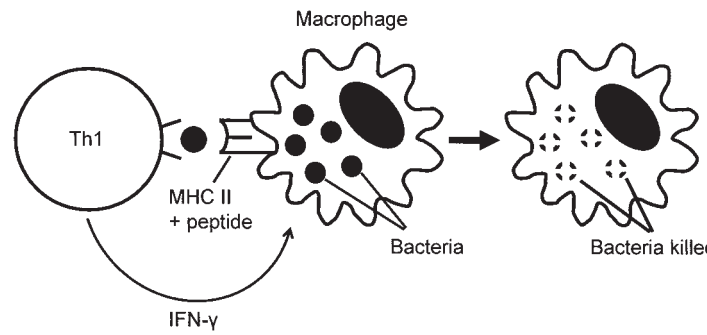
memory cells that, on second encounter with antigen, will develop into IgG-producing plasma cells.

Follicular germinal centers (Fig. 7) are the sites where B-cell proliferation, class switching, and memory cell production take place in lymphoid tissues. In addition, selection of B cells that have higher affinity for antigen takes place here, resulting in a progressive increase in antibody avidity in secondary and tertiary responses to the same antigen.

T-Cell Mechanisms in Host Defense: Cell-Mediated Immunity

We have already seen how Th2 cells help B cells to make antibodies and the way they work in protection against invading microbes and even large worms. Th1 cells directly interact with macrophages containing intracellular microbes that are proliferating inside them, e.g., mycobacteria. IFN-γ produced by them is a macrophage activation factor that stimulates the macrophage to kill the intracellular invader (Fig. 23). This is not always successful, and overstimulation of Th1 cells can lead to hypersensitivity

FIGURE 23 Th1-mediated activation of macrophages. Mycobacteria (e.g., *Mycobacterium tuberculosis* or *Mycobacterium leprae*) phagocytosed by macrophages are difficult to kill. However, antigenic pieces of the intracellular mycobacterium are processed, and small peptides are displayed on the surface to specific Th1 cells. Intracellular killing is enhanced by Th1 cells releasing IFN-γ that activates the macrophages. Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Garland Science, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f23



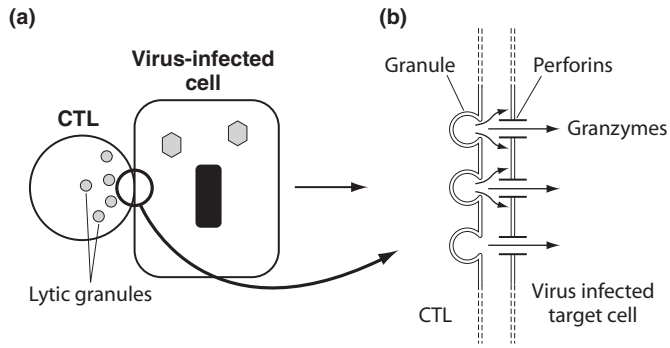


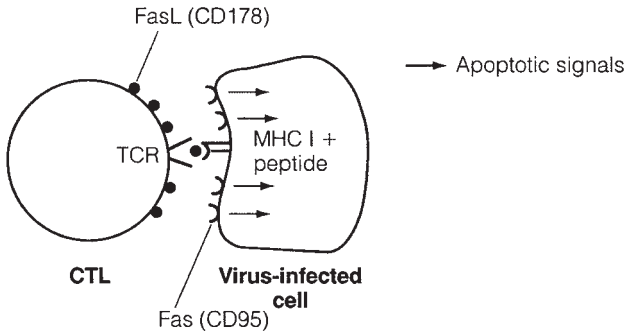
FIGURE 24 Perforin-mediated cytotoxicity by cytotoxic T cells. (a) Virus-infected cells displaying viral peptides in their MHC class I molecules are recognized by mature specific cytotoxic T cells (CTL) that, on contact, release lytic granules containing perforin that forms pores in the target cell membrane. (b) Enzymes (granzymes) pass into the target cell, causing the infected cell to commit suicide (apoptosis). Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Garland Science, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f24

and formation of granulomata (immunopathology). It is these granulomata that often cause death in tuberculosis, since they obliterate large areas of respiratory tissues.

Mature cytotoxic cells derived from cytotoxic precursor T cells interact with virus-infected cells that are presenting viral peptides through their HLA class I molecules. Killing of the infected cells occurs via two mechanisms, both designed to induce the cells to commit suicide (apoptosis).

1. Perforin-mediated cell death: Mature cytotoxic T cells (Tc) (also called cytotoxic lymphocytes, or CTL) have cytoplasmic granules containing perforin and granzymes that induce apoptosis (Fig. 24).
2. Fas-mediated apoptosis: Virus-infected cells increase a molecule Fas (CD95) on their surface; mature activated Tc cells have the ligand for Fas on their surface. Cross-linking of the Fas leads to apoptosis (Fig. 25).

FIGURE 25 FasL-mediated cytotoxicity by cytotoxic T cells. Mature cytotoxic T cells (CTL), upon attachment to target infected cells, become activated and express FasL. Interaction of this molecule with Fas on the surface of the infected cell also induces cell death by apoptosis. Reprinted from P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes in Immunology*, 3rd ed. (Garland Science, Taylor and Francis Group, Oxford, United Kingdom, 2011), with permission. doi:10.1128/9781555818906.ch2.f25



In this way, the cell shrivels up without release of the viral particles to infect further cells. The apoptotic cells are taken up by phagocytic cells and digested,with the viral particles being killed by effective intracellular killing mechanisms.

Regulation of the Immune Response

The immune system has to be "turned on" in an appropriate response to danger signals, fine-tuned, and "turned off" again when the threat has been removed. Danger signals come mainly from invading nonself microorganisms but also come from within, in the form of self antigens (autoimmunity), when immunological tolerance breaks down.

In the innate immune system, phagocytes normally only recognize self cells when these are damaged or dying. Natural killer cells do not normally go around killing self cells, since inhibitory receptors on their cell surface recognize specific surface molecules and prevent this. Likewise, inhibitory molecules on the surface of self cells prevent complement from being activated by them.

A number of mechanisms regulate the adaptive immune response. These include both the amount and physical structure of the foreign antigen. Thus, small amounts of antigen given over a period of time tend to produce better adaptive responses and very large amounts can produce unresponsiveness (tolerance). Aggregated antigens or those presented on alum particles (adjuvants) tend to produce larger responses. In addition, the adaptive response is regulated by cell interactions in both positive and negative ways. For example, Th1 cells regulate the expansion of Th2 cells and vice versa (through cytokines they produce). Tregs regulate the production of antibodies and T-cell responses as well as helping to prevent the breakdown of self tolerance in the periphery. Last but not least, there is increasing evidence that the neuroendocrine system also plays an important role in modulating immune responses.

KEY POINTS

Physical barriers, such as the epithelial lining of the main tracts of the body and the skin, together with antimicrobial proteins, e.g., defensins, and other secretions, such as lysozyme and short chain fatty acids, are the first line of defense against microorganisms.

Microorganisms that penetrate these barriers encounter:

- Phagocytic cells that ingest and kill microorganisms—PMNs circulate in the blood, and macrophages are tissue associated
- NK cells that attack virus-infected cells
- Mast cells that release inflammatory mediators
- Complement that is activated by microbes or antibodies and, through a cascade of enzymatic reactions, helps initiate inflammation and produces opsonins and components that induce lysis of bacteria by punching holes in their membranes
- Acute-phase proteins that can contribute to host defense and repair damage caused by infection
- Cytokines, messenger (communication) molecules that regulate immune activity; interferons also have antiviral activity

The adaptive immune response is the second level of defense.

Lymphocytes are the key cell type in the adaptive system. T cells are produced in the thymus, and B cells are produced in the bone marrow.

Lymphocytes recirculate in the blood and are also found in specialized lymphoid tissues such as the lymph nodes and spleen. Lymphoid tissue associated with mucosal surfaces is known as MALT.

Differentiated B cells (plasma cells) produce antibody, Y-shaped molecules that bind foreign antigens through hypervariable domains in their heavy and light chains at the front end of the molecule.

There are five major classes of antibodies based on the type of heavy chain in the molecule. IgA, IgG, IgM, IgD, and IgE all have different functional properties.

KEY POINTS

(Continued from previous page)
General functions of antibodies include preventing attachment of microbes and toxins, agglutination and immobilization of organisms, opsonization, activation of the complement cascade, and induction of extracellular release of antimicrobial enzymes.

B cells have antibody receptors of predetermined specificity. Binding of complementary antigen causes proliferation and maturation into antibody-producing cells (clonal selection). Some of each type of B-cell clone will become memory cells that can react more rapidly to infection with the same organism.

T cells have receptors that recognize microbial antigens but only when they are presented by an MHC molecule (called HLA molecules in humans). Cytotoxic T cells recognize and kill virus-infected nucleated cells by TCR binding to small antigenic peptides presented with HLA class I molecules. Helper T cells facilitate microbial killing inside macrophages and recognize slightly longer antigenic peptides (with HLA class II molecules) than those presented by HLA class I molecules. Differential processing inside cells directs peptides to either the HLA class I or II molecules.

The innate and adaptive systems interface with each other. Antigen-presenting cells such as dendritic cells and macrophages recognize antigens through their pattern recognition receptors. Processed antigens in conjunction with HLA class II molecules are presented to CD4 on Th cells. The T cells become activated and proliferate clonally to produce memory cells with the same specificity and also secrete cytokines. A subset of Th cells (Th1) produces cytokines that condition dendritic cells with HLA class I molecules that then stimulate $CD8^+$ Tc cells. In addition, cytokines are produced that stimulate intracellular killing by macrophages. Another subset of Th cells (Th2) produces cytokines that mainly stimulate B-cell proliferation and class switching of antibodies (from IgM to IgG and IgA). A newly identified subset known as Th17 plays a role in host defense against extracellular pathogens by orchestrating innate immunity and inflammation. There is a significant cross-regulation among the different T-cell subsets. This, together with the function of Tregs in influencing T-cell function and production of antibodies is important in the control (regulation) of immune responses.

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The Oral Environment

Frank A. Scannapieco

INTRODUCTION

Bacteria that reside in the oral cavity occupy a unique and fascinating habitat. The mouth is the only part of the body where hard tissues (the teeth) are naturally exposed to the external environment. Like barnacles attaching to a ship's hull, a diverse ensemble of bacteria firmly adhere to and grow on the teeth to form a complex biofilm known as dental plaque. The complexity of the oral microbial ecology is magnified by the fact that the mouth also possesses a variety of other surfaces, including the buccal and vestibular mucosa, hard palate, tongue, and the floor of the mouth, all of which provide unique habitats for microbial colonization. Considerable fluctuations in oral environmental parameters also occur, such as temperature, oxygen availability, pH, and variability in the composition and frequency of exposure to dietary constituents. Finally, the oral tissues are bathed in saliva, which provides physical cleansing by virtue of fluid flow and dilution effects as well as host immune and nonimmune defense factors that together have profound consequences for the microbial ecology. The goal of this chapter is to provide an overview of the environment in which oral bacteria live and to describe some host defense factors oral bacteria face and the mechanisms the bacteria use to overcome them.

GENERAL FEATURES OF THE ORAL ENVIRONMENT

Teeth

Uniquely in the mouth, the mineralized enamel surfaces of the teeth are exposed to the external environment. In a healthy person, the tooth emerges from the supporting tissues that cover the root of the tooth apical (toward the apex) of the cementoenamel junction; as such, only enamel is exposed within the oral cavity. The tooth itself is composed of four tissues: enamel, dentin, cementum, and pulp (Fig. 1). (The reader is urged to consult textbooks and reviews of oral histology for more information on the histology of these tissues. Some of these are listed at the end of this chapter.) Like bone, each of these tissues is formed from the mineralization of an organic matrix secreted by specialized cells during the process of development. This matrix is composed of proteins involved in

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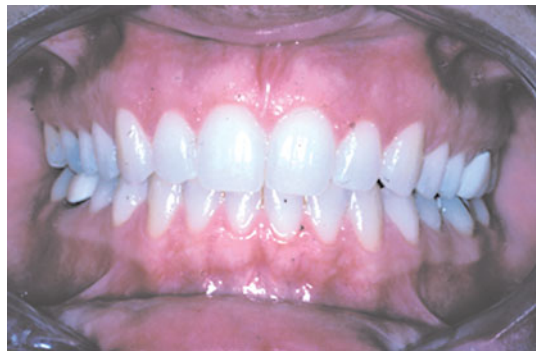

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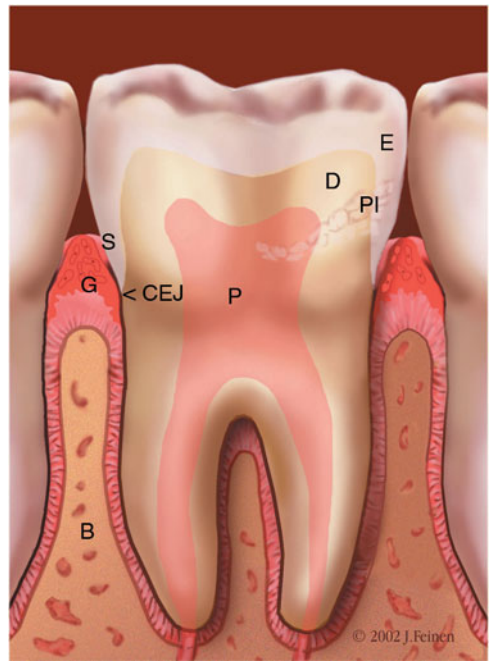

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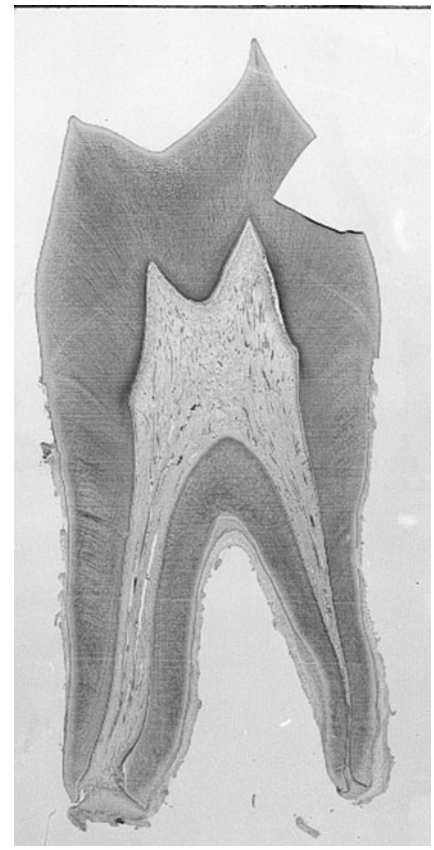

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FIGURE 1 The mouth and teeth in health. (A) Clinical picture of a healthy mouth. Note absence of deposits on the teeth and the pink gingival tissues. (B) Artist's depiction of the structures of the healthy tooth. E, enamel; D, dentin; B, bone; P, pulp; G, gingiva; Pl, plaque; CEJ, cemento-enamel junction; S, sulcus. Connective tissue fibers of the periodontal ligament span the space between the cementum and the supporting bone. (C) Axial section through maxillary first molar showing prominent pulp space surrounded by dentin. (Courtesy of H. E. Schroeder, Institute of Oral Structural Biology, University of Zurich, Zurich, Switzerland.)
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extracellular mineralization. They include amelogenins, tuftelins, ameloblastins, enamelins, and proteinases.

The crown of the tooth is composed of enamel, which is the most highly mineralized tissue in the body (containing 96% inorganic material). As the result of developmental processes, enamel displays a variety of structural features conspicuous on the surface of tooth crowns. For example, horizontal lines across the crown, called perikymata, can lend an undulation to the tooth surface. Each type of tooth (anterior, premolar, and molar) has unique patterns of fissures and grooves specific to each tooth type. These grooves can be shallow or deep, depending on

the individual. Enamel itself is composed of an assemblage of numerous rods that extend at roughly right angles from the dentoenamel junction toward the outer surface of the tooth. The average diameter of each rod is about 4 mm. Each enamel rod, itself the product of the ameloblast, a specialized cell responsible for the development of the enamel and tooth crown, is organized as arrays of hydroxyapatite crystals. The outline form of each keyhole-shaped rod is visible on a slightly demineralized enamel surface. Taken together, these diverse structures may have clinical significance insofar as they may subtly influence dental plaque development and susceptibility to dental caries and periodontal disease.

The surface of the enamel is covered by the pellicle, a film composed of salivary and other proteins. This pellicle plays an important role in the interaction of bacteria with the tooth surface and in preventing mineral loss from the tooth and is discussed in greater detail below.

Dentin, which is 70% mineral and 30% water and organic matrix, comprises the bulk of each tooth. It underlies the enamel and surrounds the tooth pulp. Dentin is not exposed in the oral cavity of a healthy individual. Dentin develops as the odontoblast secretes a set of matrix proteins at the mineralization front that becomes calcified. This matrix is composed of collagen and other proteins common to mineralized tissues such as osteopontin, acidic glycoprotein-75, dentin matrix protein 1, bone sialoprotein, decorin, and biglycan. The odontoblast also produces proteins unique to dentin, including dentin phosphoprotein and dentin sialoprotein. Certain oral bacteria, especially those implicated in the process of dental caries, can interact specifically with dentinal proteins such as collagen. This interaction may assist bacterial invasion of dentin during the process of dental caries.

As a result of development, the odontoblast produces a dentinal tubule that extends from the pulp to the dentinoenamel or cementoenamel junction of the tooth. In health, each tubule is filled with the odontoblastic process, an extension of the odontoblast. The odontoblast is also responsible for the formation of secondary or reparative dentin. During the process of dental caries, bacteria can invade the dentinal tubules to injure the odontoblast (for a more complete discussion of dental caries, see chapters 11 and 12). Reparative dentin is often produced in response to such chronic infection to isolate the bacteria from the pulp. Should the bacteria spread into the pulp, the resulting infection and inflammation are often the cause of serious pain and the possible spread of infection into the bone surrounding and supporting the teeth. In most cases, pulpitis requires root canal therapy or tooth extraction. Before the advent of antibiotic use, such dental infections could cause a severe and sometimes fatal infection.

The pulp space is filled with soft tissues that are enclosed in dentin. The pulp is the only nonmineralized tissue of the tooth. It contains several types of cells, including fibroblasts, macrophages, monocytes, and other immune competent cells, mesenchymal cells, and odontoblasts. The latter cells line the pulp-dentin interface and project the odontoblast processes into the dentinal tubules. The intercellular space within the pulp is composed of collagen fibrils and a variety of proteins and proteoglycans. The pulp also contains nerve fibers and blood vessels that provide sensory innervation, nutrition, and metabolite disposal. The pulp under normal circumstances is sterile but can be invaded by bacteria during the process of dental caries.

The Oral Soft Tissues (Periodontium, Oral Mucosa, and Tongue)

The gingiva surrounds the teeth to provide a seal that prevents microbial invasion into the underlying tissues. The surface of the gingiva is covered by stratified squamous keratinized epithelium. Underlying the epithelium is fibrous connective tissue (the lamina propria) that is directly attached to the underlying alveolar bone via collagen fibrils.

The gingiva forms a collar around the teeth that defines the gingival sulcus. The portion of the gingiva not attached to the tooth is called the free gingiva. The inner wall of the sulcus is the tooth surface. The outer wall is the sulcular epithelium that is continuous with the outer epithelial surface of the gingiva. The base (toward the tooth apex) of the gingival sulcus is continuous with the junctional epithelium that is directly attached to the tooth surface. It is the junctional epithelium that seals the external environment from the underlying tissues that support the teeth. The junctional epithelium attaches to the tooth surface by structures (hemidesmosomes) that resemble those that attach the epithelial basal cell membrane to the basement lamina. In health, the depth of the gingival sulcus rarely exceeds 2 mm. As an individual ages, the teeth slowly but continuously erupt and the gingival tissues may migrate slowly apically. The tissues that cover the tooth root can become exposed to the oral cavity environment as a result of physical abrasion of the gingival tissues (for example, by aggressive oral hygiene procedures) or as a consequence of the pathological process of periodontal disease that results in loss of the tooth's connective tissue attachment and supporting alveolar bone (see chapter 13).

The cementum and periodontal ligament are specialized tissues that surround and support the root surface of each tooth. Cementum is a mineralized tissue that overlies and is attached to the root dentin. Collagen fibers (Sharpey's fibers) from the periodontal ligament extend into the cementum. It is these Sharpey's fibers that are responsible for the attachment of the tooth root via the cementum to the periodontal ligament and the tooth socket of the alveolus. Cells within the cementum (cementoblasts) also provide reparative functions following trauma. The periodontal ligament is a layer of connective tissue that surrounds the tooth root between the cementum and alveolar bone. It is composed of cells (mostly fibroblasts, with some nerve cells, macrophages, and lymphocytes), connective tissues (primarily collagen), blood vessels, and interstitial fluids.

The tissues lining the buccal mucosa and vestibule, floor of the mouth, hard and soft palate, and tongue in many ways resemble skin from a histologic point of view. The surface of the oral mucosa is covered by epithelium, which is supported by an underlying connective tissue (or lamina propria). Interspersed throughout the oral mucosa are numerous minor salivary glands of the mucous type. Oral mucosa differs from skin in that the epithelium is not keratinized (except on the tongue, gingiva, and palate), and it is kept moist by saliva rather than by sebaceous oils and sweat. Passing through the oral mucosa are ducts of the major salivary glands (parotid, submandibular, and sublingual) as well as the minor glands.

The surface of the tongue is different from other oral epithelia in that it demonstrates a complicated surface, with a variety of structures including filliform, fungiform, and circumvallate papillae and lingual tonsils

that furnish numerous crypts, trenches, and other protected sites providing sheltered habitats for bacterial colonization.

All oral surfaces are covered with a layer of components mostly derived from the salivary glands. These oral pellicles serve to lubricate and hydrate the tissues and modulate the microbial flora. Of added significance to the microbiology of the mouth, the epithelial cells also express a variety of potential bacterial receptors, such as glycolipids, on their surfaces. As described below, the pellicle and cell surface proteins may provide adhesion receptors to which oral bacteria may attach. Epithelial cells also respond to bacterially induced inflammation by producing a variety of antimicrobial peptides, such as human beta-defensins, adrenomedullin, and calprotectin, that likely limit bacterial invasion of the soft tissues.

PHYSICAL AND HOST PARAMETERS AFFECTING ORAL MICROBIAL COLONIZATION

Temperature

The temperature in the mouth of a healthy person may vary considerably. For example, during a standardized drinking regimen of hot black coffee (72.5°C) followed by cold orange juice, a maximum intraoral temperature of 68.0°C and a minimum of 15.4°C were recorded. The maximal intraoral temperature differences between upper and lower extremes following such a regimen were measured to be 29.6°C at the base of a coronal restoration, 27.1°C on the facial surface of teeth, and 11.8°C within the root canal. Localized inflammatory processes may also modulate oral tissue temperature. The mean temperature of healthy gingival sulci has been measured to range from 33.7 to 36.6°C, depending on the tooth, whereas the temperature within a diseased periodontal pocket may be several degrees higher. These temperature fluctuations may influence oral microbes, since it is known that such fluctuations can influence the synthesis of global regulators of gene expression in bacteria known as transcriptional regulators (for example, sigma factors). These regulatory proteins can then direct the expression of the so-called heat shock proteins. It is thought that heat shock proteins may modulate the virulence of certain pathogens subjected to temperature stress, as occurs during the process of infection.

pH

It is well known that bacteria in dental plaque are capable of producing copious amounts of lactic and other organic acids from the metabolism of simple dietary sugars. This is discussed in greater detail in the context of the pathogenesis of dental caries in chapters 11 and 12. The classic studies of Robert Stephan in the 1940s illustrate the central role of dental plaque acid in the caries process. Those studies showed that the resting plaque pH of caries-free subjects is slightly alkaline (~7.2). However, the resting plaque pH of subjects with severe caries could be measured to be as low as 5.5. Stephan also found that plaques of caries-susceptible subjects challenged with a glucose rinse reduce pH levels from above 6 to well below 5 within 10 min, presumably due to the effects of bacterial metabolism of the sugar to organic acids. These shifts in dental plaque

pH have profound effects on plaque ecology. When a person frequently eats foods rich in carbohydrates (especially simple sugars such as sucrose), acid-sensitive bacteria are eliminated and acid-tolerant bacteria such as the mutans group streptococci and lactobacilli are enriched within the plaque microbiota. This ecologic shift likely causes an increase of the pH-lowering and cariogenic potential of plaque.

Acid-resistant (aciduric) bacteria such as oral streptococci and lactobacilli possess interesting mechanisms to maintain appropriate intracellular pH levels in the face of very low extracellular pH. For example, *Streptococcus mutans* has been shown to have a membrane proton-translocating ATPase, a pump that exports H^+ ions out of the cell. Bacteria such as *S. mutans* can also grow at low pH, a property not seen in other more acid-sensitive species. Other bacteria may also protect themselves from low pH conditions by producing compounds that can buffer acid. Thus, *Streptococcus salivarius* is capable of producing significant amounts of the basic compound urea.

Oxygen

The concentration of oxygen varies enormously in the mouth, depending on the location in which it is measured. Obviously, inspirated gases contain the same concentration of O_2 as does ambient air. However, its concentration rapidly diminishes as it approaches the oral surfaces. Early dental plaque is relatively rich in oxygen, but mature plaque is relatively anaerobic. More important to the colonization of the mouth by bacteria than the oxygen content of breathed air are the production and metabolism of highly reactive oxygen species by the bacteria and the host. They include the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). Such reactive species are highly toxic to bacteria, causing irreversible damage to membranes and proteins. The plaque biofilm, however, adjusts environmental conditions to allow anaerobic bacteria to flourish. These anaerobes also possess mechanisms to remove toxic oxygen, for example, enzymes such as superoxide dismutase, catalase, and NAD oxidase.

Mechanical Abrasive Forces

Inspection of the typical pattern of dental plaque on teeth reveals that plaques are not homogeneously spread over the teeth but appear to be localized to interproximal, buccal, and lingual tooth surfaces adjacent to the gingival margin. Typically, buccal and lingual surfaces of teeth at or above the height of contour are free of plaque, even in patients with very poor oral hygiene habits. This is due to the potent abrasive cleansing action of the movement of the lips, buccal mucosa, and tongue over the surfaces of the teeth. This leads to desquamation of the surface cells of the epithelia, explaining why plaque does not form to a great extent on mucosal surfaces.

Fluid Flow

It is clear that saliva is important in the control of dental plaque formation and in the pathogenesis of dental caries and periodontal disease. Subjects with compromised salivary function display enhanced plaque formation and have a greater risk for oral diseases such as dental caries

TABLE 1 Mean flow rates from various salivary glands[d]

Gland	Unstimulated flow rate[a]	Stimulated flow rate[a]
Parotid[b]	47.0 ± 1.2 μl/min/gland	619 ± 56 μl/min/gland
Submandibular[c]	58 ± 9 μl/min/gland	254 ± 25 μl/min/gland
Palatal	0.74 ± 0.35 μl/min	0.75 ± 0.30 μl/min
Labial	0.96 ± 0.55 μl/min	1.00 ± 0.39 μl/min
Buccal	2.64 ± 0.98 μl/min	2.52 ± 0.92 μl/min
Whole saliva	300 μl/min (range, 250–350)	1,500 μl/min (range, 1,000–3,000)

[a]Mean ± standard deviation.

[b]Data for middle-aged males.

[c]Data for young males.

[d]Information from B. J. Baum, p. 126–134, *in* D. B. Ferguson (ed.), *The Aging Mouth* (Karger, Basel, Switzerland, 1987); I. L. Shannon et al., *Saliva: Composition and Secretion* (Karger, Basel, Switzerland, 1974); and R. J. Shern et al., *J. Dent. Res.* **69:**1146–1149, 1990.

and periodontitis. One of the most important functions of saliva is the physical cleansing action of the fluid on the oral cavity.

The contribution of each gland to whole saliva is quite variable (Table 1). Normal flow rates for whole saliva have been calculated to be 0.3 ml/min unstimulated and ≥1.0 ml/min stimulated. Most of the saliva is secreted from the major glands, with minor glands contributing around 5% of the volume of whole saliva. Frequent swallowing combined with continuous salivary flow constantly replenishes the fluids in the oral cavity and promotes the dilution and clearance of acid and bacterial toxins from plaque into saliva and eventually away from the oral cavity.

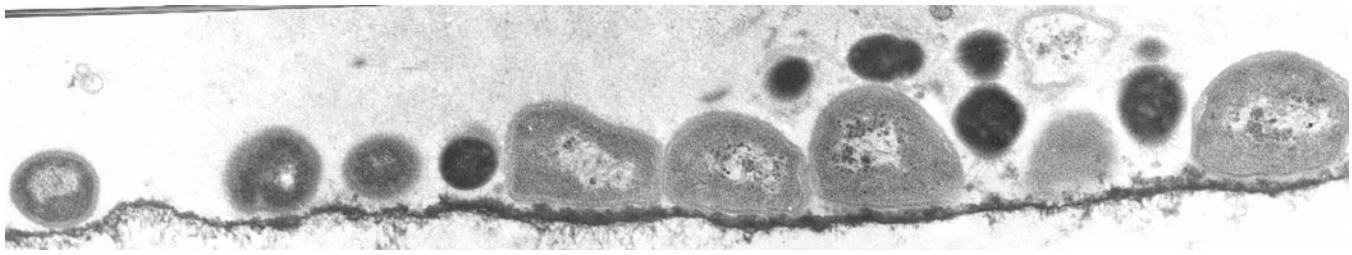
Host Age

A variety of studies have suggested that the mouth is susceptible to colonization by different bacterial species at different times over the course of the human life span. For example, early studies suggested that anaerobic bacteria such as *Porphyromonas gingivalis* do not colonize the mouths of children in appreciable numbers. These organisms begin to colonize the mouth around the time of adolescence. Recent longitudinal studies of transmission and acquisition of oral bacteria within mother-infant pairs found that the permanent colonization of the mouth by oral streptococci occurs during a discrete "window of infectivity" (around 9 months of age). This group of bacteria, which includes the mutans group streptococci, requires the presence of teeth or other nonshedding surfaces (for example, a denture) to colonize the mouth. Thus, these bacteria are found only transiently in the mouths of children before tooth eruption.

THE ORAL MICROBIOTA

Tooth (Dental) Plaque: Early Determinants of Plaque Formation

Dental plaque is a dense bacterial mass (also known as a biofilm) that is tightly adherent to the tooth surface. Bacterial attachment to the tooth is mediated by receptors in the thin salivary coating of the tooth, termed the acquired pellicle (Fig. 2). The pellicle and plaque matrices are composed of host-derived and bacterial products. The tightly adherent dental plaque should be differentiated from the so-called materia alba, a loosely



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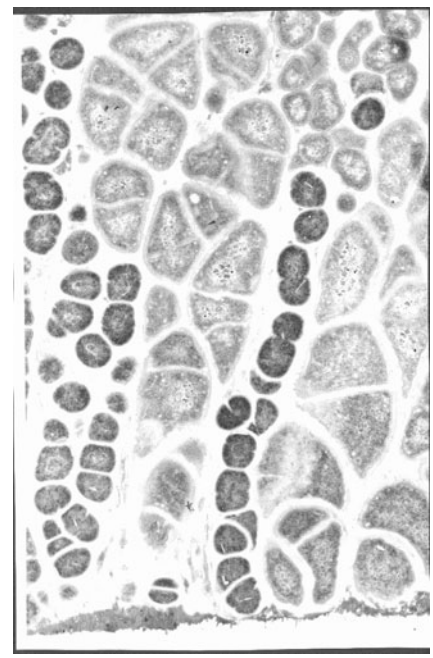

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FIGURE 2 (A) Transmission electron micrograph of the initial adhesion of cocci to supragingival enamel pellicle. The dark line of material is the salivary pellicle adsorbed to enamel. (B) Growth of the initial layer of adherent bacteria on the tooth to form columnar microcolonies. (Courtesy of H. E. Schroeder, Institute of Oral Structural Biology, University of Zurich, Zurich, Switzerland.)
doi:10.1128/9781555818906.ch3.f2

adherent soft, white material composed of food debris, bacteria, leukocytes, and desquamated oral epithelial cells that accumulates on the oral surfaces of an unclean mouth and that is easily removed from the oral surfaces by a strong water spray.

A critical determinant of bacterial colonization to any surface is the ability of the bacteria to adhere to that surface. Bacterial adhesion to host tissues may occur by a variety of mechanisms. One general mechanism involves nonspecific forces (e.g., ionic, hydrophobic, hydrogen bonding, and van der Waals) between the microbial and host surfaces. Another mechanism involves specific or stereochemical interactions between bacterial surface adhesins and the host components in pellicle. The latter interactions, similar to antibody-antigen or enzyme-substrate interactions, depend on recognition of molecular shapes between proteins. These interactions are highly specific and, when superimposed on the nonspecific forces, account in part for selective colonization of the host tissues. Specific adherent interactions are mediated by specialized bacterial surface proteins, or adhesins, that bind to receptors on the host surface. The receptor may be another protein or a carbohydrate (often attached to a glycoprotein or glycolipid).

Dental plaque maturation, the stage of plaque development following initial bacterial adhesion, is thought to depend to a large extent on the adsorption of bacteria in saliva to bacteria already adherent to the tooth. This phenomenon has been studied in vitro by allowing two or more strains of different species to interact, which results in clumping together of the two cell types. This can occur between two species in solution (coaggregation) or between one bacterium in solution adhering to an already adherent bacterium (coadhesion).

Plaque maturation also depends on bacterial growth and multiplication. Plaque bacteria derive their nutrition from metabolism of host products (for example, salivary proteins) or dietary constituents (for example, sugar or amino acids from foods) to grow and divide to form microcolonies on the tooth surface. Often, these bacteria produce products that can injure the host tissues. Thus, the bacteria in plaque can ferment sugar to form lactic acid, which can then cause demineralization of the tooth substance, resulting in dental caries.

Following thorough mechanical cleansing of the tooth (thus removing all plaque deposits), the first morphologically identifiable structure to immediately form on the tooth surface is the acquired enamel pellicle. Bacteria then adhere to the pellicle and proliferate. The first bacteria to adhere are usually cocci. With time, plaque becomes morphologically diverse, with filaments, flagellated and motile forms, and spirochetes all taking up residence in plaque. In all cases, an amorphous substance, or interbacterial matrix, surrounds bacteria in plaque. This matrix is composed of both bacterial and host products.

Histomorphologic, electron microscopic, culture, and molecular biological investigations of dental plaque have together provided important information on the microbiologic composition of plaque associated with health and a variety of disease states. It is now estimated that over 700 species have the potential to inhabit the human oral cavity. The bacterial taxa found in the mouth are listed in Table 2, and species commonly associated with oral health and various disease states are presented in Table 3. It should be noted that recent studies that use culture-independent molecular methods suggest that many of the genetic types are not cultivable.

This approach has also suggested that bacteria heretofore not recognized as human pathogens may be involved in the pathogenesis of oral disease. For example, members of the TM7 group, a recently described division of the bacteria known only from environmental 16S ribosomal DNA sequence studies, have been identified as prominent members of subgingival dental plaque. This phylotype was originally found in sludge wastewater treatment systems. Such findings suggest that many of the older experiments that identified only cultivable bacteria associated with health and disease conditions may not have provided an accurate picture of the microbial diversity associated with these clinical states.

The initial or early colonizers of plaque are primarily commensal species such as streptococci (*S. sanguinis*, *S. gordonii*, and *S. oralis*) and actinomyces. Oral health begins to deteriorate as plaque becomes colonized with other species. Gingivitis is defined as inflammation of the epithelial and connective tissues around the teeth but without loss of the connective tissue attachment or alveolar bone supporting the tooth. Plaque

TABLE 2 Some common bacterial taxa in the oral cavity[a]

Bacterial phyla	Representative species
Obsidian Pool OP11	
TM7	
Deferribacteres	
Spirochetes	*Treponema medium*, *T. denticola*, *T. maltophilum*, *T. socranskii*
Fusobacteria	*Fusobacterium naviforme*, *F. nucleatum* subsp. *animalis*, *F. nucleatum*, *Leptotrichia buccalis*
Actinobacteria	*Actinomyces naeslundii*, *A. israelii*, *A. odontolyticus*, *Rothia dentocariosa*, *Atopobium*, *Bifidobacterium dentium*, *Corynebacterium matruchotii*, *Propionibacterium propionicum*
Firmicutes	Class "Bacilli": *Streptococcus oralis*, *S. mitis*, *S. gordonii*, *S. mutans*, *S. sobrinus*, *S. sanguinis*, *S. parasanguinis*, *S. salivarius*, *S. intermedius*, *S. constellatus*, *S. anginosus*; *Abiotrophia adiacens*, *A. defectiva*, *Gemella haemolysans* Class "Mollicutes": Mycoplasma; *Solobacterium moorei* Class "Clostridia": *Catonella morbi*, *Dialister*, *Eubacterium brachy*, *E. sabbureum*, *Megasphaera*, *Peptostreptococcus anaerobius*, *P. micros*, *Selenomonas*, *Veillonella dispar*, *V. parvula*, *Eubacterium saphenum*, clone PUS9.170, *Filifactor alocis*, *Catonella morbi*, *Megasphaera elsdenii*, *Dialister pneumosintes*, *Selenomonas sputigena*
Proteobacteria	*Haemophilus parainfluenzae*, *Campylobacter rectus*, *C. gracilis*, *C. concisus*, *Neisseria mucosa*, *Desulfobulbus* oral clone R004, *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens*, enteric species (*Escherichia coli*), *Pseudomonas aeruginosa*
Bacteroidetes	*Porphyromonas gingivalis*, *P. endodontalis*, *Bacteroides forsythus*, *Prevotella denticola*, *P. oris*, *P. tannerae*, *Capnocytophaga ochracea*, *C. gingivalis*

[a]Information from W. E. C. Moore and L. V. H. Moore, *Periodontol*. 2000 **5**:66–77, 1994; B. J. Paster et al., *J. Bacteriol*. **183**:3770–3783, 2001; and J. M. Tanzer et al., *J. Dent. Educ*. **65**:1028–1037, 2001.

TABLE 3 Microbiota of the human mouth in health and disease

Health

Teeth
- Streptococci
- *Streptococcus mitis* bv. 1
- *Streptococcus gordonii*
- Veillonellae
- *Streptococcus sanguinis*
- *Streptococcus oralis*
- *Actinomyces*

Tongue
- *S. mitis* bv. 2
- *Streptococcus salivarius*

Disease

Dental caries
- *Streptococcus sanguinis*
- *Streptococcus oralis*
- Mutans streptococci
- Veillonellae
- *Streptococcus mitis* bv. 1
- *Streptococcus gordonii*
- *Actinomyces*
- Lactobacilli

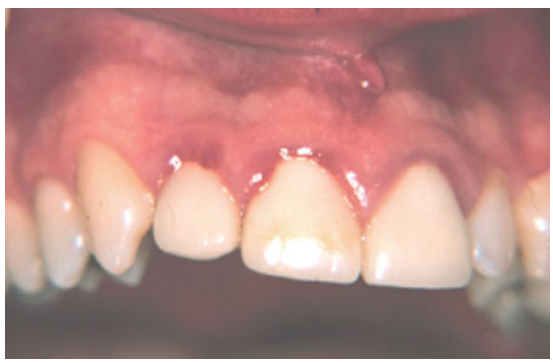
Gingivitis
- *Actinomyces naeslundii*

Gingivitis (*continued*)
- *Actinomyces viscosus*
- *Streptococcus sanguinis*
- *Fusobacterium nucleatum*
- *Selenomonas sputigena*
- *Haemophilus parainfluenzae*
- *Actinomyces israelii*
- *Streptococcus mitis*
- *Peptostreptococcus*
- *Prevotella intermedia*
- *Campylobacter sputorum*
- *Veillonella* species

Chronic periodontitis
- Clone I025
- TM7
- *Fusobacterium nucleatum* subsp. *animalis*
- *Atopobium parvulum*
- *Eubacterium* sp. strain PUS9.170
- *Abiotrophia adiacens*
- *Dialister pneumosintes*
- *Filifactor alocis*
- *Selenomonas* sp. strain GAA14
- *Streptococcus constellatus*
- *Campylobacter rectus*
- *Tannerella forsythia*

Chronic periodontitis (*continued*)
- *Porphyromonas endodontalis*
- *Wolinella recta*
- *Treponema* sp. strain 1:G:T21
- *Fusobacterium nucleatum*
- *Atopobium rimae*
- *Megasphaera* sp. clone BB166
- *Catonella morbi*
- *Eubacterium saphenum*
- *Gemella haemolysans*
- *Streptococcus anginosus*
- *Campylobacter gracilis*
- *Haemophilus parainfluenzae*
- *Prevotella tannerae*
- *Porphyromonas gingivalis*
- *Peptostreptococcus micros*

Localized aggressive periodontitis
- *Eikenella corrodens*
- *Capnocytophaga sputigena*
- *Aggregatibacter actinomycetemcomitans*
- *Prevotella intermedia*



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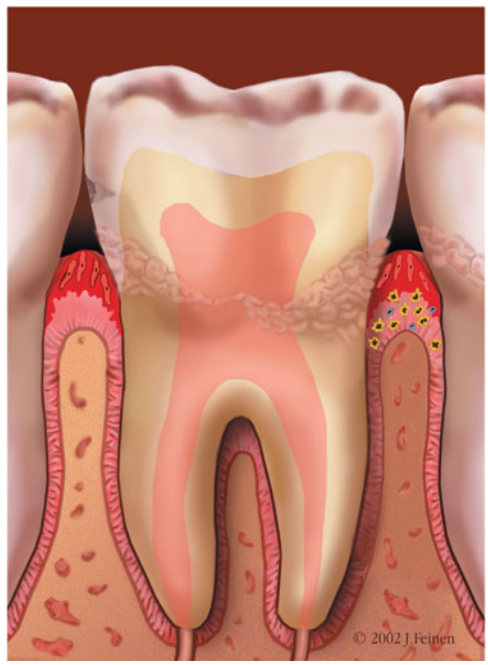


B

FIGURE 3 (A) Clinical presentation of gingivitis. Note red, swollen gingiva adjacent to crown restorations of maxillary anterior teeth. (B) Artist's depiction of gingivitis and initial interproximal caries formation. Plaque formation has become extensive. Gingival tissues are infiltrated with inflammatory cells (right gingival tissues). Incipient carious lesion is found in enamel under the left interproximal contact point.
doi:10.1128/9781555818906.ch3.f3

associated with gingivitis appears somewhat thicker than that from normal healthy sites (Fig. 3). Bacteria in deeper layers often appear lysed ("ghosts"). Mineralized deposits within these plaques are common (eventually developing into dental calculus or tartar). Greater proportions of filamentous and gram-negative bacteria (for example, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Selenomonas sputigena*, *Campylobacter sputorum*, and *Haemophilus parainfluenzae*) reside in these plaques.

Chronic periodontitis is defined as the loss of connective tissue and alveolar bone support of the teeth that is the result of both the direct activity of pathogenic bacteria and plaque-induced inflammation. Periodontitis-associated plaque, by definition, always extends onto the cementum. Plaques are as thick as or thicker than those associated with gingivitis (Fig. 4). Filamentous gram-positive and motile gram-negative bacteria and spirochetes predominate. Many distinct configurations of bacterial interactions occur in subgingival plaque, including interesting interactions between bacteria of different species resulting in "corncob" and "test-tube brush"-like formations (Fig. 5). These interactions occur between a central filamentous bacterium and numerous bacterial cells of a different species that adhere along the length of the filament. Anaerobic species such as *P. gingivalis*, *P. intermedia*, *Tannerella forsythia*, and spirochetes are common in the subgingival plaque of periodontitis patients. A transitional zone is noted between supra- and subgingival plaque, with filamentous and flagellar forms in greater proportions in subgingival

A B

FIGURE 4 (A) Clinical presentation of severe periodontitis. Note abundant plaque deposits that have, in many places, mineralized to form stained calculus (tartar). (B) Artist's depiction of severe periodontitis and dentinal caries. Plaque has become mineralized calculus. The action of plaque and of the host inflammatory response has resulted in severe alveolar bone destruction. Caries has penetrated the dentinal-enamel junction and is spreading through the dentinal tubules toward the pulp.
doi:10.1128/9781555818906.ch3.f4

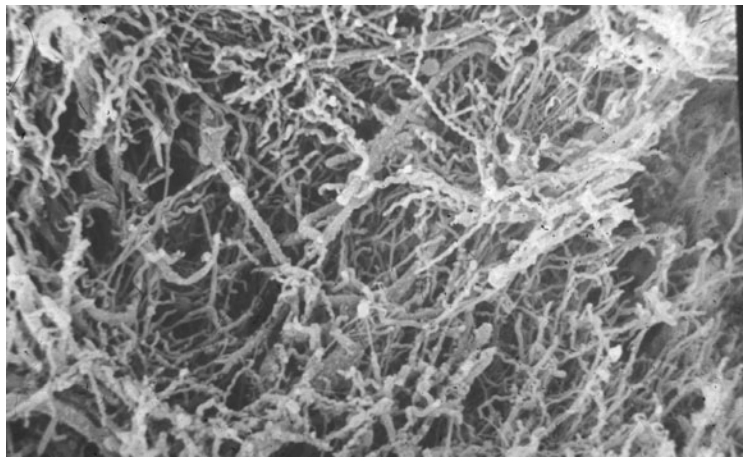
plaque. This plaque displays all of the features of a climax community with a stable, mature habitat.

The plaque of patients with aggressive periodontitis (such as localized aggressive periodontitis) appears much different from that seen in chronic periodontitis. The plaque associated with localized aggressive periodontitis is relatively sparse and morphologically simple. The predominant bacterial morphologic type appears to be small gram-negative coccoid forms. Clinical microbiological findings indicate that the primary pathogen associated with this disease is *Aggregatibacter actinomycetemcomitans*.

The dental plaque associated with acute necrotizing ulcerative gingivitis presents a unique morphology. It appears that spirochetes, fusobacteria, and other microorganisms invade the nonnecrotic lamina propria of the gingiva of patients demonstrating symptoms of acute necrotizing ulcerative gingivitis. Similar findings have been described for the necrotizing gingiva seen in patients with human immunodeficiency virus (HIV) infections.

Calculus

Dental calculus is calcified dental plaque, composed primarily of calcium phosphate mineral salts deposited between and within remnants of formerly viable microorganisms. Viable bacteria cover calculus deposits (Fig. 6). Supragingival calculus is that which forms coronal to the gingival margin, and subgingival calculus forms below the gingival margin. In



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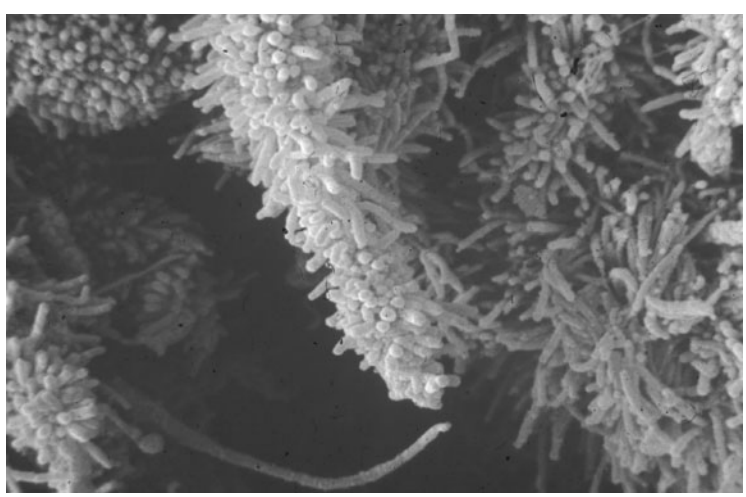

C

FIGURE 5 Scanning electron micrographic study of subgingival plaque from a patient with severe periodontitis. (A) Spirochetes and filamentous bacteria; (B) "corncobs"; (C) "test-tube brushes." (Courtesy of H. E. Schroeder, Institute of Oral Structural Biology, University of Zurich, Zurich, Switzerland.)
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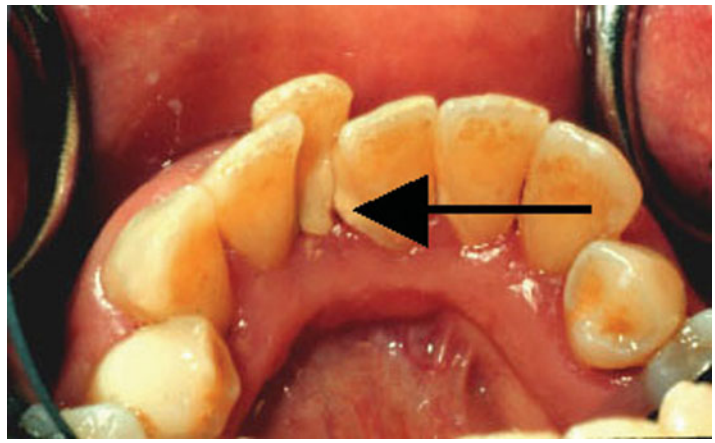

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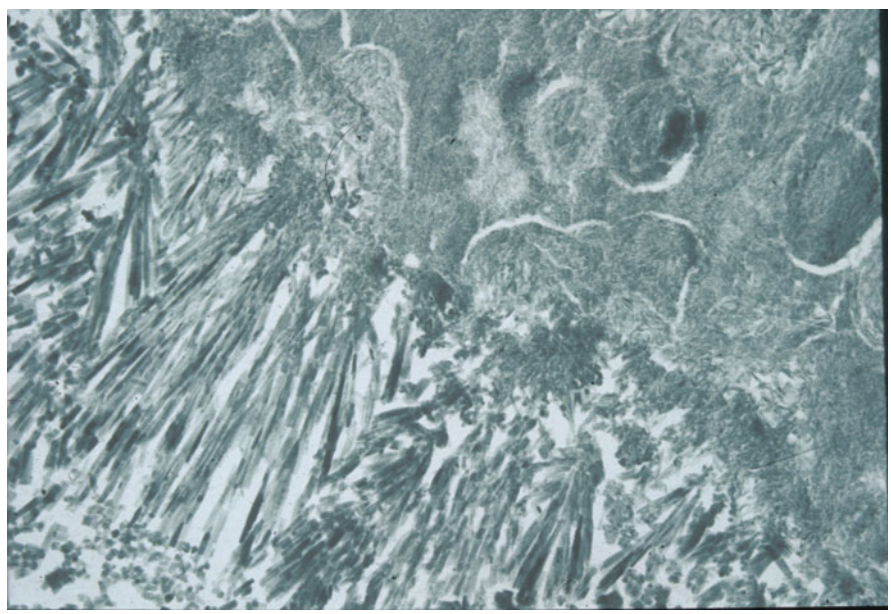


B

FIGURE 6 (A) Clinical presentation of supragingival calculus (at end of arrow), which often forms on the lingual surfaces of the mandibular anterior teeth. These tooth surfaces are in close proximity to Wharton's duct that drains the submandibular and submaxillary glands to deliver mineral-laden saliva to the teeth. (B) Transmission electron micrograph of calcified bacteria and a portion of a crystal aggregate in supragingival calculus. (Courtesy of H. E. Schroeder, Institute of Oral Structural Biology, University of Zurich, Zurich, Switzerland.)
doi:10.1128/9781555818906.ch3.f6

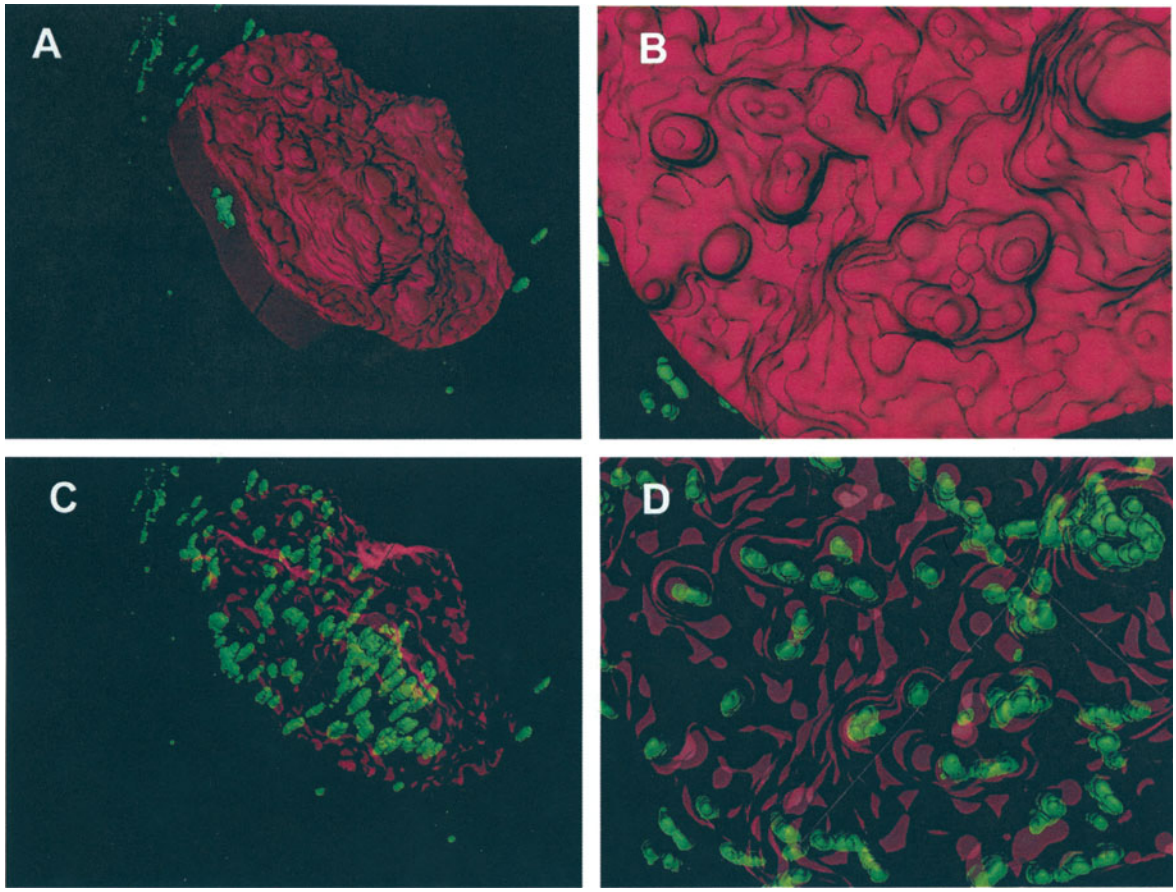
patients who do not practice regular hygiene and who do not have access to professional care, supragingival calculus often is present throughout the dentition and can be extreme. Although calculus itself is not thought to cause periodontal disease (it is the viable bacteria growing on the surface of the calculus that incite the disease), removal of subgingival plaque and calculus remains the cornerstone of periodontal therapy, since good oral hygiene can only be maintained on calculus-free teeth.

Calculus formation can be controlled by inhibitors of biomineralization. It is known that certain salivary proteins are effective inhibitors of mineralization in dental plaque. Such mineralization is also affected by changes in plaque pH as well as by fluctuations in the concentrations of ions and molecules in the solution phase of the plaque. How these salivary proteins function in the process of mineralization is not yet completely understood, especially in subjects in whom heavy calculus forms.

The Mucosal Microbiota

The epithelium covering the gingival, buccal, and palatal tissues is also a habitat for microbes. Because the surface cells of epithelia desquamate at a regular rate (thus shedding attached bacteria), the soft tissues do not support the voluminous microbiota seen on the surface of the teeth. Stable colonization of the mucosa therefore requires a continuous process of attachment, growth, and generation of daughter cells that detach and readhere to freshly exposed epithelial cells. An interesting recent study that used fluorescent in situ hybridization with probes to conserved and variable regions in the ribosomal 16S subunit suggests that human buccal epithelial cells contain intracellular bacteria, including the periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis*, as well as other species of bacteria (Fig. 7). The invasion of these epithelial cells may allow

FIGURE 7 Three-dimensional reconstruction of a buccal epithelial cell from a single subject. (A) Surface contour of the target cell, with the surface rendered opaque in red. Green bacteria, which appear to be extracellular, were in fact contained within the cells that were edited out. (B) A close-up view of the opaque host cell surface reveals a very irregular contour. (C) The surface of the target cell is rendered transparent with red highlights. This reveals clusters of green bacteria, which appear to be intracellular, since they cannot be seen otherwise. (D) The close-up transparent view shows that some surface protuberances were associated with bacterial clusters. However, bacteria in those clusters seemed to be located below the surface. Reprinted from J. D. Rudney et al., *Infect. Immun.* **69:**2700–2707, 2001, with permission. doi:10.1128/9781555818906.ch3.f7



bacteria to establish themselves in the mouth and find a habitat protected from host defense factors such as salivary antimicrobial systems.

The tongue offers another unique habitat for bacterial growth. Because the tongue surface is covered with papillae, there are numerous protected sites that provide sheltered habitats for bacterial colonization. Indeed, with the exception of the teeth and gingival sulcus, the dorsum of the tongue harbors a greater microbial biomass than any other site in the mouth. It is therefore interesting that relatively few comprehensive studies of the microbiology of the tongue have been reported. From the few studies that are available, it appears that the tongue microbiota includes bacterial species that also colonize the teeth as well as those that are relatively selective for the tongue. Thus, the dorsum of the tongue harbors streptococci such as *Streptococcus mitis* that typically colonize teeth as well as organisms such as *S. salivarius*, whose primary habitat in the mouth appears to be the tongue. The tongue of medically compromised subjects, for example, patients with Sjögren's syndrome who suffer from dry mouth (xerostomia), may be more susceptible to colonization by potential pathogens such as *Candida albicans*, *Staphylococcus aureus*, enteric gram-negative bacteria, and enterococci than healthy subjects are.

RECENT CONCEPTS OF DENTAL BIOFILM FORMATION

Our conception of dental plaque formation has expanded in recent years as a consequence of the development of several powerful methodologies to study microbial ecosystems. The development of confocal scanning laser microscopy and other advanced microscopic techniques, as well as molecular approaches to identify and quantify noncultivable bacterial species, has revolutionized the study of dental plaque. The confocal microscope allows computer analysis of confocal laser images and can produce repeated three-dimensional reconstruction of microbial systems over time. Morphologic, biochemical, and molecular biologic studies demonstrate that dental plaque is an example of a biofilm, a polymer-encased community of microbes that accumulates at a surface interface (Fig. 8). Microbial biofilms are complicated structures that appear to undergo specific developmental events as they mature. It has been suggested that most bacteria do not survive in nature as unicellular organisms but as biofilms. The bacteria within the biofilm organize to distribute metabolic activities among the different members of the biofilm. In addition, many bacteria use small soluble molecules to signal the expression of multiple target genes in neighboring cells and this coordinates metabolic activities (see chapter 5). It is also clear that biofilms demonstrate intrinsic resistance to antibiotics and host defense systems. Thus, biofilm-induced diseases are difficult to treat. This antibiotic resistance may be due to slow bacterial cell growth and/or induction of a genetically directed stress response. Bacterial exopolysaccharides or other aspects of biofilm architecture could also confer resistance by exclusion of antibiotics from the bacteria.

SALIVA AND THE SALIVARY PROTEOME

Whole saliva ("spit") is actually a mixture of fluids from several sources. Per unit volume, the bulk of whole saliva is derived from salivary gland secretions. The major salivary glands are the paired parotid, submaxillary,

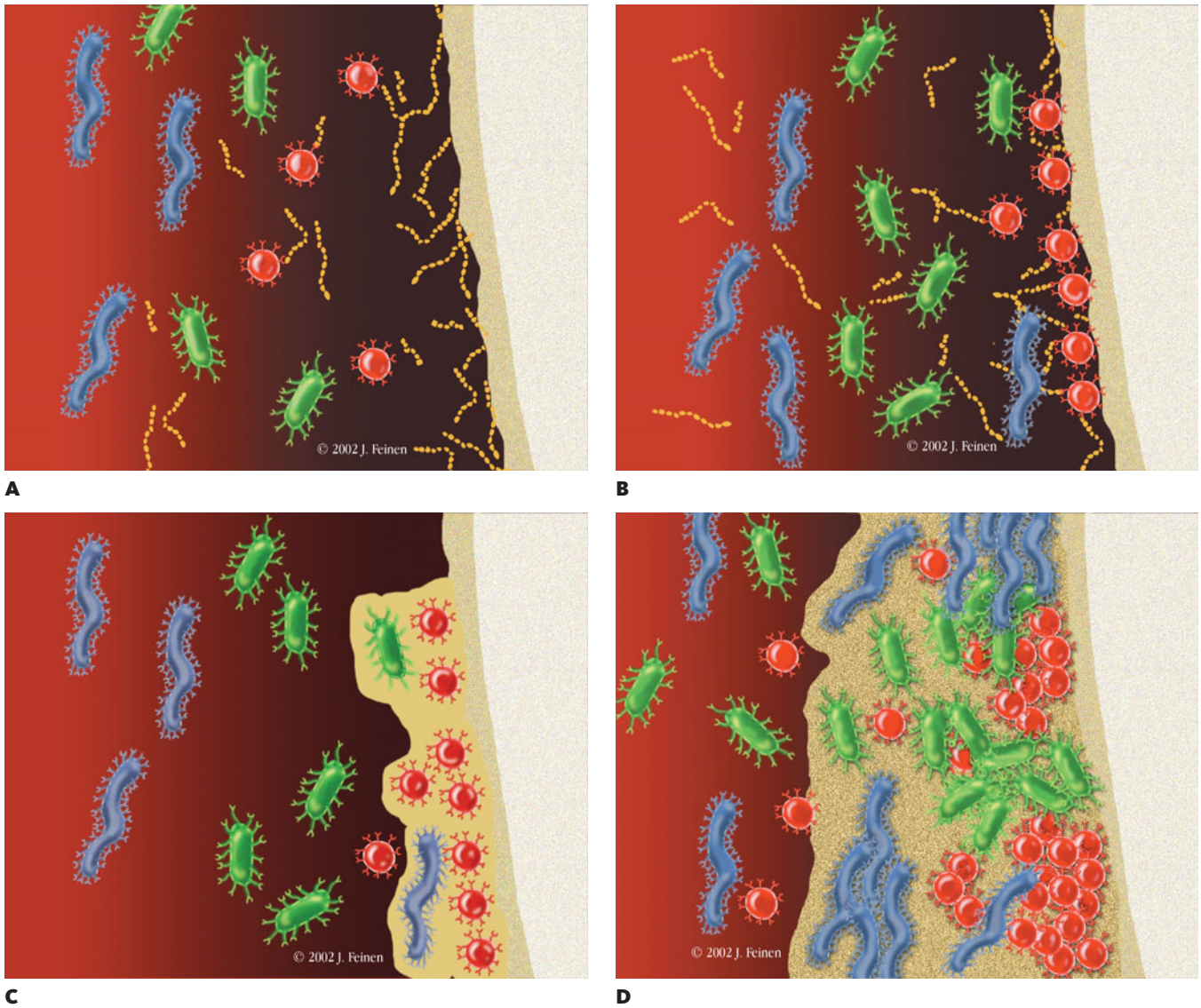


FIGURE 8 Events during biofilm formation. (A) Proteins in solution adsorb to the solid substratum to form pellicle (in the case of dental plaque, the tooth). (B) Bacteria adsorb to proteins in pellicle. (C) Bacteria of dissimilar species interact on the tooth surface. Bacteria synthesize polymers to form plaque matrix. (D) Adherent bacteria divide to form microcolonies. doi:10.1128/9781555818906.ch3.f8

and sublingual glands. In addition, a multitude of minor glands scattered within the oral mucosa (labial, buccal, glossopalatine, palatine, and lingual) contribute substantially to the total output of whole saliva. The saliva of most people, especially those with periodontal disease, also contains constituents of gingival crevicular fluid (GCF), which is derived from serum and whose flow rate from the gingival sulcus is related to the severity of gingival inflammation. Degradation products of bacterial and oral cells along with components from gastric and respiratory reflux can also be found in whole saliva.

The relative concentration of various components in saliva varies with their source. Some components, such as mucin glycoproteins, are

produced for the most part by mucous glands (e.g., the submaxillary, sublingual, and minor glands). Mucins are essentially absent in parotid secretions. In contrast, parotid secretions have higher concentrations of serous proteins, such as amylase, parotid agglutinin (gp340), and proline-rich proteins, than do secretions from mucous glands.

Once saliva exits the gland ducts, the distribution of its components within the oral cavity is probably not homogeneous. For example, parotid components released into unstimulated whole saliva are distributed primarily within the region vestibular to the maxillary molars (where roughly 61% of all saliva is from the parotid glands). Only 7% of whole saliva found vestibular to the upper incisors is from the parotid glands. This localization of specific salivary components within the mouth may have subtle but real effects on bacterial interactions that are responsible for dental plaque formation at different anatomical sites within the mouth.

Recently, the methodologies of proteomics, the large-scale study of proteins, have been applied to the study of human saliva. The salivary proteome is defined as the total complement of proteins found in saliva. The number and amount of each protein in saliva will vary with the individual, with disease state, and with time. A complete assessment of all of the protein constituents of saliva will allow for a more complete understanding of the functions of saliva. Such information may also serve to identify biomarkers associated with specific disease states.

Evidence from the preproteomic era suggested that ductal saliva contained about 100 distinct proteins. However, with the use of sophisticated technologies such as two-dimensional gel electrophoresis, liquid chromatography-mass spectrometry, and complex computer analysis of large protein databases, it has been estimated that close to 1,000 proteins are present in ductal saliva and many more are present in whole saliva. Of these, about 200 appear to be derived from plasma, vascular leakage, or the contribution of fluid from the interstitial compartment. Additional sources of proteins in whole saliva include the gingival crevicular fluid, sloughed epithelial cells, or other host cells such as neutrophils and oral microbes.

This information could prove useful by suggesting potential biomarkers as indicators of the onset or progression of specific oral or systemic diseases. Since saliva is readily accessible for non-invasive collection and analysis, salivary biomarkers have been sought for a number of diseases. To date, few single biomarkers have been identified that correlate with specific disease activity. However, a number of promising protein biomarker candidates have been identified, such as selected protein-degrading enzymes and cytokines with periodontal disease, statherin and cystatin-S with dental caries, and carbonic anhydrase VI (CA6) with breast cancer.

SALIVA-MICROBE INTERACTIONS

Saliva is involved in a diverse range of functions (Table 4). It plays a role in food digestion by solubilizing food components to facilitate taste, by preparing the food bolus for swallowing, and by providing enzymes to initiate digestion. Saliva is also thought to function, in part, by forming tenacious films, or pellicles, on the oral surfaces such as the tooth (enamel, dentin, and cementum), the epithelium (oral, lingual, alveolar, and palatal

TABLE 4 Functions of salivary molecules with reference to dental plaque formation

Molecule	Microbial agglutination	Promotion or inhibition of microbial adhesion	Antimicrobial	Microbial nutrition
Amylase	–	+	–	+
β_2-Microglobulin	+	?	?	?
Fibronectin	–	+	–	?
Lysozyme	+	+	+	?
Mucin	+	+	+	+
Parotid agglutinin	+	+	–	?
Proline-rich proteins	–	+	–	?
S-IgA	+	+	+	?
Statherin	–	+	–	?

mucosa and gingiva, etc.), and plaque itself. Salivary pellicles help maintain a balance between tooth demineralization and remineralization. The salivary molecules controlling these processes have high affinities for the tooth surface through possession of negatively charged domains. These molecules appear to function by inhibiting the precipitation of calcium phosphate salts from solution and thus maintaining calcium and phosphate in a supersaturated state in saliva.

The adsorbed salivary molecules also lubricate tissues to facilitate chewing, speaking, and swallowing and prevent the oral tissues from drying. Other important functions of salivary molecules include buffering of acids and neutralization of toxic products produced by oral microorganisms.

The function of individual salivary molecules is dependent on their conformation or molecular shape. For example, proline-rich proteins only promote bacterial adhesion when they are adsorbed onto a surface. Interestingly, when these proteins are in solution, they do not interact with bacteria. This suggests that the adsorption of these molecules to a surface causes a shape change that exposes otherwise hidden domains capable of interacting with bacteria. Another interesting molecule with conformational requirements is human salivary amylase. This relatively large protein contains 496 amino acids with 5 interchain disulfide bonds that are distributed over the overall length of the molecule. Its biological activities include the enzymatic digestion of starch, interaction with oral streptococci, and binding to hydroxyapatite tooth surfaces. Cleavage of this enzyme's disulfide bonds results in the disruption of its suprastructure and loss of all biological activities.

The protective functions of saliva are enhanced by its built-in redundancies. In other words, many of the molecules in saliva have overlapping functions. Indeed, most salivary molecules are multifunctional. Thus, mucins play a role in lubrication, tissue coating, digestion, and agglutination of microorganisms. This results in functional compensation and may explain why most cross-sectional studies find a high degree of variability in the concentration of individual salivary molecules within study populations and often no correlation with the prevalence of oral disease. A single salivary molecule may have both protective and detrimental properties ("amphifunctionality"). For example, statherin and acidic proline-rich

proteins work at the enamel surface by inhibiting the formation of primary and secondary calcium phosphate salts. However, when adsorbed to the enamel surface, these components may also promote adhesion of potentially cariogenic microorganisms to the tooth surface.

Comparisons of purified salivary molecules with intact secretions have shown that functional relationships must exist between different molecules in saliva. These functional relationships are likely predicated on complexing between molecules. Two types of complexing can occur: homotypic ones between similar molecules and heterotypic ones between different molecules. Complexes can occur by either covalent or noncovalent interactions. Mucins provide an interesting example. These molecules may form homotypic complexes, or end-to-end oligomers, with themselves via interchain disulfide bonds. Mucin oligomer formation is required for this molecule to exert its lubrication and viscoelastic properties. Mucins that coat the various oral tissue surfaces can also form heterotypic complexes with other salivary molecules, including antimicrobials such as secretory immunoglobulin A (S-IgA), lysozyme, and cystatins (see chapter 10). These complexes are mediated primarily by noncovalent ionic forces and function to concentrate these antimicrobials at the saliva-tissue interface. It is possible that the complexes themselves may have additional functions over and above those of the individual molecules comprising the complex.

Covalent bonding can be important in other salivary complexing mechanisms. Proline-rich proteins can cross-link into higher-molecular-mass complexes by the action of buccal epithelial cell transglutaminase on the lysine residues of proline-rich proteins. This enzyme also cross-links statherin to proline-rich proteins. These observations suggest that such cross-linking reactions may permit other proteins in the oral cavity to be incorporated into oral pellicles.

Many salivary components have been shown to interact with oral microorganisms. The interactions between bacteria and salivary components can be divided into four major categories: (i) interactions causing aggregation or agglutination of bacteria, (ii) interactions that foster adhesion of bacteria to surfaces, (iii) interactions that kill or inhibit the growth of microorganisms, and (iv) interactions that contribute to microbial nutrition. Together, these interactions probably control to a considerable extent the microbial ecology of the oral cavity. The following discussion provides examples of individual salivary molecules that display each of these four general functions (Table 4).

CLEARANCE OF BACTERIA FROM THE ORAL CAVITY: AGGLUTININS

Forces that promote bacterial clearance from the oral cavity are powerful. When suspensions of pure cultures of bacteria are experimentally introduced into the oral cavity, most are soon removed, with only a small fraction able to attach and persist. Clearance occurs by mechanical flushing due to physiologic movements (e.g., swallowing, chewing, and speaking) and dilution effects by continuous salivary flow. In addition, many species of bacteria are rapidly agglutinated (that is, clumped or aggregated) when suspended in saliva (Fig. 9). It is thought that agglutination promotes

bacterial clearance from the oral cavity. The binding of salivary components to the bacterial surface may block the adhesion of bacteria to the tooth, mucosa, or plaque. Salivary molecules that have been shown to agglutinate bacteria include mucins, S-IgA, parotid agglutinin, lysozyme, β_2-microglobulin, and Ca^{2+} ions.

Salivary mucins are large-molecular-weight glycoproteins originating from mucous acinar cells. By definition, mucin glycoproteins contain 30 to 80% carbohydrate. Several mucins in human submandibular-sublingual saliva have been identified. Initial biochemical studies identified two mucins: mucin-glycoprotein 1 (MG1, also known as MUC5B) and mucin-glycoprotein 2 (MG2, also known as MUC7). MG1 was found to be composed of monomers having a molecular mass greater than 1×10^6 kDa, whereas MG2 was found to have a molecular mass of about 200 kDa. More recent molecular and genetic studies have clarified the chemical nature of these mucins. It is now recognized that human salivary glands make membrane-bound mucins, MUC1 and MUC4, in addition to the gel-forming mucin MUC5B. MG1 has been shown to be composed mostly of MUC5B, which appears to be the major subunit secreted from sublingual and submandibular glands, as well as by gallbladder, colon, female reproductive tract, and respiratory tract epithelium. MUC5B monomers interact to form dimers via disulfide linkages between cysteine residues in the C-terminal cysteine knot motif. The dimers are then O glycosylated in the *cis*-Golgi and form trimers during passage to the secretory granules. Tissue-bound MUC1 is expressed as a single polypeptide chain that is cleaved in the endoplasmic reticulum into two subunits, one the extracellular portion and the other the membrane-spanning and C-terminal cytoplasmic domains. The extracellular portion is probably shed to form the soluble form of MUC1. MUC4 is the largest mucin gene product so far described. Like MUC1, MUC4 is synthesized as a single polypeptide that is cleaved and reassembled into extracellular and membrane-spanning domains. Both MUC1 and MUC4 are also expressed by minor glands and, surprisingly, in low amounts by parotid glands and buccal epithelial cells. It is likely that these forms of mucin are involved in the physical protection of the epithelial surfaces from which they are secreted.

High-molecular-weight salivary glycoproteins, including mucins, can aggregate oral bacteria. Removal of the terminal sialic acid of the mucin oligosaccharide side chains abolishes its interaction with some, but not all, species of oral streptococci. The interaction of these bacteria with mucin is thus mediated by a sialic acid-binding protein, or lectin, on the bacterial surface.

If mucins participate in clearance of bacteria from the oral cavity, it would make sense that people with higher mucin concentrations in their saliva would have fewer bacteria in their mouth. This hypothesis was tested with respect to the relationships between the number of *S. mutans* organisms in the oral cavity and mucin concentrations in saliva. The results of this study suggested that an elevated level of *S. mutans* is significantly associated with diminished concentrations of MG2 in unstimulated whole saliva. This suggests that *S. mutans* may not be cleared from the mouth due to low concentrations of MG2 in saliva.

Another important agglutinin in saliva is S-IgA (see chapters 2 and 10). S-IgA is the predominant immunoglobulin found in all mucosal

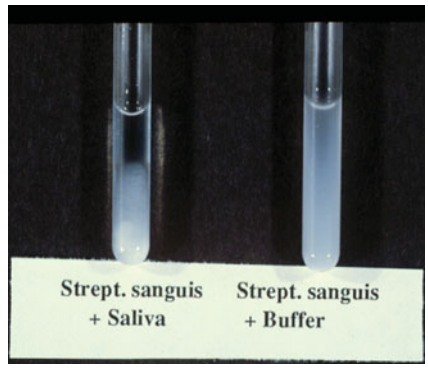


FIGURE 9 Incubation of *S. sanguinis* with parotid saliva (left tube) or buffer (right tube). Agglutinins in parotid saliva cause the bacteria to clump and fall to the bottom of the test tube within minutes. Bacteria remain suspended in buffer.
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secretions, including saliva. S-IgA is composed of an IgA dimer (300 kDa), J chain (15 kDa), and secretory component (70 kDa). J chain is a glycoprotein that polymerizes the two IgA molecules into a dimer. Polymeric IgA containing the J chain is secreted by local plasma cells. Polymeric IgA is taken up by the polyimmunoglobulin receptor, which is expressed on the basolateral surface of the secretory epithelial cells in the glands. The complex is transported in membrane vesicles through the epithelial cells, and the membrane part of the receptor is cleaved off when the complex reaches the surface. The remaining part of the receptor is secretory component. Secretory component functions to protect the molecule from attack by acids or proteases in the oral environment.

A major function of S-IgA appears to be the inhibition of microbial adherence to the host surface, thus preventing colonization. Inhibition is usually specific, requiring that S-IgA be directed against antigenic determinants on the microbial surface. Indeed, like other antibody species, S-IgA in saliva is a mixture of many S-IgA molecules, each synthesized by a B-cell clone and directed to a specific antigen. It is this function that forms the basis for a number of proposed mucosal vaccines, the immunogens being various components of pathogenic bacteria such as the cariogenic mutans group streptococci. It is also possible that certain bacteria may interact with S-IgA through lectin binding to the oligosaccharide structures of the immunoglobulins. It has also been suggested that bacteria may subvert the inhibitory function of S-IgA via specific proteases. Indeed, several oral bacteria, including important initial colonizers of tooth surfaces, produce an IgA1 protease similar to that produced by the bacterial species that are the three principal causes of bacterial meningitis. However, the exact role of the S-IgA-cleaving enzymes in the pathogenesis of oral disease remains to be determined.

Another high-molecular-weight salivary glycoprotein agglutinin is the parotid agglutinin that is identical to lung (lavage) gp340, a member of the scavenger receptor cysteine-rich protein family. This glycoprotein binds to the surface of oral streptococci through the interaction of its sialic acid residues with a lectin protein on the bacterium's surface. This lectin is a high-affinity calcium-binding protein that binds 1 mol of calcium per mol of protein.

PELLICLE ADHESION RECEPTORS

Saliva influences the attachment of bacteria to the tooth surface via the enamel, cemental, or dentinal pellicle. Such surfaces have been modeled in vitro using experimental pellicles formed by incubating saliva with beads of hydroxyapatite (HA), enamel, or dentin powders. The adhesion of bacteria to saliva-coated hydroxyapatite (sHA) has been shown to be complex. Whereas some species of bacteria adhere in similar numbers to naked HA, coating HA with saliva markedly alters the adhesion of other species. *S. mutans*, for example, adheres in lower numbers to sHA than to uncoated HA. Furthermore, *S. mutans* first suspended in saliva will attach in even lower numbers to sHA than untreated *S. mutans*. In contrast, *S. gordonii* and *Actinomyces* species attach in higher numbers to sHA than to bare HA. These in vitro investigations must, however, be interpreted with some caution. For example, pellicles formed on HA in

vitro have been shown to differ from those formed in vivo. As an example, albumin is found in greater amounts within in vivo pellicles than within in vitro pellicles (probably contributed from periodontal inflammation through GCF). There appears to be less proline in in vivo pellicle than in in vitro pellicle, probably due to a reduction in the content of acidic proline-rich proteins in the in vivo pellicles. Thus, constituents of the oral environment, such as enzymes released from GCF into whole saliva, may alter oral surfaces to account for the differences in bacterial adhesion observed between in vivo and in vitro systems.

Despite these limitations, the few studies that have attempted to study these phenomena in vivo support the modulatory role of saliva in bacterial colonization. Thus, the implantation of *S. mutans* in the oral cavity of humans is modified by pretreatment of the bacteria with saliva. Saliva from caries-active individuals enhanced the implantation of *S. mutans* in volunteers compared to *S. mutans* suspended in saliva from caries-free controls. This suggests that there may be a component in the saliva of some individuals that promotes the colonization of *S. mutans*.

Cataloging the salivary components in pellicles has consistently identified several components, including proline-rich proteins, lysozyme, albumin, histatins, statherin, mucins, S-IgA, and α-amylase. Each of these proteins probably serves as a pellicle receptor for one or more species of oral bacteria that adhere to the tooth surface. It is also interesting that enamel pellicles display a distinctive structure consisting of a sponge-like meshwork of microglobules.

Salivary components do not, however, appear to be homogeneously distributed within pellicles sampled in different parts of the oral cavity. In fact, enamel pellicles eluted from the premolar teeth (bathed mostly by saliva from the parotid glands) are qualitatively different from pellicles that form on mandibular anterior teeth (bathed primarily by secretions from the submandibular/sublingual glands). Thus, parotid agglutinin is found in maxillary premolar pellicles in greater amounts than in mandibular anterior pellicles, whereas mucins show the opposite pattern of localization. The observed variations in salivary protein localization might be important to the establishment of microbiota and tooth-related disease patterns in various parts of the dentition. A more complete discussion of bacterial adhesion to receptors in salivary pellicle can be found in chapter 5.

ANTIMICROBIAL COMPONENTS IN SALIVA

Several salivary components have been shown to kill or inhibit the growth of bacteria in vitro. Lysozyme, one of the first salivary antimicrobial components to be described, is a 14-kDa protein that hydrolyzes the β-1,4 glucosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan of the bacterial cell wall (see chapter 1). The resultant weakening of the cell wall of susceptible bacterial species, mostly gram-positive bacteria, results in the lysis of the bacteria. Interestingly, many oral species are insensitive to the action of lysozyme. However, other mechanisms of lysozyme bactericidal activity have been described. One involves the activation of endogenous bacterial enzyme(s) by lysozyme, which can kill bacteria that are insensitive to the muramidase activity of lysozyme. Lysozyme can also bind to oral bacteria and aggregate

them, thus facilitating their clearance from the oral cavity. Finally, lysozyme possesses small amphipathic sequences within the C terminus that have antimicrobial effects.

Histatins (also known as histidine-rich proteins) comprise an interesting family of cationic low-molecular-weight proteins found in abundance in submandibular/sublingual and parotid salivas (~10 to 150 mg/ml of saliva, depending on the source and degree of stimulation). At least a dozen human salivary molecules with similar properties have been described, all of which have large amounts of the basic amino acid histidine (up to 41%). Variants of histatins differ with respect to primary sequence, chain length, and phosphorylation. A variety of functions have been ascribed to the histatins, including histamine release from mast cells, inhibition of HA crystal growth, and tanin binding. However, the function paid most attention by investigators is the antimicrobial activity of these peptides. The first antimicrobial property described for histatin was antifungal; however, these peptides also have less potent but measurable antibacterial properties and have been noted to prevent bacterial coaggregation. Recent evidence suggests that key steps in the histatin 5 antifungal mechanism involve a bioenergetic collapse of *C. albicans*, likely from a decrease in mitochondrial ATP synthesis. Additionally, histatins may serve as a competitive inhibitor of several proteinases, including cysteine proteinases from mammals and bacteria. By inhibiting such enzymes, the histatins may affect the course of diseases such as periodontitis where there is extensive proteolytic destruction of the periodontal tissues.

Epithelium- and myeloid-derived antimicrobial peptides are also found in whole saliva. For example, β-defensins are induced in gingival epithelium by selected bacteria. These peptides exhibit broad-spectrum activity against gram-positive and gram-negative bacteria and fungi. The human cathelicidin, LL-37, is produced by monocytes, T cells, and airway epithelia. LL-37 has antimicrobial activity similar to that of defensins. Salivary peroxidase and thiocyanate act together with hydrogen peroxide (produced by bacteria) to generate oxidized thiocyanate ion derivatives, which inhibit the growth of bacteria as well as inhibit acid formation by oral streptococci. Recent evidence also suggests that the lactoperoxidase-hydrogen peroxide-thiocyanate antimicrobial system inhibits respiration in gram-negative species such as *Escherichia coli* by inhibiting membrane dehydrogenases. Together with lysozyme, lactoperoxidase may also inhibit the adhesion of bacteria to teeth.

Lactoferrin (Lf) is a glycoprotein of 75 kDa synthesized by glandular acinar and epithelial as well as inflammatory cells. Lf binds two atoms of iron per molecule, with the simultaneous binding of two molecules of bicarbonate. The primary function of Lf may thus be to bind and sequester iron from bacteria. In addition, iron-free lactoferrin (apoLf) may also possess a direct, iron-independent, bactericidal effect on various strains of oral bacteria such as *P. gingivalis*. It is possible that apoLf forms a complex with essential iron-containing nutrients such as hemin. Moreover, the 25-residue N-terminal peptide from Lf, called lactoferricin, itself has antimicrobial activity. Functional studies suggest that this peptide causes a depolarization of bacterial cytoplasmic membranes, loss of the pH gradient, and a resultant bactericidal effect. In addition to this bactericidal activity, apoLf may also serve to inhibit the adhesion of bacteria to oral surfaces such as hydroxyapatite.

ANTIVIRAL COMPONENTS IN SALIVA

Saliva also appears to possess potent antiviral factors. For example, the infectivity of HIV has been shown to be significantly suppressed in the presence of human saliva, and antiviral factors have been isolated from saliva. The protein secretory leukocyte protease inhibitor appears to possess substantial anti-HIV type 1 (anti-HIV-1) activity at physiological concentrations. The human parotid basic proline-rich proteins also inhibit HIV-1 activity. The mechanism of action may involve the binding of the basic proline-rich proteins to the HIV-1 gp120 coat of the virus before the interaction of the virus with host target cells. Recent work suggests that soluble salivary gp340 can function as a specific inhibitor of infectivity of HIV-1 and influenza A.

SALIVA AS A SOURCE OF BACTERIAL NUTRITION

Some bacteria such as certain oral streptococci and actinomyces are able to grow on chemically defined medium supplemented with saliva. A number of protein components of saliva are degraded following growth of these bacteria, suggesting that these bacteria are unable to utilize free amino acids in saliva for growth but instead metabolize salivary proteins. Bacteria grown in saliva also appear to have elevated levels of cell-associated hydrolytic activities, including glycosidases, exo- and endopeptidases, esterases, and neuraminidase. The bacteria may therefore obtain nutrients by the enzymatic breakdown and subsequent metabolism of oligosaccharides and peptides from salivary glycoproteins. Other studies have extended these findings by demonstrating that the utilization of salivary peptides may be more dependent on their physical properties (hydrophobicity) than their size. That is, hydrophilic peptides stimulated growth of streptococci far better than hydrophobic peptides.

Another example of how saliva may influence bacterial nutrition involves amylase. Salivary amylase binds with high affinity to certain species of oral streptococci that are abundant in dental plaque. It appears that the bound enzyme remains enzymatically active and thus may facilitate dietary starch hydrolysis to provide additional glucose for metabolism by plaque microorganisms in close proximity to the tooth surface.

GINGIVAL CREVICULAR FLUID

GCF is an inflammatory exudate that can be collected at the gingival margin or within the gingival crevice. When the gum tissues are absolutely healthy, the flow of crevicular fluid from the sulcus is so low that it can hardly be measured. However, GCF flow increases as gingival inflammation increases, and significant amounts of GCF can be collected from patients with severe disease. GCF is a mixture of plasma proteins such as albumin, inflammatory products, and constituents released from phagocytic cells. Many attempts have been made to relate the presence or amount of certain proteins in GCF with the progression or activity of periodontal disease. Inflammatory mediators such as prostaglandin E2, beta-glucuronidase, neutrophil elastase, aspartate aminotransferase, and matrix metalloproteinases, especially collagenase-2 (matrix metalloproteinase 8), have all been studied in this regard, although thus far none have proved to be reliable markers of the disease. The significance of GCF

in the context of oral microbiology relates to the role of GCF constituents in oral bacterial colonization. For example, a variety of plasma proteins such as intact fibrin, as well as degradation products of fibrin and fibronectin, were seen in both healthy and diseased GCF samples. These proteins are known to interact with oral bacteria, and such interactions may influence bacterial colonization of host surfaces.

KEY POINTS

The oral cavity contains the mineralized hard tissues of the teeth along with the soft tissues of the oral mucosa, all of which are continuously bathed in saliva. The environment of the oral cavity experiences fluctuations in temperature, pH, oxygen levels, and availability of nutritional substrates.

The crown of the tooth (that part above the gum) is composed of enamel, a highly mineralized, substituted hydroxyapatite. Beneath the enamel is located dentin, which is less mineralized and surrounds the pulp chamber. The tooth root is anchored in the gum and has a cementum surface.

The gum (gingiva) forms a collar around the tooth with a gap (sulcus) between the root and the sulcular epithelium. Junctional epithelium attaches the inner surface of the sulcus to the cementum of the root. The periodontal ligament attaches the tooth to the underlying alveolar bone. A serum exudate called GCF collects in the gingival sulcus.

To first colonize teeth, bacteria must attach to salivary receptors in the pellicle of salivary molecules that coats the enamel surface. This allows bacteria to resist the mechanical shearing forces that would tend to dislodge them. Shearing forces arise from tongue and lip movement and from salivary fluid flow.

Primary colonizers of the tooth are mainly streptococci and actinomycetes. Subsequently, more actinomycetes and related organisms arrive. Finally, increasing numbers of gram-negative anaerobes and spirochetes colonize. The pattern of plaque accumulation is driven by bacterial adherence to host surfaces and to other bacteria, nutrient availability that will modulate multiplication, and environmental factors such as oxygen levels.

Bacterial colonization of mucosal surfaces is less abundant as the epithelial cells are continually dying and sloughing off, thus removing the attached bacteria.

Saliva contains numerous components that can either promote or inhibit bacterial colonization. Salivary receptors for bacterial adherence, along with molecules that can act as a carbon and nitrogen source for bacteria, will assist bacterial colonization. Antibacterial components include agglutinins, lysozyme, histatins, peroxidase and thiocyanate, and lactoferrin.

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Isolation, Classification, and Identification of Oral Microorganisms

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INTRODUCTION

The title of this chapter, "Isolation, Classification, and Identification of Oral Microorganisms," until recently would have reflected an approach to oral microbiology where oral samples were taken and the bacteria were dispersed and plated on various media. Those bacteria that grew were isolated and characterized, while species unable to grow on the media were overlooked. Over the last several years, our ability to obtain a more comprehensive look at complex microbial communities has steadily increased due to advances in molecular techniques. Today, using high-volume sequencing techniques, we are able to get a detailed, accurate description of the composition of oral biofilm communities, including uncultivated members. Therefore, a common scheme today is to first identify bacteria by analyzing DNA from oral samples, and then to use molecular methods to characterize new bacteria by sequencing genes and even whole genomes. Determining where a new species grows in the mouth (subgingival, supragingival, etc.) and under what conditions (substrate availability, cocolonizing species) can guide attempts to culture these new species in the laboratory. This can be challenging, especially considering that most of the easily cultivable species have been isolated. At this time there are still a number of species of interest to oral microbiologists that have yet to be cultured. Although important new species have been added to the list of cultivated bacteria in recent years, entire phyla, such as the TM7s, are still missing from our cultivated collection.

DIVERSITY OF THE ORAL MICROBIOTA

The bacterial community in the oral cavity is one of the most complex known. More than 700 bacterial species routinely colonize the human oral cavity. This is the oral microbiota. The totality of the microbiota, their genomes, and their interactions with each other and the human host is called the oral microbiome. Although the most abundant species of the oral microbiota have been cultured, almost half remain uncultured and are

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known only by their molecular signatures. In addition, a large number of transient species have also been observed in oral samples, although their natural habitat is not the oral cavity. There have been attempts to estimate the number of species that could potentially be found; however, with the wide variety seen from sample to sample, the number appears to be open ended. It is likely at this time that all or almost all of the common species have been observed. Up to 200 to 300 species are likely to be found in any individual, and within individuals, the profile varies from site to site, depending on the local environment. Bacterial community composition can be radically different from person to person, with functionally similar but genetically distinct species occupying a given ecologic niche.

Because of the complexity of the oral microbiome, until recently it was necessary to use a large number of technical approaches to analyze oral samples. Many of these approaches were developed by oral microbiologists to study the unique features found in the oral cavity, and microbiologists studying complex mixtures of bacteria in other environments have developed additional techniques. With a number of technical advances including next generation DNA sequencing, new bioinformatic approaches to analysis of microbial communities, and growing databases cataloging microbial diversity, it has become possible to obtain a comprehensive quantitative as well as qualitative picture of the composition of complex mixtures of bacteria. With these techniques, a new picture of the oral microbiome has emerged. Bacterial communities are complex organizations composed of many species, and consortia of several species may be required for disease. Previously overlooked species have emerged as potential pathogens, and some of the species traditionally regarded as pathogens that were first identified because they grow readily in culture appear to be less dominant than previously thought. Using next generation sequencing, it is even possible to look beyond species and examine all the genetic material present in a sample, consisting of the genomes of many individual bacteria, the metagenome.

THE RIBOSOMAL 16S GENE AND BACTERIAL IDENTIFICATION AND CLASSIFICATION

The most powerful techniques for bacterial identification are based on comparisons of an essential gene, the ribosomal 16S gene. This gene codes for the RNA present in the small subunit of the ribosome. Ribosomes are the machinery that translates DNA sequence into proteins using the universal genetic code. Because fidelity and maintenance of this translation function are critical, some regions of the rRNA gene are so highly conserved that they can be used to align genes from dissimilar organisms. Other regions less critical to translation of the code are under less selective pressure and show enough variation that each species has a unique sequence (Fig. 1). This variation allows similar species to be distinguished. Currently hundreds of thousands of bacterial 16S sequences have been deposited in the major public DNA databases, GenBank, the Ribosomal Database Project, and SILVA. An unknown bacterium can be identified by obtaining its 16S gene DNA sequence and comparing it to sequences in these databases with a search engine called BLAST. The process is commonly referred to as "BLASTing." In addition, the entire genomes of

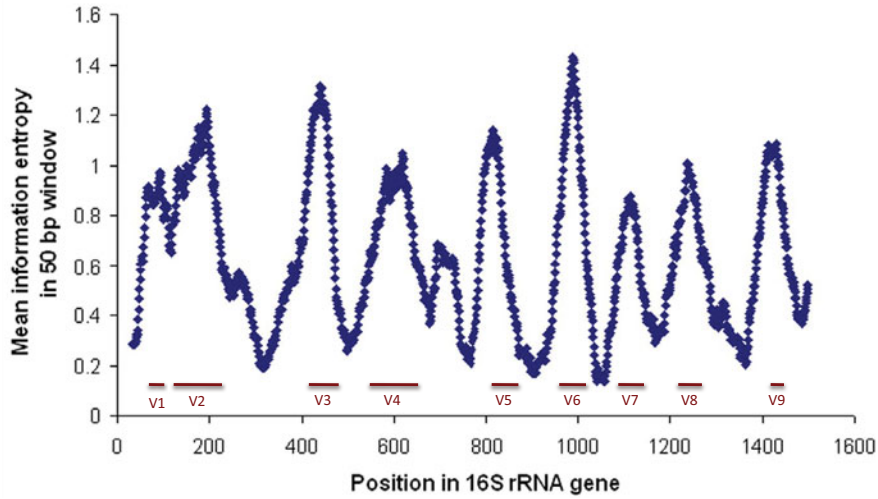


FIGURE 1 Plot of variable and conserved regions along the 16S rRNA gene. The *y* axis is a measure of the sequence diversity among all oral bacteria. The *x* axis is a representation of the 16S gene from the 5′ to the 3′ end. The bars indicate the regions, labeled V1 through V9, of the most highly variable region along the gene. Numbers below each bar are the locations of each variable region on the *E. coli* 16S gene. doi:10.1128/9781555818906.ch4.f1

thousands of bacteria have been deposited into DNA databases. In many cases, several strains have been entirely sequenced so that comparisons of genomic content can be made.

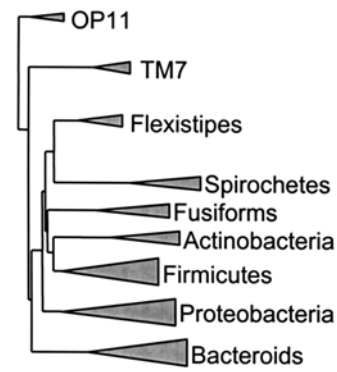
The conserved and variable regions of the 16S gene provide targets for PCR (see chapter 8). The 16S DNA from all bacteria can be amplified using universal primers homologous to conserved regions of the 16S gene. This allows previously unknown bacteria to be studied. Species-specific primers homologous to variable regions can also be designed to allow the detection of specific bacteria in clinical samples.

16S and Phylogeny

Comparison of 16S gene sequences allows more than just the identification of bacteria. The longer two species have diverged over their evolutionary history, the greater will be the difference between their 16S genes. The 16S sequences from multiple species can be aligned based on conserved regions, and computations based on the number of differences found in variable regions can be used to construct a tree diagram of evolutionary relationships. This technique is the basis for modern bacterial phylogeny. Figure 2 shows a tree diagram of the nine bacterial phyla (also called divisions) commonly found in the oral cavity. With this approach, the relationship among bacteria at the genus and species level can also be determined. This phylogenetic information is useful in designing PCR primers for groups of bacteria, for example, a primer that is homologous to all species of a genus such as *Streptococcus*.

Ribosomal 16S phylogeny is the most widely applicable technique currently available, but the inference of evolutionary relationships based on ribosomal genes has some limitations. Ribosomal gene-based phylogeny traces direct lines of descent, but bacteria often undergo lateral transfer of genes. Mixed ancestry from lateral transfer of distant genes may

FIGURE 2 Phylogenetic tree of the nine bacterial phyla that have been detected in the human oral cavity. (Adapted from B. J. Paster et al., *J. Bacteriol.* **183:**3770–3783, 2001.) doi:10.1128/9781555818906.ch4.f2



not be reflected in 16S-based phylogeny, and these genes could be critical determinants of important functions such as virulence factors. Complete genome sequences are known for a large number of bacteria, and more are becoming available daily. As the list has expanded, genomic comparisons are providing a more comprehensive basis for phylogenic classification of bacteria.

SAMPLING ORAL BACTERIA

The oral cavity contains a variety of different niches that harbor distinctive communities of bacteria. For example, the gingival sulcus is a physically separated environment sequestered from the rest of the oral cavity and bathed in gingival crevicular fluid, and the supragingival surface of the tooth is bathed in saliva and periodically exposed to ingested food. As a result of these environmental determinants, the bacterial communities found in the sulcus and on the crown of the tooth are distinct. One feature they do share, however, is that they are biofilms (made up of sessile bacteria). Unattached (planktonic) bacteria are quickly lost from the oral cavity due to the constant washing of biological fluids. Studies of various niches in the oral cavity have shown distinctive microbial profiles for the tongue, tooth surfaces, the buccal mucosa, and the gingival crevice. Saliva is easily sampled, but it contains a mix of bacteria shed from these many ecosystems. Having patients chew paraffin prior to collecting saliva to dislodge bacteria from the tooth surface is commonly used to enrich the tooth-derived bacteria in a saliva sample. When patients are too young to provide a stimulated saliva sample, a sterile tongue blade may be applied to the dorsum of the tongue to collect a bacterial sample. These approaches are often used for convenience in large studies, but collecting the biofilm (dental plaque) from specific sites yields a more representative sample.

There are two commonly used techniques for sampling dental plaque. One approach is to use a curette to scrape the biofilm off the tooth. This is the most commonly used technique for sampling supragingival plaque. For subgingival plaque, the curette method is frequently used, but there are difficulties in sampling deep pockets in patients with periodontal disease. The curette cannot physically be inserted deeper than about 6 mm. The alternative approach is to use an endodontic paper point that consists of a very thin paper rolled into a tapered stick. The paper point can be inserted into a periodontal pocket where it wicks up fluid containing bacteria. A large number of bacteria can be recovered with this approach, and the paper point has the potential to obtain material from even the deepest pocket. This approach may obtain more of the planktonic (or free-floating) bacteria than the bacteria within the biofilm. At this time, very little work has been done to compare these two methods.

IDENTIFYING ORAL BACTERIA

As mentioned, traditional approaches to identifying and classifying oral bacteria involve culturing of the organisms, whereas more recent molecular techniques do not require culturing. Over the last few decades, molecular techniques have acquired a more prominent role in the analysis

of oral bacteria, and they have become the standard. This approach has resulted in the identification of hundreds of new species that have yet to be cultured. With the identification of these new bacteria, many of which could play important roles in oral health or disease, it is time for the reemergence of culturing to further our understanding of oral microbiology. The "taming" of these yet-to-be cultivated bacteria will require the development of new methods or media.

Until recently, there was no one technique that could be used to characterize the full complement of organisms in an oral sample; therefore, a variety of techniques were used to give us a picture of oral microbial communities. All of these techniques offer their own advantages, drawbacks, and biases. Both molecular and cultivation approaches provide vital information for understanding oral bacteria.

Molecular Techniques for Bacterial Identification

The accurate identification and quantitation of complex mixtures of bacteria such as those found in the oral cavity are difficult processes. All techniques that are used for the characterization of mixed cultures of bacteria are potentially subject to some experimental bias. Many of the molecular techniques involve the analysis of DNA isolated directly from oral samples without culturing. Although this will circumvent the bias introduced by culturing, which will overlook more than 50% of the species present in the sample, it can introduce bias due to differential recovery of DNA from the hundreds of different organisms in the sample.

RECOVERY OF BACTERIAL DNA FROM CLINICAL SAMPLES

To extract the DNA from the bacterial cell so it can be analyzed, the bacterial cell wall must be lysed without damage to the DNA. There are several methods used to lyse bacteria, and these methods can result in relative differences in yields from different organisms. Methods that result in high recovery of one group of bacteria may not recover similar amounts of DNA from other groups of bacteria. There are a number of commercially available kits for isolating bacterial DNA, using a variety of methods to lyse the cells, although these kits often target gram-negative bacteria. One common approach is to use detergent and proteinase K to lyse bacterial cell walls. This approach generally lyses a wide spectrum of bacteria, but there is evidence suggesting that some gram-positive bacteria such as the streptococci are resistant to this method. For this reason, it is used primarily for subgingival samples. Over the past few years, the use of bead-beating has become more popular. Bacteria are mixed with a slurry of tiny glass beads. The beads are projected through the bacteria by vibration in an apparatus much like an amalgam triturator. The duration of vibrating and the composition of the beads can be varied to make the process more or less severe. This procedure will lyse even the most sturdy bacteria; however, care must be taken to recover the more fragile bacterial species, such as spirochetes. Centrifugation of the bacteria is generally avoided because DNA from lysed bacteria will be lost. Bead-beating has become the method of choice for isolating DNA from supragingival samples that are likely to contain high levels of streptococci. It is likely that new methods will continue to be developed in an effort to obtain a more representative DNA profile of complex oral bacterial samples.

Preparation of bacterial DNA for molecular analysis after lysis can be accomplished in a number of ways. The simplest approach is to use the lysate directly in PCR or some alternative analysis. But the use of crude preparations can be problematic, so the DNA is usually purified. Commercial kits for isolation of DNA from small sample volumes are available and are widely used. Many of them rely on adhering DNA to silica (ground glass) and removing impurities by washing.

PCR DETECTION

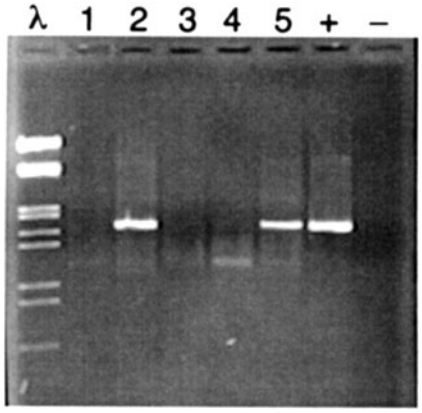
Almost every DNA-based method that does not require culturing uses PCR at some point during the procedure. The amplification of DNA with PCR has allowed the detection of bacteria that are present in very low levels, theoretically as low as one bacterial cell. PCR has also made it possible to do extensive, detailed analysis with very small samples.

One of the simplest approaches to identifying bacteria is to use species-specific primers in a PCR. This allows the specific amplification of DNA from a target species even in the presence of the hundreds of different species. It can be used to detect cultivable and yet-to-be cultivated species.

Primer design

With the availability of DNA sequence data for the 16S genes from thousands of bacteria, it is possible to design species-specific primers without any laboratory work and to do a virtual test of their specificity. First, sequences from closely related species are downloaded from any of several available databases and aligned, and regions that are unique to the species of interest are identified. The candidate sequence for primer construction can then be checked against the entire GenBank database to confirm its uniqueness. The likelihood of there being undiscovered bacteria that contain the presumed unique sequence is becoming smaller each day with the availability of 16S sequences from more species. However, it is still theoretcially possible that unknown species may cross-hybridize with the chosen primers.

FIGURE 3 An agarose gel showing the results of PCRs with ribosomal 16S primers specific for *Porphyromonas gingivalis* on five clinical samples and positive and negative controls. Size markers are shown in lane λ. *P. gingivalis* was detected in samples 2 and 5. doi:10.1128/9781555818906.ch4.f3



PCR assay

The presence or absence of a species-specific PCR product from a sample is generally determined by agarose gel electrophoresis (Fig. 3). Very rapid detection for clinical assays can also be obtained with monitored or real-time quantitative PCR systems that detect the appearance of amplification product during the reaction. Real-time PCR is described in more detail in the next section. It is also common to develop PCR assays based on genes other than 16S by targeting genes that are unique to an individual bacterium. An example is the leukotoxin gene of *Aggregatibacter actinomycetemcomitans*, a bacterium associated with localized forms of aggressive periodontitis.

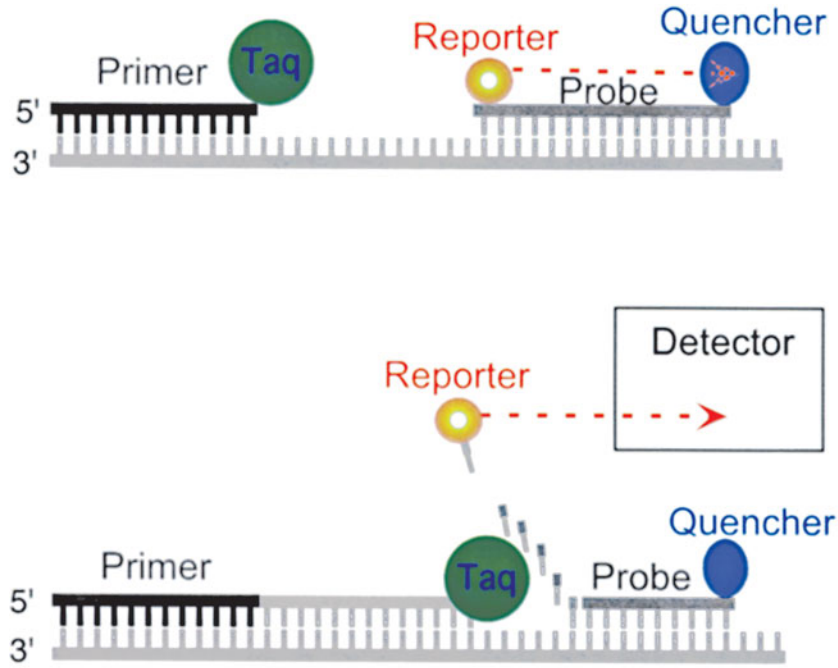
PCR provides an extremely sensitive and efficient approach to identifying oral bacteria. Once unique primers are identified and tested, large numbers of samples can be analyzed. It is still the fastest, simplest, and most sensitive method for detecting the presence of bacteria. This approach, however, does not provide quantitative information. Quantitative real-time PCR, an adaptation of PCR using specialized monitoring technology, allows the monitoring of bacterial levels. This approach can

only monitor one species at a time. These targeted approaches are rapidly being replaced with open-ended methods that can identify any species present in the sample.

QUANTITATIVE PCR

PCR can be used to determine whether an oral sample contains a bacterial species, but it normally does not give quantitative information. This is because the final amount of product in a PCR is not directly dependent on the amount of target DNA in the original sample. However, with the addition of a fluorescent indicator dye, the progress of the reaction can be monitored in real time and quantitative information can be obtained. One commonly used quantitative PCR approach is the TaqMan system (Fig. 4). For the TaqMan assay, in addition to the two primers needed for PCR, an oligonucleotide probe is added. This probe binds to the template internal to the two primers. The probe has two fluorescent dyes attached, a reporter dye at the 5′ end and a quencher at the 3′ end. When the probe is intact, because of the proximity of the two dyes, fluorescence by the reporter is quenched. The TaqMan system relies on the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase, the enzyme used in PCR. During the extension phase of PCR, the probe that is hybridized downstream of the primer is cleaved into nucleotides, releasing the reporter dye from the quencher. The resulting fluorescent signal, which is proportional to the amount of DNA synthesis, is measured by a fluorometer attached to the PCR thermocycler. Samples with unknown amounts of bacteria can be compared to a standard curve to determine the amount of target sequence that was present in the original sample (Fig. 5). Real-time PCR thermocyclers can measure fluorescence in 96 samples simultaneously.

FIGURE 4 The TaqMan method for real-time PCR. See the text for a description of the method. doi:10.1128/9781555818906.ch4.f4



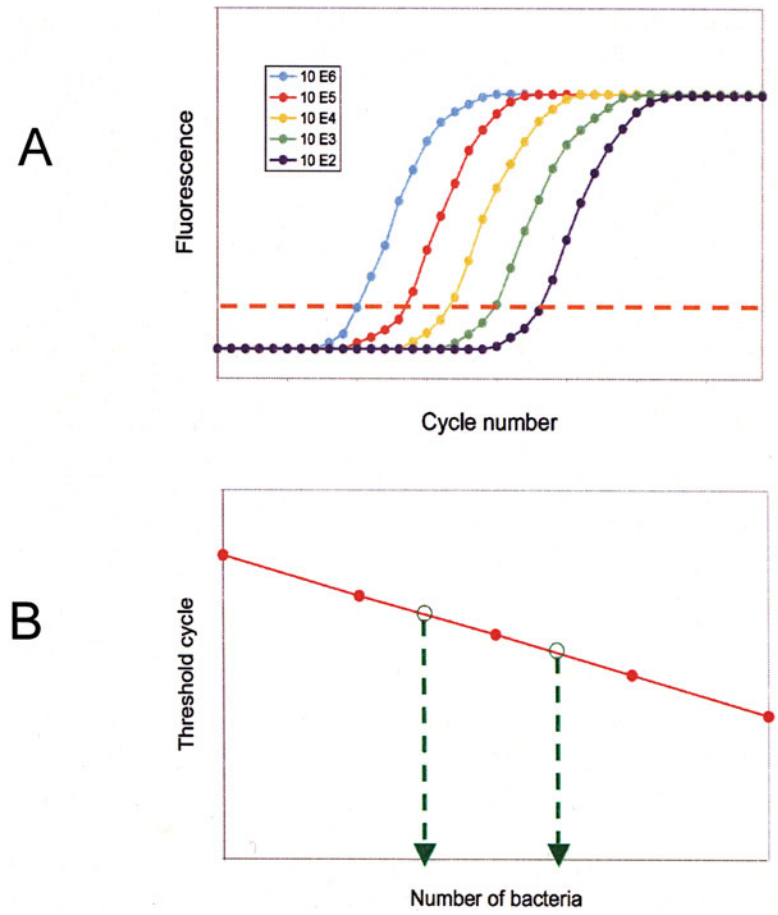


FIGURE 5 (A) Real-time PCR measurement of fluorescence from five 10-fold serial dilutions of bacterial DNA. Fluorescence is monitored at every cycle during the PCR. The number of cycles it takes for the fluorescence to reach the threshold level shown by the dashed line (threshold cycle) is proportional to the quantity of DNA in the standard. Fluorescence is detected earliest in the samples with the most DNA. (B) A standard curve obtained from plotting the number of bacteria in the serially diluted standards against the threshold cycle determined in panel A. This standard curve is used to determine the DNA concentration in samples. The threshold cycle for samples (green circles) is fit to the line and used to calculate the number of bacteria in the sample (green arrows).
doi:10.1128/9781555818906.ch4.f5

An alternative to the TaqMan quantitative PCR approach is to use a dye that fluoresces only when it is bound to double-stranded DNA, such as SYBR green. By adding this dye to the PCR mixture and monitoring the appearance of the fluorescent signal during PCR, the amount of synthesis of new DNA can be measured. Standard curves are generated from known amounts of the target DNA. While easier to carry out and less expensive than the TaqMan method, the SYBR green method is more subject to artifacts resulting from the amplification of extraneous DNA. Therefore, the TaqMan approach is more often used for complex mixtures of similar DNA sequences, such as oral samples.

DNA HYBRIDIZATION ASSAYS

Because of the complexity of the oral microbial community, to study the bacterial etiology of oral infections, it is necessary to identify many species of bacteria from a single sample. Checkerboard analysis is a DNA hybridization assay that was developed to identify many bacterial species from a sample at the same time.

Whole-genomic checkerboard analysis

Whole-genomic, species-specific hybridization probes are used for this analysis. DNA from bacterial samples is attached to nylon membranes in long strips. Species-specific probes constructed from whole-genomic DNA

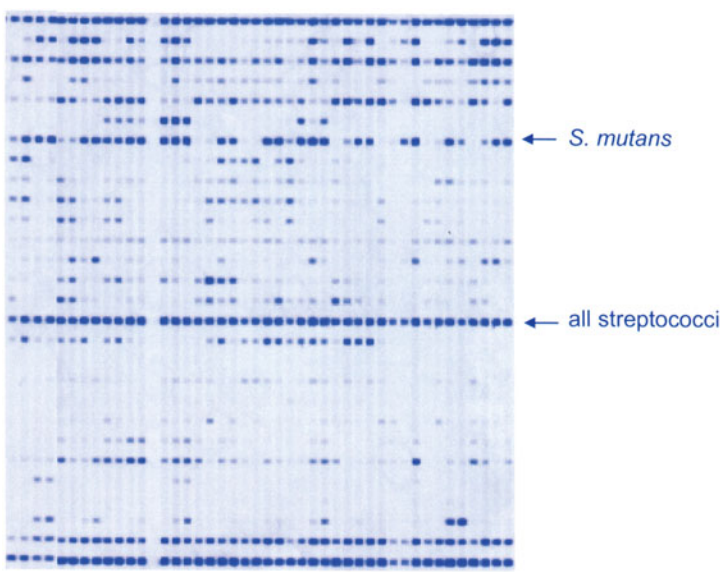
are labeled and hybridized in long strips at right angles to the samples, creating a checkerboard pattern. With this approach, 30 to 40 samples can be analyzed for the presence of 30 to 40 species at the same time. By using controls containing known amounts of DNA, this technique can be semiquantitative.

Only cultivated species can be analyzed with this type of checkerboard analysis, since whole-genomic DNA probes must be constructed. This technique can also be subject to cross-reactivity with species that are closely related to the target species, due to the presence of sequences in the probe that are not species specific, despite the care taken in making the original probes to minimize cross-reactivity. With the checkerboard technique, it is possible to generate large amounts of data in a relatively short time, allowing the comparison of multiple species in studies that involve very large sample sizes.

Oligonucleotide checkerboard analysis

In this modification of the original checkerboard technique, oligonucleotides homologous to 16S ribosomal genes are used as hybridization probes instead of the whole-genomic probes (Fig. 6). Because the probe, rather than the sample, is attached first to the membrane, this approach is sometimes called "reverse capture checkerboard." PCR with labeled universal 16S primers is used to amplify and label samples before hybridization. This approach has the potential to be more specific than the original checkerboard method. However, finding hybridization conditions that work well for the many different probes used in an assay can be difficult.

FIGURE 6 Reverse capture checkerboard hybridization blot. Probes were applied in horizontal rows, and samples and standards were then hybridized in vertical columns. Each spot on the blot represents hybridization of one probe to one sample. The intensity of the spot is proportional to the amount of DNA in the sample. The rows indicated by the arrows represent an *S. mutans* probe that gave positive results for most samples, and a multiple-species streptococcal probe that gave a positive result for every sample tested. (From M. R. Becker et al., *J. Clin. Microbiol.* **40:**1001–1009, 2002.) doi:10.1128/9781555818906.ch4.f6



The reverse capture technique offers the advantage that probes can be made for species that have never been cultivated as long as the sequence of the 16S gene has been determined.

Microarray hydridization

Microarrays are essentially a miniaturization of the checkerboard approach, potentially allowing investigators to probe for extremely large numbers of species at the same time. Microarrays have the potential to identify almost unlimited numbers of species in complex oral samples. The ultimate goal is to establish arrays containing probes to every species found in the oral cavity. Microarrays based on 16S sequences are limited by the amount of sequence diversity in 16S genes and are subject to the same limitations as other hybridization methods.

Of the approaches to bacterial detection discussed so far, both PCR and checkerboard allow the detection of species that have not been cultivated. As long as the sequence of the 16S gene or another unique gene is known, probes or primers can be constructed for the assays. However, neither technique allows the discovery of new species or the detection of unexpected species. To detect new species, another approach, 16S sequencing, is widely used. As sequencing has become cheaper and the amount of information obtained increases, hybridization approaches have become less favored.

RIBOSOMAL 16S CLONING AND SEQUENCE ANALYSIS

Ribosomal 16S cloning and sequence analysis (Fig. 7) is an open-ended molecular technique that allows the identification of any bacterium present in a sample, regardless of whether it can be cultivated or has been previously discovered. This approach relies on PCR amplification of bacterial community DNA with universal bacterial 16S primers that hybridize to all eubacteria. The amplified product, containing 16S sequences from all the bacteria present in the sample, is ligated into an *Escherichia coli* plasmid (vector), and *E. coli* cells are transfected with the plasmid. Each successfully transformed *E. coli* cell now includes the 16S gene from one bacterium. The transformed *E. coli* cells are plated onto media and grown. The vector contains a reporter gene system so that colonies with successful integration of a bacterial 16S gene appear white (those without inserts are blue). DNA from the *E. coli* colonies with inserts of bacterial 16S DNA is sequenced. Sequences are matched to 16S profiles in public databases for identification of bacterial species. Sequences that do not match any sequence in a public database may indicate the discovery of a new species. The number of bacteria that have been found in the oral cavity has more than doubled from the results of this approach. It is possible to use 16S cloning and sequencing as a quantitative technique if conditions that maintain the original composition of the 16S genes are used.

Subgroups of bacteria can be targeted for cloning and sequencing by using a subgroup-specific primer prior to cloning. This is analogous to using selective media to culture specific groups of bacteria. The targeted technique has been useful in identifying many of the *Bacteroides* spp. and treponemes that are present at low levels in the subgingival biofilm.

Over the last few years there has been a major effort to characterize the human microbiome, including the National Institutes of

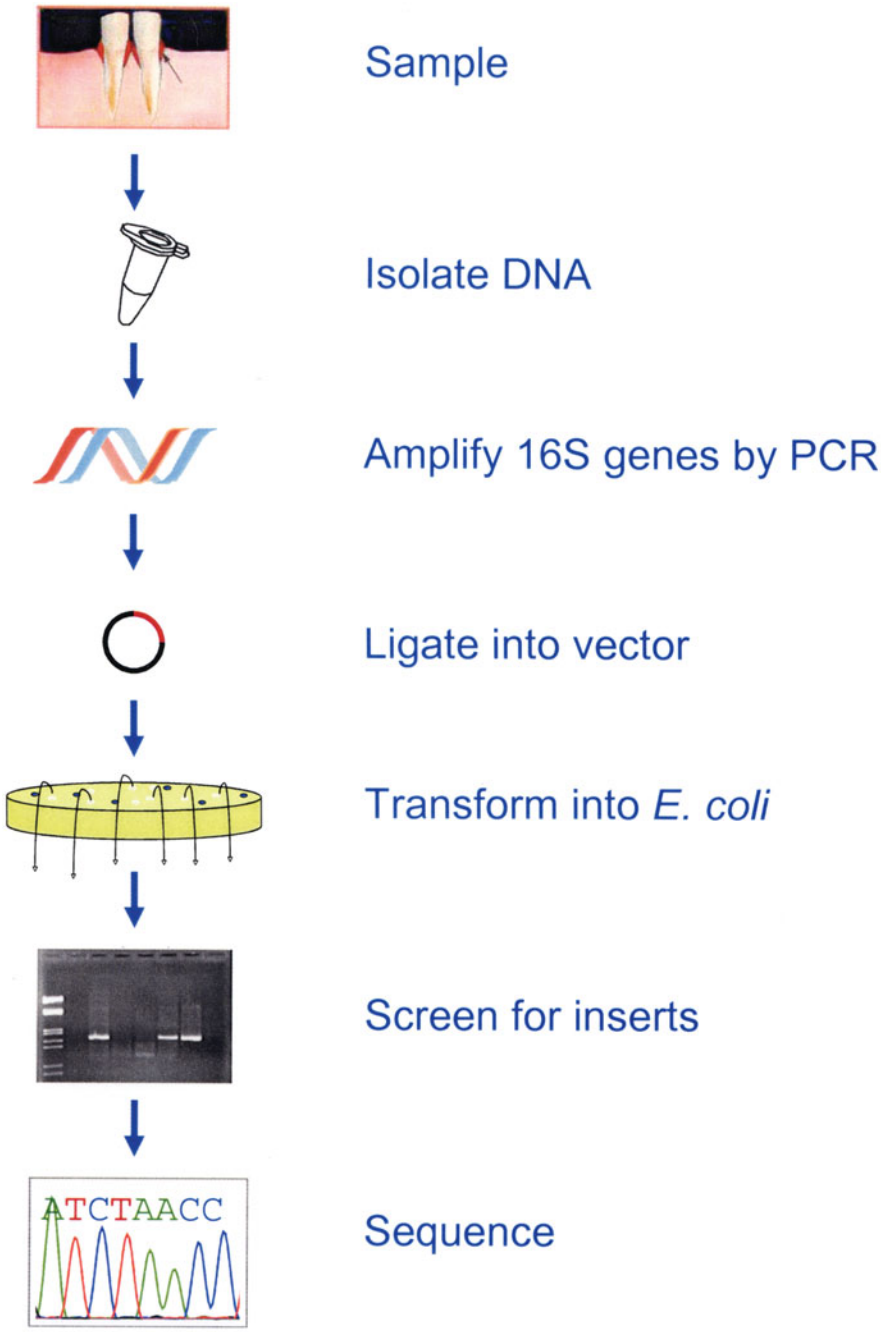


FIGURE 7 Cloning and sequencing of bacterial 16S genes. DNA is isolated from samples, and the 16S rRNA gene is amplified from all the bacteria present in the sample. The 16S fragments are ligated into an *E. coli* plasmid (vector), and *E. coli* cells are transfected with the plasmid. Each successfully transformed *E. coli* cell now includes the 16S gene from one bacterium. The transformed *E. coli* cells are plated onto media and grown. The vector contains a reporter gene system so that colonies with successful integration of a bacterial 16S gene appear white (those without inserts are blue). DNA from the *E. coli* colonies with inserts of bacterial 16S DNA is sequenced for identification of bacterial species. doi:10.1128/9781555818906.ch4.f7

Health-sponsored Human Microbiome Project. With this intense effort, the oral cavity is one of the most comprehensively characterized regions of the human body. The development of comprehensive oral 16S databases such as CORE (www.microbiome.osu.edu) and the HOMD (www.homd.org) has allowed accurate indentification of bacteria at the species level from 16S sequence data. In contrast, the lack of databases for other areas of the human body has made it difficult to analyze the bacterial composition beyond the genus level, and this may not be sufficiently informative, since we know that species within the same genus can have very different pathogenic or other biologic potential.

NEXT GENERATION SEQUENCING OF 16S rRNA GENES

Next generation sequencing refers to a family of modern, high-throughput sequencing technologies that have recently been applied to characterizing bacteria from oral samples. The most common version utilizes the Roche 454 sequencing technology, which is also known as pyrosequencing.

In the process of pyrosequencing, as in 16S gene cloning, whole-genomic DNA is isolated from oral samples and PCR is carried out with universal primers that amplify 16S genes from all species. However, the primers for pyrosequencing have two additional components not present in cloning primers. Immediately adjacent to the 16S complementary region is a short "barcode" sequence. This sequence varies between versions of the primer that are used on different samples. Multiple samples can therefore be mixed together and sequenced in a single lane of the sequencer. After the data are generated, the barcode sequences allow the assignment of each 16S sequence to the appropriate sample. During the preparation, individual DNA molecules are attached to beads and amplified on the bead. The beads are distributed on a plate so that the entire mixture of DNA can be sequenced together, avoiding the labor-intensive process of cloning each molecule separately. Each primer also contains an adapter sequence that is used during the 454 process. The Human Microbiome Project (http://www.hmpdacc.org) has published suitable 16S primer and barcode sequences on the web. Human Microbiome Project primers are available for two different regions of the 16S gene.

Currently, 454 sequencing runs can generate up to a million sequence reads in a run with read lengths of 500 to 600 bases. Improvements are increasing both the number of sequences per run and the read length. Therefore, the technology allows bacterial populations to be surveyed at orders-of-magnitude-greater depth than is possible by clone sequencing.

The large number of sequence reads generated with the 454 method can be processed through a bioinformatic pipeline to identify the bacteria present in the sample and the relative amount of each. Data sets must be filtered to remove bad sequences and artifacts. Early studies done before these filters were routinely used vastly overestimated the number of species found in each sample.

Although less used up until now, other high-throughput sequencing technologies can be employed for 16S sequencing and microbial community analysis. One is the Ion Torrent system, which is a newer technology with some similarities to 454 sequencing that is developing rapidly. Another is the Illumina system that can give very high numbers of sequence reads (see below) but with somewhat shorter read length.

METAGENOMIC SEQUENCE ANALYSIS

A metagenome refers to the mixture of all genomes present in a population of organisms. Scientists have shown, through efforts such as the Global Ocean Sampling Expedition, that it is possible to derive insights about bacterial populations (including uncultured organisms) by sequencing and analyzing metagenomes much as one would for individual organism genomes. Such efforts have been greatly aided by the high throughput of next generation sequencing and a large decrease in the cost.

The general protocol for metagenomic sequencing is similar to next generation sequencing of 16S genes except that random fragments of DNA are prepared instead of targeted 16S sequences. This typically involves ligation of specific adapter sequences to the genomic DNA. The present method of choice is Illumina sequencing. The current version allows the decoding of billions of bases per run, with paired end lengths of over 100 bases. Sequences can be read from each end, doubling the amount of sequence read from each fragment.

Analysis of the enormous data sets from metagenomic sequencing can be carried out by several approaches. One is to analyze sequence reads directly. Each read is compared to DNA sequence databases to identify the function of the gene containing that sequence. Comparing each sequence to whole-genomic databases can identify the source of each fragment. There are currently hundreds of whole-genomic sequences from oral bacteria available, with more being continually added. Reads that code for ribosomal RNAs can be identified and searched against 16S databases to determine the origin. An analysis of the taxonomic character of the sample should give insight into the population, although the resolution may be lower than with methods that produce longer reads.

It is also possible to examine the raw reads by looking at their coding potential. Since bacterial genomes tend to be fairly tightly packed with protein-coding genes, many of the raw reads will be derived from protein-coding regions. They can be searched by a module of the BLAST program called BLASTx. This module translates nucleic acid queries in all six frames, looking at sequence similarities to a protein sequence database such as Genbank nr. This method can find similarities that might not be apparent at the nucleic acid level. With this approach, it is possible to get not only taxonomic information but also functional information about the encoded proteins.

If the metagenomic sequencing is performed to a sufficient depth, it may be possible to perform sequence assembly with the next generation sequence reads. In this process, overlaps between reads are identified and used to discern the sequence of longer fragments, termed contigs. Sequence assembly algorithms have been extensively used on whole-genome sequences from isolated organisms. In some cases, the same algorithms are applied to metagenomes, though there are some issues that can make metagenome assemblies difficult. Because of these issues, there has been much work recently on metagenome-specific assembly algorithms. A successful assembly of large contigs can allow the study of complete genes and operons and can aid in taxonomic and functional identification.

The amount of sequence information that can be generated continues to increase at a rapid pace. This is shifting the focus to whole-genomic approaches that can better define species and identify metabolic processes

that are present under curtain conditions. More-detailed sequence information will allow identification at the subspecies or strain level. This will make it possible to determine differences within species and the overall genomic composition of mixed populations, furthering our understanding of how different bacteria work together in a polymicrobial community to establish a healthy environment or cause disease.

NAMING OF BACTERIA AND MOLECULAR ANALYSIS

The classification and naming of bacteria are ongoing processes that are continually being refined and updated. There is a Judicial Commission that decides on any changes or additions to the current list of known bacteria, and proposed changes in the classification of bacteria are continuously being considered and acted on. Official recognition of a bacterium occurs when it has been described in the *International Journal of Systematic and Evolutionary Microbiology*, based on observations of the organism in culture.

The many species of bacteria that have been detected based only on their DNA sequence do not have official names. So uncultivated bacteria are unofficially named, usually with the genus of their closest neighbor and a letter and number code, such as *Veillonella* sp. oral clone X042.

Bacterial names are often changed when new information about genetic lineage is discovered. For example, *Fusobacterium alocis* was originally named on the basis of its phenotypic similarity to the fusobacteria. It was later given a genus of its own, *Filifactor alocis,* when 16S sequencing showed it should be placed in a different phylum than the fusobacteria.

DIRECT OBSERVATION OF ORAL BACTERIA

Oral bacteria can be directly observed by microscopy. Basic morphotypes and motile and nonmotile bacteria can be distinguished in unstained specimens viewed under phase-contrast or dark-field microscopy. The Gram stain further divides bacteria into two groups based on cell wall structure. Species-specific probes are used to label bacteria so that individual species or groups of bacteria can be visualized and identified. For example, antibodies raised by injecting cultured bacteria into animals can be fluorescently tagged and used to label bacteria for visualization by microscopy. Bacteria can even be counted as a method of quantitation. This approach is very sensitive to variations in technique, and since the development of oligonucleotide probes, it is seldom used.

Fluorescent in situ hybridization (FISH) is a technique that allows targeted labeling of bacterial cells with fluorescently labeled oligonucleotide probes (Fig. 8). Probes that hybridize to ribosomal 16S RNA are most commonly used. The 16S rRNA is an excellent target for labeling, since there are many ribosomes in a cell, and the 16S sequence has variable regions that can be used for construction of probes that are unique for individual bacteria or groups of bacteria. FISH is performed on glass slides or microscopic wells, and the labeled cells are visualized under a microscope. Conventional epifluorescence microscopes are used to visualize monolayer slides. However, thicker samples, such as biofilms, can be

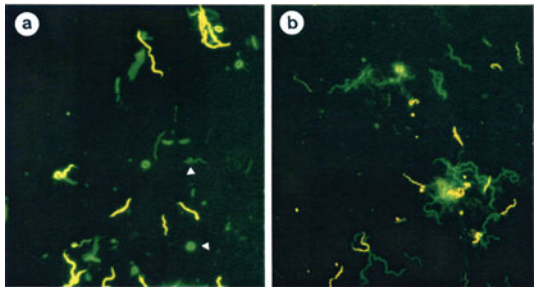


FIGURE 8 FISH of subgingival plaque material. Multiple colors can be used simultaneously, and the specificity of probes can be controlled. (a) Microphotograph showing simultaneous hybridization of a universal eubacterial probe (green) and a spirochete-specific probe (yellow). (b) Microphotograph showing hybridization with two different spirochete probes, one yellow and one green. (From A. Moter et al., *J. Clin. Microbiol.* **36:**1399–1403, 1998, with permission.)
doi:10.1128/9781555818906.ch4.f8

viewed using confocal laser-scanning microscopy. Serial optical sections are collected from a thick specimen, and the serial images are used to reconstruct a three-dimensional image. Multiple species-specific probes, each labeled with differently colored fluorescing compounds, can be used at the same time. This approach is useful for determining the relationship of multiple species or groups of bacteria in a biofilm. The specificity of the probe is critical to the accuracy of FISH, and it can be challenging to differentiate closely related species.

CULTIVATION OF ORAL BACTERIA

The diverse bacterial inhabitants of the oral cavity have a broad spectrum of physical and chemical requirements. To successfully grow bacteria in the laboratory, cultivation conditions must be adjusted to suit these varied requirements.

The general scheme for the cultivation of oral bacteria (Fig. 9) is to collect a sample and place it immediately into a transport medium. The medium is taken to the laboratory for processing. Bacteria tend to clump together, so they are usually dispersed by agitation or sonication prior to plating onto petri dishes. There are many more bacteria in an oral sample than could be grown on one plate, so serial dilutions are generally carried out before plating to obtain discrete colonies rather than a solid lawn of bacteria. The natural habitat of oral species is at approximately 37°C, so oral bacteria are usually grown in incubators. Further specific conditions for cultivation are described below. Once bacteria are grown from a clinical specimen, they may be isolated for growth in pure culture by picking out a colony and subculturing it.

Oxygen Requirements

The amount of oxygen in the atmosphere is a critical determinant of bacterial growth. Most bacteria in the oral cavity are either facultative anaerobes that can grow in the presence or absence of oxygen or anaerobes that can grow only in a reduced oxygen atmosphere. Strict anaerobes cannot tolerate

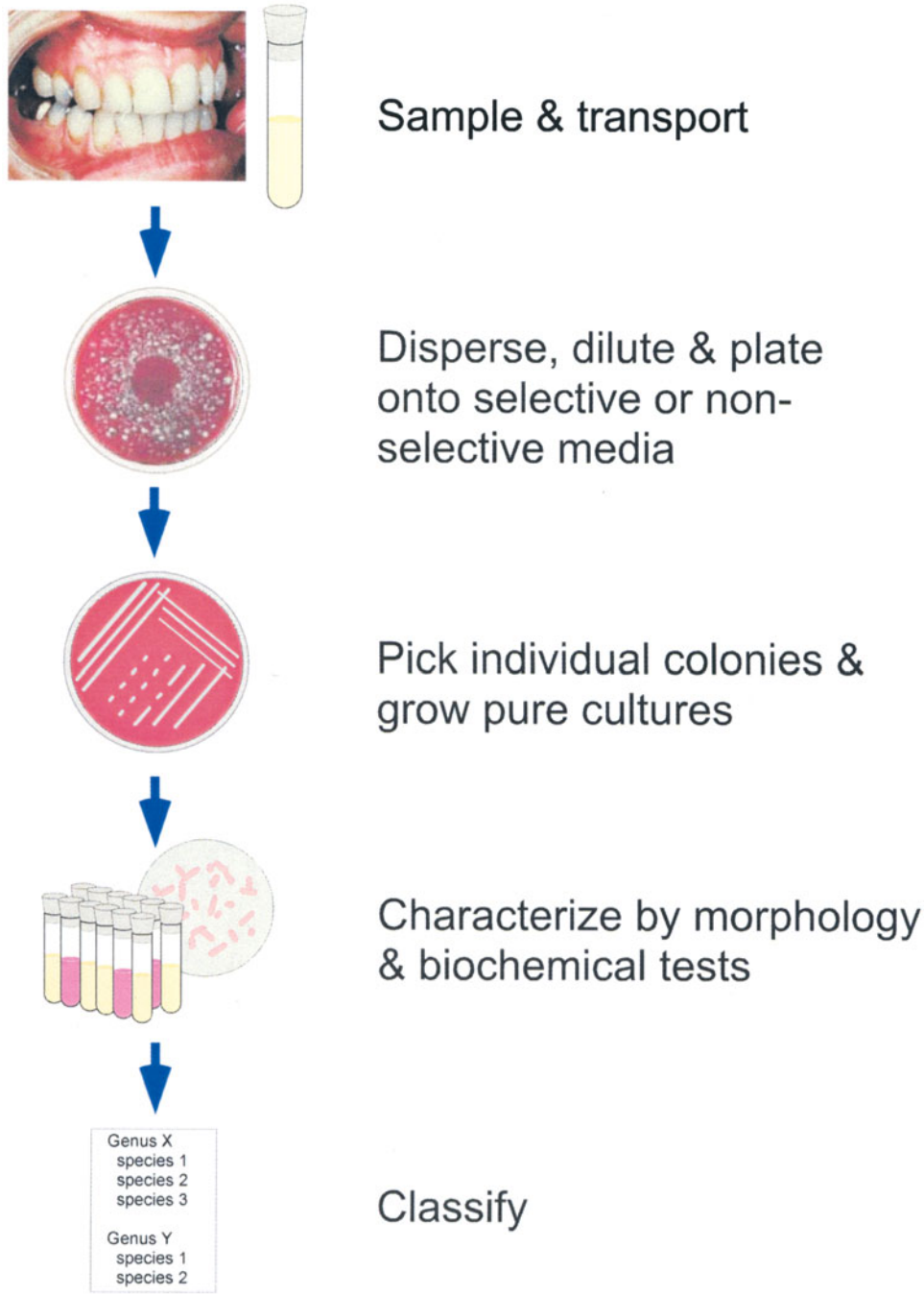


FIGURE 9 Process for identification and classification of bacteria by culturing. Samples are collected and placed immediately into a transport medium. Bacteria are dispersed by agitation or sonication prior to being plated onto petri dishes. There are many more bacteria in an oral sample than can be grown on one plate, so serial dilutions are generally carried out before plating to obtain discrete colonies rather than a solid lawn of bacteria. Bacteria may be isolated for growth in pure culture by picking out a colony and subculturing it. Bacteria are characterized and classified by biochemical tests and other methods. doi:10.1128/9781555818906.ch4.f9

any oxygen, and microaerophilic anaerobes require low levels of oxygen. In addition, many capnophilic bacteria, such as the periodontal pathogen *A. actinomycetemcomitans*, require carbon dioxide in the atmosphere.

Many facultative bacteria important in dental caries, such as *Streptococcus mutans* and lactobacilli, can be grown in normal atmospheric conditions. But subgingival species are almost exclusively anaerobic and must be grown in special chambers that provide a reduced oxygen environment. Most anaerobic growth chambers contain low levels of CO_2 to promote the growth of capnophilic bacteria. Care is often taken to remove oxygen from the media used to transport the bacteria from the mouth to the anaerobic chamber. This is accomplished by boiling the media or flushing it with oxygen-free gas. Prereduced media may be purchased from commercial sources. Many oral anaerobes will grow even after being stored in transport fluid for hours. There are several methods for creating and maintaining an oxygen-free environment. After inoculation, plates can be placed into an anaerobic jar or bag. The container is sealed, and the oxygen is removed with a chemical reaction involving hydrogen generation and formation of water from hydrogen and oxygen in the presence of a palladium catalyst. Indicator strips are generally used to monitor for oxygen. The jar or bag is placed in a 37°C incubator. The use of small containers to grow anaerobic bacteria requires that any manipulations are carried out on the bench top in an aerobic environment. This is generally not a problem for all but the most fastidious anaerobes. Anaerobic chambers are also available for manipulation and growth of anaerobic bacteria. Flexible plastic bags that are big enough to contain a small 37°C incubator can be used. These are entered through gloves attached to the chamber. This makes it easy to manipulate cultures without disturbing the atmosphere. Rigid gas-tight cabinets can also be used (Fig. 10). These cabinets are often heated to 37°C and can therefore serve as an incubator as well as a place to work on the bacteria. They also may contain gloveless

FIGURE 10 An anaerobic chamber. This rigid gas-tight cabinet is heated to 37°C and serves as an incubator as well as a place to work on the bacteria. It contains gloveless sleeves that seal around the operator's arms. This eliminates the dexterity problem associated with gloves that tend to be cumbersome. The gloveless cabinet requires that the air in the sleeves be evacuated before opening the entrance to the chamber. The airlock doors are open and visible in the picture.
doi:10.1128/9781555818906.ch4.f10

sleeves that seal around the operator's arms. This eliminates the dexterity problem associated with gloves that tend to be cumbersome. The gloveless cabinet requires that the air in the sleeves be evacuated before opening the entrance to the chamber.To maximize recovery of the greatest number of bacteria, both aerobic and anaerobic cultures can be established from the same samples.

Culture Media

Cultivation on broad-spectrum, nonselective media such as blood agar supports the growth of many oral species. An oral sample typically produces a bewildering array of colony morphologies. It can be difficult to sort out individual species from the mix, and species that comprise only a small percentage of total bacteria may not even be seen. Selective media that contain ingredients that inhibit the growth of all but a few species can be very useful for isolating individual species. For example, mitis salivarius agar with the addition of the antibiotic bacitracin (MSB agar) is highly selective for mutans group streptococci. The use of selective media to enhance recovery is often necessary for the detection of bacteria, such as *Bacteroides* spp., that are present in low levels.

Some bacteria have specific requirements for nutrients and can be difficult to culture until those requirements are determined and the media are supplemented. *Tannerella forsythia*, an organism that is strongly associated with periodontitis, has a metabolic requirement for *N*-acetylmuramic acid, a component of the cell wall synthesized by most other bacteria. It was not until this requirment was recognized that it was possible to isolate *T. forsythia* in culture. The particular nutritional requirements of many species have so far eluded oral microbiologists; based on data from 16S cloning and sequencing, less than half of known oral bacteria have been cultured.

SINGLE-CELL GENOMICS

An additional way to study uncultured bacteria is to perform genome sequencing of single cells. This technique involves isolating single bacterial cells and then amplifying their genomes by a technique called multiple displacement amplification. The genome can be sequenced by techniques similar to those used for genome sequences of pure cultures. Single bacterial cells may be isolated by a number of different techniques. One study used a specially designed microfluidic chip to isolate uncultured oral bacteria from phylum TM7, but other studies have used flow sorting or micromanipulation to isolate cells.

Cells of particular interest, such as currently uncharacterized organisms or those implicated in pathogenesis can be identified by in situ hybridization as discussed earlier, with specific antibodies, or, following genome amplification, by PCR and sequencing of the 16S rRNA gene.

As with metagenome sequencing, sequence assembly algorithms and programs designed for cultured organisms can be used on single-cell genomes. Analysis of gene profiles gives insight into the metabolic functions present in these poorly understood species. The availability of genomes from uncultured organisms will also aid in the analysis of metagenomic data. In addition, the genomic sequence may help to identify metabolic deficiencies that have made culturing difficult and therefore may help to design successful conditions for culturing the bacterium.

Classification of Cultured Bacteria

Before the development of molecular approaches to bacterial classification and phylogeny, bacteria were classified exclusively based on a series of morphological and biochemical tests. These tests are still an important part of the classification process. After a new bacterium is obtained in pure culture, it is first described morphologically. Colony morphology is documented; the size, shape, and color of colonies are noted. Growth of the bacterium on a different medium, however, can produce a totally different morphology. Microscopic examination is used to determine the shape of individual cells (rods, spheres, etc.) and whether the bacterium is positive or negative by Gram staining.

Bacteria are also characterized based on their biochemical profiles. One method is to test for the presence of metabolites using gas chromatography. As with colony morphology, the results may be influenced by the culture conditions. This method is less commonly used today, partially because specialized equipment is needed to perform the analysis. Other approaches test for activity of several enzymes or the ability to ferment various sugars. Commercial kits are sold for these purposes. The biochemical profiles of even closely related species may differ, and individual species can be characterized by their distinctive fingerprints.

Using cultivation-based techniques, microbiologists were able to make tremendous progress in characterizing and classifying bacteria. The advent of molecular techniques has allowed the refinement of bacterial classifications based on phylogeny, and now new cultivation techniques are needed to move ahead with the study of the yet-to-be-cultivated species. Over the last few years, several important oral bacteria have been cultured for the first time. These include members of the previously uncultured group *Synergistes*.

Antibiotic Susceptibility

Antibiotic susceptibility tests can be performed on cultured bacteria. Most commonly, the disk diffusion test is used. Disks impregnated with antibiotic are applied to a petri dish that has been inoculated with the bacterium to be tested, and the plate is incubated. The zone of growth inhibition around the disk is proportional to the susceptibility of the bacterium to the antimicrobial agent. Antibiotic susceptibility testing is used only rarely in dentistry, usually for patients with serious odontogenic infections that are refractory to empirical antibiotic therapy.

OTHER ORAL MICROORGANISMS

Although this chapter has focused on bacteria, there are also viruses, fungi and other eukaryotes routinely found in the oral cavity. While much is known about the fungus *Candida albicans* (chapter 17) and several viruses (chapter 16), these groups have not been systematically characterized. This is in large part due to technical difficulties. The majority of oral viruses are bacterial viruses or phage, and viruses may be even more diverse than oral bacteria. Because there is no gene that is found in all viruses, there is no common target, like the 16S gene of bacteria, that can be exploited. Instead, attempts have been made to physically isolate the viruses before isolation of DNA and sequencing. With the development of high-volume sequence techniques, viruses may be identified by analysis of metagenomic sequencing data.

KEY POINTS

The oral microbiota is one of the most diverse microbe communities known. More than 700 species of bacteria have been identified in the oral cavity. Many oral species have never been grown in culture.

Bacteria can be identified with both culture-based and molecular techniques. Of course, only molecular techniques allow the detection of yet-to-be-cultivated species.

Molecular identification methods for bacteria are most often based on sequence analysis of the ribosomal 16S genes. Ribosomal gene analysis also allows evolutionary relationships to be inferred and so is useful for bacterial classification.

Common techniques for molecular detection of bacteria:
- PCR with specific primers
- Quantitative PCR
- DNA hybridization assays (checkerboards)
- Ribosomal 16S amplification/cloning and sequence analysis
- High-throughput sequencing of genomes
- FISH and microscopy

Cultivation of many oral species requires special conditions such as an anaerobic environment, CO_2 in the atmosphere, and incubation at 37°C. Some oral bacteria have specialized nutritional requirements, and this may be the reason many have not yet been cultivated. New culture techniques must be developed to grow the many species that have been identified but not yet cultured.

Cultivated bacteria may be identified and classified based on morphology and biochemical tests.

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Oral Microbial Ecology

Howard F. Jenkinson and Richard J. Lamont

INTRODUCTION

An eclectic and diverse assemblage of microorganisms call the oral cavity home. At least 700 species of bacteria are supported by the habitats within the mouth, such as the surfaces of the teeth and oral mucosa, although in any one individual the number is closer to 200. Distinct microbial communities accumulate on these surfaces through successive colonization events. On the tooth surfaces, for example, early or primary colonizers are mainly streptococci and actinomyces. Over time, the proportions of these gram-positive facultatively anaerobic bacteria decrease and eventually gram-negative anaerobes become more established, especially at the interface of the teeth and gums (gingival margins). The resulting mixed-species community, familiar as dental plaque, is a complex and dynamic entity, and populational shifts within plaque contribute to oral diseases. Overrepresentation of bacteria tolerating more acidic conditions (acidophiles) increases susceptibility to caries, whereas elevated numbers of gram-negative obligately anaerobic bacteria, particularly in the gingival crevice, can lead to periodontal disease.

Although an immense variety of organisms are displayed in the oral microbiota, many more bacteria pass through the oral cavity than are retained. A cursory comparison between the bacterial inhabitants of the mouth and those within the small intestine will attest to this. Successful colonizers of the oral cavity must, therefore, possess certain attributes that allow them to survive and thrive in this unique environment (Fig. 1). The ability of microorganisms to adhere to oral surfaces is especially important in the oral system. Continuous flow of saliva together with the mechanical shearing actions of the tongue and lips act to dislodge and expel bacteria, so adhesion is essential for retention. Furthermore, the mouth can experience rapid and dramatic changes in physical parameters such as temperature, oxygen tension, pH, osmolarity, and nutrient availability. Bacteria that are capable of sensing and responding to these stresses have a selective advantage over those that are less adaptable and are more likely to increase in number. Organisms that lack a critical number of these survival attributes are likely to be present in the mouth only temporarily.

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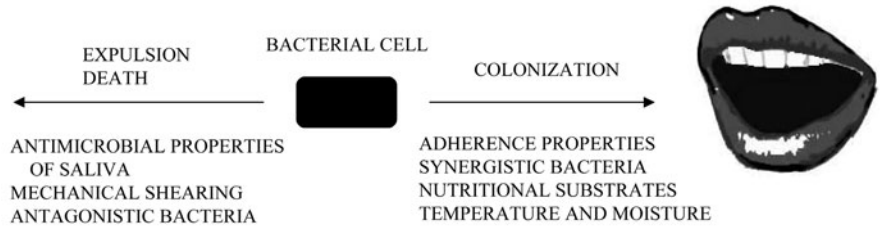

FIGURE 1 Constraints acting on bacteria entering the mouth. Certain factors favor colonization while others tend to eliminate bacterial cells.
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ACQUISITION OF ORAL BACTERIA

The oral cavity generally lacks significant bacterial colonization at birth. However, beginning shortly after birth, bacteria are continually introduced into the mouth from contaminated animate and inanimate objects. While the majority of these bacteria are transients, successful oral colonizers will be obtained from exogenous saliva. Primary caregivers or other close contacts, such as siblings, are usually the source of this bacterium-containing saliva. After a few months, most mouths possess a microbiota consisting of recognizable oral organisms. The next major ecological event is the eruption of the deciduous teeth at around 6 months of age. The appearance of these hard nonshedding surfaces allows the colonization of organisms that are exquisitely adapted to this environment. Bacteria such as the oral streptococci and actinomyces thus comprise a significant proportion of the organisms in dental plaque on tooth surfaces. The oral microbiota continues to develop, changing with age in composition and overall activity. Hormonal changes during puberty can contribute to increased colonization by groups of gram-negative anaerobes and spirochetes, with some hormones possibly acting as nutritional sources. In adults, gradual age-related changes, physical exercise levels, and psychological stress can all influence the numbers or proportions of oral bacteria, often through effects on immune function or salivary flow rate. Similarly, lifestyle events such as smoking, frequency of carbohydrate consumption, or pregnancy can affect the microbial composition. In later years, the decline in salivary flow rate and in general health status leads to changes in microbial colonization, such as increased carriage of the yeast *Candida albicans*, with subsequent higher risk of oral candidiasis.

The excessive buildup of dental plaque is unsightly and malodorous and causes inflammation of the gingival tissues (gums). Moreover, among the oral microbiota there are a number of bacterial species that are considered undesirable because they clearly contribute to oral disease. Nonetheless, many of the microorganisms present within plaque may not be especially undesirable and in fact provide a protective function for the host. There are many examples of how nonpathogenic resident (commensal) bacteria can restrict establishment and growth of pathogenic organisms in microbial communities, such as by competing for adhesion receptors and by producing toxic metabolic products. The temporal acquisition of oral streptococcal species by infants provides an excellent example of how components of the normal microbiota can be beneficial to the host. Shortly after birth, the infant acquires an oral microbiota on the mucosa that after some weeks consists generally of *Streptococcus*,

Haemophilus, and *Neisseria* species. Many of these species, e.g., *Streptococcus mitis* and *Streptococcus oralis*, etc., produce immunoglobulin A (IgA) proteases that specifically cleave secretory IgA antibodies. It is speculated that this provides an early selective advantage for survival of these bacteria in an environment rich in secretory IgA from the mother's milk. These species of bacteria are able to colonize the mucosal surfaces. It is not until after the emergence of the first teeth that species such as *Streptococcus sanguinis* and *Streptococcus mutans* are found in the oral cavity. Colonization by *S. sanguinis* is dependent on the presence of teeth and occurs at a median age of about 9 months. This is referred to as the window of infectivity, and levels of these bacteria increase with the age of the infant, as more teeth emerge. Colonization by *S. mutans*, the principal agent associated with dental caries, generally occurs much later (around 26 months) and is associated with a decline in *S. sanguinis* numbers. Thus, these two bacteria antagonize or compete with each other. Early colonization of infants with *S. sanguinis* results in delayed colonization by *S. mutans* and reduces the incidence of carious lesions. This is an important concept because it implies that initial adhesion events (primary colonization) during the acquisition phase can shape the microbial plaque community and directly influence oral disease potential.

COLONIZATION BY ORAL BACTERIA

A microorganism first entering the oral cavity is all at sea, buffeted in the waves and currents of saliva that continually flow through the mouth and across the oral surfaces. As discussed in chapter 3, saliva contains direct antimicrobial components as well as molecules (agglutinins) that can aggregate or clump bacterial cells such that they are more readily removed from the mouth by expectoration or swallowing. To resist this innate host protective mechanism, and to resist physical dislodgement by mechanical shearing forces, oral bacteria adhere to the available surfaces. Localization at surfaces provides the additional advantage that such sites often concentrate nutrients and so will promote bacterial growth. In this regard, a tooth in the mouth is similar to a rock in a stream; both sites favor bacterial colonization and the accumulation of cells into multiple-species communities that are known as biofilms.

The oral cavity provides a variety of surfaces for bacterial attachment and colonization. These include the saliva-coated surfaces of the teeth, along with the epithelium of the cheeks, gums, and tongue. Moreover, as these surfaces become colonized by bacteria, adhesion between cells of different species becomes important. Such interbacterial coaggregation (or coadhesion) drives the temporal development of mixed-species biofilms on oral surfaces and facilitates nutritional relationships among organisms. To fully appreciate the genesis and development of the oral ecosystem, it is necessary to consider what bacteria stick with, how they stick, what they stick to, and what happens after they are stuck.

Surface Structures and Molecules Involved in Adhesion

The surfaces of both gram-positive and -negative bacteria are structurally complex (see chapter 1). Proteins, glycoproteins, lipoproteins, lipopolysaccharides (gram negative), and lipoteichoic acids (gram positive) can all be present to varying degrees. Protein subunits can be assembled

into flagella, which provide motility, while other subunits (designated fimbrillins or pilins) can be assembled into thread- or hair-like appendages known as fimbriae or pili. Another type of cell surface structure, known as a fibril, is much shorter than a fimbria or pilus. Fibrils are commonly found on oral streptococcal cells and extend up to 200 nm from the cell surface, as opposed to fimbriae that are up to 1 mm in length. Fibrils have tapered ends and may be peritrichous (evenly distributed over the bacterial cell surface) or localized to a lateral crust or polar tuft. Some bacteria are also closely surrounded by a loose polysaccharide layer known as a capsule. The question then arises: which of these surface-exposed molecules or structures are responsible for bacterial adhesion? The answer, unfortunately, is not a simple one. Adhesion is unlike motility, for example, because it is not usually defined by one specific surface component. Rather, bacteria utilize different and often multiple surface structures for adhesion, and any of the available surface components can be involved in the adhesive process. The contribution of an individual component to the overall binding process can involve a physicochemical aspect, often charge or hydrophobicity dependent, or a more specific ligand-receptor type aspect (as discussed in more detail below). From a bacterial perspective, the presence of multiple adhesion-mediating molecules (designated adhesins) with distinct binding partners (receptors) on host surfaces is likely both to improve the chances of adhesion occurring and to result in stronger binding.

Although all bacterial surface components could potentially be involved in adhesion, certain trends have emerged for the well-studied oral bacteria. Major adhesins are often fimbriae or major outer membrane proteins in gram-negative bacteria and fibrils, fimbriae, pili, or prominent cell wall-linked surface proteins in gram-positive bacteria. *Actinomyces oris*, for example, possesses two major classes of fimbriae, type 1 and type 2. Type 1 fimbriae are associated with adhesion to the tooth surfaces through interactions with salivary acidic proline-rich proteins and statherin deposited within the salivary pellicle that coats enamel. Adhesion may not be mediated by the fimbrial subunit protein itself but instead by accessory proteins displayed on the fimbrial support structure. The adherence-promoting activity of the proline-rich proteins is enhanced after deposition on the enamel surface, probably through conformation changes exposing previously hidden binding domains (cryptitopes). Type 2 fimbriae are involved in adhesion through a lectin-like mechanism to glycosidic (sugar) receptors on epithelial cells, and in this case, the fimbrial structural protein is the adhesive unit.

Streptococcal adhesion is also a complex process and streptococcal cell surfaces are decorated with multiple adhesins with differing specificities. Many of these adhesins are conserved across species, a testament to their importance to the organisms. *Streptococcus parasanguinis* fimbriae (composed of a protein named Fap1) and *Streptococcus gordonii* fibrils (composed of a protein designated CshA) are involved in adhesion to saliva-coated substrates. Fap1 is a serine-rich glycoprotein required for both adhesion and biofilm formation by *S. parasanguinis*. The CshA protein forms the fibrillar component in several species of mitis group streptococci and also mediates attachment of these cells to some other oral bacteria and to human fibronectin. The tufts of fibrils on *Streptococcus*

cristatus are associated with the binding of these bacteria to *Fusobacterium nucleatum* to form characteristic corncob aggregates of bacteria seen in mature dental plaque (Fig. 2). *S. mutans* adhesion to the salivary pellicle appears to involve a major surface protein of the antigen (Ag) I/II family. This protein is well conserved across a number of streptococcal species and possesses multifunctional domains that have been adapted by different species for binding to salivary pellicle receptors, salivary agglutinins, and other bacteria. They thus provide a pivotal role in the development of dental plaque that is usually initiated by the primary colonizing streptococci. The AgI/II protein of mitis group streptococci is responsible, in part, for initial adhesion to the salivary pellicle, and it also allows streptococci to bind to collagen present in dentin that can be exposed after carious destruction of the enamel. This can lead to bacteria penetrating the dentinal tubules and infecting the tooth root canals and pulpal tissues. AgI/II polypeptides also provide a mechanism for binding the salivary agglutinin glycoprotein to the streptococcal cell surface. This in turn generates a receptor to which other bacteria can adhere (Fig. 3). The AgI/II protein also participates in the direct bacteria-bacteria binding (coadhesion), such as occurs between streptococci and the gram-negative pathogenic *Porphyromonas gingivalis*, thus enabling these secondary colonizing organisms to become incorporated into plaque communities (Fig. 3). Coadhesion with *P. gingivalis* is directed by a discrete domain that protrudes from the surface resembling a handle for attachment. This domain is not conserved in the AgI/II homologs in streptococcal species that do not colonize tooth surfaces.

Another example of streptococcal adhesion to salivary receptors, again illustrating the multiplicity of adhesion mechanisms, involves the

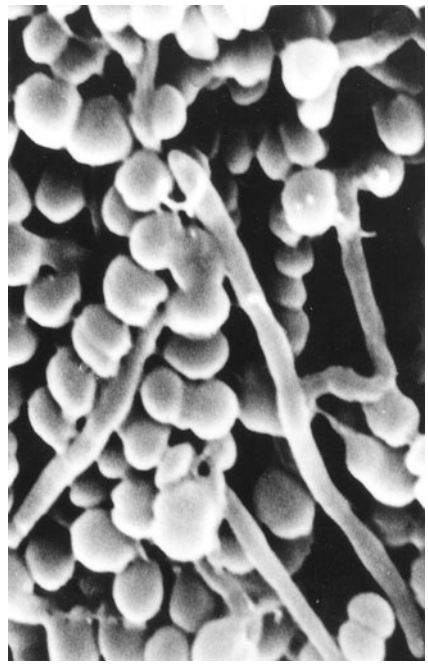
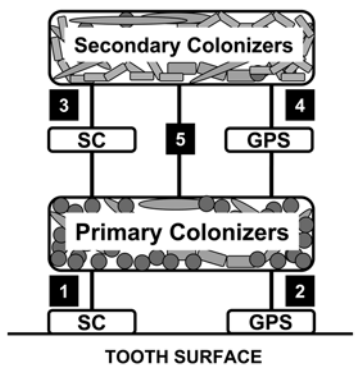


FIGURE 2 Scanning electron micrograph of "corncob" bacterial aggregates often seen in mature dental plaque. A number of coccal *Streptococcus cristatus* cells bind along the surface of a central *F. nucleatum* rod-shaped cell. Such adhesive interactions are thought to aid colonization and retention of the participating bacteria. doi:10.1128/9781555818906.ch5.f2

FIGURE 3 Microbial adhesion in the development of oral microbial plaque communities. Primary colonizers (e.g., mitis group streptococci and *A. oris*) adhere with high affinity to salivary components (SC), such as proline-rich proteins, statherin, α-amylase, and mucin glycoproteins, that are bound to the enamel surface (1). Glucan polysaccharides (GPS), generated by the activities of bacterial glucosyltransferase enzymes, also become incorporated into the salivary pellicle and provide adhesion receptors for bacteria expressing glucan-binding proteins (2). Secondary colonizers (e.g., *F. nucleatum, P. gingivalis*, and *T. forsythia*), which do not effectively compete with the primary colonizers in binding to the tooth surface, adhere to SC and GPS that are deposited onto the surfaces of the primary colonizers (3, 4). Direct cell-to-cell adhesion (coaggregation) of secondary and primary colonizers (5), often through protein-carbohydrate (lectin-like) interactions, promotes the development of spatially defined groups of microorganisms. doi:10.1128/9781555818906.ch5.f3



salivary starch-degrading enzyme α-amylase. Amylase, which is abundant in saliva, binds to several species of oral streptococci by at least two specific amylase-binding proteins (called AbpA and AbpB) present on the bacterial surface. Mutant strains of bacteria deficient in amylase binding adhere less well to amylase-coated enamel than do wild-type strains. While the wild-type strain produced large microcolonies in a flow cell biofilm model, a mutant strain made deficient in AbpA (and shown not to bind amylase) grew much more poorly and produced relatively small microcolonies. It is possible that in addition to a role in adhesion, amylase may support microbial nutrition by degrading dietary starch in proximity to the cell surface. The products of starch breakdown (glucose and maltodextrins) can be rapidly transported into the cell to be used as a source of energy.

Many streptococcal species, as well as *A. oris* and *Actinomyces naeslundii*, have the ability to synthesize extracellular polysaccharides. Glucans, which are synthesized from sucrose through the activities of secreted glucosyltransferase enzymes, are thought to play a key role in the formation of plaque because they adhere to smooth surfaces and mediate the coadhesion of bacterial cells (see chapter 12). The accumulation of mutans group streptococci within plaque is enhanced through the activities of cell surface-associated glucan-binding proteins. These proteins act as adhesins, mediating attachment of streptococci to glucans incorporated into the salivary pellicle, as well as mediating coadhesion of streptococci (Fig. 3). Since the glucan-binding proteins and AgI/II proteins are so important for the adhesion of streptococci, there is considerable interest in developing inhibitors of their functions, such as adhesin or substrate analogues, or specific antibodies through vaccination that might be utilized in the future to control plaque development.

Mechanisms of Adhesion

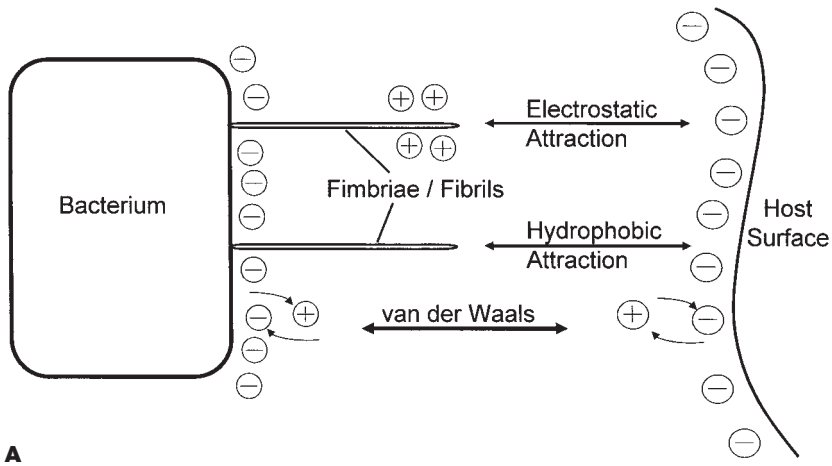
To adhere to an oral surface, bacteria that are initially suspended in saliva must first come in close proximity to the substratum. Many factors contribute to the approach of a microorganism to a surface. When salivary flow is high, convective transport by fluid dynamic forces causes accumulation of bacteria at solid-liquid interfaces where there is a viscous boundary layer. Frictional drag and turbulent downsweeps also assist contact with the surface. Irregularities present on surfaces provide shelters in which the bacteria can be protected from shear forces. When salivary flow is low, diffusive transport resulting from Brownian motion can serve, albeit more slowly, to concentrate suspended bacteria at a surface. Some bacterial species, such as the spirochetes (e.g., *Treponema denticola*), can actively participate in their surface localization by chemotactic motile responses.

Following arrival at a surface, the next challenge is to avoid displacement. It is instructive, although an oversimplification, at this point to consider bacteria as inert negatively charged particles and the host surface as similarly inert and negatively charged. The physical interaction between two such entities can then be predicted by the Derjaguin-Landau-Verwey-Overbeek theory of energetic interactions in biocolloidal systems. Simply put, the tendency for two negatively charged surfaces to repel each other can be overcome by fluctuating dipoles (van der Waals attraction) within

individual molecules on the approaching surfaces. If the surfaces become too close, there will again be a repulsive force due to overlapping electron layers. Although the Derjaguin-Landau-Verwey-Overbeek theory provides a theoretical framework for initial attachment, it is unable to fully accommodate the complexity of bacterial adhesion. Other factors that contribute to this early attachment phase include bridging by divalent cations (e.g., Ca^{2+}) in saliva, hydrophobic interactions between nonpolar regions of molecules on the surfaces, and hydrogen bonding (Fig. 4).

The sum total of these forces still produces only a relatively weak association of bacteria and substratum, and the bacteria cycle onto and off the surfaces. To remain more firmly attached for an extended period,

FIGURE 4 Bacterial attachment to host surfaces such as the saliva-coated tooth. (A) The bacteria first localize at sites on the salivary pellicle that are most thermodynamically favorable. This depends on interactions between the bacterial surface and the tooth surface that include van der Waals forces, along with electrostatic and hydrophobic interactions. Bacteria can concentrate positively charged or hydrophobic amino acids on their fimbriae/fibrils to strengthen the adhesive forces. (B) As the bacterial cells get closer to the surface, hydrogen bonds and divalent cation bridges can also stabilize the interaction. The sum total of all these weak interactions allows initial attachment but is not enough to retain the bacteria on the surface for long periods. Thus, after initial localization at the surface, bacteria must then form a higher-affinity bond between complementary adhesins and receptors (see text for details). The figure is not to scale. doi:10.1128/9781555818906.ch5.f4



bacteria need to form higher-affinity bonds utilizing specific surface molecules that interact stereochemically with cognate receptors, much as in an antibody-antigen interaction. The common arrangement whereby such adhesins are localized on fimbriae or fibrils that can extend several microns out from the bacterial wall facilitates engagement of a receptor while keeping the bacteria at an optimal distance from the host surface. Stronger, essentially irreversible, short-range associations can be mediated by protein-carbohydrate (lectin-like) interactions and by protein-protein interactions, as mentioned above. In either event, the requirement for complementary adhesins and receptors imparts a high degree of specificity to the adhesion mechanism. This is the basis for the tissue-specific binding (tropism) demonstrated by many oral bacteria. For example, a major reason that *S. sanguinis* and related organisms are found almost exclusively on the tooth surface, whereas the closely related *Streptococcus salivarius* resides on the dorsum of the tongue, is due at least in part to the specificity of the adhesins displayed by these organisms. Coordinated expression of a number of bacterial cell activities may be required for optimal adhesion. For example, partial degradation of host cell receptors by bacterial proteinases can expose binding domains that would otherwise remain hidden within the molecule and unavailable for attachment purposes.

Host Surface-Specific Constraints on Bacterial Adhesion

Teeth are highly mineralized structures that reside for extended periods, if not permanently, in the oral cavity. A newly erupted or professionally cleaned tooth becomes coated with a layer, or pellicle, of salivary proteins and glycoproteins within minutes. It is to this saliva-coated surface, rather than enamel itself, that bacteria adhere. Once established, pioneer colonizers of the tooth (such as streptococci, which can account for up to 80% of early plaque, and *A. oris*) become long-term inhabitants. These organisms, together with their products such as insoluble polysaccharides, provide adhesion substrates for secondary colonizers. Epithelial surfaces, in contrast, continuously turn over, and cells along with their attached bacteria are lost from the mouth (swallowed or expectorated). As living cells, the epithelial layer expresses surface molecules and is embedded in a matrix that comprises fibronectin and laminin, along with other proteins and proteoglycans. Given these discrete challenges, it is not surprising that the microbiota of the oral epithelia differs from that of tooth surfaces, both qualitatively and quantitatively. Moreover, there are distinct consequences of adhesion to living epithelial cells in that bacterial and host cells can both sense and respond to each other, as discussed below. The multiplicity of adhesins displayed by oral bacteria thus increases the likelihood and strength of receptor binding and provides the means by which bacteria can manipulate host cell signaling pathways that are stimulated through individual receptor engagements.

Adhesion and Metabolism

There is an enormous diversity of substrates present within the oral cavity that provide receptors for bacterial adhesion. There is also an equally large, or larger, diversity of substrates for bacteria to metabolize. These two processes, adhesion and metabolism, are not often considered together, but they are intricately linked. The composition and activities

of oral microbial communities are shaped by environmental (extrinsic) influences, such as temperature, shear forces, and nutrient supply, etc., and by intrinsic influences from both adherence capabilities and microbial metabolism. These intrinsic effects may result in the establishment of close metabolic, usually nutritional, interrelationships between groups of microorganisms. The interrelationships in catabolism develop mainly through differences in the efficiency of substrate utilization. Thus, it becomes beneficial for bacteria to assemble into groups that can utilize a complex substrate to maximum efficiency. Many metabolic interactions between bacterial pairs or groups have now been defined, and it is of considerable significance that these interactions are often associated with specific coadhesion of the bacteria involved. The outcome is a close physical association of microorganisms that comprises a more efficient, and thus more successful, metabolic unit.

The complex streptococcal microbiota of the nasopharynx provides an example of how diversity in metabolic function benefits a community. It is thought that the availability of sugars for utilization is scarce on the mucosal surfaces, in contrast to the oral cavity where sugars are often readily available from dietary food intake. Sugar utilization by streptococci in the nasopharynx may depend to a considerable extent on the abilities of bacteria to cleave, and then metabolize, sugar residues from oligosaccharide substitutions on host glycoproteins or from mucins. Cleavage of sugars from these substrates is achieved through the production of secreted enzymes known as glycan hydrolases. These enzymes include sialidase (neuraminidase), galactosidase, and fucosidase that cleave off sialic acid, galactose, or fucose residues, respectively, from oligosaccharides. Not all streptococci produce all of these enzymes, so defined groups of organisms become established that can assist, and feed off, each other to make the most efficient use of the complex oligosaccharide substrates. Not only do different species of streptococci come together in these associations but other bacteria such as *Haemophilus*, *Neisseria*, and *Staphylococcus* species benefit from metabolic synergies. Moreover, the modification of oligosaccharide structures on the mucosal cell surfaces continually provides new adhesion receptors for many of these bacteria.

Coaggregation itself may facilitate metabolic interactions between microorganisms. Perhaps the most frequently cited example is the utilization by *Veillonella* species of organic acids, e.g., lactic acid, produced by streptococci. Removal of lactate from the immediate environment results in a transient rise in pH that then drives more carbohydrate fermentation by the streptococci and more lactate production. This metabolic communication is enhanced by a short-range diffusible signal produced by *Veillonella atypica* that increases amylase production, and thus degradation of starch in streptococci. High lactate-producing streptococci, such as *S. mutans*, coaggregate with *Veillonella* species, and communities of these two species are found in carious lesions. *Veillonella* species are also found in associations with *S. salivarius* on the dorsum of the tongue. As another example, contact between the coaggregating species *S. gordonii* and *A. oris* induces expression of genes involved in streptococcal arginine biosynthesis and transport. As a result, *S. gordonii* becomes less dependent upon environmental arginine, which is scarce in saliva. Moreover, *A. oris* produces catalase that can remove H_2O_2 from the local environment and

TABLE 1 Mutually beneficial adhesion and nutritional interactions among oral bacteria

Interaction	Benefits
S. gordonii-P. gingivalis	Adhesion, reduced oxygen and redox potential for *P. gingivalis*
S. gordonii-F. nucleatum	Adhesion, reduced redox potential, tricarboxylic and fatty acids for *F. nucleatum*
F. nucleatum-T. forsythia	Growth factors, e.g, *N*-acetylmuramic acid, for *T. forsythia*
P. gingivalis-T. forsythia	Peptides and hemin for *T. forsythia*
P. gingivalis-T. denticola	Succinate for *P. gingivalis*; isobutyric acid for *T. denticola*

consequently decrease the level of oxidation and damage to *S. gordonii* proteins.

Gram-negative anaerobic bacteria such as *F. nucleatum*, *P. gingivalis*, and *Tannerella forsythia* demonstrate a variety of associations with each other and with gram-positive bacteria. These associations can be theorized to provide multiple benefits for the nutritionally and metabolically fastidious gram-negative bacteria. For example, *P. gingivalis* binds only weakly to *S. gordonii* cells in suspension but adheres avidly to *S. gordonii* cells when they are deposited onto a surface. Once attached to the streptococci, *P. gingivalis* cells can accumulate rapidly into a biofilm. Since *P. gingivalis* is an obligate anaerobe, it survives only at sites where the redox potential is low and oxygen is scarce. Regions of plaque composed of streptococci provide such sites. These kinds of interactions can form the basis for more complex interbridging events (Table 1), each generating a perceived benefit for one or more bacteria within the community.

GENE REGULATION

The ever-changing environment of the oral cavity provides an impetus for bacteria to regulate gene expression to maintain optimal phenotypic properties. Consider the journey an organism can undertake in the mouth (Fig. 5). Upon first entry, bacteria encounter the prevailing conditions of saliva with temperatures, pH, and osmolarity less than the levels in blood and tissues. In addition, there are extremes of nutrient availability depending on host feeding patterns. Once in the gingival crevice, the amount of oxygen decreases, osmolarity and pH increase, and nutrients can be obtained from gingival crevicular fluid. During periodontal disease progression, the temperature increases as a result of inflammation and there is elevated iron (or heme) availability because of bleeding. In addition to the changes in parameters in the oral cavity, bacteria that gain access systemically (see chapter 19) face a new set of challenges. The ideal numbers and types of adhesins, for example, expressed in the mouth may not be the most advantageous configuration in the tissues where such adhesion might promote uptake by the host professional phagocytic cells. To survive in different host environments, therefore, a successful oral organism has to rapidly sense and respond to the prevailing environmental circumstances. The mechanistic basis of gene regulation in oral bacteria remains to be determined in most cases. In general, however, bacteria often regulate gene expression at the level of transcription. This can occur through transcriptional activators that bind to DNA in or near the promoter regions of genes and then interact with RNA polymerase to stimulate the

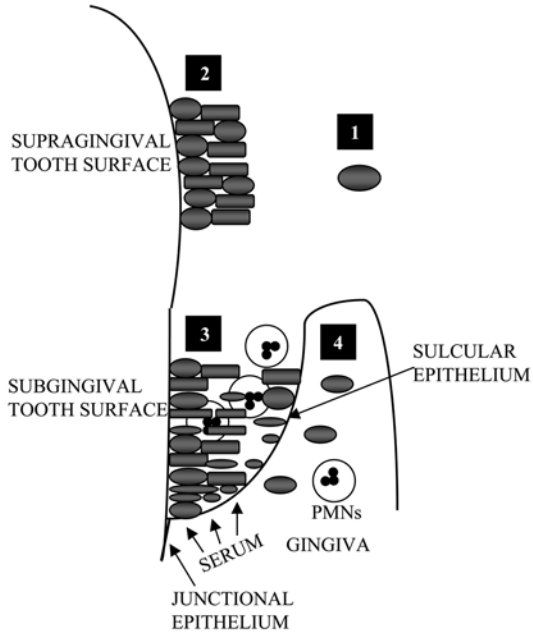


FIGURE 5 Differing environmental conditions that may be encountered by bacteria in the various oral niches. (1) Upon entry into the mouth, bacteria are suspended in saliva and experience lower pH, osmolarity, and temperature than internal body levels. Saliva also contains antimicrobial factors including antibodies, along with proteins and glycoproteins that can be metabolized by bacteria that express the necessary enzymes. (2) Once attached to the salivary pellicle coating the supragingival tooth surface, bacteria accumulate in a densely packed multispecies biofilm containing metabolic products, signaling molecules, and host dietary components. Temperature varies between extremes, for example, upon consumption of hot coffee or ice cream. (3) Bacteria that colonize the gingival crevice are bathed in crevicular fluid that is a serum exudate. Host polymorphonuclear leukocytes (PMNs) and other immune cells are present. As inflammatory periodontal disease progresses, there is an increase in temperature and bleeding into the periodontal pocket, which increases the levels of, among other things, heme-containing proteins. (4) Certain oral bacteria are able to invade the host tissues, both intercellularly and intracellularly. Within the gingival tissue there are normal body physiologic conditions, and the bacteria are also challenged with host immune cells and immune effector molecules.
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rate of initiation of transcription. Conversely, transcriptional repressors prevent transcription, usually by binding to DNA in the promoter region and physically impeding the action of RNA polymerase. Both activators and repressors often have active and inactive forms, depending on the binding of a ligand. For example, repressors for iron (Fe)-regulated genes may only bind DNA when complexed with Fe^{2+} or Fe^{3+} ions. Transcriptional activators are often turned on by phosphorylation, frequently as the result of a series or cascade of effector molecules. In this manner, gene regulation can be connected to events at the cell surface where signaling is initiated by phosphorylation of a sensor molecule. A common mechanism adopted by bacteria to translate environmental changes to changes in gene expression is the two-component regulatory system (TCS). The typical TCS consists of a membrane-bound sensor kinase, which responds to extracellular signals, and an intracellular response regulator, which usually interacts with gene promoter regions to modulate DNA transcription.

When sensor kinases become activated, they are phosphorylated on a histidine and then the phosphate is transferred to an aspartate in the response regulator. Bacteria also utilize phosphorylation/dephosphorylation of tyrosine residues as a means to transmit information. For example, the tyrosine phosphatase Ltp1 in *P. gingivalis* is involved in controlling the development of dual-species communities with *S. gordonii*. Many other means and levels of gene regulation are also available to bacteria that will allow both fine-tuning of gene expression and rapid on/off responses to major environmental changes.

BACTERIAL COMMUNICATION

Bacteria in complex communities such as biofilms communicate with one another through a variety of chemical signaling molecules. Intercellular communication blurs the distinction between single- and multicellular organisms and allows bacteria to coordinate gene expression and thus the behavior of the entire community. In this way, bacteria in biofilms such as plaque can derive benefits from their physical proximity that would not be available to organisms suspended in flowing fluids such as saliva.

One means by which bacterial cells communicate is through quorum sensing: cell density-dependent regulation of gene expression. This process involves the production and secretion of a signaling molecule called an autoinducer (AI). Upon reaching a threshold concentration, the AI triggers a change in gene expression, sometimes through interacting with a TCS. Quorum sensing can control a broad range of bacterial activities, including virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation. There are three major classes of quorum-sensing systems. Gram-negative bacteria use acylated homoserine lactones as autoinducers (AI-1), whereas gram-positive bacteria utilize oligopeptides (discussed below). Both gram-negative and gram-positive bacteria can also use an autoinducer-2 (AI-2) molecule. AI-2 is formed chemically from 4,5-dihydroxy-2,3-pentanedione that is generated by the action of LuxS autoinducer synthase on *S*-ribosylhomocysteine. AI-1 signaling has not been detected among oral gram-negative bacteria; however, many oral organisms possess AI-2 signaling. In *P. gingivalis* and *Aggregatibacter actinomycetemcomitans*, LuxS-based signaling regulates expression of iron acquisition systems and/or virulence factors such as proteases in *P. gingivalis* and leukotoxin in *A. actinomycetemcomitans*. In *S. gordonii*, aspects of carbohydrate metabolism are controlled through this pathway. The AI-2 signal also transmits information between bacterial species, as *P. gingivalis* genes respond to *A. actinomycetemcomitans* AI-2. The extent to which AI-2 signaling truly represents quorum sensing (i.e., is strictly cell density dependent) or is partially a reflection of the metabolic state of the cell remains to be determined. In either event, since plaque bacteria accumulate to a high cell density in metabolically synergistic communities (discussed above), AI-2 signaling can be expected to play a role in plaque development.

A feature of many species of oral streptococci is that they are able to exchange segments of genetic material (DNA) with high frequency. This leads to considerable adaptability because new populations of cells that have a selective advantage may arise relatively quickly. The process of

uptake and incorporation, by one cell, of DNA extruded by another is termed transformation, and streptococci are able to undergo this process in the natural environment only when they are in a state of competence. Competence is generated through a signaling mechanism involving an extracellular peptide pheromone designated competence-stimulating peptide (CSP). CSPs are produced by a wide range of oral streptococci and by *Streptococcus pneumoniae*. Cells in a population secrete low levels of CSP during growth, and CSP gradually accumulates in the environment to reach a critical threshold level. Once the bacteria sense this threshold level, the expression of genes that are involved in CSP production are up-regulated (so that the population as a whole is stimulated) and so are genes involved in the processes of DNA uptake and recombination. Different species of streptococci produce slightly different pheromone peptides, so that more closely related strains or species are better stimulated than those more distantly related. Originally, it was thought that CSP production was uniquely involved in competence development. However, it was discovered later that competence pheromones promote other intracellular changes not necessarily linked to DNA uptake and recombination. Many of these changes occur through induction of expression of an alternate RNA polymerase sigma factor that transcribes new sets of genes. Thus, CSP production and sensing by *S. mutans* in biofilms have been found to be necessary for the bacteria to express proteins necessary for the cells to tolerate acid stress and adapt to low pH. In mitis group streptococci, it is possible that CSP-mediated induction of the DNA repair enzymes, including RecA, may be part of a general stress or SOS response. These observations allow speculation that competence pheromones may actually be alarm signals that prime whole bacterial communities to oncoming stress conditions.

Oral streptococci also secrete a wide range of peptides (named bacteriocins) that are inhibitory to bacterial growth. Generally, bacteriocins are able to kill competing organisms by forming pores in the cytoplasmic membrane of sensitive gram-positive bacterial cells but do little or no harm to human cells. Bacteriocins that contain the thioether amino acid lanthionine are termed lantibiotics and tend to be synthesized and secreted through a complex machinery of proteins encoded by a number of genes. These genes are clustered on the streptococcal chromosome and include the structural gene for the bacteriocin (lantibiotic) precursor, genes encoding modification and transport proteins, genes for immunity, and sensor and regulatory protein genes. Immunity peptides provide protection for the producing strain against its own lantibiotic, while the sensor and regulatory proteins provide a mechanism for sensing and responding to the presence of lantibiotic. In this way, lantibiotics may also act as signaling molecules between streptococci. While sensing of CSP may signal impending general stress conditions, sensing of lantibiotic may signal the presence of competing streptococci and stimulate a defensive response within the population. Strains of *S. mutans* and *S. salivarius* produce potent lantibiotics, called mutacin and salivaricin, respectively, that act on other species of oral streptococci. These lantibiotics may thus play important roles in determining the composition of oral microbial communities.

Hence, bacteria that find themselves neighbors as a result of specific adhesion mechanisms and nutritional requirements also have means by

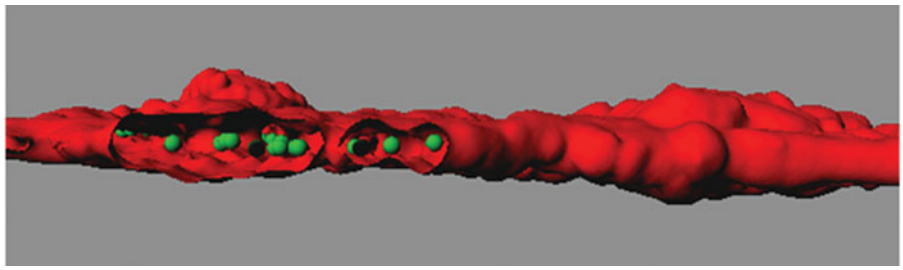
which they can communicate. Different species may be able to effectively talk to each other, but the extent to which this occurs is unknown. Cross talking between similar organisms clearly may help promote subpopulations or societies of organisms that then have a growth advantage over individual cells. In addition, the killing effects of some signaling molecules will be likely to provide an added advantage to those bacteria that are resistant.

COMMUNICATION WITH HOST CELLS

The epithelial cells that line the gingival crevice provide an important barrier function and physically prevent the ingress of bacteria into the highly vascularized gingival tissues. In addition to this mechanical role, the epithelial cells are capable of sensing and responding to the presence of bacteria and there exists an elaborate communication network between bacterial and host cells. This molecular dialogue conveys information among the bacteria, epithelial cells, and underlying immune and inflammatory cells in the mucosa. The results of this communication have a significant effect on the overall health status of the gingiva. Processes that can be affected by host-bacteria cross talk range from the production of immune effector molecules such as cytokines to cell life-or-death decisions through both necrotic and apoptotic pathways.

One of the most visible and dramatic outcomes of the intimate association between bacteria and epithelial cells is internalization of the organisms into the host cells. As epithelial cells are not professional phagocytes, the bacteria themselves provide the molecular instructions to allow membrane invaginations of the host cells that accommodate bacterial cell uptake. An intracellular location may benefit bacteria by providing a nutritionally rich environment that is partially sheltered from the ravages of the immune system.

In the case of *P. gingivalis*, invasion is initiated through fimbria-mediated adhesion to epithelial cell receptors. A series of signal transduction events then occur that lead to reorganization of both actin microfilament and microtubule cytoskeletal components, facilitating subsequent bacterial entry into the cytoplasm (Fig. 6). Both bacterial and epithelial cells remain viable following internalization, indicating that this is a long-term stable association. The phenotypic results of *P. gingivalis* having seized control of a variety of epithelial cell intracellular pathways have immediate relevance to the disease process. Regulation of matrix metalloproteinase production by gingival epithelial cells is disrupted following contact with the organism, and this interferes with extracellular matrix repair and reorganization. Invasion of *P. gingivalis* also has implications for innate host immunity. Secretion of interleukin 8 (IL-8) by gingival epithelial cells is inhibited following *P. gingivalis* invasion. Inhibition of IL-8 accumulation by *P. gingivalis* at sites of bacterial invasion will debilitate innate host defense in the periodontium where bacterial exposure is constant. The host will have difficulty detecting the presence of bacteria and directing leukocytes for their removal. The ensuing overgrowth of bacteria could then contribute to an episodic recurrence of periodontal disease activity.



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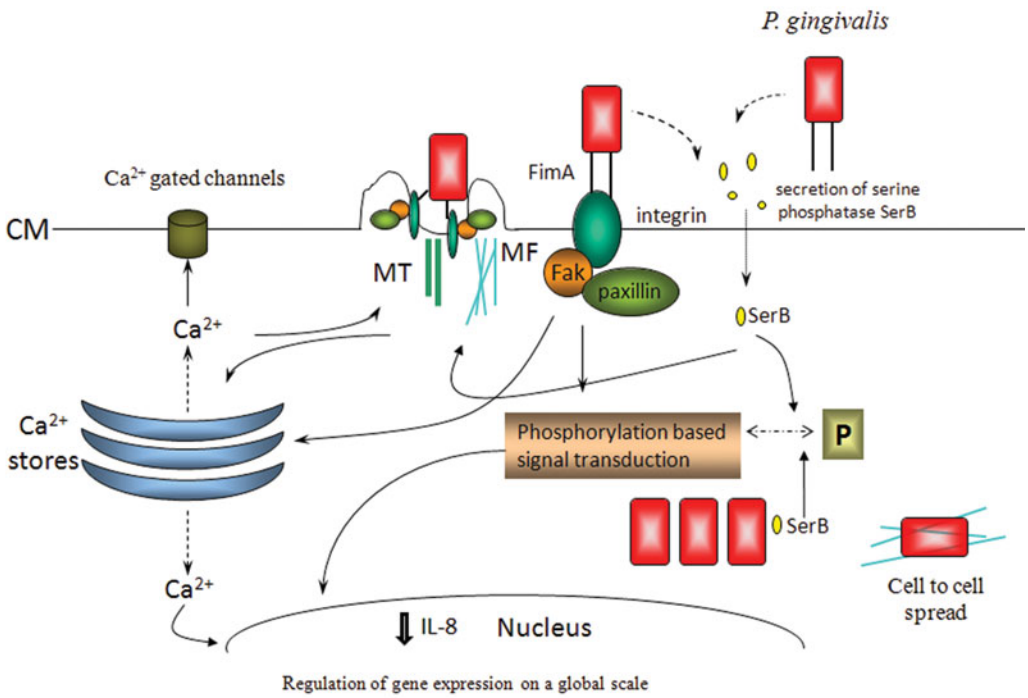

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FIGURE 6 Invasion of gingival epithelial cells by *P. gingivalis*. (A) Cutaway side view of gingival epithelial cell (red) showing internalized *P. gingivalis* (green). (B) Schematic (not to scale) representation of the molecular interactions between *P. gingivalis* and gingival epithelial cells. In proximity with epithelial cells, *P. gingivalis* secretes a serine phosphatase (SerB) that is functional within epithelial cells and can modulate phosphorylation/dephosphorylation-based signal transduction and cytoskeletal structure. *P. gingivalis* cells bind integrins on the epithelial cell surface through fimbrial adhesins. FAK and paxillin are recruited, and microtubules and microfilaments are rearranged to facilitate invagination of the membrane that results in the engulfment of bacterial cells. *P. gingivalis* cells rapidly locate in the perinuclear area where they replicate. Calcium ions are released from intracellular stores and regulate calcium-gated pores in the cytoplasmic membrane. Calcium ion- and phosphorylation-based signal transduction converge on gene expression, and regulation of immune mediators such as IL-8, cell death- and survival-related genes, and matrix metalloproteases is disrupted. *P. gingivalis* ultimately enters adjoining epithelial cells in an actin-dependent process. Ca, calcium; CM, cytoplasmic membrane; IL-8, interleukin 8; MF, actin microfilaments; MT, tubulin microtubules; NM, nuclear membrane; P, phosphate; solid arrow, pathway with potential intermediate steps; dashed arrow, release; two-headed arrow, reversible association.
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Internalization is not always an outcome of bacterial-host cell cross talk. For example, the oral spirochete *T. denticola* can induce depolymerization and rearrangement of actin microfilaments along with a loss of cell-cell adhesion and substratum detachment independent of host cell invasion. In addition, a spirochetal chymotrypsin-like enzyme can degrade junctional complexes and can be transported into the cell where it disturbs the actin cytoskeleton. A major surface protein of *T. denticola* can also be translocated into the epithelial cell membrane where it forms a conductance ion channel and depolarizes the membrane. In light of this onslaught on normal epithelial cell physiology, it is unsurprising that elevated numbers of treponemes are associated with periodontal disease.

Oral bacteria and host cells can be seen to be involved in an intricate and orchestrated series of interactive events. Both sets of cells maneuver and manipulate each other to obtain a physiological advantage. The adaptability of host cells to encroachment by bacteria plays a large part in determining whether the bacteria will be constrained or will evade host innate defenses and cause tissue destruction.

KEY POINTS

Dental plaque that develops on tooth surfaces is a diverse and complex microbial community (biofilm) in which over 700 species can participate.

Colonization of bacteria is ordered and sequential. Initially, plaque is rich in commensals such as streptococci and actinomycetes. Populational shifts ultimately result in higher levels of gram-negative potential periodontal pathogens such as *P. gingivalis*, *A. actinomycetemcomitans*, and *T. denticola*.

Bacterial binding to host surfaces and to other oral bacteria is important in the development of plaque. Lower-affinity attachment occurs through physicochemical forces such as van der Waals, hydrophobic, and electrostatic. Higher-affinity adhesion occurs through complementary adhesin-receptor interactions. These closely fitting molecules can be on the surfaces of bacteria, in the salivary pellicle on the enamel surface, or on the surfaces of host cells. Hence, bacteria can form stable associations with themselves and with the oral hard and soft tissues. The pattern of expression of bacterial and host cell adhesive molecules imparts specificity of adhesion and colonization.

Complexes of bacteria often participate in complementary metabolic interactions that ensure optimal substrate utilization.

Bacteria can also be antagonistic to one another through competition for nutritional substrates and attachment sites and by the production of toxic metabolites and bacteriocins.

Bacteria in close proximity can communicate with one another, resulting in modulation of phenotypic properties and, potentially, coordination of gene expression throughout multispecies communities. One such signaling system is mediated by AI-2, produced through the action of the LuxS enzyme. Gram-positive bacteria also signal through short peptides such as the CSPs and lantibiotics.

Certain oral bacteria such as *P. gingivalis* can manipulate host cell signal transduction pathways and locate intracellularly within epithelial cells. Internalized bacteria are protected from the immune system and can affect host immune status by modulating cytokine expression. Bacteria within host cells may serve as a reservoir of infection.

FURTHER READING

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Oral Microbial Physiology

Paul G. Egland and Robert E. Marquis

OVERVIEW

The physiology of organisms in the oral cavity is geared to their need to survive in crowded conditions and to manage the stresses associated with existence in biofilms. These stresses can include low nutrient availability, oxidative stress, and accumulation of harmful waste products. The enamel of teeth is a unique nonshedding biological surface; therefore, it can provide a substratum for undisturbed plaque growth. As the plaque biofilm matures, it can become dense enough to limit diffusion of factors that affect growth of the organisms.

Plaque biofilms develop in protected areas, for example, in pits and fissures or in interproximal and subgingival regions. In these protected sites, the organisms can look forward to long-term coexistence with the host. However, life can be tough for the bacteria because of slow inward movement of nutrients and slow outward movement of toxic wastes. In these protected environments, organisms such as lactic acid bacteria can compete, and because of their high levels of acid tolerance, probably cause major damage to the teeth in pit and fissure caries. Our knowledge of the physiology of plaque bacteria in these protected sites in the mouth is very limited, but fissure caries is important because it currently makes up a major part of the total caries of humans and appears to be less amenable to the anticaries actions of fluoride than smooth-surface caries.

While well adapted to manage the stresses of the oral environment, the majority of oral bacteria are not well equipped to deal with the world outside the mouth. They are specialists, fit for a specific lifestyle. Conversely, the oral environment is not suitable for all organisms. While there are transient organisms in the mouth, they are only temporary colonizers and are not capable of competing with the specialized organisms of the autochthonous microbiota, unless there is a fairly severe physiological upset of the host.

Bacteria such as *Streptococcus mutans* are prime examples of organisms that are fit for the oral environment. Not only is *S. mutans* adapted to life in the mouth, it is narrowly adapted to life only on hard surfaces in the mouth. Prior to eruption of the teeth, it is not usually a permanent member of the oral microbiota. If there is loss of all the teeth and no implant or denture replacement, it vacates the premises. Furthermore, like other

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streptococci, *S. mutans* has multiple nutritional dependencies and derives needed nutrients from the host, either from saliva or from the diet. It is multiply auxotrophic, meaning that it does not have genes encoding enzymes required for synthesis of many required nutrients, including many amino acids and vitamins. Thus, they must obtain these nutrients from their environment. Consequently, the commonly used, defined, minimal medium for growth of *S. mutans* contains some 42 separate ingredients, plus trace minerals, needed to meet minimal nutritional needs for growth.

Organisms such as the oral spirochete *Treponema denticola* are even more dependent on their host. There is a defined, minimal medium on which *T. denticola* can grow. However, the spirochete requires very specialized growth conditions, including an anaerobic environment. Its physiology is adapted specifically to life in periodontal pockets or along inflamed gingival margins. It appears from sequence analyses of genes for 16S rRNA in extracts from periodontal plaque that many spirochetes related to *T. denticola* have never been cultivated. Thus, there is currently no medium that provides their required nutrition for in vitro growth, and our knowledge of these other organisms is scant. Of the 700 or more species of bacteria detectable in the mouth by analyses of 16S rRNA gene sequences in extracted DNA, only a limited number have been cultivated in the laboratory.

While oral bacteria are specialized for life in the mouth, they regularly enter the circulatory system of the human body. Bacteremia occurs as a result of activities such as brushing the teeth, flossing, dental prophylaxis, other dental procedures, or minor daily traumas. This dissemination does not generally lead to septicemia (growth of microbes in the bloodstream) but only bacteremia (presence of bacteria in the bloodstream). However, oral bacteria may lodge in specific sites in the body, such as defective heart valves and artificial joints, or may be involved in mixed-organism abscesses. Currently, there are also questions regarding whether oral bacteria can be involved in long-term colonization of the host, for example, in blood vessel walls associated with atherosclerotic plaques (see chapter 19). In all of these situations, adaptation of the oral organisms to growth in surface-associated biofilms enhances their abilities to colonize the host and evade normal host defenses such as phagocytosis or the complement-antibody-mediated lytic system.

There are exceptions to the crowded existence of bacteria in the oral environment. For example, surfaces of epithelial cells of the buccal mucosa are only sparsely colonized by bacteria. Therefore, the environment of the epithelial surface is less competitive in regard to crowding and also more buffered against stresses such as acidification. However, the organisms have a limited existence there because of regular shedding of the mucosa into saliva and then passage of the shed cells with their attached microorganisms. Saliva itself has a moderately dense microbiota with some 10^6 to 10^8 organisms/ml, dependent on how long it has been in the mouth and how much shedding from surfaces has occurred. In the stick-or-be-swallowed world of the oral cavity, saliva provides only a very transitory home for microbes. Bacteria in saliva enter the gut after swallowing or are transferred from the mouth to the external environment by expectoration. In the gut, the environment is hostile, with pH values in the stomach as low as 1. Relatively few oral bacteria survive the rigors of

the passage and competition with intestinal microbes to emerge viable in the feces.

In the plaque community, organisms are densely associated with each other, often actually binding to each other via coaggregation. This allows the physiological activities of one organism to modify the environment for neighboring organisms. In a successful community, neighboring organisms are expected to benefit from these modifications. This is the case in the oral microbial environment. Saliva is a critical source of nutrition and energy for oral bacteria. While some organisms, such as *Streptococcus gordonii*, are able to survive with saliva as a sole nutrient, others cannot. Many rely on the successive actions of one or more other bacteria to break down salivary glycoproteins to make the nutrients accessible. Others organisms require the fermentation products of community members as a source of energy. The study of such symbioses is currently an active area of research. Some important physiological properties that are required for these symbioses and community building, such as adherence, coaggregation, and cell-cell signaling, are addressed in other chapters.

With over 700 species of bacteria in the oral cavity, the physiological and metabolic capabilities of the complete oral bacterial community is far from defined. In this chapter, no attempt is made to consider all aspects of the physiology of oral microorganisms. Instead, it includes a survey of some important nutritional and catabolic capabilities of bacteria in the oral bacterial community and specific aspects of physiology considered to be important for oral infectious diseases.

SURVEY OF METABOLIC ACTIVITIES IMPORTANT TO THE ORAL BACTERIAL COMMUNITY

Carbohydrate Fermentation

Sugar fermentation is one of the most important and best understood mechanisms of energy generation by oral bacteria. It has received attention, in large part, due to the destructive nature of the acidic end products of fermentation and the role of the acid in caries formation. While many groups of oral bacteria are able to use sugars for growth, the cariogenic capabilities of the streptococci and other lactic acid bacteria make them an important and commonly regarded group of oral bacteria. These organisms ferment host-derived sugars as well as dietary sugar. Of the host-derived sugars, most are acquired by the action of bacterial exoglycosidases that cleave the sugars from host salivary glycoproteins. In addition, some oral bacteria can access sugars from host hyaluronic acid, an extracellular matrix component composed of repeating disaccharide subunits, by excreting hyaluronidases that break down the polymer. The liberated sugars can be brought into the cell by transport mechanisms including the sugar-phosphotransferase systems (PTS), as described in chapter 1.

While many groups of oral bacteria are able to ferment sugars, *Streptococcus mutans* is the most thought of member of the oral community in terms of sugar fermentation, due to its long-recognized association with caries. When grown with excess glucose, *S. mutans* is largely homofermentative, meaning it produces a single fermentation product, lactate. Under these conditions, energy is generated by the Embden-Meyerhof-Parnas

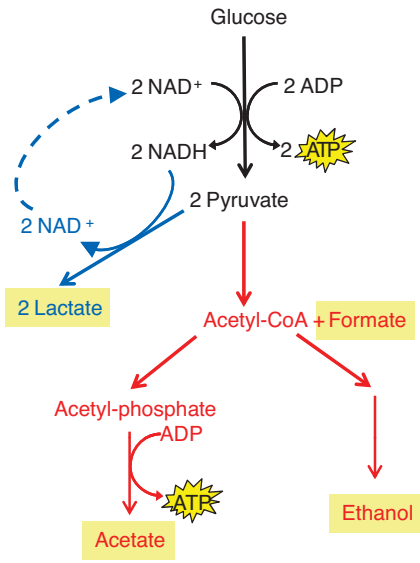

FIGURE 1 Sugar fermentation by *S. mutans*. Summary pathways of glycolysis via the Embden-Meyerhof-Parnas pathway (black), lactate fermentation (blue), and mixed-acid fermentation (red) are shown. The sugars that enter glycolysis are phosphorylated as they enter the cell when transported via the phosphotransferase system. Depending on sugars available and oxygen levels, cells may produce lactate only or lactate as well as formate, acetate, and ethanol.
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pathway of glycolysis. This is followed by fermentation of pyruvate to lactic acid to regenerate electron-accepting NAD^+ needed for further ATP formation. However, when grown on sugars other than glucose, *S. mutans* performs mixed acid fermentation, producing formate, ethanol, and acetate in addition to lactic acid (Fig. 1). Furthermore, the balance between mixed-acid and homofermentative growth is also dependent on the amount of oxygen present in the environment. The presence of oxygen forces cells to use the homofermentation pathway due to the oxygen-sensitive nature of some of the enzymes needed for mixed-acid fermentation.

While host-tissue-derived sugars are sufficient for many oral bacteria, the diet of the human host clearly provides fermentable sugars. In terms of caries formation, sucrose is thought to be of critical importance. *S. mutans* uses sucrose to synthesize extracellular glucans that are important for adherence to the enamel surface. It also utilizes sucrose as a source of energy. The importance of sucrose to *S. mutans* is illustrated by its multiple transport systems for the sugar, including PTS systems and an ATP-binding cassette transporter. When taken up by the PTS system, sucrose is converted to sucrose-6-phosphate as it enters the cell and is hydrolyzed to yield glucose-6-phosphate and fructose. When imported by the ATP-binding cassette transport system, the enzyme sucrose phosphorylase converts the sucrose to glucose-1-phosphate and fructose, both of which can be subject to glycolysis.

Fermentable sugars can also be obtained from complex carbohydrates in the human diet. For example, starch is hydrolyzed to sugars for use by the host by human salivary amylases. To gain access to these sugars, oral streptococci have surface-localized amylase binding proteins that bind to the human salivary amylase. Once bound, the human amylase is localized to the surface of the bacterial cell. Sugars liberated by the amylase can then be transported into the cell and utilized as a source of energy for the streptococci. Pectin, another plant-derived carbohydrate, can be used as a source of sugar by some oral spirochetes as well. To access the sugar in this macromolecule, the pectin-degrading enzymes, pectin methylesterase and pectate lyase, are exported from the bacterial cells. These enzymes break pectin down to its component sugars, which can be imported and used as substrates.

To protect themselves from the feast-or-famine nature of the oral environment, some oral bacteria including *Streptococcus* spp., *Actinomyces* spp., and *Fusobacterium nucleatum* are able to produce intracellular polysaccharide (IPS) for carbohydrate storage. This glycogen-like carbohydrate polymer is formed during conditions of excess sugar. The IPS is hydrolyzed to fermentable sugar when exogenous sugar is not available. This ability is likely to be important for other organisms in the community, since, as described later, a *Streptococcus gordonii* amylase gene thought to be responsible for liberating sugar from IPS is induced by signaling from *Veillonella parvula*.

Metabolism of Organic Acids

While sugar fermentation by lactic acid bacteria is thought of as a hallmark of the oral bacterial community, the oral cavity is home to a variety of organisms with diversity among their substrates used for energy

generation. Some of these organisms utilize waste products from sugar fermentation, such as lactate (lactic acid) and formate, for growth substrates. Other organisms use amino acid fermentation as the primary means for generation of energy. Some important examples of the use of organic acids for growth are described here.

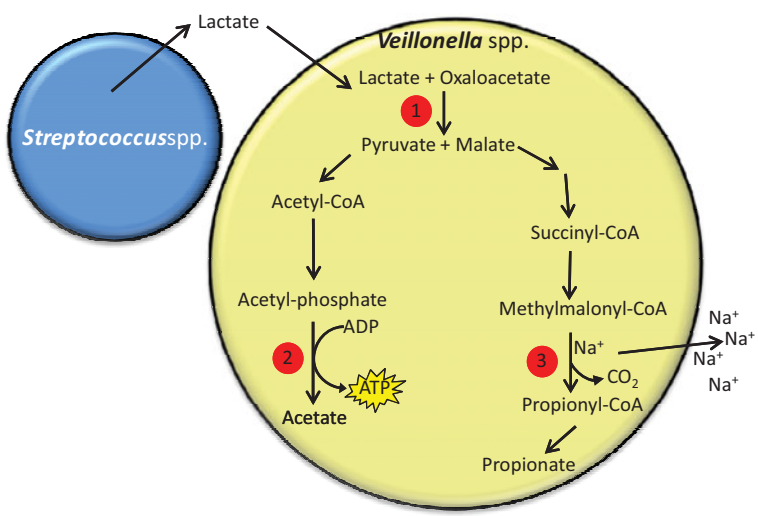
Energy Generation Using Lactate

One group of organisms that can use lactate as an energy source is the genus *Veillonella*. The veillonellae are asaccharolytic, anaerobic cocci that are found in dental plaque as part of the normal microbiota and are also present in disease states. The taxonomy of this group is unusual. The veillonellae have an outer membrane containing lipopolysaccharide and, thus, are gram negative. However, based on 16S rRNA gene sequences, they are members of the phylum Firmicutes, which contains the low-G+C gram-positive bacteria. Much of what is known about the physiology of this group is based on studies of *Veillonella parvula*, but several other species of *Veillonella* are found in dental plaque and many uncultivated phylotypes have been detected in human oral samples.

The overall pathway for lactate consumption by *V. parvula* results in the conversion of lactate to propionate and acetate (Fig. 2). This is thought to be protective of tooth enamel because of the relative weakness of these acids. Lactic acid has a lower pK_a (3.86) than propionic acid ($pK_a = 4.87$) and acetic acid ($pK_a = 4.79$). Thus, lactic acid dissociates more fully in the oral environment and is more harmful to enamel that the weaker acids.

As lactate consumers, *Veillonella parvula* and other members of the genus are important members of the oral bacterial community because they consume the major waste product of the glucose-fermenting organisms. As such, they are able to participate in symbioses with the oral streptococci and other lactate producers. *Veillonella* spp. coaggregate with oral streptococci, putting them in close proximity to the lactate produced.

FIGURE 2 Lactate metabolism by *V. parvula*. Key enzymes are indicated: 1, malic lactic transhydrogenase; 2, acetate kinase, which is responsible for ATP formation; 3, methyl-malonyl-CoA decarboxylase, the enzyme responsible for generation of the sodium ion gradient. doi:10.1128/9781555818906.ch6.f2



Further, *V. parvula* has been shown to signal to *S. gordonii*, resulting in increased expression of an amylase gene that is thought to hydrolyze intracellular storage polysaccharide. The liberated sugars can then be fermented, providing *V. parvula* with lactate.

V. parvula uses lactate in an unusual reaction scheme in which energy is generated from the substrate by both substrate-level phosphorylation and by creation of an electrochemical gradient across the bacterial plasma membrane. Lactate is converted to pyruvate and malate by malic-lactic transhydrogenase. Pyruvate and malate then enter separate energy generation pathways. In the substrate-level phosphorylation pathway, high-energy phosphate is transferred from acetyl-phosphate to generate ATP. The latter pathway involves the use of a sodium ion-dependent decarboxylase, methylmalonyl-coenzyme A (CoA) decarboxylase, which translocates sodium ions across the plasma membrane, creating an electrochemical gradient as propionyl-CoA is formed. The sodium ion gradient generated by such decarboxylases has been shown to drive ATP formation in other organisms. Although there have been reports of increased ATP produced from succinate by *V. parvula* growing with lactate, such a sodium ion-driven F-type ATPase has not yet been described from *Veillonella* spp. It may be that the energy from this electrochemical gradient could also be used for sodium ion-based transport of growth substrates. Such substrates could include amino acids, as has been shown for other organisms, or perhaps lactate itself.

Aggregatibacter actinomycetemcomitans is a gram-negative, coccobacillus-shaped, facultative anaerobe that is proposed to be the etiologic agent in localized aggressive periodontitis. Like *Veillonella* spp., *A. actinomycetemcomitans* can obtain lactate from oral streptococci fermenting sugars. However, *A. actinomycetemcomitans* employs a pathway of lactate use that involves conversion of lactate to pyruvate by lactate dehydrogenase. Pyruvate can then be used for generation of ATP by substrate-level phosphorylation in a scheme similar to that used by *V. parvula*. However, *A. actinomycetemcomitans* does not use the methylmalonyl-CoA pathway employed by *Veillonella*.

In addition to use of lactate, *A. actinomycetemcomitans* is able to ferment glucose and other sugars. Although more energy can be obtained using sugars, *A. actinomycetemcomitans* has been shown to have a preference for lactate. It is thought that the mechanism for lactate preference is based on an exclusion mechanism in which pyruvate formed by the lactate dehydrogenase inhibits the phosphotransferase system of glucose transport.

Metabolism of Amino Acids

Amino acids are an essential source of energy and nitrogen for the growth of many oral bacteria. In fact, many oral bacteria are asaccharolytic and use amino acids as their only source of energy. Furthermore, many organisms are auxotrophic for several amino acids and must obtain some of the amino acids needed for protein synthesis from the environment. While the host diet includes proteins, these are virtually unavailable to many bacteria. Not only are dietary proteins transitory, many of the amino acid-utilizing anaerobes are in locations that have nearly no access to dietary components. For example, the bacteria comprising subgingival plaque do not have access to host dietary proteins or even salivary proteins. Thus,

most of the amino acids used by bacteria are derived from either gingival crevicular fluid or are obtained by degradation of host tissues by bacterial proteases. As these proteinases are typically secreted or surface associated, their cleavage products may be available to the entire bacterial community and not just the protease-producing organisms. This proteolytic activity is not only important for bacterial nutrition but is also the basis of tissue destruction of periodontal disease. While the exact pathways for amino acid use have not been elucidated for all of the oral bacteria, a few mechanisms by which amino acids are used for energy generation and two important protease producers are described below.

The Role of Proteases in Energy Generation

Of the several species thought to be periodontal pathogens, proteinase production by *Porphyromonas gingivalis*, a gram-negative, black-pigmented, coccobacillus, is the best characterized. *P. gingivalis* has an expansive suite of proteolytic enzymes that can degrade host extracellular matrix proteins, including collagen, laminin, and fibronectin. A group of cysteine endopeptidases, known as the Lys-Xaa and Arg-Xaa gingipains based on their specificity, are major contributors to this activity. In addition, *P. gingivalis* proteinases activate host matrix metalloproteinases, further contributing to the tissue damage seen in periodontitis. Through the concerted effects of the gingipains and other endopeptidases and exopeptidases, host proteins are degraded to successively smaller peptides, eventually to the level of di-and tripeptides. These peptides can be transported into the cell, cleaved into monomers by intracellular peptidases, and used for growth.

In addition to their role in proteolysis, the *P. gingivalis* gingipains are important for adhesion to host tissues and colonization. Two of the gingipains have protein domains that contribute to their roles in hemagglutination and adhesion. These protein domains have specificity for the connective tissue proteins fibrinogen and fibronectin. Furthermore, one of the gingipains can bind to hemoglobin and is thought to be involved in acquisition of heme.

An interesting feature of the proteinases from *P. gingivalis*, and those from *T. denticola* mentioned below, is their release from the cells in vesicles generated from the outer membrane. The presence of gingipains in these vesicles not only allows export of proteinase activity into the oral microbial community where other organisms may benefit from their activities but targets substrates by means of the adhesive domains of these proteins. The released vesicles bind fibrinogen, fibronectin, and laminin, which could serve as substrates for their enzymatic activity. Further, these gingipains have domains that mediate coaggregation with other oral species.

T. denticola, one of the few cultivatable oral spirochetes, is also associated with periodontitis. *T. denticola* also possesses several proteinases that are important for the nutrition of the bacterium as well as its virulence. Combined with its motility, *T. denticola* proteases are thought to contribute to its ability to migrate though host basement membranes by degrading laminin, fibronectin, and collagen. Further, *T. denticola* proteases contribute to virulence by cleaving human proteins involved in immunity such as immunoglobulins and transferrin as well as several bioactive peptides.

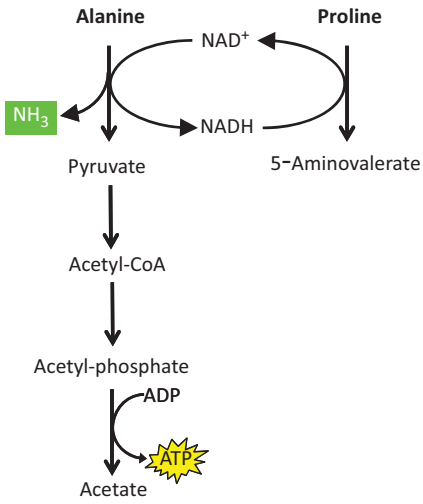


FIGURE 3 The Stickland reaction for amino acid metabolism. In this example, alanine and proline are used as the oxidized and reduced pair. Proline serves as the electron acceptor for the regeneration of NAD^+, which is needed for oxidative NH_3 removal. ATP generation occurs by substrate level phosphorylation.
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Amino Acid Metabolism by the Stickland Reaction

After proteases have degraded extracellular proteins, bacteria can import peptides for use in energy generation. The Stickland reaction is a mechanism by which bacteria can use amino acids as a source of energy. This is actually a coupled pathway in which one amino acid serves as an electron donor while another serves as an electron acceptor. There are several different amino acid pairs that can participate in energy generation by this scheme. These reactions have been well described in the genus *Clostridium*, members of which are able to utilize several different amino acid pairs for this type of fermentation.

The Stickland reaction has been detected in plaque samples and characterized in the oral bacterium *Peptostreptococcus anaerobius*, a member of the phylogenetic class *Clostridia*. *P. anaerobius* is able to use the Stickland reaction to gain energy using alanine and proline as the oxidized and reduced pair of amino acids (Fig. 3). The first step in the Stickland reaction is oxidative deamination of alanine using proline as the ultimate electron acceptor. The released NH_3 combines with free protons to yield NH_4^+, which contributes to alkylation of the bacterial cytoplasm. The pathway generates ATP by substrate-level phosphorylation, as acetyl-phosphate derived from alanine is dephosphorylated by acetate kinase.

Amino Acid Fermentation by *Fusobacterium nucleatum*

Fusobacterium nucleatum, a gram-negative, spindle-shaped anaerobe, is highly associated with periodontal disease. *F. nucleatum* has several physiological traits that may be important for development of the bacterial community in terms of modifying the pH and oxidative stress of the local environment. *F. nucleatum* generates energy primarily by fermentation of amino acids, especially glutamate, which is abundant in dental plaque. This breakdown of amino acids results in the release of nitrogen in the form of NH_3. ATP is generated by substrate-level phosphorylation.

While there are several known bacterial pathways for the use of glutamate, *F. nucleatum* uses the pathway of glutamate fermentation characterized by the involvement of 2-hydroxyglutarate as an intermediate (Fig. 4). This pathway includes two especially noteworthy reactions. The first is catalyzed by 2-hydroxyglutaryl-CoA dehydratase, which catalyzes an energetically difficult removal of water from its substrate to produce glutaconyl-CoA. This enzyme is interesting in that it requires the use of an oxygen-sensitive activator protein that reduces the actual dehydratase enzyme with a low potential electron. Once activated, the enzymatic component can go through multiple catalytic cycles. This reaction is followed by a decarboxylation by glutaconyl-CoA decarboxylase. Like methylmalonyl-CoA decarboxylase of *V. parvula* mentioned above, this enzyme forms an electrochemical Na^+ gradient across the plasma membrane. It has been proposed that energy from this electrochemical gradient is used to reduce the activator protein needed for the dehydratase reaction.

While *Fusobacterium* spp. strains are most known for their ability to ferment amino acids, some are able to derive energy, to various degrees, from sugars as well. *F. nucleatum*, the species most prevalent in the oral bacterial community, is known to generate glycogen-like IPS reserves when growing using amino acids in the presence of sugars, especially fructose. In the absence of amino acids, the glycogen can be mobilized for use as an energy source.

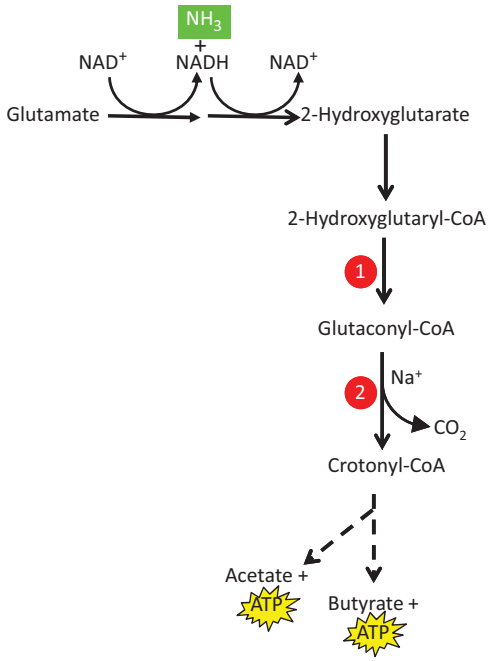

FIGURE 4 Abbreviated pathway of glutamate fermentation by *F. nucleatum*. Nitrogen from the amino acid is removed as ammonia, and two ATPs are formed by substrate-level phosphorylation. Reaction 1 is catalyzed by 2-hydroxyglutaryl-CoA dehydratase, an enzyme that must be activated by a reduced activator protein. Reaction 2 is catalyzed by a glutaconyl-CoA decarboxylase, which translocates Na^+ across the plasma membrane, generating an electrochemical gradient that can be used to reduce the activator of protein required for the dehydratase in reaction 1.
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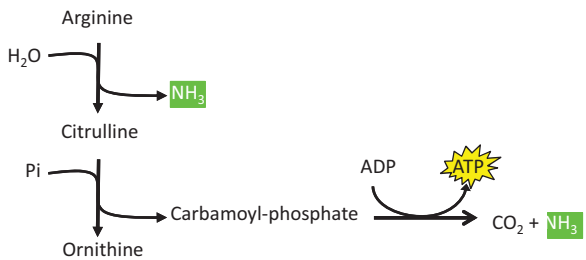
Arginine Metabolism by the Arginine Deiminase System

Many streptococci and other oral bacteria are able to metabolize arginine using the arginine deiminase system (ADS). This series of reactions converts arginine to ornithine, CO_2, and ammonia while generating ATP (Fig. 5). While the system does provide energy, it is also important to acid tolerance, as the ammonia generated can combine with free protons to generate ammonium ions, thus neutralizing the cytoplasm. There is also a variant of the usual arginine deiminase enzyme described for *Porphyromonas gingivalis*. This variant enzyme acts on the N-terminal arginine residues of proteins and peptides to release ammonia and leave a citrulline residue. The role of ADS and other systems of acid tolerance is discussed later in this chapter.

FIGURE 5 Arginine metabolism by the ADS. The pathway generates ammonia, which deacidifies the cytoplasm by combining with free protons and generates ATP.
doi:10.1128/9781555818906.ch6.f5



ACID-BASE PHYSIOLOGY OF ORAL MICROORGANISMS

Acid-Base Cycling in the Mouth

Acid-base physiology of oral bacteria is of particular significance in relation to dental caries. To be cariogenic, a bacterium such as *S. mutans* must be able to colonize plaque, mainly preexisting plaque. *S. mutans* is a secondary colonizer rather than a pioneer. After initial attachment, it must be able to become sufficiently well established in plaque to make up a significant fraction of the total population. Finally, it must be able to function at low pH values. Caries occurs only at acid pH values, below about 5, especially when acidification is prolonged. The range of pH in the mouth is from values somewhat below 4 in plaque in pits or fissures of the teeth or near contact points between teeth, to close to 8 in periodontal pockets. The harmful effects of acid to teeth in causing demineralization are well known. There appear also to be harmful effects of alkali in regard to calculus formation and in gingivitis or periodontal diseases. In the latter diseases, a major harmful factor may be specifically ammonia, which can be cytotoxic, rather than just alkaline conditions.

The Range of Acid Tolerance among Oral Bacteria Related to Oral Ecology

The range of acid tolerance among oral bacteria extends from certain strains of *Actinomyces naeslundii* or many periodontal organisms, which cannot function at pH values much below about 6, to *Lactobacillus* organisms able to carry out glycolysis at pH values close to 3. Even within a single genus or species, there can be an ecologically significant range of tolerance. For example, one well-studied strain (NCTC 10904) of *Streptococcus sanguinis* has low acid tolerance and does not function well at pH values much below 5. In contrast, other strains of *S. sanguinis* have sufficient acid tolerance to compete with *S. mutans* at pH values as low as 4 and are considered to be cariogenic organisms. Fungi in general are adapted to function best in acidified environments, and organisms such as *Candida albicans* can survive in the confines of denture plaque, where acids can build up because of poor diffusion into the oral cavity and low local salivary flow.

Periodontal pockets generally have nearly neutral pH or are slightly alkaline. This is, in part, because the microbiota are not highly acidogenic and because crevicular fluid is buffering. Although some periodontal organisms, including *F. nucleatum*, *T. denticola*, and others, can degrade sugars to produce acids, many are asaccharolytic and cannot catabolize sugars. Organisms in subgingival plaque catabolize amino acids to produce the ATP they need to grow and function. As described above, oral bacteria have a variety of known pathways for catabolism of amino acids. Our knowledge of specific pathways for periodontal organisms is currently sketchy. Many of the organisms have the ADS from which ATP can be derived, as depicted in Fig. 5. *T. denticola* has the ADS, and in addition, can ferment glycine, alanine, cysteine, and serine. Unfortunately, our knowledge of the overall metabolism of periodontal organisms still has many gaps.

Catabolism of amino acids generally results in a rise in pH. The CO_2 produced diffuses away. The pK_a for the reaction $HCO_3^- + H^+ \rightarrow H_2CO_3$

is about 6.5, and H_2CO_3 readily dissociates into $CO_2 + H_2O$. In contrast, the ammonia produced is generally retained as ammonium because the pK_a for the reaction $NH_3 + H^+ \rightarrow NH_4^+$ is about 9.6. Thus, the organisms of subgingival plaque have not had to adapt to compete in acidified environments and generally are not major components of the microbiota of supragingival plaque, although they can be detected regularly in small numbers in supragingival plaque, especially near the contact points of teeth. In general, supragingival plaque is made up mainly of gram-positive, facultative anaerobes, while subgingival plaque is very much enriched for gram-negative anaerobes. A commonly accepted sequence for development of periodontal disease starts with accumulation of supragingival plaque at the gingival margin leading to irritation. Subsequent host responses, involving inflammation, lead to a shift in the microbial population to include anaerobic, gram-negative rods and spirochetes, and finally, development of periodontal pockets and outright disease.

Acid Tolerance Related to Specific Functions

The terms acid tolerance and aciduricity are relative—one organism is more or less acid tolerant than another. Highly acid-tolerant bacteria, such as *Thiobacillus* organisms, can function at pH values as low as 0. They are commonly obligate acidophiles not able to function well at pH values close to neutrality. Most of the organisms in the mouth are neutrophiles, that is, they operate best at pH values close to neutrality. However, the more acid-tolerant organisms, especially *Lactobacillus* strains and fungi, function better at lower pH values of 3 to 4, and can be considered moderate acidophiles. Bacteria such as *S. mutans* generally grow better at pH values on the acid side at a pH value of about 6.

The term acid tolerance is relative in another sense, in that it pertains to specific functions. The key acid tolerance in relation to virulence and caries is the capacity of an organism to carry out glycolysis at low pH values but not necessarily grow at that pH. For example, *S. mutans* strains do not grow at pH values lower than about 5. They can carry out glycolysis at pH values close to 4 but cannot use the ATP produced for growth at this low pH value. Thus, the metabolism of the organism is uncoupled—catabolism is uncoupled from anabolism. The organism continues to carry out glycolysis and to synthesize ATP but then simply degrades it. This is mainly catalyzed by the F-ATPase of the cell membrane. This breakdown of ATP is not a waste of resources because the F-ATPase excretes protons across the cell membrane in association with ATP hydrolysis. The pumping out of protons prevents severe acidification of the cytoplasm, which would result in inactivation of key enzymes by acid. In fact, most of the glycolytic enzymes are severely inhibited at pH values much below about 6. Thus, the only way the organism can carry out glycolysis in an environment at a pH as low as 4 is to maintain a cytoplasmic pH higher than the environmental pH. The difference in pH between the cytoplasm and the environment is termed ΔpH, which may be one full pH unit or even greater when the bacteria are operating in acidified plaque. Clearly, the proton-pumping activity of the F-ATPase is key to acid tolerance and, therefore, to cariogenicity because the cariogenic potential of plaque is highly and inversely correlated with pH value. The damage to tooth mineral is exponentially related to the pH drop in plaque. The solubility of

tooth mineral in aqueous solutions is exponentially related to the fall in pH in plaque during sugar metabolism. Since the relation is exponential, small reductions of plaque pH can have major effects.

The acidification of plaque by bacteria such as the mutans group streptococci also gives them an advantage over less acid-tolerant organisms. In the extreme, organisms with low acid tolerance can be killed or severely compromised in acidified plaque. Prolonged acidification of plaque favors the more acid-tolerant organisms of the community. However, it should be realized that growth of these organisms occurs mainly during the more alkaline phases of the plaque pH cycle, above pH values of about 5. Organisms disabled as a result of acid damage are less likely to be able to grow rapidly when plaque pH rises to levels allowing for growth of undamaged organisms.

Constitutive and Adaptive Acid Tolerance

Not only are organisms such as the mutans group streptococci constitutively acid tolerant but they also can respond to acidification adaptively. In other words, when they grow in acidified media at a pH value of 5, they become even more acid tolerant than when they grow in environments at pH close to neutrality. Constitutive acid tolerance depends on a number of physiologic characteristics of the organism, but the most important is the level of F-ATPase activity. Acid-adapted cells have higher levels of F-ATPase activity and higher capacities to pump protons out of the cytoplasm and reduce internal acidification during glycolysis. Adaptive acid tolerance involves up-regulation of genes not only for F-ATPase but also for a variety of other genes, including those responsible for changes in membrane fatty acid composition, for synthesis of chaperonin proteins involved in renaturation and proteolysis of damaged proteins, and for increased activities of DNA or protein repair systems. These changes enhance the capacities of the organisms to produce acid at low pH values and increase their cariogenic potentials. Therefore, continued acid challenge in the mouth associated with activities of the host, such as consuming high-sugar snacks, would not only serve to select for the more acid-tolerant bacteria in plaque but also enhance the caries-producing potential of the selected organisms by inducing adaptive increases in acid tolerance.

Newly synthesized F-ATPase units cannot be inserted into old membranes but need to be incorporated into growing membranes. Therefore, for acid adaptation, new membrane has to be synthesized by the organisms, and full adaptation takes a number of generations. F-ATPases in their fully functional state are actually aggregates of proteins and lipids. The lipids associate mainly with the F_o part of the enzyme, which is embedded in the membrane and contains a pore through which protons move into or out of the cell. The diagram in Fig. 6 shows the basic structure of an F-ATPase. The older name for the enzyme was F_1F_o ATPase. The enzyme is often called the H^+-ATPase to distinguish it from ATPases that function to transport metal cations, rather than H^+, across the cell membrane. The F_1 component sticks out from the membrane into the cytoplasm and can catalyze either hydrolysis or synthesis of ATP. It consists of three α subunits, three β subunits, and single copies of the γ, δ, and ε subunits. The β subunits have the active sites for ATP hydrolysis or synthesis. The F_1

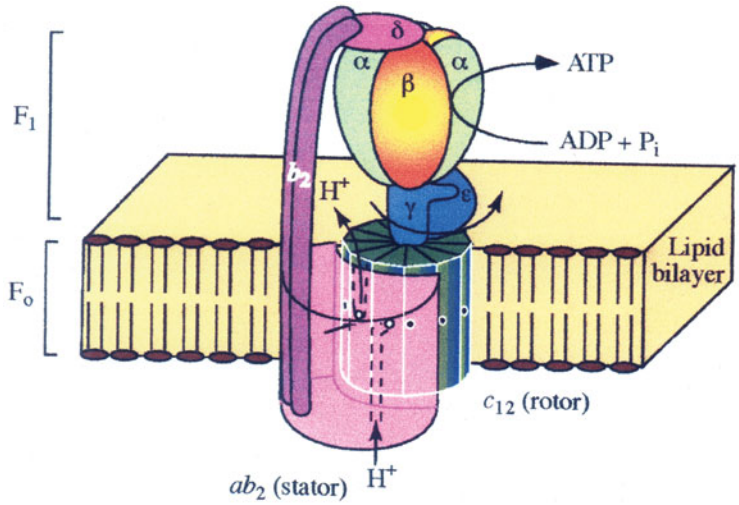


FIGURE 6 Rotary model for F_1F_o ATPase. *S. mutans* uses the system in reverse to increase cytoplasmic pH by pumping protons from the cytoplasm at the cost of ATP. Reproduced from *The Journal of Experimental Biology* with permission. (**R. H. Fillingame, W. Jiang, and O. Y. Dmitriev.** 2000. Coupling H^+ transport to rotary catalysis in F-type ATP synthases: structure and organization of the transmembrane rotary motor. *J. Exp. Biol.* **203:**9–17. http://jeb.biologists.org/) doi:10.1128/9781555818906.ch6.f6

complex, when separated from the cell membrane, can catalyze hydrolysis of ATP but cannot carry out coupled proton transport. The F_o part of the enzyme in the membrane consists of one α, two β, and usually about 12 γ subunits. The enzyme is the major energy-transfer agent in living cells. It is able to transform electrical energy, associated with transmembrane electrical potentials, into mechanical energy involved in enzyme rotation and then into chemical energy involved in ATP synthesis or hydrolysis. In respiration, protons are excreted across the cell membrane by respiratory catalysts to acidify the environment. The protons then move back in across the membrane through the ATPase, and this movement is coupled to ATP synthesis. The movement involves a mechanical process in which the c subunits rotate in relation to a stator made up of ab_2 subunits. The γ and ε subunits spin with the c rotator and translate the spin to the complex of alternating α and β subunits. This movement results in transfer of energy with synthesis of ATP from ADP and P_i. In the oral streptococci, the enzyme functions mainly in the reverse direction. ATP is synthesized at the substrate level, primarily through glycolysis, and can be hydrolyzed by the F-ATPase with concomitant movement of protons out of the cell and maintenance of a cytoplasmic pH higher than the environmental pH.

Acid tolerance responses are of the global type, that is, expression of many genes are up-regulated while expression of many others is down-regulated in response to growth in acidified environments. Often in stress responses of microorganisms, cells become more resistant not only to the inducing stress but also to other stresses. A good example here is the stress response of *S. mutans* to acidification, which results in the organism becoming more tolerant not only to acidification but also to oxidative stresses, such as those associated with exposure to peroxides, which are frequently in oral care products. Acid adaptation also leads to increased

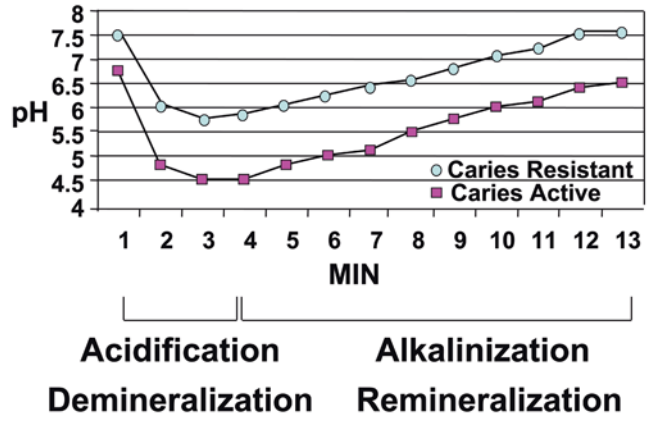
levels of long-chain and unsaturated fatty acids in the cell membrane and changes in general membrane physiology. Thus, the acid-adaptive response is a complicated response involving global regulators, multiple genes, multiple proteins, and lipids.

Most organisms in plaque undergo stress adaptation, notably to acid but also to other stresses associated with life in crowded conditions. In addition, many plaque bacteria are capable of the cell density-dependent regulation of gene expression called quorum sensing (see chapter 5). They can sense crowded conditions and respond by up-regulating certain genes and down-regulating others. For oral streptococci, the biofilm state results in enhanced genetic exchange among cells, and this enhancement appears also to be related to quorum sensing and to responses to environmental stress.

Alkali Production and Tolerance

Much of the focus of interest on bacterial metabolism in relation to caries has been on acid production. However, alkali production is important for moderation of caries. In the standard Stephan curve for changes in plaque pH after a sugar challenge, there is generally a rapid pH drop followed by a slow rise back to the original pH for resting or fasting plaque or even to somewhat higher values (Fig. 7). The rise in pH is thought to involve multiple factors, including the washing action of saliva, buffering by salivary bicarbonate, and alkali production by plaque bacteria. The washing effect may be moderated because of the slow, diffusion-controlled movement of acids out of plaque. In addition, bicarbonate in saliva is not a very good buffer for plaque, again because of diffusion problems. However, plaque itself has a high buffering capacity, mainly because of the high concentration of bacteria embedded in the plaque matrix. The bacteria are buffering against acid drop because of their content of phosphate ($pK_a = 6.5$) and of side chain carboxyl groups such as those of aspartyl or glutamyl residues

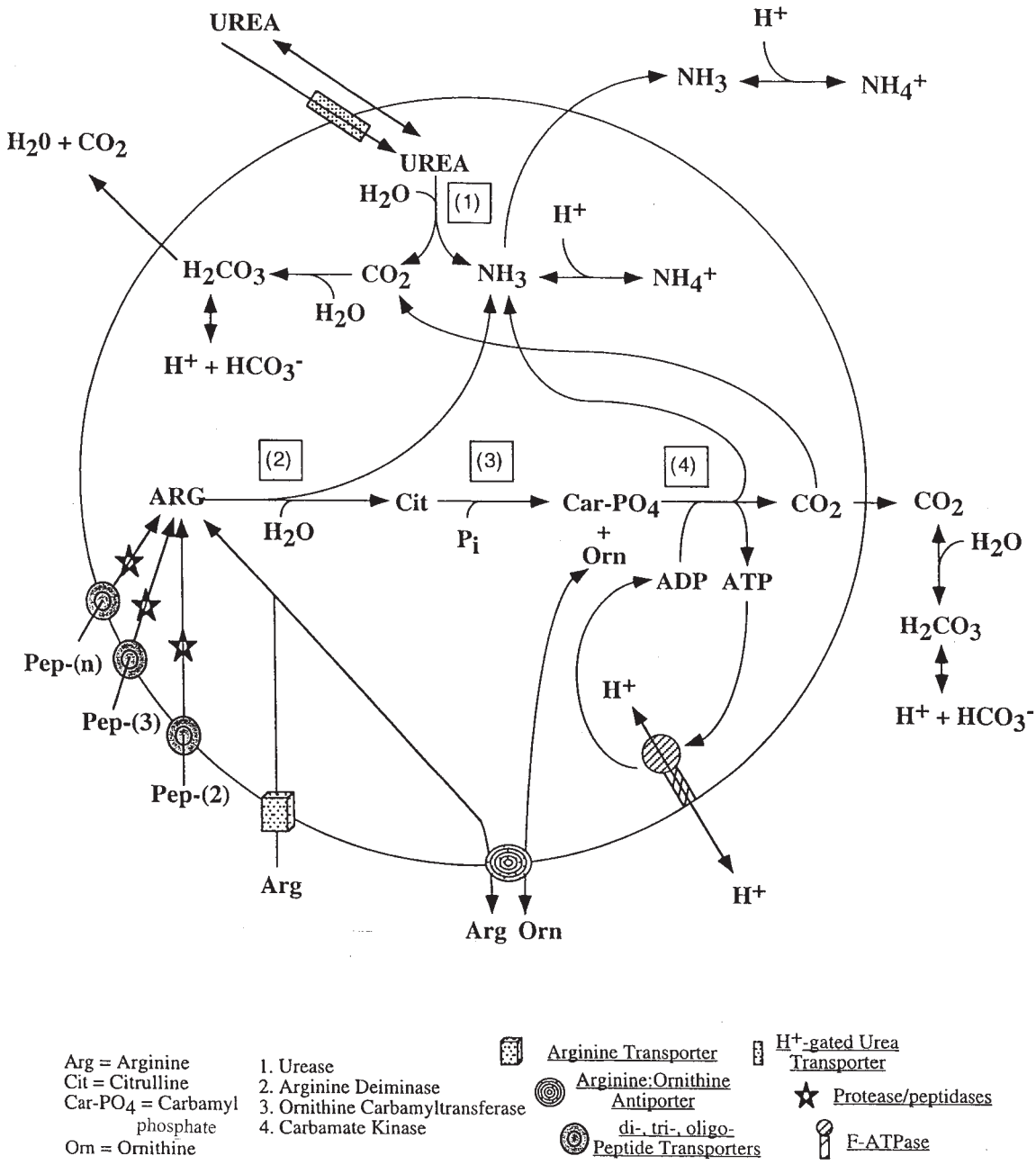
FIGURE 7 Representative pH changes in plaque following ingestion of a carbohydrate source. Time periods involving demineralization of enamel by low pH and subsequent remineralization as the pH rises are indicated. Note that in addition to a more acidic resting pH, plaque from caries-susceptible individuals drops to a lower pH value and has a slower recovery time than plaque from caries-resistant individuals. Adaptation of Stephan curve provided by Ann Griswold, University of Florida.
doi:10.1128/9781555818906.ch6.f7



(pK_a = 4.5). A significant part of the pH rise is considered to be due to production of ammonia from arginine via the ADS and from urea through the action of urease (Fig. 8). Prominent ADS-positive bacteria include *S. gordonii*, *S. sanguinis*, *Streptococcus ratti*, *Streptococcus anginosus*, *A. naeslundii*, *Lactobacillus fermentum*, *P. gingivalis*, and *T. denticola*.

The major urease-positive bacteria in supragingival plaque are the actinomycetes and organisms such as *Haemophilus parainfluenzae*. However, other oral bacteria not so prominent in plaque, such as *Streptococcus*

FIGURE 8 Overview of the major alkali-producing reactions of a composite oral streptococcus. (From R. A. Burne and R. E. Marquis, *FEMS Microbiol. Lett.* **193:** 1–6, 2000.) doi:10.1128/9781555818906.ch6.f8



salivarius and *Streptococcus vestibularis*, are also urease positive and may degrade urea in saliva rather than in plaque. Saliva contains high levels of urea, from 3 to 10 mM, approximately the same as those in serum. Arginine is present as the free amino acid at average levels of only about 50 μM in saliva. However, the major source of arginine for oral bacteria is considered to be in peptides and proteins, which can be degraded by proteases and peptidases of saliva and those secreted by oral bacteria. Many oral bacteria have multiple transport systems for uptake of peptides from proteolysis, including those as large as octapeptides.

Acid-Base Physiology, Virulence, and Disease

The main reactions involved in ammonia production by oral bacteria are shown in Fig. 8. There are other reactions that can release ammonia in plaque, including reduction of proline or ornithine to 5-aminovalerate via Stickland reactions (Fig. 3) and reactions catalyzed by deaminases. However, urease and ADS are considered the main agents for ammonia release. Base production and the associated pH rise are considered to be beneficial in supragingival plaque in relation to dental caries. Base production in plaque is thought to have beneficial ecological effects in favoring the survival of less acid-tolerant organisms in the oral microbiota. The effect then would be to lower the cariogenic potential of plaque.

Alkali production can also have negative influences on oral health. It can enhance production of calculus or tartar because of enhanced precipitation of calcium salts at higher pH values. Ammonia may also be damaging in gingivitis and periodontal disease, as it can be cytotoxic. Other damaging effects of ammonia may also exist.

OXYGEN METABOLISM, OXIDATIVE STRESS, AND ADAPTATION

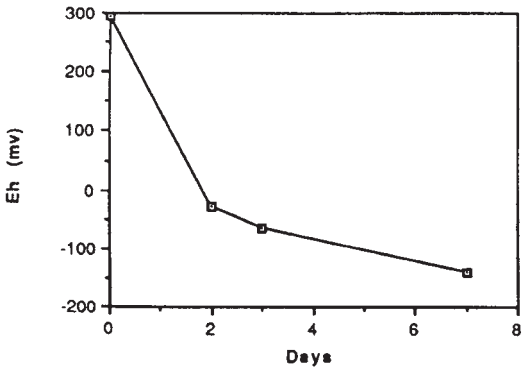
Sources of Oxygen for Oral Bacteria

There are two main sources of O_2 for oral bacteria. One is the gas atmosphere in the mouth, which is basically air with periodic depletion of O_2 and enrichment in CO_2 as a result of respiratory exchange. The gases in the mouth are important for the physiology of supragingival plaque and of the organisms on the soft tissues of the mouth, including the tongue, which is a major site for microbial growth and metabolism. Saliva can be a direct source of O_2 because of the oxygen it contains when secreted or an indirect source because it transfers O_2 from the gas phase in the mouth to the tissues and biofilms it bathes. The other source of O_2 for the mouth is crevicular fluid. This fluid has approximately serum levels of O_2 (1.3 ppm of O_2, 0.04 μmol of O_2/ml, or an average P_{O2} of about 30 mm Hg, values similar to those for venous blood) and supplies oxygen to subgingival plaque. The presence of subgingival plaque during the development of periodontal disease is a stimulus for enhanced flow of crevicular fluid as the host attempts to respond to inflammatory products of bacteria and to rid itself of invaders. Host-produced inflammatory molecules also play important roles in enhancing crevicular fluid flow. The situation in gingivitis is intermediate with some inflammation and fluid flow but also some access to the gas in the mouth.

Oxygen Levels and Oxidation-Reduction Potentials in Dental Plaque

Mature dental plaque is often considered to be highly anaerobic because of its dense biomass. However, plaque is actually a thin film with a large surface-to-volume ratio so that O_2 can diffuse readily into it. Electrode measurements of O_2 levels in periodontal pockets indicate P_{O2} at some 10% of the value for air-saturated water, or an average partial pressure of 13.3 mm Hg. Thus, even subgingival plaque is not highly anaerobic. Measurements have also been made of oxidation-reduction potentials (E_h) of supragingival plaque as it develops on the teeth. E_h is a measure of oxidation or reduction state, somewhat analogous to pH as a measure of acid or base state. The pH value indicates the activity of protons in the system under study, whereas E_h indicates the potential in the system for electrons to be transferred, that is, the potential for oxidation or reduction. As shown in Fig. 9, the E_h value drops as plaque develops but not to values indicative of highly anaerobic conditions. Fig. 9 also shows what are called E_0' values for biologically important oxidation-reduction couples. These values are similar to pK values for acids and bases; only they indicate the E_h value at which the concentration of the oxidized form is equal to that of the reduced form. Thus, for the lactate/pyruvate couple, the E_0' is the E_h value at which the lactate and pyruvate concentrations are equal. Positive values for E_0' indicate an oxidizing couple, for example, the O_2/H_2O couple (E_0' = +818 mV), whereas negative values indicate more reducing couples, for example, the NAD/NADH couple (E_0' = –320 mV). In cells, NADH can serve to transfer electrons to O_2; that is, NADH can serve to reduce O_2. This reduction may involve cytochromes and an electron transport system coupled to oxidative phosphorylation. For many oral bacteria, including the oral streptococci, respiration involves mainly NADH oxidases, which are flavin enzymes able to catalyze transfer of reducing equivalents from NADH through sulfenic acid and flavin to O_2 to produce either H_2O_2 or H_2O. Flavin enzymes typically transfer single electrons, and so there is a propensity for formation of superoxide radicals ($O_2^{\cdot -}$) during catalysis.

FIGURE 9 E_h drop in developing supragingival plaque and E_0' values for some of the oxidation-reduction couples pertinent to plaque. doi:10.1128/9781555818906.ch6.f9



Couple	E_0'(mV)
O_2/H_2O	+818
O_2/H_2O_2	+300
Fe^{3+}/Fe^{2+}	+110
$FAD/FADH_2$	–60
pyruvate/lactate	–186 (–6 at pH 4)
NAD/NADH	–320 (–140 at pH 4)
$O_2/O_2^{\cdot -}$	–330
$\cdot O_2, H^+/HO_2\cdot$	–460

Even in mature plaque, the E_h value falls only to about –100 mV, close to the E_0' value for the pyruvate/lactate couple. Highly anaerobic systems have E_h values in the range of –400 to –500 mV. Thus, plaque is a reducing system overall but not highly reducing. However, it should be realized that the plaque biofilm is stratified. The general finding is that the outer strata are more aerobic and the deeper strata more anaerobic. Thus, E_h in plaque would be expected to decrease with depth away from the saliva-plaque interface or the boundary between plaque and crevicular fluid.

Another oral malady associated with the development of an environment conducive to growth and metabolism of anaerobic bacteria is halitosis or oral malodor. It is associated particularly with conditions such as gingivitis and periodontitis. Malodor can occur without these inflammatory states but is related to metabolism of the tongue microbiota. Malodor is due mainly to putrefactive actions of bacteria on endogenous or exogenous proteins and peptides. The major offending compounds are hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH), and to a lesser extent, dimethylmercaptan (CH_3SSCH_3). These sulfides are produced mainly from cysteine, cystine, and methionine by enzymes such as L-cysteine desulfhydrase, which catalyzes cysteine hydrolysis with production of H_2S, ammonia, and pyruvate. The enzyme is produced by gram-positive bacteria, such as *S. anginosus*, or by *F. nucleatum*, which is commonly associated with gingivitis. *T. denticola* and other gram-negative anaerobes prominent in periodontal disease also produce the enzyme. In addition, compounds such as indole and skatol may contribute to malodor. Currently, there are no good inhibitors of sulfide production for use in the mouth. Control measures, such as brushing of the tongue, are mainly oriented to general plaque reduction. However, there is need for effective antimicrobials against malodor organisms to extend the time between mechanical removal of organisms and regrowth.

Oxygen Metabolism in Oral Bacteria, Reactive Oxygen Species, and Oxidative Damage

In a biological system such as dental plaque, the residual molecular oxygen (O_2) is not the biologically most important form of oxygen, other than serving as an indicator that not all of the incoming O_2 is metabolized. The relevant O_2 is that which is metabolized by the cells. This respiratory metabolism is of importance in transferring energy for the various functions of oral microbes. Respiration involving O_2 as a terminal electron acceptor and that involving alternative acceptors such as nitrate, fumarate, and sulfide can occur in the mouth. Estimates of O_2 flow into plaque with a 1-cm^2 surface area and 0.1-cm thickness are about 15 μmol of O_2/ml/min. This flux can be compared with measured respiratory capacities of 2 to 5 μmol of O_2/ml of wet cells/min for organisms such as *S. mutans*. Anaerobes in plaque also are able to respire. In fact, if anaerobes did not metabolize O_2 at all, they would not likely be anaerobes. It is mainly when O_2 is metabolized that it becomes toxic through production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide radical, perhydroxyl radical ($HO_2^{\cdot}$), and hydroxyl radical (HO·), which are capable of causing oxidative damage. The indicated ROS can be produced directly during oxygen metabolism except for hydroxyl radical, which is thought to be produced mainly through the Fenton reactions indicated

below involving hydrogen peroxide and reduced transition metal cations, mainly Fe^{2+} and Cu^{+} in biological systems. As shown in reaction 1, the oxidized forms of the metal cations can be reduced through reaction with superoxide radical. Then, the reduced cations react with H_2O_2 produced by metabolism or added from outside the mouth to yield hydroxyl anion and hydroxyl radical, as shown in reaction 2.

$$O_2^{\cdot -} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot} \quad (2)$$

The example here is with iron cations, but copper cations in biological systems are also involved commonly. Nickel and cobalt cations are generally at low levels in biological systems, but they also can take part in Fenton reactions. $OH^{\cdot}$ is considered one of the most damaging radicals produced as a result of O_2 metabolism. To avoid having it formed in significant amounts, most cells, especially those of organisms able to grow aerobically, produce catalases and peroxidases to catalyze degradation of H_2O_2 and superoxide dismutases to catalyze removal of the superoxide radical. The reactions catalyzed by these enzymes are included in Table 1. In addition, levels of free transition metal cations are highly regulated by means of various binding proteins so that free Fe and Cu cations do not occur in the cytoplasm. Nitrogen-based radicals, such as peroxynitric acid (HOONO), or organic radicals can also be produced secondarily through reactions involving ROS. They also can be major agents of oxidative damage.

By definition, anaerobes cannot grow in the presence of air in which O_2 is present at a level of 0.21 atm (ca. 160 mm Hg) because they metabolize the gas at a level that overwhelms their limited defense mechanisms

TABLE 1 Examples of enzymes and proteins of oral bacteria protective against oxidative damage[a]

Enzyme or protein	Protective reaction or function
Catalases	$2H_2O_2 \rightarrow 2H_2O_2 + O_2$
Mn-peroxidase	$2Mn^{2+} + H_2O_2 \rightarrow 2Mn^{3+} + H_2O + 0.5O_2$
NADH peroxidases	$NADH + H^+ + H_2O_2 \rightarrow NAD + 2H_2O$
Alkyl hydroperoxidases	$NADH + H^+ + ROOH \rightarrow NAD^+ + H_2O + ROH$
Superoxide dismutases	$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$
Hypothiocyanite reductase	$NADPH + H^+ + OSCN^- \rightarrow NAD^+ + H_2O + SCN^-$
Peroxynitrite reductase (AhpC)	$HOONO + NADH \rightarrow NO_2^- + H_2O$
Glutathione reductase	$NADPH + H^+ + GSSG \rightarrow NADP^+ + 2GSH$[b]
Disulfide reductase	$NADPH + H^+ +$ protein (S-S) $\rightarrow NADP^+$ protein$(SH)_2$
Iron-binding proteins, such as Dpr protein	Bind iron cations
Copper-binding proteins	Bind copper cations
Endonuclease IV, exonuclease III	Apurinic/apyrimidinic endonucleases
Endonuclease III	Removes thymine glycols
Excision nuclease	Excision of damaged DNA
DNA polymerase	DNA synthesis after excision
Glucose-6-P-dehydrogenase	Production of NADPH
SoxRS, OxyR, RpoS, RecA	Regulators of oxidative stress genes

[a]From R. E. Marquis, *Sci. Prog.* **87:**153–177, 2004.

[b]GSH, glutathione (glutamyl-cysteinyl-glycine); GSSG, oxidized glutathione.

against ROS stress. While anaerobes generally have protective enzymes and other mechanisms against oxidative damage, the problem is that they do not have sufficient protection to allow for growth or survival in the presence of the 0.21 atm of O_2 in air. There are also microaerophilic organisms in plaque. They require O_2 for growth but cannot manage the stress of the full 0.21 atm of O_2 in air.

Plaque appears to be stratified in regard to aerobic and anaerobic organisms. There are very few, if any, strict aerobes in plaque. Most of the oral organisms able to grow aerobically are actually facultative anaerobes. The main possible exceptions are the neisseriae, although even they can carry out functions such as glycolysis under conditions of restricted oxygen supply. Most of the organisms in supragingival plaque are facultatively anaerobic, including oral streptococci, most *Actinomyces* and related organisms, *Haemophilus, Aggregatibacter*, and others. Anaerobic bacteria, including veillonellae, can routinely be isolated from supragingival plaque. Gram-negative anaerobes can also be isolated from interproximal areas and deep plaque, presumably in part because of restricted access to O_2 in these areas. In addition, facultative anaerobes can protect strict anaerobes against damage by ROS. For example, many facultative organisms produce catalases, which are highly effective in degrading H_2O_2. Catalase-positive bacteria can protect catalase-negative bacteria against H_2O_2 damage. Even so, in supragingival plaque, the gram-negative anaerobes are generally only a minority population.

In subgingival plaque, anaerobes flourish. They include organisms of the genera *Porphyromonas*, *Prevotella*, and *Fusobacterium*, many *Actinomyces* organisms, and a variety of oral spirochetes. It was once thought that oral anaerobes such as *T. denticola* were among the most extremely anaerobic organisms. However, the results of more recent work with improved culturing methods suggest that they actually are not all that extreme. Although they cannot grow when cultures are aerated, they can grow in complex media in static cultures under an atmosphere of air. They do have protective mechanisms against oxidative damage, including enzymes such as superoxide dismutase. Moreover, in mixed cultures, they can be protected against peroxide damage by other bacteria capable of rapidly degrading peroxides. Likewise, *P. gingivalis* can tolerate oxygen-containing environments when grown in the presence of *F. nucleatum*, since the latter organism has the enzymes needed to remove toxic oxygen compounds from the environment.

In the mouth, there are relatively high levels of thiocyanate (SCN^-), which can react with H_2O_2 in reactions catalyzed by peroxidases to produce hypothiocyanite ($OSCN^-$). Hypothiocyanite can be highly damaging for oral bacteria, and the thiocyanate-H_2O_2-peroxidase system is considered a major defense against infection. There are questions about which is more toxic—hypothiocyanite or hydrogen peroxide. Conceivably, production of hypothiocyanite by peroxidases could be protective for bacteria because it reduces the level of H_2O_2. Moreover, many oral bacteria produce the protective enzyme hypothiocyanite reductase (Table 1).

In environments where halogens are in greater supply than thiocyanate, peroxidases can couple peroxide degradation to form compounds such as hypochlorite or hypoiodite. These compounds are toxic to microorganisms, and their formation is part of the innate immune response of

the host. As mentioned above, nitric oxide (NO) produced by phagocytes and other host cells from arginine can induce oxidative damage. It can also react with superoxide radical to produce toxic peroxynitrous oxide (HOONO), which can be lethal for bacterial cells. HOONO is a weak acid with a pK_a value of 6.8. It can move readily across membranes in the protonated state and then dissociate in the relatively alkaline cytoplasm to yield H^+ and $OONO^-$.

ROS and other highly reactive radicals can react with lipids, nucleic acids, and proteins to yield a variety of damaged products. Lipid peroxidation is important in damage to eukaryotic organisms and involves peroxidation of polyunsaturated fatty acids with possible formation of secondary lipid radicals. Bacteria generally do not produce polyunsaturated fatty acids, with only a few exceptions, for example, certain deep-sea bacteria. Therefore, damage to lipids is not likely to be major for oral bacteria. However, damage to proteins and to nucleic acids does occur. In fact, ROS can be mutagenic as a result of damage to DNA, which the cell then attempts to repair by means of multiple repair systems. Many of these are error prone and introduce genetic changes. Thus, oxygen metabolism presents a mutagenic challenge to organisms and is a major force for variation leading to evolution and biodiversity.

Damage to enzymes and other proteins has also been well documented. For example, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase is highly sensitive to H_2O_2, and damage is enhanced by the presence of reduced transition metal cations. The enzymes or proteins usually most sensitive to oxidative damage are those to which transition metal cations can bind and those with disulfide bonds, especially in the form of Fe-S clusters. Many amino acid residues can be damaged by ROS, and so the products of damage are multiple. However, the range of sensitivity to damage among proteins is wide. For example, dehydratase enzymes are particularly sensitive. This is related to their metal-binding sites, which often involve oxidizable iron-sulfur clusters.

Repair Systems

There may be questions about just how important oxidative stress and damage are in a system such as dental plaque. The physiology of plaque bacteria tells us clearly how important they are. Oral microbes possess an impressive array of defense systems against oxidative damage. They would not have multiple systems for protection if oxidative stress were not a part of day-to-day life, either because of their own oxidative metabolism or a need to neutralize oxidative products secreted by their neighbors or produced by the host. Moreover, as indicated earlier, oral bacteria are generally specifically adapted to life in the mouth and do not have much of a life outside of the mouth. Therefore, their protective mechanisms against oxidative stress are indicative of stress conditions in the mouth.

Table 1 presents examples of some of the protective systems of oral bacteria against oxidative damage. Basically, there are two prominent modes of protection; either getting rid of the damaging species by reduction or dismutation and repairing the damage after it has already been done. There are other possible protective strategies, one of which is just not to metabolize O_2. As discussed earlier, this avoidance does not seem to be a common strategy. Another strategy is to develop metabolic pathways

that do not result in production of ROS. The usual sort of cytochrome aa_3 oxidase is a good example. It catalyzes a 4-electron reduction of O_2 with little or no production of ROS. In contrast, many flavin-based enzymes carry out 1-electron reductions of O_2 and are prone to produce superoxide radicals. When the metabolic systems are under stress or when compounds such as the herbicide paraquat are present, normal electron transport can be disrupted and the production of ROS increased. Many oral bacteria produce H_2O_2, for example, as a result of NADH oxidase action. Then the H_2O_2 can react with reduced transition metal cations to yield the highly toxic hydroxyl radical. Oral bacteria may also produce NADH oxidases that yield water instead of hydrogen peroxide, and these enzymes are considered protective against peroxide damage.

PHYSIOLOGY OF ORAL BIOFILMS

As mentioned previously and discussed in chapters 3 and 5, the microorganisms of the mouth exist generally in biofilms, closely knit communities on surfaces. Oral bacteria adhere to the acquired pellicle on supragingival tooth surfaces, root surfaces, and to cells of soft tissues, especially on the dorsum of the tongue. They also adhere to the epithelial cells in the gingival crevice, which in periodontal disease may be enlarged to form pockets filled with aggregates and biofilms of oral microbes. The most common surfaces in the mouth for adherence are other bacterial cells. Bacteria stick to each other by a variety of mechanisms. The bacteria to which they adhere and the metabolic processes and products of these organisms shape the bacterial community. The study of biofilms is not new, but it is currently of great interest in many microbial systems. Oral microbiology has been a leader in this field because of many decades of research on dental plaque, much of it even before the term biofilm was widely used to describe dental plaque. One key to our understanding of oral microbes and their roles in health and disease is to better understand the nature and activities of biofilms associated with oral surfaces.

The ability to physically interact with surfaces and other bacterial species is an important physiological property of oral bacteria. While interaction with surfaces can occur via a variety of mechanisms, coaggregation, the cell-to-cell interaction between genetically distinct cell types, is especially important. The importance is underscored by the fact that nearly all oral bacteria tested are able to coaggregate with other organisms. Coaggregation generally is mediated by the binding of a protein adhesin on one cell type to a carbohydrate receptor on another cell type. In many cases, the surface molecules mediating the interaction between pairs of organisms has been defined.

Coaggregation is important in the development of dental plaque biofilms because it allows the juxtaposition of organisms that participate in physiological interactions. A popular model of dental plaque formation involves early colonizing bacteria binding to the acquired pellicle on the enamel surface of teeth. These bacteria create a new surface to which other species bind via coaggregation interactions, allowing organisms that participate in physiological interactions to come in close contact with each other. For example, coaggregation is common between early

colonizing *Streptococcus* spp. and *Veillonella* spp. This physical interaction can be detected in samples grown in situ in humans and can be studied using in vitro model systems. The ability to bind to the streptococci allows veillonellae access to lactate produced by streptococci before it is diluted in the flow of saliva. Furthermore, this close proximity between streptococci and veillonellae is important for cell-cell interactions that result in changes in gene expression in the streptococci.

It is clear that the physiological activities of bacteria in dental plaque are governed by environmental factors causing changes in gene expression. These factors include diffusible signaling molecules, autoinducers used for quorum sensing, and competence-stimulating peptides, which are produced by bacteria and are detected by other cells of the same or different species. In some cases, these signaling molecules are needed for mutualistic growth of oral bacteria on saliva. Communication using these systems is described in other chapters of this text.

While much is known regarding the importance of adherence and a seemingly sessile lifestyle to the survival of bacteria in the oral cavity, little is known about the importance of motility of oral bacteria. In some nonoral systems, motility plays a role in biofilm development. Further, bacterial motility is known to play a role in pathogenesis in some nonoral infections. All of the characterized means of bacterial motility are represented among the oral microbiota. These include flagellar motility by *Selenomonas* spp., gliding motility of *Capnocytophaga* spp., twitching motility of organisms including *Eikenella corrodens*, and endoflagellar motility of the spirochetes, including *T. denticola*. A role for motility in invasion of host tissues by *T. denticola* is established, and it seems likely that motility of other oral organisms will have physiological importance and play an as yet undefined role in biofilm community formation.

Physicochemical Gradients in Oral Biofilms and Concentrative Capacities of Biofilms for Fluoride and Other Antimicrobials

Entry and exit of solutes into and out of plaque biofilms tend to be diffusion limited, especially for larger solutes. Very large solutes, such as biopolymers from the environment, are generally excluded from biofilms. Slow diffusion can limit metabolism in biofilms by restricting supplies of nutrients and allowing buildup of inhibitory products such as lactic acid. However, some biofilm structures may contain water channels penetrating deep into biofilms. These channels will increase the surface-to-volume ratio of biofilms and enhance diffusion. Nutrients coming into plaque from saliva or crevicular fluids are at their greatest concentrations at the biofilm surface exposed to the environment. Nutrient levels then decrease with depth in the biofilms as the nutrients are metabolized by surface organisms. Products of metabolism by more superficial organisms may then diffuse out of plaque but also into the depth of plaque, where metabolism is thought to be of a more anaerobic nature. Thus, damaging molecules such as organic acids can move into deep plaque to affect the tooth. In caries development, dissolution of tooth enamel generally initiates below, rather than at, the tooth surface, probably because pellicle can act as a buffer as well as a modulator of solute movement into and out of the tooth. The mineral ions released through acid dissolution of

tooth structure also tend to be concentrated deeper in the plaque biofilm and diffuse only slowly to the surface. In general, salivary influences are separated from the tooth surface by the plaque biofilm but also by pellicle. The dynamics of solute movements through biofilms clearly are of major importance in terms of caries development and likely also periodontal diseases, although less is known regarding the latter.

Biofilms are noted for being resistant to a variety of antimicrobials. It appears that there are multiple mechanisms for their resistance. Some types of resistance, for example, to higher-molecular-weight antibiotics, appear to depend on diffusion barriers in the biofilms. Other types of resistance can depend simply on the high biomass concentration within the biofilms, especially for many disinfectants for which cells have many binding sites not involved in the damage done to the cell by the agent. Typically, the potency of these agents is very biomass dependent with potency inversely related to biomass concentration. Still other types of resistance depend on the physiology of the biofilm organisms. For example, biofilm cells tend to be resistant to β-lactam antibiotics simply because they are growing so slowly, and β-lactams target peptidoglycan synthesis by growing cells. Moreover, biofilms have microenvironments in which microbes are in nongrowing or stationary-phase states, which generally lead to enhanced levels of protective resistance against environmental stresses, including those caused by antimicrobials. Growth in biofilms is thought to occur predominantly in peripheral regions where nutrient levels are highest. Thus, there is heterogeneity within the population that can affect overall sensitivity to antimicrobials and can also result in high levels of persistor organisms not greatly damaged by the agents. However, long-term exposure of plaque to agents such as fluoride has not resulted in development of a resistant microbiota, probably because of the constant fluctuations and turnover of organisms in the mouth.

Biofilms also have a tendency to concentrate many chemicals. This concentrative action can be of advantage to biofilm organisms, especially in the early stages of biofilm formation, because nutrients are concentrated in the developing film, mainly through adsorption. However, these concentrative effects can also be negative, for example, in terms of concentration of heavy metal ions in biofilms. In oral biofilms, fluoride is concentrated to levels some 100 times those in saliva. It appears that the concentration of fluoride is mainly related to its weak-acid character (pK_a = ca. 3.15). Fluoride moves into bacterial cells in relation to ΔpH across the cell membrane, with the cytoplasm alkaline relative to the environment. In the relatively acidified environment outside a cell, fluoride becomes protonated to yield HF. The cell membrane is highly permeable to HF, some 10^7 times more permeable than it is to F^-. Therefore, HF moves readily into the cell where the pH value is higher than that of the environment. Once in the relatively alkaline cytoplasm, HF dissociates to yield F^-, which may act as an enzyme poison, but also H^+, which acts to acidify the cytoplasm. Cytoplasmic acidification is inhibitory for many cytoplasmic enzymes, including those of glycolysis. As long as there is ATP in the cell, and the F-ATPase can function to move protons back out of the cell, some level of ΔpH across the membrane will be maintained, and fluoride will remain concentrated within bacterial cells. Thus, fluoride

acts to thwart the action of the F-ATPase in moving protons out of the cell by bringing extruded protons back across the membrane with HF acting as a carrier. This return of protons to the cytoplasm reduces the acid tolerance of the cell and, presumably, also the cariogenicity of organisms such as *S. mutans*. Other weak acids are also concentrated by dental plaque biofilms, including some used as food preservative, such as benzoate and sorbate, and weak-acid, nonsteroidal, anti-inflammatory agents, such as ibuprofen or indomethacin.

Fluoride may, under certain conditions, precipitate in plaque as calcium fluoride. This type of concentration is relatively short term but is considered important for increasing the levels of fluoride associated with plaque.

Plaque Nutrition Related to Biofilm Physiology

As mentioned, many nutritional interactions occur in plaque biofilms. A well-known example is production of lactic acid by oral streptococci and its subsequent use as a major catabolite by *Veillonella* spp. Another major example involves hydrolysis of salivary glycoproteins by certain oral streptococci and *Actinomyces* spp., resulting in release of sugars. Other organisms, such as the pathogen *S. mutans*, can then use these sugars for catabolism, resulting in acid production. In subgingival plaque, cross-feeding interactions involving oral spirochetes and *P. gingivalis* have been well demonstrated. In this relationship, *T. denticola* excretes succinate, a product of amino acid fermentation, which is consumed by *P. gingivalis*.

While biofilm development relies on the cooperative interactions between oral bacteria, antagonistic interactions occur in oral biofilms as well. For example, organisms such as *S. sanguinis* are able to produce H_2O_2 and can be antagonistic to nonproducers such as *S. mutans*, which generally are more peroxide sensitive. In addition, bacteriocins play a role in interactions between oral bacteria as well. Bacteriocins are protein toxins that are narrow in their killing spectrum, acting on organisms that are similar to the bacterium producing the bacteriocin. It is thought that bacteriocins are produced by one organism to protect its niche from similar organisms that may compete for resources. Bacteriocins or bacteriocin-like activities have been detected from many bacterial species, including *F. nucleatum*, *A. actinomycetemcomitans*, *P. gingivalis*, *Haemophilus influenzae*, and several species of *Streptococcus*. *S. mutans* alone produces at least five different lantibiotic bacteriocins, known as mutacins, that are involved in competition with other oral streptococci. Lantibiotics are posttranslationally modified peptides that contain intramolecular rings formed by thioether cross-linking alanine residues. These peptides are amphipathic and interact with the plasma membrane of target cells to cause leakage of cell contents.

A very important point when considering the physiology of oral biofilms is that a biofilm truly is an integrated community. Its physiology and, therefore, its capacity to cause either disease or protect against disease are related to the entire community and not just to one of the members. This community view is important in terms of strategies to control oral infectious diseases and highlights the roles for the so-called good oral microbes in maintaining oral health.

KEY POINTS

A variety of challenging conditions are faced by the oral microbiota—from the need for the capacity to catabolize a wide variety of substrates in saliva and the diet to tolerance of major fluctuations in environmental conditions that place considerable stress on the populations.

Carbohydrates serve as a major source of energy for oral bacteria. These can be acquired from the host diet as well as through the action of bacterial enzymes that hydrolyze carbohydrates from host glycoproteins.

Lactic acid, a common product of sugar fermentation, is an important substrate for energy generation by other oral bacteria including *Veillonella* species and *A. actinomycetemcomitans*.

Many oral bacteria, especially gram-negative anaerobes, use amino acids as a source of energy. These amino acids are acquired from host proteins through the successive actions of bacterial proteases. Periodontal pathogens, including *P. gingivalis* and *T. denticola*, are known for their numerous proteinases that contribute to the peptides available to the oral bacterial community. The amino acids are fermented for energy by several different pathways. In addition, many oral bacteria are unable to synthesize all of the amino acids needed for protein translation and must obtain them from their environment as well.

The pH of the mouth fluctuates often and to a great extent. Oral bacteria have multiple physiologic and genetic mechanisms to cope with sudden drops in pH and in sustained acidification, which favor the growth of aciduric, cariogenic plaque bacteria.

Pumping protons to maintain a cytoplasmic pH that is neutral relative to the extracellular environment, production of ammonia by, for example, arginine deiminase, and adaptive acid tolerance are critical factors in low pH tolerance.

A primary mechanism of action of fluoride is to dissipate the proton gradient ($pH_{out} < pH_{in}$) by carrying protons into the cell as HF at low plaque pH values.

Oxygen itself is not particularly toxic for bacteria. Instead, it is the single-electron reductions of oxygen by the oxidative enzymes of bacteria that generate ROS that can damage lipids, proteins, and DNA. Anaerobes cannot tolerate oxygen because they either have very low levels of, or lack, the enzymes needed to detoxify ROS.

Growth of oral bacteria in biofilms, which have an ordered three-dimensional structure that creates gradients of nutrients, end products, and stressors, fosters biodiversity and allows complex, degradative communities of bacteria to persist in the mouth. Growth in biofilms offers bacteria considerable protection from host immune and nonimmune defenses and from antimicrobial agents.

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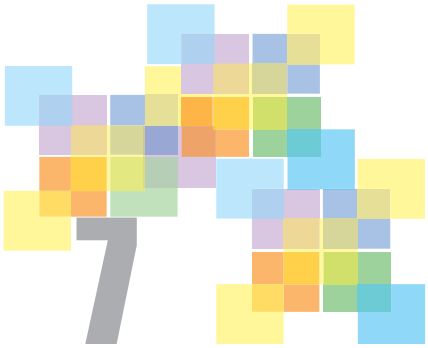

Genetics and Molecular Biology of Oral Microorganisms

Susan Kinder Haake, Donald J. LeBlanc, and Gena D. Tribble

INTRODUCTION

Fundamental Terms in Bacterial Genetics

The genetics of oral microorganisms as an area of study, relative to the broader field of microbial genetics, is still much less well developed. Genetic analysis of the oral microbiota really only began in earnest in 1973 with the first reports of finding plasmid DNA in a strain of *Streptococcus mutans*. In the 1980s, mechanisms by which exchange of genetic information occurred between oral streptococcal species were described. The isolation of naturally occurring streptococcal plasmids led to the development of new molecular tools for genetic studies. By the late 1980s and 1990s, it was possible to genetically manipulate other oral microorganisms such as *Actinomyces* spp., *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis*. Genetic studies were then extended to strains of *Treponema denticola*, soon followed by genetic and molecular analyses of *Fusobacterium nucleatum* and *Prevotella intermedia* strains. More recently, the genomic sequences of over 30 oral bacterial species have been determined. With the advent of high-sensitivity, high-throughput DNA sequencing technologies, the genetic composition of biofilm communities taken directly from the oral cavity can be obtained, bypassing the need for cultivation and manipulation of fastidious oral bacteria.

It is assumed that the reader has at least a basic understanding of the fundamentals of genetics. Some terms, as they apply specifically to microorganisms, and more specifically to bacteria, are defined below. For a more thorough review of microbial genetics and molecular biology, the reader is referred to chapters 14 through 20 in J. W. Lengeler et al. (ed.), *Biology of the Prokaryotes* (Blackwell Science, Malden, MA, 1999). The term microbial genetics refers to studies of the processes by which all the qualities of a microbial species are transmitted from one generation to another by mechanisms associated with DNA replication and with the expression of the information contained in that DNA. The genotype of a microbial strain is the total complement of genetic information that is carried by that strain on the chromosomal, plasmid, or viral DNA molecules

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that comprise its total genome. The phenotype of a strain refers to those characteristics encoded by the genome that are actually expressed at any one time, and under a particular set of environmental conditions, as observable or measurable traits. The genetic information of bacteria is organized in operons, which are contiguous segments of DNA that encode single mRNA molecules containing one or more structural genes and a single set of regulatory elements that coordinate the expression of all the genes within an operon. A structural gene is a segment of DNA, also referred to as an open reading frame, that encodes a protein, and the segment of an mRNA transcript from which the protein will be translated is referred to as a cistron. Thus, an operon may yield a monocistronic or polycistronic mRNA. The expression of structural genes within an operon is governed by regulatory elements that may be *cis*-acting nucleotide base sequences in the DNA or RNA, or *trans*-acting molecules such as proteins. The more common regulatory elements that may be associated with operon expression include a promoter, or region of DNA at which RNA polymerase binds and initiates transcription, and a sigma factor, which is a subunit of RNA polymerase that ensures proper recognition of a specific promoter sequence. An operator is the region of DNA at the 5′ end of an operon at which either a repressor, which inhibits transcription (negative control), or an activator, which potentiates transcription (positive control), binds for control of operon expression. An operon inducer may be a molecule or environmental condition that serves to remove a repressor from an operator to allow transcription or converts a repressor to an activator of transcription. Groups of operons, some of which are induced while others are repressed by the same environmental stimuli, are referred to as regulons. Operons that are part of the same regulon need not be adjacent to each other on the chromosome, and the expression of an operon may be influenced through more than one regulon. Progress toward a more comprehensive understanding of the genetics of microorganisms, as well as correlations between genotypes and phenotypes under a variety of environmental conditions, has been accelerated by the development of recombinant DNA technology and the publication of the nucleotide base sequences of an ever increasing number of bacterial genomes. The application of these technologies and advances to studies of the oral microbiota is the topic of this chapter and chapter 8.

BACTERIAL DNA INHERITANCE

Gene Transfer Mechanisms

DNA can be acquired by either vertical or horizontal (also referred to as lateral) transmission (Fig. 1). Vertical transmission (Fig. 1A) refers to the transfer of a copy of the parental DNA to daughter cells upon cell division. Parental DNA is copied prior to bacterial cell division by DNA polymerases, and this copy of the parental DNA is partitioned into the daughter cell. DNA acquired by vertical transmission is generally identical to that of the parental cell, and therefore, the daughter cells are most often phenotypically indistinguishable from the parental cells. However, genetic changes can occur in the context of vertical DNA transmission, as a result of minor errors in the DNA replication process, resulting in

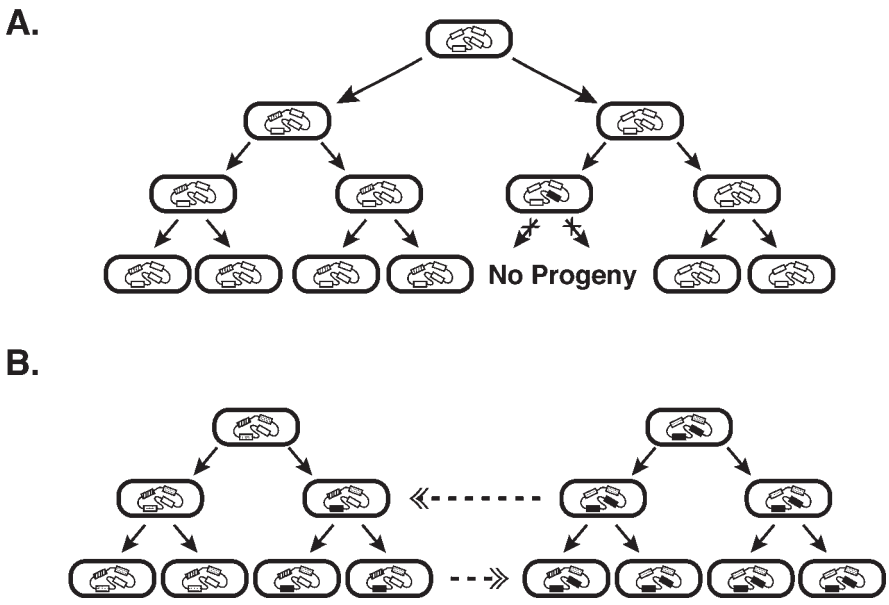

FIGURE 1 Schematic illustration of the effects of vertical or horizontal transmission of genes. (A) Vertical transmission involves the transfer of a duplicate copy of the parental DNA to each of the daughter cells. Genetic diversity is dependent on mutations (hatched and black blocks) that yield variants of a given gene, which follow a clonal distribution. Selective pressures that can influence the population distribution include lethal mutations; cells receiving such lethal mutations do not produce progeny. (B) Horizontal transmission involves the transfer of DNA between cells of a population, enabling a shuffling of genes or DNA segments. The occurrence of a given genetic variant does not follow a clonal pattern of distribution when transfer is by horizontal transmission. (Adapted from Fig. 1 in M. C. J. Maiden, *FEMS Microbiol. Lett.* **112:**243–250, 1993, with permission from Elsevier.)
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single-base-pair (point) mutations in the daughter cell genome. These spontaneous and random mutations, if they occur in an open reading frame, can result in a change in protein amino acid sequence (missense mutations), or early termination of a protein can result from introduction of a stop codon (nonsense mutations). Additionally, point mutations in promoter regions can result in changes in the level of gene expression. These seemingly minor changes to the DNA sequence can sometimes result in significant phenotypic changes in the daughter cell. For example, the antibiotic rifampicin acts by binding to a specific site on the bacterial RNA polymerase enzyme, blocking transcription of mRNA. Spontaneous rifampin resistance occurs if the binding site amino acid is altered due to a single missense point mutation. These random changes resulting from DNA polymerase errors occur at an extrememly low frequency, usually on the order of less than one nucleotide per 10^7 processed, because of proofreading functions of the polymerase enzyme.

Horizontal transmission refers to the acquisition of entirely new segments of DNA, which can occur between strains of the same species or among different species, and can involve large regions of DNA such as plasmids or transposons (Fig. 1B). The significance of horizontal DNA transfer became evident in the 1960s when the spread of antibiotic resistance genes among bacterial pathogens was correlated with the acquisition

of plasmid DNA by the resistant strains. At a molecular level, two basic approaches are used to identify new regions of DNA acquired by horizontal transmission. Phylogenetic analyses involve molecular characterization of specific genes and DNA or amino acid sequence comparisons. These analyses reveal identity or similarity of genes with homologues in other strains or orthologues in other species and provide evidence for horizontal transfer. Mosaic genes, which demonstrate blocks of DNA that appear to have arisen by lateral transfer of DNA from homologous genes in related species based on sequence analyses, have also been described (Fig. 2). More recently, sequence-based parametric analyses have been used to examine DNA regions in the context of the genome in which they occur. Bacterial genomes have a characteristic average percentage of G/C content, which can range from approximately 25 to 75%. For example, the *P. gingivalis* genome has an average G/C content of 48%, whereas *F. nucleatum* strain genomes range from 26 to 27% and *Streptococcus mutans* genomes average 36% G/C content (Table 1). Within the chromosome of a given species, genes are typically relatively uniform in the distribution of this DNA base composition. Thus, identification of a gene or region of DNA with a base composition markedly different from that of the genome as a whole suggests that the atypical region of DNA may have been acquired by horizontal transmission from a species with a different G/C content. Similar analyses are conducted using codon bias and dinucleotide frequencies. The identification of segments of DNA specifically

FIGURE 2 PBP genes of sensitive and resistant streptococci. The coding region of the PBP2B gene of penicillin-sensitive *S. pneumoniae* strain R6 is represented by the open bar A, with the transpeptidase domain and active site serine residue indicated above the bar. The sequenced regions of the PBP2B genes from penicillin-resistant streptococci are represented in bars B to E. Two distinct blocks, one black and one hatched, each represent homologous DNA sequences that are highly diverged from the sequence of the penicillin-sensitive wild-type strain. The percentage of divergence from the sequence of the gene from the penicillin-sensitive strain is indicated above the relevant region of the gene. The distribution of homologous blocks of divergent DNA in the resistant strains, forming "mosaic genes," indicates horizontal transfer of DNA as a mechanism of resistance development. (Adapted from C. G. Dowson et al., *Proc. Natl. Acad. Sci. USA* 87:5858–5862, 1990.)
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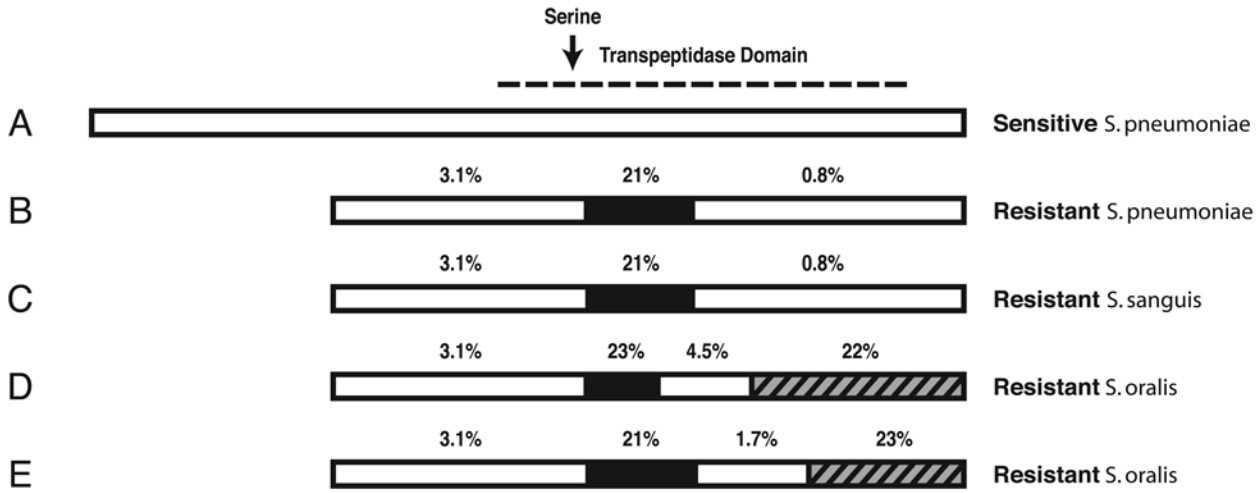

TABLE 1 Genome and plasmid data for oral bacteria

Microorganism[a]	No. of strains sequenced[b]	Avg genome size (Mb)	Avg % G+C	Sequenced native plasmid(s)[c]
A. actinomycetemcomitans	6	2.2	44.3	pVT745
F. nucleatum subsp. *polymorphum*	2	2.4	26.8	pFN1
F. nucleatum subsp. *nucleatum*	1	2.1	27.1	pKH9, pPA52
F. nucleatum subsp. *vincentii*	1	2.1	27.3	
P. gingivalis	3	2.3	48.2	
P. intermedia	1	2.6	43.4	pPI1, pYHBi1
Prevotella melaninogenica	2	3.2	40.8	
S. gordonii	1	2.1	40.5	
S. mitis	6	2.0	40.4	
S. mutans	2	2.0	36.8	pUA140, pLM7
S. sanguinis	12	2.3	43.4	
Tannerella forsythia	1	3.4	46.9	
T. denticola	2	2.7	37.8	pTS1
Veillonella parvula	3	2.1	38.6	

[a]Sites for comparative analyses of oral pathogens may be found at The J. Craig Venter Institute (JCVI) Comprehensive Microbial Resource site (http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi), the Los Alamos National Laboratory Human Oral Genomic and Metagenomic Resource (http://semiglobe.lanl.gov/),The Human Oral Microbiome Database (HOMD) (http://www.homd.org/), and The Human Microbiome Project (HMP) (http://www.hmpdacc.org/).

[b]As of 2012.

[c]From the Los Alamos National Laboratory Human Oral Genomic and Metagenomic Resource, Oral Plasmid Collection (http://semiglobe.lanl.gov/microbe.php?class=oral).

associated with mobile genetic elements, such as plasmids, bacteriophage, or transposons, and flanking genes with disparate base composition provides additional evidence of a foreign origin of the DNA.

Horizontal gene transfer requires the uptake and establishment of the DNA in the recipient cell, either by integration into and subsequent replication as part of the chromosome or as an extrachromosomally replicating element, such as a plasmid or bacteriophage. The known mechanisms responsible for horizontal gene transfer are transformation, conjugation, and transduction. Transformation involves the uptake of cell-free DNA from the environment by the recipient bacteria. The ability of a bacterium to actively take up DNA is referred to as "competence," and specific environmental conditions influence competence development. Transduction refers to the introduction of DNA into a bacterial cell by a bacteriophage (bacterial virus). Bacteriophages have been found in a variety of bacterial species and are produced within a bacterial cell and then released from this donor for subsequent infection of a new recipient cell. However, transduction requires specific receptors on the recipient strain that are recognized by the bacteriophage, and this requirement limits the bacterial host range of bacteriophage. Transduction, like transformation, does not require contact between the donor and recipient bacterial cells. The third mechanism, conjugation, involves the transfer of DNA in a process that requires cell-to-cell contact. This contact is often mediated by formation of a tube or pore between cells, through which DNA is actively transferred. A DNA element that possesses all the genes necessary for transferring itself is referred to as "conjugative." In some cases, DNA is "mobilizable," possessing one or more properties necessary for conjugative transfer but with most of the genes required for conjugation encoded

by another coresident conjugative plasmid or on the host chromosome. DNA elements transferred by conjugation may be conjugative plasmids or transposons and mobilizable plasmids or transposons. Most plasmids transferred by conjugation remain as extrachromosomally replicating elements upon transfer to a new host. Transposons and some plasmids integrate into the recipient cell chromosome and are replicated along with the chromosomal DNA. Conjugation is responsible for the transfer of genetic elements between similar microbial species as well as between organisms as diverse as bacteria and plants.

Gene Transfer in Nature

Evidence of gene transfer involving oral microorganisms consists of documentation of the acquisition and spread of phenotypic properties such as antibiotic resistance, documentation of DNA sequences in different strains and species consistent with horizontal transfer, and demonstration of naturally occurring mechanisms of gene transfer in oral species. Issues regarding antibiotic resistance are discussed in chapter 21 and are not discussed in detail here. The discussion below focuses on natural mechanisms of gene transfer and evidence for gene transfer based on analyses of DNA sequence.

Natural transformation appears to be widespread in bacteria and common to many oral species. The details of naturally occurring transformation have been described for many oral streptococci and for the Gram-negative periodontal pathogen *P. gingivalis* (Table 2). In streptococci, competence develops during the early to mid-logarithmic phase of growth, and there are considerable differences in the optimal conditions for any specific strain or species. The development of competence involves a quorum-sensing process encoded by three genes, *comC*, *comD*, and *comE* (Fig. 3). The *comC* gene encodes a small peptide precursor of the signaling molecule (competence stimulating peptide) responsible for cell-to-cell communication. The precursor molecule is cleaved during export from the bacterial cell by the products of *comA* and *comB*. This mature signaling peptide is a 19-amino-acid competence factor in *Streptococcus gordonii* and 17- or 21-amino-acid competence-stimulating peptides in *S.*

TABLE 2 Natural transformation in oral microorganisms

Microorganism	Strain(s)	Mechanisms involved in competence and DNA uptake
A. actinomycetemcomitans	D7S	Competence induced by cAMP; preferential uptake of DNA possessing USS
P. gingivalis	W83, ATCC 33277, ATCC 53977	Increased transformation efficiency in biofilm cells compared to planktonic cells; *comF* dependent
Streptococcus cristatus	CC5a, CR3, CR311, PSH1a, PSH1b	Competence induced by competence factor
S. gordonii	Wicky, M5, DL1	Competence induced by competence factor
S. mutans	UA159, NG8, JH1005, BM77, GB14, CT11	Competence induced by competence factor; 10- to 600-fold increased transformation efficiency in biofilm cells compared to planktonic cells
S. oralis	CN3410	Competence induced by competence factor
S. sanguinis	ATCC 10556	Competence induced by competence factor

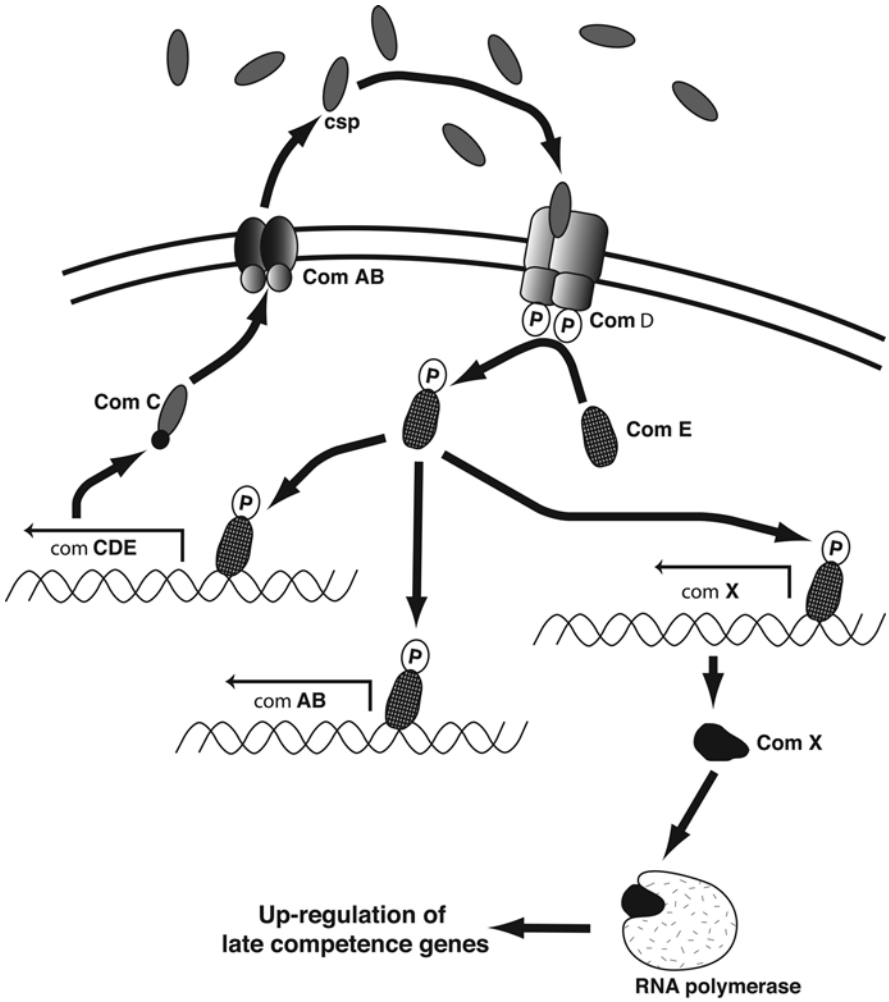

FIGURE 3 Schematic illustration of pathways involved in competence development in streptococci. The pathway of competence development in *S. pneumoniae* is illustrated. Homologues of many of the specific genes and gene products involved have been identified in the chromosomes of competent oral streptococci, including *S. mutans*. (Adapted from Fig. 2 in D. G. Cvitkovitch, *Crit. Rev. Oral Biol. Med.* **12:**217–243, 2001.) See text for details of pathways.
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mutans and *Streptococcus pneumoniae*, respectively. Concentrations of competence factors or competence-stimulating peptides in the extracellular milieu above a threshold level are detected by neighboring bacterial cells by a sensor kinase (histidine kinase) encoded by *comD*, which then phosphorylates a response regulator encoded by *comE* within the bacterial cell. Phosphorylated ComE interacts with a specific DNA sequence upstream of genes involved in transformation, thereby up-regulating their expression and inducing a signaling cascade within the bacterium. These include early-competence genes such as *comCDE*, whose products function in cell-to-cell signaling, as well as an alternate sigma factor gene *comX*, which up-regulates late-competence genes with gene products involved in DNA uptake and recombination.

The transformation process is initiated with binding of double-stranded DNA to the bacterial cell surface. This binding is not dependent on the DNA sequence and is sensitive to DNase treatment. Following nicking of the DNA, one strand is transported into the bacterial cell in a 3′-to-5′ direction, while the other strand is degraded. Within the bacterial cell, the DNA may incorporate into the recipient cell chromosome in a RecA-dependent process if it contains a sufficient region of homologous

DNA. Plasmid DNA may circularize and become established as an extrachromosomally replicating element, but this process is limited in efficiency due to the recircularization requirement. Recent results obtained with *S. pneumoniae* demonstrate that DNA is released from a subfraction of cells simultaneously with the development of competence. Thus, natural competence development in streptococci appears to involve a complex coordination of mechanisms providing a pool of donor DNA as well as the uptake of environmental DNA.

Natural transformation in several gram-negative species, including *Haemophilus* species and *A. actinomycetemcomitans*, differs from that found in the streptococci. Two key differences are the existence of specificity in the uptake of DNA and regulation of competence development. The presence of an uptake signal sequence (USS) in the DNA confers specificity as to which DNA molecules will be taken up. The USS of *Haemophilus influenzae* and *A. actinomycetemcomitans*, characterized by a 9-bp core sequence (5′-AAGTGCGTT), is found at much greater frequency in the genome than would be expected if its occurrence were random. Competent cells efficiently and preferentially take up DNA molecules possessing the USS sequence. The addition of a cytoplasmic regulatory molecule, 3′-5′ cyclic AMP (cAMP), to cultures of these gram-negative species stimulates the development of competence in both species, suggesting that catabolite repression regulates competence development. Catabolite repression is a metabolic sensing system in which an abundance of nutrient substrate is linked to low levels of cAMP and minimal expression of the regulated metabolic pathways. In the relative absence of nutrients, increased levels of cAMP result in increased expression of selected backup metabolic pathways. In *H. influenzae*, only a small fraction of cells become competent in late-exponential- or early-stationary-phase cultures in rich medium, whereas all cells become competent by shifting the culture to starvation conditions, and competence is inhibited by providing nucleic acid precursors.

Bacterial competence and the uptake of environmental DNA are a highly regulated process. It has been speculated that competence functions as a mechanism for acquisition of DNA as a nutrient as well as a mechanism for ensuring genetic diversity. Documentation of mosaic genes and demonstrations in vitro of transformation processes contributing to gene rearrangement support the latter hypothesis. Both oral gram-negative and gram-postitive bacteria are thought to utilize competence mechanisms to generate genetic diversity and adapt to changes in the oral cavity. One example is *P. gingivalis*, which has been shown to exchange extracellular DNA between strains during biofilm formation, which may result in reassortment of virulence alleles to form new pathogenic isotypes. Another example involves penicillin-binding proteins (PBP) in oral streptococci. Resistance to penicillin in *S. pneumoniae* was first reported in the 1960s and is now evident in strains worldwide. Penicillin resistance is found in strains harboring low-affinity variants of the PBPs. The variant PBP genes demonstrate discrete blocks of DNA responsible for resistance, with sequences nearly identical to homologous genes of commensal streptococci including *Streptococcus mitis* and *Streptococcus oralis* (Fig. 2). In vitro studies demonstrating transformation of *S. pneumoniae* with DNA from *S. mitis* or *S. oralis* yielded transformants with increased penicillin

resistance and recombination in the PBP gene consistent with the pattern seen in mosaic genes in nature. Analyses suggest that in vivo resistance develops in commensal species prior to lateral transfer to pneumococci by transformation. There is also evidence of the transfer of a mosaic PBP gene conferring resistance from *S. pneumoniae* to *Streptococcus sanguinis*, another member of the normal oral microbiota. These results demonstrate the relevance of transformation in normal oral ecology and pathogenesis, and the importance of commensal microorganisms as a "repository" for genetic determinants.

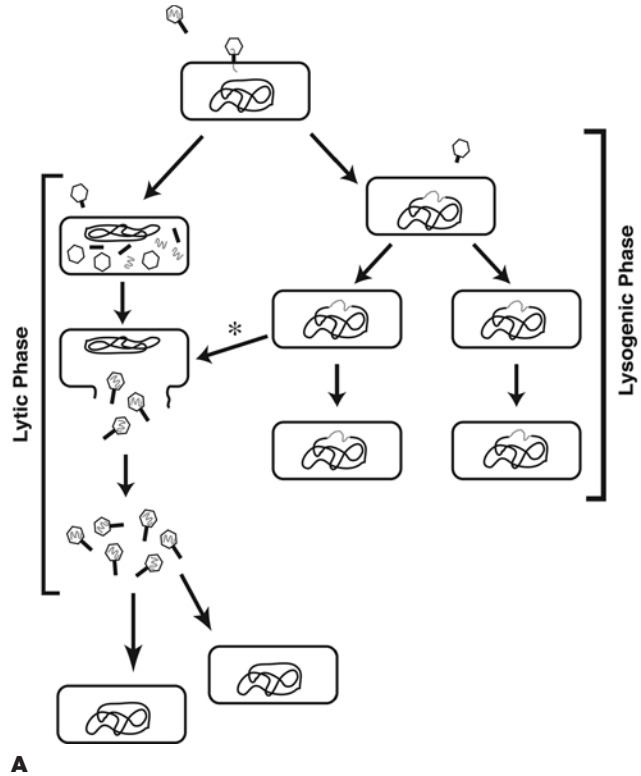
With the advent of whole-genome sequencing techniques, evidence for genetic exchange by transduction among oral microorganisms is growing. Bacteriophages have been detected in a number of oral microorganisms, and the ability of many to replicate in their respective hosts has been demonstrated (Table 3). Bacteriophages are termed "virulent" or "temperate" depending on characteristics of their life cycle in the host bacterial cell (Fig. 4A). Virulent bacteriophages uniformly lyse the infected bacterial cell and release phage particles in what is termed lytic growth. Temperate bacteriophages are also capable of lytic growth but, alternatively, may exist in a quiescent phase when the DNA integrates into the bacterial chromosome or replicates extrachromosomally. This quiescent

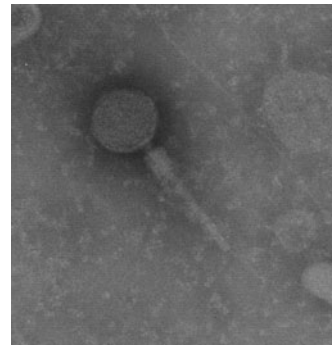
TABLE 3 Bacteriophages of oral microorganisms

Microorganism	Phage designation(s)	Phage distribution	Phage properties	Phage structure	Nucleic acid composition
A. actinomycetemcomitans	AaΦ23 (type strain of predominant class of bacteriophage), others[a]	Bacteriophages are detected in 33–54% of strains examined; the majority of bacteriophages are related to AaΦ23 and the majority of strains harboring AaΦ23 phage are serotype a	Temperate phage; generalized transducing phage	Isometric head, 60-nm diameter; contractile tail of 115 nm	Linear dsDNA,[b] 44 kb; prophage is integrated into the host chromosome
Actinomyces viscosus, *A. naeslundii*	Multiple bacteriophages have been isolated, including Av-1, CT2, BF307, and Φ224	Bacteriophages isolated from dental plaque (10% of samples tested) and sewage	Temperate (Φ224) and virulent bacteriophages	Av-1, CT2, and BF307: polyhedral head 40 nm in diameter, tail of 26 nm	dsDNA ranging from 16–60 kb; Φ224 replicates in strain MG-1 as a plasmid
S. mutans	ΦPK 1	Bacteriophage isolated from *S. mutans* PK 1	Temperate bacteriophage	Hexagonal head with contractile tail	DNA, 27–29 kb
S. mitis	SM1	Isolated from strain SF100; encodes adhesins for platelet binding	Temperate phage, *Siphoviridae* family		dsDNA, 34 kb
E. faecalis	No designation	Isolated from saliva (22% of samples tested) or swabs of tongue and teeth	Virulent and temperate bacteriophages	Spherical, enveloped, spiked structure, 70-nm diameter	Undetermined
Treponema denticola	varphitd1	Isolated from biofilm model strain			Circular
Fusobacterium nucleatum	FnpΦ02, FnpΦ13	Isolated from two clinical isolates	*Siphoviridae* family	Icosahedral head and segmented tail	dsDNA, 59 kb

[a]Numerous temperate bacteriophages have been isolated from *A. actinomycetemcomitans*. AaΦ23 appears to predominate, but the genetic relationship of AaΦ23 with others such as ΦAa has not been determined.

[b]dsDNA, double-stranded DNA.

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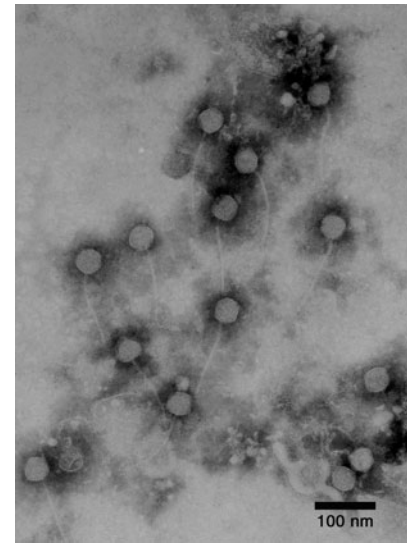

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FIGURE 4 (A) Schematic illustration of transduction pathways. Bacterial recipients of bacteriophage DNA enter lytic or lysogenic phases. Virulent bacteriophages use lytic growth (left side), which involves the replication and assembly of progeny bacteriophage followed by their release upon lysis of the host bacterial cell. Temperate bacteriophages may use the lytic phase or the lysogenic phase. In the lysogenic phase (right side), the bacteriophage DNA is replicated within the host cell, either integrated within the chromosomal DNA (illustrated) or extrachromosomally (not illustrated). Bacterial cells in the lysogenic phase do not undergo lysis but may be induced to enter the lytic phase (asterisk). However, when temperate phages enter into a lytic phase, during the maturation process, fragments from virtually any part of the host chromosome may be packaged into new heads in place of the phage genome (generalized transducing phage), or a segment of host chromosome adjacent to the specific site of insertion of the prophage may be excised along with the phage DNA and subsequently packaged into new heads (specialized transducing phage). In either case, bacterial DNA from one bacterial host may be transferred to a new bacterial host during subsequent rounds of phage infection (transduction). (B) Phage with contractile sheath (Bradley [1967] group A) isolated from *A. actinomycetemcomitans* from sites undergoing rapid periodontal destruction in patients with Papillon-Lefèvre syndrome. The head of the phage is 60 nm in diameter. Image kindly provided by E. Namork, Norwegian Institute of Public Health, and H. R. Preus, Faculty of Dentistry, University of Oslo, Oslo, Norway. (C) Electron micrograph of *E. faecalis* bacteriophage ÆEf that was isolated from an *E. faecalis* strain originating in an infected root canal. The negative staining reveals the phage head and long tail structures. Image kindly provided by R. H. Stevens, O. D. Porras, and A. L. Delisle.
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"prophage" DNA may be induced to lytic growth with subsequent production of phage particles and bacterial cell lysis.

Bacteriophages may transfer bacterial DNA from one host to another by one of two mechanisms, i.e., either generalized or specialized transduction. In generalized transduction, virtually any segment of host cell DNA becomes incorporated into the phage head in place of the phage genome. When a phage particle containing such bacterial DNA injects its nucleic acid into a new host following attachment, some or all of the DNA may

then become part of that cell's genomic makeup. In specialized transduction, a temperate phage excises imprecisely from the bacterial host chromosome. In this manner, a segment of phage DNA is left behind, and a segment of chromosomal DNA adjacent to either end of the specific site of integration of the phage DNA may be excised along with the remaining phage DNA and become packaged in new phage heads during the lytic phase. Following release of phage particles during cell lysis, if such a phage particle attaches to a bacterial cell, the excised segment of the previous host's chromosome will be injected into the new host as part of the phage DNA and incorporated into the new host's chromosome during the formation of a prophage state (lysogeny).

Among oral bacteria, prophages harbored in strains of *A. actinomycetemcomitans* and *S. mutans* have been isolated (Table 3). In addition, bacteriophage specific to *Actinomyces naeslundii*, *Enterococcus faecalis*, *T. denticola*, and *F. nucleatum* have been isolated from dental plaque, saliva, oral swabs, and sewage samples (Table 3). Temperate bacteriophages are common in strains of *A. actinomycetemcomitans*, with detection rates from 33 to 54% of strains examined. The commonly isolated bacteriophages comprise a genetically related family with AaΦ23 representing the type strain (Fig. 4B). AaΦ23 is a temperate phage containing a linear 44-kb double-stranded DNA molecule that integrates into the bacterial host chromosome in the prophage state. When induced, the bacteriophage enters a lytic phase with production of phage particles that are released upon lysis of the bacterial cell. In the laboratory, AaΦ23-like bacteriophages are capable of transferring antibiotic resistance determinants to nonresistant strains, indicating that they may contribute to lateral gene transfer among strains of *A. actinomycetemcomitans*. In *S. mitis*, bacteriophage SM1 encodes two adhesin proteins that allow specific binding of the bacteria to platelets, a proposed central mechanism for the initiation of endocarditis. Finally, studies of the salivary virome reveal a substantial number of bacteriophage types present in saliva (approximately 2,000), and many encode genes for lysogeny as well as potential virulence factors for their host bacteria, such as genes involved in degredation of complement.

Further evidence of bacteriophage activity in the oral cavity is the discovery of clustered regularly interspaced short palindromic repeats, or "CRISPRs," in the genomes of oral bacteria. CRISPRs are found in many different species of bacteria from a wide variety of environments and provide a defense mechanism or "immunization" against repeated infection by bacteriophages. CRISPRs are short noncoding DNA regions associated with a DNA/RNA processing enzyme complex ("CRISPR-associated," or cas, proteins.) The sequence of the CRISPR DNA is originally derived from an infecting bacteriophage; after the infection is resolved, the host bacteria inserts a fragment of viral DNA into a genomic CRISPR site. The CRISPR region then produces RNA that is bound by the cas proteins and used to recognize any future infecting bacteriophage and block infection. Recognition of infecting phage DNA is determined by homology between the CRIPSR RNA and incoming phage DNA; the cas enzyme complex can then target and destroy the phage. Bacteria may have many CRISPR regions and, thus, immunity or resistance to many types of phage.

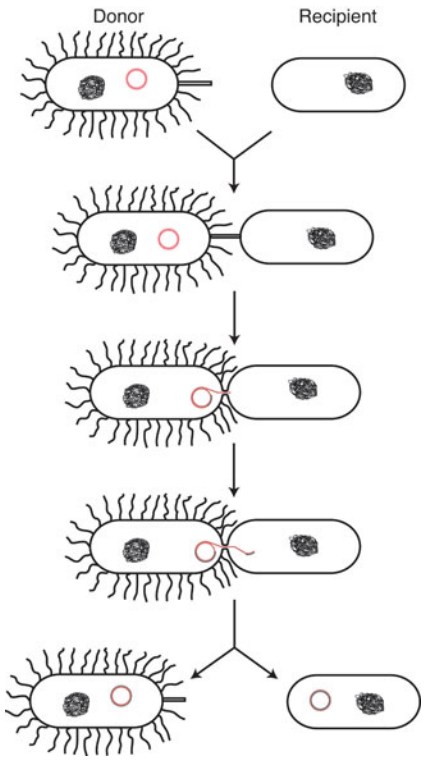

FIGURE 5 Schematic illustration of conjugative DNA transfer. The donor bacterium on the left possesses a double-stranded conjugative plasmid, which is transferred to the recipient bacterium on the right via a single-stranded DNA intermediate, after contact between the donor and recipient bacteria is established. The double-stranded conjugative plasmid is established in both donor and recipient cell following replication of a second strand.
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A third mechanism of bacterial genetic transfer is conjugation, a complex process involving intercellular contact, DNA replication, and DNA transport (Fig. 5). Evidence for gene transfer by conjugation in oral species includes the demonstration in oral bacterial isolates of genetic elements capable of conjugative transfer and demonstrations of conjugation in vitro in which donor, recipient, or both have been oral species. Among the most common types of genetic elements transferable by conjugation are plasmids. Many gram-negative bacteria harbor plasmids that encode the production of sex pili that initiate contact between recipient and donor cells, leading to the formation of aggregates possessing an intercellular membrane pore that enables DNA transport. The initial phase at the DNA level involves a single-strand cleavage at the origin of transfer mediated by a relaxase enzyme complex. This is followed by transport of the nicked strand of DNA from the donor to the recipient cell and synthesis of complementary DNA in both cells to yield a double-stranded product. In some gram-positive bacteria, e.g., strains of *E. faecalis*, the initial contact involves aggregation factors other than pili. A large number of gram-positive, and some gram-negative, bacterial species harbor conjugative plasmids that mediate their own DNA transfer to and replication in a recipient host but do not encode functions required for the formation of donor-recipient pairs or aggregates. For these plasmids to be transferred from a donor to a recipient cell, it is necessary that potential donors and recipients come into contact incidentally on a solid surface. In nature, such incidental contact might occur in a biofilm such as dental plaque.

Plasmids are extrachromosomally replicating genetic elements. The vast majority of plasmids are present in their host cells as double-stranded covalently closed circular DNA molecules, although linear double-stranded plasmids have been described in a few bacterial species. The first plasmid to be studied to any great extent was the F factor, a conjugative genetic element that may exist in its host as an extrachromosomal element, or it may be integrated into the host chromosome. However, most studies that led to an understanding of the molecular and genetic nature of plasmids were initiated in bacterial strains with recently acquired antibiotic resistance, underscoring their significance in horizontal gene transfer. Plasmids have been found, or shown to replicate, in a number of oral species (Tables 1 and 4), although surveys of oral isolates for plasmids generally document a relatively low rate of occurrence. For example, plasmids have been reported to occur in approximately 5% of strains of *S. mutans*, *P. intermedia*, and *A. actinomycetemcomitans*. However, native plasmids have not been detected in strains of *Actinomyces* spp. or *P. gingivalis*. Plasmid DNA was detected in 18% of *F. nucleatum* strains examined, and in oral *Treponema* spp., reports of plasmid detection range from 13 to 44%. The occurrence of related or identical plasmids in distinct strains and species from geographically diverse sources is evident in several oral species, including *F. nucleatum, Treponema* spp., *S. mutans*, and *Eikenella corrodens*. These findings may reflect the presence of natural genetic transfer systems within these species or may have resulted from vertical transfer before the evolutionary divergence of these species.

To ensure their survival, plasmids must encode the elements required for autonomous replication, including an origin of replication and a

TABLE 4 Selected native plasmids

Host species of origin	Plasmid	Plasmid size (kb)	%G+C content (if known)	Mode of replication (if known)	Relevant phenotype and other properties of interest	Host range[a]
Enterococcus faecalis	pAMβ1	26.5		Theta replication	Macrolide, lincosamide, streptogramin B alpha resistance; conjugative, broad-host-range, low-copy-number plasmid; replicon related to that of pIP501	11 different genera, including such oral species as *E. faecalis*, *S. sanguinis*, *S. salivarius*, *S. mutans*, and *S. sobrinus*
Streptococcus agalactiae	pIP501	30.2		Theta replication	Resistance to chloramphenicol and erythromycin; conjugative, broad-host-range plasmid; replicon related to that of pAMβ1	7 different genera including such oral species as *E. faecalis*[a] and *S. sanguinis*[a]
Streptococcus ferus (previously *S. mutans*)	pVA380-1	4.2	37.0	RC replication	Cryptic, mobilizable	*S. sobrinus*,[a] *E. faecalis*,[a] *S. gordonii*,[a] *S. pyogenes*,[a] *S. sanguinis*,[a] *S. agalactiae*[a]
Streptococcus mutans	pUA140	5.6	32.7	RC replication	Cryptic	*S. mutans*
Prevotella intermedia	pYHBil	5.1	38		Cryptic, mobilizable	*P. intermedia*, *E. coli*[a]
Fusobacterium nucleatum	pFN1	5.9	23		Cryptic	*F. nucleatum*, *E. coli*[a]
Aggregatibacter actinomycetemcomitans	pVT736-1	2.0	38.3	RC replication	Cryptic	*A. actinomycetemcomitans*[a]
	pVT745	25.1	39.0		Cryptic, conjugative	*A. actinomycetemcomitans*, *E. coli*[a]
Eikenella corrodens	pFM739	9.4			Resistance to streptomycin, penicillin, and sulfonamide; RSF1010 replicon; mobilizable	*Neisseria* spp., *E. corrodens*, *E. coli*
Treponema denticola, *Treponema socranskii*	pTS1	3.7	34.2		Cryptic	*T. denticola*,[a] *E. coli*[a]

[a]Plasmid derived from the native plasmid.

contiguous gene(s) encoding replication proteins and other controlling elements. Some native plasmids encode genes that confer host strain resistance to antibiotics, and selective pressures associated with the use of antibiotics may facilitate the spread of these plasmids. Other phenotypes that may be encoded by plasmids include virulence traits that contribute to the bacterium's ability to cause disease, metabolic capabilities, and the production of bacteriocins. Some plasmids have no detectable phenotype other than autonomous replication and are referred to as "cryptic." Plasmids may also be either conjugative or mobilizable. Conjugative plasmids are large, due to the requirement for genes sufficient to confer all the properties involved in conjugative transfer. For example, the conjugative plasmid pVT745 from *A. actinomycetemcomitans* is composed of 25.4 kb, and the genes necessary for DNA processing and mating are encoded in two gene clusters. The *E. faecalis* plasmid pAMβ1 is a broad-host-range conjugative plasmid of 26.5 kb that has been introduced into a number of distinct oral *Streptococcus* spp. (Table 4). Several smaller plasmids can be transferred by conjugation when the genes encoding conjugative functions are provided by another plasmid or chromosomally located genes.

These plasmids are termed "mobilizable," and examples include pYHBil from *P. intermedia* and pVA380-1 from *Streptococcus ferus*.

Another type of bacterial genetic element is the transposon, or "jumping gene." Transposons are discrete nonreplicative segments of DNA capable of inserting into and being replicated as part of a bacterial replicon, such as a chromosome or plasmid. They may also move from one site in a DNA molecule to another. Insertion of a transposon into DNA is mediated by insertion sequence elements, which are located at the ends of transposons and encode enzymes termed transposases that catalyze the insertion events. Transposon insertion is potentially random, i.e., may occur at virtually any site on a DNA molecule, independent of extensive homology, and can occur in a recombination-deficient (*recA*) host cell. In reality, most transposons have recombination hotspots. DNA between the insertion sequence elements of a transposon may encode virtually any type of bacterial trait, such as antibiotic resistance, or metabolic activity. Some transposons are conjugative and encode the genes necessary for transfer from one bacterial cell to another. One of the best characterized of the conjugative transposons is Tn*916*, which is an 18-kb element first isolated from *E. faecalis* DS16. Tn*916* possesses a *tetM* gene, which confers tetracycline resistance to the host bacterium. Conjugative transfer begins with excision of Tn*916* from a donor host replicon and formation of a circular intermediate, which is transferred to a recipient cell and is replicated as part of the new host's genome following insertion into a replicon. In the recipient cell, the transposon reinserts into the host DNA preferentially at sites that are AT rich. Relatively little is known about the conjugation process, but regions of Tn*916* required for intercellular transposition have been identified. Tn*916* is an example of a promiscuous element based on its tremendously broad host range. Tn*916*-like elements have been identified in a number of oral species, including *Streptococcus* spp., *Enterococcus* spp., *E. corrodens*, *F. nucleatum*, and *Veillonella parvula*. Other broad-host-range transposons, related to Tn*916*, encode resistance to tetracycline as well as other antibiotics, such as erythromycin.

Based on the myriad of examples listed here, it is clear that horizontal DNA transfer mechanisms are widespread among bacteria that inhabit the oral cavity. Conjugative transfer of plasmids and transposons is important in lateral gene transfer, as exemplified by the spread of antibiotic resistance genes. Natural transformation and transduction in specific groups of oral species are important for transfer of virulence factors and reassortment of alleles. The acquisition of new DNA may contribute to environmental and evolutionary diversification, allowing the bacterium to survive under a wider range of environmental conditions and to evolve over time. Among oral microorganisms, the increased incidence of antibiotic-resistant strains, demonstration of gene transfer in vitro, and identification of elements involved in gene transfer support the role of these processes in genetic transfer in the natural environment. Moreover, since the mouth is the first entry point for microorganisms associated with foods, there is potential for uptake of antibiotic resistance traits from extrachromosomal elements carried by food-associated bacteria, e.g., in yogurt, cheese, fermented meat products, and unwashed salads. These

findings additionally indicate that members of the normal oral microbiota may provide a reservoir for resistance and other virulence-related determinants.

Oral bacteria commonly reside in mixed species, organized biofilm communities adhered to solid surfaces. The conditions in dental plaque biofilms likely enhance the opportunity to exchange DNA, as bacteria are in close contact with other strains and species, and undisturbed plaque may persist for long periods of time in protected sites such as subgingival pockets and enamel pits and fissures. The close physical proximity between bacterial cells and the protection provided by the biofilm matrix provide ideal conditions for conjugation, which requires stable formation of a mating tube between the donor and recipient bacteria. Additionally, bacterial biofilms, including dental plaque, have been shown to include extracellular DNA (eDNA) as a component of the biofilm matrix. This eDNA is known to contribute to the structural stability of the biofilm; however, it also likely serves as a source of DNA for uptake by competent bacteria within the mixed-species community. Studies with the oral pathogens *S. mutans* and *P. gingivalis* have shown that the ability to form biofilms, secrete eDNA, and express competence are important and interlinked survival mechanisms in the oral cavity. Finally, the presence of bacteriophage in the oral cavity has been shown to be more widespread than previously thought and confirms the importance of DNA transfer to the ecology of the oral biofilm.

MOLECULAR MANIPULATION AND ANALYSIS OF ORAL MICROORGANISMS

Examination of two isolates of a bacterial strain, one that expresses a gene of interest, i.e., the wild-type parent, and one that does not, i.e., a mutant, helps define the contribution of the corresponding gene to the bacterial phenotype. A bacterial mutant in which only a defined gene has been altered is termed an isogenic mutant, and a change in sequence, total inactivation, or deletion of a gene from its native host background will lead to a change or loss of function specific to that gene. Restoration of the gene and/or its expression, referred to as complementation of the mutant, should restore function. Complementation serves as an important control in ruling out the possibility that a loss of function has occurred from effects of the insertion or deletion on downstream genes or from an undetected spontaneous mutation in another gene. Studies with *Escherichia coli* and other enteric species led the field in achieving these goals through the development of systems for the introduction of DNA into bacterial hosts that were not naturally competent, for the construction of DNA molecules that could be used to inactivate chromosomal genes, and for the complementation of inactivated genes. These technologies also facilitated studies of gene regulation, particularly with respect to gaining an understanding of how a bacterium responds to different environmental stimuli and conditions. In this regard, however, cellular functions encoded by bacterial genes, the expression of which may be regulated by such stimuli, are generally not easy to measure. Thus, another important aspect of molecular analysis has involved the use of reporter genes, which encode

proteins that are easily assayed enzymatically or visually. DNA molecules are constructed in such a way that the expression of a reporter gene will be controlled by the particular regulatory element under study following its introduction into a bacterial host. The most commonly used reporter genes encode the proteins β-galactosidase (detected with a colorimetric substrate), luciferase (detected by luminescence), and the green fluorescent protein or its derivates (detected with fluorescence).

The genetic manipulations required to construct the types of bacterial strains described above became possible with the emergence of recombinant DNA technology, which began in the 1970s following the discovery that restriction endonucleases are enzymes that reproducibly cleave DNA at specific sites. Most bacteria produce restriction endonucleases, typically in association with a modification enzyme responsible for methylation of specific nucleotides that prevents cleavage of the host cell DNA. The ability of a bacterial system to target "nonself" DNA for degradation is postulated to function in protection against invading DNA but may also provide a nutrient pool for synthesis of host cell nucleic acids. The percentage of bacterial strains that encode restriction endonucleases may be as high as 80%, and restriction endonucleases have been identified in all the bacterial genomes of oral species published to date. Multiple restriction modification systems may occur in a single strain. For example, up to seven possible restriction modification systems were identified in the genome sequence of one strain of *F. nucleatum*. The existence of restriction endonucleases can be a significant barrier to the development of efficient genetic transfer in specific bacterial strains.

The type II restriction endonucleases are commonly used in recombinant DNA techniques. These enzymes are characterized by their ability to cleave DNA within, or close to, a specific palindromic recognition sequence of 4 to 8 bp (Table 5). The ability to reproducibly cleave DNA at a defined base pair sequence provided the necessary foundation for the development of DNA mapping and sequencing techniques. In addition, each enzyme leaves characteristic ends on the cleaved DNA molecule, which may be blunt or overhanging with extensions of either 3′ or 5′ ends. The overhanging ends, referred to as "sticky" or "cohesive" ends, provide a powerful template for reannealing of the DNA based on the specificity of the base pair sequence. Recognition of the utility of cohesive ends led to recombinant DNA technology. The insertion of heterologous DNA into a plasmid and its propagation in *E. coli* is the process known as cloning. A description of the general steps involved in recombinant DNA technology (cloning) can be found in chapter 8. The cloning and characterization of genes and gene products of oral bacteria were first reported in the early 1980s (Table 6).

Early recombinant DNA technology relied to a large extent on naturally occurring restriction endonuclease sites. This proved cumbersome when convenient sites were not present, but this limitation has largely been overcome with a major technological advance in the 1980s, the development of the PCR. PCR is a process by which millions of copies of a specific region of DNA can be synthesized using a minute quantity of DNA template, a pool of the four individual deoxyribonucleotides, and short oligonucleotide primers complementary to DNA sequences flanking

TABLE 5 Selected restriction endonucleases

Restriction endonuclease[a]	Microorganism from which restriction enzyme was originally isolated	Recognition sequence and cleavage pattern[b]	Comments
*Fnu*DII	*Fusobacterium nucleatum*	5′... CGCG...3′ 3′... GCGC...5′	*Fnu*DII has a 4-bp recognition sequence and cleavage leaves blunt ends; religation of DNA with blunt ends is less efficient than religation of DNA with compatible cohesive ends
*Eco*RV	*E. coli*	5′... GATATC...3′ 3′... CTATAG...5′	*Eco*RV has a 6-bp recognition sequence and cleavage leaves blunt ends
*Fse*I	*Frankia* spp.	5′... GGCCGGCC...3′ 3′... CCGGCCGG...5′	*Fse*I has an 8-bp recognition sequence and cleavage leaves a 3′ overhang “GGCC”
*Xba*I	*Xanthomonas badrii*	5′... TCTAGA...3′ 3′... AGATCT...5′	*Xba*I has a 6-bp recognition sequence and cleavage leaves a 5′ cohesive end “CTAG” that is compatible with that of *Spe*I; ligation of *Xba*I- and *Spe*I-cleaved DNA is not recleavable with either enzyme but may be cleaved with *Bfa*I
*Spe*I	*Sphaerotilus* spp.	5′... ACTAGT...3′ 3′... TGATCA...5′	*Spe*I has a 6-bp recognition sequence and cleavage leaves a 5′ cohesive end “CTAG” that is compatible with that of *Xba*I; ligation of *Xba*I- and *Spe*I-cleaved DNA is not recleavable with either enzyme but may be cleaved with *Bfa*I
*Bfa*I	*Bacteroides fragilis*	5′... CTAG...3′ 3′... GATC...5′	*Bfa*I has a 4-bp recognition sequence and cleavage leaves a 5′ cohesive end “TA”; the recognition sequence corresponds to the cohesive overhang generated by digestion with *Xba*I or *Spe*I; a site generated from one *Xba*I-digested and one *Spe*I-digested DNA fragment can be cleaved with *Bfa*I
*Sts*I	*Streptococcus sanguinis*	5′... GGATGNNNNNNNNNNNNNNNNN...3′ 3′... CCTACNNNNNNNNNNNNNNNNN...5′	*Sts*I has a 5-bp recognition sequence and cleaves the DNA at 10 and 14 bp from this sequence as indicated, leaving a 5′ overhang of unspecified sequence
*Smu*EI	*Streptococcus mutans*	5′... GGWCC...3′ 3′... CCWGG...5′	*Smu*EI has a 5-bp recognition sequence with the central residue as either an A or T; cleavage leaves a 5′ cohesive end of “GACC” or “GTCC”
*Fnu*4HI	*Fusobacterium nucleatum*	5′... GCNGC...3′ 3′... CGNCG...5′	*Fnu*4HI has a 4-bp recognition sequence with a central residue that may be any base; cleavage leaves a 5′ cohesive end with the specificity of the central residue

[a]By convention, the first three letters of the restriction endonuclease derive from the first letter of the genus followed by the first two letters of the species designation of the microorganism from which the restriction endonuclease originates. The designation *Sts*I is an exception to this convention.

[b]Cleavage pattern is indicated by shading of bases. Abbreviations: N = A, C, G, or T; W = A or T.

the region of interest (see Fig. 3 in chapter 8). The desired DNA fragment is amplified in a cyclical thermal process of denaturation, annealing of oligonucleotide primers, and synthesis of complementary DNA with incorporation of nucleotides using a thermostable DNA polymerase. A single DNA fragment can be amplified to hundreds of millions of copies in 30 to 40 cycles, and the resulting DNA molecule is referred to as an “amplimer.” A powerful use of PCR technology involves the addition of novel base pairs into the oligonucleotide primers and, thus, the amplimer. This can be done to introduce new restriction endonuclease sites or regions of homology to facilitate subsequent molecular manipulations. One example of this approach is the construction of an erythromycin resistance cassette that has been widely used in the transformation of oral bacterial

TABLE 6 Milestones in the development of genetic systems for oral species

Species	Development	Description
S. mutans, *S. sanguinis/gordonii*	Gene cloned	1982: Cloning of *S. mutans* aspartate-semialdehyde dehydrogenase gene, *asd*
		1982: Cloning of *S. mutans* surface protein antigen gene, *spa*A
		1982: Cloning of *S. mutans* tetracycline resistance determinant in *S. sanguinis*
	Native plasmids identified	1973: Plasmid isolated from *S. mutans* strain LM-7
		1978: Plasmid isolated from *S. sanguinis* encoding resistance to erythromycin and lincomycin
	Transformation	1976: Natural transformation of *S. sanguinis* strain Challis with plasmid DNA from *Streptococcus faecalis*
		1976: Natural transformation of *S. sanguinis* strain Challis with DNA from heterologous streptococcal strains
		1981: Natural transformation of *S. mutans* with DNA from homologous and heterologous
	Conjugation	1978: Transfer to *S. mutans* and *S. sanguinis/gordonii* of pAMβ from *S. faecalis* by conjugation
	Shuttle plasmid	1982: *Streptococcus-E. coli* shuttle plasmid pVA838
		1982: *Streptococcus-E. coli* shuttle plasmid pVA856
	Site-directed mutagenesis	1986: Defined mutation of glucosyltransferase gene of *S. mutans*
		1990: Defined mutation of a 76-kDa cell surface protein of *S. gordonii*
	Transposon mutagenesis	1988: *Tn*916 used to generate mutations in *S. mutans* chromosome, with auxotrophs identified
		1995: *Tn*4001 used to generate lactose-negative mutants in *S. gordonii*
		1996: *Tn*917 used to generate mutations in genes involved in acid tolerance, nutrition, and bacteriocin production
	Complementation	1992: Low glucosyltransferase activity in *S. gordonii* complemented with a chimeric plasmid pAM5010 possessing the regulatory gene *rgg*
		1996: Complementation of lactate dehydrogenase deficiency in *S. mutans* with heterologous alcohol dehydrogenase on plasmid pCR3-8
Actinomyces spp.	Gene cloned	1987: Cloning and expression of the type I fimbrial subunit gene, *fimP*, of *Actinomyces viscosus*
	Native plasmids identified	Native plasmids have not been found
	Bacteriophage identified	1978: A lytic phage for *A. viscosus* was isolated from sewage
	Transformation	1994: Transformation of *A. viscosus* and *A. naeslundii* with broad-host-range plasmid pJDR215
	Transfection	1997: Transfection of *A. viscosus* and *A. naeslundii* strains with phage isolated from dental plaque
	Conjugation	Not reported
	Shuttle plasmid	1994: Transformation of *A. viscosus* and *A. naeslundii* with broad-host-range plasmid pJDR215
	Site-directed mutagenesis	1995: Defined mutation in the *fimP* gene of *A. viscosus*
	Transposon mutagenesis	Not reported
	Complementation	Not reported
P. intermedia	Gene cloned	1999: Cloning of *P. intermedia* DNA conferring hemolytic activity in *E. coli*
	Native plasmids identified	1990: Identification of multiple cryptic plasmids, including the 5-kb pYHBil
	Transformation	Not reported
	Conjugation	2002: Conjugation with chimeric shuttle plasmid pDRD5904
	Shuttle plasmid	2002: Shuttle plasmid pDRD5904 engineered using the native plasmid pYHBil and a kanamycin resistance determinant
	Site-directed mutagenesis	Not reported
	Transposon mutagenesis	Not reported
	Complementation	Not reported

F. nucleatum	Gene cloned	1996: Cloning and expression of the major outer membrane protein gene, *fomA*
	Native plasmids identified	1995: Identification of cryptic homologous plasmids, including pSG2717 and pSG4544
	Transformation	2000: Transformation by electroporation with shuttle plasmid pHS17
	Conjugation	1990: Transfer of tetracycline resistance and *tetM*-like DNA sequences to *F. nucleatum* strains
	Shuttle plasmid	2000: Shuttle plasmid pHS17 engineered using the native plasmid pFN1 and an erythromycin resistance cassette
		2003: Shuttle plasmid engineered using the native plasmid pKH9
	Site-directed mutagenesis	2003: Defined chromosomal insertions in the *rnr* (exoribonuclease R) and NADH oxidase genes
	Transposon mutagenesis	Not reported
	Complementation	Not reported
A. actinomycetemcomitans	Gene cloned	1989: Cloning of the leukotoxin gene, *ltxA*
	Native plasmids identified	1989: Multiple plasmids identified ranging in size from 4 to 20 MDa
	Transformation	1990: Natural transformation
		1991: Transformation via electroporation with pDL282
	Conjugation	1993: Conjugation with broad-host-range IncP and IncQ plasmids
		2001: Conjugation with native plasmid pVT745
	Shuttle plasmid	1991: Shuttle plasmid pDL282 constructed from native plasmids
	Site-directed mutagenesis	1995: Defined mutations in the genes of the leukotoxin operon
	Transposon mutagenesis	1992: *Tn*916 mutagenesis to isolate mutants lacking serotype b-specific polysaccharide antigen
		1995: *Tn*5 mutagenesis
		1999: IS903ϕkan mutagenesis to isolate catalase-negative mutants
	Complementation	2000: Complemented a *tad* gene mutation with a broad-host-range IncQ expression vector, pJAK16
P. gingivalis	Gene cloned	1988: Cloning of the fimbrial subunit gene, *fimA*, in *E. coli*
	Native plasmids identified	Native plasmids have not been found
	Transformation	1993: Transformation by electroporation with pE5-2 and derivate pYT7
	Conjugation	1989: Conjugation with plasmid pE5-2
	Shuttle plasmid	1989: Shuttle plasmid pE5-2 with *E. coli* and *Bacteroides eggerthii* replicons
		1997: Shuttle plasmid pYH400, engineered with *P. asaccharolytica* replicon
	Site-directed mutagenesis	1993: Defined mutation in a protase gene (*tpr*) of strain W83
	Transposon mutagenesis	1998: *Tn*4351 mutagenesis to isolate nonhemolytic and nonpigmented mutants
		2000: *Tn*4400′ mutagenesis shown to yield large numbers of mutants with simple insertions
	Complementation	1997: Complemented a *tpr* gene mutation in strain W83 using *Bacteroides* shuttle plasmid pNJR12 with intact *tpr* gene
T. denticola	Gene cloned	1990: Cloning of a chymotrypsin-like protease gene, *prtA* (Que and Kuramitsu)
	Native plasmids identified	1991: Isolated 2.6-kb plasmid pTD1
		1996: Isolated 4.2-kb plasmid pTS1
	Transformation	1996: Transformation by electroporation with broad-host-range plasmid pKT210
	Conjugation	Not reported
	Shuttle plasmid	1996: Transformation by electroporation with broad-host-range plasmid pKT210
		2002: Novel shuttle plasmid pKMCou conferring coumermycin resistance
	Site-directed mutagenesis	1996: Defined mutation of the flagellar hook protein *flgE*
	Transposon mutagenesis	Not reported
	Complementation	2002: Complementation of *flgE* mutant using novel shuttle plasmid pKMCou

species (Fig. 6). Today the basic processes of specific DNA cleavage and religation in combination with PCR allow the design and engineering of almost unlimited combinations of DNA fragments of diverse biological or synthetic origin.

Molecular analyses in the native bacterial cell also require a means by which to introduce and establish DNA molecules in the bacterium. The natural processes of transformation, conjugation, and transduction have been successfully used for these purposes in many bacterial species and strains (Table 6). Another option for delivery of DNA into bacterial cells is to make them competent for transformation by some artificial means, such as electroporation. Electroporation is a process in which a brief high-voltage pulse is applied to a suspension of bacterial cells and DNA, resulting in reversible membrane permeability and DNA uptake by a fraction of the surviving cells. This technique has been widely used for both gram-negative and gram-positive bacteria.

The need for molecular analyses has only increased with the emergence of genomic information, both for confirmation of gene function initially inferred by DNA sequence homology or phenotypic analyses and for investigation of genes of unknown function. The importance of these systems in the study of microbial ecology and pathogenesis is further illustrated in chapter 8, which focuses on applied molecular biology in oral microorganisms. The remainder of this chapter focuses on molecular tools designed specifically for the development of and use with genetic transfer systems in oral microorganisms and on the use of transposons for generalized mutagenesis in oral species.

Vectors and Their Utility

The cloning and characterization of DNA from oral bacterial species in *E. coli* can provide considerable information regarding individual genes and, in some cases, their gene products. Ultimately, however, it is critical to be able to conduct molecular manipulations in the bacterial species of interest so that gene function can be evaluated in the native host background. One requirement for the majority of such studies is the availability of vectors onto which DNA can be added and introduced into the host of choice. The most basic property of such a vector is the ability to function as a replicon in the host of interest. Other properties common to vectors include the presence of at least one, and preferably several, unique restriction endonuclease recognition sequences, i.e., cloning sites, for the introduction of homologous or heterologous DNA, and a selectable trait, such as resistance to an antibiotic, to detect the presence of the vector in a host into which it has been introduced. Another feature might include a gene flanking the cloning site, the function of which would be interrupted by the insertion of DNA, for the screening of selected transformants for those carrying the vector with inserts. In some instances, the inclusion of a transcriptional terminator downstream of the insertion site may be desirable to prevent readthrough beyond the cloned DNA and overexpression of potentially lethal vector-encoded traits. Another useful feature of a vector, depending on the specific purpose, might be an inducible promoter from which the expression of cloned genes can be regulated, particularly if the objective is to obtain large quantities of the gene product. Finally, if the purpose is to study conditions that modulate expression of a cloned

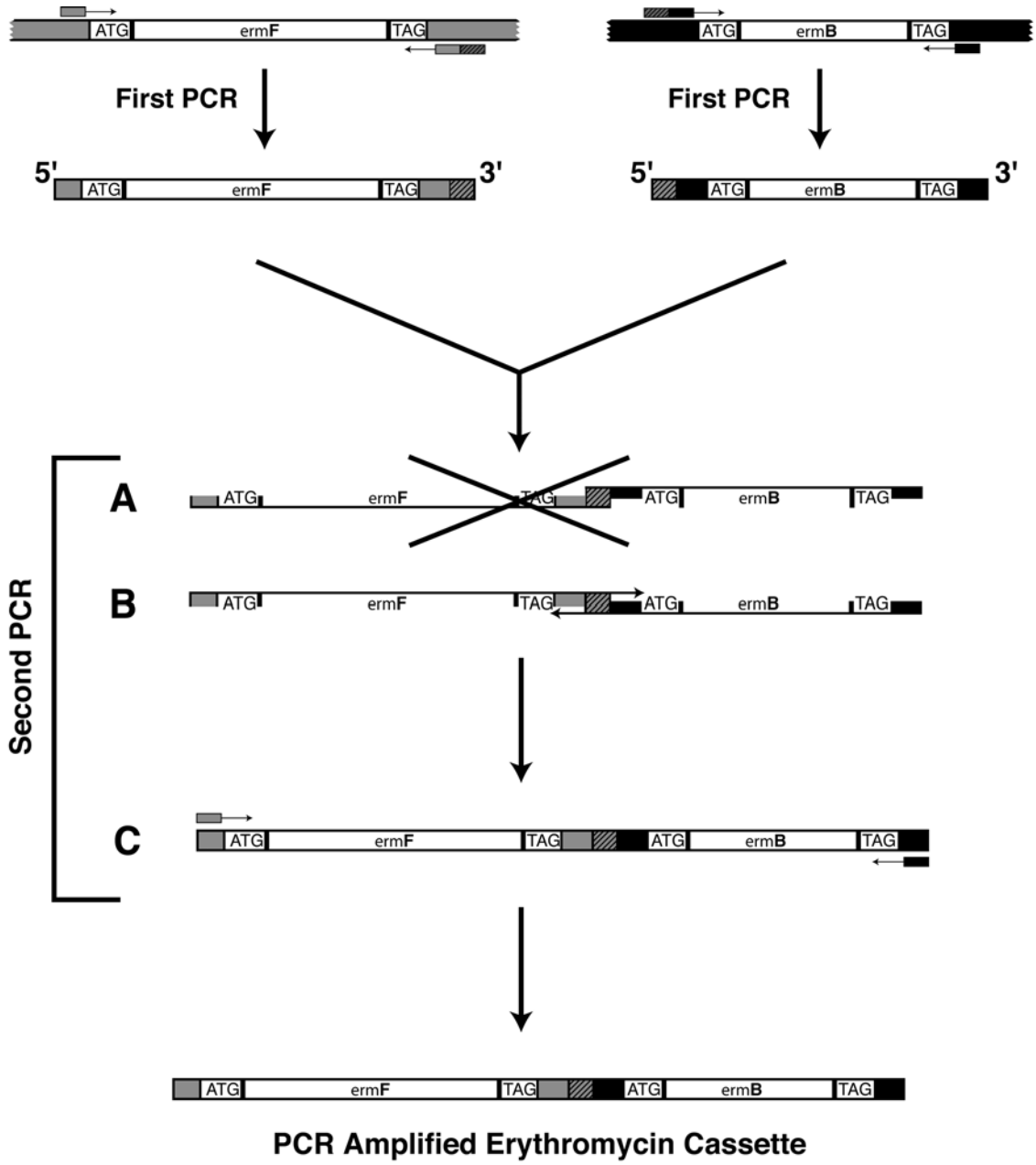

FIGURE 6 Construction of an erythromycin resistance cassette using the PCR-based overlap extension method. The erythromycin resistance cassette was constructed using a fusion PCR approach to combine erythromycin resistance genes that would function in *E. coli* (*ermB* gene [previously designated *ermAM*]) and in *P. gingivalis* (*ermF*). PCR was first used to amplify the individual erythromycin resistance genes, including their regulatory regions. The 3′ primer for the *ermF* amplicon and the 5′ primer for the *ermB* amplicon incorporated a complementary overlapping sequence (indicated by the shaded box with hatch marks). The products of the first PCR were purified and combined along with primers for the 5′ region of the *ermF* amplicon and the 3′ region of the *ermB* amplicon. In the second PCR, denatured amplicons from the first reaction may hybridize with the homologous amplicon (not illustrated) or with the heterologous amplicon (A and B) due to annealing of the inserted overlapping sequence (hatched box). The combination of annealed strands illustrated in panel A was not amplified because DNA polymerase functions in a 3′ to 5′ direction. The combination of strands as seen in panel B enabled DNA synthesis complementary to the annealed strand to proceed from the free 3′ end of the overlapping region. The resulting product yielded a DNA molecule possessing both *ermF* and *ermB*, further amplified by the flanking primers (C). (Adapted from H. M. Fletcher et al., *Infect. Immun.* **63:**1521–1528, 1995.)
doi:10.1128/9781555818906.ch7.f6

promoter, then it may be useful to have on the vector, downstream of the cloning site, a promoterless reporter gene for the easy quantitation of promoter activity.

Extrachromosomally replicating elements, particularly plasmids, that have been isolated directly from oral species, or at least shown to replicate in them, have provided the starting points in the development of new molecular and genetic systems for several oral bacterial species. One important application of plasmids has involved the development of genetic transfer systems for species in which natural mechanisms either do not exist or have not been identified. For example, transformation with a plasmid of a species in which that plasmid will replicate enables optimization of the methodology for introducing DNA into the microorganism, for the testing of selectable markers and reporter genes, and ultimately for the construction of a useful vector molecule based on the plasmid. Data from such experiments also provide a foundation for all other molecular analyses in the native host, such as the mutagenesis of specific chromosomal genes as a means to examine the function of the gene and its gene product, i.e., the construction of isogenic mutants. A common approach to generating isogenic mutants is to use a vector that does not replicate in the bacterium of interest (a "suicide" vector) into which has been cloned DNA homologous to a region of the targeted gene as well as a resistance determinant that provides a marker for selection. Phenotypic analyses are then used to evaluate the effect of the gene disruption. When a chromosomal gene disruption (knockout) blocks gene function as assessed phenotypically, it is important to rule out the possibility that the loss of function actually occurred because of a "polar effect," or inactivation of a downstream gene due to the insertion in the upstream gene. In such an experiment, an intact copy of the disrupted gene would be provided on a replicating vector in the mutant strain to demonstrate that it can restore the lost function, a process described above as complementation. Unless a gene is the only or last one in an operon, a specific function can be attributed to that gene only if the loss is restorable by complementation. In addition, if the gene function under study is essential to the bacterial strain, then complementation would have to precede its knockout. Vectors that function in oral species also enable the cloning and expression of genes in the native host background for assessment of the phenotypic properties. This is particularly critical for genes that are not expressed or the products of which are not functional in *E. coli*, including, for example, many antibiotic resistance genes found in oral species. Additionally, extrachromosomal replicating elements provide a vehicle for delivery of reporter genes to the host species, which may be used in studies of gene regulation or as a biological marker for the microorganism in multispecies systems such as biofilms and in interactions with host tissue cells.

Features of Plasmids Essential for Vector Construction

The only absolutely essential feature of a plasmid is its ability to self-replicate, i.e., to function as an independent replicon, and the most useful component of a plasmid in terms of its potential as a vector is the region required for extrachromosomal replication, referred to as the minimal replicon (see discussion below).

Initially, plasmids were categorized on the basis of their inability to coexist within a single bacterium, referred to as "incompatibility groups" (e.g., IncP and IncQ group plasmids). In essence, plasmids in a given incompatibility group cannot survive as extrachromosomal elements in the same bacterial host due to similar mechanisms controlling their propagation. More recently, plasmids have been categorized according to the particular mechanism by which they replicate. Two general modes of replication identified for circular plasmids are theta (also the mechanism used by most bacteria for replication of their chromosomes) and rolling-circle (RC) replication. Historically, theta-replicating plasmids were associated with gram-negative bacteria and RC-replicating plasmids were associated with gram-positive bacteria. However, it is now known that either mechanism of replication can be employed by plasmids from both gram-positive and gram-negative bacteria, but any given plasmid replicates by one mode or the other. The features of a minimal replicon are typically contiguous on the plasmid and include an origin of replication and a gene encoding a replication initiation protein. Additional elements of importance involve regulation of replication termination, regulation of the number of copies of the plasmid per bacterial chromosome (termed the "copy number"), and mechanisms to ensure that as the bacterial cells divide each of the progeny inherits at least one copy of the plasmid (referred to as "partitioning" or segregational stability). The origin of replication, termed *ori*, is characteristic of the replicon and includes the site at which DNA replication is initiated. Many plasmids encode a replication initiation protein, termed a Rep protein, which binds to a DNA sequence in the corresponding *ori* that is characteristic of that replicon. Host proteins required for replication are provided in *trans* from genes on the host bacterial chromosome. A wide array of systems within the broad grouping of theta and RC replication are found in bacterial plasmids.

Basic processes in the initiation of theta replication include binding of a plasmid-encoded replication protein (Rep) and the melting of an AT-rich region of the parental DNA strands to form an "open complex," followed by initiation of DNA synthesis. Once initiated, DNA synthesis is continuous on one strand ("leading strand") and discontinuous on the other ("lagging strand"), and replication may proceed in one or both directions. The term "theta replication" derives from the θ (theta)-like appearance of replication intermediates viewed by electron microscopy (Fig. 7A).

Iteron-regulated replicons, a subgroup of theta-replicating plasmids, are commonly found in gram-negative bacteria but may also be found in gram-positive species. These plasmids are characterized by a series of tandem repeats, or "iterons," in the *ori*, which provide binding sites for the corresponding plasmid-encoded Rep proteins (Fig. 7A). Additional DNA sequences in the *ori*, known as DnaA boxes, provide specific binding sites for the host-encoded DnaA protein. Oligomerization of the Rep proteins that are bound to the iterons leads to local distortion of the DNA molecule and melting of the adjacent adenine- and thymine-rich DNA region to form the open complex. This is followed, with contributions from additional host replication proteins, by initiation of DNA synthesis. One example of a native plasmid demonstrating features consistent with iteron-regulated plasmids is pFN1, isolated from a strain of *F. nucleatum*. The structural features of the putative pFN1 *ori* include six identical

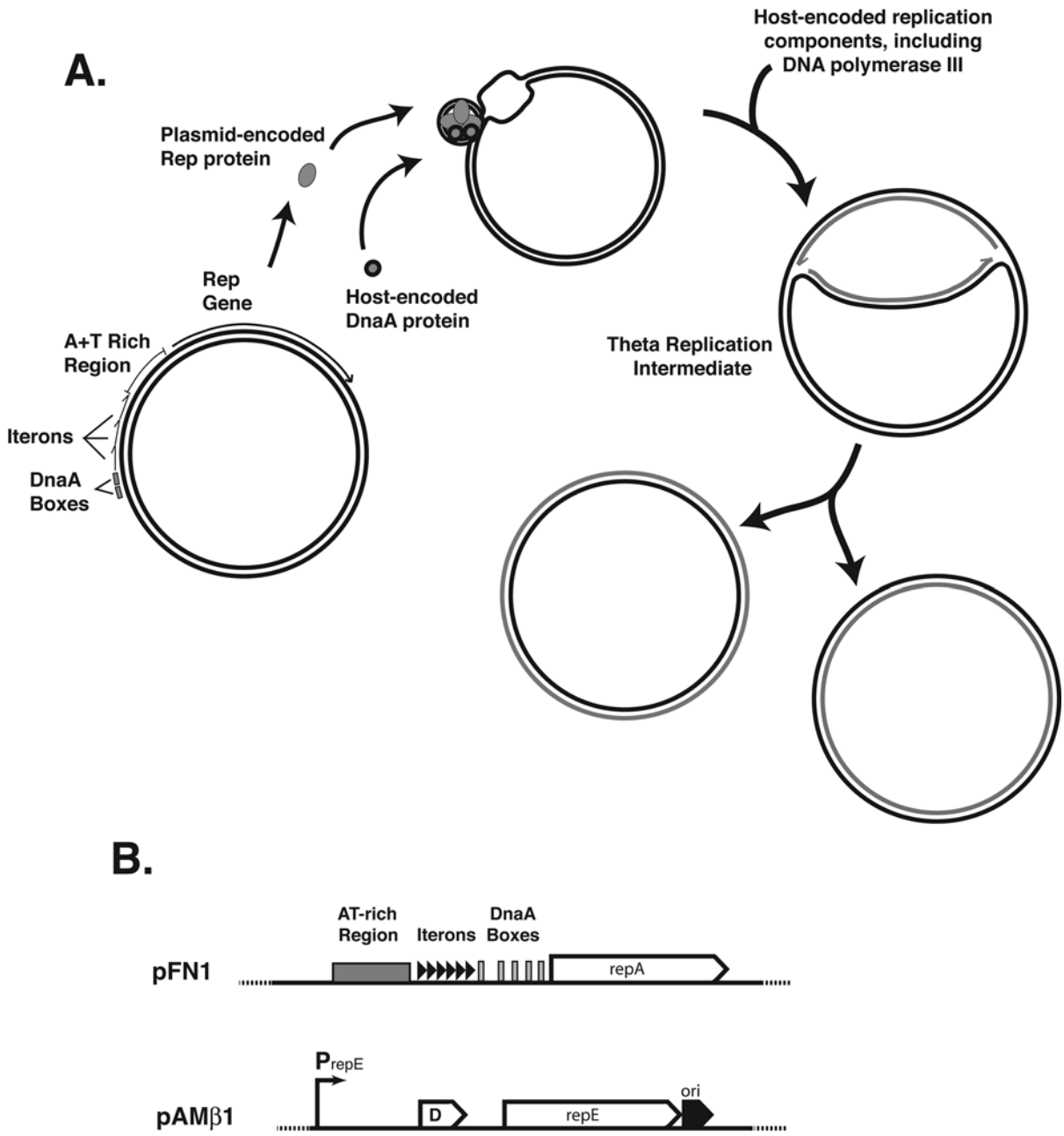

FIGURE 7 (A) Schematic representation of theta replication. The basic scheme of theta replication in an iteron-regulated plasmid is illustrated schematically. The *ori* of iteron-regulated plasmids typically possesses a series of tandem repeats at 22-bp intervals known as iterons, an AT-rich region, and DnaA-binding sequences. The plasmid-encoded Rep proteins and host-encoded DnaA proteins bind to specific DNA sequences within the iteron, inducing a melting of the parental DNA strands in the adjacent AT-rich region and forming a preinitiation complex. The DnaA and/or Rep protein facilitates the incorporation of additional host replication components needed for initiation. Replication proceeds in one or both directions and leads to the characteristic theta replication intermediate structure that can be observed on examination by electron microscopy. (B) Structural features of plasmid replicons. The features of the pFN1 replicon (based on Fig. 2B in S. Kinder Haake et al., *J. Bacteriol.* **182:**1176–1180, 2000) are consistent with those of iteron-regulated plasmids. The putative *ori* contains an AT-rich region, six perfect 22-bp repeats (iterons), and several DnaA-binding sites (DnaA boxes); the downstream *repA* homologue is related to a known theta-replication initiation protein. The features of the theta-replicating plasmid pAMβ (based on Fig. 8 in D. R. Helinski et al., p. 2295–2324, *In* F. C. Neidhardt et al. [ed.], Escherichia coli *and* Salmonella: *Cellular and Molecular Biology*, 2nd ed., vol. 2 [ASM Press, Washington, DC, 1996]) include the *repE* gene encoding the replication initiation protein RepE, the upstream *repE* promoter, and the small *ori* located downstream of the *repE* gene. See text for details of pAMβ replication initiation. doi:10.1128/9781555818906.ch7.f7

tandem repeats or iterons, an adjacent AT-rich region, and several putative DnaA boxes. The adjacent *repA* is homologous to a plasmid replication initiation gene, the protein product of which is known to initiate theta replication (Fig. 7B). Experimental evidence of theta replication involves analysis of replication intermediates by either electron microscopy or two-dimensional gel electrophoretic analysis of restriction enzyme-digested replication intermediates.

The broad-host-range plasmid pAMβ1, which replicates in numerous streptococcal species including *S. mutans*, *S. sanguinis*, and *Streptococcus salivarius*, utilizes a unidirectional theta mechanism that is distinct from the iteron-regulated plasmids. The basic replicon of pAMβ1 consists of a Rep protein gene, its upstream promoter (P_{repE}), and a small *ori* located downstream of the rep gene (Fig. 7B). The pAMβ1 *ori* does not possess repeat sequences but provides a binding site for the cognate Rep protein (RepE) and also contains a 16-bp AT-rich region. Transcription from P_{repE}, located 2 kb upstream of the *ori*, leads to expression of *repE*. The binding of RepE to the double-stranded *ori* induces DNA melting and open complex formation. RepE additionally binds to single-stranded DNA in the open complex, and RNA transcription through the origin is required for replication initiation, possibly by providing the RNA primer needed for initiation of DNA replication.

Plasmid replication by the RC mechanism is distinct from theta replication. RC replication is described as asymmetric due to the uncoupling of leading and lagging strand synthesis, which results in the presence of a single-stranded DNA intermediate. Replication is initiated by the Rep protein (Fig. 8), which nicks the supercoiled DNA at a site known as the double-stranded origin (*dso*). This cleavage leaves a DNA strand with a free 3′ hydroxyl that provides a primer for leading strand DNA synthesis by the host DNA polymerase III. As the newly synthesized DNA strand is elongated, the parental nicked strand is progressively displaced. Elongation continues to the newly synthesized *dso* site where the Rep protein cleaves and rejoins DNA strands, yielding one double-stranded product and one single-stranded intermediate. The single-stranded intermediate is converted to a double-stranded product by synthesis of an RNA primer at the single-stranded origin (*sso*), which is physically distinct from the *dso*, followed by DNA synthesis of the complementary strand. The conversion of the single-stranded intermediate to the double-stranded product is accomplished with host-encoded enzymes.

Both theta- and RC-replicating plasmids have been isolated from and used in molecular analyses of oral microorganisms. Plasmids that replicate by the RC mechanism are typically smaller than 10 kb in size. However, small size does not mean that a plasmid uses an RC replication mechanism, as theta-replicating plasmids less than 10 kb have also been identified. Large plasmids, such as the 25.4-kb conjugative plasmid pVT745 from *A. actinomycetemcomitans*, are believed to replicate by a theta mechanism. Future investigations of plasmid ecology in the oral microbiota are likely to result in the identification of other types of extrachromosomal elements, e.g., linear plasmids.

The second most important feature of a vector is the presence of a marker that enables selection for cells that possess it. Quite often, the presence on a plasmid of a selectable marker is what has led to its discovery.

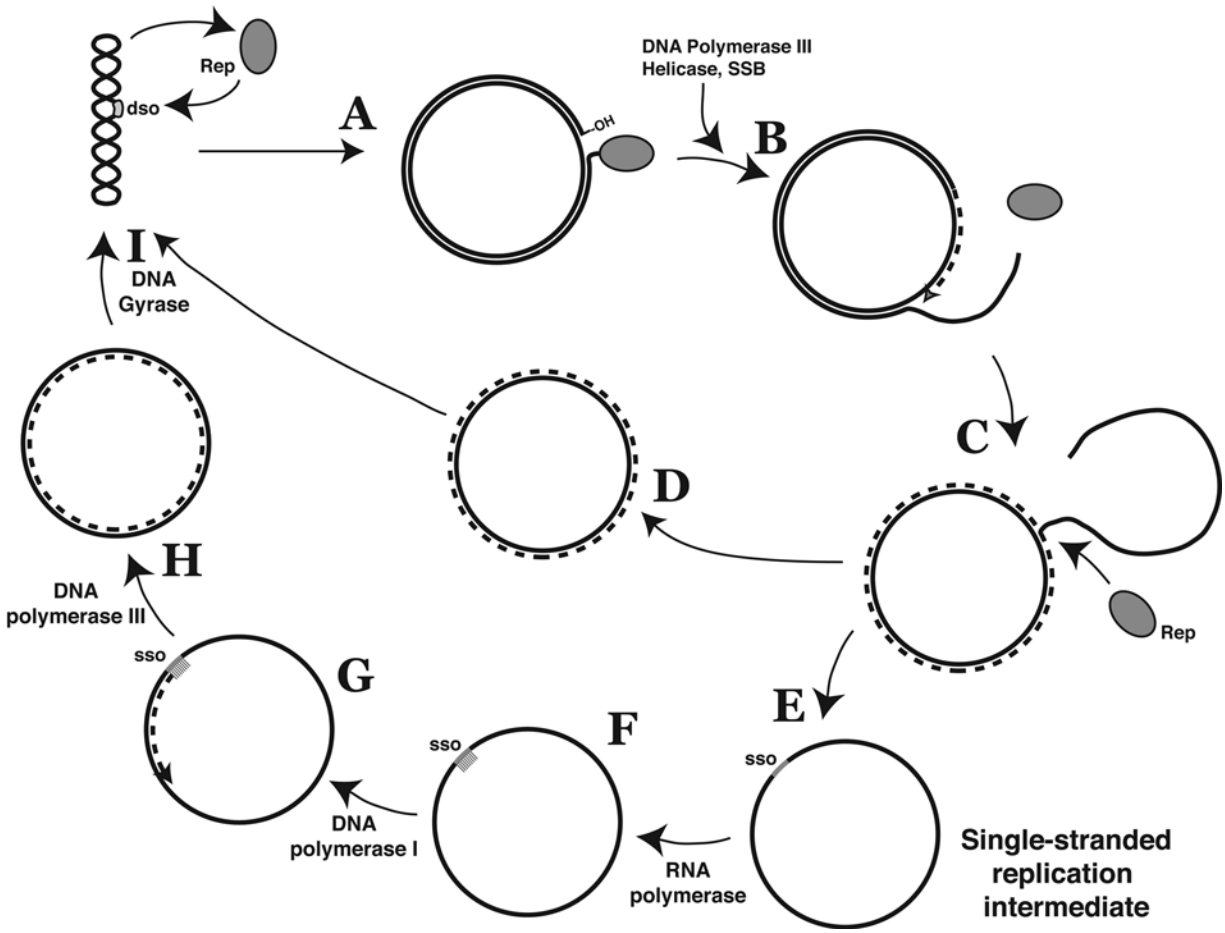

FIGURE 8 Schematic representation of RC replication. The sequential steps in RC replication are illustrated schematically. One strand of the supercoiled plasmid DNA is nicked (A) at the double-strand origin (*dso*) by the plasmid-encoded Rep protein, leaving a 3′ hydroxyl end, which serves as a primer site for the initiation of DNA synthesis (B) by host cell enzymes. DNA synthesis progresses while displacing the nicked DNA strand (C) until the newly formed *dso* is reached. The Rep protein mediates cleavage and rejoining of DNA ends to yield one double-stranded plasmid (D), consisting of one parental and one newly synthesized strand, and one single-stranded DNA intermediate (E). An RNA primer is synthesized at the single-strand origin (*sso*) site (F) that provides a primer for DNA synthesis (G), which proceeds to yield a second double-stranded plasmid product (H). The double-stranded DNA is converted to the supercoiled form by DNA gyrase (I). (Adapted from Fig. 6 of G. del Solar et al., *Microbiol. Mol. Biol. Rev.* **62:**434–464, 1998.)
doi:10.1128/9781555818906.ch7.f8

Useful selectable markers typically encode resistance to antimicrobial agents that can be incorporated into media. This enables growth of cells harboring the resistance determinant while inhibiting the growth of cells lacking the element. The most common selectable markers encode resistance to antibiotics, but resistance to other agents toxic to bacteria, such as heavy metals, may also be plasmid encoded. The choice of a selectable marker to use in vector development is based on consideration of several factors: the susceptibility profile of the host species and strain as well as the known occurrence of resistance and resistance determinants in the

species. If native selectable markers are not available for a given species, a reasonable alternative is to test markers that are known to function in related species. However, ethical considerations prevent the de novo introduction of determinants encoding resistance to antimicrobial agents that are used in the clinical treatment of infections involving the target species. Antibiotic resistance in the oral microbiota is discussed in detail in chapter 21 and will not be discussed further here.

Use of Native Plasmids in Molecular Analyses

Understanding the fundamental mechanisms of plasmid biology facilitates the development of genetic tools for molecular analysis of bacterial species. Identification of native plasmids in oral bacterial species provides researchers with genetic tools to artifically manipulate these species. A common tool used by microbiologists is the "chimeric plasmid," which combines features of native plasmids plus those of plasmids from *E. coli*, the bacterial workhorse of genetics. DNA cloning, sequencing, and other molecular techniques are easily accomplished in *E. coli*. In a chimeric plasmid, the genetic manipulations may be performed in *E. coli* and then the modified DNA introduced into the oral bacteria of interest. For example, the *E. coli-Streptococcus* shuttle vector, pVA838, is able to replicate as a plasmid in both *E. coli* and *S. gordonii*. Construction of pVA838 involved combining the *E. coli* plasmid pACYC184 and the streptococcal plasmid pVA749. The ability of pVA838 to replicate in *E. coli* was provided by pACYC184, which could be selected by propagation in the presence of chloramphenicol or tetracycline, resistance to each being encoded by pACYC184. The ability of pVA838 to replicate in streptococci was provided by pVA749, which had previously been constructed by the cloning of an erythromycin resistance gene, selectable in either streptococci or *E. coli*, onto intact pVA380-1, the native streptococcal plasmid. The availability of pVA838 made it possible, at least theoretically, to clone virtually any streptococcal gene, selectable or not, directly in *E. coli*, in which cloning is considerably more efficient than in most other bacterial species. The resultant recombinant molecule could be isolated from *E. coli*, and the cloned gene, and any traits encoded by it, could then be studied after inserting the chimeric shuttle vector by electroporation or transformation into a streptococcal host. A wide range of chimeric plasmids is now available for shuttling genes between *E. coli* and gram-positive bacteria in general.

Suicide vectors contain a replication origin and selectable marker for growth as a plasmid in *E. coli* but contain a selectable marker but no replication origin for the partner bacterial species. Such a vector cannot survive as a plasmid when introduced into the oral bacterial strain, but if DNA with homology to chromosomal DNA has been cloned onto it, the entire plasmid can become integrated into the transformed host chromosome at the site of homology. The suicide plasmid DNA will now be replicated as part of the host bacterial chromosome. Suicide vectors can be used to "knock out" or mutate genes of interest by cloning a small fragment of the gene into the suicide vector in *E. coli*. The vector is then transferred to the oral bacterial species and must insert into the chromosomal gene to be maintained. Insertion of the suicide plasmid into the gene of interest interrupts expression of the gene, resulting in the knockout mutant.

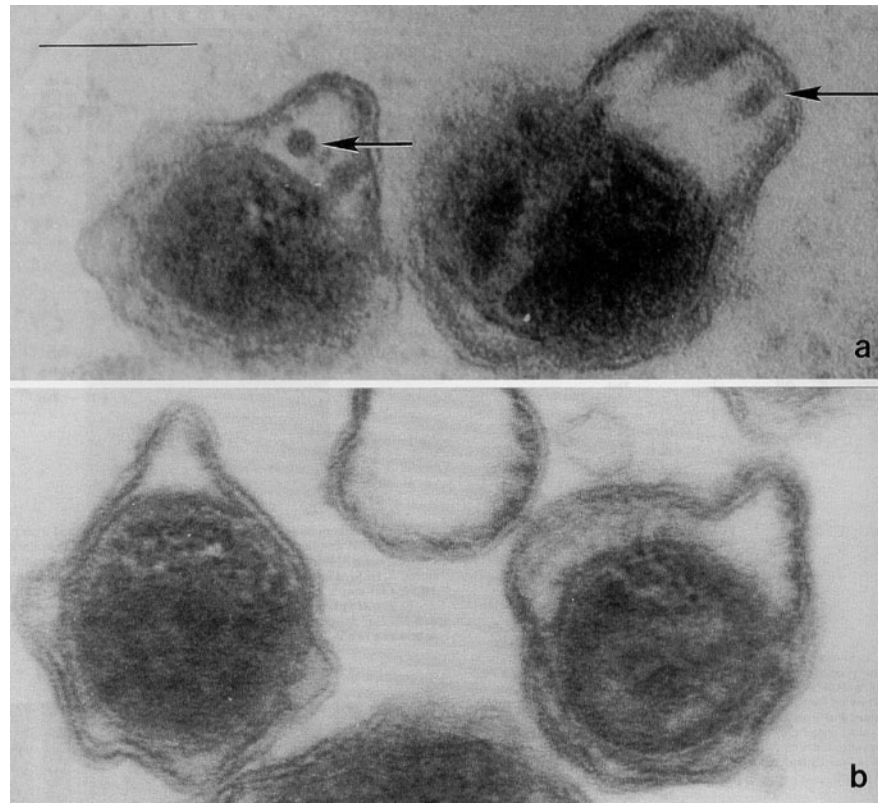

FIGURE 9 Phenotypic properties of wild-type and *flgE* mutant strains of *T. denticola*. Electron micrographs of a wild-type (a) and an *flgE* mutant (b) strain of *T. denticola* are shown. The *flgE* gene encodes the hook protein responsible for attachment of the endoflagella to the bacterial membrane. The periplasmic endoflagella, evident in the wild-type strain (arrows), are not found in the mutant strain. Bar in panel a, 0.1 mm. (Reproduced from H. Li et al., *J. Bacteriol.* **178**:3664–3667, 1996, with permission.)
doi:10.1128/9781555818906.ch7.f9

Shuttle vectors and suicide vectors have been important in studies of oral spirochetes, a group of microorganisms involved in disease processes such as chronic periodontitis. Mutation of the chromosomal *flgE* gene, which encodes the flagellar hook protein, was accomplished in an oral spirochete, *T. denticola*, a species that can be cultivated in vitro. A plasmid unable to replicate in *T. denticola* but containing a portion of *flgE* interrupted by an erythromycin resistance cassette was used to create the mutant by allelic exchange. Because pHLfE lacked a *T. denticola* replicon and was a suicide plasmid, erythromycin-resistant transformants could only arise by recombination of the plasmid DNA into the bacterial chromosome. Analysis of transformants demonstrated disruption of the chromosomal *flgE* gene with the erythromycin resistance cassette and loss of the periplasmic endoflagella (Fig. 9). This chromosomal mutation was then complemented using an *E. coli-T. denticola* shuttle vector possessing a replicon from the native *T. denticola* plasmid pTS1 and an intact copy of the *flgE* gene. The mutant strain was shown to be nonmotile, in contrast to both the wild-type strain and the complemented mutant. Demonstration that disruption of the *flgE* gene caused loss of motility and that complementation of the chromosomal mutation restored motility confirms that loss of motility is due to disruption of the *flgE* gene.

Use of Nonnative or Broad-Host-Range Plasmids in Molecular Analyses

The use of native plasmids in the development of systems for gene transfer provides the advantage of a replicon known to function in the species of interest by virtue of the isolation of the plasmid from that species. However, there may be specific applications for which the properties of native plasmids are not desirable. In addition, native plasmids are not found in some species of interest, such as *P. gingivalis*. In these instances, the use of plasmids known to function in a broad range of species may be of benefit. There are a substantial number of broad-host-range plasmids from nonoral species that function in oral microorganisms. *A. actinomycetemcomitans* is an example of an oral pathogen for which both native and broad-host-range plasmids have been used. *P. gingivalis* provides an example of an oral pathogen found to be lacking in native plasmids but amenable to the use of selected plasmids from related bacterial species.

The first reports of transformation of *A. actinomycetemcomitans* used broad-host-range conjugative (pRK2525 and pRK212.2) and mobilizable (pBK1) plasmids (Table 7). Although these plasmids are stable in *A. actinomycetemcomitans*, they are large and thus cumbersome for use as cloning or shuttle plasmids. The native plasmid pVT736-1 is a 2-kb element that provides an *A. actinomycetemcomitans*-specific replicon that has been used in the construction of several vectors designed for cloning in *A. actinomycetemcomitans* (pDMG1 and pDMG2 in Table 7). However, the *E. coli-A. actinomycetemcomitans* shuttle plasmid pDL282 using the pVT736-1 replicon was found to be structurally unstable, suffering deletion on passage without antibiotics. pDL282 was additionally unstable segregationally, meaning that the plasmid did not efficiently segregate into daughter cells and was lost from the population after passage without antibiotics. Due to these difficulties, a systematic investigation of replicons functioning in *A. actinomycetemcomitans* was undertaken. Alternate replicons, including p15A from the *E. coli* vector pACYC184 and the replicon of pWV01 from *Lactococcus lactis*, were found to function in *A. actinomycetemcomitans*. The shuttle plasmid pDMG4 was of particular interest based on its small size (3.4 kb), with a single replicon (p15A) that functions in both *A. actinomycetemcomitans* and *E. coli*. pDMG4 proved to be structurally and segregationally stable with selection and was demonstrated to function in cloning of *A. actinomycetemcomitans* DNA in both species. Thus, pDMG4, which possesses the heterologous p15A replicon, appears to be better suited for use as a shuttle plasmid than constructs using the native *A. actinomycetemcomitans* plasmid replicon.

P. gingivalis has been the focus of intensive investigation of putative virulence determinants due to its strong association with periodontitis. The cloning of *P. gingivalis* genes encoding proteases and adhesins has led to investigation of their properties in the heterologous *E. coli* host system.Targeted mutations in selected genes in strains of *P. gingivalis* have greatly enhanced our understanding of the properties of the gene products in the native host environment. A limitation of many of these studies, however, has been the lack of complementation of the chromosomal mutation to demonstrate restoration of function with the intact gene. Numerous studies have failed to detect plasmids in strains of *P. gingivalis*, and the development of plasmids that function in this species has relied on the use of native plasmids from closely related species (Table 7). The *E.*

TABLE 7 Selected plasmids for use in *A. actinomycetemcomitans* and *P. gingivalis*

Bacterial host	Plasmid(s)	Relevant properties	Replicon[a]	Plasmid size (kb)	Plasmid stability	Comments
A. actinomycetemcomitans	pRK2525, pRK212.2	Ampicillin and kanamycin resistance; conjugative	RK2	60 (pRK2525) to 67 (pRK212.2)	Structurally stable	Transformation of *A. actinomycetemcomitans* with these IncP plasmids provided proof of principal for conjugal transfer to *A. actinomycetemcomitans* and functioning of the RK2 replicon; the large size of these plasmids limits their use as vector systems
	pBK1	Chloramphenicol and ampicillin resistance; mobilizable	RSF1010	12.5	Structurally stable	Mobilization of pBK1 (an IncQ plasmid) by a chromosomally integrated IncP plasmid in *E. coli* SM10; large size in comparison with other plasmids a potential disadvantage
	pDL282	Spectinomycin resistance	ColE1/replicon from *A. actinomycetemcomitans* plasmid pVT736-1	5.8	Structurally and segregationally stable with selection	Potential use in *A. actinomycetemcomitans* and *E. coli*, but without selection, plasmid is structurally and segregationally unstable
	pDMG1[b]	Erythromycin resistance	pVT736-1 replicon	3.7	Structurally and segregationally stable	Potential use in cloning directly in *A. actinomycetemcomitans* (does not replicate in *E. coli*)
	pDMG2[b]	Spectinomycin resistance	pVT736-1 replicon	3.1	Structurally and segregationally stable	Potential use in cloning directly in *A. actinomycetemcomitans* (does not replicate in *E. coli*)
	pDMG4	Spectinomycin resistance	p15A	3.4	Structurally stable and segregationally stable with selection	Demonstrated use in cloning of *A. actinomycetemcomitans* DNA in *E. coli* and stable maintenance of the recombinant molecule in *A. actinomycetemcomitans*
	pGA14/spc	Spectinomycin resistance	pWV01	5.6	Structurally stable and segregationally stable with selection	May be of use, but attempts to clone into the plasmid have not yielded positive results
	pDL293	Spectinomycin resistance	RSF1010	9.9	Structurally and segregationally stable	Large size in comparison with other plasmids a potential disadvantage
P. gingivalis	pE5-2	Erythromycin and clindamycin resistance; mobilizable	RSF1010/replicon from *Bacteroides* spp. plasmid pB8-51	17.1	Structurally and segregationally unstable	This plasmid is mobilized from *E. coli* by a coresident plasmid R751 to *P. gingivalis*; pE5-2 harbors a transposon, *Tn4351*, that is observed to insert in the chromosome in a significant percentage of transconjugants
	pYH400	Ampicillin and erythromycin resistance	ColE1/replicon from *P. asaccharolytica* plasmid pYHBA1	12.8	Structurally and segregationally stable	High rates of transformation in selected recipient strains; large size and limited convenient restriction endonuclease sites may limit utility
	pYH420[c]	Erythromycin resistance	pYHBA1 replicon	8.9	Structurally and segregationally stable	Smaller size and unique cloning sites provide some advantages; high rates of transformation in selected recipient strains

[a]Notes on replicons: RK2 is a broad-host-range conjugative plasmid of the IncP family; RSF1010 is found on broad-host-range plasmids of the IncQ family that are not conjugative but are mobilizable; pVT736-1 is a 2-kb plasmid native to *A. actinomycetemcomitans*; ColE1 is an *E. coli* replicon; p15A is a replicon from the *E. coli* cloning vector pACYC184 which is compatible with ColE1; pWV01 is a native plasmid of *Lactococcus lactis*.

[b]Note that pDMG1 and pDMG2 possess only the replicon from the *A. actinomycetemcomitans* native plasmid pVT736-1 which does not function in *E. coli*.

[c]Note that pYH420 lacks the *E. coli* replicon.

coli-Bacteroides shuttle plasmid pE5-2 was mobilized to *P. gingivalis* but is not stably maintained. pE5-2 possesses a transposon that inserts into the host cell chromosome under selective conditions, and the plasmid is lost under nonselective conditions. Studies with pE5-2 clearly demonstrated that plasmid DNA isolated from *P. gingivalis* was much more efficient in transforming *P. gingivalis* than plasmid DNA isolated from *E. coli*. This presumably is due to an indigenous restriction-modification system, so that the methylation of DNA in *P. gingivalis* protects it from cleavage by the host cell restriction endonuclease. However, *P. gingivalis* strains amenable to transformation with DNA from any source, presumed to be deficient in restriction endonuclease activity, have been isolated. These strains were then used to identify replicons that would function in *P. gingivalis* from plasmids of related species. A native plasmid of the related species *Porphyromonas asaccharolytica* was successfully used to generate the *E. coli-P. gingivalis* plasmid pYH400 and the *P. gingivalis* plasmid pYH420. These plasmids, which are stably maintained in *P. gingivalis*, along with the selected strains amenable to transformation, provide a feasible host vector system for investigation of this oral pathogen. More recently, pT-COW, based on the *Bacteroides-E. coli* shuttle vector pVAL1, has successfully been used for complementation in *P. gingivalis*.

Integration Vectors

The recombinant DNA vectors and shuttle plasmids described above, able to replicate in the oral bacterial species of interest or in both *E. coli* and the species of interest, facilitated the cloning of oral bacterial DNA and made possible the study of oral bacterial gene functions in their native host species. Descriptions of vectors that either are improvements on existing molecules or extend the spectrum of oral bacterial species amenable to genetic analysis continue to appear in the literature. However, certain types of studies cannot be conducted with genes of interest that have been cloned onto replicating plasmids. For instance, one may wish to conduct studies on a bacterial species in the absence of a particular gene product to learn more about the contribution of that product to the overall phenotype of the species. It may also be advantageous to determine if the product of a gene is essential for survival of the bacterial cell, as in the case of potential targets for new antimicrobial agents. One approach to eliminating a gene function is to attempt to interrupt or delete the gene, i.e., construct a functional knockout. If this can be done, then the gene clearly is not essential and studies on its contributions to the host species phenotype can be pursued. If attempts to knock out the gene fail, it can only be considered putatively essential, since failure to knock it out may have been due to faulty experimental design or the essentiality of a downstream gene on the same operon. Therefore, it is necessary to add to the strain a second, complementing copy of the gene, e.g., on a vector, and then show that under these circumstances the chromosomal copy can be knocked out by conducting the original knockout experiment on the strain carrying the complementing gene copy. The use of suicide and shuttle vectors to accomplish this experimental design in *T. denticola* was described earlier in this chapter. This same experimental approach can be applied to virtually any oral bacterial species that is transformable, due either to the ability to achieve a natural state of competence or via artificial

means such as electroporation, as long as the integration vector encodes a resistance gene that is expressed in that species. Numerous integration vectors with a variety of resistance markers have been described. Two or more such suicide vectors may be used in the same bacterial species to construct a strain with multiple gene knockouts.

Very often, it is not advantageous to examine gene functions when they are expressed on a plasmid vector, possibly because the vector is structurally unstable in the host species of interest or because the plasmid is rapidly lost in the absence of antibiotic selection, and such selection may interfere with or alter the phenotype(s) expressed by the cloned gene. In other instances, the expression of multiple copies of the cloned gene, as is the case when the gene is carried on a plasmid vector, may not provide an accurate picture of its true physiological function in the cell when present in a single copy on the host chromosome. And, in some cases, overexpression of a gene may be toxic to the bacterial cell. Several genetic systems have been devised by which a cloned gene can be integrated into a bacterial host chromosome so as to examine the effects of a single copy expression of that gene. One such system is of particular interest because it provides a mechanism by which a cloned fragment of DNA can be transferred to, and integrated into, the chromosome of either a transformable or nontransformable streptococcal species, and it is applicable to DNA that may or may not share homology with the chromosome of the target bacterial species. The system is composed of a specialized integration vector and one or more transformable streptococcal strains in which the vector can integrate into the chromosome. The integration vector pDP36 consists of pVA891 flanked by DNA from a conjugative element, V6001, which is similar to the conjugative transposon Tn*916*. The construction of pDP36 resulted in a 1.1-kb deletion of V6001 DNA that caused a deletion of its *tetM* gene. Virtually any DNA, with or without homology to the target host species, can be ligated to pDP36 and cloned in *E. coli* by selection for resistance to chloramphenicol, which is encoded on the pVA891 plasmid. Subsequently, the resultant recombinant plasmid can be isolated from *E. coli* and transferred to a naturally transformable streptococcal species, e.g., *S. gordonii* that carries V6001 in its chromosome. Since pDP36 cannot replicate in a streptococcal host, the only way that it, or a recombinant DNA molecule derived from it, will survive in such a host is to integrate into the new host chromosome, due to the extensive homology on either side of pVA891 to V6001. Transformants are selected in the presence of erythromycin, resistance to which is also encoded by pVA891. Such transformants will also be susceptible to tetracycline, due to the replacement of a large segment of V6001, including its functional *tetM* gene, with the portion of this element containing the inactivated *tetM* gene. The function(s) encoded by the DNA cloned into pDP36 in *E. coli* can now be studied in the *S. gordonii* transformant, in which they are present in this host's chromosome in single copy. Also because of the integration of the recombinant pDP36 molecule into V6001, it has now become an integral part of a conjugative element that can be transferred to nontransformable streptococcal strains via conjugation. Thus, the pDP36 recombinant DNA system could theoretically be used to clone virtually any bacterial gene and to subsequently study, and utilize for various purposes, its expression by any species of *Streptococcus*.

Genes of bacterial species that are naturally transformable, such as many of the oral streptococcal species and *P. gingivalis*, can be manipulated without a requirement for vectors of any kind. Linear double-stranded DNA can be used to transform such species, and transforming molecules of virtually any composition can be constructed by a method referred to as fusion PCR. For instance, if one wished to delete a gene designated gene B completely, in the order ABC on the host chromosome, then it would only be necessary to construct an amplicon composed of an antibiotic resistance gene, e.g., gene X, flanked on one side by DNA from gene A and on the other side by DNA from gene C. If such an amplicon was used to transform a strain containing gene B, followed by selection on a medium containing the antibiotic to which resistance is encoded by gene X, then transformants would be selected in which gene B has been displaced by gene X, due to a double-crossover event based on the homology between genes A and C in the amplicon and the host chromosome. In fusion PCR, the transforming amplicon composed of genes AXC is constructed as follows. Pairs of oligonucleotide primers would be designed such that for the first pair, one would consist of perhaps the first 20 bases at the 5′ end of gene A, for synthesis of the 5′-to-3′ strand of gene A, and the second would contain 10 or so bases from the 5′ end of gene X and 10 or so from the 3′ end of gene A for synthesis of the 3′-to-5′ strand of gene A, such that the amplicon synthesized would consist of the 3′ end of approximately 1,000 bp of gene A DNA with about 10 bp of homology to the 5′ end of gene X. Oligonucleotide primers for the synthesis of gene X would be designed so that the 5′ end of the gene amplicon would have approximately 10 bp of homology to the 3′ end of gene A and the 3′ end of it would have approximately 10 bp of homology to the 5′ end of gene C. The gene C amplicon would contain approximately 1,000 bp of the 5′ end of the gene with approximately 10 bp at its 5′ end of the 3′ end of gene X. Because of the overlapping homologies, the amplicon comprising the 3′ end of gene A and the gene X amplicon would be combined and used as a template for synthesis of a gene AX amplicon (similar to the PCR processes described in Fig. 6). This AX gene fragment can subsequently be used as a template, along with the gene C amplicon, for the synthesis of the final gene AXC amplicon using a similar approach. This would then be used as the transforming DNA for the isolation of a transformant in which gene B has been deleted and replaced with the antibiotic resistance-encoding gene X. This fusion PCR approach can be employed for the synthesis of transforming amplicons encoding virtually any combinations of DNA segments from the same source or different bacterial sources. If gene B is essential to the host species, or part of an operon in which essential downstream genes are not expressed as a result of the chromosomal insertion, then its deletion would not be possible by transformation with the AXB gene amplicon, as described above. Approaches to address these situations would also use fusion PCR to generate DNA fragments possessing the essential genes and their promoters, or other functional promoters, for insertion elsewhere in nonessential regions of the chromosome. Once a functional essential gene is inserted elsewhere, one would then show that the allele could now be deleted from its original chromosomal site by the insertion of the AXB amplicon.

TRANSPOSON MUTAGENESIS

Integration vectors are useful for creating knockout mutations in situations where the DNA sequence of a gene is known. In other circumstances, it may be desirable to identify genes associated with a particular phenotype, without prior knowledge of the DNA sequence. The identification and analysis of genes that encode a variety of phenotypic traits have been aided by application of transposons to the random insertional mutagenesis of bacterial genomes. When a transposon inserts into a bacterial genome, if the site of insertion is within a structural gene, a defective gene product (protein) will be made, if any product at all is synthesized. The phenotypic trait(s) imparted by that gene product (or an operon, if the interrupted gene is followed by other genes within the operon and the insertion resulted in polar effects) can be identified by comparing the properties of the isogenic mutant to its wild-type parent strain. A variety of transposons have been employed in the random mutagenesis of oral bacterial genomes, followed by studies of the phenotypes affected. One of the most useful transposons for insertional mutagenesis has been the conjugative transposon Tn*916*. The use of Tn*916* for this purpose was prompted by observations that it, or closely related conjugative elements, had either been shown to transfer to a variety of oral bacterial species or was present in tetracycline-resistant isolates of these species from the oral cavity. The presence of these elements has been reported in such oral streptococcal species as *S. mutans*, *S. sanguinis*, *S. mitis*, and *S. oralis* as well as gram-negative species such as *F. nucleatum*. Selection for the transfer of Tn*916* via conjugation involves the use of media selective for recipient strains that have become resistant to tetracycline, followed by screening of the resistant isolates for alterations in detectable phenotypes. The site of transposon integration can be detected by the cloning, from the mutant strain, of Tn*916*-associated DNA carrying the *tetM* gene and flanking chromosomal DNA. Among the oral streptococci, Tn*916* has been used in the identification and study of genes encoding intergeneric coaggregation in a strain of *S. gordonii*, a fibronectin-binding protein in *S. sanguinis*, and the ability to produce rods, exhibit aciduricity, and accumulate intracellular polysaccharides by strains of *S. mutans*.

Tn*916* was also employed for insertional mutagenesis of *A. actinomycetemcomitans*, but the transposon was not transferred by conjugation but rather by electrotransformation. The plasmid, pAM120, into which Tn*916* had been cloned, was isolated from *E. coli* and used to transform the Y4 strain of *A. actinomycetemcomitans* by electroporation, followed by selection for resistance to tetracycline. Since pAM120 cannot replicate in *A. actinomycetemcomitans*, bacterial cells were able to become resistant to tetracycline only if the *tetM*-encoding Tn*916* inserted into the new host chromosome via transposition. The results of Southern hybridization experiments showed that Tn*916* had inserted at a variety of chromosomal locations among several tetracycline-resistant Y4 transformants examined. Several tetracycline-resistant isolates were subsequently shown to be defective in the production of the capsule-like serotype b-specific antigen, SPA, due to the insertion of the transposon into the corresponding gene.

The methods of transposon insertional mutagenesis described above are dependent on transposition, a low-frequency event, immediately

following the introduction of the transposon to the recipient cell via conjugation or following transformation with a suicide plasmid, with both transfer methods also being low-frequency events. Thus, the number of mutant isolates obtainable from each transfer experiment is generally quite low. Frequencies of mutant isolation can be increased dramatically by the construction of a transposon delivery plasmid that is temperature sensitive for replication. One such plasmid, designated pTV1-OK, consists of (i) a mutant *L. lactis* plasmid, *repAts*-pWV01, able to replicate in numerous gram-positive and gram-negative species at temperatures around 30°C but unable to replicate if the temperature of incubation is shifted to 40 to 45°C; (ii) the enterococcal transposon Tn*917*, which encodes resistance to erythromycin and can be induced to transpose at a higher than normal frequency in the presence of subinhibitory concentrations of erythromycin; and (iii) *aph3A*, a gene encoding resistance to high levels of kanamycin in both gram-positive and gram-negative species. pTV1-OK is introduced into the bacterial strain of interest by transformation, followed by selection for resistance to kanamycin. The plasmid is distributed to all cells in a culture if propagated at a temperature permissive for its replication. If the culture is also incubated in the presence of subinhibitory concentrations of erythromycin, then transposition of Tn*917* will be induced, with many copies of it inserting into various sites on the host chromosome. If such a culture is diluted and then incubated at a temperature nonpermissive for replication, the plasmid will be lost as the cells divide. Subsequent culture with a concentration of erythromycin selective for the transposon causes colonies to appear that have lost pTV1-OK but contain Tn*917* in their chromosomes. Evidence for the loss of pTV1-OK is demonstrated by showing that the erythromycin-resistant isolates have become susceptible to kanamycin. This transposon delivery vector has been used to interrupt, identify, and study a variety of genes involved in the metabolism of *S. mutans* as well as a number of genes whose expression is regulated by different environmental conditions.

Two transposons originally described in strains of *Bacteroides fragilis*, Tn*4351* and Tn*4400*, have been used for the transposon insertional mutagenesis of *P. gingivalis*. Tn*4351* is delivered on the plasmid R751::*Ω4 to strains of *P. gingivalis* from *E. coli* by conjugation. R751 is a broad-host-range conjugative plasmid that can transfer to, but cannot replicate in, *P. gingivalis*, whereas R751::*Ω4 is R751 containing a partial tandem duplication of Tn*4351*. Theoretically, the only way Tn*4351* can survive in *P. gingivalis* following transfer on R751::*Ω4 is via transposition to the *P. gingivalis* chromosome, which can be selected for in the presence of erythromycin, as resistance to this antibiotic is encoded by the transposon. However, in at least one strain of *P. gingivalis*, among the majority of mutants obtained, plasmid R751 had accompanied Tn*4351* into the chromosome via cointegration, rather than a simple transposition event that would have resulted in the insertion of Tn*4351* only. Although the mutations that were caused by a cointegration event were stable, the presence of the large plasmid (53 kb), in addition to the transposon, at the site of insertion made it more difficult to characterize the interrupted gene. The second *B. fragilis* transposon, Tn*4400*, is transferred to *P. gingivalis* strains on the delivery vehicle, pYT646B, which, in addition to Tn*4400*,

consists of the *E. coli* plasmid pBR322 and *oriT* from RK2, which facilitates transfer from *E. coli* to *P. gingivalis* by the mobilizing plasmid RK231. Integration of the transposon is confirmed by selection for tetracycline resistance encoded by the transposon-mediated, anaerobically expressed *tetQ* gene.

Integration or suicide vectors enable targeted mutagenesis of genes of interest. In contrast, transposons enable mutagenesis at undefined sites, so mutants that display the loss of a phenotype of interest can be characterized to determine which genetic loci contribute to that phenotype. The use of transposons could then be considered to be phenotypically targeted, as opposed to the use of integration vectors, which are genetically targeted. With the wealth of genome sequence data available, genetically targeted mutagenesis is facilitated by knowledge of the sequence and organization of genes predicted by their DNA sequence to be of interest. However, it is significant that often more than a third of the genes in an annotated genome are unassigned in function. This highlights the need for phenotypically targeted mutagenesis, which provides a powerful approach to delineate novel genes contributing to a phenotype.

CONCLUSIONS

Analyses of the genomic DNA sequences of oral microorganisms provide important information for investigation of microbial taxonomy, metabolism, and virulence mechanisms. Comparative sequence analyses of microbial genomes allows recognition of the tremendous diversity in genetic composition among species and the importance of horizontal gene transfer in the evolution of microorganisms. The pace of such studies is, and will be, hastened by the availability of the total genomic sequence of an ever-increasing number of oral bacterial species. Such sequences provide important information as to which genes of a species are present and likely to contribute to its role in the oral environment or, in some instances, its ability to cause disease. Investigations into natural gene transfer mechanisms in oral bacteria reveal the importance of horizontal DNA transfer to survival in the oral cavity and provide genetic tools for the advanced analysis of oral bacteria. The application of genetic and molecular biological tools to the study of oral microbiology now includes important oral bacterial genera such as *Aggregatibacter*, *Porphyromonas*, *Actinomyces*, *Prevotella*, *Fusobacterium*, and *Treponema*. With the availability of the genetic and molecular tools described in this chapter, it is possible to confirm a function predicted by the sequence of a gene, to examine environmental conditions that regulate its expression, or to elucidate the function in each species of the 30 to 40% of genes whose function cannot be predicted by sequence alone.

KEY POINTS

Investigations of the ability of strains of a few species of oral streptococci to achieve a natural state of competence for genetic transformation were being conducted in the early 1960s. However, the true origin of the genetics and molecular biology of the oral microbiota can be traced to the isolation of plasmid DNA from a strain of *S. mutans* in 1973, followed shortly thereafter by demonstration of the transfer of plasmids between oral streptococci.

DNA can be acquired by bacterial cells through either vertical or horizontal (lateral) transmission. In vertical transmission, there is transfer of a copy of the parental DNA to daughter cells upon cell division. In horizontal transmission, there is acquisition of foreign DNA. The known mechanisms responsible for horizontal gene transfer are transformation, the uptake of naked DNA by competent recipient cells; conjugation, the transfer of DNA through cell-cell contact; and transduction, the introduction of DNA into a bacterial cell by bacteriophages containing either random (generalized transduction) or specific (specialized transduction) pieces of DNA from the previous host of the phage.

The development of competence (the ability to uptake DNA by transformation) in streptococci can involve quorum sensing with small peptide-signaling molecules that induce phosphorylation-dependent signaling cascades. In some gram-negative organisms, there is specificity of DNA uptake with the presence of USS being required. Regulation of competence in gram-negative organisms can occur by catabolite repression.

Plasmids are extrachromosomally replicating genetic elements. Most plasmids are present in their host cells as double-stranded, covalently closed, circular DNA molecules. Plasmids must encode the elements required for autonomous replication, including an origin of replication and contiguous gene(s) encoding replication proteins and other controlling elements. Some native plasmids encode proteins that confer resistance to antibiotics. Other phenotypes that may be encoded by plasmids include virulence traits, metabolic capabilities, and the production of bacteriocins. Plasmids may be either conjugative or mobilizable. Conjugative plasmids possess all the genes necessary for conjugative transfer. Mobilizable plasmids require genes encoding conjugative functions to be provided by another plasmid or the chromosome. Circular plasmids replicate by theta and RC mechanisms.

Transposons are discrete nonreplicative segments of DNA capable of inserting into, and being replicated as part of, a bacterial replicon such as a chromosome, plasmid, or bacteriophage genome. They may also move from one site in a DNA molecule to another. Insertion of a transposon into DNA is mediated by insertion sequence elements, which are located at the ends of transposons and encode the enzyme transposase, which catalyzes the insertion events.

Transposon insertion is usually random, i.e., it may occur at virtually any site on a DNA molecule, independent of extensive homology. Thus, transposons can be used for random mutagenesis where screening for a phenotypic property is possible. Some transposons can be transferred by conjugation.

A bacterial mutant in which only a defined gene has been altered is termed an isogenic mutant, and this will lead to a change or loss of function specific to that gene. Restoration of the gene and/or its expression, referred to as complementation of the mutant, should restore function.

A common approach to generating isogenic mutants is to use a vector that does not replicate in the bacterium of interest (a suicide vector) but into which has been cloned DNA homologous to a region of the targeted gene as well as a resistance determinant that provides a marker for selection. A single recombination (or crossover) event will result in integration of the entire plasmid and disruption of the gene. A mutation in a gene can also be created by a double recombination event between two identical DNA regions of the plasmid (constructed to flank the selection marker) and the chromosome, resulting in an allelic replacement, essentially a swapping of a portion of the gene with the selection marker. A similar double recombination event can also be used to create a deletion mutation, a loss of a region of a gene without introduction of a selection marker. Phenotypic analyses are used to evaluate the effect of the gene disruption. In all cases, it is important to assess any effects on genes downstream of the target gene.

Shuttle vectors that replicate in both *E. coli* and another species facilitate cloning (in *E. coli*) and transfer of genetic material into the species of interest. These vectors can be used to generate double recombination mutants or to provide an intact copy of a gene for complementation. Restoration of gene function by complementation ensures that the phenotypic effect observed was due to inactivation of the gene, not to a polar effect on a downstream gene or other unintended mutation.

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Applied Molecular Biology and the Oral Microbes

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INTRODUCTION

The development of recombinant DNA technology in the 1970s proved significant in advancing knowledge of the biochemical, physiological, and virulence properties of oral microorganisms. The advent of molecular genetics as a research tool in oral microbiology provided new insights into the mechanisms by which bacteria colonize the host and cause disease. These advances also led to the development of molecular diagnostic protocols for more rapid identification of oral microbes.

Since the initial cloning of a streptococcal gene in the late 1970s, genes relevant to specific characteristics of many other oral microbes have been cloned and expressed. More recently, the nucleotide sequences of the entire genomes of a range of oral bacteria have been obtained, including those of *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola*, *Actinomyces naeslundii*, *Streptococcus mutans*, and *Prevotella intermedia*. The postgenomic era, with continually developing technologies, allows genome sequence and proteome comparisons for different strains of a single bacterial species, facilitating identification of potential candidates for virulence genes, vaccine and antimicrobial targets, and diagnostics. High-throughput methods have become available for more rapid ecological analysis and genetic characterization of all of the bacteria present in the oral cavity (the oral microbiome).

In this chapter, we describe some of the basic recombinant DNA techniques which have been applied to the study of oral microbes. The continued progress and success of these technologies will bring many pragmatic benefits, particularly in therapeutics for oral and related systemic infections.

INVESTIGATING GENE EXPRESSION: GENETIC APPROACHES

DNA Cloning

The elucidation of the genetic code, and of the structure of DNA and its role as the genetic material, made it clear that unraveling the secrets of biological processes would require an ability to propagate specific DNA segments and determine their sequences. A first step in this process is the

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cloning of a gene into a vector that can be propagated in bacterial or viral host cells (Fig. 1). This step takes advantage of several types of enzymes that can cleave, copy, or chemically modify DNA and, in some instances, RNA. Important among these enzymes are restriction endonucleases that catalyze site-specific cleavage of DNA. The enzymes recognize base pair sequences within DNA that are palindromic, that is they read the same sequence 5′-phosphate to 3′-hydroxyl on both strands. The enzymes can leave single-stranded overhanging sequences, or they can leave blunt ends with no overhanging sequences (Fig. 1). The overhanging or blunt-ended sequences of the vector can be joined with corresponding sequences, formed by enzymic digestion of the DNA to be cloned by an enzyme termed ligase. Another enzyme, termed reverse transcriptase, can generate a DNA copy from an RNA template, while a variety of DNA polymerases are available now to catalyze the synthesis of complementary DNA strands from single-stranded templates.

As a first step in the cloning of a gene, purified genomic DNA from an organism is digested with a restriction endonuclease to produce fragments. The numbers and sizes of fragments produced will depend upon the frequency of sites along the genomic DNA molecules that are recognition sequences for the enzyme used. The vector DNA, within which there is only a single cut site for the enzyme used, is also digested with the same or a compatible enzyme. Depending on the type of restriction enzyme used, fragments may be generated with "sticky" (or complementary) ends that may have a 5′ or 3′ single-stranded overhang with 1 to 5 bp exposed (Fig. 1). Because the digested fragments have complementary sequences on their ends, they adhere to each other by hydrogen bonding. Fragments from the host genome can adhere to each other, and two or more digested vector molecules may also adhere to each other. However, a single fragment of chromosomal DNA may also adhere to a single digested vector molecule. In the presence of DNA ligase from the *Escherichia coli* bacteriophage T4, the ends of the combined fragments are covalently linked, thus producing new recombinations of DNA molecules (hence "recombinant DNA"). When the DNA molecules have been digested with an enzyme that produces complementary single-stranded ends, the ligated junctions of the recombinant molecules will contain the original restriction sites (Fig. 1), which facilitates the future isolation and purification of cloned fragments. Alternatively, blunt-ended fragments, which are without a single-stranded overhang, can be generated by specific restriction enzymes or by the "filling in" of the 5′ single-stranded overhangs or digestion of 3′ → 5′ single-stranded overhangs with an enzyme such as phage T4 DNA polymerase (or S1 nuclease). These blunt-end fragments may also come together, be joined by DNA ligase, and generate new recombinant molecules that may or may not contain the original restriction site. The mixture of ligated recombinant molecules is then introduced into a bacterial host strain by transformation or electrotransformation (electroporation). The plasmid vector contains an origin of replication that is functional in the host of choice and also encodes a gene that mediates resistance to an antibiotic. By spreading the transformation mixture onto an agar-based medium containing the antibiotic, cells that have received recombinant plasmids will be resistant to the antibiotic and grow on the nutrients in the medium. Colonies of

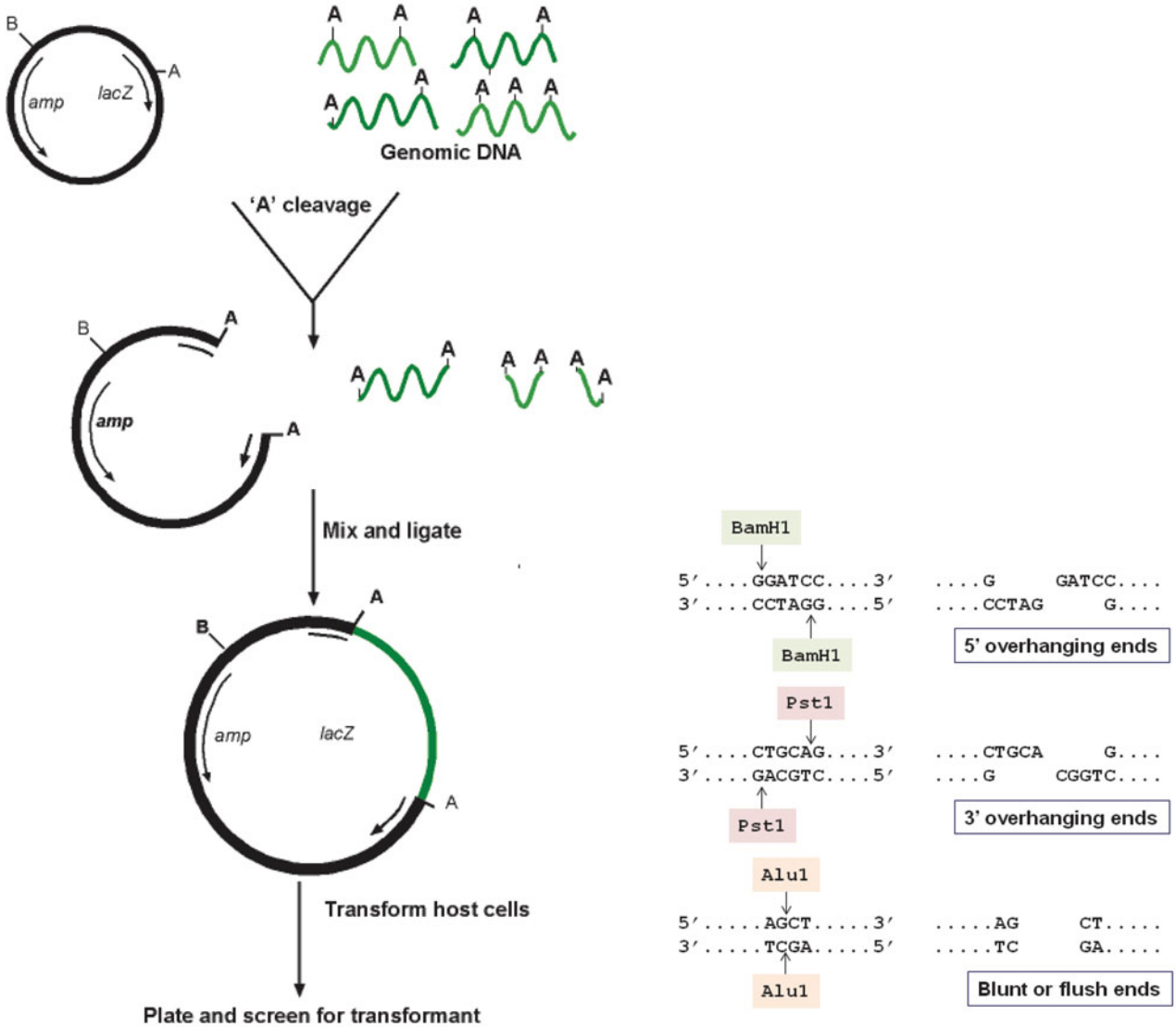

FIGURE 1 General method for cloning a DNA fragment into a plasmid vector. Double-stranded plasmid vector and genomic DNA are purified separately, and each is digested with the same or a compatible restriction endonuclease, in this example, recognizing sequence A. The digested DNAs are mixed together, and the complementary ends are ligated (covalent bonds are formed) by phage T4 DNA ligase. The ligated recombinant molecules are used to transform a host cell. The plasmid vector contains an origin of replication and an antibiotic resistance marker (in this case ampicillin, *amp*). Thus, selection of transformants that have received the vector will be resistant to the antibiotic, whereas cells that are not transformed will not survive on antibiotic-containing medium. In the transformants, the plasmid can replicate independently of the host chromosome, and these cells can then be screened for a predicted phenotype imparted by a gene or genes that were inserted into the vector. In this case, vector with insert will no longer express *lacZ* and so will not produce β-galactosidase. Vector DNA with no inserts, or with miscellaneous inserted genomic DNA, will not impart the desired phenotype. See text for other methods for screening for specific cloned DNA. The right side of the figure shows examples of restriction endonuclease cutting sites for three different enzymes *Bam*H1, *Pst*1, and *Alu*1, generating DNA fragments with single-stranded nucleotide overhanging ends or blunt ends. doi:10.1128/9781555818906.ch8.f1

transformants that have received plasmid vector DNA, with or without cloned genomic DNA fragments, may be subsequently screened for the presence of a cloned gene of interest.

A direct way of identifying a DNA fragment carrying a gene of interest is by detecting an expressible phenotype from the cloned gene. This allows the recombinant clone to be differentiated from others that do not carry the fragment. A limitation associated with this method of detection is the inability of some genes to have an expressible phenotype in *E. coli*. This limitation has been overcome in some instances by the construction and availability of shuttle vectors. These vectors carry two origins of replication, one for replication in *E. coli* and one for replication in another bacterial host of interest. The vector contains an antibiotic resistance gene that is expressed in both bacterial hosts or two antibiotic resistance genes, one for each host. Thus, initial cloning is performed in *E. coli* (the best host system for most cloning experiments), and then DNA is purified from a library of recombinant clones and used to transform the host of interest, in which the desired gene can be expressed.

Another approach for identifying desired clones is based upon detecting the gene product by using an antibody. Transformant colonies can be applied to a nitrocellulose membrane and reacted with antibody, followed by detection reagent, to identify clones expressing the protein of interest.

Clones carrying the desired DNA fragment may also be identified by methods that do not require expression. With this approach, DNA from a cloned source is transferred onto a nitrocellulose filter membrane. The DNA is then denatured to single strands by alkali treatment. Since single strands of DNA are fixed to the surface of the filter, they will not reanneal with each other. They will, however, anneal to complementary DNA (the probe) in solution applied to the filter-bound target DNA. The extent of the hybridization (annealing) to the probe will be directly proportional to the stringency of the hybridization conditions and the degree of homology between the probe and the target DNAs (Fig. 2 illustrates hybridization). The probe may be labeled radioactively or with a chemiluminescent compound for detection of binding.

When a single recombinant molecule composed of a vector plus an inserted DNA fragment is introduced into an appropriate host cell, large numbers of recombinant DNA molecules are produced. In this way, cloning of a variety of genes from oral microorganisms has been accomplished with a wide range of cloning vectors and systems.

A more favorable method of preparing genomic DNA for insertion into a vector is by amplification of specific genes of interest from genomic DNA by PCR (see below). The fragments can be joined to a vector by ligase to generate a new recombinant molecule. A major advantage of this method is the ability to clone only the gene, or DNA fragment, of interest, to the exclusion of all other genomic DNA from the host of origin. Another advantage is the ability to engineer into the DNA fragment to be cloned any restriction endonuclease site of choice.

PCR

The primary objectives of genetic cloning are to isolate and amplify genes or DNA fragments for functional analyses or further manipulations. PCR is a powerful tool for the amplification of specific DNA fragments from

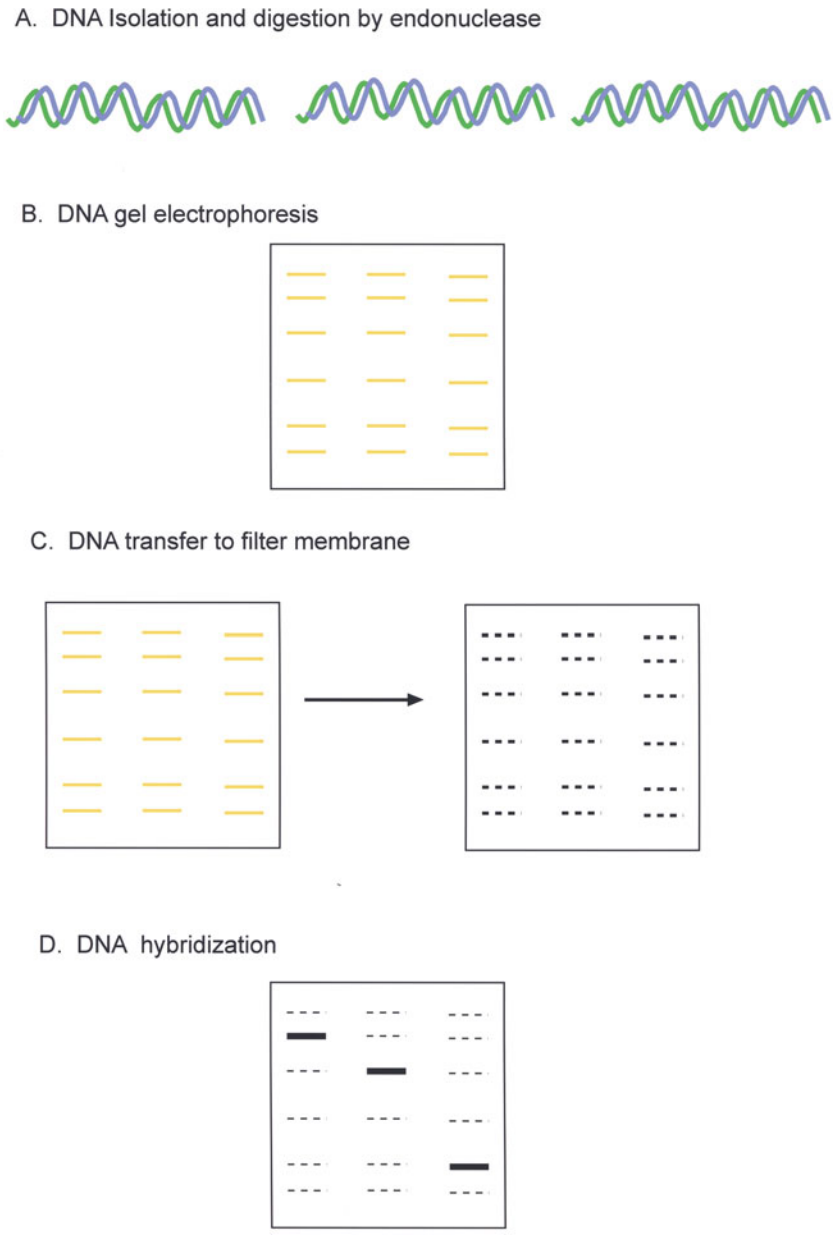

FIGURE 2 General method of Southern blot hybridization. (A) DNA is isolated and digested with a restriction endonuclease. The DNA fragments are separated by gel electrophoresis (B) and transferred and fixed to a filter (C). (D) The filter is hybridized with a probe that has been labeled either with a radioactive isotope or a reactive chemical. A signal detection procedure (e.g., autoradiography) reveals bands where the target DNA is complementary to the probe. doi:10.1128/9781555818906.ch8.f2

any DNA molecule in which they are present (Fig. 3). For PCR amplification, two oligonucleotide primers (typically 20 to 30 nucleotides in length) are added in vast excess compared to the DNA to be amplified. They hybridize to opposite strands of the DNA and are oriented with their 3′ ends facing each other to enable synthesis of the DNA segement between them by the DNA polymerase, which adds on nucleotides to the 3′ end of the growing strand. The first cycle of synthesis results in new strands of indeterminate length, which, like the parental strands, can hybridize to the primers upon denaturation and annealing. The second cycle of denaturation, annealing, and synthesis produces two single-stranded products that together compose a discrete double-stranded product that is exactly the length between the primer ends. Each strand of this discrete product

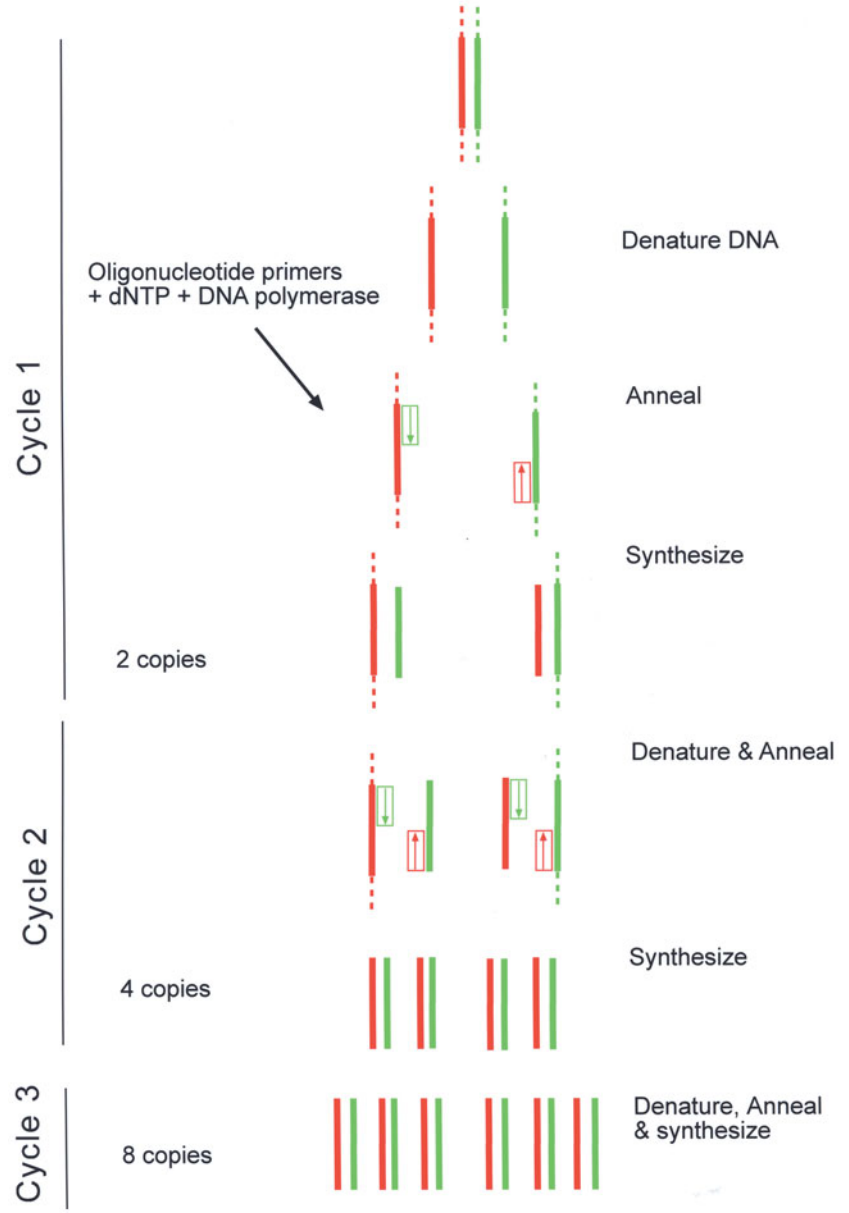

FIGURE 3 PCR. A solution containing the DNA in which the target sequence to be amplified is present is heated to a temperature that will allow it to denature. The solution is then cooled to a temperature that will permit renaturation, and a reaction mixture is added. This mixture contains the heat-stable DNA polymerase, the four nucleotides dATP, dGTP, dCTP, and dTTP, and a pair of short oligonucleotide primer sequences that are known to be complementary to base sequences on each strand at either end of the target sequence. The mixture is incubated (minutes) at a temperature that will allow annealing of the target sequence to the appropriate primer sequence, followed by the synthesis of complementary strands in the direction of 5′ to 3′ (i.e., nucleotides added to the 3′ ends of the single-stranded DNA molecules). This produces two copies of the target sequence. A cycle of heating for a few minutes to a temperature that permits denaturation, followed by cooling to a temperature that will permit reannealing and synthesis, is repeated numerous times (generally 30 to 40 cycles), giving rise to millions of copies of the target DNA in a few hours.
doi:10.1128/9781555818906.ch8.f3

contains the end sequence complementary to one of the two primers and can therefore participate as a template in subsequent cycles. The amount of this product doubles with every subsequent cycle of synthesis, denaturation, and annealing, accumulating exponentially so that 30 cycles can result in a 2^{28}-fold (270 million-fold) amplification of the discrete product. The PCR product can then be detected by electrophoresis on agarose gels, staining with a dye, such as ethidium bromide, that binds specifically to DNA, and visualization using ultraviolet light. Specific DNA fragments may also be detected by Southern hybridization (Fig. 2).

Since its initial development, PCR technology has been extended to numerous applications. For example, PCR products can be sequenced directly without the need for cloning. Because of the small quantity of template DNA required, in addition to the specificity in amplification that can be achieved by using unique oligonucleotide primers, PCR has been applied to the direct identification of oral microorganisms. Unique primers based upon the nucleotide sequences of genes encoding the 16S rRNA subunit, which contains species-specific regions, are used in PCR to detect oral microorganisms without the need to culture. This technique has been used for the study of microbial communities, including unculturable species. Thus, PCR analysis of biological samples using oligonucleotide primers that recognize species-specific 16S rRNA gene sequences, in conjunction with other species-specific genes, has facilitated the identification of cultivable oral microorganisms, including *Streptococcus* spp., *Porphyromonas* spp., *Aggregatibacter*, *Treponema* spp., and *Actinomyces*, and not yet cultivable organisms, such as *Dialister* spp., TM7, and *Synergistes*. Direct amplification of 16S rRNA sequences by PCR from mixed culture biomasses followed by purification and sequencing has also allowed the analysis of complex communities. Novel taxa identified by phylogenetic analysis in this way are designated "phylotypes." Sequence data from noncultivable organisms can be used to design specific PCR primers and DNA probes for rapid detection of the organisms in clinical specimens. These can then be used to determine the prevalence of the organisms in healthy or diseased tissues and, if specific associations are found, may provide useful markers of disease activity.

PCR has also been adopted for use with RNA templates for the production of cDNA using reverse transcriptase (RT). RT-PCR has been useful for gene regulation studies by quantifying levels of mRNAs produced under different conditions, a technique named real-time PCR. Incorporation of dyes into the reaction mixture that bind to the amplimer (PCR product) allows the quantitation of the PCR product after each amplification cycle.

INVESTIGATING GENE FUNCTION

Directed or site-specific mutagenesis is a powerful technique for dissecting the genetic basis of bacterial functions (e.g., the virulence of bacterial pathogens). Before the advent of recombinant DNA technology, chemical and physical methods of mutagenesis were used to alter the phenotype of bacterial strains. However, these techniques lacked specificity in that mutations produced could occur anywhere on the bacterial genome and were not confined solely to the gene of interest. Thus, new phenotypes could

have been the result of mutations in one or more genes, making analysis difficult. The development of intergeneric shuttle plasmids and efficient DNA transfer systems has provided the tools necessary to determine and analyze gene functions in oral microorganisms.

Directed mutagenesis can involve insertion-duplication mutagenesis, allelic exchange, or transposon mutagenesis. In insertion-duplication mutagenesis, a nonreplicating plasmid containing a cloned segment of a gene is utilized to construct specific isogenic mutants. The type of plasmid used, called a "suicide vector," usually contains an *E. coli* origin of replication, which permits replication in *E. coli* but not in the species under study. The plasmid also carries one or more antibiotic resistance markers, such that selection for antibiotic resistance can be made in *E. coli* and in the bacterial species of study. An internal subcistronic fragment of the gene of interest is cloned into the vector in *E. coli*, and the recombinant plasmid is propagated and isolated. The plasmid DNA is then used to transform the strain of interest, with selection for the appropriate antibiotic resistance phenotype. Because the plasmid is unable to replicate in the strain of interest, the resulting antibiotic-resistant transformant colonies must contain the vector that has become integrated into the genome, thus disrupting expression of the gene at the site of insertion. This is accomplished by a reciprocal recombination event between the plasmid and chromosome involving the cloned subcistronic DNA fragments which disrupts the target gene. Because this method will give rise to duplication during the recombination event, it is critical that the subcistronic DNA fragment be internal to the gene of interest. A limitation of insertion-duplication mutagenesis is usually the need to maintain selective pressure, i.e., growth in the presence of antibiotic, to maintain the integrated plasmid.

Allelic exchange mutagenesis (Fig. 4) is another technique that uses cloned gene sequences to generate a specific isogenic mutant. Construction of such mutants requires the exchange of gene alleles either between strains, such as from a plasmid, or by direct transformation or electroporation, into the appropriate chromosomal position. The ability to partly or entirely replace a wild-type chromosomal copy of a gene with an antibiotic resistance gene allows the phenotype of the insertion (disruption) to be studied under a defined set of in vitro or in vivo conditions. Several widely used allelic exchange systems have been developed for gram-negative and gram-positive oral bacteria. Nearly all constructs used in these systems rely on the positive selection of an antibiotic resistance gene (e.g., erythromycin or *erm*) that is also used to interrupt expression of the target gene. Insertion of the antibiotic resistance marker must be within the coding sequence of the gene. When replacement constructs are linearized, the drug resistance gene is flanked by two regions of homology to the target gene. Thus, allelic exchange mutagenesis occurs by a double-crossover event. Selection with the appropriate antibiotic eliminates the great majority of cells that have not stably incorporated the construct. Antibiotic-resistant transformants are those mutants that now have a defective gene as a result of additive integration. The net effect is that the defective version of the gene replaces the wild-type copy, creating a gene-specific mutant strain. Unlike insertion-duplication mutagenesis, allelic exchange mutagenesis is stably maintained even in the absence of selective pressure.

To bypass the cloning step in the above procedure, overlapping extension PCR can be utilized. This considerably reduces time and effort

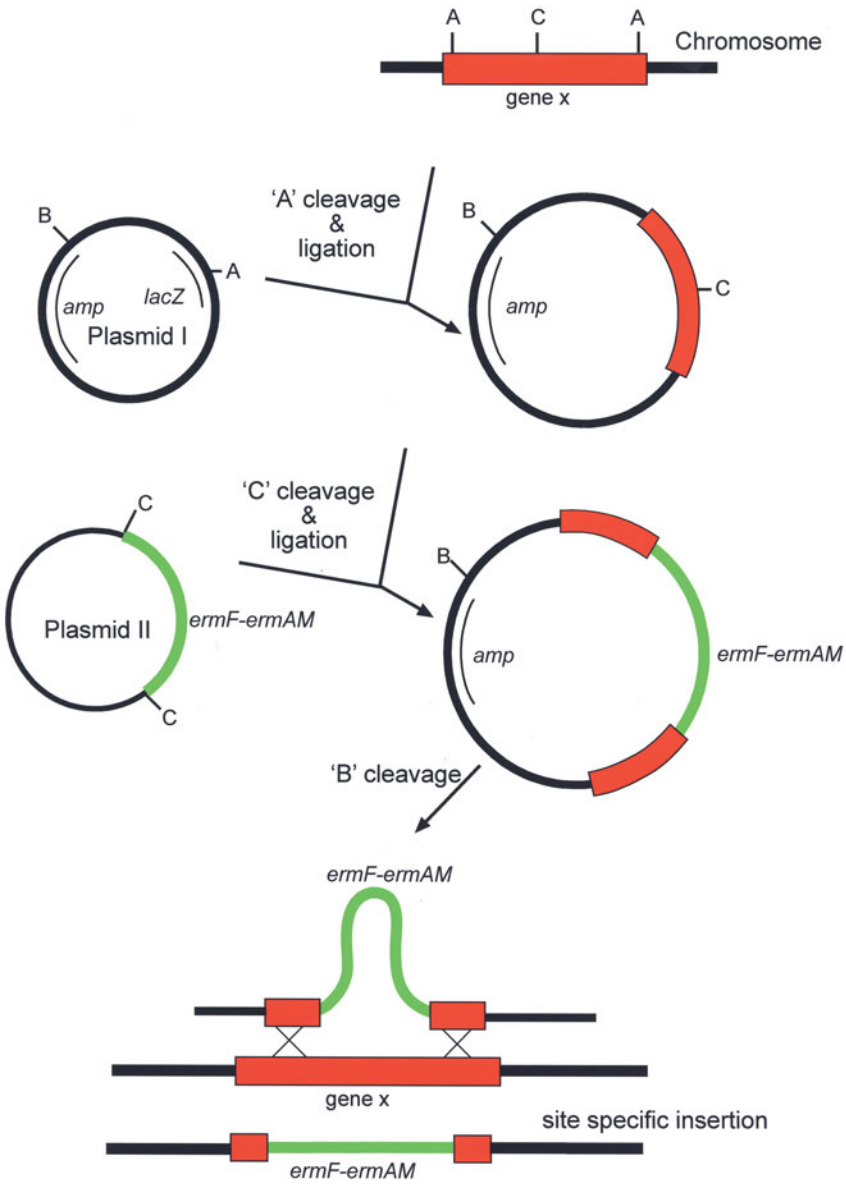

FIGURE 4 Example of the use of allelic exchange mutagenesis to construct a site-specific mutant. The chromosome of the organism under study and *E. coli* plasmid vector I are digested with restriction endonuclease A. This liberates numerous chromosomal fragments, one from each copy of the chromosome that encodes gene x. Alternatively, a chromosomal fragment containing only gene x can also be amplified from the chromosomal DNA by PCR. Insertion and ligation of DNA fragments into the cleaved site of plasmid vector I inactivate the *lacZ* gene. Plasmids containing fragment inserts are selected by screening for ampicillin-resistant transformants of *E. coli* that form white colonies on solid medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, the chromogenic substrate of β-galactosidase). Recombinant plasmids that have been identified as those encoding gene x and plasmid vector are digested with restriction endonuclease C. This enzyme is chosen because it either cleaves within gene x or results in the excision of a segment of gene x, which allows the insertion of an *erm* cassette (encoding erythromycin resistance) from plasmid vector II. Plasmids with the insertionally inactivated gene x are selected by screening for erythromycin-resistant colonies. This plasmid is then introduced (usually by electrotransformation or by natural transformation, depending on species and strain) into the original wild-type host cell. The inactivated gene shares homology with the host cell chromosome via the sequences of gene x that flank the *erm* gene. Thus, the functional allele of the host is exchanged for the inactivated allele by homologous recombination. Successful integration of the inactivated gene into the host chromosome is monitored by acquisition of erythromycin resistance and loss of the phenotype encoded by the gene.
doi:10.1128/9781555818906.ch8.f4

compared to a cloning-based approach. Custom made primers with complementary sequences at their 5′ ends, together with two separate DNA fragments amplified from regions flanking a target gene, can be fused to form a single product by means of primer extension without relying on restriction endonucleases or ligation reactions (Fig. 5). This method uses high-fidelity DNA polymerase, thus limiting unwanted mutations in the final construct that might otherwise become incorporated into the flanking regions.

Another useful technique for generating isogenic mutants without a need for first cloning the gene of interest is transposon mutagenesis. This involves the insertion of a transposable element into the host chromosome, giving rise to specific insertion mutations. Although some transposable elements may have "hot spots" for insertion, many can insert into target DNA more or less randomly. These elements usually contain an

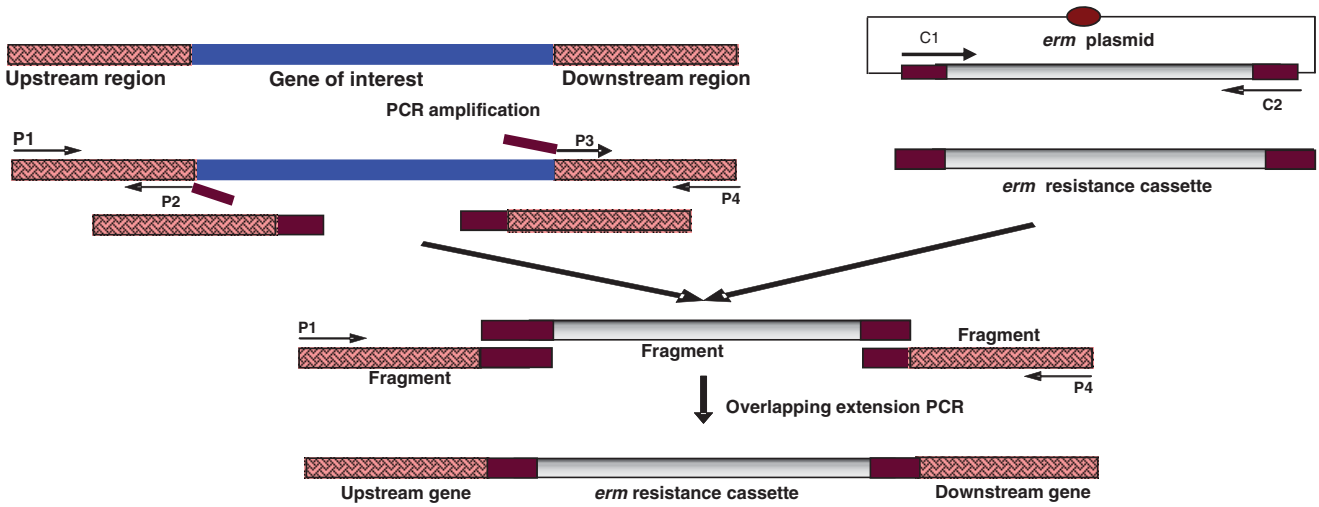

FIGURE 5 The overlapping extension PCR method uses two flanking and two mutagenic primers. Each primer pair will amplify upstream and downstream of the gene of interest to be mutated. Based on the orientation of the gene in the genome, the forward or the reverse primer of both the up- and downstream primers correspond to the first half and the last half of the antibiotic cassette sequence to be used for mutation. The 3′ end of the reverse primer corresponding to the upstream fragment should be palindromic to the 5′ sequence of the antibiotic gene of interest. Likewise, the 5′ end of the forward primer corresponding to the downstream fragment should be palindromic to the 3′ sequence of the antibiotic gene of interest. In the first round of amplification, three individual PCR experiments give rise to three fractions, namely the amplification of the upstream gene, the downstream gene, and the antibiotic gene of interest. The second round of PCR uses all three DNAs and creates a fusion product in the orientation similar to the gene in the original genome. An effective fusion product is limited to the primers that correspond to the antibiotic gene in the middle. Fusion PCR is possible by using an annealing temperature of approximately 5°C less than the melting temperature (T_m) of the primers used. As a rule of thumb, the elongation time should be 1 min for every 1 kb of length of the PCR product to be amplified, and the real success of fusion lies on the high fidelity of the PCR mix that contains the DNA polymerase. doi:10.1128/9781555818906.ch8.f5

antibiotic resistance marker that facilitates direct selection. An advantage of transposon mutagenesis is that no preconceived idea of gene function is necessary and it is possible to identify the gene inactivated in a mutant based upon the nucleotide sequence flanking the transposon. For example, genes involved in biofilm formation of a *Streptococcus gordonii* strain were characterized by screening mutants generated by Tn*916* transposon mutagenesis for defective biofilm formation. By using inverse PCR and DNA sequencing of the regions flanking the transposon in these mutants, it was shown that genes required for biofilm formation included, among others, those that are involved in signal transduction and quorum sensing, peptidoglycan biosynthesis, osmoadaptation, and adhesion.

Transposons can also be modified to facilitate rapid cloning of the inactivated gene. An example of this is a mutagenesis system for *P. gingivalis* based on Tn*44009*, a modified version of the *Bacteroides* transposon Tn*4400*. This transposon contains a pBR322 replicon and a beta-lactamase gene (conferring ampicillin resistance). Therefore, cloning of a disrupted genomic DNA region from a transposon insertion is easily accomplished by restriction endonuclease digestion of genomic fragments, ligation, and transformation into *E. coli*.

These transposon mutagenesis methods are limited to species with well-developed genetic systems and, therefore, cannot be applied readily to a number of oral microorganisms. New transposon mutagenesis approaches to overcome insertional hot spots and generate more highly saturated mutations have been developed in nonoral bacteria. One method,

termed GAMBIT (genomic analysis and mapping by in vitro transposition), can identify essential genes through the application of PCR, in vitro transposition, transformation, and genetic footprinting. This approach uses the Mariner family transposons (e.g., *Himar1*) that are know to have a broad host range and are highly promiscuous, only requiring a TA nucleotide region in the host genome for insertion. The Mariner transposon carried on a suicide plasmid vector can be utilized to create transposon mutant libraries. To adapt this approach to the study of oral pathogens, a Mariner-based transposon modified with a promoter recognized by the appropiate oral pathogen could be easily developed to create transposon mutant libraries in specific oral bacterial species. An advantage of this Mariner transposon system is the ability to combine saturating transposon mutagenesis with sequencing of the transposon-chromosome junctions. This technique, known as "Tn-seq," can facilitate the immediate identification of essential genes and the fitness contribution of nonessential genes in any test condition of interest.

A caveat associated with gene inactivation by any of the above methods is the possibility that insertions can affect the expression of downstream gene sequences. This can complicate interpretation of the function of disrupted genes. To overcome this potential problem, antibiotic resistance markers have been produced that do not contain transcriptional termination sequences. Thus, the provision for readthrough of the antibiotic resistance gene into the downstream sequence alleveiates potential disruption of downstream gene expression. To check that downstream gene expression has not been influenced, this can be monitored by RT-PCR or by Northern blot analysis if DNA sequences are available. It is also desirable to complement the mutant if such systems are available. A mutation can be complemented by introducing a replicative plasmid containing a complete wild-type copy of the target gene into the mutant. Provided that the gene is expressed from the plasmid at a suitable level, the wild-type phenotpye should be regenerated. This rules out the possible introduction of other mutations into the strain of interest. In reality, however, it is quite rare that a complementation experiment utilizing a plasmid (in *trans*) restores the wild-type phenotype completely. This is because expression levels of the complementing gene are hard to control and are not identicial in the complemented mutant and wild type, and greater or lesser expression levels result in upset of regulatory mechanisms. Of course, if polar effects on gene expression are suspected, mutagenesis on downstream sequences may be performed and the effects evaluated.

UNDERSTANDING GENE REGULATION

The regulation of a gene, e.g., induction or repression, in response to environmental signals can shed light on its function. For example, genes that encode iron-scavenging proteins are often upregulated in response to low iron concentrations. The activities of most bacterial gene products are generally difficult to assay or detect. Thus, surrogate genes, referred to as "reporter genes," that encode well-studied and easily assayed enzymes are often used in regulation studies. Hence, to assess the effects of environmental signals on the expression of a gene of interest, a construct is designed such that a reporter gene is incorporated immediately downstream of (and at times as a replacement for) the gene of interest. The result is a

transcriptional fusion in which the regulatory region of the gene of interest now controls the expression of the reporter gene. This type of fusion is easily created by cloning a promoterless reporter gene downstream of the promoter of the gene of interest. One of the more common reporter genes used to study gene regulation in oral microorganisms is *lacZ*, which encodes β-galactosidase, an enzyme that hydrolyzes the disaccharide lactose to glucose and galactose. Another commonly used reporter gene is *cat*, which encodes chloramphenicol acetyltransferase, an enzyme that inactivates the antibiotic chloramphenicol via the addition of an acetyl group to the molecule. The reporter is particularly useful because the activity of the enzyme can be measured directly or by determining the level of antibiotic resistance. Chloramphenicol is one of the very few antibiotics for which the MIC is directly correlated with the level of expression of the encoding gene, i.e., enzyme copy number. Other useful reporter genes include *galK*, a gene whose product, galactokinase, phosphorylates galactose, an activity that can be measured by a radioactive assay using galactose and radiolabled phosphorous as substrates. Other reporters that can be used include *xa*, a gene encoding a bifunctional xylosidase/arabinosidase enzyme easily assayed by using β-nitrophenol derivatives as substrates; a gene (*gfp*) encoding the green fluorescent protein, a fluorescent reporter; or bacterial luciferase (*luxAB*), a chemiluminescent reporter. The activities of fluorescent and chemiluminescent proteins can be detected visually and can also be quantitated with specialized instruments, such as fluorimeters.

A transcriptional fusion may be carried on a plasmid vector or can be inserted into the chromosome of the organism. However, in most cases, it is more desirable for the transcriptional fusion to be inserted in the chromosome via the insertion-duplication method (see above). This is important when it is necessary to maintain the gene dosage, i.e., a single copy on the chromosome, as opposed to multiple copies of a recombinant plasmid, or when no stable vector is available for the organism of interest. In a transcriptional fusion, the RNA polymerase starts transcription from the promoter region of the gene of interest. The fusion is expressed only under environmental conditions where the gene of interest is normally expressed.

The identification of unknown genes regulated by similar environmental stimuli can be accomplished with a transposon-based strategy. Transposons having a selectable antibiotic resistance marker can be engineered to also carry a promoterless *lacZ* gene. Selection of antibiotic-resistant isogenic mutants will generate a library of clones in which the transposon is inserted randomly into the chromosome of the bacteria. The screening of clones for the desired regulation of the *lacZ* reporter gene facilitates identification of those clones in which the transposon would have placed *lacZ* downstream of a promoter that is responsive to the environmental signal of interest. Once a clone is obtained, the nucleotide sequence flanking the transposon can be readily determined and will provide valuable information about the original gene(s) regulated by that promoter.

Although multiple approaches for the global analysis of genes and gene function are available for certain organisms, their adaptation for use in oral microorganisms is limited. For example, reporter genes that can be used to detect expression in vivo, such as those encoding luciferase and green fluorescent protein, have not yet been successfully adapted for

use in *P. gingivalis*. Approaches for detection of gene expression in novel environments can involve the use of several techniques including in vivo expression technology (IVET), signature-tagged mutagenesis, and differential display. IVET has been adapted for use in *P. gingivalis*. The IVET vector for *P. gingivalis* (pPGIVET) is a 9.3-kb self-transmissible suicide plasmid containing a cloning site upstream of two tandem promoterless reporter genes that encode tetracycline resistance [*tet*A(Q)2] and galactokinase (*galK*). Heterodiploids of *P. gingivalis* 381 in which the reporter genes are under the control of either the *hagB* or *hagC* promoter provided the first direct evidence that these genes are expressed during the infectious process in the mouse abscess model.

The lack of availability of animal models is one limitation for the study of host-induced genes of oral pathogens. Additionally, in those cases for which an animal model is available, it is often not a close approximate to the human condition. In vivo induced antigen technology (IVIAT) was developed to identify in vivo expressed genes, without reliance on animal models. Instead, it identifies genes expressed during an actual human infection. IVIAT utilizes sera from patients with the infection or disease of interest to probe for genes specifically expressed in vivo. The use of pooled sera allows the identification of the widest possible array of antigens produced during different stages of infection and, when appropriate, from patients infected via different routes. Clones containing DNA fragments from the pathogen are screened with the pooled sera, and the positive reacting clones are the ones that encode in vivo induced antigens. Of course some antigens that are identified by IVIAT may also be expressed under laboratory conditions. IVIAT was initially developed to study the oral pathogen *A. actinomycetemcomitans* and has now been used to identify in vivo produced proteins from other pathogens. A noteworthy feature of IVIAT is the potential for defining a timeline for antigen production by pathogens associated with subacute or chronic infections, such as periodontal disease. Identification of in vivo induced genes could lead to the discovery of new targets or pathways for the development of antimicrobials or new diagnostic tests and could suggest new targets for vaccine strategies.

GENOMICS: APPROACHES AND CHALLENGES

Genomics is the study of all genes, including their functions and regulatory elements, present in the genomes of organisms. The genome of an organism consists of its chromosomes and any extrachromosomal elements that may be present. With the advent of whole-genome sequencing, a new revolution in the search for an understanding of host-bacterium interactions began. Determination of the nucleotide base sequence of an organism's complete genome is a major goal of genomics and was made possible by recent advances in DNA sequencing automation and informatics. The completed genome sequences of organisms are available for analysis through the internet (www.tigr.org, www.ncbi.nlm.nih.gov/bioproject). Given the ease with which genomes may now be sequenced, many thousands of bacterial genomes will be sequenced over the next few years.

Knowledge of the contiguous DNA sequence of a bacterium's chromosome provides important insights into its genetic makeup. Interpretation

of completed genome sequences is accomplished by first identifying the predicted protein coding sequences or open reading frames (ORFs). While an ORF is identified as any stretch of codons that does not include a chain termination triplet, only a subset of all the ORFs present in the genomic sequence actually encode proteins. This prediction is based on the detection of regulatory sequences including a ribosomal binding site. In addition, many untranslated RNAs (mainly tRNA and rRNA genes) are identified and annotated. Various other features may be part of the annotation, including elements of the predicted protein structure, such as secondary structure motifs, which may help in the identification of function, and membrane spanning regions, which would suggest an association with the cell surface.

The mining of genomic sequences for genes that encode virulence factors and other functions of interest may be conducted by several approaches. The comparison of predicted coding sequences to sequences in databases (e.g., GenBank) with the BLAST program identifies matches to known genes. Typically, however, approximately 20% of the predicted ORFs in a genome do not match any other ORFs in GenBank, whereas another 10 to 70% match genes of unknown function, often discovered in other genome projects. It is important to stress, however, that genes annotated on the basis of sequence similarities do not necessarily encode proteins with similar functions. Other databases, such as BLOCKS (a database of conserved regions of protein families obtained from multiply aligned sequences), provide information on different motifs within a protein. Hits in databases such as these are based on much smaller conserved regions of DNA and do not require extensive similarity elsewhere in the sequence, as may be the case with whole-gene matches. Other sequence-based clues as to function may also be unearthed in this type of analysis. For example, tandem repeats of sequences are often found in or near certain virulence genes, and motifs commonly associated with binding sites for regulators, such as inverted repeats, can sometimes be identified in regions of a genome that control genes involved in pathogenesis.

In all of the completed genome sequences, a high proportion of genes with no assigned function have been identified. These genes classified as FUN (*f*unction *un*known) range from 10 to 70% of the genome. Because the assignments of ORFs are based on homology searches of previous databases of known gene sequences, one caveat of the annotation process is the potential misassignment of some ORFs. Thus, physiological characterization of gene products will always be necessary to verify their functions.

TRANSCRIPTOMICS AND PROTEOMICS

Transcriptomics

The availability of several oral microbial genomes deposited in databanks (e.g., Los Alamos Oral Pathogens Database, Human Oral Microbiome Database) has opened the door for in-depth postgenomics investigations. Facilitated by new generation sequencing technology (e.g., Illumina), these studies have made advances that have led to extraordinary discoveries in bacterial transcriptomics. Because a gene is usually transcribed only when

and where the function of its product is required, determining the environmental conditions under which a gene is expressed allows inferences to be made about its function. The techniques for evaluating gene expression have advanced greatly from analyzing one or a few genes, using Northern blotting, quantitative RT-PCR, or nuclease protection assay, to analyzing a large number of genes, possibly through subtractive hybridization, differential display, serial analysis of gene expression, and microarray. DNA microarray hybridization has been shown to be a most valuable method owing to its simplicity, comprehensiveness, data consistency, and high throughput. First described in 1995, microarray experiments are based on the principle that labeled nucleic acid molecules in solution hybridize, with high sensitivity and specificity, to complementary sequences immobilized on a solid substrate, thus facilitating parallel quantitative measurement of many different sequences in a complex mixture.

Microarrays are usually made by deposition of spots of single-stranded DNA sequence on a solid support such as a coated glass surface. The DNA spots that are homologous to regions of each ORF in the genome are obtained by PCR amplification using ORF-specific oligonucleotides. Analysis using homology-searching algorithms before oligonucleotide design is important to choose regions of genes that will not cross-hybridize with other regions of the genome. After a simple purification step, PCR fragments can be deposited on a coated glass surface by a robotic arrayer. The flatness of the glass surface makes it possible to array molecules in a parallel fashion, to miniaturize the procedure, and to allow the use of fluorescent dyes for detection. There is no diffusion of the applied material into the support, thus allowing reading of the slides by laser scanning microscopy.

For measuring relative gene expression, RNA is prepared from two or more culture samples to be compared, each having been propagated under a different environmental condition or for a different amount of time. Labeled cDNA is prepared by reverse transcription, incorporating different fluorescent cyanine dyes for each sample (e.g., Cy3 [green] or Cy5 [red]). The two labeled cDNA mixtures are combined and hybridized to the microarray, and the slide is scanned. With the use of image analysis software, signal intensities are determined for each dye at each element of the array, and the logarithm of the ratio of Cy5 intensity to Cy3 intensity is calculated. Cluster analysis of the data set can identify coregulated genes, which can become important in such instances as identifying sets of virulence genes. Because many virulence-associated genes are coordinately and tightly regulated, clustering gene expression profiles across a number of environmental conditions that can mimic infection, and precisely monitoring their coregulation, can reveal subtleties of regulation that may lead to the identification of virulence genes or groups of genes that are similarly regulated.

Microarray technology has become a standard tool for assessing environmental parameters that influence gene expression. Examples of genes that have been identified by this technology with *P. gingivalis* are those that are either up- or downregulated in response to iron concentrations, genes regulated directly or indirectly by autoinducer-2, and genes that are regulated upon entry and invasion of host cells. Because microarrays cannot measure expression of genes that are absent from the reference strain,

one limitation of the approach is their inability to identify genotypic differences between closely related strains that may be due to horizontal genetic transfer (as can occur with virulence factors). In addition, as with any method that quantifies transcription, posttranscriptional regulatory events, such as whether or not a message is translated, or the translational product is subsequently modified, cannot be detected. The large and comprehensive data sets that are generated from microarray experiments require careful analysis. For example, as is demanded for candidate genes identified by any expression screening approach, a role in pathogenesis must be subsequently confirmed by mutation and assays of virulence.

The technical limits of microarray technology are now being superseded by new generation sequencing (Roche 454, Illumina, and ABI SOLiD) of RNA on an "ultra large scale." This approach, termed "RNA-seq," has been utilized to represent the transcriptome revealed by sequencing cDNA molecules through new generation sequencing. This method has high sensitivity and is useful for discovering new transcripts and identifying mutations, deletions, and insertions. It provides excellent coverage and can generate more than 600 million reads in a single run. RNA-seq technology has been shown to be highly precise in defining the start of transcription of each gene under multiple environmental conditions and in the quantification of transcription levels, giving results similar to those provided by quantitative PCR. It is able to detect from one to numerous copies of RNA per cell. While the differentiation of transcriptional and posttranscriptional events can present technical challenges, the cost for RNA-seq analysis is considerably lower than previous methods; thus, its widespread use is expected to rise.

Proteomics

In some instances, genes may be transcribed but not translated into protein. Furthermore, numbers of mRNA copies may not necessarily reflect the number of functional protein molecules. Thus, insights into such factors as the relative abundance of a protein product, posttranslational modification, subcellular localization, turnover, and interaction with other proteins can only be monitored by analysis of the protein profile. The technology for monitoring protein expression is named proteomics, defined as the identification and quantitation of the complete set of proteins, both in space and time, that is synthesized under a given set of conditions.

There are two main approaches to proteomics: one is the expression model in which all proteins are analyzed, and the other is the cell map model in which only a selected set of proteins, like complexes, are studied. In the expression model, changes in the patterns of expression in response to different conditions, or in a mutant strain, are observed quantitatively for a large number of proteins within a cell. This makes it possible to identify differentially expressed proteins on a global scale. It also allows deductions of entire regulatory networks by identifying proteins that may undergo coordinated changes of expression. One goal of the cell map model is to identify proteins that interact with or form complexes with other proteins. By documenting the physical interaction of proteins, the associations of proteins in particular pathways can be deduced.

The analysis of genome-encoded proteins relies on the use of several technologies. One of the more commonly used techniques is two-dimensional gel electrophoresis. This technique provides higher resolution than one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) analysis, as it separates proteins both in terms of their isoelectric points and molecular mass. To establish a pH gradient, for separation on the basis of isoelectric point, two-dimensional PAGE technology utilizes carrier ampholytes (amphoteric compounds) or immobilized pH gradients, which are an integral part of the polyacrylamide matrix. After separation on the basis of charge, the proteins within the horizontal gel are then run vertically by sodium dodecyl sulfate-PAGE, thus producing a pattern of protein spots. Methods of protein identification include immunoblotting, peptide sequencing following Edman degradation, determination of amino acid composition, matrix-assisted laser desorption/ionization mass spectrometry, and electrospray ionization. The last two methods, which rely on the comparison of peptide mass fingerprints, are fast and require only picomole amounts of proteins. The peptide sequences obtained can be matched with the predicted amino acid sequences of the genomic ORFs to identify the protein spot.

Mass spectrometry is one of the versatile methods of studying proteins in a whole cell. This method avoids two-dimensional gels altogether and can determine the whole protein complement of the cell including identification, modification, quantification, and localization of proteins. This is the most comprehensive and versatile tool in large-scale proteomics. After subjecting whole cells or cellular fractions to proteolytic digestion (usually trypsin), the resulting peptide mixtures are separated by liquid chromatography and then electrosprayed for tandem mass spectrometric analysis allowing determination of amino acid sequence. Tens of thousands of peptides elute over a relatively short time, and mass spectrometers have been developed to provide higher sensitivity, sequencing speed, and resolution. Use of state-of-the-art instruments like the Orbitrap mass spectrometer is most advantageous, as it identifies and quantifies the protein as well as being able to identify posttranslational modifications.

Another analytical approach to protein quantitation is the use of labeling tags. Proteins in metabolically active bacteria can also be differentially labeled by incorporation of ^{15}N or ^{13}C, which can then be discriminated from unlabelled peptides through the incremental increase in mass. Alternatively, stable isotopes can be introduced into proteins by chemical derivatization of either the C or N terminus; for example, ^{18}O can be incorporated into the C terminus of peptides by protease-catalyzed cleavage of the amide bond. Isobaric tags are compounds with the same mass but which fragment differently to yield reporter ions of different mass. The results obtained with tags can show the relative abundance of the whole proteome of the pathogen under various experimental conditions. At the same time, they can also identify host proteins that may be up- or downregulated. The relative quantification of proteins produced by microbe and by host aids in the understanding of the infectious process and of host cell responses. With the increasing availability of complete genome sequences for oral bacteria, global proteomics analyses are becoming standard in the laboratory.

KEY POINTS

Restriction endonucleases that cut DNA at specific sequences of bases, along with vectors such as plasmids that can be propagated, allow individual genes to be excised and recombined with vectors (cloned), thus facilitating their manipulation and study.

PCR is an iterative process of amplification of a stretch of DNA (such as a gene) using short oligonucelotides (primers) that bind to regions flanking the DNA of interest. PCR amplification of regions of DNA that are specific to individual organisms allows their detection in clinical samples without recourse to culture. Real-time (or quantitative) PCR allows for detection and quantitation of mRNA after the enzyme reverse transcriptase has been used to convert the mRNA to cDNA.

The activity of a gene (the amount of mRNA produced) can be measured by cloning the promoter region (the binding site for RNA polymerase), along with upstream regulatory sequences next to a reporter gene, and returning this construct to the chromosome.

The reporter gene generally encodes an enzyme for which substrate accumulation is easily measured. Bacterial strains containing such promoter-reporter constructs can be tested in animal models of disease, allowing the detection of genes that are expressed in vivo.

Many bacteria, including important oral microorganisms, have had their genomes sequenced, and their total genetic makeup is available. It is thus possible to measure gene activity on a global, or whole-organism, scale using microarray technology. In a microarray, DNA sequences corresponding to every gene in a bacterium are deposited onto a solid support such as a glass slide. mRNA from the organism under test conditions is converted to cDNA and labeled with a fluorescent dye. The cDNA is hybridized (allowed to bind to matching sequences) to the target DNA on the array. After washing, the amount of fluorescent label remaining bound to the array for each target gene gives a measure of the amount of mRNA from that gene present in the test sample, which in turn reflects gene activity.

High-throughput sequencing of cDNA (RNA-seq) provides even more sensitive and genome-wide analysis of RNA expression.

The availability of sequenced genomes has led to global analysis of protein production. An organism's total proteins can be separated by electrophoresis or by chromatography and each protein identified by mass spectrometry. As proteins are the main effector molecules of the cell, the expressed proteome provides a basis for understanding the physiology and pathogenicity of bacteria.

FURTHER READING

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Population Genetics of Oral Bacteria

Mogens Kilian

INTRODUCTION

One of the results of the application of molecular genetics to bacteriology is recognition of an overwhelming genetic diversity within species of bacteria. It has become clear that bacterial species must be viewed as populations of individual strains that share basic housekeeping functions but otherwise may have very different properties. Analysis of the genetic structure of bacterial populations can elucidate the genetic mechanisms that cause this diversity. More importantly, population genetics analysis may identify particularly virulent variants within a species, thus providing a better background for the identification of important virulence factors. Such approaches may also yield a more detailed understanding of host-parasite relationships and may explain why temporal variations may occur in the prevalence of bacterial infections.

The goal of this chapter is to provide a brief overview of the molecular basis of bacterial population genetics and to demonstrate how the application of population genetics analysis to oral microbiology makes it possible to address many important questions about patterns of acquisition, transmission, and dynamics of the oral microbiota; whether particularly virulent forms of oral bacteria are responsible for oral diseases; and what the molecular mechanisms behind geographic and temporal variations in oral disease frequency and severity may be.

BACTERIAL SPECIES SHOW DIFFERENT PATTERNS OF EVOLUTION

Bacteria multiply by binary fission, a process that results in identical offspring. It was assumed previously that the major mechanism of genetic diversification is accumulation of point mutations in the bacterial genome. It is now clear, however, that horizontal gene transfer and homologous recombination contribute significantly to diversification but to different extents in different groups of bacteria. As a result, individual genera, species, and even subpopulations within species may display different population genetics structures.

Species in which accumulation of mutations is the dominant mechanism of genetic diversification consist of discrete phylogenetic lineages

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or clones. It has been estimated that the number of distinct evolutionary lineages within a given pathogenic species may range, at the global level, within a few hundred. Although members of individual clones are descendants of the same cell, they are not necessarily identical. Maynard Smith, a renowned population geneticist, defines a clone as "a set of genetically similar cells, recently derived from a common ancestor, without chromosomal recombination." What "recently" means in the evolutionary context is not clearly defined, but it may easily be thousands of years. The clonal population structure, which is the result of this evolutionary scenario, is characterized by linkage disequilibrium in the genome, i.e., particular alleles at individual gene loci co-occur (Fig. 1). For example, verotoxin production in *Escherichia coli* was for a long time associated with the serotypic trait O157:H7, the inability to ferment sorbitol, a particular DNA fingerprint pattern, and many other genotypic and phenotypic traits. As a practical matter, any of these properties could be used to trace bacteria with this particular virulence factor.

In most bacterial species, interstrain homologous recombination or acquisition of new genes (horizontal transfer) by conjugation, transformation, or transduction is an additional, and often more important, source of genetic diversification. If recombination in a bacterial population is very frequent compared to the mutation rate, a panmictic population structure arises. This is characterized by a random (or nearly random) assortment of alleles, and distinct phylogenetic lineages are no longer discernible. As a result, isolates with identical serotype, biotype, or other phenotypic trait are not necessarily genetically related. For example, genetically dissimilar isolates of *Neisseria meningitidis* or *Streptococcus pneumoniae* may express the same capsule serotype and yet show strikingly different pathogenic potential. Other examples of medically important species that show a panmictic population structure are *Neisseria gonorrhoeae* and *Helicobacter pylori*. In practical terms, no single property will be able to identify a virulent phenotype in such bacteria unless that property is uniquely responsible for the pathogenic potential.

A bacterial population characterized by frequent recombination is constantly undergoing changes, with new variants emerging and disappearing. This process may occasionally result in an explosive increase of a particularly successful variant, which may predominate for a time, possibly spread worldwide, and then eventually disappear as a result of

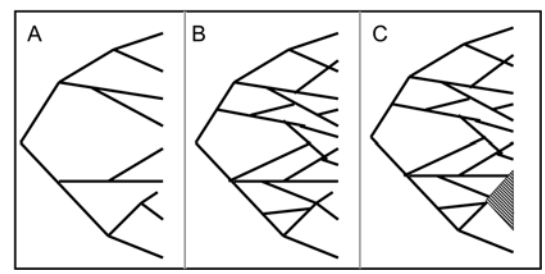


FIGURE 1 Three different genetic structures of bacterial populations: (A) clonal population structure; (B) panmictic population structure; and (C) epidemic population structure. The hatched area in panel C depicts a recently emerged, successfully spreading clone. doi:10.1128/9781555818906.ch9.f1

erosion of its evolutionary success by recombination or resistance in the host population. A cross-sectional analysis may, mistakenly, interpret this as evidence of a clonal population structure because repeated isolates from different geographic locations of one or more successful variants will show the same combination of alleles. This evolutionary pattern, which accounts for the fluctuating prevalence of, for example, meningococcal meningitis, has been termed epidemic population structure. Another medically important species that shows this pattern is *Pseudomonas aeruginosa*.

It is not unusual that a single bacterial species includes subpopulations with different population structures. For example, serogroup A strains of *N. meningitidis* appear to have a largely clonal population structure, whereas the remaining part of this species is characterized by panmixia. Thus, the serogroup A population, which for unknown reasons has become genetically separated, may, in the long run, become a species distinct from *N. meningitidis*, as it happened to the gonococcus many years ago when some genetic event(s) allowed it to take up a separate ecological niche.

LOCALIZED SEX IN BACTERIA

In most bacteria, the situation is more complex than described above. Different parts of a bacterial genome or even of a single gene may have a phylogenetic history different from that of the remaining genome. Thus, even in species that show a basically clonal population structure, it is usual to find that genes encoding virulence factors or surface proteins reveal a mosaic-like structure as a result of local recombination. This localized process results in antigenic diversity of the encoded proteins, which confers ecological advantage under the selection pressure exerted by the immune system of the host.

Also, complete virulence genes or whole "pathogenicity islands" may spread through a basically clonal population of bacteria by horizontal genetic transfer to confer the same virulence properties on otherwise evolutionarily distinct lineages. Pathogenicity islands are large genomic regions 10 to 200 kb in size that often have G+C content and codon usage that differ from those of the rest of the genome, suggestive of a "foreign" origin. Their location in the genome adjacent to highly conserved gene sequences facilitates their insertion in foreign genomes by homologous recombination. Pathogenicity islands usually contain multiple genes encoding adhesins, toxins, invasins, protein secretion systems, iron acquisition systems, and other proteins associated with virulence. The structure of the genes indicates that pathogenicity islands are generated through evolution by a multistep process. Similar mobile clusters of genes or genomic islands that enhance ecological fitness are also found in many nonpathogenic bacteria.

Other mobile genetic elements that may spread across evolutionary lineages and introduce new virulence-associated properties or antibiotic resistance are plasmids, bacteriophages, and conjugative transposons (see chapter 7).

As a result of such inserts, individual strains of bacterial species may show remarkable differences. For example, comparisons of sequenced

genomes of multiple strains of *E. coli* revealed that the mean number of genes in any strain is approximately 4,700, of which less than half are shared by all strains of *E. coli*, while the total gene pool of *E. coli* includes approximately 18,000 genes. Thus, bacterial genomes consist of a core of genes necessary for basic metabolism and survival and a flexible gene pool consisting of an assortment of strain-specific genetic information that may determine virulence or ecological success.

DIFFERENCES IN PATHOGENICITY OF STRAINS

Given the mentioned differences in genomes of closely related bacteria, it is not surprising that strains of a species may vary significantly in pathogenic potential. Even within strains of a subspecies or sero- or biotype that look identical when examined with traditional diagnostic tests, remarkable diversity may occur. This explains the observation that the majority of cases of serious disease observed worldwide are often caused by a small proportion of the total number of extant clones. Even within a recognized bacterial pathogen like *Haemophilus influenzae* serotype b (Hib), there are distinct phylogenetic lineages within the basically clonal population that rarely, if ever, are associated with disease. Likewise, most of the genetic variants of meningococci that constantly emerge never cause disease but are found in healthy carriers, where they contribute to the induction of resistance to more virulent clones.

From bacteria that evolve without significant recombination, one may expect a relatively stable occurrence of infections over long periods, as long as there is no significant intervention from the environment. Conversely, in pathogenic species undergoing frequent recombination, either involving the entire genome or localized areas associated with virulence, clones with altered virulence will continuously emerge and disappear. Whereas evolution leading to new species (macroevolution) is a very slow process (*E. coli* and *Salmonella* separated more than 100 million years ago!), generation of new variants with altered virulence or resistance to antibiotics may take only minutes or days (microevolution). These fluctuations in virulence explain temporal variations in the prevalence and severity of infections caused by some bacterial pathogens.

SPECIFIC HOST ADAPTATION OF BACTERIAL CLONES

There is convincing evidence in biology for the coevolution of parasites and their hosts and for the hypothesis that they have speciated in synchrony. A result of this close mutual adaptation is that many species of bacteria exclusively cause disease or colonize in one host species. The evolutionary process has included optimization of the bacterial genome, often leading to loss of metabolic versatility and, thereby, enhanced dependency on the host. Examples are found in the *Haemophilus-Actinobacillus-Aggregatibacter-Pasteurella* group of bacteria (the family *Pasteurellaceae*), which include both pathogens and commensals associated with humans or various animal species. A closer look at the individual species in this group of bacteria reveals that they have developed strategies for iron acquisition and evasion of host defenses that work only in their respective

hosts. Other known examples of strict host adaptation can be explained by specific interaction of adhesins of the bacteria with receptors uniquely present in their host.

Recent population genetics analyses of bacteria associated with humans suggest that this host adaptation may be even subtler in some species of bacteria. Comprehensive studies of some pathogens strictly associated with humans have noted significant differences in the occurrence of individual clones in different parts of the world. One well-studied example is the invasive pathogen Hib. Before introduction of the Hib vaccine in most industrialized countries, Hib was an important cause of meningitis worldwide, though with markedly different prevalences of disease in different human populations. Analysis of disease isolates of Hib revealed that different clones were responsible for disease in different populations, even within the same country. While single clones were responsible for disease in ethnically homogeneous countries, a variety of clones were isolates from patients in North America. A comprehensive look revealed that individual clones were distributed in the world in patterns resembling those of the very large population movements which occurred in the Middle Ages. Adaptation of individual clones of *H. influenzae* serotype b to hosts with a particular genetic constitution over many years of coevolution is an attractive hypothesis to explain this observation and is in accordance with occurrence of multiple clones in the ethnically heterogeneous North American population.

Likewise, population genetics analyses of the periodontal pathogen *Aggregatibacter actinomycetemcomitans* (see below) and of the gastric pathogen *H. pylori* further emphasize that significant differences may occur between ethnic groups (and thus geographic locations). Different clones of these species have been isolated from different ethnic groups of humans. This can be partially explained in the case of *H. pylori* by the presence of particular blood group determinants in the host that serve as receptors for specific adhesins required for efficient colonization.

The pattern of colonization, in which only hosts of a specific ethnicity or blood group are colonized, is not seen universally. For example, in the case of the pathogen *Bordetella pertussis*, the etiologic agent of whooping cough, and toxic shock syndrome-associated *Staphylococcus aureus*, a limited number of clones are known to have caused disease worldwide. Furthermore, metabolically versatile bacteria like *E. coli* and *P. aeruginosa* have multiple habitats.

POPULATION SIZES OF PATHOGENIC AND COMMENSAL BACTERIA

As mentioned above, most exogenous bacterial pathogens are represented worldwide by a relatively limited number of clones, although with frequently recombining bacteria, it is necessary to operate with complexes of types to reach the same conclusion. The individual clones disseminate successfully in the human population and may cause disease in nonimmune individuals. Many bacteria that form part of the commensal microbiota on mucosal membranes show a very different pattern. The population sizes are enormous, as indicated by the observation that it is difficult

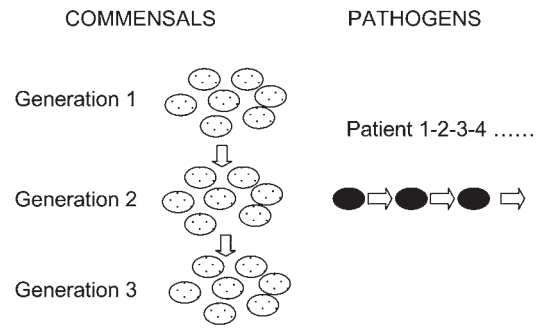


FIGURE 2 Different patterns of transmission and population sizes of commensal and pathogenic bacteria. Whereas commensals primarily spread from parents to their offspring (vertical transmission) and usually as a diversity of clones, pathogenic bacteria spread from patient to patient (horizontal transmission). Most pathogens consist of a limited number of clones. doi:10.1128/9781555818906.ch9.f2

to find two individuals who carry the same clone unless they belong to the same family or otherwise are in close contact. This indicates that the major mechanism of transmission of commensal bacteria is vertical (i.e., from parents to their children), whereas lateral transfer is limited and restricted to intimate contacts (Fig. 2). Constrained evolution and diversification of clones within families over millions of years may explain the immense number of distinct clones of commensal bacterial species, in contrast to pathogens, which spread horizontally from one individual to another. Furthermore, studies of the commensal bacteria in the oral cavity, pharynx, and gut demonstrate that multiple clones of some of the species often coexist in one individual.

ORAL BACTERIA SHOW VARYING DEGREES OF GENETIC DIVERSITY

The oral microbiota provides a remarkable example of biodiversity within a small habitat. Several hundred bacterial species have been isolated from the oral cavity and characterized in varying detail. However, analyses based on sequencing of 16S rRNA genes that may be amplified from dental plaque indicate that over half of the more than 700 species that may occur in the oral cavity are yet to be cultured and characterized. In addition to this complexity, application of sensitive DNA-based typing methods reveals considerable genetic diversity within species harbored by a single subject. This diversity makes it possible to disclose species-specific patterns of acquisition, population dynamics within the individual, and transmission between individuals.

It is clear that the degree of genetic diversity differs between species, although the available information is still incomplete. Much of the actual diversity is likely to be missed in culture studies, which disclose only the predominant clones, even when selective isolation media are employed. Furthermore, results of such studies depend on the sampling technique, the number of isolates examined, and the resolving power of the typing method used. Extensive diversity has been demonstrated in populations of bacteria that are "true" commensals, i.e., *Streptococcus mitis*,

Actinomyces naeslundii, *Fusobacterium nucleatum*, *Haemophilus parainfluenzae*, *Eikenella corrodens*, and some of the *Prevotella* species. In contrast, plaque populations of putative oral pathogens like *Porphyromonas gingivalis*, *A. actinomycetemcomitans*, and *Streptococcus mutans* usually consist of a single or a very limited number of genotypes at any one time.

Comparison of isolates of oral bacteria has demonstrated that the same genotypes of oral bacteria often are present in members of the same family, whereas unrelated individuals harbor distinct genotypes. In this respect, putative oral periodontal pathogens like *P. gingivalis* and *A. actinomycetemcomitans* present a combination of patterns usually associated with commensal bacteria and exogenous pathogens, respectively. As discussed below, the JP2 clone of *A. actinomycetemcomitans* has been isolated from patients with juvenile periodontitis in virtually all continents of the world but restricted to individuals of northwest African descent, while the remaining part of the species shows extensive diversity. Likewise, *P. gingivalis*, which shows extensive diversity and evidence of recombination, also includes clones that appear to have disseminated successfully in the human population.

THE ORAL MICROBIOTA IS A DYNAMIC POPULATION UNDERGOING CONSTANT CHANGES

It has long been recognized that the composition of the dental plaque microbiota is highly dependent on oral hygiene and dietary habits. Longitudinal studies of *A. actinomycetemcomitans* and *P. gingivalis* show that both species establish stable colonization in dental plaque of adults, although their proportions may differ between individuals and may change over time. However, recent studies in children using very sensitive detection by PCR show considerable instability. *A. actinomycetemcomitans* was detected in more than 50% of healthy children and *P. gingivalis* in more than 40%, but with random concordance between results obtained 1 to 3 years apart. Furthermore, in children who were colonized at both samplings, different clones were present. Thus, in most children, these two species colonize only transiently, and a succession of colonizing clones may be observed during childhood before the more stable colonization of adulthood.

S. mitis is one of the pioneers of the oral microbiota in infants. Within the first days of life, several clones of this species may already be detected by culture studies. Throughout life, a significant number of clones of this species inhabit the mucosa of the oral cavity and pharynx, but constant fluctuations occur in the relative proportions of the individual clones. While no clone remains detectable for more than 3 months in infants, more stability is present in adults.

S. mutans seems to be an exception to this pattern. This species colonizes the oral cavity predominantly during a brief "window of infectivity" after eruption of teeth. One or two clones of this species then seem to be stably present in dental plaque throughout life. In general, it is conceivable that clones of bacteria colonizing dental plaque are more stable than clones colonizing mucosal surfaces in the oral cavity. The more stable colonization in adults may also be explained by more undisturbed microhabitats of dental plaque associated with the adult dentition.

Collectively, these studies emphasize the immense biodiversity of the oral microbiota and demonstrate the individuality of the microbiota in different subjects. The observed differences in population dynamics of oral bacteria raise important questions about mechanisms driving the fluctuations and how some bacteria are capable of coping with local selection pressures, including the mucosal immune system, whereas others are being rapidly eliminated.

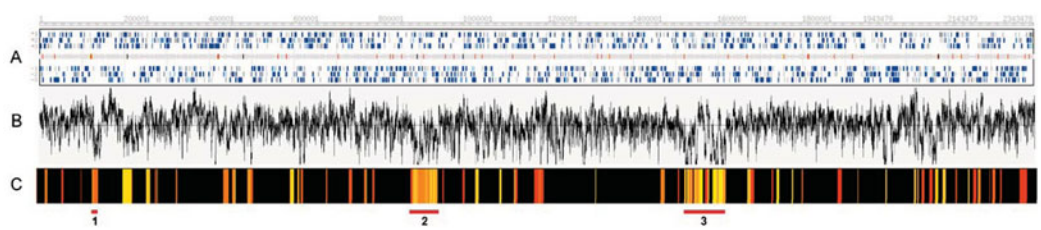
VIRULENCE DIFFERENCES WITHIN SPECIES OF ORAL BACTERIA?

From a disease perspective, the individuality of dental plaques, particularly at the clonal level, has very significant implications. Thus, individual genotypes of the bacterial species present in dental plaques may have very different virulence properties. The putative periodontal pathogen *P. gingivalis* is an illustrative example. Studies of individual isolates of this species have revealed significant differences in virulence in experimental infection models in animals and in the expression of various properties that are likely to be of significance in the pathogenesis of periodontal disease (Table 1). Likewise, analysis of the complete genome of a strain of *P. gingivalis* (W83) reveals numerous areas that resemble pathogenicity islands or "foreign" DNA by their significantly divergent G+C contents. Furthermore, the genome of strain W83 contains at least 96 complete or partial copies of insertion sequence (IS) elements that fall into 12 families. These IS elements may potentially inactivate genes and provide sequence homology that enables intrachromosomal recombinations, which may result in deletions and inversions. A comparison of the genomes of two strains with different pathogenic potential revealed numerous genes that are highly divergent or absent in the type strain and confirm that "hot spots" coincide with regions of lower G+C ratios (Fig. 3). Variably present proteins encoded by such regions include the putative virulence factor

TABLE 1 Strain-dependent differences in *P. gingivalis*

Virulence in animal infection models
Biological activities of cell wall lipopolysaccharide
Resistance to phagocytosis
Capsule production and capsule structure
Cytotoxic activity
Invasion of cells
Proteolytic activity
Expression and function of fimbria
Expression of RagB protein
Autoaggregation
Glycosylation pattern of expressed glycoproteins

FIGURE 3 Distribution of divergent genes in the genomes of two strains of *P. gingivalis*, W83 and the type strain ATCC 33277. (A) W83 genome and six-frame distribution of predicted genes. Dark blue, genes annotated with names or function; light blue, conserved hypothetical genes; gray, hypothetical genes and intergenic regions; red, tRNAs; yellow, rRNA. (B) GC distribution based on the G+C content of a 500-bp window and showing significantly divergent areas suggestive of recent acquisition by horizontal gene transfer. (C) Simulated heat map of the distribution of genes categorized from slightly (yellow) to highly (red) divergent between strains W83 and ATCC 33277. The black background indicates areas where genes are present in both genomes. Reproduced with permission from T. Chen, Y. Hosogi, K. Nishikawa, K. Abbey, R. D. Fleischmann, J. Walling, and M. J. Duncan, *J. Bacteriol.* **186:**5473–5479, 2004. doi:10.1128/9781555818906.ch9.f3



RagB, enzymes involved in capsular polysaccharide biosynthesis, and numerous proteins of yet unknown function.

In *A. actinomycetemcomitans*, among 12 genotypes detected by restriction fragment length polymorphism (RFLP) (see below), one type was exclusively associated with periodontal disease, whereas some others were exclusively associated with health. Furthermore, significant differences in the expression of leukotoxin, a toxin that kills human phagocytes (see chapter 14), have been demonstrated among *A. actinomycetemcomitans* strains (see below).

Experimental studies in animal models show striking differences in the cariogenic activity of human isolates of *S. mutans* and *Streptococcus sobrinus*, which are considered principal causes of dental caries (see chapter 11). However, it is notable that some isolates of the species *S. mitis*, which has been considered nonpathogenic, are as acidogenic and aciduric as these mutans group streptococci.

Studies of several other oral bacteria have revealed different allelic versions of genes encoding suspected virulence properties, but the functional significance of this genetic polymorphism has received only limited attention.

METHODS OF STRAIN DIFFERENTIATION AND SEARCH FOR VIRULENT CLONES

Application of DNA technology to the typing of microorganisms has resulted in numerous methods for strain differentiation. In contrast to traditional typing methods such as biotyping and serotyping, the discriminatory power of most of these DNA-based methods is very high. The choice of method depends primarily on the question being asked and on the genetic diversity within the population analyzed.

Single-locus typing targeted on a gene that shows significant polymorphism (e.g., genes encoding virulence properties or surface proteins under strong selection) usually has a high discriminatory power. The diversity may be demonstrated by nucleotide sequencing or by RFLP analysis using frequently cutting restriction enzymes and a labeled probe for demonstration of fragments. Ribotyping is a popular version of RFLP typing because a universal probe, e.g., from the *E. coli* rRNA operon, can be used for all bacterial species due to the conservation of parts of the rRNA gene. This fact may also be exploited to sequence or RFLP analyze the highly variable spacer region between 16S and 23S rRNA operons. In general, these techniques are suitable for short-term epidemiology, whereas in most cases they are inappropriate for global epidemiology because the high rates of evolution will obscure the overall genetic relationships between isolates. Furthermore, the plasticity of many bacterial genomes and the fact that some species are characterized by a random assortment of alleles at individual gene loci imply that studies focusing on single genes or limited parts of the bacterial genome, in most cases, will not provide an accurate picture of the overall phylogeny or genetic relationships within a bacterial population.

Methods that reveal the polymorphism in multiple random sections of the genome combine high discriminatory power with a more comprehensive view of the genome. The most commonly used methods are DNA

fingerprinting achieved by restriction enzyme analysis or amplification of random DNA sequences by PCR. Restriction enzyme analysis is generally performed with frequently cutting restriction enzymes and separation of DNA fragments in agarose gels. Analysis of results obtained by this technique is often hampered by the complexity of the banding patterns, which makes it difficult to compare many strains. This problem may be overcome by pulsed-field gel electrophoresis, which allows separation of very large DNA fragments obtained by the use of rare-cutting restriction enzymes. An alternative restriction enzyme analysis method, which has been successfully applied to the typing of *A. actinomycetemcomitans*, is based on restriction fragment end labeling with a radioactive isotope. Separation of the fragments is performed in a polyacrylamide/urea sequencing gel, and visualization is by autoradiography. With an appropriately chosen restriction enzyme, this method produces very distinct patterns of fragments.

Amplification of random DNA segments with single primers of arbitrary nucleotide sequence is also used for genomic DNA fingerprinting. This technique is usually referred to as arbitrary primer PCR or random amplified primer DNA fingerprinting. Although this is a sensitive typing method, a number of experimental parameters have been found to influence the amplicon profiles, and the reproducibility of the technique has been questioned. A method that circumvents the inherent problems of the arbitrary method is rep-PCR, which targets repetitive DNA motifs. By detecting differences in copy number and chromosomal location of the repetitive element used as a target, this method produces distinct and reproducible patterns.

The mentioned nontargeted multilocus methods are excellent for typing, as they have high discriminatory power, but they do not provide a quantitative measure of genetic relatedness and are not suited for phylogenetic analysis. Thus, introduction of insertion sequences or recombination in part of the genome may blur the overall genetic relationships. Even more importantly, genome rearrangements, which are frequent events in some bacteria (e.g., *H. pylori*, *P. aeruginosa*, *Salmonella typhi*, and *A. actinomycetemcomitans*) but do not necessarily affect their phenotype, may be mistaken for mutations at restriction sites. Finally, the methods are often too discriminatory to reveal overall relationships.

The most popular method for population genetic analysis is multilocus sequence typing (MLST), a further development of multilocus enzyme electrophoresis (MLEE), which for a long time was a standard method in eukaryotic population genetics. In principle, these methods map the genetic polymorphism and relationships in a population of microorganisms by analysis of samples of the core genome. The targets are intracellular housekeeping enzymes in which mutations are assumed to be evolutionarily neutral as long as they do not affect function. Instead of characterizing the housekeeping genes indirectly by comparing electrophoretic mobilities of the gene products as done with MLEE, MLST characterizes the genes directly by nucleotide sequencing. The important advantages of this is the unambiguity of nucleotide sequence data, that results of MLST are electronically portable, and that centralized databases consisting of data generated in laboratories throughout the world can provide

unique insight into the epidemiology of bacterial clones. The method was originally developed and validated in a study of *N. meningitidis* and subsequently has been applied to several other bacterial pathogens, including some oral species such as *S. mutans* and *P. gingivalis*. MLST databases are directly accessible on the Internet (http://mlst.net and http://pubmlst.org/general.shtml) where sequences can be directly compared.

MLST sequences (usually fragments of seven genes) may be combined into one concatemer sequence and subjected to phylogenetic analysis (multilocus sequence analysis) using a variety of softwares. Provided that the population structure is basically clonal, the resulting tree will reflect the phylogeny of the population.

Analyses based on entire bacterial genome sequences generated by "next-generation" technologies is already feasible and will provide detailed insight into the evolution of bacterial species, including mechanisms of short-term changes in pathogenic potential and antibiotic resistance.

The crucial factor in any attempt to study the population genetic structure of bacteria is the sample. It must faithfully represent the population chosen for analysis and reflect the parameters under study, whether this is disease association or geographic or temporal variations. In contrast to many other infections in humans, studies of the etiology of oral diseases like periodontitis and caries pose significant problems due to the complexity of the microbiota. Isolation of a strain of *A. actinomycetemcomitans* or *P. gingivalis* or any other suspected pathogen from a patient with periodontal disease does not necessarily imply etiologic involvement. Conversely, an isolate from an apparently healthy subject is not necessarily nonpathogenic. The same problem applies to studies of dental caries etiology.

To approach the problem of identifying particularly virulent clones or subpopulations, clinical information on the isolates is compared with the clustering obtained by MLST or multilocus sequence analysis. If the bacterial population under study has a clonal population structure, this will usually disclose particular disease-associated clones or subpopulations if such occur. Conversely, in a population characterized by frequent recombination, pathogenic isolates may not cluster together but are characterized by distinct genes that encode virulence.

POPULATION GENETICS STRUCTURE OF ORAL BACTERIA

So far, only a few species of oral bacteria, *P. gingivalis*, *A. actinomycetemcomitans*, *S. mutans*, *S. mitis*, and *S. oralis* have been subjected to population genetics analyses. These studies utilized large collections of isolates that were clinically, geographically, and temporally diverse. The *P. gingivalis* MLST database is available at http://pubmlst.org/pgingivalis/. The oral *Streptococcus* MLST website at http://pubmlst.org/soralis/ contains data for *S. oralis* and *S. mutans*.

Studies of *P. gingivalis* populations demonstrate that virtually all isolates are genetically distinct and that isolates from cats, dogs, sheep, and New World monkeys belong to evolutionary lineages distinct from human isolates. In contrast, isolates from Old World monkeys are closely related to human isolates. In agreement with the conclusion that the population

structure of *P. gingivalis* is panmictic, i.e., characterized by frequent recombination, there is no clear clustering within human isolates. Isolates for which pathogenicity has been demonstrated in animal models are randomly distributed across the population. These observations suggest that pathogenicity in *P. gingivalis* is associated with particular recombinant forms, presumably depending on genes in mobile chromosomal elements such as pathogenicity islands. Screenings of comprehensive collections of isolates from clinically well-defined situations using microarray technology are required to identify specific combinations of genes that determine a virulent phenotype.

Studies of *A. actinomycetemcomitans* reveal a picture with both similarities and surprising differences from that observed for *P. gingivalis*. Cluster analysis of results obtained by MLEE and sequencing of selected gene loci demonstrate extensive genetic diversity, as in *P. gingivalis*, with only a few examples of isolates being identical. However, the population structure is clearly clonal with only limited evidence of genetic recombination. This conclusion is based on the observation that strains cluster according to serotype and that there is clear evidence of genetic linkage disequilibrium. In an analysis based almost entirely on isolates from Europeans, there was no evidence of single clonal types being responsible for multiple cases of periodontitis or systemic infections, nor was there evidence of disease-associated isolates clustering separately from isolates obtained from healthy individuals.

These observations are compatible with the conclusion that, in the examined human populations, both *P. gingivalis* and *A. actinomycetemcomitans* behave like commensals that spread among individuals primarily by vertical transmission. Thus, if etiologically involved in the pathogenesis of periodontal disease, they play the role of an opportunistic (endogenous) pathogen.

However, subsequent studies surprisingly revealed that a large number of isolates of *A. actinomycetemcomitans* from certain geographic regions belong to a single clone, i.e., the JP2 clone. The JP2 clone belongs to serotype b and differs from other strains of the species by significantly enhanced leukotoxic activity, by its hemolytic activity on horse blood agar, and by aberrant mechanisms of iron acquisition as a result of deleterious mutations in the gene encoding the hemoglobin-binding surface protein. The enhanced production of leukotoxin is due to a single deletion of 530 bp in the promoter region of the leukotoxin gene operon. Interestingly, in a Japanese isolate from juvenile periodontitis, similar enhanced activity was due to an insertion sequence interrupting the normal promoter of leukotoxin expression (Fig. 4).

The JP2 clone is strongly associated with juvenile, aggressive periodontitis in patients of northwest African descent living in the United States and in a variety of countries in South America and Europe. The clone is endemically present in Morocco, where it is associated with an unusually high prevalence of periodontal disease (15%) among adolescents (odds ratio, 29.4). It has also been detected in dental plaque from a group of Israeli children with an unusually high prevalence (38%) of early-onset periodontitis. Interestingly, other clones *of A. actinomycetemcomitans* showed no association with disease in the Moroccan population. Limited

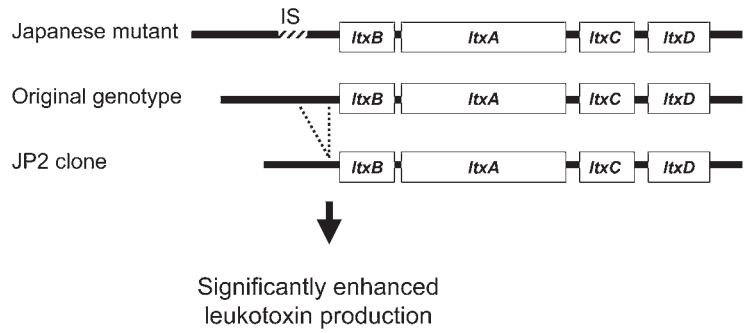


FIGURE 4 The structure of the leukotoxin gene operon in *A. actinomycetemcomitans*. Two different genetic events have resulted in enhanced leukotoxin production in particular clones of *A. actinomycetemcomitans*. In the JP2 clone associated with aggressive periodontitis in patients of northwest African descent, a 530-bp deletion interrupts the regular promoter. In a Japanese isolate an IS element has affected the normal promoter of the Ltx operon. The epidemiology and disease association of this clone is not known. doi:10.1128/9781555818906.ch9.f4

longitudinal observations confirmed the association of the JP2 clone with disease progression.

The characteristic epidemiology of the JP2 clone suggests that it originally arose in Africa and subsequently disseminated to most parts of the world but was still restricted to the original host population. Whether this restricted pattern of dissemination is due to adaptation of the JP2 clone to individuals of a particular genetic constitution or to predominantly vertical transmission is unknown. Its very strong association with early-onset aggressive periodontitis strongly supports its etiologic significance in this disease.

These findings emphasize that observations obtained in one geographic locality or in human populations consisting of individuals of a certain racial or ethnic background are not necessarily applicable worldwide. They further suggest that early-onset aggressive periodontitis may be a disease with dual etiology and epidemiology. In at least some persons of African descent, disease is associated with a particular clone of *A. actinomycetemcomitans*, which has the epidemiological characteristics of an exogenous pathogen. The etiology in Caucasians is less clear. If etiologically involved, diverse *A. actinomycetemcomitans* clones may be acting as opportunistic pathogens. If this assumption is correct, it has important implications for treatment strategies. While eradication of the pathogenic clone with antibiotics or through vaccination may be relevant in the former situation, the most logical treatment in the latter situation would be to attempt to restore the natural balance in the commensal microbiota by hygienic measures.

KEY POINTS

Bacterial species can be viewed as populations of individual strains that share basic housekeeping functions but otherwise may have very different properties.

Genetic diversification can result from accumulation of point mutations in the bacterial genome or from recombinational replacements.

Species in which accumulation of mutations is the dominant mechanism of genetic diversification consist of discrete phylogenetic lineages or clones.

If recombination in a bacterial population is very frequent compared to the mutation rate, a panmictic population structure arises. This is characterized by a random (or nearly random) assortment of alleles and the absence of distinct phylogenetic lineages. Hence, no single property will be able to identify a virulent phenotype in such bacteria unless that property is uniquely responsible for the pathogenic potential.

Different parts of a bacterial genome or even of a single gene may have a phylogenetic history different from that of the remaining genome. For example, surface proteins often demonstrate antigenic diversity as a result of local recombination. In addition, complete virulence genes or whole pathogenicity islands may spread through a basically clonal population of bacteria by horizontal genetic transfer to confer the same virulence properties on otherwise evolutionarily distinct lineages.

Many bacteria appear to have coevolved with their hosts and have speciated in synchrony. A result of this close mutual adaptation is that many species of bacteria exclusively cause disease or colonize in one host species or even subgroups of that host species. The evolutionary process has included optimization of the bacterial genome, often leading to loss of metabolic versatility and, thereby, enhanced dependency on the host.

For bacteria that form part of the commensal microbiota on mucosal membranes, the major mechanism of transmission is vertical. Constrained evolution and diversification of clones within families over millions of years may explain the immense number of distinct clones of commensal bacterial species, in contrast to pathogens, which spread horizontally from one individual to another.

Molecular genetics has demonstrated a degree of biodiversity in the oral microbiota far exceeding earlier expectations and has provided new insight into the patterns of acquisition, transmission, and dynamics of oral bacteria. Combined with the recent realization that individual clones within bacterial species may have widely different properties, including widely differing virulence, this implies a remarkable degree of individuality of dental plaques. This new realization implies that previous attempts to identify etiologic agents of oral diseases by searching for associations between presence of particular cultivable bacteria and disease activity were too simplistic. Not all members of "pathogenic species" are likely to be virulent. Conversely, species considered nonpathogenic have been almost neglected, though there may be functionally important differences within such species. Even ubiquitous species, which are usually dismissed as lacking interest as potential pathogens, may include virulent subpopulations. Population genetics analysis of oral bacteria on a broad scale can reveal new insight into the genetic mechanisms of the genetic and phenotypic diversity that exists in the oral microbiota and provide a better basis for our understanding of the etiology of oral diseases.

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Immunology of the Oral Cavity

Evlambia Hajishengallis and George Hajishengallis

INTRODUCTION

The mucosal surfaces of the body constitute a major interface with the microbial world, and the majority of pathogens that infect humans invade through mucosal routes. The mouth forms the entry to the alimentary tract and is, moreover, in continuity with the pharynx. The physical and functional integrity of the oral mucosa is therefore important both for oral and systemic health. In the oral cavity, soft shedding mucosal surfaces coexist with hard nonshedding tooth surfaces, both of which are protected by innate and adaptive immune mechanisms (Fig. 1). A particularly susceptible area, however, is the junction between the teeth and the gingiva, which constitutes a breeding ground for periodontal bacteria. The immune mechanisms and molecules of the oral cavity derive either from the secretory or the systemic immune system. The former enter the oral cavity through the secretions of the major and minor salivary glands, whereas the latter enter through the gingival crevicular fluid (GCF) (Fig. 1). The GCF is a serum exudate that originates in the gingival capillaries and flows into the gingival crevice carrying locally produced mediators of innate and adaptive immunity. This chapter is divided into two sections, oral secretory immunity and subgingival immunity, to better reflect the peculiarities of the respective anatomical areas and the immune mechanisms operating therein.

ORAL SECRETORY IMMUNITY

Every day, an estimated 0.5 to 1.5 liters of saliva is produced and secreted in the oral cavity by the paired major (parotid, sublingual, and submandibular) and the numerous minor salivary glands throughout the mouth. About 65% of the whole saliva is produced by the submandibular gland, 23% by the parotid, and 4% by the sublingual gland. The minor salivary glands collectively contribute the remaining 8% of the salivary production. The basic secretory unit of salivary glands is represented by the acini, a cluster of epithelial cells that secrete a fluid comprising water, electrolytes, mucins, and proteins including enzymes. There are two basic types of acinar epithelial cells, the serous cells that produce a watery fluid devoid of mucus material and the mucous cells that produce a mucus-rich

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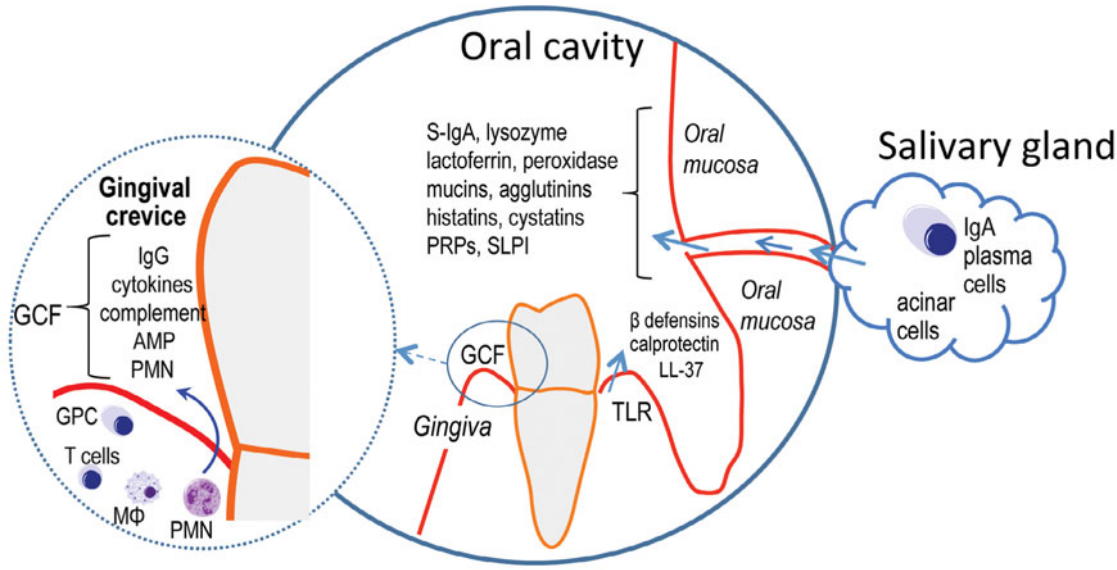


FIGURE 1 Defense mechanisms of the oral cavity. Salivary glands secrete mucins and other innate antimicrobial factors which protect mucosal and tooth surfaces. The salivary glands also constitute a mucosal effector site where B cells terminally differentiate into polymeric IgA-secreting plasma cells. The IgA is secreted in saliva in the form of S-IgA. T and B cells also localize in inflamed gingiva, and B cells differentiate into plasma cells that secrete mainly IgG (also IgM or monomeric IgA). These, as well as immunoglobulins derived from the circulation, can transude into the gingival crevice. Toll-like receptors are expressed in the gingival epithelium and, in response to bacterial challenge, induce the production of antimicrobial peptides (e.g., β-defensins, calprotectin, and cathelicidin LL-37). Antimicrobial peptides and cytokines are also produced by leukocytes present in the gingival connective tissue, the junctional epithelium, or the gingival crevice, where leukocytes are chemotactically recruited. The gingival crevice also contains functional complement which is activated by subgingival bacteria. AMP, antimicrobial peptides; GCF, gingival crevicular fluid; GPC, gingival plasma cells; Mφ, macrophages; PMN, polymorphonuclear neutrophils; PRPs, proline-rich proteins; SLPI, secretory leukocyte protease inhibitor; TLRs, Toll-like receptors. doi:10.1128/9781555818906.ch10.f1

secretion. The acini in the parotid glands and the von Ebner's glands (minor salivary glands in the posterior dorsal and lateral side of the tongue) are exclusively of the serous type. The other salivary glands contain both types of acini, although acini of the mucus type predominate in the sublingual glands. From the acini, secretions are initially collected into small collecting ducts, which lead to larger ducts and finally to a single large duct that secretes the salivary contents into the oral cavity. These include both inorganic (electrolytes, such as chloride, potassium, sodium, and bicarbonate) and organic components. The latter include a number of proteins such as the digestive enzyme amylase, mucous glycoproteins, acidic proline-rich and tyrosine-rich proteins, and numerous humoral host defense factors.

Innate Host Defense Factors in Saliva

The oral cavity contains an array of innate antimicrobial factors that are secreted by salivary glands but also by epithelial cells and neutrophils. These antimicrobial molecules can kill or inhibit the growth of microorganisms and have broad-spectrum antibacterial, antifungal, and antiviral properties. Therefore, at least in principle, they can efficiently protect the

oral mucosal surfaces from pathogens. Some of these molecules are high-molecular-weight adhesive (glyco)proteins which can promote microbial adherence by acting as receptors (when adsorbed to solid surfaces, such as teeth) or, conversely, mediate microbial clearance through agglutination and swallowing (when present in the fluid). Below is a brief description of major innate host factors found in the oral cavity (Table 1).

TABLE 1 Important innate antimicrobial factors in the oral cavity

Cationic antimicrobial peptides
α- and β-defensins
Cathelicidin (LL-37)
Histatins 1 and 3
Adrenomedullin
Azurocidin
Adhesive proteins mediating bacterial agglutination
Mucin-7
Salivary agglutinin
Surfactant protein A
β2 microglobulin
PRPs
Fibronectin
Metal ion chelators
Calgranulin A and B (calprotectin)
Lactoferrin
Protease inhibitors
Cystatins
SLPI
SKALP/elafin
Enzymes acting against bacterial cell walls
Lysozyme
Peptidoglycan recognition proteins 3 and 4
Peroxidases
Salivary peroxidase
Myeloperoxidase (in neutrophils)

CATIONIC ANTIMICROBIAL PEPTIDES

These are small 15- to 20-amino-acid-long peptides with a net positive charge, owing to an excess of basic amino acids, such as arginine, lysine, and histidine. At least 50% of the amino acids in these molecules are hydrophobic. This property allows them to interact with bacterial membranes, which constitutes their common target, despite different modes of action. Cationic antimicrobial peptides are collectively active against gram-positive and gram-negative bacteria, fungi, parasites, enveloped viruses such as human immunodeficiency virus (HIV), herpesvirus, vesicular stomatitis virus, and also cancerous cells. Examples of cationic peptides found in the oral cavity are the α- and β-defensins, cathelicidin (LL-37), histatins 1 and 3, adrenomedullin, statherin, C-C motif chemokine 28, and azurocidin.

Several other peptides, originally appreciated for their neural or neuroendocrine signaling functions appear to also exhibit, at least in vitro, potent antimicrobial activities. Such peptides include the calcitonin gene-related peptide, substance P, neuropeptide Y, and vasoactive intestinal peptide. Their exact mechanism of action is not known and their biological role is not clear, as their concentration in the oral environment may range below the level needed for an antimicrobial inhibitory effect.

ADHESIVE PROTEINS WITH BACTERIAL AGGLUTINATION PROPERTIES

Mucin-7

Mucin-7 is a relatively small salivary glycoprotein (357 amino acid residues) which is produced by the mucous acinar cells of the salivary glands. It has been shown to promote bacterial agglutination and prevention of penetration of HIV into the host tissue. Its concentration in stimulated saliva is reduced in patients with periodontitis.

Salivary agglutinin

Salivary agglutinin is a large glycoprotein (340 kDa), derived from the parotid secretion, which can agglutinate a large number of oral bacteria (including cariogenic streptococci) via its multiple scavenger receptor cysteine-rich repeats. Gene polymorphisms of the salivary agglutinin have been associated with a high incidence of caries. It is identical to the scavenger receptor gp-340 present in bronchoalveolar fluid.

Surfactant protein A

Surfactant protein A belongs to the collectin family and is a major protein component of lung surfactant. It is also expressed in human salivary glands and can agglutinate bacteria and neutralize the influenza virus via its sialic acid residues. Its levels in the salivary glands are upregulated in patients with chronic sialadenitis. This might suggest that surfactant protein A plays a role in the innate host defense of salivary glands.

β2 microglobulin

β2 microglobulin can agglutinate *Streptococcus mutans* and a number of other bacteria, and its levels are upregulated in periodontal disease. Moreover, it is a salivary biomarker in Sjögren's syndrome.

PRPs

Proline-rich proteins (PRPs) are produced by the parotid and submandibular glands and constitute nearly 70% of the total protein of human saliva. Proline accounts for about 25 to 40% of the amino acid content of PRPs. The larger PRPs bind to tooth surfaces and promote bacterial adherence, whereas the smaller PRPs antagonize bacterial adherence and hence colonization by agglutinating bacteria in the fluid phase.

Fibronectin

Fibronectin is a large glycoprotein expressed by many cells, including hepatocytes and epithelial cells. It is also found in saliva, where it mediates bacterial agglutination and therefore serves to control bacterial colonization. Fibronectin binds to the fimbriae of *Porphyromonas gingivalis* and inhibits the fimbria-induced expression of inflammatory cytokines in macrophages. Low levels of fibronectin are correlated with periodontitis in adults and with high levels of *S. mutans* in children.

METAL ION CHELATORS

Calgranulin A and B (calprotectin)

Calgranulin A and calgranulin B are metal ion-binding proteins that inhibit bacterial growth by scavenging divalent cations, such as Mn^{2+} and Zn^{2+}. The dimer of calgranulin A and B, termed calprotectin, is expressed in the cytosol of neutrophils, monocytes, and keratinocytes. The expression of calprotectin is upregulated in the GCF of patients with periodontal disease, and in saliva of patients with candidiasis, implying a host defense mechanism to control infection.

Lactoferrin

Lactoferrin is an 80-kDa glycoprotein produced by mucosal epithelial cells and neutrophils and found in all major mucosal secretions, including saliva and the GCF. Lactoferrin binds iron (Fe^{3+}) in association with bicarbonate and, therefore, can deprive microorganisms (bacteria, viruses, fungi, and parasites) of this essential nutrient. It has also been shown to be bactericidal against *Aggregatibacter actinomycetemcomitans*. Gene polymorphisms of lactoferrin have been associated with aggressive periodontitis. Lactoferrin also has anti-inflammatory effects by binding and neutralizing the lipid A component of bacterial lipopolysaccharide, a major proinflammatory molecule.

PROTEASE INHIBITORS

Cystatins

Cystatins comprise a family of proteins involving at least 14 genes. Seven of these genes express salivary proteins that are contributed by the submandibular and sublingual salivary glands. Their antimicrobial action involves inhibition of bacterial cysteine proteases, such as the gingipains

of *Porphyromonas gingivalis*. As bacterial proteases play a major role in nutrient acquisition, their inhibition by cystatins can lead to suppression of bacterial growth. Cystatins also inhibit human lysosomal cathepsins, which under inflammatory conditions contribute to periodontal tissue destruction.

SLPI

Secretory leukocyte protease inhibitor (SLPI) is an 11.7-kDa cationic protein expressed by gingival keratinocytes and other mucosal epithelial cells. SLPI is therefore present in mucosal secretions including saliva. It exerts antibacterial, antifungal, and anti-inflammatory properties through its serine protease inhibitor properties. SLPI can block the entry of HIV into host cells, although this action is independent of its protease inhibitor activity.

SKALP/elafin

Skin-derived antileukoproteinase (SKALP)/elafin is a 12-kDa salivary protein expressed in the human submandibular gland. It acts as a transglutaminase substrate, has antielastase activity, and can kill both gram-positive and gram-negative bacteria.

ENZYMES ACTING AGAINST BACTERIAL CELL WALLS

Lysozyme

Lysozyme is a 14.6-kDa protein present in all major body fluids, including saliva, milk, lacrimal fluids, and nasal and bronchial secretions. Salivary lysozyme is derived from the salivary glands and the GCF. In terms of enzymatic activity, lysozyme is a muramidase that hydrolyses the β(1-4) glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine residues in the peptidoglycan of the cell wall of gram-positive bacteria. This action may lead to osmotic breakage of the cell membrane. Other functions of lysozyme include activation of autolysins in the cell wall, agglutination of microorganisms, and synergistic interactions with lactoferrin, salivary peroxidase, and secretory immunoglobulin A (S-IgA). The levels of lysozyme appear to increase in aggressive periodontitis.

Peptidoglycan recognition proteins 3 and 4

The peptidoglycan recognition proteins are a family of large (90 to 115 kDa) antimicrobial proteins found also in insects. Four of these peptidoglycan recognition proteins are present in humans, and members 3 and 4 are expressed in the salivary glands. Their mechanism of action is unclear, as they bind to the cell wall peptidoglycans without permeabilizing the bacterial membrane. They can be either bactericidal or bacteriostatic, depending on the bacterial target.

THE PEROXIDASE SYSTEM

Peroxidases are found in all mucosal secretions. The peroxidase system of saliva includes the salivary peroxidase (which is similar to lactoperoxidase found in milk) and the myeloperoxidase expressed in neutrophils. These enzymes catalyze the oxidation of thiocyanate ions (SCN^-) by hydrogen peroxide to hypothiocyanite ($OSCN^-$), which is bactericidal. The

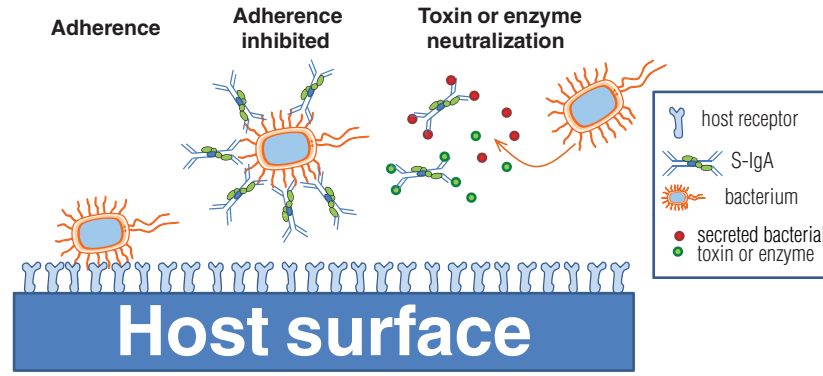
thiocyanate ion is a normal component in saliva, plaque fluid, and GCF, while hydrogen peroxide is produced during the aerobic metabolism of glucose by bacteria. Therefore, the availability of H_2O_2 is a major limiting factor for SCN^- peroxidation in human saliva. The hypothiocyanite oxidizes bacterial enzymes in the glycolytic pathway and, therefore, inhibits the growth of and acid production by oral microorganisms, including streptococci, lactobacilli, and fungi. Hydrogen peroxide is toxic also to eukaryotic cells, and its reduction to H_2O by salivary peroxidase protects the oral mucosa. The peroxidase system seems to interact synergistically with other antimicrobial molecules, such as lysozyme, lactoferrin, and S-IgA.

Specific Host Defense Factors in Saliva: S-IgA

In terms of adaptive immunity, the oral mucosal surfaces in humans and other mammals are protected by a sophisticated system which is largely independent of the circulatory immune system and is known as the mucosal immune system. The single most important humoral mediator of specific oral mucosal immunity is the S-IgA, which enters the oral cavity through the salivary secretions. Antibodies of this isotype provide host defense by binding to specific antigenic targets, thereby inhibiting microbial adherence or neutralizing toxic substances (Fig. 2). S-IgM is another secretory immunoglobulin which acquires particular significance in cases of IgA deficiency, as it is thought to replace the missing S-IgA.

In contrast to the plasma IgA which is monomeric, S-IgA is polymeric (primarily dimeric). In the secretory immunoglobulin molecule, polymeric IgA and pentameric IgM are linked to two additional protein components, the joining (J) chain and the secretory component (SC). Polymeric IgA and IgM with incorporated J chain are produced by plasma cells found in the lamina propria of mucosal epithelia or the salivary glands. These J chain-containing immunoglobulins interact noncovalently via their J chain with the polymeric immunoglobulin receptor (pIgR) expressed at the basolateral surface of the epithelial cells lining the mucous membranes or the serous-type glandular epithelial cells (Fig. 3). Following transcytosis, S-IgA (or S-IgM) is exocytosed at the apical epithelial surface after cleavage of the pIgR. The extracellular part of the pIgR remains

FIGURE 2 Major functions of S-IgA antibodies. S-IgA antibodies can bind and block microbial adhesins, resulting in reduced adherence of microorganisms to receptors on host surfaces, such as mucosal epithelial cells or salivary pellicle-coated tooth surfaces (middle). S-IgA antibodies can also bind and neutralize virulence factors such as microbial toxins or enzymes (right). doi:10.1128/9781555818906.ch10.f2



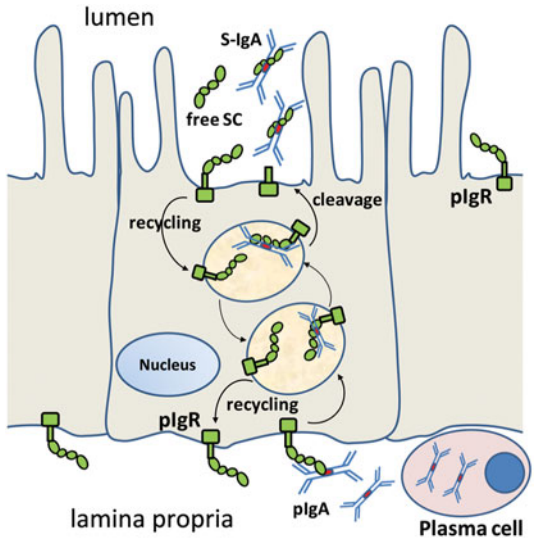


FIGURE 3 Transport of polymeric IgA (pIgA) across the mucosal epithelium. B cells home to mucosal effector sites, such as the salivary glands, where they terminally differentiate into polymeric IgA-secreting plasma cells. Polymeric IgA is secreted by epithelial cells in the form of S-IgA. Specifically, the epithelial cells express the polymeric immunoglobulin receptor on their basolateral membrane which binds the joining chain and other regions of polymeric IgA. This complex is then internalized and traffics to the apical membrane where it is secreted to the lumen as S-IgA. In the S-IgA molecule, the polymeric immunoglobulin receptor becomes a secretory component which represents its extracellular portion following proteolytic cleavage. The secretory component can also be found in a free (unoccupied) form in secretions. J chain, joining chain; pIgR, polymeric immunoglobulin receptor; SC, secretory component. doi:10.1128/9781555818906.ch10.f3

covalently linked to the S-IgA as bound SC, whereas the intracellular part of the pIgR undergoes intracellular degradation. Unbound SC produced by the same proteolytic cleavage of unoccupied pIgR is also found in saliva and other secretions, where it can exert innate neutralizing effects against pathogens and their toxins. Similar innate immune functions are shared by the bound SC, which moreover renders the S-IgA molecule resistant to proteolytic degradation. The SC also appears to target S-IgA to mucins for the formation of S-IgA-mucin complexes, which are particularly effective in microbial clearance. In the S-IgM molecule, the SC is not covalently bound and S-IgM is less resistant to proteolysis than is S-IgA. Furthermore, the epithelial transport of IgM is less efficient than that of IgA, owing, at least in part, to diffusion constraints of the large pentameric IgM molecule.

There are two subclasses of IgA, IgA1 and IgA2. IgA1 antibodies seem to be induced primarily against protein antigens, whereas IgA2 are induced against polysaccharides. In the plasma, most of the IgA is of the IgA1 subclass, whereas in saliva the proportion of S-IgA2 may reach 35 to 50% of total IgA. Both IgA subclasses seem to be transported equally well through the epithelial tissues. Intriguingly, the hinge region of IgA2 lacks a 13-amino-acid segment with O-linked glycans. This deletion renders IgA2 resistant to IgA proteases produced by certain oral bacteria, whereas IgA1 is susceptible to IgA protease-mediated cleavage, yielding intact Fab and Fc fragments.

THE MUCOSAL IMMUNE SYSTEM AND INDUCTION OF S-IgA ANTIBODIES IN SALIVA

The induction of B-cell responses which can lead to production of S-IgA antibodies in saliva is initiated at specialized inductive sites of the mucosal immune system, such as the intestinal Peyer's patches and the tonsils and adenoids forming the Waldeyer's pharyngeal ring. These lymphoid structures are collectively known as mucosa-associated lymphoid tissues. Microbial antigens are taken up by specialized epithelial cells overlying mucosa-associated lymphoid tissues and are passed through transcytosis to the underlying antigen-presenting cells (APC), such as dendritic cells. After intracellular processing of microbial antigens, APC present antigenic peptides and provide costimulatory signals to naive T cells which thereby become activated. These antigen-sensitized T cells activate B cells, which preferentially switch to express IgA and emigrate through the draining lymphatics into the circulation. These IgA-committed cells, along with cognate T cells, eventually home to various effector sites (such as the intestinal lamina propria, as well as salivary, lacrimal, and mammary glands) that may be remote from the inductive site where they were originally stimulated. At the effector sites, the IgA-committed B cells differentiate into polymeric IgA-secreting cells. These plasma cells secrete large quantities of polymeric IgA close to the basolateral surfaces of the epithelia under which they now reside. The mucosal epithelia-expressed pIgR binds J-chain-containing polymeric IgA (and IgM, if present) and transports it—through a vesicular transport mechanism—to the apical surface as described above (Fig. 3).

It was initially thought that induction of immune responses at any mucosal inductive site could result in uniform appearance of antibodies in all effector sites (hence the original name "common mucosal immune system"). However, later studies demonstrated a considerable degree of compartmentalization within the mucosal immune system. In this regard, B and T cells induced in a particular inductive site emigrate selectively to a particular subset of effector sites. Regarding the salivary glands, the most relevant inductive sites appear to be the lymphoid tissues in the Waldeyer's ring, the nasal lymphoid tissue, and to a lesser extent, the Peyer's patches in the intestine. Selective expression of mucosal homing receptors, which recognize mucosal cell adhesion molecules, and of chemokine receptors, which recognize specific mucosal chemokines, have been identified and may at least in part explain the compartmentalization within the mucosal immune system.

BIOLOGICAL ACTIVITIES OF S-IgA ANTIBODIES

S-IgA is a relatively stable immunoglobulin that can maintain its antibody activity for a long time in the proteolytic environment of the oral cavity. The major defense function of S-IgA involves immune exclusion of soluble and particulate antigens, which is an effective anti-inflammatory mechanism to protect mucosal surfaces. In this respect, moreover, S-IgA does not activate the complement system and therefore is considered to be an anti-inflammatory immunoglobulin. By binding and blocking microbial adhesins, S-IgA antibodies can inhibit microbial colonization, whereas they can neutralize the action of microbial enzymes or toxins (Fig. 2). In this regard, S-IgA antibodies are considerably more efficient than monomeric antibodies, such as IgG. Intriguingly, pIgA antibodies can neutralize

pathogens by intercepting them inside secretory epithelial cells, during their pIgR-mediated exocytosis. S-IgA also synergizes with and promotes the bacteriostatic effect of lactoferrin and the peroxidase system.

S-IgA is absent in the saliva of newborns. However, its levels emerge rapidly in the postnatal period and by 1 to 2 years of age approximate, and by 4 to 7 years of age reach, the adult levels. Besides age, there are other important factors that can influence the salivary levels of S-IgA, including the salivary flow rate, cigarette smoking, pregnancy, and other stressors. Interestingly, most of S-IgA comes from the submandibular glands, which contain approximately two times more IgA-secreting plasma cells per tissue unit than the parotid gland. The minor salivary glands also contain numerous IgA-secreting plasma cells (3 times more cells are found in the labial glands per tissue unit than in the parotid glands) and contribute about 30 to 35% of the total S-IgA in saliva.

Although S-IgA antibodies exert important defense functions, the results of studies which attempted to correlate levels of S-IgA antibodies with dental caries have not been conclusive. Nevertheless, mucosal application of caries vaccines in rodents, orally inoculated with mutans streptococci and fed a high-sucrose diet, induces protective S-IgA antibody responses that suppress mutans streptococcal colonization and inhibit formation of carious lesions. Moreover, human S-IgA antibodies inhibit the adherence of mutans streptococci to models of the salivary pellicle-coated tooth surfaces, although S-IgA1 antibodies lose this protective effect after cleavage with bacterial IgA1 proteases.

In addition to secretory immunoglobulins, the saliva may also contain monomeric IgA and IgG, which enter the oral cavity via the GCF. Therefore, their levels are expected to be higher in periodontitis relative to health. GCF-derived IgG antibodies can be protective against caries through opsonization of mutans streptococci with subsequent phagocytosis by neutrophils in a limited area of the tooth that is bathed with GCF (i.e., in the vicinity of the gingival margins).

SUBGINGIVAL IMMUNITY AND INFLAMMATION

The junction between the teeth and the gingival mucosa forms an interface between the mucosal and systemic immune systems. The GCF, which bathes the gingival crevice and accumulates around the necks of the teeth, also enters the oral cavity and mixes with saliva at an estimated dilution of 1:500 to 1:1,000. In this context, humoral antimicrobial molecules and antibodies present in the GCF may impact the bacterial colonization of the teeth, at least in the vicinity of the gingival margins. However, the transition from the isotonic gingival crevice to hypotonic saliva is not conducive for the normal function of immune cells, such as phagocytic cells or lymphocytes, if they enter the oral cavity. On the other hand, the GCF and the immune mechanisms operating in the gingival crevice are intimately associated with periodontal health or disease.

Innate and Adaptive Immune Players below the Gums

In a clinically healthy periodontium, the tooth-associated biofilm is usually confined to the gingival margin. Under these conditions, the GCF represents a slow-flowing transudate of plasma proteins. However, the flow of GCF into the gingival crevice increases considerably if the biofilm

is left undisturbed for 2 to 4 days (i.e., time required for the initiation of gingivitis). This is because the biofilm enters the crevice (by proliferation and spreading or by relocation of dislodged bacteria) and the host senses the invading danger and responds with increased inflammation. The initial host response to the maturing biofilm involves increased vascular permeability (of the subepithelial blood vessels) and flow of inflammatory exudate, as well as chemotactic recruitment of inflammatory phagocytes, such as neutrophils and—to a lesser extent—macrophages. The migration of cells into the crevice occurs through the junctional epithelium which, under inflammatory conditions, is largely occupied (by about 60%) by trafficking neutrophils.

In the gingival crevice, the recruited inflammatory cells, the flowing GCF, and the immediate surroundings (oral sulcular epithelium, junctional epithelium, subgingival tooth surface, and the attached biofilm) form a complex network of immune players (Fig. 1). The innate immune players include phagocytes, epithelial cells, and soluble inflammatory mediators, such as cytokines and complement activation products. As adaptive immunity becomes involved, other factors entering the picture are antibodies and lymphocytes (T cells, B cells, and plasma cells), which, however, are largely confined to the underlying connective tissue. The outcome of the interactions between these cellular and humoral elements may be steady-state homeostasis or, conversely, persistent inflammation that may eventually develop into destructive periodontitis.

ANATOMY AND PHYSIOLOGY OF THE SUBGINGIVAL ENVIRONMENT: ROLE IN HOST RESPONSE

It is a cliché to say that the host immune response is a double-edged sword, but nothing could be truer in the field of immunology. An unwarranted host response to the subgingival biofilm inflicts collateral damage upon the periodontal tissues. On the other hand, inadequate or impaired immunity leads to the overgrowth of the bacteria and similar unfavorable outcomes, as suggested by the increased susceptibility to periodontitis of individuals with neutrophil deficiencies with regard to numbers, trafficking capacity, or killing function (see below). By contrast, periodontal health represents a dynamic balanced state where proinflammatory and antimicrobial activities are optimally regulated to prevent unwarranted host reactions. This homeostatic balance may be disrupted, however, by congenital immune deficiencies or by immune subversive tactics of periodontal bacteria. Moreover, there may be anatomical and physiological factors rendering periodontal homeostasis a delicate and challenging task for the host. To better understand the peculiarities of the subgingival environment, it would be helpful to contrast it with another tissue or organ.

The intestine is often cited as an example of an organ that has optimally evolved to accommodate beneficial host-microbe interactions. Indeed, although the intestinal immune system is faced with an unusually high microbial burden compared to most other tissues or organs, humans—and mammals in general—are normally healthy and free of clinically significant gut inflammation. Important mechanisms that contribute to the control of adverse inflammatory responses in the intestine include the production of a thick mucus layer that covers the intestinal epithelium and the induction of S-IgA antibodies. The inner mucus layer

remains essentially sterile and, therefore, provides a protective buffer zone. Moreover, S-IgA antibodies reinforce this protection by sequestering gut bacteria. In contrast, the sulcular and junctional epithelium that lines the gingival crevice is not covered by mucin and is not bathed by S-IgA, which, as outlined above, is a salivary secretion bathing supragingival areas. The GCF contains primarily IgG antibodies, but this is a proinflammatory isotype, in stark contrast to the anti-inflammatory properties of S-IgA. Moreover, the junctional epithelium is highly porous, since these epithelial cells are interconnected by only a few desmosomes and occasional gap junctions. Thus, the junctional epithelium is potentially permeable by inflammatory bacterial products. By contrast, the intestinal epithelial cells are connected by tight junctions that contribute to their barrier function. Therefore, when the crevice is populated with periodontal bacteria, it may be very challenging to prevent overt inflammation.

CREVICULAR NEUTROPHILS

Subgingival biofilms containing *P. gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, or other putative pathogens, have been associated with persistent inflammation. Given the panoply of the host periodontal response (e.g., neutrophils, activated complement, cytokines, antibodies, and the adaptive immunity in general), one may wonder why the host often fails to control the bacteria and resolve subgingival inflammation. We start with the neutrophils, which are the first cells to be recruited to handle the subgingival bacterial challenge.

Neutrophils are professional phagocytes that can be found in great numbers in the gingival crevice, comprising ≥95% of total leukocytes. The extravasation of circulating neutrophils depends on a well-coordinated adhesive cascade, including interactions of β2 integrins, such as LFA-1, with endothelial counterreceptors, such as intercellular adhesion molecules (ICAMs). The recruitment of neutrophils to the gingival crevice is facilitated by a gradient of chemokines and cell adhesion molecules, such as interleukin 8 (IL-8) and ICAM-1, respectively, which directs the migration of neutrophils from the vasculature. The gradient becomes denser toward the upper epithelial cell layers, which are closer to the bacterial challenge (Fig. 4). The coordinated and regulated recruitment of neutrophils is vital for periodontal tissue defense. This is suggested by clinical observations in individuals with neutrophil deficiencies in terms of numbers (chronic or cyclic neutropenia), trafficking capacity (leukocyte adhesion deficiencies), or killing function (Chédiak-Higashi and Papillon-Lefèvre syndromes); these individuals display increased susceptibility to and severity of periodontitis. However, neutrophils can also be involved in periodontal tissue destruction. At least in principle, this may occur when they are recruited to the periodontium in excessively high numbers (e.g., due to host immunoregulatory defects) or when their functions are subverted by periodontal pathogens.

As alluded to above, the interaction of the LFA-1 integrin on neutrophils with ICAM-1 on endothelial cells is critical for the recruitment of neutrophils to peripheral tissues. This step is regulated by an endothelial cell-derived glycoprotein, termed Del-1 (for developmental endothelial locus 1). Mice that are genetically deficient in Del-1 spontaneously develop periodontitis attributed to unwarranted recruitment of high numbers

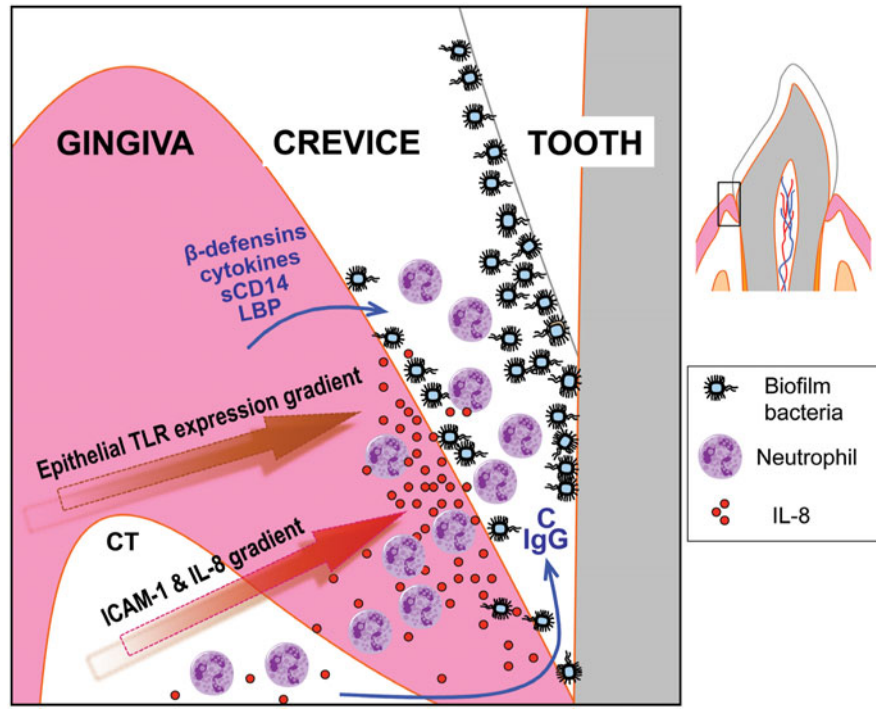


FIGURE 4 Important host immune factors in the gingival crevice. The presence of subgingival biofilm elicits host immune responses aiming to control the bacteria. Neutrophils are recruited from the vasculature to the gingival crevice following a pathway that is defined by a gradient of chemokines and cell adhesion molecules, such as IL-8 and intercellular adhesion molecule-1. The gradient becomes denser toward the upper epithelial cell layers which are closer to the bacterial challenge. Toll-like receptor expression in the gingival epithelia also becomes denser in the spinous epithelial layers compared to the basal layers, which are more remote from the biofilm. The induction of production of antimicrobial peptides (e.g., β-defensins) by gingival epithelial cells depends on the activation of Toll-like receptors by periodontal bacteria. Epithelial cells also express IL-8 and other cytokines, as well as soluble CD14 and lipopolysaccharide-binding protein, which potentially contribute to bacterial clearance. Neutrophils, as well as other leukocytes present in the junctional epithelium or the connective tissue, produce cytokines and antimicrobial molecules (e.g., α-defensins) which transit to the gingival crevice. The crevice also contains functional complement and IgG antibodies derived from the circulation or from local gingival production. C, complement; CT, connective tissue; ICAM-1, intercellular adhesion molecule-1; LBP, lipopolysaccharide-binding protein.
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of neutrophils. Normal mice lose the ability to express adequate levels of gingival Del-1 in old age, and this correlates with the appearance of neutrophil-dependent periodontal tissue destruction. These observations underscore the importance of periodontal homeostatic mechanisms for the control of the local inflammatory response and the prevention of periodontitis.

In addition to host factors, bacteria may also contribute to the disruption of periodontal tissue homeostasis by subverting the host response. Histological and electron microscopy studies show that gingival crevicular neutrophils form what looks like a defense wall against the subgingival biofilm bacteria. However, despite maintaining viability and capacity to elicit inflammatory responses, including induction of the oxidative burst and formation of extracellular DNA traps (web-like scaffolds that

concentrate released antimicrobial molecules, resulting in the trapping and extracellular killing of bacteria), the neutrophils may often fail to control dental plaque bacteria. The reasons why neutrophils may fail to control periodontal infection and prevent nonresolving inflammation are not fully understood. At least in part, however, this may be the result of microbial immune evasion, which is specifically dealt with in chapter 15. When neutrophils fail to control the subgingival biofilm, this causes additional problems. Toxic substances such as the reactive oxygen species do not discriminate between microbial and host cells. Therefore, when chronically released at high levels, these toxic molecules are likely to cause collateral damage to periodontal tissues. By contrast, certain bacteria that are associated with periodontitis appear to be resistant to killing mechanisms dependent on reactive oxygen species. In conclusion, the neutrophils are required for periodontal health but their presence does not necessarily guarantee a homeostatic balance between the host and the periodontal biofilm. In fact, their presence at excessive numbers is very likely to contribute to periodontal disease pathogenesis.

GINGIVAL CREVICULAR EPITHELIUM

In addition to the neutrophils that attempt to wall-off the bacteria in the gingival crevice, front-line defense is also contributed by the gingival sulcular and junctional epithelium, both as a mechanical barrier (though highly porous) and as the site of production of immune mediators and antimicrobial molecules (Fig. 4). Gingival epithelial cells, as well as leukocytes present in the junctional epithelium, contribute to host defense by expressing an array of antimicrobial peptides. These include α- and β-defensins and cathelicidin (LL-37), which can directly kill a broad spectrum of microbes. The β-defensins are contributed by the gingival epithelial cells in a characteristic expression pattern: Human β-defensin-1 (hBD-1) and hBD-2 are predominantly expressed in the upper portion of the gingival epithelium (granular and spinous layers), whereas hBD-3 is primarily expressed in the basal layer. On the other hand, α-defensins (also known as human neutrophil peptides) and LL-37 are contributed by the neutrophils and can thus be found both in the gingival crevice and the junctional epithelium.

The induction of production of antimicrobial peptides by gingival epithelial cells depends largely on the activation of Toll-like receptors (TLRs) by oral bacteria. The gingival expression of TLRs is denser in the spinous epithelial layer than in the basal layer (Fig. 4), and is upregulated in diseased (pocket) epithelia. It has been proposed that commensal bacteria stimulate epithelial cells to induce the production of antimicrobial peptides at levels that would kill opportunistic or pathogenic organisms but would spare the commensal bacteria themselves. For example, the gut commensal *Bacteroides thetaiotaomicron* induces the antimicrobial peptide angiogenin which has microbicidal activity against pathogens, while *B. thetaiotaomicron* and other commensals are relatively resistant. This differential susceptibility suggests an elegant mechanism by which the host can specifically target pathogenic bacteria. However, in the context of periodontal immunity, several studies have shown that antimicrobial peptides can be induced by both commensal and pathogenic oral bacteria, whereas several pathogens display resistance to various such molecules, often by proteolytically inactivating them. Even when periodontal

pathogens are inherently sensitive to a given antimicrobial peptide, they may use protease-independent mechanisms to overcome or attenuate this challenge. For example, *P. gingivalis* is highly susceptible to killing by hBD-3. However, the pathogen can inhibit TLR4-induced mRNA expression of hBD-3 (also of hBD-1 and -2) by means of its tetra-acylated lipid A-containing lipopolysaccharide which blocks the activation of TLR4.

In addition to attempting to neutralize the secreted antimicrobial products of epithelial cells, periodontal bacteria also appear to exploit gingival epithelial cells as a refuge that could shield them from immune surveillance in the extracellular space. Specifically, certain periodontal species were shown to actively invade and replicate within gingival epithelial cells in vitro. In this context, *P. gingivalis*, *A. actinomycetemcomitans*, *Tannerella forsythia*, and *Treponema denticola* can be detected intracellularly in human crevicular epithelial cells from subjects with chronic periodontitis.

PERIODONTAL COMPLEMENT

Complement is an antimicrobial enzyme system present in serum and inflammatory exudates, such as the GCF. Complement components can be found in active form in GCF at up to 70 to 80% of their concentration in serum, although certain components can be found at much higher levels in GCF, reflecting local generation in the periodontium. The triggering of the complement cascade involves sequential activation and proteolytic cleavage of a series of proteins and proceeds via distinct pathways, namely, the classical, lectin, or the so-called alternative pathway. All three pathways converge at the third component of complement (C3), which is activated by pathway-specific C3 convertases and leads to the generation of effector molecules that mediate recruitment and activation of inflammatory cells (anaphylatoxins C3a and C5a), microbial opsonization and phagocytosis (opsonins such as C3b and iC3b), and direct lysis of targeted pathogens (the C5b-9 membrane attack complex). However, complement has functions above and beyond its traditional role of tagging and eliminating microbes. Research in the last decade has shown that complement can orchestrate critical events during immune and inflammatory responses, including regulation of other systems such as Toll-like receptor signaling pathways.

Despite its potential role in host defense, certain complement activation pathways have actually been associated with periodontal inflammation and tissue destruction. Activated complement fragments, including the chemotactic anaphylatoxins (C3a and C5a), are abundantly found in the GCF of periodontitis patients and in inflamed gingiva, whereas they are absent or present at significantly lower levels in periodontally healthy individuals. Induction of experimental gingival inflammation in human volunteers causes progressive elevation of complement cleavage products correlating with increased clinical indices of inflammation. On the other hand, the central complement component C3 is among the top 5% of genes that are most strongly downregulated following periodontal therapy. Moreover, mice genetically deficient in the C5a receptor are protected against experimental periodontitis. This suggests that the C5a receptor, far from being protective, mediates destructive inflammatory responses in the periodontium.

The reason why complement may be involved primarily with periodontal tissue destruction, rather than protection from the disease, might

also have to do with the ability of certain periodontal bacteria to resist or manipulate the complement system. Some species are inherently resistant to complement-mediated lysis. Other bacteria can proteolytically inactivate or proactively activate specific complement components. This apparent contradiction actually makes sense if one considers that complement mediates diverse functions, some of which could have effective antimicrobial action against periodontal bacteria (targets of inactivation by the bacteria), while other complement actions could stimulate inflammatory pathways that benefit the periodontal bacteria (targets of activation; see below).

The Arg-specific gingipains of *P. gingivalis* mimic the host C5 convertase and generate high levels of C5a. This voluntary generation of C5a is puzzling considering that C5a is the most powerful inflammatory mediator of complement. However, *P. gingivalis* exploits C5a to instigate a subversive crosstalk between the C5a receptor and TLR2 in leukocytes, which selectively inhibits an antimicrobial pathway that could eliminate *P. gingivalis*. Moreover, C5a-induced periodontal inflammation may be beneficial to asaccharolytic and proteolytic bacteria in the gingival crevice. Specifically, C5a-induced vasodilation and increased vascular permeability leads to increased flow of GCF, which is a rich source of nutrients, such as hemin (source of essential iron) and tissue breakdown products (e.g., protein fragments). In direct support of this notion, anti-inflammatory treatments in animal models of periodontitis not only block inflammatory tissue destruction but also inhibit the growth of periodontal bacteria. These examples serve to illustrate that several features of the host immune and inflammatory response, rather than being detrimental to the bacteria, are actually exploited by the bacteria to promote their adaptive fitness.

PERIODONTAL TLRs AND THEIR CORECEPTORS

In addition to elevated complement activity, the inflamed periodontium is infiltrated by TLR-expressing inflammatory cells (neutrophils, macrophages, and dendritic cells). Particularly abundant are TLR2- and TLR4-expressing cells which are mostly found in the connective tissue subjacent to the pocket epithelium. On the other hand, healthy gingiva display a significantly decreased presence of TLR-expressing cells. Besides professional immune cells, gingival epithelial cells (see above) and fibroblasts also express TLRs, and the level of expression correlates with disease activity. In terms of function, TLRs mediate important immune and inflammatory responses to periodontal bacteria in vivo and in vitro and form a link between innate and adaptive immunity. The precise roles, whether protective or destructive, played by the TLRs in periodontal infection and inflammation are poorly understood. Several studies have investigated single-nucleotide polymorphisms of TLRs in periodontally healthy individuals and patients with chronic periodontitis. However, these studies yielded overall inconclusive results as to the role of TLRs in periodontitis. On the other hand, at least in animal models of periodontitis, TLRs appear to play a predominantly destructive inflammatory role.

CD14 and the lipopolysaccharide-binding protein (LBP) are important partners of TLRs (especially TLR4 and TLR2). For example, lipopolysaccharide binds to soluble (e.g., serum-derived) LBP and is then transferred to CD14 which acts as a coreceptor that facilitates TLR4

activation by lipopolysaccharide. Although low concentrations of soluble CD14 (sCD14) and LBP enhance responses to bacterial lipopolysaccharide, high concentrations of the same molecules—such as those achieved during inflammation—can inhibit lipopolysaccharide bioactivity in vitro and in vivo. At least in part, sCD14 may inhibit inflammatory responses by sequestering lipopolysaccharide from membrane-bound CD14 and diverting it to lipoproteins present in serum or extravascular fluids, such as the GCF. The levels of sCD14 in the GCF are higher than in serum, which suggests local synthesis of sCD14 in the periodontium. Interestingly, high levels of sCD14 in the GCF have been correlated with reduced number and depth of periodontal pockets. Moreover, LBP is expressed at higher mRNA and protein levels in healthy tissues than in diseased tissues. Therefore, it appears that both LBP and sCD14 may contribute to periodontal homeostasis, either by mitigating inflammation as alluded to above, or even by opsonizing bacteria and promoting their clearance, as also shown for myeloid differentiation protein-2 (MD-2). MD-2 is an essential component of the TLR4 activation complex which confers responsiveness to lipopolysaccharide in the form of TLR4-bound MD-2. In addition, soluble MD-2 functions as an opsonin that promotes the phagocytosis and intracellular killing of gram-negative bacteria. Intriguingly, MD-2 mRNA expression is significantly elevated in healthy gingiva compared to diseased tissue, but whether it promotes the phagocytosis and clearance of periodontal bacteria in the periodontium has not been examined.

THE ADAPTIVE IMMUNE RESPONSE IN THE PERIODONTIUM

The gingival mucosa contains organized lymphoid/myeloid aggregates rich in $CD4^+$ T cells, B cells, and APC, mostly dendritic cells, which can instruct the development of T-cell effector responses. The periodontal adaptive response is highly complex and may encompass both protective and destructive elements, although there is more evidence available for the latter. The destructive potential of the adaptive response in periodontitis is supported by studies in animal models. For example, severe combined immunodeficient mice, which lack both T and B lymphocytes and cannot mount adaptive immune responses, display substantially less inflammatory periodontal bone loss than immunocompetent mice. This is consistent with observations in human periodontitis: Specifically, in the bone destructive lesions of active periodontitis, T and B cells are the primary sources of receptor activator of nuclear factor-κB ligand (RANKL), which induces osteoclastogenesis by interacting with RANK, its functional receptor on osteoclast precursors. Almost 100% of activated B cells and 30 to 50% of activated T cells produce RANKL, which underscores the potential of adaptive immunity to stimulate RANKL-mediated osteoclastogenesis. This may be counteracted by osteoprotegerin (OPG), which is a decoy receptor that inhibits the RANKL-RANK interaction. RANKL levels are low in health but increase in periodontitis, whereas the converse is true for OPG. In fact, the RANKL/OPG ratio in GCF is predictive of the disease.

Mice deficient in major histocompatibility complex class II-restricted $CD4^+$ T cells also display greater resistance to experimental periodontitis than normal mice or mice deficient in major histocompatibility complex

class I-restricted $CD8^+$ T cells. These findings imply that at least one of the $CD4^+$ T-cell subsets mediates destructive inflammation in the periodontium. $CD4^+$ T helper (Th) cells comprise three major effector cell subsets, Th1, Th2, and Th17. Th1 cells are primarily responsible for cell-mediated immunity to intracellular pathogens. Th2 cells, on the other hand, mediate humoral immunity including production of IgE and activate mast cells, which mediate immune responses to helminths. The newly described Th17 subset mediates responses that reinforce innate immunity and protect against extracellular pathogens. In addition to protective effects, the same T-lymphocyte subsets can mediate harmful responses. Th1 cells were implicated in delayed-type hypersensitivity and inflammatory diseases, whereas Th2 cells were implicated in allergic reactions. After the discovery of the Th17 subset, however, the role of Th1 in destructive inflammation has been questioned in some diseases, such as rheumatoid arthritis and multiple sclerosis, where harmful responses have now been attributed to Th17.

Similarly, the original Th1 versus Th2 paradigm had not been very successful in describing the role of adaptive immunity in periodontitis. Despite some evidence for a protective Th1/destructive Th2 model, other studies found that expression of Th1-type cytokines (e.g., gamma interferon) predominate over Th2-type cytokines (e.g., IL-4) in diseased periodontal tissue, indirectly suggesting Th1 involvement in the disease. Current evidence suggests that Th17 cells play an important role in bone immunopathology; therefore, this subset is also suspected to mediate pathological bone resorption in periodontitis. IL-17, a signature cytokine of the Th17 subset but also expressed by innate immune cells, is found at increased levels in the GCF and in inflamed periodontal tissue. This suggests a potential role for IL-17 in human periodontitis, a concept that is supported by experimental evidence from animal models. In this context, IL-17 can cause connective tissue destruction and bone resorption by inducing matrix metalloproteinases in neutrophils and fibroblasts and RANKL in T cells. Ultimately, clinical studies on polymorphisms in Th17-associated cytokines, or clinical treatments with anti-IL-17 biologics may help conclusively establish the role of IL-17 and Th17 in the periodontium.

KEY POINTS

The immune mechanisms and molecules of the oral cavity derive either from the secretory or the systemic immune system. The former enter the oral cavity through the secretions of the major and minor salivary glands, whereas the latter enter through the GCF.

The oral cavity contains an array of innate antimicrobial factors secreted not only by salivary glands but also by epithelial cells and neutrophils. These antimicrobial molecules can kill or inhibit the growth of microorganisms and have broad-spectrum antibacterial, antifungal, and antiviral properties.

The single most important humoral mediator of specific oral mucosal immunity is S-IgA, which enters the oral cavity through the salivary secretions. Antibodies of this isotype provide host defense by binding to specific microbial targets and thereby inhibiting microbial adherence or neutralizing toxins.

The induction of B-cell responses which can lead to production of S-IgA antibodies in saliva is initiated at specialized inductive sites of the mucosal immune system, such as the intestinal Peyer's patches and the tonsils and adenoids, forming the Waldeyer's pharyngeal ring.

The initial host response to the maturing subgingival biofilm involves increased vascular permeability and flow of inflammatory exudate (GCF) as well as chemotactic recruitment of inflammatory phagocytes, mostly neutrophils.

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KEY POINTS

(Continued from previous page)

Elements of innate immunity in the gingival crevice include recruited phagocytes, epithelial cells (oral sulcular epithelium and junctional epithelium), and soluble inflammatory mediators, such as cytokines and complement activation products. As adaptive immunity becomes involved, other factors include antibodies elicited in response to the biofilm and lymphocytes (T cells, B cells, and plasma cells), which are largely confined to the underlying connective tissue.

Neutrophils can be found in great numbers in the gingival crevice, comprising ≥95% of total leukocytes. The recruitment of neutrophils to the gingival crevice is facilitated by a gradient of chemokines and cell adhesion molecules which directs the migration of neutrophils from the vasculature.

The coordinated and regulated recruitment of neutrophils is crucial for periodontal tissue defense, as suggested by the increased susceptibility to periodontitis of individuals with neutrophil deficiencies with regard to numbers (chronic or cyclic neutropenia), trafficking capacity (leukocyte adhesion deficiencies), or killing function (Chédiak-Higashi and Papillon-Lefèvre syndromes). However, when present in excessively high numbers, or when they fail to control dental plaque bacteria, neutrophils may cause collateral tissue damage through the release of inflammatory and toxic substances as well as tissue-degrading enzymes.

In addition to forming a mechanical barrier, gingival epithelial cells contribute to host defense by expressing an array of antimicrobial peptides (e.g., β-defensins) or regulatory molecules (cytokines and chemokines). The induction of these host defense molecules depends largely on the activation of epithelial TLRs by oral bacteria.

Complement is an antimicrobial enzyme system present in serum and inflammatory exudates, such as the GCF. Moreover, complement can orchestrate immune and inflammatory responses, often through crosstalk with TLR signaling pathways. Despite the potential for host defense, certain complement activation pathways have actually been associated with periodontal inflammation and tissue destruction.

TLRs mediate important immune and inflammatory responses to periodontal bacteria in vivo and in vitro and form a link between innate and adaptive immunity. Studies investigating single-nucleotide polymorphisms of TLRs in periodontally healthy individuals and patients with chronic periodontitis yielded inconclusive results as to the role of TLRs in periodontitis. On the other hand, TLRs appear to play a predominantly destructive role in experimental animal periodontitis.

The gingival mucosa contains organized lymphoid/myeloid aggregates rich in $CD4^+$ T cells, B cells, and APC which instruct the development of T-cell responses. The periodontal adaptive response is highly complex and may encompass both protective and destructive elements, although there is more evidence for the latter. In the bone-destructive lesions of human periodontitis, T and B cells are the primary sources of RANKL, a key factor in inducing osteoclast differentiation and maturation.

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SECTION II INFECTION-DRIVEN ORAL DISEASES

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Dental Caries: General Concepts

Robert G. Quivey, Jr., Hyun Koo, José Lemos, and Dorota T. Kopycka-Kedzierawski

OVERVIEW

Dental caries (tooth decay) is an infectious disease of the human dentition, characterized by complex interactions that occur between specific oral microorganisms, the products produced by these organisms, salivary constituents, and dietary carbohydrates on a tooth surface. These interactions modulate the formation of biofilms (clinically known as dental plaque) on susceptible tooth surfaces, which eventually leads to loss of the mineralized tooth enamel. The surfaces become permanently damaged, and the underlying dentin is then at risk of being damaged. The disease has been characterized as an ecological collision in the mouth, involving infectious bacteria and the ready availability of sugars in our diet. Specific microbial populations utilize sugars to produce extracellular polysaccharides and destructive organic acids, thus forming highly adhesive and acidic biofilms. If allowed to persist, the acidification brought about by bacteria within the biofilm results in permanent demineralization of the adjacent enamel. Thus, the formation of an extracellular polysaccharide matrix and acidification of the milieu are major virulence factors modulating the development of cariogenic biofilms.

Carious lesions which occur as a result of the dissolution of tooth enamel are caused by the prolonged exposure to bacterially derived organic acids; the disease does not occur in the absence of bacteria. Dissolution of enamel by nonbacterial acids is called acid etching, or erosion. Tooth enamel is normally bathed in saliva, which at physiological pH levels is supersaturated with respect to calcium and phosphate, the primary minerals in enamel, and is highly buffered at neutral pH. If sugar is present in sufficient quantities and frequency in the diet, oral streptococci will preferentially produce and secrete lactic acid. The milieu across the biofilm, and that in close proximity of the tooth surface, become highly acidic and are isolated from the neutralizing effects of surrounding saliva. The ionization constant of lactic acid ($pK_a = 3.5$) is below that of tooth enamel. When plaque pH values remain below a critical point, approximately pH 5.5, the balance between enamel remineralization and demineralization is disrupted and the caries process is initiated. With prolonged high levels of acid confined at the enamel-biofilm (plaque) interface, mineral dissolution proceeds to the point when crystal structure is lost, and teeth can become permanently damaged.

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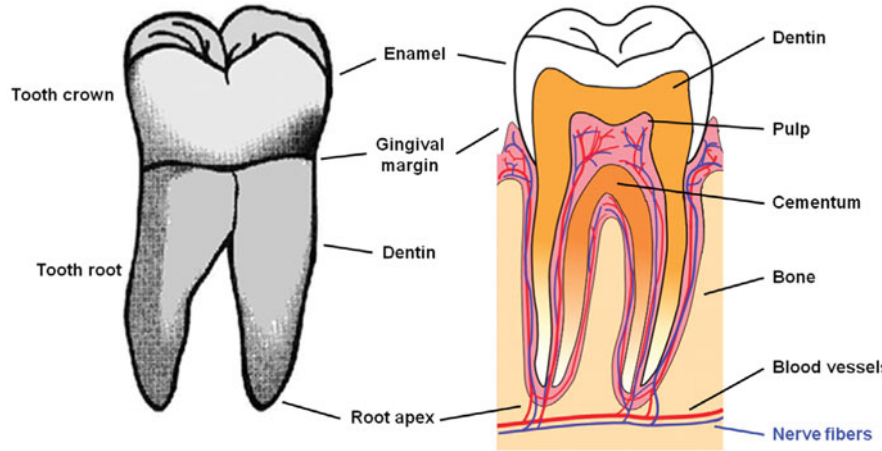
TOOTH STRUCTURE AND DEVELOPMENT

The tooth consists of a supragingival structure or crown above the gumline and a root structure below the gum (Fig. 1). The white, visible part of teeth is enamel, which covers the dentin, pulp, and cementum. Enamel is one of the most dense biologic tissues known. It is a mineralized tissue consisting initially of two proteins, amelogenin and enamelin, and protein-bound minerals, predominantly calcium, with phosphate, carbonate, and small quantities of many other minerals. As enamel is secreted, prisms of solid enamel are formed by the action of multiple cells called ameloblasts. Enamelin is elevated in aspartic and glutamic acid residues and is phosphorylated. Amelogenin, on the other hand, contains high levels of proline and histidine as well as glutamate and leucine. As enamel develops, most of the amelogenin content is lost, whereas enamelin remains bound in the crystal structure.

The mineral content of enamel has been of great interest for many years, not only for the role of trace elements in the structure and function of enamel but also as reporter compounds for the effects of the environment on human development. Two important examples should be mentioned. The first is the element lead (Pb), which at high levels of exposure in women, can lead to developmental disabilities in their children. Exposure to lead can also result in relatively softer enamel and a greater likelihood of caries formation. The second example is fluorine, which becomes incorporated into the crystal structure of enamel as fluoride and leads to a stronger crystal structure. This is a particular benefit of water fluoridation. Saliva normally promotes remineralization of enamel, and during this process, fluoride ions become incorporated and potentially strengthen the tooth surface.

Dentin, the material directly under enamel (Fig. 1) and supporting the enamel, also has a high mineral content. Dentin is much more closely related to bone and also originates from mesodermal tissue. Dentin arises from the secretion of odontoblasts and contains approximately 20%

FIGURE 1 Anatomical and structural features of the tooth in whole view (left panel) and vertical setion (right panel). doi:10.1128/9781555818906.ch11.f1



protein, primarily type I collagen, together with phosphoproteins and proteoglycans. Cementum acts to join the teeth to their sockets, arises from mesoderm, and has similar chemical composition to dentin.

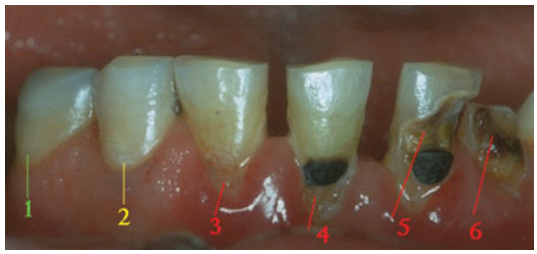
Dentin contains multiple closely packed microscopic channels, termed dentinal tubules. These tubules contain fluid and cellular structures, and they radiate outward from the pulp to the exterior cementum or enamel border. Dentin therefore has a degree of permeability as well as compressability. These features are critical for tooth function but can increase the sensation of pain and the rate of tooth decay when acid-producing bacteria enter the tubules. Dentin is produced throughout life, unlike enamel, and reparative dentin is laid down in response to stimuli such as breaching of enamel by bacteria or by attrition.

SITES OF CARIOUS LESIONS

Coronal Caries

Dental caries occurring on the region of the tooth that is above the gingival (gum) margin is termed supragingival caries, or coronal caries (Fig. 2). The smooth surfaces of the tooth are accessible to salivary flow, are easier to clean, and are subjected to mechanical abrasive forces from the tongue and lips. Therefore, bacteria need specialized colonization mechanisms to attach and accumulate on these surfaces. By contrast, bacteria can become embedded in the pits and fissures of the tooth crown without specialized adherence mechanisms. If demineralization occurs at this site, it follows the direction of the enamel rods (the basic unit of enamel structure) and runs perpendicularly from the tooth surface to the dentin. This is termed pit and fissure caries, and the advancing front is a zone of acid-demineralized dentin that does not contain bacteria. Caries that is left untreated can progress right through the dentin and into the pulp (Fig. 2). Widespread and severe lesions, which are usually associated with predisposing host factors such as xerostomia (reduced salivary flow or dry mouth), are known as rampant caries. Caries that occurs around previously treated and restored sites is known as recurrent caries.

FIGURE 2 A patient presenting with caries in multiple stages. The stages of tooth decay are described as follows. (1) A tooth surface without caries. (2) A "white spot" lesion, which is not classified as a carious lesion but, rather, as incipient caries. (3) The tooth enamel has been penetrated; this lesion is referred to as a decayed surface. (4) A decayed tooth containing a restoration, or filling, in which the lesion has continued to expand. (5) Demineralization has proceeded, leading to and undermining the tooth. (6) Damage to the tooth has become severe enough to lead to a fracture. Image and legend were generously provided by Douglas Bratthall, Malmö University, Malmö, Sweden. doi:10.1128/9781555818906.ch11.f2



Root Surface Caries

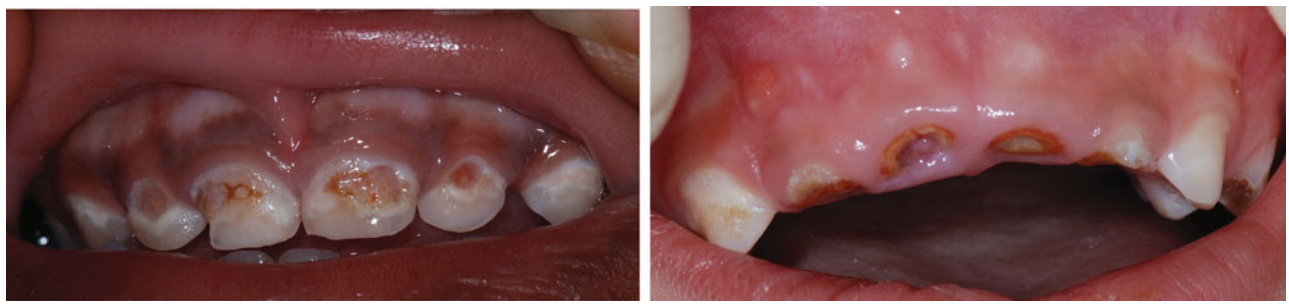
Root surface caries occurs on surfaces of the teeth that are normally covered by the gums. The disease is a significant oral health concern and is likely to become more problematic in the future because people are living longer and retaining more of their natural teeth but with receding gingiva. The cementum of the root surface is more soluble and more easily demineralized than enamel. As dissolution of the cementum progresses, the dentin will also demineralize, eventually leading to exposure of the dental pulp and loss of the pulp's collagenous matrix. The progression of root surface caries is paralleled by discoloration (Fig. 2) and changes in texture. There is transition from a hard to a softer or more leathery surface and then to a soft material as the pulp is exposed, weakening the entire structure and leading to the possibility of fractures.

EARLY CHILDHOOD CARIES

Early childhood caries (ECC) (Fig. 3) refers to severe tooth decay in infants or young children (under 71 months of age). It is epidemic in malnourished and underpriviledged populations and is principally associated with frequent consumption of liquids containing fermentable carbohydrates, such as fruit juice, soda drinks, and sweetened formulae. Prolonged contact between sugars in the liquids and cariogenic bacteria on the teeth promotes extensive demineralization. This entire process is exacerbated by malnutrition (or obesity, which is a form of malnutrition) in the pregnant mother. In these cases, children are more likely to have hypomineralized enamel in their primary dentition, thus making the teeth even more susceptible to demineralization.

Severe ECC (S-ECC) is defined by the presence of smooth-surface caries in children under 3 years. Previously termed nursing bottle caries, the disease is acute and progressive. The habit of leaving a bottle containing fruit juices, sweetened teas, or milk containing fermentable carbohydrates with a child while sleeping has a high influence on the development of S-ECC. Moreover, S-ECC has been linked to increased risk of caries development in the permanent dentition. The premature loss of primary teeth as a result of dental caries can have a profound effect on dental arch

FIGURE 3 S-ECC. In the right panel, caries has progressed to the point where the teeth have decayed and been lost at the gumline. Images generously provided by Ed Zapert, Florida State Department of Health. doi:10.1128/9781555818906.ch11.f3



status with both social and health consequences. If untreated, the disease process can rapidly lead to infection of the dental pulp tissue and possibly life-threatening fascial space involvement. These infections can result in a medical emergency requiring hospitalization, application of antibiotics, and extraction of the affected tooth or teeth. Children with S-ECC usually require operating room treatment under general anesthesia that is costly and poses potential risks to the child.

CARIES IN POPULATIONS

The prevalence of caries in large populations has closely paralleled the industrialization of nations. As wealth has increased, so too has sugar consumption in the form of foods and confections. In modern times, fluoridation of water systems (although not all communities have fluoridated water), as well as the presence of fluoride in packaged foods and beverages, has greatly reduced the prevalence of dental caries in industrialized countries. Nevertheless, reducing the risk of caries onset depends on many factors such as improving oral hygiene, dietary factors, and access to dental care. As described in the first Surgeon General's Report on Oral Health in America, summarizing a large number of studies and presenting the most recent, comprehensive snapshot of the prevalence of dental caries in the U.S. population, dental caries remains the most common chronic childhood disease in the United States, with 28% of 2- to 5-year-old U.S. children classified as having caries experience in the primary dentition.

The main dental caries dilemma in the United States and some other industrial countries today is not so much overall dental caries levels but the disparities in dental caries experience and treatment among various socioeconomic and racial-ethnic communities. Dental caries is unevenly distributed across the U.S. population with regional variations. In the early 1930s, Native Americans in the Northwest experienced severe dental caries more commonly than tribes in the Southwest. Similar regional patterns are still seen today in the general American population, and other countries also have large regional variations in caries incidence. Overall, by age 20, more than 85% of U.S. individuals have some caries, and by age 35, almost 95% of U.S. individuals have experienced dental caries. The prevalence of dental caries in the permanent dentition among dentate U.S. seniors 65 years of age and older was almost 94%. Moreover, nearly half of the U.S. dentate population over the age of 75 had at least one tooth exhibiting root caries. About 24% of U.S. seniors aged 65 to 74 years experience edentulism (all teeth lost), rising to 32% of those over 75 years old.

Socioeconomic status (SES) is also strongly associated with dental caries experience. In the United States, the higher SES groups have experienced the sharpest decline in caries experience. Among older adults, those in the higher SES groups have fewer missing teeth and more filled tooth surfaces than similar-aged adults from the lower SES strata. In parallel with this, children from families without dental insurance are three times more likely to have unmet dental needs than children with either government or private dental insurance.

BACTERIAL ETIOLOGY OF DENTAL CARIES

W. D. Miller, in the l890s, published the parasitic germ theory of dental decay in which he articulated a cogent argument that acid-producing bacteria were the causative agents of dental disease. Moreover, he also cautioned that good dental hygiene with thorough removal of the organic matter on teeth was essential to prevent disease, many years ahead of his time. The organism that presently enjoys the most attention with respect to dental caries, *Streptococcus mutans*, was first identified as an isolate from a human in 1924 by J. K. Clarke. The bacterium took its name from the varying morphologies that were exhibited during growth on different sugars. Later in that decade, *S. mutans* was identified in a high percentage of subjects with dental caries, suggesting a possible causal role in tooth decay. Definitive evidence for *S. mutans* as an etiological agent in caries was obtained in the late 1950s when Orland, Fitzgerald, Keyes and coworkers showed that strains of *S. mutans* could be used to inoculate germ-free rats and hamsters and that infection would subsequently lead to the formation of caries.

In the late 1960s and early 1970s, it was shown by Bratthall and coworkers that *S. mutans* itself was actually a group of organisms that could be distinguished on the basis of serology. Five serological groups were determined at first (groups a to e), and two more serotypes (*f* and *g*) were added later. The type most commonly associated with human disease is the serotype *c* group. As technology advanced and came to include DNA-DNA reassociation kinetics based on G:C content, Coykendall and colleagues proposed establishing four subgroups of *S. mutans*, which later became species in their own right: *S. mutans* (serotypes *c*, *e*, and *f*; genetic group I), *S. rattus* (serotype *b*; genetic group II), *S. sobrinus* (serotypes *d* and *g*; genetic group III), and *S. cricetus* (serotype *a*; genetic group IV). *S. downei* was isolated from monkeys in 1981, is very closely related to *S. sobrinus*, and has been designated serotype *h*. In 2004, a new *S. mutans* serotype, designated serotype *k*, was described in Japan. These species are known collectively as the mutans streptococci. In human dental caries, *S. mutans* and *S. sobrinus* are the predominant pathogenic species.

Epidemiological studies have shown that children are colonized by mutans streptococci early in life, soon after the eruption of the deciduous teeth. Evidence suggests that bacteria are transmitted most frequently from the mother or primary caregiver to child through salivary contact. Bacteria can be also be acquired from other members of a family or other children, especially in childcare settings. In the United States, it would appear that virtually the entire population is infected to some extent with mutans streptococci. Other acidogenic (acid producing) and aciduric (acid tolerant) organisms that can be isolated from carious lesions include species of *Lactobacillus* and *Actinomyces*. More recent molecular analyses of the genomes present within cariogenic dental plaque (metagenomic analysis, see below) have revealed the presence of many additional bacterial species, e.g., *Bifidobacterium* that may also be involved in the initiation or progression of dental caries.

THE SUPRAGINGIVAL ORAL MICROBIOME

Investigation of the numbers and identities of bacterial species present in the oral cavity, representing the oral microbiome, has been ongoing for decades. For many years, characterization of bacterial species was limited to cultivation methods involving agar growth media that would permit differential growth of specific microbial genera or metabolic groups. However, it is now accepted that approximately 50% of oral microbial species (or phylotypes) are not yet cultivable in the laboratory. Methodology now exists for identifying bacteria that does not rely on the ability to grow them in axenic culture. One technology detects specific DNA sequences of individual species that are present in complex mixtures of microorganisms. Almost all bacteria contain at least one gene that encodes a ribosomal RNA, referred to as the 16S RNA. The 16S RNA genes contain highly conserved stretches of DNA sequences that are found in all bacteria, interspersed with well-conserved sequences that can be assigned to single species (or phylotypes). Thus, bacterial barcodes can be generated representing different species. The abundance of each barcode in complex communities of bacteria can provide an approximate percentage of different bacterial species present. However, this technology is now being superseded by high-throughput sequencing, which allows sequencing of millions of short DNA sequences within a complex biological sample to determine the genomic composition (metagenome). An international project, termed the Human Microbiome Project, was initiated in 2007 to determine the complete microbial composition of the human body at multiple sites, including the skin, urogenital tract, gastrointestinal tract, nose, and oral cavity. As of 2012, the project has identified approximately 10,000 different microbial species, estimated to contribute 1 to 3% of the human body's total mass. The number of species in the oral cavity has been estimated at approximately 700 to 800 species, with about 80 to 100 species being common to most people. The microorganisms that have been identified in the oral cavity, referred to as the oral microbiome, can be viewed on the Internet at the Human Oral Microbiome Database at http://www.homd.org/.

Not surprisingly, bacteria occupy specific niches within or on the surfaces of the human body. The oral cavity contains many distinct ecological sites in which bacteria are found, including soft tissues of the gums, cheeks, and tongue, and the hard surfaces of the supragingival portion of teeth and the subgingival root surface. Importantly, the occurrence of disease has been shown to have a major impact on bacterial communities at all sites in the human body, generally resulting in reduced diversity of species. This is also true in the oral cavity. Multiple studies investigating the numbers and types of bacteria present in dental plaque samples from carious lesions at all stages, and in periodontal pockets, have shown that diversity of species can diminish substantially. In the case of dental caries, a clear progression occurs with respect to the members of the plaque microbiota. The lactic acid-producing bacteria (*Streptococcus* and *Lactobacillus*, etc.) increasingly dominate samples isolated from carious lesions as the disease progresses. Multiple studies now show that the microbiota

of dental plaque from healthy sites can contain over 100 species, including members of the genera *Streptococcus*, *Gemella*, *Abiotrophia*, *Granulicatella*, *Rothia*, *Neisseria*, *Prevotella*, and *Proteobacteria*. However, analyses of dental plaque samples from children with dental caries reveal fewer overall species present, with *Streptococcus mutans* and species of *Veillonella*, *Actinomyces*, *Granulicatella*, *Leptotrichia*, *Thiomonas*, *Bifidobacterium*, and *Scardovia* becoming more abundant. The changes in composition of the plaque microbiota as caries progresses supports the hypothesis that an ecological microbial population shift is associated with progression of the disease.

ROOT CARIES

In bacteriological terms, development of root caries is very similar to coronal caries in that the etiology involves predominance by aciduric bacteria. However, the specific bacterial etiology of root surface caries has been somewhat troublesome to establish. Greater abundance of *Actinomyces* species was for many years considered to be a characteristic of root surface caries. More recent data indicate that streptococci, as well as bifidobacteria, are more abundant on root carious lesions than on healthy surfaces in the same subject. Moreover, levels of *Actinomyces* species are often essentially equivalent on healthy versus diseased sites in the same patient. It seems that the aciduric bacteria, such as mutans streptococci and *Lactobacillus*, are the main agents associated with root caries. The higher incidence of *Prevotella* species in root carious lesions suggests that these bacteria might also be significant in the disease process. As root caries progresses into the pulp, and the collagenous matrix is lost, proteolytic bacteria like *Prevotella* species would also find a ready nutritional source in this advanced stage of disease. Metagenomic studies have also been conducted on root surface caries. These studies show that *S. mutans* is usually present in carious tooth dentin, and that additionally *Lactobacillus casei*, *L. paracasei*, and *L. rhamnosus* as well as *Pseudoramibacter alactolyticus* are frequently present.

CARIES RISK ASSESSMENT

In medical practice, risk assessment procedures are used to indicate a patient's disease susceptibility and plan for preventive measures. Dental caries risk assessment models that are currently used involve a combination of factors including diet, fluoride exposure, susceptibility of the host, composition of the oral microbiota, and various social, cultural, and behavioral factors. Assessment of a patient's caries risk entails determination of the likelihood of the occurrence of the new carious lesions in the specific time period (caries incidence) or the likelihood that there will be a change in the size or activity of the carious lesion already present in the patient's mouth. Currently, there are no caries risk factors or combination of such that have achieved high levels of positive and negative predictive values in individuals, although these parameters do reflect caries trends in populations. The best predictor for future caries experience is past caries experience, which of course is not useful in young children. For this

group, amount of plaque accumulation, mutans streptococci levels, and the age of the child when first colonized by mutans streptococci are indicators strongly associated with caries development. Notwithstanding the shortcomings of such caries risk assessments, they can help general dental practitioners identify, refer, and treat high-risk children efficiently and appropriately.

PREVENTION OF DENTAL CARIES

Fluoride

The use of fluoride to reduce dental caries in populations is one of the leading success stories of public health policy in the history of the United States in terms of its clinical effectiveness, cost-effectiveness, and distribution across the nation. The history of how community-based water fluoridation arose as a mechanism to reduce dental decay in populations is a fascinating story involving early observations by local dentists that children presenting with dark spots on their teeth also exhibited significantly reduced dental decay compared to children without the spots. Subsequent investigation led to the finding that drinking water consumed by these children contained high levels of fluoride. Formal studies into the role of fluoride as an agent capable of preventing, or reducing, dental caries were conducted on a national scale in a number of countries, representing an early public health investigation of substantial magnitude. The data from those studies provided overwhelming evidence that fluoride, at approximately 1 ppm in drinking water, leads to a substantial reduction in the prevalence and progression of caries. This finding led to the broader use of fluoride in community water supplies where it was feasible to do so.

There are a number of mechanisms by which fluoride protects tooth enamel. Fluoride present in the aqueous phase in the matrix of the enamel will substitute for hydroxyl ions in the crystal structure, a process facilitated by low pH during acid challenge. Fluoridated mineral has a lower solubility product than natural enamel and is thus more resistant to demineralization. Fluoride also interacts with calcium in the calcium-phosphate matrix of enamel and promotes remineralization of enamel by attracting calcium and phosphate ions. Finally, fluoride interferes with bacterial acid production by inhibiting metabolic enzymes and by disrupting the proton gradient across bacterial membranes and rendering the organisms more susceptible to low pH. Thus fluoride acts to maintain, stabilize, repair, and somewhat strengthen enamel.

Topical fluorides (gels, pastes, and varnishes containing fluoride compounds) have been aggressively pursued for a number of decades as a way to provide locally higher levels of fluoride-containing compounds more directly to tooth enamel. Particularly in the case of gels and pastes, an additional benefit of application derives from the debridement of dental plaque by the act of brushing or polishing of the tooth surfaces prior to application of the fluoride product. Available evidence indicates that the benefit from these products is in elevating the locally available fluoride concentrations in saliva, which then acts to promote calcium and phosphate deposition on partially dissolved hydroxyapatite crystals.

Despite the success of fluoride in reducing dental caries in many parts of the world, fluoridation of community water supplies is not always feasible. In addition, high levels of carbohydrate consumption mean that caries remains a significant public health problem in virtually all of the industrialized nations and with growing issues in developing nations. In addition to the delivery of fluoride in drinking water or in topical form, studies have been conducted with fluoride salts and fluoride in foods. As a practical matter, many foodstuffs are already fluoridated because the water used in their preparation contains fluoride. There are general health concerns that arise about the amounts of fluoride available for consumption, and additional approaches are greatly needed to control caries on a wider scale. In that vein, current research is largely directed towards developing methods for longer-term delivery of fluoride in situ and for preventing growth of lactic acid-producing bacteria on tooth surfaces.

Natural Products

The use of natural products to treat dental maladies has been common throughout history and across world cultures. Based upon the belief that there are undiscovered antimicrobial compounds available in nature, there has been considerable interest in the possibility of identifying anti-caries agents present in foods, plants, and even insect products. The types of compounds that have been explored have been grouped into general categories, which most often relate to their chemical properties.

Inhibitors of growth include a broad range of plant metabolites such as phenolic acids, anthraquinones, flavonoids, tannins, terpenoids, and alkaloids. The mechanisms by which these compounds affect growth of bacteria vary, although disruption of the cell membrane and inhibition of metabolic processes are the most common. The catechins found in green and oolong teas fall into this group, and catechin derivatives have been identified that inhibit *S. mutans* and *S. sobrinus*, although the exact mechanism of catechin-mediated activity is not yet fully established. The so-called essential oils are included in many commercial oral rinses. The terpenoids represent the largest fraction of oils, followed by aromatic and aliphatic oils. The mechanism by which oils inhibit bacterial growth appears to be through disruption of membrane function. This leads to depolarization and breakdown of membrane permeability, loss of cellular contents, and cell death.

Propolis, a product found in bee hives and in honey, is thought to function as a natural antimicrobial and antifungal agent to protect hives and contains flavanones and terpenoids, specifically tt-farnesol. Like the essential oils and oleic and linoleic acids, tt-farnesol is highly effective against *S. mutans* growing in suspension. It has also been shown to be effective against biofilm-associated *S. mutans*, and it reduces disease levels in the rat model of dental caries.

Inhibitors of acid production and acidurance are potentially useful for reducing caries levels. Several compounds that disrupt membrane function in *S. mutans* are also effective in reducing the glycolysis rate and thus lactate production. The formation of intracellular storage polysaccharides is important for survival of mutans streptococci in the oral

cavity during times of nutrient starvation. These polysaccharides are broken down to provide a source of fermentable sugars. Compounds such as fluoride, which inhibits the glycolytic enzyme enolase, may inhibit acid production as well as the formation of intracellular storage polysaccharides. Likewise, catechins from green tea also disrupt central metabolic enzymes, leading to reduced acid production as well as the ability to grow in more acidic conditions.

Polyphenolic compounds have been identified that inhibit the synthesis of extracellular polysaccharides by mutans streptococci and, thus, the formation of biofilms. Typically, however, these compounds are present as crude mixtures and additional work is needed to establish the mechanisms by which they reduce glucan formation. Interestingly, proanthocyanidin oligomers from cranberries can reduce glucan formation, and the structures of the active antiglucan compounds have been characterized. Key requirements for inhibitory activity include the position of hydroxyl groups and double bonds in the flavone structure, which may provide sites for nucleophilic attack on the glucosyltransferase enzymes that synthesize the extracellular polysaccharides.

Specifically Targeted Antimicrobial Peptides

S. mutans grown in biofilms or dense planktonic cultures produces a small peptide referred to as competence-stimulating peptide (CSP). The CSP peptide is produced when the organism is starved for nutrients, although the biological aspects of CSP are now known to include effects on the organism's ability to respond to oxygen and to transport foreign DNA into cells. A key feature of the CSP molecule, and CSP molecules in general in the bacterial world, is that they are signaling molecules specifically for the organisms that produce and secrete them. Thus, CSP from *S. mutans* sends signals only to other *S. mutans* organisms. This key insight has facilitated the development of a new type of antimicrobial peptide, one that is organism specific. Synthetic peptides are now being assembled and tested for their abilities to inhibit the growth of *S. mutans*. The general approach is to combine the *S. mutans*-specific portion of the CSP peptide with peptides derived from established, broad-spectrum antibiotics. The result is a synthetic peptide with a domain that targets *S. mutans* (via the CSP peptide region), and kills the organism via the antibiotic peptide domain. Results from testing of prototype peptides indicate that the molecules are effective in killing *S. mutans* with specificity and at relatively low concentrations. Future research will investigate the effectiveness of these compounds on microbial biofilms and in inhibiting dental caries induced by *S. mutans* in animal models of infection. There are appealing aspects to the approach of using organism-specific antimicrobial agents because they potentially preserve commensal bacterial biofilms on tooth surfaces.

KEY POINTS

Caries is a physicochemical process whereby the mineralized tissues of the tooth become demineralized as a result of the metabolic activity of bacteria. Hence, caries requires a susceptible host, pathogenic bacteria, and a nutritional substrate for the bacteria.

Caries can occur on smooth surfaces, pits and fissures, and roots of teeth. Untreated caries can extend into the dentin and pulp chamber. Severe early childhood caries is rampant caries of the primary dentition, associated with prolonged nighttime exposure to sugar-containing drinks.

Dental caries is the most common chronic childhood disease in the United States, and 28% of 2- to 5-year-old U.S. children have caries in the primary dentition. The prevalence of dental caries in the permanent dentition among dentate U.S. seniors 65 years of age and older is over 90%.

Cariogenic bacteria include mutans streptococci and lactobacilli. Mutans streptococci include the predominant human pathogens *S. mutans* and *S. sobrinus*. High-throughput sequencing has identified a number of other organisms that increase in number in carious lesions, including *Veillonella*, *Actinomyces*, *Granulicatella*, *Leptotrichia*, *Thiomonas*, *Bifidobacterium*, and *Scardovia*.

Fluoride can inhibit the metabolic activity of cariogenic bacteria, prevent demineralization of enamel, and promote remineralization. Crystals of enamel incorporating fluoride are more resistant to dissolution by acid.

Natural products, including essential oils and propolis made by bees, can inhibit cariogenic bacteria. Engineered peptides containing specific *S. mutans* binding domains and antimicrobial domains show promise as anti-*S. mutans* agents.

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Pathogenic Mechanisms in Dental Caries

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BACKGROUND

Dental caries (tooth decay) is very often found in the industrialized nations, where sugar consumption is the highest. Not surprisingly, the most extensively studied pathogen, *Streptococcus mutans*, is recognized as a highly efficient sugar-processing engine, utilizing extracellular functions and intracellular metabolism to ferment the sugars that are common in our diets. In particular, sucrose, the ordinary table sugar and the historical sweetener in cooking, is useful to the organism. Early studies involving human volunteers as well as animal models showed conclusively that irreversible colonization of the mouth by mutans streptococci occurred only in the presence of sucrose and not in the presence of monomeric sugars. Some years later, after the advent of molecular techniques, the reasons for the sucrose dependence of adherence to the tooth surface and biofilm development became clearer, as we shall explore in this chapter.

The focus on *S. mutans* as a mediator of dental caries has come about because of its (i) repeatedly high correlation with disease in epidemiological surveys of children and adults, (ii) low abundance in the absence of disease, (iii) demonstrated ability to grow at low pH values in vitro and to rapidly produce large amounts of insoluble exopolysaccharides, and (iv) ability to cause dental caries in animal models. The virulence factors of *S. mutans* are summarized in Table 1. There are two key features of *S. mutans* physiology that characterize its role in dental disease. The first is that *S. mutans* readily metabolizes dietary sucrose to form insoluble glucose polymers (glucans). These aid in the permanent colonization of hard surfaces and in the development of the extracellular polymeric substance matrix in situ, leading to the formation of highly adhesive and cohesive biofilms. The second feature is that *S. mutans* can survive at acidic pH values that are lethal to most other bacterial species present in the mouth. In fact, the organism is capable of utilizing a large number of different sugars, which are rapidly metabolized to organic acids. The ability to adapt to acidic conditions is a key attribute of the organism's pathogenic potential. In the presence of high levels of sugar, the predominant acid produced is lactic acid. This rapidly acidifies the biofilm, resulting in the selection of highly acidogenic and aciduric microbiota. If bacteria are not removed physically from the tooth surface, a cariogenic plaque will be formed. This

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TABLE 1 Virulence factors of *S. mutans*

Virulence attribute	Location	Function
SpaP (AgB, AgI/II, P1)	Surface, cell wall anchored	Adherence, binding to saliva-coated tooth surfaces and salivary agglutinin
Glucosyltransferases GtfB, -C, and -D	Secreted, often cell associated	Production of α1,3/α1,6-linked polymers of glucose from sucrose; important for adherence and biofilm accumulation
Glucan-binding proteins GbpA, -B, and -C	Surface, cell wall anchored	Binding of glucans produced by the glucosyltransferases; adherence to teeth, biofilm accumulation
Fructosyltransferase Ftf	Secreted, sometimes cell associated	Production of β2,1/β2,6-linked polymers of fructose from sucrose that can serve primarily as an extracellular reserve of fermentable carbohydrate; possibly implicated in adherence
Fructanase	Secreted and cell wall anchored	Hydrolysis of fructan polymers produced by Ftf; extends depth and duration of acidification
Dextranase	Secreted and sometimes cell associated	Endo-hydrolytic cleavage of α1,6-linked glucans; remodeling of glucan polymers to make them more water insoluble, releases glucose from polymers that can be utilized for growth and acid production
Intracellular polysaccharides	Cytoplasm	Glycogen-like polymer of glucose used as a storage polysaccharide when exogenous sources are depleted; extends depth and duration of acidification
Phosphoenolpyruvate sugar:phosphotransferase	Membrane/cytoplasm	Catalyzes high-affinity and high-capacity uptake of multiple different sugars; critical for growth and acid production
ATPase (F_1F_O ATPase or H^+ ATPase)	Membrane (F_O) and cytoplasm (F_1)	Large enzyme complex that uses ATP to pump protons from the cytoplasm; critical in acid tolerance
Acid tolerance and adaptation	Global, multifactorial	Allows organisms to develop reduced sensitivity to acids and to grow more effectively at low pH

involves accumulations and interactions of microbial cells, together with salivary proteins and food particles, with concomitant increases in plaque retention and in acid production.

COLONIZATION OF THE ORAL CAVITY BY MUTANS STREPTOCOCCI

S. mutans and *Streptococcus sobrinus* colonize the hard surfaces present in the oral cavity. While other species of streptococci can colonize the mucosal surfaces as well as enamel surfaces, *S. mutans* appears to colonize only the hard tissue surfaces. The bacteria do of course become shed into saliva, and salivary samples form the basis for quick estimations of *S. mutans* carriage in children. The colonization process can be divided into three phases: (i) adherence of bacterial cells to salivary pellicle, (ii) formation of microcolonies expressing signaling molecules and extracellular polymeric substance, and (iii) accumulations of bacteria cemented by polysaccharides (and salivary glycoproteins) (Fig. 1).

Initial adherence of *S. mutans* to the tooth surface involves the action of a major surface protein adhesin termed antigen I/II. This is produced by all strains of *S. mutans* and *S. sobrinus* that have been examined, and the adhesin recognizes glycoproteins present in saliva as receptor molecules. The salivary factor for which antigen I/II has highest affinity is a glycoprotein named salivary agglutinin. This is now referred to as gp-340 (glycoprotein with molecular mass of 340 kDa) and is also found in tears and

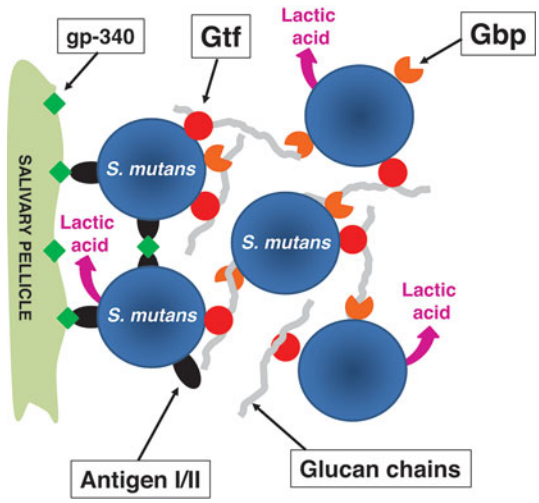

FIGURE 1 Essential features of the mechanisms by which *S. mutans* adheres to salivary pellicle, accumulates to form dental plaque, and produces dental caries. Streptococci adhere to salivary glycoproteins, such as gp-340, present within the salivary pellicle that coats the tooth surface. In the presence of sucrose, glucosyltransferase enzymes (Gtfs) produce glucans and these are trapped at the streptococcal cell surfaces by glucan-binding proteins (Gbps). In animal models, the GtfB and GtfC enzymes and glucan-binding proteins A and C are essential for biofilm formation and development of caries. doi:10.1128/9781555818906.ch12.f1

secreted by the lung and gut epithelia. The primary function of the salivary agglutinin is to aggregate bacteria and viruses so that they can be disposed of, either swallowed or engulfed by macrophages. However, when the agglutinin protein (gp-340) becomes deposited upon the tooth surface, it acts as a receptor for antigen I/II protein and promotes adherence of *S. mutans*. The antigen I/II is linked to the streptococcal cell wall through the carboxy terminus of the protein, and it forms an elongated structure composed of a globular base region, a stalk, and a head. Each of these regions has been shown to react with gp-340, suggesting that it might be possible for the bacteria to be aggregated by gp-340 while they are attached to gp-340, hence forming microcolonies. Interest in the antigen I/II protein has been sustained because peptide sequences derived from the amino-terminal region of the polypeptide can be used to vaccinate rodents and provide protection against *S. mutans* colonization and subsequent dental caries. In addition, monoclonal antibody reacting with a portion of the neck region of antigen I/II has been used in passive immunization studies to protect human volunteers against recolonization by *S. mutans*.

POLYSACCHARIDE PRODUCTION

S. mutans synthesizes and secretes four extracellular enzymes, referred to as glucosyltransferases (Gtfs) and a fructosyltransferase (Ftf) (Fig. 2). These enzymes split (hydrolyze) sucrose into glucose and fructose and concomitantly form polymers of glucose (glucans) or fructose (fructans), respectively. There are three *S. mutans* glucosyltransferases, designated GtfB, GtfC, and GtfD. These are themselves diverse in that one of the enzymes, GtfB, acts to form insoluble glucans with a predominance of

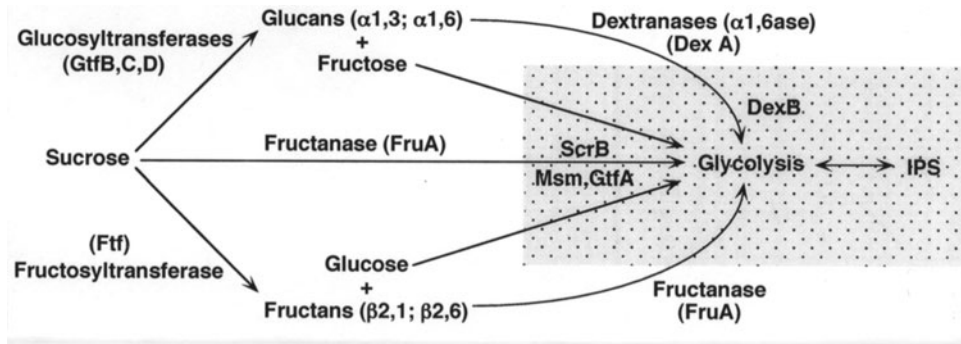

FIGURE 2 Metabolism of sucrose by *S. mutans*. The disaccharide sucrose can be converted to the polymer glucan or fructan by the enzyme glucosyltransferase (B, C, or D) or fructosyltransferase, respectively. Energy is provided by splitting of the glucose-fructose bond, and in both cases, monomers of the nonpolymerized monosaccharide (fructose or glucose) are produced. Fructanase can split sucrose or fructan and provide monsaccharides for glycolysis inside the bacterial cell. Similarly, dextranase can convert glucan into glucose for glycolysis. Intracellularly, glucose can also be polymerized into intracellular polysaccharide, which can be mobilized for glycolysis as necessary. doi:10.1128/9781555818906.ch12.f2

α1,3 linkages between the glucose monomers. GtfC produces a mixture of insoluble and soluble glucans (rich in α1,6 linkages), whereas GtfD functions to produce primarily soluble glucans. The Ftf catalyzes synthesis of a fructan polymer, which is soluble and serves as an extracellular, readily metabolizable, reserve energy source. The Gtf enzymes contain two domains; one possesses glucan-synthesizing activity and the other functions as a glucan-binding domain. Mechanistically, the catalytic domain hydrolyzes sucrose and the glucose produced is added to a glucan chain that is attached to the glucan-binding domain. The enzymes can therefore be considered polymerases, adding glucosyl or fructosyl residues onto glucan or fructan primers. The functions of these enzymes are to produce extracellular polysaccharides that potentially provide a storage of carbohydrate that can be utilized when nutritional conditions are unfavorable. The more soluble polysaccharides can be degraded rapidly by dextranase enzymes to produce glucose for energy production. The less-soluble polysaccharides act to cement the cells together in a matrix.

Accumulation of *S. mutans* into a resilient biofilm requires the activity of a third set of protein factors designated glucan-binding proteins (Gbps). The functions of these proteins are to hold the polysaccharides that have been synthesized by the Gtf enzymes close to the bacterial cell surface. There are four Gbps identified to date, designated GbpA to GbpD. The production of Gbps is essential for *S. mutans* to form a biofilm or accumulate as plaque because they effectively cross-link the bacterial cells through binding of the extracellular glucans (Fig. 1). GbpA, GbpB, and GbpC have each individually been shown to be necessary for caries production in rodent models. The really essential function of GbpB in the process of caries development is suggested by observations that immunization of rodents with selected domains of GbpB provides protection against *S. mutans* colonization.

Gtf enzymes, derived from streptococci, are found in saliva and are readily adsorbed into experimental pellicle and onto bacterial cell surfaces. These are then able to synthesize glucans from available sucrose and

therefore provide additional adherence sites for streptococci expressing Gbps on their surface. Although all three Gtf enzymes can bind to pellicle, their affinities are quite different, with GtfC displaying the strongest binding. *S. mutans* (and other oral microorganisms) bind avidly to glucan-coated surfaces, particularly those synthesized in situ by GtfB and GtfC, through interactions with cell-associated Gbps. Interestingly, Gtfs become bound to other oral bacteria and also to the fungus *Candida albicans*. The enzymes retain their activities when adsorbed to other microorganisms, thereby converting non-Gtf producers into de facto glucan formers. The simultaneous synthesis of glucans by surface-adsorbed GtfB and GtfC (rich in insoluble α1,3 linkages) enhances bacterial adherence and provides opportunity for aggregation and accumulation of bacterial cells. In combination with data that show a disruption of normal biofilm development and caries induction by mutant strains defective in Gbps and Gtfs, the conclusion is inescapable that glucan production and glucan-binding are critical elements of the *S. mutans* infectious process. Interestingly, Gtf enzymes are not expressed constitutively (all the time) but are produced at higher levels during growth of bacteria at lower pH, a setting that would also promote the dissolution of enamel and the initiation of carious lesions.

In summary, the picture that has emerged collectively from three decades of study is the following. *S. mutans* binds to salivary pellicle and takes advantage of the presence of sucrose to bind irreversibly to surfaces through the ability to produce and adhere to glucans. The formation of highly adhesive and cohesive biofilms closely attached to the tooth surface generates a positive feedback cycle. The acidic microenvironment selects for aciduric-acidogenic organisms, which in turn ensures continued, localized acid production. So long as the biofilm remains undisturbed, it becomes more acidogenic with consequent enamel demineralization.

ACID PRODUCTION

The final piece in the jigsaw of *S. mutans*-induced dental caries is the production of lactic acid (Fig. 1). The processes of adherence and accumulation would not lead to tooth decay without production of lactic acid. *S. mutans* can ferment a range of sugars, generating weak acids including lactic, formic, and acetic acids as metabolic end products (see Fig. 1 in chapter 6). Lactic acid is among the strongest of these acids and is capable of reducing the plaque pH to below 5.5, levels at which enamel can begin to be solubilized. Sucrose is the most cariogenic sugar because the component hexoses (glucose and fructose) are very efficiently fermented to produce lactic acid (in addition to providing the building blocks for extracellular polysaccharide production). *S. mutans* utilizes a high-affinity and high-capacity phosphenolpyruvate sugar-phosphotransferase uptake system to transport sugars into the cells. Sucrose accumulates as sucrose-6-phosphate, which is then hydrolyzed to glucose-6-phosphate and fructose, which are metabolized through the glycolytic pathway. *S. mutans* has additional sugar uptake systems including proton motive force-driven transport. Glycolysis of one C_6 (hexose) sugar produces two molecules of pyruvate (C_3). Lactate dehydrogenase then converts pyruvate into lactic acid using NADH as an electron donor. Organisms such as *S. mutans* are considered homofermentative, as almost all pyruvic acid is converted to lactic acid. There are, therefore, four points of attack that could be

targeted to prevent *S. mutans*-induced dental caries (Fig. 1). These are adherence to the tooth surface, production of extracellular glucans, expression of glucan-binding proteins, and production of lactic acid.

ACID TOLERANCE AND STRESS RESISTANCE

After bacteria have colonized the tooth surface, their survival depends upon the ability to cope with several types of environmental stress. For example, fluctuations in the source and availability of carbohydrates will affect growth rates. The presence of oxygen and its metabolites would be inhibitory to anaerobic metabolism, and accumulation of organic acids will lead to feedback and inhibition of glycolysis. Since the caries process is clearly linked to the ability of *S. mutans* to thrive under stressful conditions, *S. mutans* has served as a model organism to study acid stress physiology. In addition, the acid tolerance mechanisms of commensal oral bacteria involve production of ammonia, thus raising the cytoplasmic and environmental pH, and conferring protection against acidic conditions.

The complete genome sequence of a serotype *c* strain of *S. mutans*, published in 2002, provided a basis for a better understanding of the acid-adaptive strategies of the organism. Unlike enteric bacteria that usually maintain internal pH (pH_i) at fairly constant 7.6 to 7.8, the pH_i in streptococci fluctuates in response to extracellular pH, with the organisms working to maintain a ΔpH of 0.5 to 1 unit above that of the external environment. The membrane-bound F_1F_0-ATPase is the primary mechanism by which protons are extruded to maintain pH homeostasis. By helping to maintain pH_i closer to neutrality, the F-ATPase ensures that acid-sensitive glycolytic enzymes are protected from inhibition and can still function in situations where the external pH is below levels that sustain growth. Acid tolerance levels correlate well with the pH optimum of the F-ATPase enzyme. For example, in the highly aciduric bacteria *Lactobacillus casei* and *S. mutans*, the optimal pHs for the F-ATPase enzymes are 5.5 and 6.0, respectively. On the other hand, for less acid-tolerant strains of *Streptococcus salivarius* and *Streptococcus sanguinis*, the pH optima of the ATPase enzymes are 7.0 and 7.5, respectively. It should be noted that acid-tolerant strains of *S. sanguinis* and *Streptococcus parasanguinis* can be found in acidogenic plaque so the aciduric phenotype is not restricted to the mutans streptococci.

Alterations in the membrane fatty acid composition is another important contributor to acid tolerance by *S. mutans*. Specifically, growth under acidic conditions causes a substantial increase in the proportion of unsaturated membrane fatty acids compared to growth at neutral pH values. Inactivation of the *fabM* gene responsible for biosynthesis of monounsaturated fatty acids in *S. mutans* results in extreme sensitivity to low pH, inability to maintain ΔpH, and substantial reduction in the numbers and severity of caries lesions in a rat model.

An important mechanism for acid protection utilized by many noncariogenic oral streptococci is the production of ammonia through the activities of urease enzymes or the arginine deiminase system (ADS). Organisms carrying these enzymes are able to convert urea or arginine, respectively, to produce CO_2 and ammonia. The latter compound neutralizes acid and provides the microorganisms with a competitive advantage in acidified biofilms. Since both urea and arginine are relatively abundant

in the oral cavity, ammonia-producing bacteria would seem to have the ability to limit reduction of pH in situ and, as a result, modulate plaque composition. Consistent with this, studies involving caries-free or caries-active subjects have showed a strong negative correlation between *S. mutans* and ammonia released by ADS or urease activities.

Although *S. mutans* is not capable of generating significant quantities of alkali, an agmatine deiminase system (AgDS) is broadly found in lactic acid bacteria, including *S. mutans*. The biochemical pathway of the AgDS yields ammonia, CO_2, and ATP, while agmatine is converted to putrescine. Whereas the ADS and urease pathways catalyze substantial environmental alkalinization, and appear to be associated with caries resistance, the AgDS of *S. mutans* is expressed at relatively low levels and is unlikely to elicit a significant alkalinization of the environment. However, the production of ammonia from agmatine appears to contribute to the competitive fitness of *S. mutans* at low pH by increasing cytoplasmic pH and generating ATP that can be used for growth, or to extrude protons.

Another potential contributor to acid tolerance of lactic acid bacteria is malolactic fermentation. This catalyzes the conversion of dicarboxylic L-malate, a major acid in fruits such as apples, to lactic acid and CO_2. The net effect of the transport and enzymatic reactions is the formation of a pH-gradient, thereby increasing the proton motive force available for the cell to take up other nutrients. Because malate utilization contributes to maintenance of ATP pools and the CO_2 generated can be rapidly converted to bicarbonate via carbonic anhydrase, malolactic fermentation enhances the buffering capacity of the bacterial cell. While malate does not serve as a catabolite for the growth of *S. mutans*, it protects the organism against acid killing by increasing the pH of the cytoplasm via production of CO_2.

Genome-wide transcriptional and proteomic approaches have also identified proteins involved in protection, repair, and degradation of macromolecules, including molecular chaperones, proteases, and DNA repair enzymes, which are an integral part of the *S. mutans* general stress response. Further, it has become clear that *S. mutans* has developed a sophisticated network of regulatory strategies to coordinate an appropriate and effective response against sudden and frequent fluctuations in the plaque environment. The strategies include utilization of two-component signal transduction systems, production of quorum-sensing signaling molecules, effective carbon catabolite repression, and expression of nutrient-sensing regulators.

ANIMAL MODELS OF CARIES

The use of the animal model, specifically the rodent caries model, has had a profound effect on the understanding of the pathophysiology and the infectious character of dental caries. Prior to the use of animal models, there were a number of different theories regarding the etiology of caries. These included a genetic predisposition for the disease, a physicochemical process, and the chemoparasitic theory. With the arrival of laboratory germ-free rats and hamsters in the 1950s, scientists had animal models that were available to test their theories. Major breakthroughs in caries research came from studies of scientists such as those of Orland and his colleagues and Fitzgerald and Keyes. Through their work using rats and

Syrian hamsters, it was discovered that dental caries was a transmissible disease. It was not likely to be related to genetic susceptibility but by combined activities of microorganisms and a high-sucrose diet. The organisms causing decay were found in the oral cavity and in the feces of the animals and were later confirmed to be mutans streptococci.

Subsequent studies using animal models have provided critical information about (i) the infectious character of the disease, (ii) the effects of diet and frequency of eating, (iii) the contribution of specific *S. mutans* genes to virulence via colonization, transmissibility, and carious lesion evaluations, and (iv) the influence of salivary proteins on dental caries development. This well-established model has also been valuable for investigating the therapeutic effects of applying topical treatments of cariostatic agents (mimicking clinical use in humans). Importantly, to date, it has never been shown that agents effective in the rodent model are not also efficacious in humans when evaluated in a comparable fashion. Furthermore, results from studies that have used surgically desalivated animals have provided information to the clinical setting for patients suffering from xerostomia.

Over the last five decades, the rat has been the most commonly employed animal for a caries model. Larger animals such as dogs, cats, horses, and pigs are now seldomly used, mainly because of cost issues but also because they are less susceptible to the disease. Primates, particularly macaques, have been used in investigations generally related to caries vaccine work because of their similarities to humans both in general physiology and in the organization of their dentition. However, the difficulties of working with macaques, the cost of maintaining them, and their availability has dramatically reduced their use in routine caries studies.

The Rat Caries Model

Rats are an appropriate model for the study of dental caries because the pathogenesis of caries is essentially the same in rats and humans. The distribution of caries lesions found in rats is similar to that found in humans when fed a diet that is rich in carbohydrate content. In addition, many of the salivary proteins found in humans are also found in rats. There are several strains of rats such as the Cotton, Osborne-Mendel, Wistar, and Sprague-Dawley that are commonly used in caries models. Two types of experimental rat models are potentially available for use. First is the gnotobiotic model, in which pups are delivered by cesarean section and raised in isolation using sterilized food, thus preventing colonization by the normal, indigenous microbiota. The far more often used alternative is the specific-pathogen-free animal model. These animals are screened for the presence of human strains of oral microorganisms and the presence of other infectious agents, such as sialodacryoadenitis virus, prior to their use in caries experiments.

In Sprague-Dawley (albino) rats, the first mandibular and maxillary molars usually erupt into the oral cavity around the 16th day of age. The second mandibular and maxillary molars erupt 1 or 2 days later, and by the 20th or 21st day, when rats are normally weaned, all four molars have erupted. The third mandibular and maxillary molars usually erupt when the pups are 33 days old. In the experimental setting, the pups are usually infected around the time of weaning from their mothers, generally in the period of 17 to 21 days following their birth. The procedure involves

dipping sterile, cotton swabs into a bacterial suspension, placing the swab carefully into the animal's mouth, and swabbing all surfaces of the molars. Difficulty in achieving a productive infection may arise with procedures that are performed more than 27 to 28 days after birth. Several studies have shown that the age of the animal can affect the ability of the bacteria to implant into the animal's mouth. There appear to be at least two reasons for the need to infect the animals relatively quickly after weaning. The first is that the indigenous microbiota may block *S. mutans* infection; the second reason is that the molars are hypomineralized upon eruption and then become mineralized over time, thereby introducing another variable into the experimental design. Reintroduction of the infectious agent is often performed on each of several successive days following the first infection, the purpose being to ensure infection. It is quite common to use antibiotic-resistant strains of bacteria, such that selective bacteriological media can be used to verify the success of swabbing.

The formation of dental caries is dependent on the availability of carbohydrates, and many foods have been examined for their ability to promote dental caries in rats. However, the model system typically relies on sucrose being present in the diet. Commercially available dietary formulations provide for a high-sucrose (56%) diet or a lower-sucrose (5%) diet. Debate continues as to the usefulness of one type of diet over the other, but in both cases, sucrose is in excess. There is overwhelming agreement for the essential role of sugar in the formation of caries in model systems and in humans.

Caries Scoring in Rats

Significant advantages of the rodent model for caries are that the depths of carious lesions can be measured and the incidence and severity of lesions can be recorded at multiple sites (sulcal, smooth, and interproximal) simultaneously, and there is an established coding method for scoring the disease, deveoped initially by Paul Keyes in 1958 and subsequently modified by Larson. The scoring system relies on judging the linear extent of the lesion and then the severity of the lesions by measuring the depth of penetration from enamel into dentin. Each molar surface is assigned units to describe the size and severity of the lesions, so from the results, the extent of decay can be determined. Examples of rat mandibles exhibiting carious lesions compared to jaws prepared from rats fed a noncariogenic diet are shown in Fig. 3.

FIGURE 3 Rat mandibular molars prepared for caries scoring. (A) Jaws prepared from a rat fed a noncariogenic diet. (B) Jaws prepared from a rat fed a highly cariogenic diet. Images were generously provided by William Bowen, University of Rochester, Rochester, NY.
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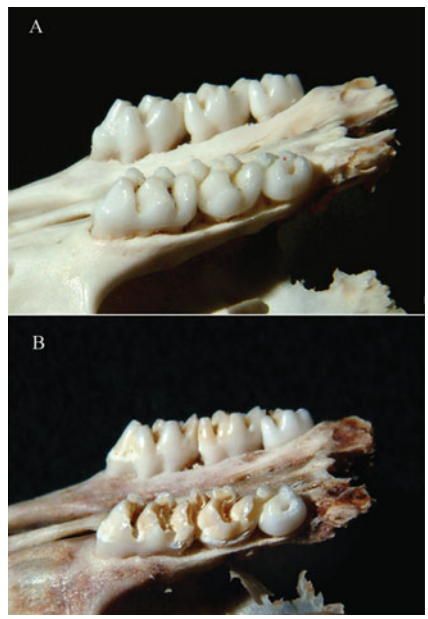

Caries Models in the Future

Considerable effort has gone into the development of alternative models for caries, including artificial mouths and the use of prosthetic devices containing enamel slabs worn in the mouths of human volunteers. Artificial mouths will require some period of time to reach a reasonable facsimile to the human mouth. These are basically in vitro models; thus, extrapolations of the outcomes to clinical relevance are limited. Nevertheless, such models may provide additional relevant information about ecological changes in the biofilm during development of acidogenicity.

Intraoral devices, worn in human mouths, have the potential for delivering pertinent information. Small slabs of enamel, dentin, or other materials may be carried in the mouth on orthodontic brackets or similar mounting appliances. The concept is to allow the materials to be bathed

in normal saliva and for the subject to grow their natural microbiota on the device. One use of such a model is to subject the device to a treatment schedule to determine effects on development of the microbial community. The principal experimental issue with intraoral devices revolves around the nature of the device used to mount the slabs. Construction of the appliance can introduce other variables or constraints into the experimental design, which in turn may introduce differences in the observations vis-à-vis normal tooth surfaces.

Another caries model, an alternative animal model to the rat, is the mouse. Mice have been used relatively infrequently in caries experiments, largely owing to their smaller size and the relatively more delicate association of molar teeth in the jaw. The advantage with mice is that the genetic stocks have become very well defined over the last two decades. As a result of mouse genomic sequencing, we have learned that mouse salivary protein genes are syntenic, that is, arranged in an identical fashion, with human salivary protein genes. This suggests a high degree of conservation in form and function of salivary proteins between mice and humans. This fact alone could prove important, in that loss of salivary functions is well known to lead to rampant caries in humans and in rodents. Thus, the ability to manipulate salivary protein genes in mice could be extremely useful for studies on the role of salivary components in the balance between infection and protection of teeth. The procedures and tools established in the rat model are available for use with mice, with the added advantage of being able to genetically manipulate mice with a far greater degree of precision than is presently possible with rats. For these reasons, the mouse offers a potentially powerful system to examine the interplay of oral bacteria with host proteins and tissues.

KEY POINTS

The physiologic process that leads to dental caries is the production of organic acids, predominantly lactic acid, by bacteria from dietary carbohydrates. The organic acids lower the pH at the tooth surface to the point where the substituted hydroxyapatite of enamel becomes soluble.

S. mutans also utilizes dietary sucrose to produce polymers of glucan (built from monomers of glucose) and fructan (built from monomers of fructose) through the action of glucosyltransferases and a fructosyltransferase, respectively. Insoluble glucan polymers help attach the bacterial cells to the tooth surface through the activities of glucan-binding proteins present on the bacterial cell surfaces. Glucan and fructan can also be used as reserves of sugars for metabolism and growth.

Cariogenic bacteria such as *S. mutans* are aciduric; that is, they can resist the adverse effects of the low pH that result from their metabolic activities. Mechanisms of acid resistance include pumping protons out of the cytoplasm, increasing the proportion of unsaturated membrane fatty acids, induction of stress response proteins, and raising pH through ammonia-producing systems.

Rat models of caries are useful for testing the pathogenicity of bacteria and the effect of anticaries agents. Mouse models have the advantage of testing caries induction in defined genetic backgrounds.

FURTHER READING

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Periodontal Diseases: General Concepts

Panos N. Papapanou

INTRODUCTION

"Periodontal diseases" is a collective term used to describe the inflammatory changes of the tooth-supporting structures, i.e., the gingiva, the alveolar bone, the periodontal ligament, and the root cementum, that may lead to tissue destruction, reduced tooth support, and ultimately, tooth loss. In periodontal health, the gingiva surrounds the teeth approximately at the level of the cementoenamel junction and forms a shallow crevice, ranging in depth between one and four millimeters, depending on local anatomy. Undisturbed accumulation of dental plaque, i.e., the bacterial biofilm that forms on the tooth surface at the area of the gingival margin, elicits a local inflammatory response. This inflammatory lesion is termed gingivitis, is manifested through alterations in gingival color and texture, and is usually accompanied by bleeding upon mechanical stimulation, typically provoked by oral hygiene procedures (tooth brushing and flossing). In susceptible individuals, prolonged accumulation of dental plaque causes longstanding gingival inflammation that may result in the gradual deepening of the gingival crevice (now termed "periodontal pocket") and in the concomitant destruction of the periodontal ligament and the alveolar bone, a pathological condition termed periodontitis. In the absence of therapy, this destructive process may progress further and reach a point at which the remaining tissues are inadequate to retain the tooth in its alveolar bone housing, and tooth loss is inevitable (Fig. 1).

This chapter provides an overview of the key features of periodontal disease. First, it offers a description of the current classification system of the different forms of periodontal disease, as well as a review of their epidemiology and risk factors. The microbial etiology of these conditions is subsequently discussed, followed by a brief description of therapeutic approaches and preventive strategies. The chapter concludes with a brief discussion of periodontal disease as a potential risk factor for nonoral pathologic conditions.

CURRENT CLASSIFICATION OF PERIODONTAL DISEASES

As is quite common in medicine, classification systems for pathologic conditions are ever-evolving schemes that are periodically revised to reflect current knowledge related to the clinical presentation, etiology,

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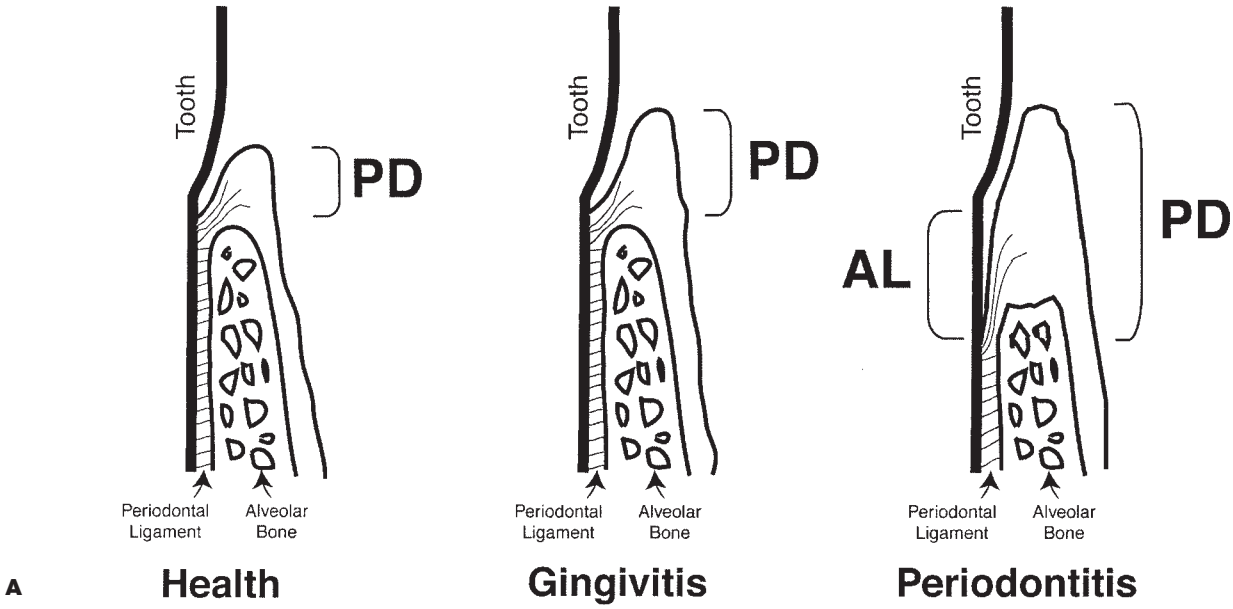

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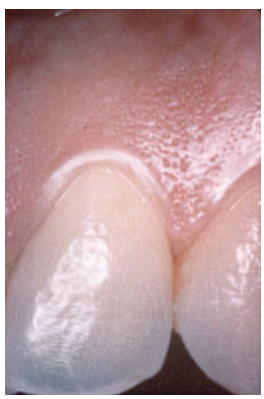

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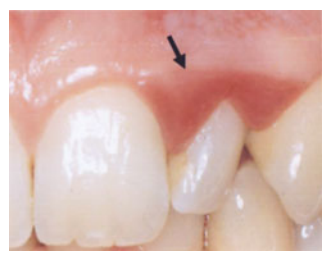

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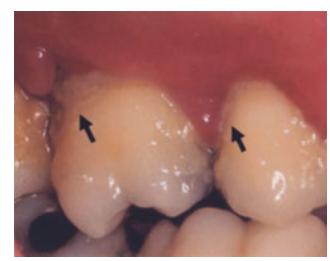

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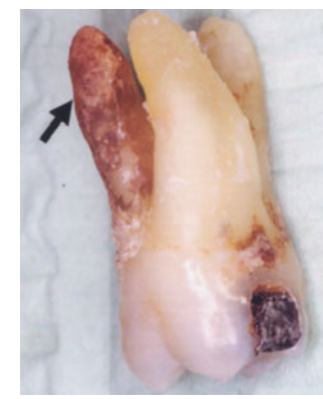

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FIGURE 1 Periodontal tissues in health and disease. (A) Schematic representation of health, gingivitis, and periodontitis. The periodontal pocket depth (PD) is increased in gingivitis due to tissue swelling associated with inflammation. In periodontitis, the PD is further increased due to the loss of the tissue attachment to the root of the tooth (attachment loss [AL]). Periodontitis is also characterized by loss of supporting alveolar bone. (B) Periodontal health. Note the lack of evidence of inflammation in the tissues, and the stippled appearance of the gingiva. (C) Gingivitis. Inflammation is present in the gingival tissues (arrow), evidenced by redness and swelling. (D) Periodontitis. Severe gingival inflammation is evident. Note the abundant accumulation of plaque and calculus (arrows). (E) Extracted molar from patient in panel D. Note the presence of calculus on the distal root (arrow) that extends to the apex of the root. (Images in panels A, C, D, and E courtesy of Susan Kinder Haake.)
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pathobiology, treatment response, or long-term prognosis of the disease in question. The classification of periodontal diseases is no exception, and after several revisions over the years, the currently accepted system reflects the consensus report of an international conference in 1999 that was specifically convened for that purpose. This scheme defines eight main categories of periodontal disease (Table 1).

TABLE 1 Current classification of periodontal diseases and conditions

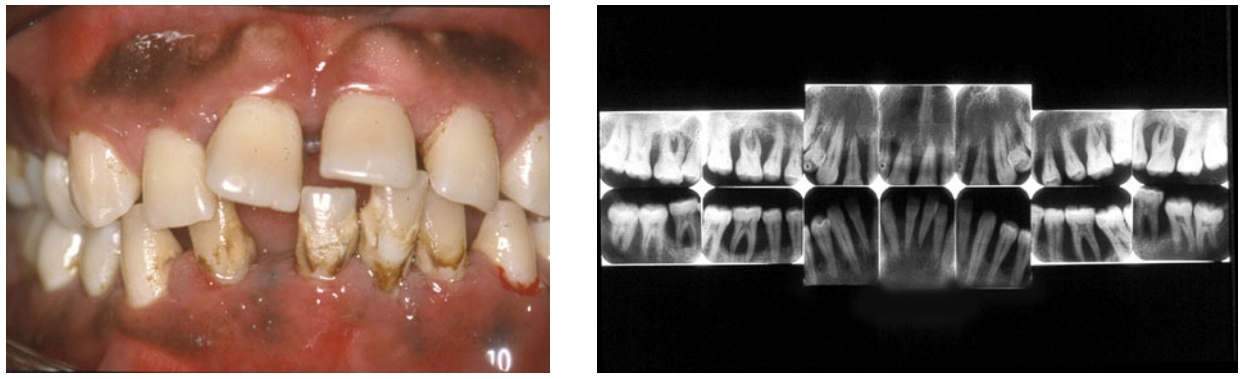
I.	Gingival diseases
	a. Dental plaque-induced gingival diseases
	b. Non-plaque-induced gingival lesions
II.	Chronic periodontitis
	a. Localized
	b. Generalized
III.	Aggressive periodontitis
	a. Localized
	b. Generalized
IV.	Periodontitis as a manifestation of systemic disease
V.	Necrotizing periodontal diseases
VI.	Abscesses of the periodontium
VII.	Periodontitis associated with endodontic lesions
VIII.	Developmental or acquired deformities and conditions

[a]Source: International Workshop for the Classification of Periodontal Diseases and Conditions. From G. C. Armitage, *Ann. Periodontol.* **4:**1–6, 1999.

The first category, gingival diseases, describes pathological alterations confined to the gingival tissues that have not yet resulted in loss of periodontal tissue support, i.e., in attachment loss or bone loss. These conditions either are induced by bacterial plaque and may be further modified by systemic factors, medications, or malnutrition or may be unrelated to dental plaque accumulation. The latter category includes lesions of distinct bacterial origin (such as the ones occurring in venereal diseases such as syphilis or gonorrhea), viral origin (e.g., herpesvirus infections), or fungal origin (candidiasis) as well as gingival lesions of genetic origin (hereditary gingival fibromatosis), systemic mucocutaneous disorders (e.g., lichen planus, pemphigoid, pemphigus vulgaris, erythema multiforme, or lupus erythematosus), or allergic reactions and traumatic lesions.

In contrast, periodontitis is by definition a plaque-induced inflammatory disorder that has resulted in loss of periodontal tissue support. The two principal categories of periodontitis are chronic and aggressive periodontitis. Either condition can be further characterized as localized or generalized, depending on the number of affected teeth in the dentition.

Chronic periodontitis is the most common form, it affects individuals of all ages, it is commensurate with the level of local etiologic factors (i.e., plaque accumulation), and it usually progresses at a relatively slow pace with increasing age. In contrast, as the term indicates, aggressive periodontitis is a destructive form of periodontal disease that is characterized by rapid bone loss and attachment loss (Fig. 2). It aggregates within families, implying that its pathobiology is strongly determined by genetic factors and common environmental exposures, and may affect young individuals, particularly in its localized form, which is not associated with conspicuous plaque accumulation or gingival inflammation. As discussed below, localized aggressive periodontitis has been closely linked to infection by a particular periodontal pathogen, *Aggregatibacter actinomycetemcomitans*. In addition, patients with aggressive periodontitis have been shown to have abnormalities in polymorphonuclear leukocyte function, although these observations have not been unanimously corroborated and are seemingly incompatible with the fact that these individuals



A B

FIGURE 2 Clinical (A) and radiographic (B) presentation of a 16-year-old patient with aggressive periodontitis. Note that the severity of bone loss extends to the apex at several teeth. doi:10.1128/9781555818906.ch13.f2

suffer no other pathological condition besides periodontitis. The high susceptibility of these individuals to periodontitis has also been postulated to be partly explained by hyperresponsive macrophages that produce high levels of proinflammatory mediators involved in tissue destruction. Again, it is difficult to determine with certainty if this altered cellular phenotype constitutes a preexisting susceptibility factor or rather is a result of the disease itself.

The fourth category, periodontitis as a manifestation of systemic disease, encompasses a number of diverse pathologic conditions, all of which also entail loss of periodontal tissue support. These include hematological disorders (e.g., acquired neutropenia or leukemias) and various syndromes of genetic origin (e.g., trisomy 21; leukocyte adhesion deficiency; Papillon-Lefèvre, Chédiak-Higashi, Ehler-Danlos types IV and VIII, and Cohen syndromes; and histiocytosis).

Necrotizing periodontal diseases manifest themselves either as necrotizing ulcerative gingivitis, if the lesions are exclusively confined to the soft tissues, or necrotizing ulcerative periodontitis, in situations where the lesions have resulted in loss of periodontal tissue support. Both conditions are characterized by typical painful, necrotic gingival lesions that harbor invading periodontal bacteria (fusobacteria and spirochetes). Psychoemotional stress and excessive smoking have been identified as risk factors. In addition, immunosuppressed patients such as those suffering from AIDS may present with necrotizing forms of periodontitis.

Abscesses of the periodonticum are localized lesions that are characterized by pronounced inflammation, swelling, and frequently, purulent exudate. They are arguably not distinct forms of periodontal disease (as they can occur during the course of any of the types of periodontal disease described above) but were recognized as individual entities due to their characteristic clinical appearance and their particular therapeutic requirements.

In contrast, periodontitis associated with endodontic lesions represents pathological conditions that bear all of the characteristics of a frank periodontal lesion (gingival bleeding, deep periodontal pocket, and loss of attachment and alveolar bone), yet their etiology originates from an

endodontic infection that has subsequently spread into the periodontal tissues through the apical foramen or a pulpal accessory canal. The correct identification of the etiology of these lesions is obviously essential for their successful therapeutic management.

Lastly, the final category, termed developmental or acquired abnormalities and conditions, encompasses a series of anatomical defects that may either predispose to the development of periodontitis or have developed as a result of periodontal pathology and are typically corrected through periodontal surgical procedures. Examples of such conditions include localized tooth-related factors (e.g., cervical root resorptions or cemental tears), occlusal trauma, and mucogingival deformities around teeth (recession defects, lack of keratinized gingiva) or on edentulous ridges, which need preprosthetic intervention.

Twelve years after the introduction of the current classification system, it has become increasingly apparent that the above scheme suffers from significant shortcomings. These include lack of diagnostic precision, resulting in substantial overlap between categories, and difficulty in applying the stipulated criteria in the everyday clinical practice. For example, it is impossible to ascertain on the basis of a single examination whether the progression of the bone loss or attachment loss has been rapid or not, an assessment that is essential for the diagnosis of aggressive periodontitis. Likewise, it is difficult to determine when the second primary classification criterion for aggressive periodontitis, familial aggregation, is fulfilled, as patients are frequently unaware of the periodontal status of their siblings or parents. But more importantly, there appears to be a lack of a solid, pathobiology-based foundation for the distinction between two main disease categories, chronic and aggressive periodontitis. Therefore, further revision of the current classification is both necessary and inevitable.

EPIDEMIOLOGY OF PERIODONTAL DISEASES

Epidemiologic studies are primarily focused on the assessment of two major features of a given disease: the frequency by which the particular disease affects the population, i.e., the assessment of its prevalence; and the identification of risk factors for the onset or the progression of the disease. Typically, the former task falls within the realm of descriptive epidemiology, while the latter is the focus of analytical epidemiology.

Assessing the prevalence of periodontitis in the population is not as straightforward a task as one would normally expect. Indeed, a number of key features of periodontitis render the definition of a periodontitis case rather complicated. The first is the fact that periodontitis is a highly site-specific disorder. In other words, the disease affects specific tooth sites in the dentition, and deep pockets, attachment loss, and bone loss do not occur uniformly within the affected individual. It is therefore necessary to determine specific thresholds for both the minimum number of affected sites required per subject and the minimal severity of the defects (i.e., the magnitude of pocket depth, attachment loss, or bone loss) in order to diagnose a particular individual as affected by periodontitis. In this context, it is important to realize that (i) factors other than plaque-induced periodontitis (such as traumatic tooth brushing, malposition of teeth, or

endodontic lesions) may also result in loss of periodontal tissue support at individual tooth sites, and (ii) the definition of periodontal pathology based on linear probing assessments must exceed the error inherent in probing measurements, in order to identify with reasonable certainty a true loss of periodontal tissue support. Unfortunately, the periodontal research community has so far failed to establish universally accepted thresholds for periodontal pathology. Therefore, it is next to impossible to reconcile worldwide prevalence estimates from different studies in geographically and ethnically diverse populations because of the variable criteria used for case definition. An additional difficulty stems from the fact that most epidemiologic studies have used partial recording methodologies; in other words, they have carried out abbreviated examinations using probing assessments at only a subset of teeth, rather than at all teeth present. Methodological research on the impact of different examination systems has made increasingly apparent that these partial recording protocols result in severe underestimation of the prevalence of periodontitis in the population, and therefore the data quoted by these studies are most likely biased. Lastly, a major additional difficulty lies with the current classification system of periodontitis described above, and particularly with the diagnosis of aggressive periodontitis based on rapid progression of bone loss and attachment loss and familiar aggregation, criteria that are often impossible to assess in epidemiologic studies. Therefore, reliable estimates of the prevalence of chronic versus aggressive periodontitis in any given population are not currently available in the periodontal literature.

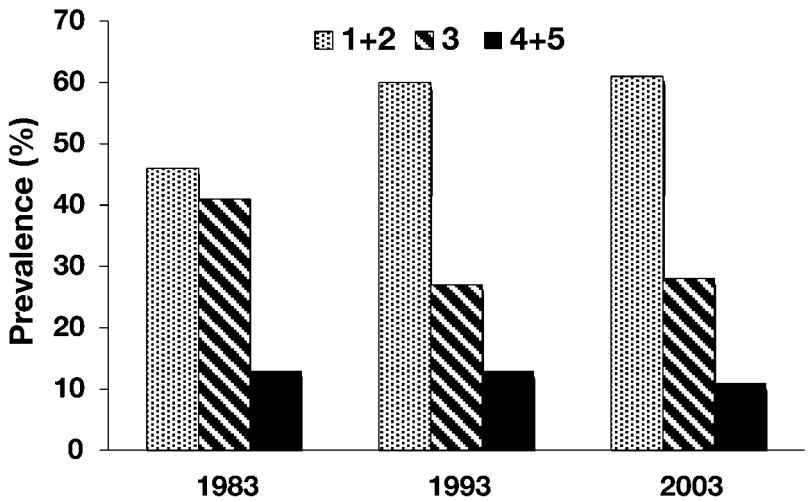
Despite these difficulties, a few conclusions related to the prevalence of periodontal diseases do emerge from the available descriptive epidemiologic studies and can be summarized as follows.

1. Signs of periodontal inflammation and attachment loss of limited magnitude are ubiquitous in all populations. It is therefore not surprising that several publications quote prevalence figures of periodontitis in excess of 70% in the population. Use of thresholds of minimal severity, e.g., loss of attachment of ≥2 mm, can easily result in prevalence figures approaching 100% in the adult population.
2. It is likely more reasonable to focus our attention to the assessment of the prevalence of severe forms of periodontitis, i.e., of periodontitis resulting in substantial loss of periodontal tissue support that may lead to tooth loss and jeopardize function and esthetics. Although a definition of severe periodontitis is also variable in the periodontal literature, it appears that these forms of advanced disease do not affect more than 10 to 15% of the adult population. Furthermore, it appears that the prevalence of these severe forms increases until the age of approximately 60 years and then reaches a plateau because of the effect of tooth loss and edentulism.

Finally, there is still considerable debate on whether the prevalence of periodontitis shows a worldwide decline, possibly due to improved health literacy, better access to oral health care, more effective control of risk factors, etc. There are indeed data available from some parts of the world, notably the United States, that are suggestive of such a trend. Nevertheless, data from a comparative study carried out in Sweden over

30 years (covering the period from 1973 to 2003) are really informative in this context, particularly because they stem from a European country of high socioeconomic status, with high levels of health literacy and easy and affordable access to health care. This particular study compared periodontal conditions in three random samples of adults drawn 10 years apart from the same geographical region, using the same examination methodology (clinical probing assessments and radiographic measurements of bone loss). The periodontal status of the participants was classified using a severity scale ranging from 1 to 5, where a score of 1 indicates periodontally healthy conditions and a score of 5 indicates severe loss of periodontal tissue support. As is shown in Fig. 3, the percentage of individuals in the two healthiest groups (i.e., those with scores of 1 and 2) increased significantly over the observation period, and this improvement occurred at the expense of the intermediate group (score 3), which experienced a corresponding decrease. However, the proportion of individuals who were mostly affected by periodontitis (those in groups 4 and 5) remained remarkably stable, at a level ranging between 13 and 11%. These data seem to indicate that an improvement of periodontal conditions may have indeed occurred over the past few decades, but the beneficiaries of this improvement are largely individuals with moderate levels of periodontitis. The proportion of individuals with severe periodontitis, arguably the highly susceptible group that requires high-intensity therapeutic intervention to shift to a lower disease level, was seemingly unaffected. These data seem to indicate that the current population-based strategies for the prevention and treatment of periodontitis do not fully address the therapeutic needs of the portion of the population that is highly susceptible to periodontitis.

FIGURE 3 Prevalence of periodontal conditions of different severity among inhabitants in a county in Sweden in 1983, 1993, and 2003. Scores 1 and 2 indicate individuals that either are periodontally healthy or suffer from gingivitis; score 3 describes individuals with moderate periodontitis; scores 4 and 5 represent individuals with severe periodontitis. Graph based on data from A. Hugoson, B. Sjödin, and O. Norderyd, *J. Clin. Periodontol.* **35**:405–414, 2008.
doi:10.1128/9781555818906.ch13.f3



Which are then the determinants of susceptibility to severe periodontitis? Is it possible to identify these periodontitis-prone individuals prior to the development of irreversible periodontal tissue damage and expose them to effective prevention programs? What are the lessons learned from analytical epidemiologic studies with respect to the major risk factors for periodontitis?

Before discussing the above questions, we need to clarify a few definitions. A risk factor is an aspect of personal behavior or lifestyle, an environmental exposure, or an inborn or inherited characteristic that, on the basis of epidemiologic evidence, is associated with the occurrence of disease. Identification of a risk factor is a multilevel process that requires (i) the putative factor to continue to be associated with disease after adjustment for (i.e., concomitant consideration of) other exposures known to affect disease development in multivariate statistical analyses, (ii) the "external validity" of the observed association to be confirmed in additional studies in other populations, and finally (iii) in case of risk factors that are amenable to intervention, evidence that a decreased exposure to the factor results in lower levels of disease in prospective studies. Note that a true risk factor is not necessarily etiological; in other words, it is not a necessary and sufficient condition for the onset of the disease. Most diseases have a multifactorial etiology, as they require a combination of conditions to be present for disease to develop. For example, an infectious disease requires colonization by a virulent pathogen but also the presence of a susceptible host that will respond to the microbial challenge in a particular way that is conducive to the development of disease. Colonization of another, nonsusceptible host by the same virulent pathogen may not lead to disease. After the onset of the disease (i.e., after its initial occurrence), progression of the disease to a more advanced state may depend on additional factors that may have little to do with the exposures that caused the disease in the first place. Thus, a risk factor can operate at any level during the course of the disease development: it may be etiological, or it may help expose the host to etiological factors; in addition, it may modify the response to the etiological factor in ways that are conducive to the onset or progression of the disease. Depending on its strength, i.e., the level of its involvement in any of the above steps, a factor may be variably associated with the disease in epidemiologic studies. Lastly, it must be recognized that a risk factor may be of paramount importance for disease development in one population but not in another: for example, an environmental exposure that is prevalent only in a particular geographic location may affect disease development in the local population but not universally; a genetic factor, such as a particular gene polymorphism, may occur only among certain ethnic groups and will consequently have no impact on disease development in other populations.

With the above in mind, let us now review the established risk factors for periodontitis. As discussed below under "Microbial Etiology of Periodontal Diseases," plaque bacteria constitute the stimulus that triggers an inflammatory response at the dentogingival region that, under certain conducive conditions, may lead to destructive periodontitis. Epidemiologic studies carried out in different parts of the world have demonstrated that colonization by particular periodontal species, including *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella*

forsythia, and *Campylobacter rectus*, confer high odds ratios for destructive periodontitis. Since the majority of the available epidemiologic studies that have examined the association between colonization by specific periodontal bacteria and periodontal status are cross-sectional in nature, i.e., they have assessed colonization contemporaneously with the clinical status, these data alone do not demonstrate that these bacteria are true risk factors for periodontitis. However, a relatively small number of longitudinal prospective studies extend these observations and demonstrate that colonization by certain pathogens or virulent clones ascertained prior to the manifestation of periodontal tissue breakdown indeed conveyed an increased risk for subsequent disease development. This has been elegantly demonstrated in studies examining the subgingival colonization by *A. actinomycetemcomitans* and the development of aggressive periodontitis. Pioneering research carried out by a Danish research group in Morocco demonstrated in a population-based prospective cohort study that adolescents free of periodontitis who were colonized by a particularly virulent, highly leukotoxic clone of *A. actinomycetemcomitans* had a relative risk of 18 to develop aggressive periodontitis over the subsequent 2-year period when compared to those not colonized by the pathogen. Similar data have been published from a longitudinal study of aggressive periodontitis in a cohort of predominantly African American, low socioeconomic status individuals in the United States.

In a more global sense, the paramount role of bacteria in the pathobiology of periodontitis is demonstrated by treatment studies that have invariably shown that antimicrobial therapies are effective in the control of periodontitis. Indeed, mechanical disruption of the plaque accumulating at the root surfaces achieved through scaling and root planing, followed by good plaque control, predictably results in resolution of the periodontal lesion and improvement of clinical periodontal status. Furthermore, administration of adjunctive antibiotics has been shown to enhance the therapeutic outcomes. Therefore, the collective empirical evidence from treatment studies of periodontitis and the data from epidemiologic studies have established the role of bacteria, and that of certain species in particular, as an important risk factor for periodontitis.

Beyond the bacterial component, two additional exposures are recognized as established risk factors for periodontitis: cigarette smoking and diabetes mellitus.

There are multiple biologically active substances associated with cigarette smoking, and their detrimental effects on the host tissues has been established in experimental in vitro studies and in vivo animal models. Epidemiologic association studies have invariably demonstrated that smokers show inferior periodontal status and suffer more tooth loss than nonsmokers. Importantly, these differences persist after adjustments for covariates such as race/ethnicity, socioeconomic status, and educational attainment. Likewise, prospective studies have shown a higher progression rate of periodontitis (i.e., longitudinal attachment loss and bone loss) and higher rates of tooth loss in smokers than in nonsmokers. Lastly, data from treatment studies clearly demonstrate that the outcome of periodontal therapy, and particularly that of more elaborate treatment modalities such as regenerative or soft tissue grafting procedures, in heavy smokers is inferior to that in nonsmokers. Interestingly, smoking cessation appears to

be associated with better outcomes, suggesting that the deleterious effect of smoking on the periodontal tissues is partly reversible.

Diabetes mellitus is a group of metabolic disorders that are characterized by abnormal glucose metabolism. In the context of periodontitis, both type 1 diabetes (characterized by deficiency in insulin production) and type 2 diabetes (characterized by impaired insulin action and utilization) appear to interfere with the ability of the host to successfully cope with the bacterial challenge through mechanisms that are increasingly well delineated. In an environment of glucose excess, host proteins undergo nonenzymatic glycation and form advanced glycation end products (AGEs). Activation of the AGE receptor RAGE by AGEs and its other ligands results in a proinflammatory cell phenotype that has been shown to be important in the development of diabetes complications, including periodontitis. In addition, diabetes appears to induce an uncoupling between bone destruction and bone repair mechanisms that may contribute to more-severe alveolar bone loss. Similarly to what was discussed above for smoking, epidemiologic evidence supporting a role for diabetes as a risk factor for periodontitis stems from cross-sectional association studies, longitudinal prospective studies, and treatment studies. Thus, patients with diabetes show more severe attachment loss and bone loss than nondiabetic controls, show enhanced progression of periodontitis over time, and respond less favorably to periodontal therapy. With respect to the reversibility of the detrimental effect of diabetes on clinical periodontal status, it is important to point out that patients with diabetes and good metabolic control appear to respond to periodontal therapy in a manner comparable to that of their nondiabetic counterparts. Interestingly, the negative effects of diabetes on the periodontium appear to manifest themselves at an early age: indeed, data from recent studies identified considerable periodontal pathology (in terms of bleeding on probing, pocketing, and attachment loss) among children under the age of 18 years suffering from predominantly type 1 diabetes.

As discussed above, aggressive forms of periodontitis tend to aggregate in families, suggesting that genetic predispositions may play a role in the disease process. Research data from twin studies have shown a considerable degree of similarity in periodontal status within pairs of monozygotic twins, even in situations where these genetically identical siblings were raised in different families. These data indicate that despite the distinct environmental effects that these siblings were exposed to, a substantial part of their periodontal phenotype was still determined by their genetic make-up. Yet, a "periodontitis susceptibility gene" remains elusive to date, most likely because such a deterministic gene does not seem to exist. In contrast to Mendelian diseases, in which the diseased phenotype relates to a particular mutation in a defined gene, periodontitis is a complex disease whose genetic determinants are coded collectively by multiple (hundreds or thousands of) genes and whose clinical phenotype is defined by an interplay between the environmental and the genetic factors. In recent years, particular attention has been paid to the role of genetic polymorphisms, i.e., in variations in gene sequence that occur relatively commonly in the population, as putative risk factors for periodontitis. Studies have examined the association of periodontitis with polymorphisms: in cytokine-encoding genes (extensively the ones located

in the interleukin-1 gene cluster, but also in the tumor necrosis factor alpha and the interleukin-10 genes); in human leukocyte antigen (HLA) genes; in genes coding for cellular receptors involved in immunity (toll-like receptors 2 and 4, Fcγ receptor, or CD14) or in metabolic processes (vitamin D receptor). With the exception of a particular mutation in the cathepsin C gene that was shown to be responsible for the development of Papillon-Lefèvre syndrome and the accompanying prepubertal periodontitis (i.e., an early-onset form of periodontitis that affects deciduous teeth as well), the association of the above polymorphisms with periodontitis has been highly variable in different studies and not consistent across ethnically/racially diverse populations. Contemporary genome-wide association studies that screen for associations between specific single nucleotide polymorphisms and pathological conditions continue to identify single nucleotide polymorphisms associated with destructive periodontitis at variable degrees, but there is no convincing evidence available today that supports the utility of any genetic test in the identification and management of periodontitis.

Additional exposures that have been identified as potential risk factors for periodontal disease and have not been fully validated yet include psychosocial stress, which can conceivably exercise a negative impact on the immune response; obesity, which is conducive to a state of systemic inflammation; and osteopenia/osteoporosis, particularly in postmenopausal women.

Collectively, the identification of risk factors and a thorough understanding of how these exposures mediate their detrimental effects on the periodontal tissues are of paramount importance in the successful control of periodontal diseases. As discussed below, while the cornerstone approach to periodontal therapy is anti-infective, control of those risk factors that are modifiable is an essential component in the prevention and management of periodontal diseases.

MICROBIAL ETIOLOGY OF PERIODONTAL DISEASES

In the aqueous environment of the oral cavity, a newly cleaned tooth surface is rapidly covered by a salivary glycoprotein film (termed acquired pellicle) to which oral bacteria attach and accumulate into a multispecies biofilm termed dental plaque. In contrast to bacterial formations on mucosal surfaces that are constantly disrupted due to exfoliation of their epithelial base, dental plaque forms on the stable, nonshedding surface of a tooth and may be disrupted solely by external mechanical or chemical action (e.g., through mastication or oral hygiene measures).

Under conditions that are conducive to plaque growth and in the absence of mechanical disruption, the dental plaque increases its mass through proliferation of its bacterial population and enhanced accumulation of extracellular matrix. Importantly, it undergoes significant qualitative shifts as well: while early dental plaque encompasses primarily aerobic and gram-positive microbial species, mature plaque includes increasing proportions of gram-negative anaerobic and facultative anaerobic species. Similar qualitative differences between the composition of dental plaque accumulating above the gingival margin (supragingival plaque) and that localized below the gingival margin, adjacent to a pathological

periodontal pocket (subgingival plaque), have been shown. Notably, both the supra- and the subgingival dental plaque can undergo mineralization and form hard deposits on the tooth surfaces (termed calculus).

In a classical experiment carried out in the mid-1960s, Harald Löe and his coworkers at the Royal Dental College in Aarhus, Denmark, demonstrated the causal association between dental plaque formation and gingival inflammation. These investigators followed a group of young, periodontally healthy individuals who volunteered to abstain from all oral hygiene measures over a 3-week period; the researchers documented that the increase in plaque accumulation over time was paralleled by the development of inflammatory changes in the gingival tissues, characterized by redness, swelling, and a greater bleeding tendency upon mechanical stimulation (gingivitis). Subsequently, dental prophylaxis was carried out and regular oral hygiene practices were reintroduced during a 2-week resolution phase, at the end of which inflammation was eliminated and gingival health was reinstituted. Thus, apart from identifying bacterial plaque as the cause of gingivitis, this proof-of-principle study also demonstrated the reversibility of the plaque-induced gingival inflammatory lesion.

A few years later, Jan Lindhe and his coworkers at the University of Göteborg, Sweden, extended these observations and demonstrated in an experimental model in the beagle dog that long-standing plaque accumulation, facilitated by the placement of cotton ligatures at the level of the gingival margin, induced an irreversible breakdown of the periodontal apparatus, i.e., in loss of connective tissue attachment and alveolar bone. These two landmark studies provided the first experimental evidence of the etiological role of bacteria in the development of periodontal diseases and formed the conceptual foundation for the development of antiplaque strategies in their treatment and prevention.

In parallel, intervention studies in humans put the above concepts to the test and soon established that treatment of periodontitis patients by means of mechanical disruption of the plaque deposits from the tooth/root surfaces accompanied by effective oral hygiene measures resulted in a predictable resolution of gingival inflammation and pocket depth reduction and successfully arrested any further loss of periodontal tissue support.

Until fairly recently, the identities of the organisms associated with periodontal lesions or with periodontal health were limited to those that could be cultured in the laboratory. On the basis of association with different disease states, these organisms were divided into groups of differing potential pathogenicity. These groupings derive from a landmark publication in 1998, in which Sigmund Socransky and his coworkers at the Forsyth Institute in Boston, MA, provided a detailed description of the microbial complexes that occur within the subgingival biofilm in various states of periodontal health and disease. Using checkerboard DNA-DNA hybridization for bacterial identification and enumeration, they analyzed approximately 13,000 plaque samples from 185 adults and used cluster analysis and community ordination techniques to identify consortia of bacteria that are likely to co-vary within the biofilm habitat. As shown in Fig. 4, six closely associated bacterial complexes were recognized, and they are commonly referred to by their color-coded designation. Hence,

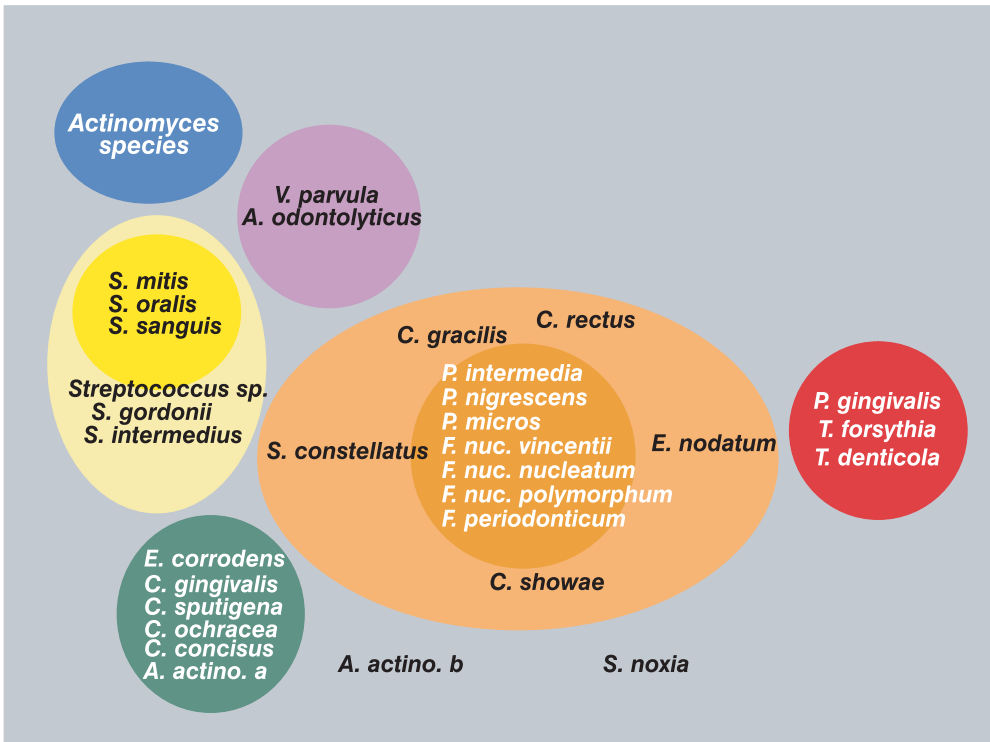


FIGURE 4 Microbial complexes in subgingival plaque. *A. actino*, *A. actinomycetemcomitans* serotype a or b as indicated. See the text for details. From S. S. Socransky and A. D. Haffajee, Periodontal infections, p. 207–267. *In* J. Lindhe, N. P. Lang, and T. Karring (ed.), *Clinical Periodontology and Implant Dentistry*, vol. 1., 2008. Blackwell Munksgaard, Oxford, United Kingdom. Reproduced by permission. doi:10.1128/9781555818906.ch13.f4

these clusters included four complexes primarily consisting of early colonizers of the tooth surfaces (a blue complex consisting of *Actinomyces* species; a yellow complex consisting of various members of the genus *Streptococcus*; a green complex consisting of *Eikenella corrodens*, a number of *Capnocytophaga* species, *Campylobacter concisus*, and *A. actinomycetemcomitans* serotype a; and a purple complex consisting of *Veillonella parvula* and *Actinomyces odontolyticus*). Two additional bacterial clusters that included several bacterial species intimately involved with pathological periodontal conditions were identified: a fairly sizeable orange complex including multiple species members of the genus *Prevotella*, *Fusobacterium*, and *Campylobacter* as well as *Streptococcus constellatus* and *Eubacterium nodatum*; and a red complex that occupied a relatively small proportion of the subgingival microbiota, consisting of three gram-negative, anaerobic bacteria (*P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola*), which are frequently elevated in plaque samples adjacent to periodontal lesions. Notably, species such as *A. actinomycetemcomitans* serotype b and *Selenomonas noxia* did not cluster within any of the above bacterial consortia, although *A. actinomycetemcomitans* is strongly associated with localized aggressive periodontitis.

With the advent of culture-independent, molecular methods of bacterial identification and enumeration such as 16S rRNA gene amplification

and high-throughput sequencing, our understanding of the bacterial composition of the periodontal region has evolved. The in-depth study of thousands of plaque samples derived from a variety of clinical periodontal conditions has demonstrated that at least 700 bacterial species are capable of colonizing the oral cavity and approximately 150 species can simultaneously colonize an individual host. The bacterial burden to the host is substantial, with deep periodontal pockets harboring between 10^8 and 10^{10} bacteria. In addition to the consensus pathogens of *P. gingivalis*, *T. forsythia*, and *T. denticola* in chronic and severe periodontitis, newly recognized noncultivable or poorly cultivable organisms that increase in number in diseased sites include the gram-positive bacteria *Filifactor alocis* and *Peptostreptococcus stomatis*; Gram stain-negative members of the *Firmicutes*, i.e., *Dialister*, *Megasphaera*, *and Selenomonas*; and species in the genera *Prevotella*, *Desulfobulbus*, and *Synergistes*.

In recognition of the fact that periodontal pathogens do not fulfill the classical Koch's postulates defining the causal relationship between an infectious agent and a disease, Socransky and coworkers introduced a list of revised criteria to be used in the identification of bacterial periodontal pathogens. These included the association criterion, fulfilled when the putative pathogen is frequently present or occurs at high levels in pathological pockets and is absent or occurs at low levels in healthy gingival crevices; the elimination criterion, requiring that eradication of the species (or suppression beyond detection) will result in an improvement in periodontal conditions; the host response criterion, i.e., the expectation that a true pathogen that gains access to the host tissues and is actively involved in the disease process will elicit a systemic antibody response while a mere colonizer will not; the virulence factor criterion, i.e., documentation of the particular properties of the alleged pathogen through which damage to the host may be mediated; and finally, evidence from experimental animal models documenting the in vivo ability of the alleged pathogen to induce disease and tissue damage. Distinction between pathogenic and nonpathogenic species should clearly be based not on the fulfillment of any single criterion in the above scheme but on a collective evaluation of the aggregate available evidence.

Admittedly, what constitutes a microbial pathogen in the context of periodontal diseases has been a subject of considerable debate in the periodontal literature. The debate has been further fueled by the recognition that causal pathogens can be encountered in biofilms of periodontally healthy individuals, casting serious doubt on the earlier proposed postulate that these microorganisms may behave as exogenous pathogens. Today, it is increasingly recognized that periodontal diseases are not bacterial infections in the classical sense, i.e., caused by a single or a limited number of pathogens that are not regular constituents of the resident periodontal microbiota, but rather occur as a result of a polymicrobial community-induced perturbation of the host homeostasis in susceptible individuals. Bacterial constituents of these communities often exhibit synergistic interactions that will enhance colonization, persistence, or virulence, and some bacteria may be involved in the breakdown of homeostasis, whereas others may trigger destructive inflammation once homeostasis is disrupted.

According to this paradigm, also referred to as the ecological plaque hypothesis, host defense mechanisms mounted to control and contain the bacterial infection at the gingival margin are derailed to result in disproportionate tissue damage, resulting in attachment loss and bone loss. What the key factors are that actually allow this hijacking of the host response and perpetuate disease is still debatable. Specific bacteria have evolved strategies that allow them to exploit specific host defense processes to their own benefit and proliferate; on the other hand, it is also possible that a human host is somehow predisposed to periodontal tissue breakdown and that the ensuing periodontal lesion provides the environment that selects for particular virulent bacterial species. A schematic overview of the bacterial etiology of the periodontal diseases described by Roy Page and Kenneth Kornman in 1997 is depicted in Fig. 5. According to this diagram, antigenic structures presented by the bacteria of the dental plaque, such as outer membrane proteins, lipopolysaccharide,

FIGURE 5 Overview of the pathogenesis of human periodontitis. The bacteria of the dental plaque challenge the host, which reacts by mounting a local inflammatory response. Under the influence of certain environmental, acquired, and genetic risk factors, inflammation leads to destruction of the periodontal tissues and establishment of a pathological periodontal pocket. The altered environment further selects for specific microbiota, propagating a pathologic cycle. From R. C. Page and K. S. Kornman, *Periodontol. 2000* **14:**9–11, 1997. Reproduced by permission.
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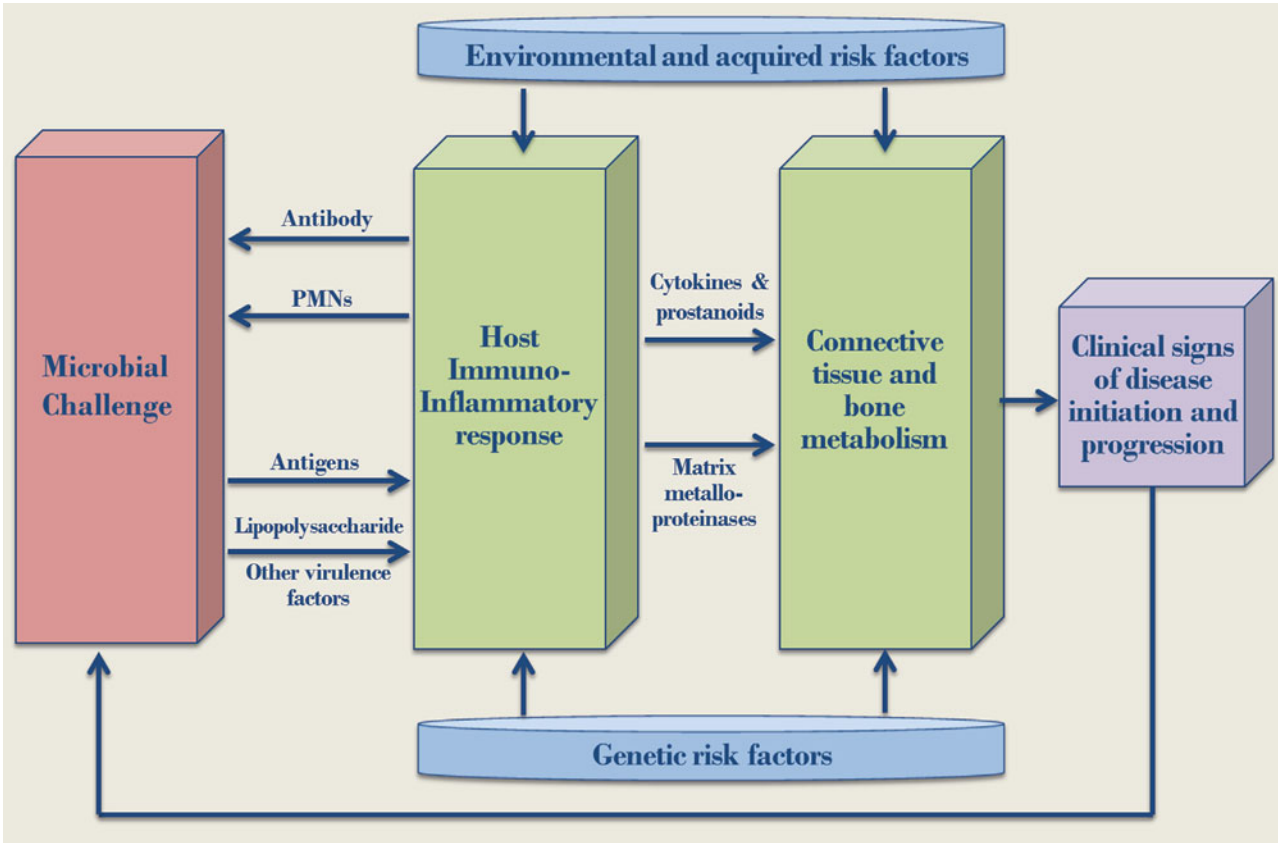

and capsular proteases, constitute the necessary condition for triggering a local inflammatory response, mediated initially by an enhanced polymorphonuclear neutrophil gradient and, subsequently, by local production of antibacterial antibody at the dentogingival niche. In nonsusceptible individuals, the ensuing gingivitis represents a largely protective type of lesion that contains the infection without concomitant loss of tissue support. However, under a combination of genetic predispositions such as specific gene polymorphisms, discussed below, systemic conditions such as diabetes mellitus, and detrimental behavioral exposures such as cigarette smoking, the pendulum between the microbial challenge and the host response may shift to the right, and proinflammatory cytokines and host-derived enzymes such as matrix metalloproteinases result in a breakdown of the tooth-supporting structures in order to maintain a safety zone between the infectious front and the body. The deepening gingival crevice now becomes a periodontal pocket, and environmental selection (anaerobic environment, increased gingival exudate flow, and altered bioavailability of nutrients) further modifies the microbial content of the subgingival biofilm as described above and generates a foul circle that perpetuates and accelerates the catabolic process. It is important to emphasize that the above-mentioned susceptibility to periodontitis, which largely dictates whether the infection will be contained as an exclusively gingival lesion or will propagate apically and result in tissue destruction, is determined at multiple levels: at the microbial level, based on the presence and in situ activity of virulent pathogens in the dental plaque that are capable of homeostasis disruption; at the host level, based on genetic predispositions that may contribute to hyperinflammatory, destructive responses; and on the level of environmental exposures that may further modify the response to the microbial challenge in either direction.

PREVENTION AND CONTROL OF PERIODONTAL DISEASES

Since bacterial accumulation at the gingival margin provides the stimulus that mobilizes the host to an inflammatory reaction that may lead to periodontitis, both the prevention and the therapeutic control of periodontal infections primarily consist of antibacterial strategies. Thus, effective oral hygiene (tooth brushing and cleaning of the interproximal spaces using dental floss or interproximal brushes at least once daily) is the standard means of achieving plaque control and an essential means of both primary and secondary prevention of periodontitis. Adjunctive chemical plaque control is commonly achieved by the use of antiseptic mouth rinses. However, once a deep periodontal pocket is established, oral hygiene alone is not capable of dispersing the subgingival biofilm and professional intervention is required. This consists primarily of mechanical debridement of the exposed tooth surfaces (scaling and root planing) followed by good plaque control, which has been shown to predictably result in resolution of the gingival inflammatory lesion and pocket depth reduction. When the periodontal destruction has propagated deeply under the gingival margin, resulting in deep periodontal pockets (usually >6 mm), debridement of the root surfaces may be carried out in conjunction with access periodontal surgery, i.e., scaling and root planing after

reflection of a mucoperiosteal flap. This procedure facilitates direct access to the apical part of the lesion and effective removal of bacterial deposits and also allows surgical reduction in pocket depth and change in the local anatomy in a manner conducive to oral home care. Given the infectious nature of periodontitis, adjunctive antibiotics are also used and are either applied locally in the periodontal pocket or are administered via a systemic route. Adjunctive systemic antibiotic therapies appear to result in enhanced therapeutic outcomes compared to those achieved by mechanical therapy alone, especially in cases of aggressive periodontitis or severe chronic periodontitis. Adjunctive pharmacological therapies may also include host modulation, i.e., systemic administration of drugs that have anti-inflammatory effects and inhibit host enzymes such as matrix metalloproteinases that mediate tissue breakdown. Such drugs have been shown to be of value in the long-term management of highly susceptible patients that are prone to additional periodontal tissue breakdown after completion of traditional periodontal therapy.

As indicated by the schematic overview of pathobiology described in Fig. 5, prevention and treatment of periodontitis must also include control of acquired and environmental risk factors amenable to intervention. For example, smoking cessation or improved metabolic control in patients with diabetes mellitus is an essential component of the armamentarium of strategies used to prevent and arrest periodontal infection/inflammation.

PERIODONTAL DISEASES AND GENERAL HEALTH OUTCOMES

Over the past decade, considerable interest has been focused on the role of periodontal diseases as an independent risk factor for nonoral diseases. The concept as such is far from novel: more than 150 years ago, the focal infection theory incriminated poor oral health as the underlying cause for a broad range of diverse diseases ranging from colitis to mental depression. As a result, scores of patients were unnecessarily edentulated before the advocated postulates were critically assessed and ultimately rejected. More recently, however, the notion that periodontal infections and the accompanying local inflammation may result in a state of systemic inflammation with subsequent development of disease at extraoral sites has been revisited. Before summarizing the current thinking regarding the association of periodontitis as an exposure with particular diseases as outcomes, it is important to outline a number of facts that substantiate the biological plausibility of such a link.

First, it must be recognized that the subgingival plaque biofilm that colonizes the root surfaces in a dentition with periodontitis is in intimate contact with the ulcerated epithelium of the periodontal pocket. In cases of generalized severe periodontitis, the total surface of the pocket epithelium is substantial and has been calculated to be approximately 8 to 20 cm^2. Therefore, the plaque bacteria are adjacent to what is essentially a wound surface, a fact that allows them to gain direct access to the underlying connective tissue because of the disruption of the epithelial barrier. In addition, certain periodontal bacteria have tissue-invading properties. As a result, bacteremias are fairly common in patients with periodontitis and have been actually shown to be triggered by considerably milder

mechanical stimuli than the invasive dental procedures that have been well recognized as bacteremia inducing. Hence, there is a requirement for antibiotic prophylaxis in special patient categories. Indeed, mastication and even oral hygiene procedures such as tooth brushing and dental flossing even in periodontally healthy subjects have been shown to induce bacteremias. Nevertheless, it must be emphasized that these bacteremias are transient and of low intensity (i.e., the number of bacteria entering the circulation is generally low), and therefore the host appears to cope with them successfully and without any untoward consequences in most cases.

An additional plausible mechanism by which periodontitis may have extraoral adverse effects is by systemic dissemination of inflammatory mediators that are abundantly produced locally in the inflamed periodontal tissues by cellular innate and adaptive immunity pathways. Thus, inflammatory cytokines, along with bacteria and bacterial products, can enter the bloodstream and reach distant organs but, importantly, also excite the vascular endothelium and provide the impetus for a pathobiological cascade of events that may lead to atherogenesis. Indeed, this so-called endothelial injury leads to increased diapedesis of circulating monocytes through the vascular endothelial lining into the intimal space, where they become tissue macrophages, uptake oxidized low-density lipoprotein cholesterol, become foam cells, and contribute to the formation of fatty streaks and atheromatous plaques. Critical to the development of thromboembolic events such as myocardial and cerebrovascular infarctions (i.e., heart attacks and strokes) is the rupture of the atheromatous plaque, which occurs when the endothelial cells that line the vasculature undergo apoptosis and expose the underlying plaque, which is degraded by enzymes such as matrix metalloproteinases.

Importantly, antibacterial antibodies to certain bacterial proteins that have a high degree of homology with mammalian proteins are, in essence, functioning as autoantibodies and contribute to the apoptosis of vascular endothelial cells. This phenomenon, termed molecular mimicry, is of particular importance in the context of periodontal infection-induced atherogenesis, as a specific heat shock protein of the periodontal pathogen *P. gingivalis* (GroEL) is highly homologous to the human protein HSP60.

Lastly, it should be remembered that several systemic conditions to which periodontitis can conceivably contribute etiologically (such as atherosclerosis) also share common risk factors with periodontitis. This fact may lead to a confounded association between the two diseases. In other words, while periodontitis may appear to be associated with atherosclerosis, in reality the two conditions may be linked due to the etiologic exposure that they share, e.g., smoking.

As implied above, one of the conditions that have been investigated as potentially linked with periodontitis is atherosclerosis and atherosclerosis-related clinical events. In vitro and in vivo experimental studies have examined the role of periodontal bacteria and bacterial products on the key atherogenesis-promoting processes mentioned above, including their ability to activate innate immune cell signaling pathways associated with atherosclerosis and to induce vascular endothelial activation and oxidative stress; their interactions with monocytes and tissue macrophages; and prothrombotic, procoagulant, and plaque-disrupting effects. Animal

studies have documented the ability of specific periodontal bacteria and bacterial products such as lipopolysaccharide to accelerate atherosclerosis. Data from human studies demonstrate that patients with periodontitis have high levels of inflammatory biomarkers in their blood (including acute-phase proteins such as C-reactive protein and proinflammatory cytokines such as interleukin-6). Importantly, periodontal therapy has been shown to result in suppression of serum inflammatory mediators and improved arterial endothelial function, suggesting that the effects of periodontitis on these surrogate markers for atherosclerosis are, to a certain extent, reversible.

With respect to clinical events, cross-sectional and prospective cohort epidemiologic studies have shown that periodontitis is associated with higher prevalence and incidence of myocardial infarction and stroke, after adjustment for potential confounders. The associations are of modest magnitude but are fairly consistent among studies. Interestingly, similar observations have been made in studies involving never-smoking individuals, which suggests that confounding by smoking cannot entirely account for the reported associations. So far, no randomized controlled trials have been carried out to test whether the control of periodontal infections may result in a reduction in the incidence of myocardial infarction or stroke. Given the fact that atherosclerosis is a process that has its onset at a relatively young age but usually requires decades until the precipitation of a clinical event, such studies are obviously difficult, time-consuming, and expensive to conduct. A pilot, secondary prevention trial, i.e., a study that examined the effects of periodontal therapy on the prevention of subsequent clinical events in individuals with established cardiovascular disease, showed no impact of periodontal therapy on the incidence of cardiovascular events.

Another area that has attracted considerable attention is the potential role of periodontal infections on precipitating adverse pregnancy outcomes, i.e., preterm birth (delivery before the 37th gestational week), low birth weight (birth weight of <2,500 g), and pre-eclampsia (a serious pregnancy complication characterized by high maternal blood pressure and proteinuria that is associated with endothelial dysfunction). Again, there is ample evidence that supports the biological plausibility of such an association, and many of the pathways outlined above in the context of atherogenesis are relevant here as well. It should be noted that maternal infections such as bacterial vaginosis and chorioamnionitis are established risk factors for prematurity. Conceivably, disseminating bacteria from the oral cavity may seed the placental membranes and induce a local inflammatory response that may initiate premature contraction of the myometrium through production of prostaglandins and other inflammatory mediators. Experimental studies in the pregnant hamster have demonstrated that infection with *P. gingivalis* causes retardation of the growth of the fetus in utero.

Corroborating the above, cross-sectional epidemiologic studies are largely—but not universally—supportive of an association between preterm birth and low birth weight in women with periodontitis. Importantly, and in contrast to what is the case in periodontitis/atherogenesis-related research, several interventions have been conducted to test whether

treatment of maternal periodontitis results in improved gestational outcomes. While earlier, limited-size, uncontrolled trials indicated that such therapy has beneficial effects, recent multicenter controlled trials that have collectively randomized approximately 3,000 pregnant women to a treatment group that received nonsurgical periodontal therapy before the completion of the second trimester or to a control group that received identical therapy after delivery showed that this particular type of periodontal intervention does not result in reduced prematurity or low birth weight rates. However, it is important to emphasize that the results of these studies should not be overinterpreted to indicate that they provide proof of no association between maternal periodontitis and pregnancy complications. This would indeed be the wrong conclusion, as randomized clinical trials only test the efficacy of a specific intervention to modify a predefined health outcome. As discussed above, intervention with a true risk factor will only lead to lower incidence of a particular outcome if the effect of the risk factor is indeed reversible. In addition, interventions against a true risk factor may still fail due to inappropriate timing (e.g., when the intervention is administered too late) or due to inadequacy of the intervention to decrease the exposure sufficiently (e.g., when the intervention fails to resolve periodontal inflammation and reinstitute periodontal health). Additional research is thus required to fully appreciate the effects of maternal periodontal infections on adverse pregnancy outcomes.

A third systemic condition investigated in this context is diabetes mellitus. We discussed above the role of diabetes as an important risk factor for periodontitis. Interestingly, a two-way association between the two diseases appears to exist: in other words, severe periodontitis may adversely affect metabolic control in diabetes. Indeed, meta-analyses of studies that have administered periodontal therapy in patients with diabetes have documented a moderate but statistically significant effect of periodontal treatment on the levels of glycated hemoglobin (HbA1C), which is the established measure of long-term metabolic control in diabetes management. Furthermore, recent studies have shown that a simple algorithm that combined medical history with measures of periodontal disease severity was effective in identifying patients with undiagnosed diabetes mellitus or with undiagnosed prediabetes (the precursor condition). Given that the estimated prevalence of undiagnosed diabetes in the United States is 3%, with an additional 7% of the population suffering from undiagnosed prediabetes, dentists can contribute decisively to the identification of these patients, whose timely diagnosis and management are essential for the prevention of the serious complications of the disease.

Lastly, preliminary evidence indicates that elderly individuals with untreated periodontitis residing in nursing homes have a higher incidence of aspiration pneumonia, a condition that can lead to significant morbidity and mortality. Data also indicate that improved oral hygiene and use of oral antiseptic mouth rinses can reduce the incidence of respiratory infections in these patients.

KEY POINTS

The principal forms of periodontal disease, chronic and aggressive periodontitis, are microbially induced inflammatory disorders of the tooth-supporting structures. While gingivitis is a reversible, potentially protective lesion, the destructive forms of periodontitis are the result of a polymicrobial perturbation of homeostatic mechanisms in a susceptible host.

Our understanding of the determinants of susceptibility to periodontitis is incomplete at present, but susceptibility appears to be conferred at multiple levels: certain microbial complexes are more conducive to disease development than others; genetic predispositions, systemic conditions such as diabetes mellitus, and environmental exposures such as cigarette smoking all play a role in determining whether the inflammatory response will be largely protective and contain the microbial infection with minimal periodontal tissue damage or whether it will result in progressive, destructive disease.

Prevention and treatment of periodontal diseases is thus based on a multipronged approach aiming at controlling the bacterial component, which is the primary etiological exposure, but also the additional risk factors that influence the course of the disease. These strategies include smoking cessation, diabetes management, and host modulation.

Lastly, periodontal diseases result in a state of systemic inflammation that appears to contribute to the development of disease at distant sites. Additional research is needed to determine whether, and to what extent, management of periodontitis may contribute to the control of extraoral pathologic conditions.

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Virulence Factors of Periodontal Bacteria

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INTRODUCTION

The etiology of periodontal diseases displays several unique features that impact pathogenicity and virulence. Periodontal diseases result from mixed infections wherein groups of organisms growing and working synergistically, coupled with host susceptibility factors (discussed in Chapter 15), are responsible for the initiation and progression of disease. Moreover, the composition of these pathogenic microbial consortia can vary from individual to individual and even from site to site in the same mouth. To add to the complexity, different organisms or groups of organisms may assume greater or lesser importance at different stages of disease, and many if not all of these pathogens can also be present in healthy individuals. Nonetheless, certain consensus pathogens have been identified (Chapter 13). While they span a number of different genera, there are common themes in their virulence properties and pathogenic mechanisms. In general, a successful periodontal pathogen has evolved to locate, attach to, and remain in the subgingival area, to overcome host defenses, and to cause damage to the periodontal tissues.

COLONIZATION

Localization in the Gingival Crevice

Upon first entry into the oral cavity, the challenge for a potential periodontal pathogen is to find a way into the subgingival crevice. Motile organisms such as spirochetes can swim directly into the subgingival area, guided by chemotactic attractants. Indeed, *Treponema denticola* is attracted into the subgingival crevice by following gradients of serum, albumin, amino acids, and glucose. These environmental signals are sensed through methyl-accepting chemotaxis proteins that traverse the inner membrane. Within the cytoplasm, the chemotaxis proteins CheA, CheW, and CheY subsequently modulate the direction of flagellar rotation.

Spirochetal motility is dependent on the presence of periplasmic flagella that propel the bacteria in a twisting motion. The advantage of this arrangement relates to the ability to retain motility in viscous environments that would impede the action of external flagella. In *Treponema*,

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up to 15 periplasmic flagella originate from the poles of the organisms, entwine the cytoplasmic compartment, and overlap in the middle of the cell. Flagellar action also involves cytoplasmic filaments located under the periplasmic flagella. Indeed, around 5% of the genome of *T. denticola* is directed to motility and chemotaxis, indicating that these are complex processes that are important to the organism.

Nonmotile organisms are at the mercy of the buffeting effects of salivary flow and are most likely to locate on surfaces remote from the subgingival area. If they possess the appropriate configuration of adhesins (discussed in more detail below), these nonmotile organisms may resist the physical forces of salivary and tongue shear that would otherwise dislodge them. At this point, there are two possible routes to the subgingival crevice. Daughter cells shed by attached organisms may reach the subgingival area through Brownian motion or at least locate at a site closer to their ultimate destination. Alternatively, if the bacteria are attached to the supragingival surfaces, they may extend subgingivally as the proliferating colony spreads outwards.

Attachment

In order to remain at surfaces, bacteria attach to available substrates (as discussed in Chapter 5). In the subgingival area, there are two major solid substrata for bacterial attachment, the root surface of the tooth and the epithelial cells that line the gingival crevice. Both of these present different opportunities and challenges for periodontal pathogens.

Root surfaces are permanent and nonshedding and are rapidly coated with a dense microbial biofilm of early colonizers before periodontal pathogens can become established. It is to these antecedent biofilm constituents and their extracellular products that periodontal pathogens must attach through coadhesive mechanisms. *Porphyromonas gingivalis* can bind to mitis group streptococci and *Actinomyces* species through the major fimbriae that are comprised of the FimA structural subunit. These fimbriae can extend up to 3 μm from the cell surface, and linear peptide domains within FimA are often responsible for mediating attachment. The *fimA* gene is a component of a gene cluster that includes the downstream genes *fimB*, *fimC*, *fimD*, and *fimE*. FimB regulates the length and expression of the fimbriae, while FimC and FimD are minor components of mature fimbriae. FimE is required for the assembly of FimC and FimD onto the fimbrillin fiber. The two genes upstream of *fimA* are involved in regulation of *fimA* expression under the control of the FimS-FimR two-component system. The FimA fimbriae can be classified into 6 genotypes (I to V and Ib), of which type II fimbriae are more often associated with disease, although type I are more common in asymptomatic disease. The shorter fimbriae of *P. gingivalis* are comprised of the Mfa1 structural subunit and are also involved in attachment to oral streptococci. The *mfa1* gene is cotranscribed with three downstream genes, the first of which, *mfa2*, encodes a protein that functions as an anchor for Mfa1 filaments as well as a regulator of filament length. *P. gingivalis*, along with *T. denticola* and *Tannerella forsythia*, also expresses nonfimbrial adhesins in the leucine-rich repeat (LRR) family of proteins that are involved in attachment and accumulation of the organisms.

Fusobacterium nucleatum binds to *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *T. denticola* through a galactose-specific

lectin-like adhesin that recognizes sugars in the capsule, lipopolysaccharide (LPS), and outer membrane proteins of the other organisms. Moreover, as an illustration of the multiplicity of adhesin expression, an arginine-inhibitable adhesin (RadA) of *F. nucleatum* is responsible for coadhesion with oral streptococci and accumulation into mixed-species biofilms. Hence, binding of *F. nucleatum* to streptococci will not occupy all of the fusobacterial adhesins, and so this configuration of adhesins will allow consortia of fusobacteria and streptococci to recruit additional gram-negative pathogens.

In contrast to root surfaces, the epithelial cells of the gingival crevice are constantly shedding, and so bacteria attached to these surfaces must have a longer-term strategy for persistence such as desorbing and reattaching elsewhere or intracellular invasion and cell-to-cell spread. The majority of successful periodontal pathogens can adhere to epithelial cells and to extracellular matrix proteins.

The FimA fimbriae of *P. gingivalis* also mediate attachment to epithelial cells through engagement of integrin receptors, an event that initiates the signaling cascade necessary for *P. gingivalis* internalization. FimA fimbriae additionally bind to fibrinogen, fibronectin, salivary proline-rich proteins, and lactoferrin. *P. gingivalis* also produces a series of hemagglutinins that can bind host cells and several proteases that possess hemagglutinin domains. *A. actinomycetemcomitans* adheres to epithelial cells through the autotransporter proteins Aae and ApiA. The extracellular matrix protein adhesin A (EmaA) is also an autotransporter and binds to collagen. The predominant epithelial cell adhesin of *T. denticola* is a major outer membrane protein, Msp, which can also bind to matrix proteins such as collagen and fibronectin. Msp acts as a porin and can become integrated into the membranes of host cells, leading to cell death. In conjunction with proteases, Msp promotes degradation of the periodontal tissues and allows *T. denticola* to invade the tissues. Other adhesins of *T. denticola* include the oligopeptide transporter OppA, which binds soluble fibronectin and plasminogen, and the LRR protein LrrA, which binds to epithelial cells. Likewise, BspA, an LRR protein of *T. forsythia*, mediates adherence to host cells and matrix proteins. BspA is also required for *T. forsythia*-induced bone loss in animals and for entry into epithelial cells. The major adhesins of important periodontal bacteria are summarized in Table 1.

Invasion

As discussed above, the attachment of periodontal bacteria to epithelial cells can induce internalization of the organism, and potential pathogens such as *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsythia*, and *F. nucleatum* enter and survive within epithelial cells. Residence within host cells provides bacteria with a nutrient-rich, low-oxygen environment that is partially protected from the host immune system. While an intracellular location is more likely a lifestyle choice than an overt virulence factor, internalized bacteria are sheltered from physical removal by scaling and root planing and from antibiotics. The intracellular population also provides a reservoir of bacteria for continual recolonization of subgingival sites. In addition, intracellular bacteria can disrupt host cell pathways that control apoptosis, the cell cycle, the production of innate immune effectors, and the secretion of matrix metalloproteinase enzymes (MMPs), all of which

TABLE 1 Major adhesins of periodontal bacteria

Adhesin	Receptor(s) and role(s)
P. gingivalis	
FimA long fimbrial subunit protein	Integrin receptors on host cells; glyceraldehyde phosphate dehydrogenase on streptococcal surfaces; *T. denticola* dentilisin; *Actinomyces* surface components; salivary proline-rich proteins and statherin; host matrix proteins; lactoferrin and hemoglobin; intercellular adhesin molecule 1
Mfa1 short fimbrial subunit protein	Streptococcal surface proteins SspA/B
Hemagglutinins HagA, B, and C; HagA domains of gingipains RgpA and Kgp	RBCs and other host cells; ECM components, including fibrinogen, fibronectin, and laminin
Hemin-binding protein 35	Hemin, gram-positive bacteria
InlJ LRR protein	Intraspecies binding
Surface polysaccharide and LPS	Interspecies binding
A. actinomycetemcomitans	
Flp1 Tad pilus subunit protein	Intraspecies binding, salivary components
EmaA autotransporter	Collagen, host cells
Aae autotransporter	Epithelial cells, collagen, fibronectin
ApiA autotransporter	Epithelial cells, collagen, fibronectin
Surface polysaccharide and LPS	Interspecies binding
T. denticola	
Msp major outer sheath porin protein	Interspecies binding, matrix proteins, works with proteases to degrade matrix proteins
LrrA	Epithelial cells, interspecies binding
OppA oligopeptide transporter	Plasminogen, fibronectin
Dentilisin protease	Epithelial cells, matrix proteins, FimA of *P. gingivalis*; cleaves bound factor H
F. nucleatum	
FadA filamentous surface protein	Attachment and invasion of epithelial and endothelial cells, colonization of placenta
FomA porin	Interspecies binding
RadA	Arginine-containing receptors, interspecies binding
30-kDa outer membrane protein	Galactose-containing receptors, interspecies binding
T. forsythia	
BspA leucine-rich protein	Epithelial cells, matrix proteins, interspecies binding, salivary agglutinin gp340; necessary for epithelial cell invasion and alveolar bone loss in mice

will disrupt tissue homeostasis and immunity. For example, intracellular *P. gingivalis* can inhibit the production of the chemokine interleukin-8 (IL-8) from epithelial cells and thus reduce the level of neutrophil recruitment into the gingival tissues. The mechanisms of intracellular invasion are discussed in Chapter 5.

Community Development

Following attachment to oral surfaces, bacteria accumulate into complex communities known as biofilms. In particular, the hard, nonshedding surfaces of the teeth harbor dynamic assemblages of multiple bacterial species. A number of interspecies communication systems exist among oral bacteria and facilitate the coordinated behavior of these heterotypic biofilms. Signaling based upon autoinducer 2, a product of the LuxS enzyme, and cell-to-cell contact induces responses in partner organisms that allow collective regulation of activities including gene expression, nutrient acquisition, and DNA exchange (discussed further in Chapter 5).

Localized areas of these biofilms can contain large numbers of individual species, and many bacterial components are utilized to facilitate

such homotypic accumulations. In *P. gingivalis*, these include the FimA and Mfa fimbriae and the LRR protein InlJ, along with the amount and composition of exopolysaccharide and LPS, all of which contribute to intercellular binding and community cohesion. In *A. actinomycetemcomitans* the *tad* (*t*ight *ad*herence) locus is necessary for bacterial adhesion to inert surfaces, autoaggregation, and biofilm formation and includes genes for the biogenesis of Flp pili. *tad*-like genes are widespread in both the *Eubacteria* and *Archaea*, and some of the proteins encoded by the *tad* locus have homology to other bacterial protein secretion systems. The Flp pili form long (around 2-μm) bundles on the cell surface and are rapidly lost on laboratory subculture.

TOXINS

Leukotoxin

Few periodontal pathogens produce potent exotoxins, the exception being *A. actinomycetemcomitans*, which produces a leukotoxin and a cytolethal distending toxin (Cdt). Leukotoxin (LtxA) is a member of the RTX (repeat in toxin) family of pore-forming cytolethal toxins. The *ltxA* gene is the second gene of an operon comprising *ltxA* through *ltxD*. Transportation of the toxin to the cell surface is accomplished by the LtxB and LtxD proteins, and the LtxC protein activates the toxin posttranslationally by fatty acid modification. Leukotoxin binds to leukocyte function antigen 1 (LFA-1) on the surface of monocytes, neutrophils, and a subset of lymphocytes. Interestingly, from an evolutionary perspective, only humans and some Old World primates are susceptible to leukotoxin. The action of the toxin causes a pore to form in the host cell membrane, and cell death can result from interference with osmotic homeostasis or through an apoptotic effect. *A. actinomycetemcomitans* strains that produce high levels of leukotoxin are more often associated with severe disease than minimally leukotoxic strains, and antibodies to the toxin correlate with reduced attachment loss in patients with early-onset periodontitis.

Cytolethal Distending Toxin

A. actinomycetemcomitans Cdt is a DNase that induces cell cycle arrest at G_2, which in some cases is followed by apoptosis. Cdt shows activity against a variety of cell types including epithelial cells, fibroblasts, and lymphocytes. Cdt holotoxin is a heterotrimer comprised of subunits CdtA, CdtB, and CdtC. The CdtB protein is the active subunit and possesses the DNase activity as well as phosphatidylinositol 3-phosphate (PIP_3) phosphatase activity. CdtB is internalized by target cells through the endocytic pathway. CdtA and CdtC are thought to interact with the target cell surface and facilitate internalization of CdtB. *A. actinomycetemcomitans* strains that possess the *cdt* operon are more frequently isolated from patients diagnosed with localized aggressive periodontitis.

PROTEOLYTIC ENZYMES

Most of the bacteria that are associated with periodontal disease produce one or more extracellular proteolytic enzymes (Table 2). *P. gingivalis* is an asaccharolytic organism and derives all its nutrients from the breakdown of proteins and peptides, thus producing an extensive suite of proteases

TABLE 2 Major proteases of periodontal pathogens

Organism and peptidase	Biochemical features	Functional characterization pertinent to pathogenicity of periodontal disease
T. denticola		
Dentilisin (trepolysin), PrtP	A serine endopeptidase referred to as chymotrypsin-like protease located on the bacterial surface. The presence of antibodies specific for the protease indicates that it is expressed in vivo.	Degrades extracellular matrix components; disrupts host signaling pathways by cytokine degradation; activates proMMP2; cleaves C3 and factor H, a central molecule of the complement activation pathway and an important regulator of complement activity, respectively; activates neutrophils, causing release of MMP-9 via interaction with complement pathway; and disrupts epithelial junctions. These PrtP activities lead to dysregulation of local homeostasis, possibly favoring the development and progression of periodontal diseases. PrtP also mediates *T. denticola* binding to fibrinogen, promoting colonization.
Prolyl oligopeptidase (POPase)	Cell surface-located serine endopeptidase, which cleaves Pro-Xaa peptide bonds only in small peptides (oligopeptides)	Degrades several bioactive peptides containing internal proline residues.
Arginine-specific oligopeptidase (trypsin-like enzyme, BAPNA-peptidase)	Cell surface-associated serine endopeptidase, which cleaves Arg-Xaa and Lys-Xaa peptide bonds exclusively in oligopeptides	Detection of this enzyme activity in GCF was the basis of a diagnostic assay used to detect the presence of *T. denticola* in the periodontal sites.
Dentipain (IdeT)	A cysteine endopeptidase degrading oligopeptides	Inactivation of the dentipain-encoding gene significantly reduced the ability of *T. denticola* to form abscesses in a murine infection model, suggesting that dentipain contributes to the virulence of *T. denticola*.
T. forsythia		
PrtH protease	A cysteine endopeptidase with the catalytic domain related to caspases, enzymes responsible for apoptosis of eukaryotic cells	Detaches adherent cells from the substratum, an activity that potentially can be implicated in disintegration of the gingival epithelium and release of chemokine IL-8 by detached cells. This activity correlates with results of clinical studies showing an association between *prtH* genotype levels and future periodontal attachment loss.
Karilysin	A metalloendopeptidase structurally closely related to MMPs. The expression of karilysin in vivo was confirmed by the RT-PCR[a] analysis of clinical samples from periodontitis sites infected with *T. forsythia*.	Abrogates the bactericidal activity of an antibacterial peptide, cathelicidin LL-37, and interferes with all three pathways of complement activation (classical, mannose, and alternative). Acts synergistically with *P. gingivalis* gingipains to inactivate complement. Releases anaphylatoxin C5a from C3. Karilysin protects *T. forsythia* from killing by innate immunity and may contribute to a sustained inflammatory reaction (anaphylatoxin production), thus eroding periodontal tissues.
Prevotella intermedia		
Interpain	Secreted cysteine endopeptidase related to streptopain (SpeB) of *Streptococcus pyogenes*	Degradation of complement in synergy with karilysin of *T. forsythia* and gingipains of *P. gingivalis*, thus bestowing *P. intermedia* with resistance to the antibacterial activity of complement. The enzyme can be detected in GCF collected from periodontal sites infected with the bacterium.

P. gingivalis		
Carboxypeptidase CPG70	Metallocarboxypeptidase exhibiting activity with peptides containing C-terminal Lys and Arg residues	Inactivation of the gene encoding CPG70 results in loss of virulence in the murine lesion model. The enzyme activity may be important for posttranslational processing of gingipains.
Dipeptidyl peptidase IV (glycylprolyl aminopeptidase), dipeptidyl peptidases 7 and 11, and prolyltripeptidyl peptidase	Serine exopeptidases associated with *P. gingivalis* surface. Activity of *P. gingivalis*-derived dipeptidyl peptidases can be detected in GCF.	These peptidases most likely have housekeeping functions. Generation of di- and tripeptides provides asaccharolytic *P. gingivalis* with nutrients used as the source of carbon and energy. The aminopeptidase activity is required for *P. gingivalis* growth on media with proteins as the sole source of carbon. Inactivation of genes encoding these enzymes attenuates virulence.
Periodontain and PrtT protease	Secreted cysteine endopeptidases related to streptopain (SpeB) of *S. pyogenes*	Role in *P. gingivalis* virulence not yet confirmed
Trp protease	Papain-like cysteine protease	Contributes to generation of peptides for *P. gingivalis* growth.
PepO	Endopeptidase with homology to endothelin-converting enzyme	Involved in epithelial cell invasion
Gingipains RgpA, RgpB, and Kgp	Caspase-like cysteine peptidases with Arg-Xaa and Lys-Xaa specificity encoded by 3 different genes (*rgpA*, *rgpB*, and *kgp*). Due to posttranslational processing and modification, gingipains occur in several different molecular forms, soluble or cell surface and outer membrane vesicle associated.	Gingipains are a major virulence factor of *P. gingivalis*. Strains with deleted gingipain genes have been found to be benign in different animal models, including the bone loss model of oral infection, which most closely mimics human periodontitis. Also, treatment with gingipain-specific inhibitors strongly attenuates *P. gingivalis* virulence. Animal immunization with purified gingipains or DNA vaccines protects against *P. gingivalis* virulence. Gingipains are detected in GCF at high concentrations, reaching 100 μM in samples collected from deep pockets (>6 mm) heavily infected with *P. gingivalis*. Although gingipains can degrade a multitude of connective tissue proteins, their major role in periodontitis pathogenicity is related to their ability to manipulate host immune defenses, signaling pathways and proteolytic cascades of kinin release, and coagulation. In addition, Rgp activity is essential for fimbria assembly, another major virulence factor of *P. gingivalis* directly linked to various pathological manifestations of periodontitis, including bone loss.

[d]RT-PCR, reverse transcription-PCR.

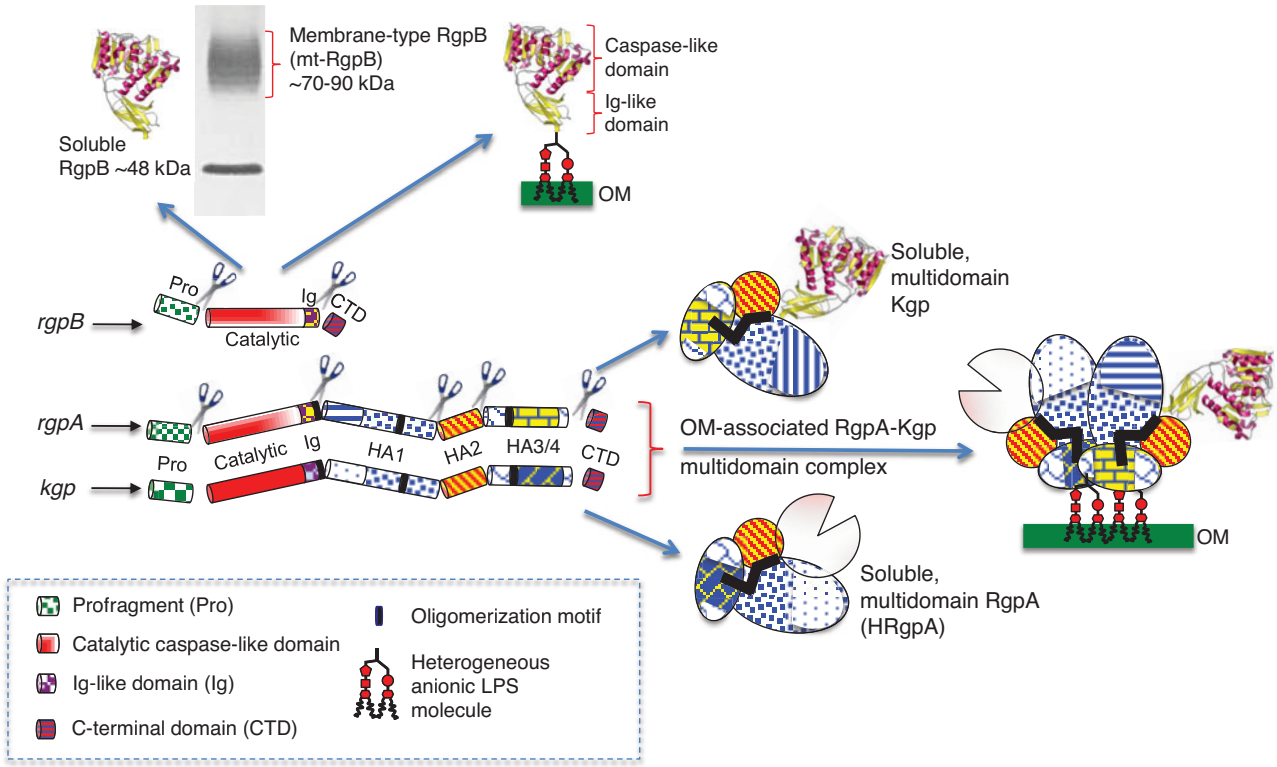

FIGURE 1 Schematic diagram of different variants of gingipains produced by *P. gingivalis*. Gingipains are products of three genes: *rgpA* and *rgpB*, encoding the Arg-specific enzymes RgpA and RgpB, and *kgp*, coding for the Lys-specific protease. RgpA and Kgp are translocated into the periplasm as polyproteins composed of a profragment, a protease domain, hemagglutinin adhesion (HA) domains, and a C-terminal domain (CTD). RgpB lacks HA domains. Proteolytic processing of RgpA and Kgp polyproteins (scissors) releases the catalytic and HA domains, which remain noncovalently associated via an oligomerization motif (black bar). Some gingipains are secreted as the individual multidomain enzymes (Kgp and HRgpA) or the soluble RgpA-Kgp complex (not shown). Similarly, soluble RgpB is also secreted. Note that profragment and CTD are absent in the mature forms of the gingipains. In addition to proteolytic processing, the majority of gingipains are posttranslationally modified by attachment of a heterologous moiety of anionic LPS and remain associated with the outer membrane (OM) on the bacterial surface (mt-RgpB and the multidomain RgpA-Kgp complex). Western blot analysis (top) of the whole culture of *P. gingivalis* using anti-RgpB monoclonal antibody illustrates the difference in molecular mass between soluble and membrane-attached RgpB. The structure of the Rgp protease domain is known. It is composed of the caspase-like catalytic domain and the immunoglobulin-like (Ig-like) domain. The Kgp catalytic domain is depicted as a "Pac-Man" shape because the structure of this enzyme is yet unknown. Nevertheless, it can be predicted to have the same fold and Rgp based on similarities at the level of amino acid sequence. The same pattern and color of shading indicate identical sequences in individual gingipains. The HA domains are responsible for nonproteolytic activity of gingipains, and sequential motives conserved in all HA1 and HA3/4 domains are responsible for hemagglutinin activity of gingipains. In addition, HA1 functions as an adhesin binding fibrinogen, fibronectin, and laminin, supporting colonization of connective tissue by *P. gingivalis*. The conserved HA2 domain (also referred to as hemoglobin receptor [HbR]) binds hemoglobin and facilitates hemin acquisition by the bacterium. Finally, an unspecified HA domain unique for RgpA sequesters endogenous plasma inhibitor of complement activation (C4-binding protein [C4BP]), contributing to *P. gingivalis* resistance to complement-dependent antimicrobial activity. doi:10.1128/9781555818906.ch14.f1

TABLE 3 Functions of *P. gingivalis* proteases

Impairment of tissue integrity	Perturbation of host defenses	Bacterial function
Degradation of extracellular matrix proteins: fibronectin, laminin	Degradation of immunoglobulins	Release of hemin and iron from host proteins
Hydrolysis of collagens I, III, IV, and V	Inactivation or activation of complement components	Exposure of host and bacterial hidden epitopes (cryptitopes)
Degradation of fibrinogen	Destruction of cytokines and chemokines	Posttranslational processing of proteases, fimbrillin, and outer membrane proteins
Inactivation of tissue and plasma proteinase inhibitors	Cleavage of leukocyte surface receptors	Involvement in epithelial cell adhesion and invasion
Activation of MMPs	Degradation of antimicrobial peptides	Provision of peptides for microbial cell growth
Activation of the kallikrein/kinin cascade		

with different specificities. The best-characterized ones are RgpA and RgpB (arginine specific) and Kgp (lysine specific), which are collectively known as the gingipains and are key virulence determinants (Fig. 1). The broad specificity of these enzymes (Table 3) allows targeting of host structural proteins (e.g., collagens, fibronectin, and laminin) and immune effector proteins (e.g., cytokines, antibodies, complement components, antimicrobial peptides, and leukocyte surface receptors). Gingipains can activate the kallikrein cascade and cause the release of kinins with subsequent induction of vascular permeability, which may allow systemic dissemination of *P. gingivalis*. The gingipains also perform multifunctional tasks for the microorganism, for example, in posttranslational processing of surface proteins such as the FimA structural component of the major fimbriae.

Another virulence-related function of the gingipains involves activation of host MMPs, particularly MMP-2 (gelatinase A-type IV collagenase), MMP-8 (neutrophil collagenase-2), and MMP-9 (gelatinase B-type IV collagenase). MMPs are a family of zinc-dependent proteinases secreted by many cell types that are activated by proteolysis and can degrade and modify matrix and basement membrane proteins in the periodontium. MMPs thus contribute to periodontal tissue destruction and failure of the periodontal lesion to heal. Gingipains can also degrade tissue inhibitors of MMPs.

RgpA and Kgp possess hemagglutinin domains (HagA) that mediate attachment to host cells. Moreover, gingipain activity can expose previously hidden adhesin binding domains (cryptitopes) on host and bacterial proteins. *P. gingivalis* releases large numbers of membrane vesicles by evagination of the outer membrane. These vesicles possess adhesins and LPS present on the bacterial outer membrane as well as entrapped periplasmic components. As vesicles contain proteases, one function may be to deliver proteolytic enzymes to the gingival tissues, which can also be penetrated by vesicles because of their small size.

ACQUISITION OF IRON

One of the hallmarks of periodontitis is inflammation and bleeding. In such environments, microorganisms that can take advantage of hemin or iron will thrive in inflamed gums. Periodontal bacteria such as *P. gingivalis*

and *Prevotella intermedia* require not only iron but also protoporphyrin IX, which is present in hemin; thus, the availability of hemin in the periodontal pocket predisposes the site for bacterial growth. Indeed, these organisms do not grow without iron or hemin, and culture under hemin limitation reduces virulence in animal models.

As most iron in mammalian hosts is bound by host proteins, the availability of the nutrient for microbial acquisition is extremely low (10^{-24} M). Such limitation of an essential nutrient is a natural defense of the mammalian host against invading and multiplying bacteria and is a way to keep microbial growth in check. In periodontal pockets, the major iron source is found in the gingival crevicular fluid (GCF). The composition of GCF resembles that of serum; it contains hemoglobin- and hemin-loaded proteins such as hemoglobin-haptoglobin, hemopexin-hemin, and hemalbumin complexes.

P. gingivalis requires hemin for growth, primarily to use as a cofactor for the cytochrome *b* subunit of fumarate reductase, a major metabolic enzyme involved in energy generation. In addition, *P. gingivalis* covers itself in hemin as protection against oxidative stress. Two hemin molecules bind oxygen, thus forming diheme polymers on the bacterial cell surface. *P. gingivalis* is well equipped to acquire hemin. The array of mechanisms available to the organism for hemin acquisition is depicted in Fig. 2. As mentioned above, the RgpA and Kgp proteases have C-terminal domains possessing hemagglutinin/adhesin capabilities and are able to bind a variety of substrates including red blood cells (RBCs). HagA possesses a sequence similar to that of the hemagglutinin/adhesin domain of the gingipains, and *P. gingivalis* also produces hemagglutinins HagB and HagC. Following binding by the array of *P. gingivalis* hemagglutinins, RBCs are lysed and hemoglobin/hemin is released. The released hemin is then bound by bacterial hemin receptors. Hemin is too large to freely diffuse across the membrane and thus requires a specialized hemin transport system. In *P. gingivalis*, multiple clusters coding for hemin acquisition mechanisms have been shown to be present. The first locus, designated *ihtABCDE* (iron-heme transport), codes for the TonB-dependent outer membrane receptor (IhtA), the lipoprotein IhtB, the periplasmic binding protein IhtC, the permease IhtD, and the cytoplasmic ATP binding protein IhtE. IhtAB forms a two-component outer membrane receptor, where the IhtB is surface located and binds hemin, while IhtA is an integral outer membrane protein that forms a pore in the membrane allowing for hemin passage across the membrane. TonB proteins provide energy for membrane transport through interactions with outer membrane TonB-dependent receptors. The second locus, designated *tlr-htrABCD*, codes for the outer membrane TonB-dependent receptor Tlr (also known as Tla) followed by a putative ATP-binding cassette hemin transport system. The third locus, designated *hmu*, is composed of six genes, *hmuYRSTUV*, coding for the outer membrane lipoprotein HmuY, the outer membrane receptor HmuR, the inner membrane chelatase HmuS, two cytoplasmic permease-like proteins (HmuTU), and the cytoplasmic membrane-attached intracellular protein HmuV. In addition, a high-affinity hemin-binding cell surface protein has been identified in *P. gingivalis*. This protein, named HusA, is proposed to function as a hemophore that can be released from bacterial

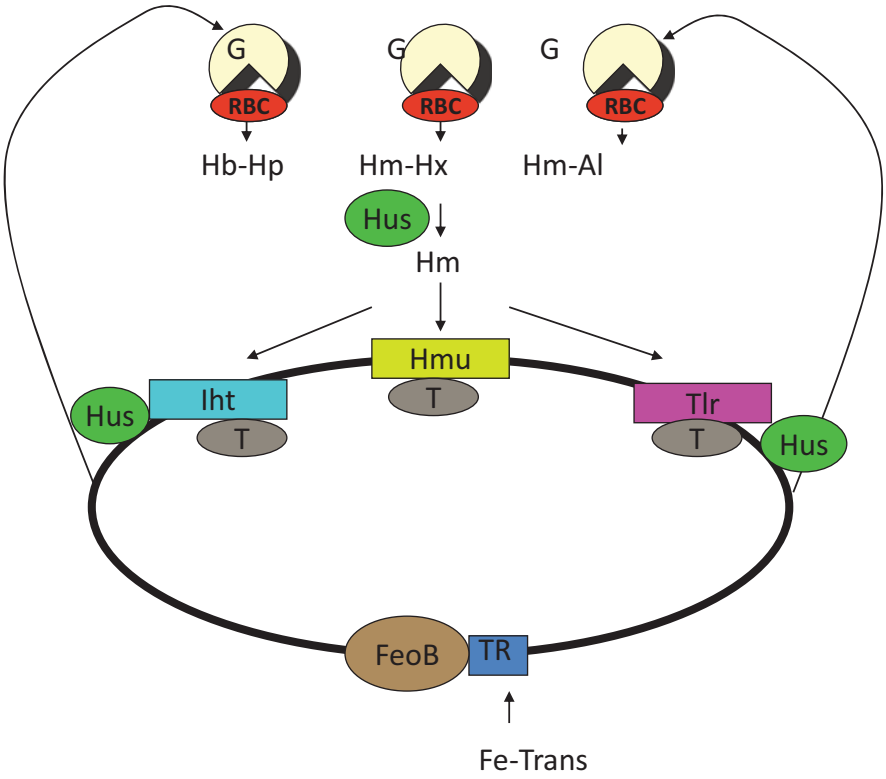

FIGURE 2 Hemin/iron acquisition in *P. gingivalis*. Gingipains (G) bind RBCs and release hemoglobin (Hb), which then is bound by haptoglobin (Hp) or HmuYR. Hemoglobin is converted from oxyhemoglobin into methemoglobin (by ArgX gingipain), which is subsequently degraded by LysX gingipain to release hemin (Hm). Then, hemin is bound by the host serum proteins hemopexin (Hx) or albumin (Al). These complexes can also be degraded by gingipains. Next, the released hemin is made available to the hemin transport systems Iht, Hmu, and Tlr, which translocate hemin inside bacterial cells. Energy for the process is provided by TonB (T), which is associated with outer membrane hemin transporters. The transport of hemin to the hemin transporters can also be aided by the hemophore HusA (Hus). In addition to hemin, *P. gingivalis* can acquire iron from transferrin (Fe-Trans). Transferrin binds *P. gingivalis* cells; however, the receptor remains unknown. Iron is then transported across the cytoplasmic membrane by the ferrous iron transporter FeoB1 (FeoB).
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cells, acquire hemin from gingipain-degraded proteins, and deliver it to outer membrane receptors that subsequently internalize the hemin. While there are a variety of hemin transport systems, the specific role of each system in hemin acquisition has yet to be determined. It is possible that the systems are differentially expressed, depending on the niche in which *P. gingivalis* is present. Furthermore, it is probable that each system acquires hemin or iron from different sources.

Although hemoglobin has been shown to be a major iron source for *P. gingivalis*, this organism can also acquire iron from transferrin. Transferrin is a serum iron-binding protein and is present in gingival crevicular fluid. Furthermore, *P. gingivalis* grows in conditions in which oxygen concentrations are reduced. In the absence of oxygen, iron can exist in

the more soluble ferrous form. Such iron is accessible to the cell and can transfer itself across the outer membrane. From the periplasmic space, ferrous iron can be transported by a ferrous iron transporter, FeoB1.

Finally, *P. gingivalis* has mechanisms to protect itself from excess intracellular iron, which is toxic to the cell. This is accomplished by the regulation of iron uptake as well as scavenging iron by two intracellular iron storage proteins, ferritin and ferritin-like Dps (DNA protection during starvation) protein.

SURFACE-ASSOCIATED BIOACTIVE COMPONENTS

Lipopolysaccharide

As many of the periodontal pathogens are gram negative, levels of LPS are increased in the periodontal pocket. In addition to cell-associated LPS, periodontal pathogens also release large amounts of membrane vesicles that contain LPS and will deliver these toxic components directly into the gingival tissues, which they can penetrate because of their small size. *P. gingivalis* and *A. actinomycetemcomitans* LPS can contribute to alveolar bone resorption by activating osteoclasts. *P. gingivalis* LPS can also contribute to bone resorption by inducing secretion of cytokines such as IL-1β and tumor necrosis factor alpha, as well as the prostanoid PGE2. Lipid A from *P. gingivalis* exhibits unique structural heterogeneity, and four lipid A structural subgroups have been identified based on acylation and phosphorylation. The tetra-acyl nonphosphorylated lipid A form is inert with respect to Toll-like receptor 4 (TLR-4) activation, potentially leading to immune evasion. In contrast, the tetra-acyl monophosphorylated lipid A is a TLR-4 antagonist that could contribute to immune suppression. This provides an underlying structural basis for the low immune-stimulatory capability of *P. gingivalis* lipid A. The penta-acyl mono- and diphosphorylated forms are TLR-4 agonists, which will stimulate the immune response. Interestingly, the level of available hemin triggers alteration in *P. gingivalis* lipid A structure. In low hemin concentrations, the penta-acyl, monophosphorylated form predominates, while at high hemin concentrations the tetra-acyl, mono-phosphorylated lipid A structure is the most abundant species. The structure of LPS also has relevance for activation of host antimicrobial peptides. Human β-defensins are upregulated by the penta-acylated forms but downregulated by the tetra-acylated species.

Capsule and Exopolysaccharide

Extracellular polysaccharide provides physical protection to organisms and masks antigens and receptors for phagocytic cells and complement activation. Exopolysaccharide can thus impede phagocytic killing, opsonization, and complement-mediated killing. In *P. gingivalis*, six capsular serotypes (K1 to K6) have been described, and these capsular types exhibit differential virulence in animal models. The K1 capsule has been found to be comprised of mannuronic acid (ManA), glucuronic acid, galacturonic acid, galactose, and *N*-acetylglucosamine. *P. gingivalis* capsule also dampens the induction of proinflammatory cytokines by the organism. *A. actinomycetemcomitans* strains can be divided into seven serotypes (a

to g) on the basis of surface carbohydrate antigens. The distribution of serotypes varies according to geographical location and ethnicity. In the United States, serotype b is associated with localized aggressive periodontitis, while in Europe and Asia there is a more equal distribution of serotypes. These capsular polysaccharides can inhibit osteoblast proliferation, induce osteoclast formation and bone resorption in animal models, and impede the production of proinflammatory cytokines.

Toxic Bacterial Components and Enzymes

Many bacterial components can exhibit properties that could contribute to the initiation and progression of periodontal disease. While these are often surface exposed or secreted, they can also be released upon bacterial cell lysis. In addition to their role in attachment, the fimbriae of *P. gingivalis* can induce the secretion of proinflammatory cytokines and contribute to bone resorption. *T. denticola* Msp, lipooligosaccharide, glycolipids, and lipoproteins can also stimulate proinflammatory cytokine secretion, and lipooligosaccharide can induce osteoclast proliferation and bone resorption. *P. gingivalis* produces novel phosphorylated dihydroceramide lipids that can activate TLR-2 and promote IL-6 secretion and also inhibit osteoblast differentiation. *T. forsythia* produces surface lipoproteins that can activate host cells through TLR-2 and cause the release of proinflammatory cytokines. These lipoproteins can also induce apoptosis in host cells.

In *A. actinomycetemcomitans*, the molecular chaperone GroEL is a potent bone-resorbing mediator. Peptidoglycan fragments of a wide range of organisms can also stimulate bone loss. *T. forsythia* possesses an S-layer that consists of serrated structural subunits in either oblique or tetragonal lattices. Two glycosylated proteins (TfsA/B) constitute this S layer, which can attenuate the host immune response by downregulation of proinflammatory cytokines and by evading bacterial recognition by the innate immune system. The S layer also mediates *T. forsythia* attachment and invasion of epithelial cells and can induce abscess formation in animal models. *Bacteroides* and related organisms express sialidase enzymes that can cleave glycosidic linkages in host oligosaccharides and proteoglycan. This will disrupt the integrity of periodontal tissues, and in addition, sialic acid can be utilized as a nutrient and as a host mimic on the bacterial cell surface to avoid immune recognition. Sialidase activity can also expose adhesive epitopes, and the enzymes themselves can act as adhesins. Many anaerobes produce volatile fatty acids as an end product of metabolism. These short-chain fatty acids, such as butyric acid, are cytotoxic and can induce DNA fragmentation and apoptosis. Anaerobic metabolism can also lead to the production of volatile sulfur compounds, such as hydrogen sulfide, methyl mercaptan, dimethyl sulfide, ammonia, and indole, all of which are cytotoxic. Methyl mercaptan is produced from methionine by methionine-α-deamino-γ-mercaptomethane lyase, which is present in *P. gingivalis* and *T. denticola*. Methyl mercaptan can inhibit protein synthesis and cell migration. Hydrogen sulfide is a metabolic end product of the fermentation of cysteine. *T. denticola* can also produce hydrogen sulfide from glutathione, utilizing the enzyme cystalysin, which can disrupt the membrane of erythrocytes.

RESISTANCE TO NEUTROPHIL KILLING

The more-pathogenic periodontal bacteria tend to exhibit resistance to phagocytosis and intracellular killing mechanisms as well as resistance to antimicrobial peptides. As *P. gingivalis* is an anaerobic organism, it possesses mechanisms to protect against reactive oxygen species and oxidative stress. These include an excision/repair system for removing oxidatively damaged DNA bases, superoxide dismutase, and rubrerythrin. These components also protect against oxidative killing by neutrophils, and indeed rubrerythrin can protect against reactive nitrogen species. The *T. denticola* Msp selectively impairs chemotaxis and phagocytosis by impacting the polymorphonuclear leukocyte cytoskeleton. *A. actinomycetemcomitans* leukotoxin kills neutrophils as mentioned above. Further discussion of the interactions between periodontal bacteria and the host immune system can be found in Chapter 15.

KEY POINTS

Periodontal diseases are polymicrobial infections involving synergistic bacterial communities. The virulence of periodontal pathogens is complex and multifactorial. A number of pathogenic mechanisms have emerged as important.

Colonization
Periodontal bacteria adhere to epithelial cells in the gingival crevice and to other bacteria in subgingival plaque. Adhesins are often associated with fimbriae on the bacterial surfaces. Adhesion to sites outside the gingival crevice can be followed by translocation to the subgingival area by spreading proliferation or translocation of dislodged progeny. Spirochetes, which are motile by means of their periplasmic flagella, can reach the gingival crevice and tissues via chemotactic attraction.

Invasion
P. gingivalis and other periodontal bacteria can direct their uptake into the otherwise nonphagocytic gingival epithelial cells. An intracellular location protects the bacteria from the immune system and can result in modulation of production of immune effectors such as cytokines. Spirochetes and other organisms invade the gingival tissues, causing inflammation and tissue damage.

Toxins
A. actinomycetemcomitans produces a potent leukotoxin that kills human neutrophils and monocytes by causing pore formation in cell membranes and by inducing apoptosis. The leukotoxin can be packaged in membrane-derived vesicles that readily penetrate the tissues. *A. actinomycetemcomitans* also produces a cytolethal distending toxin that causes cell cycle arrest.

Enzymes and toxic products
P. gingivalis produces a number of proteolytic enzymes to provide peptides for nutrition. These proteases degrade immune effector molecules, structural component of tissues, and iron- and hemin-sequestering molecules. Fatty acids and sulfur compounds produced by bacteria can inhibit host cell division.

Cell constituents
LPS interacts with monocytes/macrophages via CD14 and TLRs on macrophages and dendritic cells to induce production of cytokines and inflammatory mediators such as IL-1. Additionally, LPS interactions with monocytes/macrophages lead to the synthesis and secretion of prostanoids, notably prostaglandin E2. These pathways can result in bone and collagen destruction. Capsule and other extracellular material can prevent phagocytosis.

Resistance to neutrophil killing
Molecules that resist oxidative stress such as superoxide dismutase and rubrerythrin can also provide resistance to neutrophil oxygen-dependent killing.

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Immunopathogenic Mechanisms in Periodontal Disease

George Hajishengallis and Toshihisa Kawai

INTRODUCTION

Periodontal disease is perhaps the most common infection-driven chronic inflammatory disease in humans. This oral disease is initiated by certain species of subgingival gram-negative anaerobic bacteria and leads to inflammatory destruction of the tooth-supporting tissues. The causative bacteria coexist within dynamic microbial communities originally termed "dental plaque" and now mostly referred to as "dental biofilm." These communities are not random collections of bacteria; rather, they represent organized consortia that have evolved through interactions that secured them a niche with adequate nutrients and protection against host immunity. Although traditionally a limited number of putative periodontal pathogens have received particular attention, it should be noted that a significant percentage of the approximately 700 bacterial species present in the human oral cavity are as yet uncultivable. Culture-independent molecular characterization of the human periodontal microbiota has shown that it alone contains over 400 species and is more heterogeneous and diverse than previously thought. In addition to the consensus "red complex" pathogens (*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*), 16S rRNA gene cloning and sequencing identified several novel disease-associated organisms, such as the gram-positive *Filifactor alocis* and *Peptostreptococcus stomatis* and species in the genera *Prevotella*, *Megasphaera*, *Selenomonas*, *Desulfobulbus*, *Dialister*, and *Synergistes*. It is now becoming apparent that the microbial etiology of periodontitis entails synergistic interactions between bacteria with different roles; that is, the pathogenicity is expressed in the context of a synergistic microbial community. For instance, some bacteria may assist other bacteria in the colonization process. Still other bacteria may be involved in the breakdown of tissue homeostasis, which in turn may allow other community members to trigger destructive inflammation leading to tissue breakdown products that serve the nutritional needs of the community at large. In this context, in a mouse model of periodontitis, *P. gingivalis* was shown to disrupt host homeostasis, leading to destructive periodontal inflammation that required the participation of the commensal periodontal microbiota.

Although a pathogenic microbial community is required for initiating periodontal disease, it should be noted that it is the undesirable side

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effects of the host immune response to this pathogenic microbial community, rather than direct microbial toxic activities, that primarily inflict damage upon the periodontium. Consistent with this, transgenic mice that overexpress the inflammatory cytokine interleukin 1α (IL-1α) in the basal layer of the oral mucosal epithelium develop spontaneous periodontitis. Moreover, mice genetically deficient in the anti-inflammatory cytokine IL-10 display significantly accelerated periodontal bone loss as they age compared to normal control mice.

The etiology and underlying mechanisms of periodontitis are quite complex. This may be due, at least in part, to the fact that periodontitis is not a classic infectious disease (i.e., one that conforms to Koch's postulates), nor does it constitute sterile inflammation. One way to simplify—at least conceptually—the immunopathogenic mechanisms of periodontitis is to define the fundamental differences between periodontal health and disease. Periodontal health represents a dynamic balanced state wherein the proinflammatory and antimicrobial activities of the host are optimally regulated to prevent unwarranted host reactions. In periodontally healthy subjects, the collective action of innate and adaptive immune responses establishes a symbiotic (or at least neutral) relationship between the host and the microbiota. This relationship maintains the integrity of the gingival epithelium, thereby preventing the entry of pathogenic bacteria into the underlying connective tissue. This physiological, immunoinflammatory surveillance also leads to the production of antimicrobial peptides by the epithelium and to the regulated recruitment of neutrophils, which together contribute to the control of potentially pathogenic bacteria. Periodontal disease occurs when this homeostatic balance is disrupted. This can be due to congenital immunodeficiencies or immunoregulatory defects. Moreover, homeostasis may be disrupted by certain pathogens that subvert the host response, thereby leading to nonprotective and nonresolving chronic inflammation. Briefly stated, favorable or predisposing conditions for the development of periodontitis may arise from either host-intrinsic factors (deficient or excessive production of immune and inflammatory mediators) or microbiological factors (a shift in the microbial composition of the biofilm that includes pathogens with the capacity to manipulate the host response).

The histopathology of periodontitis shows that the disease includes elements of both innate and adaptive immunity, which form a complex network of interactions leading eventually to inflammatory bone loss (Fig. 1). Innate immunity forms the first line of defense and, moreover, instructs the development of the adaptive arm of immunity. Our discussion will thus start with innate immunity and its role in periodontal pathogenesis.

DISRUPTION OF PROTECTIVE INNATE IMMUNITY

In the clinically healthy periodontium, the innate immune system is maintained in an active state, which could be characterized as an "armed peace" with the microbiota. This physiological inflammatory state is, to a large extent, the result of host interactions with commensal bacteria, which are in close contact with (but do not invade) the periodontal tissues. In this context, the healthy human gingiva displays a coordinated gradient of

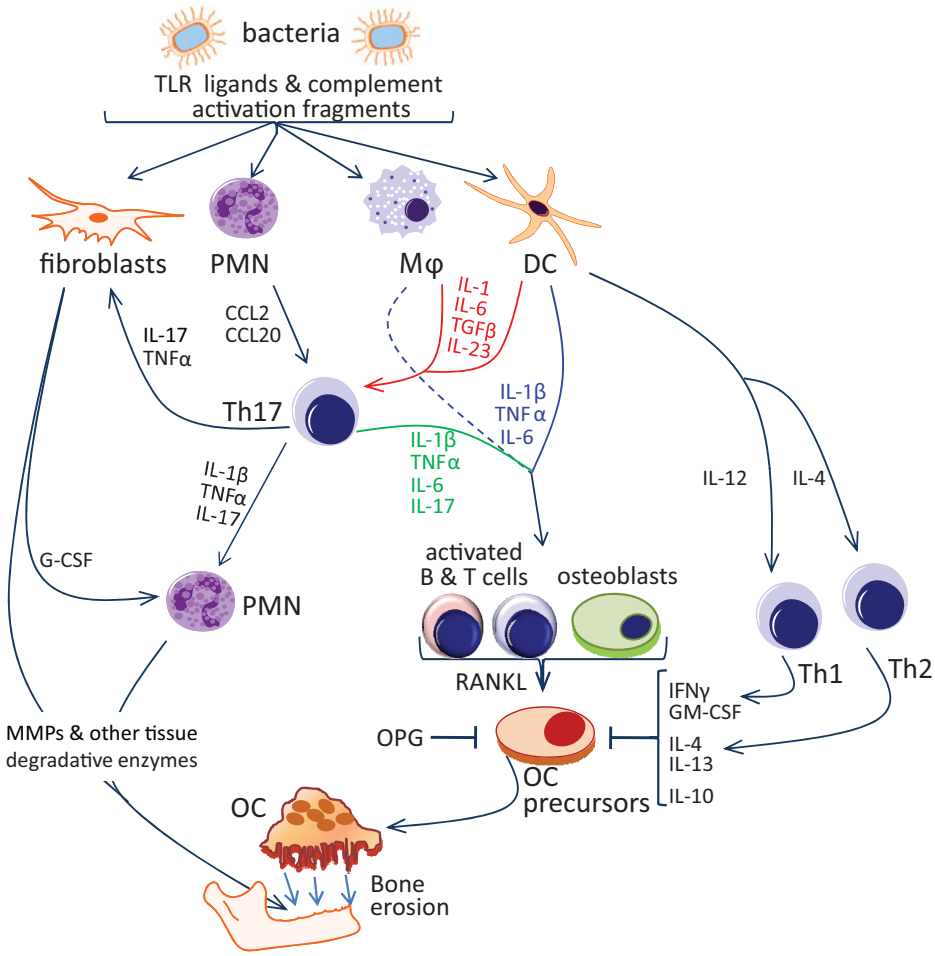

FIGURE 1 Interplay of innate and adaptive immunity in inflammatory periodontal bone loss. Periodontal bacteria interact with complement and TLRs, which in turn regulate the activation of innate immune cells. Antigen-presenting cells (APC), such as macrophages (Mφ) and DCs, regulate the development of Th cells, including Th17 cells, a subset acting as a link between innate and adaptive immunity. APC production of IL-1, IL-6, and TGF-β mediates Th17 cell differentiation, while IL-23 is involved in the survival and expansion of Th17 cells. Polymorphonuclear neutrophils (PMN) produce the chemokines CCL2 and CCL20, which selectively recruit Th17 cells to sites of infection and inflammation. PMN themselves are recruited to the periodontium through complement anaphylatoxins and chemokines, whereas fibroblast-secreted granulocyte colony-stimulating factor (G-CSF) induces PMN mobilization from the bone marrow. Th17 cells secrete IL-17 and other inflammatory cytokines, which activate innate immune and connective tissue cells (e.g., PMN and fibroblasts) that contribute to periodontal tissue damage via expression of metalloproteinases (MMPs) and other degradative enzymes. Inflammatory cytokines such as TNF, IL-1β, and IL-17 stimulate the expression of receptor activator of nuclear factor-κβ ligand (RANKL) by osteoblastic stromal cells, which thereby drive the maturation of osteoclast (OC) precursors. Activated lymphocytes (B and T cells) play a major role in pathologic bone resorption through the same RANKL-dependent mechanism, whereas OPG is a soluble decoy receptor that inhibits the interaction of RANKL with its functional receptor (RANK). The RANKL/OPG ratio increases with increasing periodontal disease activity. The anti-inflammatory cytokine IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, IL-4, and IL-13 inhibit osteoclastogenesis. Although IFN-γ and IL-4 are signature cytokines of Th1 and Th2 cells, respectively (the differentiation of which depends on APC production of IL-12 and IL-4, respectively), the roles of Th1 and Th2 cells are not necessarily protective. In fact, Th1 cells express RANKL, whereas Th2 cells have been associated with the established periodontal lesion. The innate-adaptive cell interplay is considerably more complex than depicted here, but this diagram serves to illustrate major destructive mechanisms operating in the context of unresolved periodontal infection and inflammation. doi:10.1128/9781555818906.ch15.f1

chemokines and cell adhesion molecules, such as IL-8 and intercellular adhesion molecule-1 (ICAM-1), respectively, which is topographically associated with the pathway of neutrophil migration from the vasculature to the gingival crevice. The gradient becomes denser toward the upper junctional epithelial cell layers, which are closer to the site of bacterial challenge. Moreover, in a periodontally healthy state, gingival epithelial cells and leukocytes present in the junctional epithelium express an array of antibacterial factors, including α- and β-defensins and the cathelicidin LL-37, as well as soluble or membrane-bound CD14 and lipopolysaccharide (LPS)-binding protein.

This armed peace can transition to open war when the homeostatic balance is disrupted. Given the roles of chemokine and cell adhesion molecules in leukocyte migration, the disruption of their characteristic gradient could impair the ability of the host to effectively mobilize leukocytes to sites of infection. One such mechanism is the so-called local chemokine paralysis. Specifically, the ability of gingival epithelial cells to coordinately induce IL-8 for the chemoattraction of neutrophils is proactively inhibited by *P. gingivalis* upon its invasion into epithelial cells. In the presence of *P. gingivalis*, gingival epithelial cells are inhibited from eliciting IL-8 responses even when exposed to other periodontal bacteria that are potent inducers of this chemokine on their own. This inhibitory effect on IL-8 is mediated by a secreted serine phosphatase (SerB) of *P. gingivalis*. Moreover, *P. gingivalis* acts on endothelial cells and inhibits the upregulation of the cell adhesion molecule E-selectin by other periodontal bacteria. Inhibition of E-selectin expression results in diminished neutrophil adhesion to endothelial cells in vitro and, along with the disruption of the IL-8/ICAM-1 gradient, could suppress the extravasation and directed migration of neutrophils to the gingival crevice. These subversive effects may be transient but may allow adequate time for *P. gingivalis* to initiate its colonization while delaying the influx of neutrophils. When eventually the neutrophils do come in large numbers in the gingival crevice, *P. gingivalis*, now a member within an organized microbial community, may be able to resist killing through additional subversive tactics (see below).

P. gingivalis expresses an atypical LPS molecule that inhibits the activation of Toll-like receptor (TLR) signaling, a major host mechanism of sensing and responding to an infection. Specifically, *P. gingivalis* produces LPS with a 4-acyl monophosphate lipid A structure, which acts as an antagonist of TLR4 and thereby competitively inhibits the activation of this innate immune receptor (Fig. 2). This is in stark contrast to typical LPS molecules (produced, for instance, by enteric bacteria such as *Escherichia coli* and *Salmonella* species) that function as strong agonists of TLR4. *P. gingivalis* also inhibits the activation of TLR4 by other periodontal bacteria. This TLR4 antagonist of *P. gingivalis* has been shown to inhibit cell activation, including the downregulation of β-defensin expression by human gingival epithelial cells, and may thus contribute to the ability of this pathogen to evade host defenses.

Complement has been traditionally viewed as an antimicrobial enzyme system operating in serum and inflammatory exudates, such as the gingival crevicular fluid (GCF). However, it is now well appreciated that complement is a fundamental component of host immunity, by virtue of its ability to orchestrate critical events during immune and inflammatory

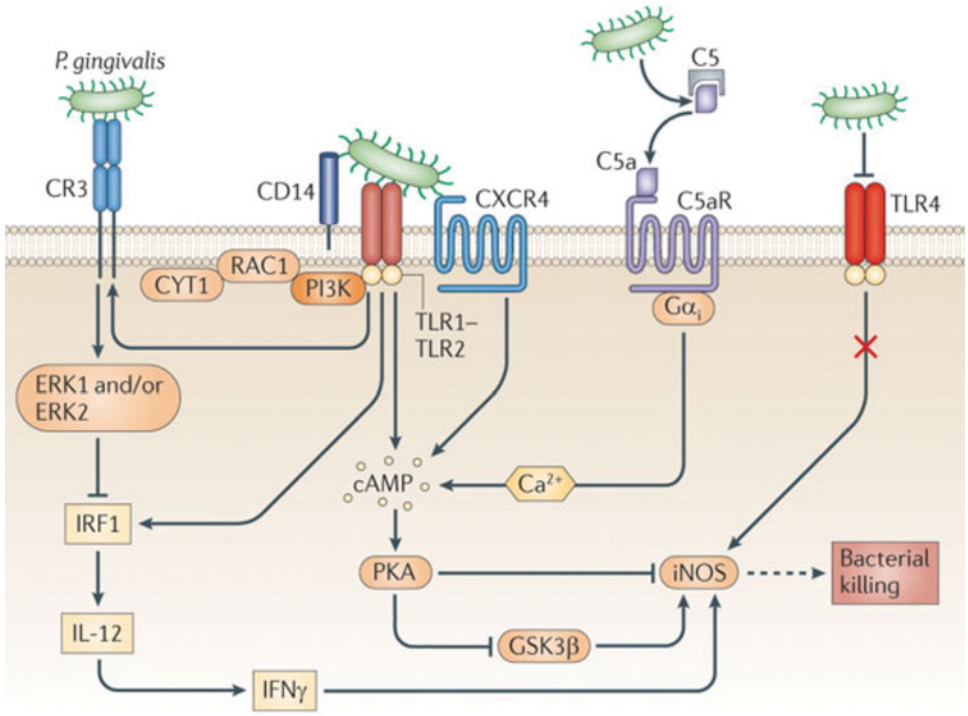

FIGURE 2 *P. gingivalis* escapes killing by manipulating host signaling pathways. *P. gingivalis* interacts with TLR2 (specifically with the CD14-TLR2-TLR1 complex) and TLR4. TLR4 is blocked by the bacterium's atypical LPS, which acts as a specific antagonist; therefore, TLR4 may not induce protective responses. The TLR2 response is proactively modified through cross talk with other receptors that are manipulated by *P. gingivalis*. *P. gingivalis* controls C5a receptor (C5aR) by virtue of Arg-specific cysteine proteinases, which attack C5 and release biologically active C5a. C5a stimulates intracellular Ca^{2+} signaling, which synergistically enhances the otherwise weak cyclic AMP (cAMP) responses induced by TLR2 activation alone. Maximal cAMP induction requires the participation of CXCR4, which is activated directly by the pathogen's fimbriae. The ensuing activation of the cAMP-dependent protein kinase A (PKA) inactivates glycogen synthase kinase-3β (GSK3β) and impairs the inducible nitric oxide synthase (iNOS)-dependent killing of the pathogen in macrophages. An additional pathway induced downstream of TLR2 is an inside-out signaling pathway, mediated by Rac1, phosphatidylinositol-3 kinase (PI3K), and cytohesin 1 (CYT1), which transactivates complement receptor 3 (CR3). Activated CR3 binds *P. gingivalis* and induces extracellular signal-regulated kinase-1 (ERK1)/ERK2 signaling, which in turn selectively downregulates IL-12, p35, and p40 mRNA expression through suppression of IFN regulatory factor 1 (IRF1). Inhibition of bioactive IL-12 and secondarily of IFN-γ leads to impaired immune clearance of *P. gingivalis*. Reproduced from G. Hajishengallis and J. D. Lambris, *Nat. Rev. Immunol.*, **11**:187–200, 2011, with permission. doi:10.1128/9781555818906.ch15.f2

responses, including regulation of other systems such as TLR signaling pathways. In principle, complement could contribute to periodontal host defense by tagging and eliminating microbes in the gingival crevice and by chemotactically recruiting and activating (in concert with TLRs) phagocytes to control the infection. However, complement is manipulated by bacteria in different ways: certain complement functions are inhibited, while others are excessively activated beyond host control. In this regard, *P. gingivalis* and *Prevotella intermedia* express proteases (gingipains and

interpain, respectively) that degrade and inactivate C3, the most central complement component shared by all three initiation pathways (classic, lectin, and alternative) of complement activation. Subversive actions by these bacteria would also be expected to protect other cohabiting bacteria that do not share the capacity to inactivate C3. In this regard, *P. gingivalis* releases readily diffusible membrane vesicles that contain critical virulence factors (e.g., gingipains) that can promote the survival and persistence of the entire subgingival biofilm, rendering it more pathogenic. This capacity of *P. gingivalis* to act as the keystone of the periodontal microbial community, i.e., to support it in a way that alters its composition and numbers, has been demonstrated in the mouse model of periodontitis.

P. gingivalis and *P. intermedia*, as well as other periodontal bacteria, such as *T. denticola*, have also developed protease-independent strategies to evade killing by complement. Specifically, these bacteria can bind and hijack host-derived complement inhibitors (such as factor H and C4b-binding protein), which are normally intended to protect host cells against the toxic actions of complement. By acquiring these negative regulators, the bacteria can now neutralize the effector functions of complement and thus protect themselves. These and other strategies by which periodontal bacteria evade complement-mediated killing or exploit complement components to proactively undermine innate immunity are summarized in Table 1.

The above are just a few examples of how the host can lose control in handling periodontal infections due to proactive interference by sophisticated pathogens. However, the homeostatic balance required for protective immunity and periodontal health may also be disrupted by host immunodeficiencies or immunoregulatory defects. This phenomenon is supported by a number of observations in humans and experimental animals. Individuals with neutrophil disorders with respect to numbers (chronic or cyclic neutropenia), trafficking capacity (leukocyte adhesion deficiencies), or killing function (Chédiak-Higashi and Papillon-Lefèvre syndromes) all have increased susceptibility to and severity of periodontitis, including forms of the disease that appear in young individuals. Similarly, mice with impaired mobilization of leukocytes to sites of infection, owing to combined P- and E-selectin deficiency or due to deficiency of the LFA-1 integrin, cannot control their oral microbiota.

TABLE 1 Exploitation of complement by oral pathogens

Mechanism	Pathogen(s)	Effector molecule(s)
1 Inhibition of complement activation through digestion of the central complement component (C3)	*P. gingivalis*	Gingipains, especially HRgpA and RgpB
	P. intermedia	Interpain (InpA)
Synergy in complement inactivation	*P. gingivalis* and *P. intermedia*	Gingipains and interpain
2 Inherent resistance to complement-mediated lysis	*P. gingivalis*	Surface anionic polysaccharide
3 Hijacking complement regulatory proteins		
C4b-binding protein	*P. gingivalis*	HRgpA gingipain
Factor H	*T. denticola*	11.4-kDa factor H-binding lipoprotein
4 Generation of specific complement fragments		
iC3b (promotes phagocytosis linked to poor microbicidal activity)	*T. denticola*	Dentilisin
C5a (inhibits TLR-induced nitric oxide-dependent killing)	*P. gingivalis*	Gingipains
5 Promotion of intracellular survival via complement receptor 3-mediated entry	*P. gingivalis*	Fimbriae

This leads to massive accumulation of dental plaque, which, in turn, results in periodontal inflammation and alveolar bone loss. Similar bacteriological findings and periodontitis features are observed in mice whose neutrophils display impaired bacterial killing owing to the genetic absence of the lysosome-associated membrane protein-2. In all these cases, induction of inflammation and periodontal bone loss are prevented by antibiotics, thus confirming the involvement of indigenous bacteria in the disease. Smoking-associated periodontal disease also appears to involve suppression of the immune system by the effect of nicotine. The immunocompromised condition of patients with HIV and recipients of organ transplantation on immunosuppressive drugs predisposes them to the development of periodontal disease as a result of impaired host immunity. These studies collectively suggest that the normal function of the immune system is important for periodontal homeostasis. Inadequate immunity allows the bacteria to thrive and induce inflammation that causes tissue damage. Conversely, hyperimmune responses may also lead to undesirable effects and initiation of periodontal disease. For example, mice deficient in Del-1, an endothelial protein that controls the recruitment of neutrophils to the periodontal tissue, develop spontaneous periodontitis characterized by heavy neutrophil infiltration.

UNRESOLVED INFLAMMATION IN PERIODONTITIS

Although *P. gingivalis* can inhibit the activation of complement, this bacterium can also "voluntarily" activate specific complement components independently of the normal complement cascade. For instance, the Arg-specific gingipains of *P. gingivalis* function as C5 convertase-like enzymes, allowing the bacterium to generate high levels of C5a (Fig. 2). Considering that C5a is the most potent effector of complement, this function is quite curious for a pathogen that goes to great lengths to inhibit all three known initiation mechanisms of complement activation. However, this unconventional behavior allows *P. gingivalis* to activate the C5a receptor in tandem with TLR2, leading to signaling cross talk that inhibits specific antimicrobial pathways in leukocytes (e.g., nitric oxide-dependent killing in macrophages) (Fig. 2; Table 1). *P. gingivalis* manipulates intracellular signaling pathways and escapes elimination also by exploiting additional innate immune receptors, such as the complement receptor 3 and the chemokine CXC receptor 4 (CXCR4) (Fig. 2). Bacteria that promote their phagocytosis through complement receptor 3, either because they can directly bind the receptor (e.g., *P. gingivalis* by means of its fimbriae) (Fig. 3) or because they can generate a ligand for the receptor (e.g., *T. denticola* by means of its dentilisin) (Table 1), may escape intracellular killing since complement receptor 3-associated phagosomes do not readily fuse with lysosomes.

Although the *P. gingivalis*-instigated C5a receptor-TLR2 cross talk inhibits leukocyte killing, it does not cause generalized immunosuppression. Indeed, the C5a receptor-TLR2 cross talk upregulates inflammatory cytokines, such as IL-1β, IL-6, and tumor necrosis factor (TNF) and enhances the oxidative burst, which, however, does not seem to harm *P. gingivalis*. Rather, these effects enhance the persistence of *P. gingivalis* in animal models of periodontitis. This is because inflammation facilitates

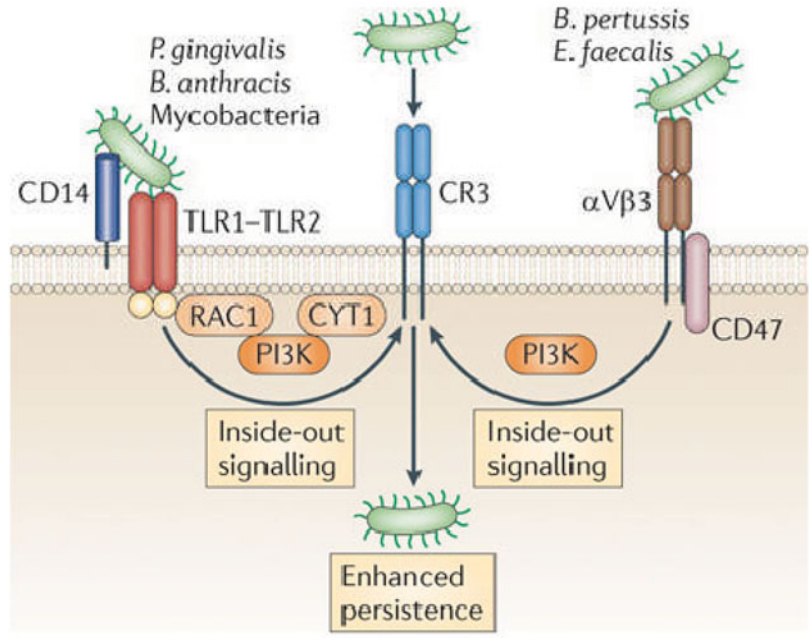

FIGURE 3 *P. gingivalis* induces "safe" phagocytosis by transactivating complement receptor 3 (CR3)-mediated internalization. *P. gingivalis* binds CD14 and induces TLR2-TLR1 inside-out signaling for activating and binding of CR3, which leads to a relatively safe uptake of the organism by macrophages. The signaling pathway that activates the high-affinity state of CR3 is mediated by RAC1, phosphatidylinositol-3 kinase (PI3K), and cytohesin 1 (Cyt1). *Mycobacterium tuberculosis* and *Bacillus anthracis* use a similar strategy to prevent intracellular killing. *Enterococcus faecalis* and *Bordetella pertussis* stimulate their uptake by CR3 through an alternative inside-out signaling pathway. This mechanism is activated by the interaction of these bacteria with a receptor complex comprising the αvβ3 integrin and CD47 and is dependent on PI3K signaling. Similarly, CR3-mediated uptake of these bacteria inhibits their intracellular killing and promotes their persistence in the mammalian host. Reproduced from G. Hajishengallis and J. D. Lambris, *Nat. Rev. Immunol.*, **11:**187–200, 2011, with permission. doi:10.1128/9781555818906.ch15.f3

the acquisition of essential nutrients by periodontal bacteria. These may include hemin (a source of iron) and protein fragments present in the GCF, the flow of which increases with increasing inflammation. Therefore, asaccharolytic and proteolytic periodontal bacteria (such as *P. gingivalis*) may have evolved to not only endure inflammation but also exploit it to serve their nutritional needs, while inflammation causes collateral periodontal tissue injury.

As alluded to above, a coordinated and topographically restricted chemokine/cell adhesion molecule expression gradient is expected to spatially limit the infiltration of inflammatory cells to sites of infection and thereby to prevent or mitigate collateral tissue damage. Conversely, disruption of this pattern of expression, accompanied by excessive complement activation and production of C5a, which is chemotactic for a broad spectrum of leukocytes, may result in out-of-control inflammation. Unwarranted and excessive recruitment of neutrophils (which constitute ≥95% of total leukocytes in the gingival crevice) mediates much of the host-mediated inflammatory tissue injury in periodontitis. Gingival crevicular neutrophils form what looks like a defense wall against the dental

biofilm bacteria. However, in periodontitis, the neutrophils largely fail to control the bacteria, even though the neutrophils remain viable and capable of eliciting inflammatory responses. The reason(s) may have more to do with microbial evasion mechanisms, as outlined above, than with the possible inherent resistance of biofilms to neutrophil phagocytosis. In fact, neutrophils can potentially attack and destroy biofilms through phagocytosis and degranulation, depending on the nature and composition of the biofilm.

The inability of crevicular neutrophils to control the subgingival biofilm and their sustained recruitment to the periodontium may be detrimental to the periodontal health of the host. This is because neutrophils can mediate periodontal tissue destruction through the release of inflammatory cytokines, oxygen radicals, and matrix-degrading metalloproteases. IL-17, a signature cytokine of the Th17 cell subset (see below) but also expressed by innate immune cells at sites of inflammation, plays a key role in neutrophil function and inflammation, as it induces or amplifies the expression of genes relevant to their recruitment and activation. Specifically, IL-17 promotes granulopoiesis and induces the chemotactic recruitment, activation, and survival of neutrophils. While IL-17 can stimulate host defenses, chronic IL-17 receptor signaling can turn an acute inflammatory response into chronic immunopathology, as occurs in rheumatoid arthritis. In this context, IL-17 can mediate connective tissue destruction and bone resorption through the induction of matrix metalloproteases and receptor activator of nuclear factor-κB ligand (RANKL) (see below).

In addition to neutrophils, IL-17 also acts on a variety of immune and nonimmune cell types including antigen-presenting cells, epithelial and endothelial cells, fibroblasts, and osteoblasts. Neutrophils cross talk with fibroblasts, which upon IL-17 activation secrete granulocyte colony-stimulating factor, which contributes to the mobilization of neutrophils from the bone marrow to the circulation and subsequently to sites of infection or tissue damage. In the context of unresolved periodontal inflammation, one may envision a network of immune and nonimmune cells that cross talk through the release of inflammatory cytokines (e.g., TNF, IL-1β, IL-6, or IL-17) or through cell-cell interactions, leading to events that destroy the periodontal connective tissue and bone (Fig. 1). The involvement of these proinflammatory cytokines in the pathogenesis of periodontitis is supported by clinical observations confirming their increased presence in disease and by interventional studies in animal models including nonhuman primates. For instance, the inhibition of IL-1 or TNF protects monkeys against experimental periodontitis. Moreover, experimental periodontitis in rabbits has been reversed by the use of lipid compounds, such as lipoxins and resolvins, which can control and resolve inflammation.

ROLE OF ADAPTIVE IMMUNITY IN PERIODONTITIS

Inflamed periodontal tissues contain organized lymphoid/myeloid aggregates rich in $CD4^+$ T cells, B cells, and antigen-presenting cells—mostly dendritic cells (DCs)—which regulate the development of adaptive immune responses. Most stages of periodontal disease include elements of

both innate and adaptive immune responses. Indeed, destructive inflammation and periodontal bone resorption are the outcome of a complex interplay between a persisting biofilm and innate and adaptive immune cells (Fig. 1). It is possible that the failure of the innate immune response to control periodontal infection leads to the recruitment of T cells and B cells to the periodontium and the development of a B-cell/plasma cell lesion, which characterizes the advanced stage of periodontitis. Topographically, the advanced lesion is not restricted to the area around the gingival sulcus but extends apically as well as laterally. One would expect that if specific antibodies with high affinity and functionality were elicited, the infection would be controlled and the disease would not progress. If, on the other hand, the result of B-cell activation leads to low-avidity antibodies or antibodies with poor antibacterial properties (e.g., poor in promoting opsonophagocytosis), then infection and ensuing inflammation would persist. Therefore, it is important to consider the role of T cells and B cells in periodontitis, starting from some basic concepts underlying their biology.

The gingival crevice provides an important function in terms of the development of adaptive immunity, namely, the sampling of bacterial antigens. Specifically, in contrast to the keratinized epithelium of the gingiva and the rest of the oral mucosa, the junctional epithelium in the crevice is not keratinized, thus permitting the direct interaction between immune cells and bacterial products. Bacteria or bacterial antigens penetrating the nonkeratinized epithelial junction are captured by innate immune phagocytes, such as macrophages and DCs, which patrol the lamina propria of the crevicular epithelium. After the capture of bacteria or bacterial antigens, macrophages and DCs process and present these antigenic peptides to T cells. DCs are particularly good at antigen presentation and serve as an innate bridge to the adaptive immune response. Protein antigens from ingested bacteria are processed, and antigenic peptides are then presented by the DCs on major histocompatibility complex (MHC) class II molecules to T-cell receptors expressed on the surface of T cells. Antigen presentation by itself, however, is not sufficient to activate T cells. The recognition of bacterial surface structures, such as the so-called pathogen-associated molecular patterns (e.g., LPS), by DC-expressed TLRs upregulates the expression of costimulatory molecules, such as B7-1(CD80) and B7-2 (CD86), on the DCs. These, in turn, provide costimulatory signals to the T cells, which along with antigen-specific stimulation lead to T-cell activation and proliferation. Activated T cells of the helper subsets (i.e., $CD4^+$ T cells) can then provide help to antigen-specific B cells. In this regard, activated $CD4^+$ T cells express CD40 ligand (CD40L), which is required to switch the immunoglobulin (Ig) isotype produced by B cells from IgM to IgG. Specifically, antigen cross-linking of the B-cell receptor and the interaction of CD40 on the B-cell surface with CD40L on $CD4^+$ T cells, as well as T-cell-secreted cytokines, collectively lead to the activation of B cells and, eventually, their differentiation into IgG antibody-secreting plasma cells.

As alluded to above, the adaptive immune response in periodontitis is potentially destructive. This is readily supported by animal studies. Indeed, severe combined immunodeficient (SCID) mice, which lack both T and B cells and cannot mount adaptive immune responses, develop reduced *P. gingivalis*-induced bone loss compared to immunocompetent

mice. With regard to T cells, it is the MHC class II-restricted $CD4^+$ T cells that are particularly involved, since $CD4^+$ T-cell-deficient mice display greater resistance to *P. gingivalis*-induced bone loss than normal mice or mice deficient in MHC class I-restricted $CD8^+$ T cells. Specific implication of B cells is supported by a study showing that adoptive transfer of *Aggregatibacter actinomycetemcomitans*-specific B cells into rats infected with the same pathogen leads to increased periodontal bone resorption.

In contrast to the T-cell-rich lesions found in gingivitis, the lesions in periodontitis are characterized by prominent infiltration of B cells and a modest level of T-cell infiltration. Therefore, the B-cell-mediated adaptive immune response, specifically an IgG-dominant B-cell lesion, appears to be associated with the pathogenesis of periodontitis, especially bone resorption. It may appear surprising that periodontal disease progresses in spite of the production of antibodies. In principle, B cells could confer protection against periodontal bacteria through their role in humoral immunity. However, naturally induced antibodies to periodontal bacteria appear to be of low affinity and poor functionality. In addition to their involvement in the activation of osteoclasts (see below), B cells contribute to periodontal inflammation through the deposition of immune complexes composed of plasma cell-secreted antibodies and antigens, seen throughout the connective tissue along with complement activation fragments.

IgG antibodies produced by plasma cells have the potential to facilitate the clearance of bacterial components through the formation of immune complexes that can be removed by phagocytosis or protease-mediated degradation. However, excess deposition of immune complexes in a tissue can cause inflammation and lead to type III hypersensitivity and disease. Deposition of immune complexes in affected organs is a prominent feature of several autoimmune diseases, such as systemic lupus erythematosus, scleroderma, rheumatoid arthritis, and Sjögren's syndrome. The deposition of IgG immune complexes in diseased periodontal tissue is remarkably high compared to that in healthy gingival tissues and might be involved in inflammatory destruction of the periodontal tissue. In this regard, the binding of multiple Fc segments present in IgG immune complexes to the receptors for the Fc component of IgG (FcγRs) expressed on phagocytic cells induces production of proinflammatory and bone-resorptive cytokines. Of the three main types of FcγRs in humans, the high-affinity receptor, FcγRI, can bind monomeric IgG, whereas the two low-affinity receptors, FcγRII and FcγRIII, bind polymeric IgG in the form of immune complexes. Interestingly, a single-nucleotide polymorphism in the human FcγRIIIB has been associated with both aggressive and chronic periodontitis, which is consistent with a possible role for IgG immune complexes in periodontal pathogenesis.

OSTEOIMMUNOLOGICAL INTERACTIONS IN PERIODONTITIS

An osteoclast is a cell type involved in bone metabolism; it can resorb bone by demineralizing it and breaking up the organic matrix. Conversely, osteoblasts are responsible for bone formation. In essence, osteoblasts are specialized fibroblasts that, in addition to fibroblastic products, express bone sialoprotein and osteocalcin. The fundamental cytokine system governing bone resorption is dependent on osteoclast differentiation

and activation. A persisting inflammatory environment may ultimately disrupt bone homeostasis, which depends on a triad of proteins within the TNF/TNF receptor family consisting of RANKL, its functional receptor, RANK, and its decoy receptor osteoprotegerin (OPG). These proteins are key factors for osteoclast maturation and activation (osteoclastogenesis). Osteoclastogenesis is promoted by the binding of RANKL (expressed by certain cell types including activated T cells and B cells and osteoblasts) to RANK on osteoclast precursors (Fig. 4). OPG serves a regulatory function by inhibiting the interaction of RANKL with RANK. However, the bone-protective OPG effect is diminished in periodontitis since the OPG/RANKL ratio decreases with increasing periodontal inflammation. The molecular events underlying RANKL-dependent osteoclastogenesis and the role of the adaptive immunity in this regard are discussed below.

RANKL is produced in two different forms, a membrane-bound form (mRANKL) and a soluble form (sRANKL). The mRANKL expressed by osteoblasts or bone marrow stromal cells induces osteoclastogenesis through cell-cell contact between these mRANKL-expressing cells and osteoclast precursors in the bone marrow. In the context of pathogenic periodontal bone destruction, however, the adaptive immune response plays

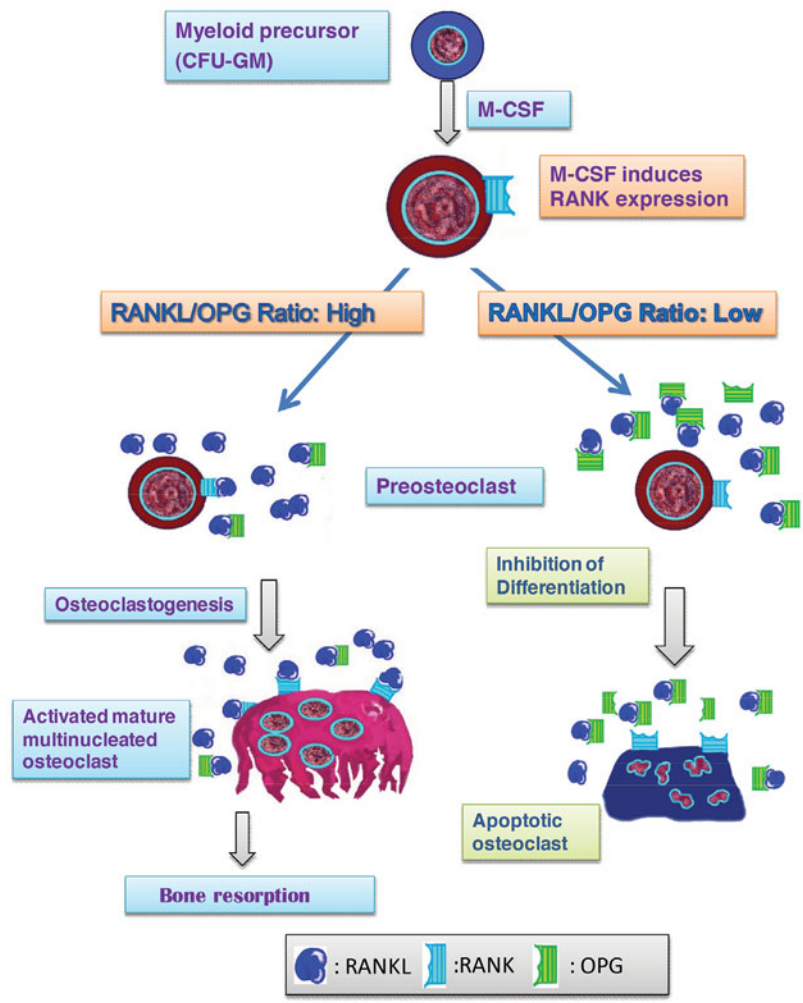

FIGURE 4 RANKL-mediated osteoclastogenesis. Osteoclast precursor cells derived from CFU granulocyte/macrophage (CFU-GM) stem cells express RANK in response to stimulation with macrophage colony-stimulating factor (M-CSF). When the levels of generated RANKL in the microenvironment of RANK-positive preosteoclasts outweigh those of OPG (a natural antagonist of RANKL), RANKL can readily bind RANK expressed on preosteoclasts, tipping the balance to favor osteoclastogenesis and bone resorption. Conversely, when the ratio of RANKL over OPG becomes low, OPG blocks the interaction of RANKL with RANK and inhibits osteoclastogenesis. The inhibition of RANKL binding to RANK by OPG also promotes osteoclast apoptosis. Therefore, the relative ratio of RANKL over OPG determines the intensity of osteoclastogenic bone resorption and plays a major role in the pathogenesis of periodontitis.
doi:10.1128/9781555818906.ch15.f4

a predominant role in osteoclastogenesis. Indeed, in the inflamed gingival tissue, nearly 100% of activated B cells produce RANKL, whereas 30 to 50% of activated T cells produce RANKL. Importantly, activated T cells and B cells produce both mRANKL and sRANKL, in contrast to osteoblasts or bone marrow stromal cells, which produce mRANKL but very little sRANKL. In periodontal lesions with active bone resorption, it is sRANKL, rather than mRANKL, that activates osteoclast precursor cells. This is because infiltrating lymphocytes have little contact with osteoclast precursors located on the surface of the alveolar bone, and therefore only lymphocyte-secreted sRANKL can effectively reach the osteoclast precursors. In contrast to the activated B and T cells, innate immune cells and periodontal ligament or gingival fibroblasts are relatively poor producers of RANKL (Fig. 5). Based on animal models of periodontal disease, few or no osteoblasts are found in the inflamed alveolar bone, suggesting that osteoblasts are not an important cellular source of RANKL in the context of inflammatory periodontal bone resorption. Although gingival epithelial cells express mRANKL, its possible functional significance is poorly understood. As gingivitis is characterized by infiltration of T cells, but not B cells, and gingivitis is not associated with bone loss, the terminal

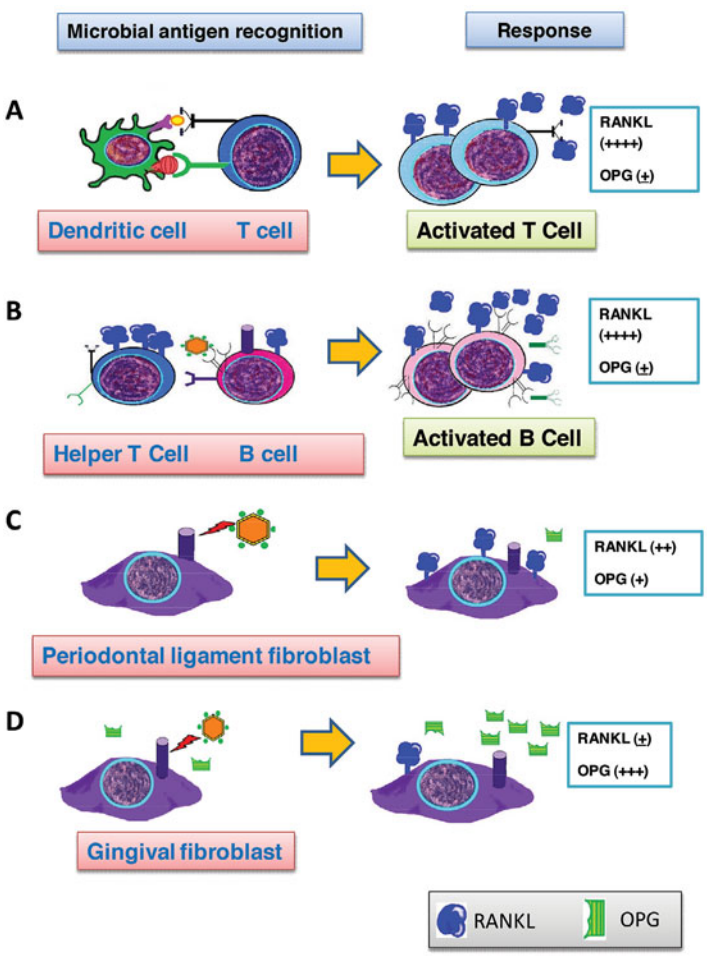

FIGURE 5 Production of RANKL and OPG by lymphocytes and fibroblasts. (A) Professional antigen-presenting cells, such as DCs, present microbial antigen to T cells and induce them to express both membrane-bound and soluble RANKL. (B) Microbial antigen engagement of the B-cell receptor induces B cells to produce not only IgG antibodies but also membrane-bound and soluble RANKL. Furthermore, the activated antigen-specific T cells provide costimulatory signals to B cells through CD40/CD40L ligation, which also upregulates RANKL expression in B cells. (C) Periodontal ligament fibroblasts express modest levels of membrane-bound RANKL but little OPG in response to microbial stimulation, increasing the RANKL/OPG ratio. (D) In contrast to periodontal ligament fibroblasts, gingival fibroblasts may downregulate osteoclastogenesis by producing high levels of OPG relative to RANKL. doi:10.1128/9781555818906.ch15.f5

activation of B cells seems to be critical for the detrimental effects of adaptive immunity in periodontitis. In this regard, the activation of B cells through B-cell receptor and CD40 promotes not only the immunoglobulin isotype switch, as outlined above, but also the secretion of sRANKL, which can readily diffuse and contact osteoclast precursors to promote osteoclastogenesis.

T-CELL SUBSETS IN PERIODONTAL DISEASE

Based on distinct cytokine production patterns and functions, $CD4^+$ T cells can be classified into five different subsets (cytokines in parentheses denote important cytokines produced from the respective T-cell subset): (i) Th1 (IL-2 and gamma interferon [IFN-γ]); (ii) Th2 (IL-4, IL-5, and IL-13); (iii) Th17 (IL-17 and IL-22); (iv) T-regulatory cells or Tregs (IL-10 and transforming growth factor β [TGF-β]); and (v) follicular helper T (Tfh) cells (IL-21) (Fig. 6). The Th1/Th2 paradigm, established in the late 1980s, elegantly explained much about T-cell-mediated immunity, but in many cases diseases of immunological etiology were pigeonholed into one category or the other, often without adequate supportive evidence. Indeed, after the identification of the Th17 subset, the role of Th1 in destructive inflammation has been questioned in some diseases, including rheumatoid arthritis. The biological properties and potential involvement in periodontitis of the various T-cell subsets is discussed below.

FIGURE 6 Th cell subsets and their functions. Naïve $CD4^+$ T cells interacting with antigen-presenting cells (APC), such as DCs, macrophages, or B cells, differentiate into various T-cell subsets depending on the generated cytokine environment. Shown are key cytokines involved in T-cell subset differentiation, as well as signature cytokines mediating important functions (indicated) of the differentiated T-cell subset. Pathological conditions, in which effector T-cell subsets have been implicated, are shown in red font. For instance, Th1 cells arise in the presence of IL-12 and secrete IFN-γ, which is a potent factor in cell-mediated immunity, although Th1 cells are implicated in delayed-type hypersensitivity. See the text for details on the other subsets.
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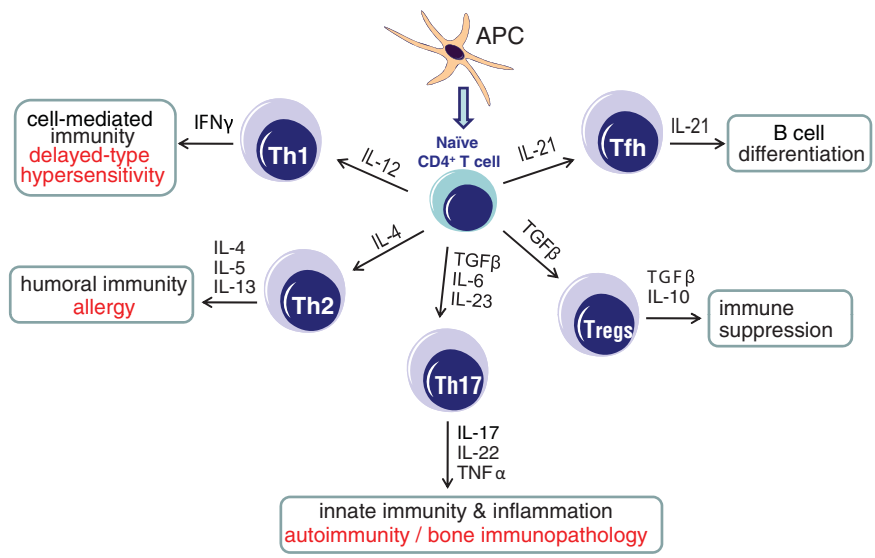

Th1 Cells

Antigen-presenting cells, such as macrophages and especially DCs, regulate the development of the various T-cell subsets and thus can influence the type of T-cell immunity. When a naïve $CD4^+$ T cell is exposed to antigens in the context of IL-12 derived from macrophages or DCs, it is driven to develop into a Th1 phenotype (Fig. 6). Th1 cells are primarily responsible for cell-mediated immunity to intracellular pathogens. Particularly important in this context is the ability of Th1 cells to secrete IFN-γ, which is a potent activator of the macrophage killing function. Although Th1 cells are not cytotoxic, their production of IL-2 and IFN-γ helps to generate cytotoxic $CD8^+$ T cells that kill host cells infected with bacteria, parasites, or viruses. Th1 cells are implicated in delayed-type hypersensitivity. Because the diseased periodontal tissue often displays high production levels of IFN-γ, Th1 cells are thought to contribute to periodontal disease. However, some studies have shown that IFN-γ can suppress RANKL-mediated osteoclastogenesis from bone marrow-derived osteoclast precursor cells (Fig. 1), although this effect appears to be dependent on the differentiation stage of the osteoclasts. On the other hand, Th1 cells may promote osteoclastogenesis by virtue of their production of RANKL, which is higher than in Th2 cells. In addition to their involvement in cell-mediated immunity, activated Th1 cells also upregulate IgG antibody responses. Therefore, it is plausible that the Th1 response in periodontitis may additionally contribute to the disease by facilitating the induction of the IgG response (see above).

Th2 Cells

When a naïve $CD4^+$ T cell is exposed to antigens in the context of IL-4 derived from antigen-presenting cells, it is induced to develop into a Th2 cell, which is characterized by the production of IL-4, IL-5, and IL-13 (Fig. 6). Th2 cells play a major supporting role in humoral immunity including production of IgE. Therefore, this T-cell subset is important for the activation of mast cells, which mediate immune responses to helminths but can also be involved in allergic reactions. However, since there are few or no basophils or eosinophils in periodontal lesions, pathogenic allergic reactions may not be important in periodontal pathogenesis. In diseased periodontal tissue, the production of Th2-type cytokines may not be as high as Th1-type cytokines. On the other hand, periodontal patients have high levels of IgG4 antibody, which is a Th2-associated immunoglobulin isotype in humans, and thus Th2-type immune responses may also play a role in periodontitis. Although the role of the Th2-type immunity in periodontal pathogenesis is overall uncertain, IL-4 and IL-13 may have a protective role through their ability to suppress RANKL-mediated osteoclastogenesis (Fig. 1).

Th17 Cells

Although early studies implicated IL-23 in inducing the differentiation of Th17 cells, it is now clear that TGF-β, IL-6, IL-1, and IL-21 are key cytokines in driving Th17 differentiation, whereas IL-23 is required for the expansion and survival of this T-cell subset. All of these cytokines are found in the inflamed periodontium.

IL-17, a signature cytokine of Th17 (but also produced by innate immune cells), acts on a number of cell types, including neutrophils, fibroblasts, endothelial cells, epithelial cells, and macrophages, which, in turn, induce the production of many other proinflammatory cytokines, such as IL-6, IL-1β, and TNF, and chemokines, including IL-8 and monocyte chemotactic protein-1. The release of these cytokines and chemokines amplifies inflammation and induces the recruitment of phagocytes, especially neutrophils, to the site of infection. These are potentially protective responses that can control infections with extracellular pathogens. Furthermore, production of IL-22 from Th17 cells stimulates epithelial cells to produce antimicrobial peptides, which underscores the potentially protective nature of this T-cell subset, at least in acute infection responses.

However, chronic IL-17 receptor signaling has been implicated in bone immunopathology. In this context, IL-17 stimulates connective tissue destruction and bone resorption by inducing matrix metalloproteinases and RANKL, which, as pointed out above, directly stimulates the maturation and activation of osteoclasts. These effects can be mediated by IL-17 efficiently and independently of IL-1β and TNF, which are also produced by Th17 cells. Th17 cells have therefore been implicated as a specialized osteoclastogenic subset that links T-cell activation to bone loss. Importantly, IL-17 is produced in the gingiva, and its concentration increases during the progression from gingivitis to periodontitis, suggesting a potential role in pathogenesis. Recent studies in the mouse model of periodontitis have established a causal link between IL-17 and periodontal bone loss. However, additional clinical studies are warranted to conclusively define the role of Th17 and IL-17 in human periodontitis.

Tfh Cells

Tfh cells are a subset of $CD4^+$ Th cells that prominently express IL-21. IL-21 also plays a role in the development of Tfh cells. Upon B-cell exposure to a bacterial antigen, Tfh cells help in the differentiation of B cells to antibody-producing plasma cells and long-lived memory B cells. In this regard, IL-21, in conjunction with CD40 costimulation, helps to activate B-cell functions including Ig class switch to IgG1 and IgG3. In addition to their signature cytokine, IL-21, Tfh cells are characterized by elevated expression of the CXC-chemokine receptor 5 (CXCR5) and a transcription factor called B-cell lymphoma 6 protein (BCL-6). In the lymph nodes, CXCR5 expressed on Tfh cells facilitates their migration in response to the B-lymphocyte chemoattractant (BLC; also known as CXCL13) produced in the germinal center. Regarding their potential role in immunopathology, Tfh cells are thought to be implicated in T-cell-dependent antibody-mediated autoimmune diseases, such as systemic lupus erythematosus and Sjögren's syndrome. CXCL13-producing cells are more prominently found in periodontitis than in gingivitis, which suggests that Tfh might be recruited in great numbers to germinal centers. However, more studies are needed to determine whether Tfh cells play a role in the development of the B-cell-rich lesions that characterize advanced periodontitis.

Tregs

Tregs are characterized as suppressor $CD4^+$ Th cells that prominently produce IL-10 and TGF-β, which exert immunosuppressive effects on Th1,

Th2, Th17, and Tfh cells (Fig. 6). Tregs express a distinct transcription factor, namely, Foxp3, which elicits gene expression in Tregs required for their immunosuppressive functions. Naturally occurring $CD4^+$ Tregs, which express CD25 and Foxp3, constitute 5 to 10% of peripheral $CD4^+$ T cells and are generated in the thymus as a functionally mature population different from conventional $CD4^+$ T cells. Some Tregs are also induced in the periphery by antigen presentation from tolerogenic DCs in the presence of TGF-β and the absence of IL-6 and/or IL-21. This is in stark contrast to Th17, the differentiation of which is induced by the combination of TGF-β with IL-6 and/or IL-21. In the inflamed periodontal tissue, TGF-β and IL-6 are produced by gingival epithelial cells and fibroblasts. Therefore, it is plausible that Th17 cells are locally generated, while Tregs migrate from the circulation. Tregs play a pivotal role in the maintenance of self-tolerance and the downregulation of excessive immune responses to commensal or pathogenic microbes. The number of $Foxp3^+$ Tregs increases in the inflamed periodontium compared to healthy tissue, which implies a host mechanism operating to mitigate collateral tissue damage caused by the overactivation of the adaptive immune cells. In this context, IL-10 produced by Tregs suppresses the induction of RANKL expression by activated T cells. Furthermore, IL-10 may suppress RANKL-mediated differentiation and activation of osteoclasts.

In summary, although the Th1/Th2 paradigm offered a productive conceptual framework for investigating the role of adaptive immunity in periodontitis, more than 2 decades of research have made it clear that the disease cannot be adequately described in simple Th1 versus Th2 dichotomous terms. The recent discovery of the Th17 subset, as well as of regulatory T-cell subsets, could contribute to a more nuanced understanding of the role of T cells in periodontal disease.

KEY POINTS

- Periodontal health represents a dynamic balanced state wherein the proinflammatory and antimicrobial activities of the host are optimally regulated to prevent unwarranted host responses while establishing a symbiotic relationship between the host and the microbiota.
- Periodontal disease occurs when the homeostatic balance between the host and the microbiota is disrupted. This may be due to congenital immunodeficiencies or immunoregulatory defects. Moreover, homeostasis may be disrupted by pathogens that subvert the host response, thereby leading to nonprotective and nonresolving chronic inflammation.
- Periodontal pathogens may have evolved not only to endure inflammation but also to exploit it as a source of nutrients (derived from the inflammatory GCF). Thus, the inflammatory consequences of host-pathogen interactions are not necessarily detrimental to the pathogens but may serve their nutritional needs while inflicting tissue damage on the host.
- The histopathology of periodontitis shows that most stages of the disease include elements of both innate and adaptive immune responses. In this regard, destructive inflammation and periodontal bone resorption are the outcome of a complex interplay between a persisting biofilm and innate and adaptive immune cells.
- Inflammatory bone resorption ultimately depends on a triad of proteins within the TNF/TNF receptor family consisting of the receptor activator of RANKL, its functional receptor, RANK, and its decoy receptor OPG. These proteins are defining factors for osteoclast maturation and activation, which are promoted by the binding of RANKL (expressed predominantly by activated T cells and B cells) to RANK on osteoclast precursors.
- The Th1/Th2 paradigm offered a productive conceptual framework for investigating the role of adaptive immunity in periodontitis, although more than 2 decades of research have made it clear that the disease cannot be adequately described in simple Th1 versus Th2 dichotomous terms. The recent discovery of the Th17 subset, which is a dedicated osteoclastogenic subset, as well as of regulatory T-cell subsets, could contribute to a more nuanced understanding of the role of T cells in periodontal disease.

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Oral Virology

Matti Sällberg

INTRODUCTION

What Is Oral Virology?

In a broad sense, oral virology could be defined as the branch of science that deals with viruses present in the oral cavity. This does not, however, imply that all these viruses infect and replicate in the tissues of the oral cavity. Rather, many viruses are present in the oral cavity merely as a consequence of infection and replication elsewhere in the body but reach the oral cavity through the circulatory system or other transport mechanisms. Thus, these viruses are directly or indirectly of interest to the dental profession. This is the definition of oral virology that will be used in this chapter.

In this chapter, the reader will be introduced to the basic concepts of virology, such as the structure of viruses and the replication cycle of viruses, and to viruses that can be defined as being related to the field of oral virology. The chapter also covers the basics of how viruses are recognized by the immune system. This is very important, since in many viral diseases it is not the virus that directly causes the clinical symptoms. Instead, it is the immune system that when fighting the infection produces cytokines that cause fever and actively kill infected cells. Finally, the chapter discusses the basics of viral vaccines and modern antiviral therapies.

What Is a Virus?

One could say that a virus is the smallest form of life. This may, however, be debated, since viruses lack the ability to replicate in the absence of a host cell. The term virus, which in Latin can mean poison, accurately reflects the behavior of many viruses. For example, a virus can be transmitted through air on invisible droplets of water exhaled by an infected person. The mere touch of the skin by an infected person may, under certain circumstances, transmit the infection. Thus, it is not difficult to understand that viral infections throughout time have been considered to behave like poison. In our history, viruses have influenced human life. A clear example of the effectiveness of this type of biological warfare was seen when the Spanish conquistadors took South and Central America starting in 1492. Despite being outnumbered by the Aztecs and the Incas, the conquistadors effectively took empire after empire. The key

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TABLE 1 Three basic characteristics common to all viruses

1. They lack their own metabolism.
2. They can replicate only inside a prokaryotic or eukaryotic cell.
3. They contain a genome and one (or more) surrounding shell that protects the genome from the environment, attaches to the cell to be infected, and determines the host range of the virus.

ingredients in this biological warfare were the smallpox and measles viruses. Because these two viruses had ravaged Europe for centuries, the conquistadors were more resistant than the Aztecs and the Incas. The indigenous populations lacked any immunity to these viruses, as this was their first exposure. The infections both killed the population and had devastating effects on the fighting morale, as the conquistadors seemed unaffected by any of this plague. Throughout history, similar examples have shown how viral infections have helped shape human development and evolution.

All viruses have some common basic characteristics, regardless of whether they infect prokaryotic or eukaryotic cells (Table 1). The components of the smallest viruses are a viral genome surrounded by a protein shell, termed capsid or nucleocapsid.

The Virus

So, now we know the basic prerequisites for viruses. The next question is what a virus looks like. All viruses have a genome. The viral genome has two basic functions. One is to code for all viral proteins, and these sequences, or regions, are called the coding regions. The second is to interact with the machinery of the host cell, such as binding transcription factors or ribosomes. These genomic regions are called the noncoding, or untranslated, regions. The coding region of the viral genome encodes two types of viral proteins. The first type is called structural proteins, and by definition these are the proteins found in the extracellular virus. Examples of structural proteins are nucleocapsid and envelope proteins. The second type of viral protein is called nonstructural proteins. These proteins are not present in the extracellular virus but can be found in the infected cell. Examples of viral nonstructural proteins are enzymes, such as DNA or RNA polymerases or proteases, and viral proteins that inhibit the host immune response. To summarize, the viral genome consists of coding regions, which encode all viral proteins, and noncoding regions, which interact with the host cell machinery. The viral proteins are either structural proteins (present in the extracellular virus) or nonstructural proteins (present only in the infected cell).

The viral genome can be one of many different types, such as those composed of a single-stranded RNA, double-stranded RNA, double-stranded DNA, and so on. To simplify the taxonomy of viruses, the two major groups of viral genomes are either RNA or DNA. Both RNA and DNA genomes can be divided into subclasses, listed in Table 2. Apart from these five subclasses, the viral genome can be linear, circular, or segmented. A segmented viral genome can be compared to the human genome in that it is composed of several chromosomes. An advantage of a segmented genome is that it can be large without risk of strand breakage. From the virus point of view, there are some other advantages to having a segmented genome. A classical example is the segmented RNA genome of influenza virus A. We all know that influenza virus A causes the respiratory disease termed influenza that appears every winter. However, every 30 to 50 years, the impact of the influenza virus A epidemic takes global proportions, and in these cases the epidemics are called pandemics.

All viruses have a shell that surrounds and protects the viral genome. However, depending on the virus type more than one layer of shells may

TABLE 2 Classes of viral genomes

Positive-sense single-stranded RNA
Negative-sense single-stranded RNA
Double-stranded RNA
Single-stranded DNA
Double-stranded DNA

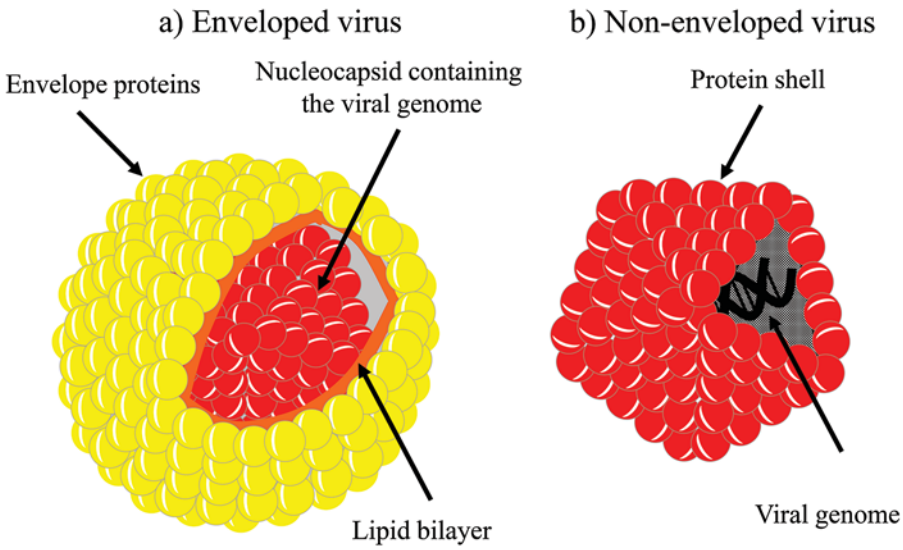

FIGURE 1 Schematic drawing of an enveloped and a nonenveloped virus.
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be present. Two major types of outer shells exist. The first is a protein shell composed of one or more proteins encoded by the viral genome and is called the nucleocapsid or capsid. Viruses that have only this type of shell are termed nonenveloped viruses. The second type of outer shell is a lipid bilayer, derived from the infected cell, that surrounds the inner protein shell, the nucleocapsid. Within the lipid envelope, viral envelope proteins have been inserted. These types of viruses are termed enveloped viruses. The schematic representation of these two groups of viruses is shown in Fig. 1.

To summarize this section, all viruses currently known contain a viral genome surrounded by a protein shell. Some viruses may also have a lipid envelope surrounding the protein shell (nucleocapsid) that contains the viral genome. The viral genome comes in many shapes and sizes, ranging from around 2,000 bases to around 200,000 bases. The viral genome can be composed of either RNA or DNA and can be linear or circular or consist of several chromosomes (i.e., be segmented).

How Viruses Change: Viral Evolution

Viruses evolve extremely rapidly to accommodate or to counteract changes in the surrounding environment. There are two ways in which a virus can change its genome, and both ways are used by influenza virus A. The first is by point mutations introduced by the viral polymerase during replication. Since RNA polymerases lack proofreading function, any error introduced during replication of the genome will be maintained in the following generations. These mutations can either be lethal for the virus or provide a survival benefit, such as escaping (i.e., escape mutations) the host immune response or an antiviral compound. The second approach is by genomic recombination. This generally means that a cell has been infected by two viruses of the same species. During replication, the two genome species are replicated. If the genome is segmented, the recombination or

reassortment, meaning the mixing of gene segments from both viruses that generates a third virus, occurs during assembly of the new virus. Alternatively, in linear genomes a template switch, or crossing-over, can occur during replication, whereby the newly transcribed genome is a mosaic of the two viral genomes present in the cell.

How can influenza virus A cause such a massive spread of infection when most individuals already have had one or more infections with the virus? The answer is directly related to the advantage of possessing a segmented genome, as described above. Influenza virus A has a very broad host range, meaning that a rather substantial number of animals can be infected by human influenza virus A. Many species, such as pigs, pigeons, and ducks, have their own species-specific influenza virus A. There are times when, for example, an avian or a pig cell is infected by both a human influenza virus A and an avian influenza virus A simultaneously. If both viruses start to replicate, this will result in a number of human and avian influenza virus A gene segments in the same cell. These human and avian gene segments can, by recombination, or reassortment, be combined and packaged into the same virus. Thus, the resulting virus will contain a new combination of human and avian influenza virus A genes. The virus now represents a new form of influenza virus A that is quite different from the influenza virus A strains that we will have previously encountered. Consequently, we lack complete protective immunity against this newly established strain. This allows for a rapid and extensive spread of the virus throughout the human population. The molecular basis for this is, of course, that the virus has a segmented genome that allows for a recombination between human and animal influenza virus A genes. From this it can be deduced that the organization of the viral genome is an important factor determining the behavior of the virus. We have been reminded throughout the ages of the medical importance of such new viruses as they appear; examples are the different coronavirus/SARS (severe acute respiratory syndrome) epidemics and the various influenza A virus pandemics in 1918, 1957, 1968, 1977, and 2009.

THE VIRAL LIFE CYCLE

The life cycle of all viruses can be generalized to some common and basic steps (summarized in Fig. 2). Of course, the exact life cycles of the different viruses vary extensively, but these general steps are relevant for most viruses. Let us consider an extracellular virus, also termed the virion, containing everything needed to transmit the viral infection. The first event that has to take place is binding to the surface of the cell to be infected. This event is called attachment. Attachment is mediated through the interaction between the surface of the virus and a molecule present on the surface of the cell. The attachment event is an important factor in determining the host range of a virus. Cells that are not permissive to infection by a particular virus can often be made permissive by genetically introducing a surface molecule to which the virus can bind.

Following attachment, the virus has to enter the cell. Depending on the structure of the virus, three mechanisms are most often used. First, if the virus has a lipid envelope, a lipid membrane of the cell can fuse with the lipid envelope of the virus. Thus, the two lipid membranes "melt" together, and the viral capsid containing the viral genome is released in the

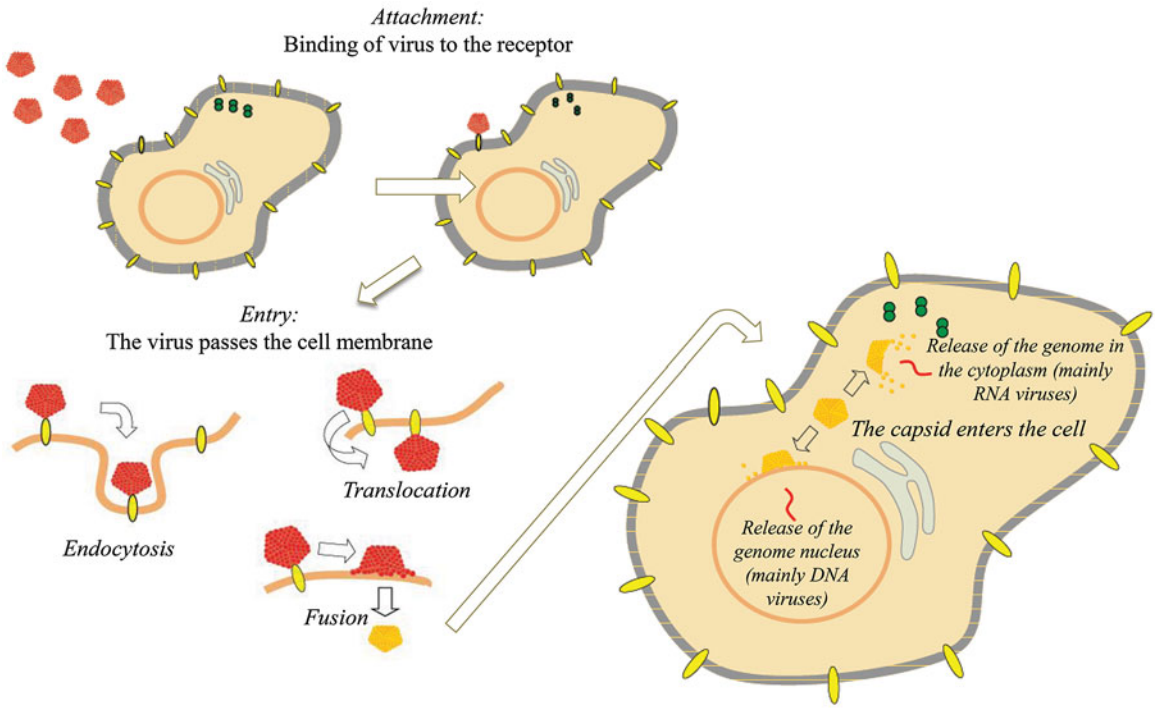


FIGURE 2 The viral life cycle. doi:10.1128/9781555818906.ch16.f2

cell. Fusion can occur at both internal and external cell membranes. Second, if the virus is a nonenveloped virus, i.e., does not have an outer lipid envelope, the virus entry into the cell is mediated either by endocytosis or by translocation. These are the common ways for cells to take up macromolecular substances from outside the cell membrane. The virus will enter the endosome and be transported into the cell cytoplasm. Release of the virus from the endosome can occur in many different ways.

After entry of the viral capsid into the cell cytoplasm, the viral genome has to be released in order to perform the needed functions. This step is known as uncoating and describes the dissociation of the capsid and the release of the viral genome into the cell. This step takes place in different compartments of the cell depending on the virus. Most DNA viruses, with few exceptions, replicate their genome in the cell nucleus. Thus, the capsid containing the viral genome is transported to the nuclear membrane, where the capsid dissociates and releases the viral genome into the cell nucleus. In contrast, the replication of most RNA viruses takes place in the cytoplasm. Thus, the viral capsid disintegrates after entry into the cell cytoplasm, where the viral genome is released.

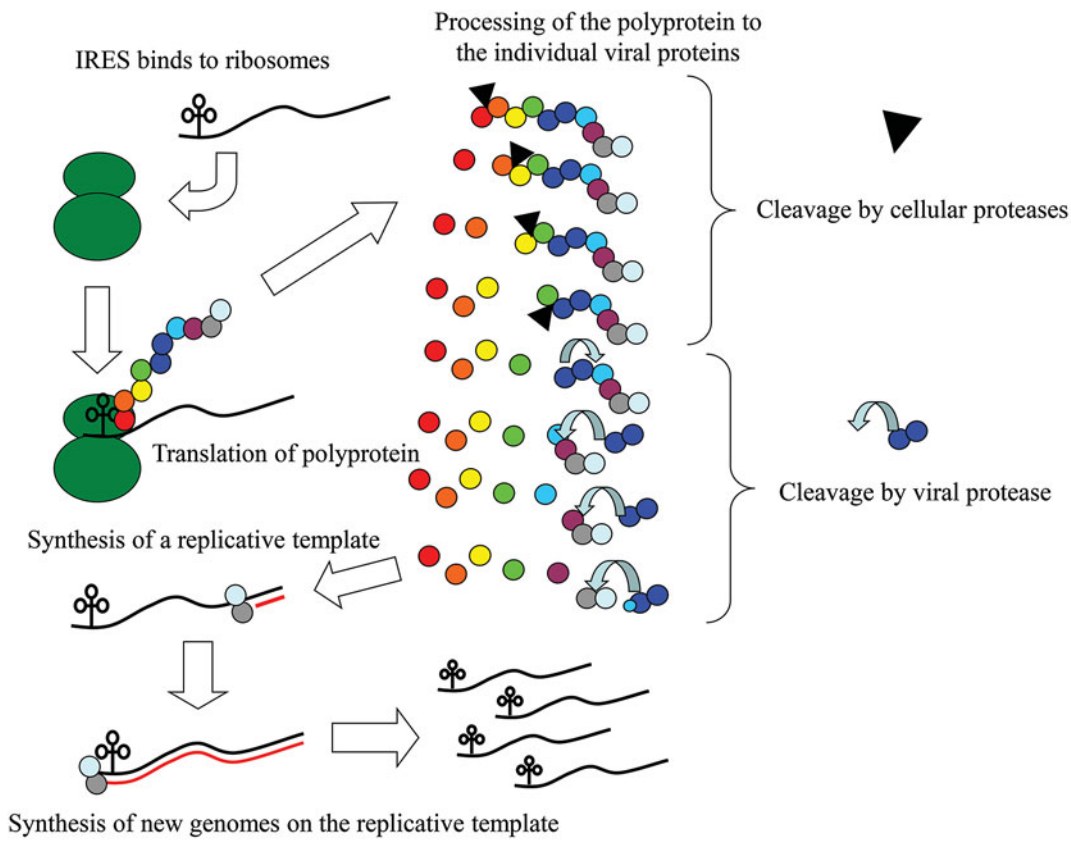
The initial steps after uncoating of the viral genome are largely dependent on the virus. In general, two events can take place, either replication/transcription or translation of the viral genome. To better describe the next steps, two different viruses will be used as examples.

As previously mentioned, most RNA viruses replicate their genome in the cell cytoplasm. One such virus family is the *Flaviviridae*, to which hepatitis C virus (HCV), GB virus C (GBV-C), and tick-borne encephalitis virus (TBE) belong. The genomes of the members of the *Flaviviridae* have some common features. All genomes are composed of one RNA strand

approximately 10,000 bases long. The coding region of the genome, the part that encodes the viral proteins, is surrounded at both ends by important noncoding regulatory sequences. They all have an RNA strand with positive polarity, meaning that the RNA can act as an mRNA and directly interact with cellular tRNA, which is an essential step in regulation in this family of viruses. An internal ribosome entry site (IRES) is present in the in the 5′-noncoding region of the genome, which allows the RNA to be translated, and is followed by the region that encodes all of the viral proteins. In addition, at the extreme 3′-end of the genome, a poly-adenosine [poly(A) tail] stretch is found. Thus, the basic structures of these viral genomes are very similar to those of cellular mRNAs and are therefore directly translated after entry into the cell cytoplasm. The ability to be immediately translated by the cellular machinery is essential for replication of the genome, since eukaryotic cells are unable to make new RNA copies from an RNA template. Let us take HCV as an example and look at what happens after the viral genome has been released in the cytoplasm.

After release of the HCV genome in the cytoplasm, the 5′-IRES directs the viral genome to ribosomes (Fig. 3). After binding, translation of the coding region is initiated, and a large precursor polyprotein corresponding to the complete coding sequence is synthesized. The first part of the polyprotein, containing the viral structural proteins, is cleaved by host cell proteases, causing release of the individual viral proteins. The remaining part of the polyprotein, containing the viral nonstructural proteins, is

FIGURE 3 Replication of the HCV genome. doi:10.1128/9781555818906.ch16.f3



cleaved by a viral protease. This releases the viral RNA-dependent RNA polymerase, required for the replication of the RNA genome. To summarize, the capsid containing the HCV genome disassembles in the cytoplasm, the viral genome is translated, the individual proteins are released, and now replication of the RNA genome can take place (Fig. 4).

The second example is hepatitis B virus (HBV), a small enveloped virus with a circular partially double-stranded DNA genome and a member of the family *Hepadnaviridae*. The capsid containing the DNA genome is transported to the nucleus, and the capsid dissociates at the nuclear membrane. The HBV DNA genome is released into the cell nucleus, and transcription of the genome is initiated (Fig. 4). The viral RNAs are either transported to the cytoplasm for synthesis of viral proteins or retained in the nucleus to be used as the RNA pregenome. The genome is replicated using the viral DNA polymerase from the RNA pregenome. This forms a DNA-RNA hybrid genome, which together with the viral polymerase is encapsidated into new viral capsids. Inside the viral capsid, the final steps of the replication are completed by the polymerase with the replacement of the RNA strand with a DNA strand to form the partially double-stranded DNA genome of HBV. Thus, since HBV can synthesize a new DNA strand from an RNA template, HBV has, like the viruses in the family *Retroviridae*, a polymerase with reverse transcriptase (RT) function.

Before, following, or simultaneous with replication of the viral genome, synthesis of viral proteins occur. Translation and protein processing

FIGURE 4 Replication of viral genomes in the cytoplasm or nucleus.
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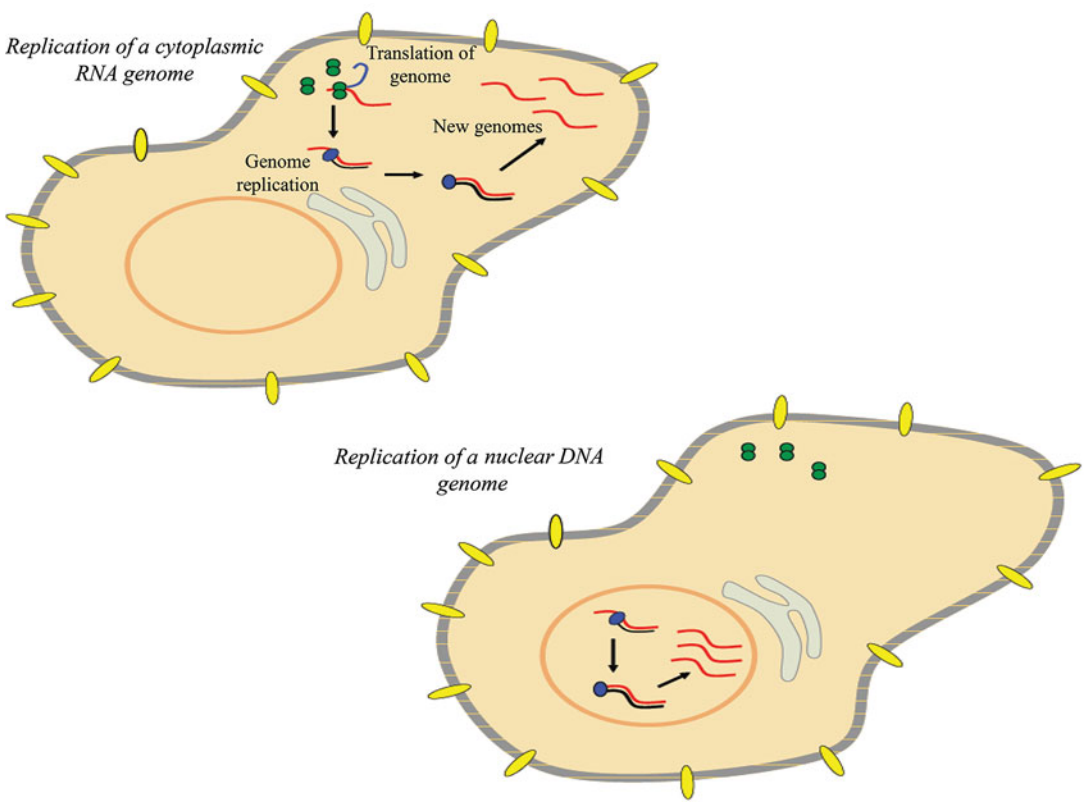

are performed by the cellular machinery. Some viruses encode their own proteases that participate in the processing of the viral proteins. Transcription of viral DNA genomes is initiated by the binding of host cell transcription factors to the promoter sequences within the viral genome. The mRNA transcripts are transported from the nucleus into the cytoplasm followed by translation (Fig. 5). The viral proteins are transported to different cellular compartments, using the same type of localization sequences as those present in normal cellular proteins.

At this stage, all components needed to build new viral particles have been generated. The process of building the new viral particles is termed assembly (Fig. 5). The different viral proteins have specific characteristics. For example, the nucleocapsid proteins contain DNA or RNA binding domains by which the proteins assemble around the viral genome to form a mature nucleocapsid. Viral envelope proteins contain membrane-binding domains, which target these proteins to cellular membranes. The envelope proteins form clusters within the cell membrane that attract the mature nucleocapsids or the precursors of the capsid proteins.

How does the virus leave the infected cell? There are two major pathways by which viruses leave cells, either by replicating and building new virus particles until the cell ruptures by lysis (lytic replication cycle) or by nonlytic pathways (nonlytic replication cycle). Nonenveloped viruses often leave the infected cell through cell lysis, but this is not always the case. Some can leave the infected cell by exocytosis, and the cell is not killed immediately.

FIGURE 5 Synthesis of viral proteins and assembly of viral particles.
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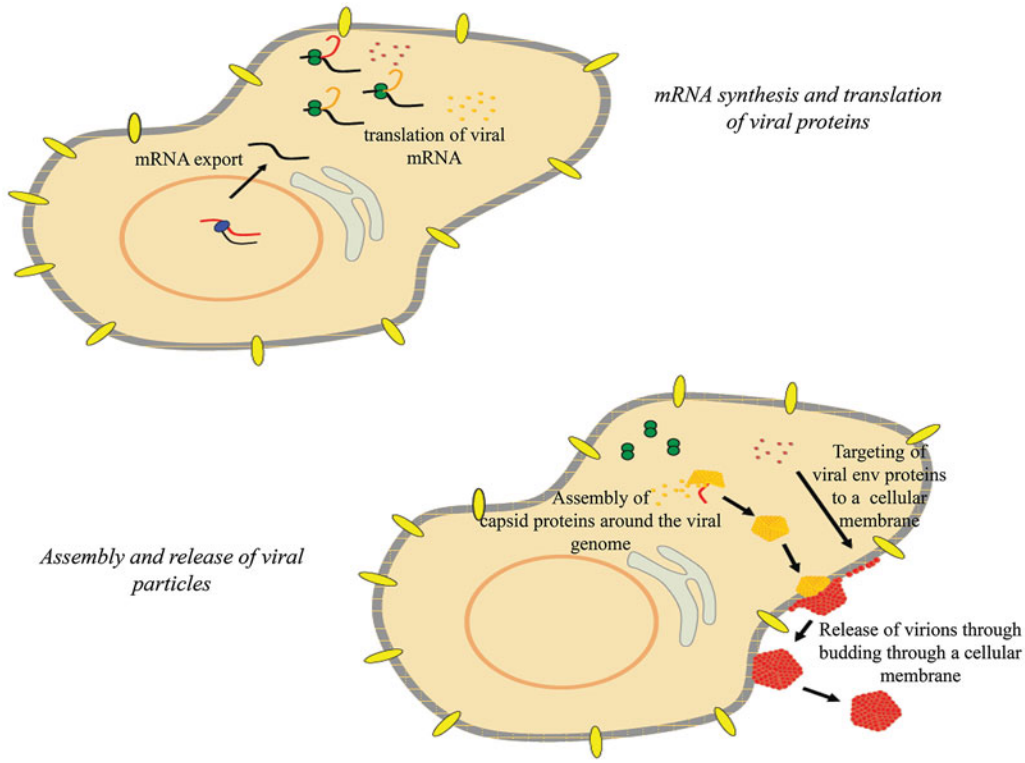


Enveloped viruses leave the cell by a process termed budding. Budding is really the reverse form of entry by fusion illustrated in Fig. 2. This means that the viral nucleocapsid buds through a cellular membrane, which allows the viral nucleocapsid to be enclosed by a lipid envelope (Fig. 5). A high level of replication of an enveloped virus may eventually kill the cell, since the cell is not able to synthesize the membrane components rapidly enough to prevent cell shrinkage and death.

Once the virus leaves the cell, the viral replication cycle is completed, and new mature extracellular viruses, or virions, can now infect new cells.

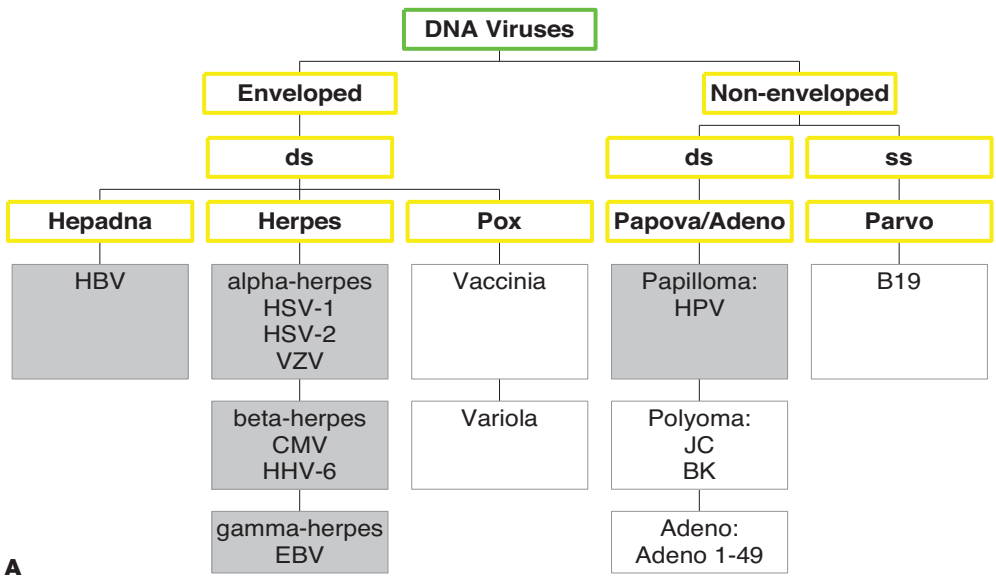
VIRUS TAXONOMY

To better understand the relationships between organisms, humans have a tendency to arrange different life forms into groups whereby each group consists of similar or closely related life forms. This is also true for viruses. There are many ways by which viruses can be grouped into different families. Presently, the most important criteria to classify viruses are based on the structure and composition of the viral genome. As previously mentioned, there are five major classes of viral genomes, which is the first and most simple way to classify a virus. During the past 2 decades, techniques in molecular biology have been developed and have revolutionized our understanding of genes and genomes. Thus, as soon as a new virus has been discovered, the first thing to do is to have a look at the viral genome. The sequence of the viral nucleic acid can tell us if it is similar to that of any known virus family. If so, we may rapidly understand the possible mechanisms of transmission and the possible host range of the virus, based on what is already known about other members of the same family. The sequence of the virus also reveals the organization of the viral genome, i.e., which proteins the virus encodes, which regulatory elements are present, and so on. Thus, by characterizing the viral genome at the nucleotide sequence level, we can predict much about the properties of the virus without having to see or grow the virus. A simplified scheme for the taxonomy of human viruses is given in Fig. 6.

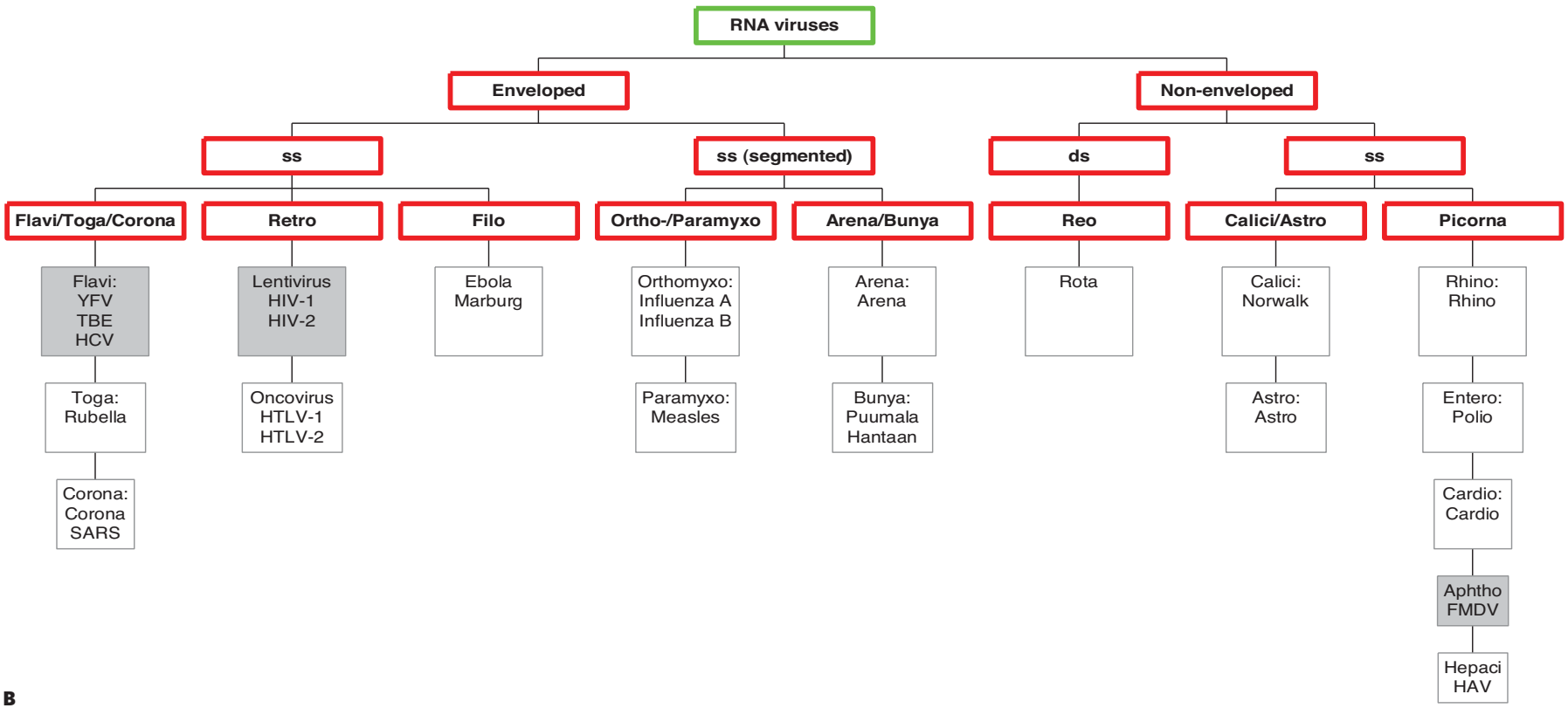
Other characteristics widely used in viral taxonomy are the basic shape and structure of the virus particle. As previously mentioned, some viruses have a lipid envelope, and some do not. The basic structure of the nucleocapsid can be either icosahedral or helical (spiral shaped). Viruses with a helical capsid structure are always enveloped. The appearance of the virus in electron microscopy allows for morphological characterization of the size and shape of the virus particle.

Different viruses give different appearances (cytopathic effects) when grown in tissue culture. As described in "The Viral Life Cycle" above, the virus can leave the cell either by budding or by lysing the infected cell. Lysis of infected cells can be determined using ordinary light microscopy to view the destruction of cells. Budding is not visible by means other than electron microscopy. However, some viruses cause syncytia (clusters of cells that merge together to form a large cell) that are visible under light microscopy.

The array of different viral proteins can also be a useful taxonomic tool. The proteins can be characterized biochemically with respect to their basic properties such as size, composition, and enzymatic functions.


DNA Viruses
Enveloped
Non-enveloped
ds
ds
ss
Hepadna
Herpes
Pox
Papova/Adeno
Parvo
HBV
alpha-herpes
HSV-1
HSV-2
VZV
Vaccinia
Papilloma:
HPV
B19
beta-herpes
CMV
HHV-6
Variola
Polyoma:
JC
BK
gamma-herpes
EBV
Adeno:
Adeno 1-49

A



B

FIGURE 6 Taxonomy of the most common and well-known human viruses. Viral families or genera that contain one or more viruses that can be related to oral virology are indicated in gray. ds, double stranded; ss, single stranded. (A) DNA viruses; (B) RNA viruses. doi:10.1128/9781555818906.ch16.f6

Some enzymes are encoded only by certain virus families. As an example, the RT enzyme, with the ability to transcribe DNA from an RNA template, is almost exclusively found among retroviruses. However, as mentioned, a polymerase with RT activity is also found among the *Hepadnaviridae*.

Discovery of New Viruses without Isolating the Virus

Viruses have traditionally been discovered by their ability to grow in tissue culture, after which the viral properties can be characterized. A somewhat recent example is HIV-1, which was first cultured in human cells in 1982. However, virus discovery in the past 20 years has been dominated by first isolating a part of the viral genome and then building "a viral puzzle." The first virus to be discovered by purely molecular techniques was HCV, in 1989. However, the identification of a viral genome can be problematical, as the following example illustrates.

In 1995 a group of scientists at Abbott Laboratories in Chicago, IL, was successful in isolating a viral genome from a small monkey, a tamarin, which had been experimentally infected by human serum. The human serum was derived from a surgeon with the initials "G.B.," who, in the 1960s, developed hepatitis due to an unknown agent. The researchers utilized a novel technique, in essence a variation of subtractive hybridization, to discover two new viral genomes, representing the GB viruses A and B (GBV-A and GBV-B). Unfortunately, neither of the viruses isolated from the tamarins could be found in human sera and thus were unlikely to be the cause of human disease. The group, armed with the sequence knowledge obtained from the tamarin viruses, started searching for a third GB-related virus in humans. This search led to the discovery of GBV-C, which clearly belonged to the *Flaviviridae* family, as does HCV. In addition, the genome of GBV-C was often detected in patients with severe liver disease. Thus, the early data suggested that it was likely that GBV-C, like its close relative HCV, might also cause hepatitis. In another twist to the story, it now appears that GBV-C is common in humans and by chance was present in the infected human at the time of testing. In total, the Abbott scientists discovered three GB viruses, GBV-A, GBV-B, and GBV-C. GBV-A and GBV-B have so far been found only in various monkey species, whereas GBV-C has been found in humans. However, after years of intensive research, it does not seem that GBV-C causes any disease in humans. GBV-C does most likely infect the human liver, but despite this it seems rather harmless. These studies have resulted in the worldwide decision not to test for GBV-C at blood banks. We now know that around 3% of the population worldwide is infected by GBV-C. Thus, if GBV-C did cause any major disease after blood transfusion, it would be evident by now. In fact, many studies suggest that a GBV-C infection may be beneficial for those infected by HIV-1, although this is still widely debated.

This is an example of a new virus being discovered, but with no known associated disease. The GBV story contrasts with how viruses have been discovered historically. A disease was first recognized, and then a virus could be identified from patients with the disease by trying to grow the virus in different cell lines. Then, the viral genome could be characterized.

ORAL VIROLOGY: THE VIRUSES

The following text will describe viruses with which dental professionals should be familiar. These viruses can cause symptoms affecting tissues in the oral cavity. Alternatively, these viruses are present in the mouth following replication and release from other tissues and organs. The presence of such viruses in the oral cavity or in the circulation may result in an increased risk of infection for the staff involved in treating the patient. These two criteria make the following viruses relevant to a discussion of oral virology. Viruses that will not be discussed in detail include those with a minor relation to pathologies in the oral cavity but with severe pathologies more relevant to the field of general medicine and others that occasionally give symptoms related to the oral cavity but whose major symptoms are found elsewhere. Examples of these are many of the childhood infections such as measles, rubella, and mumps. These can be further explored in textbooks dedicated to virology.

VIRUSES THAT CAN CAUSE PATHOLOGIES IN THE ORAL CAVITY

Picornaviridae

One virus family commonly related to oral ulcerations is the *Picornaviridae* family, which contains over 70 different viruses. A characteristic of the *Picornaviridae* is a single-stranded RNA genome of positive polarity consisting of around 10,000 bases. The viruses are small, nonenveloped, relatively resistant to environmental factors, and easily transmitted from host to host by several routes. The most famous members of the *Picornaviridae* family are the polioviruses. Fortunately, due to mass vaccination schemes, we may be close to the eradication of the polioviruses. Picornaviruses cause a wide spectrum of diseases ranging from a subclinical or mild respiratory disease to myocarditis, encephalitis, or meningitis.

A good example of the *Picornaviridae* is the hepatitis A virus (HAV) (which does not cause any oral pathologies) in that it is highly contagious. During the acute phase of the disease, HAV is excreted in large amounts in the feces. Thus, in areas of the world where the purity of drinking water is suboptimal, waterborne HAV infection is a widespread childhood disease.

Some picornaviruses have been related to pathologies in the oral mucosa. Hand, foot, and mouth disease is caused by two members of the *Enterovirus* genus, coxsackie A virus and enterovirus 71 (EV71). These viruses are transmitted by contact and cause blisters in the mouth (Fig. 7) and on the hands and feet of small children. This disease is in general quite harmless. However, in Asia there have been reports of hand, foot, and mouth disease cases caused by EV71 that have a fatal outcome. When discussing hand, foot, and mouth disease, it is important to mention a similar infection, foot and mouth disease, seen in cattle. This disease is caused by a picornavirus named foot and mouth disease virus (FMDV). FMDV cannot infect humans, and thus the veterinary disease, although of great importance, is not the same as hand, foot, and mouth disease seen in humans.

A rather common lesion or ulceration seen in the oral mucosa is the aphthous lesion. These lesions seem to recur at varying intervals in

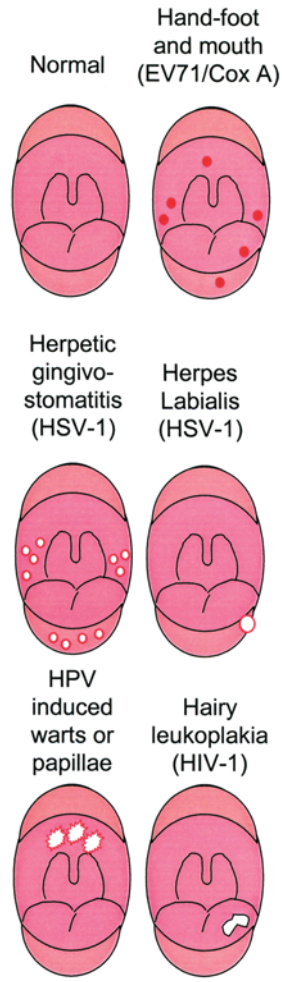


FIGURE 7 Schematic drawings of the clinical appearance of viral infections in the oral cavity (from top in each schematic: upper lips, palate, tongue, lower lips). Red spots indicate ulcerations, and white spots with a red border indicate blisters.
doi:10.1128/9781555818906.ch16.f7

different subjects and can be quite painful. It has been proposed that there may be a relationship between aphthous lesions and picornaviruses. However, despite numerous attempts, there are only a few reports of picornaviruses actually being isolated from such lesions. Thus, a causal relationship between picornaviruses and aphthous lesions has not been established.

Human Herpesviruses

The family *Herpesviridae* contains eight, or nine, known human pathogens. The first five members discovered were given names describing either their appearance in tissue culture or the names of the discoverers. Later on, a new taxonomy was agreed upon in which all new members were given the name human herpesvirus (HHV) followed by a number based on the order of discovery.

CHARACTERISTICS OF THE *HERPESVIRIDAE* FAMILY

Herpesviruses have large double-stranded DNA genomes. The genomes vary from 150 kb to around 220 kb in size. The genome is surrounded by an icosahedral nucleocapsid. Herpesviruses leave the infected cell by budding through intracellular membranes, whereupon the mature virion contains an outer lipid envelope. The lipid envelope contains glycoproteins encoded by the genome and membrane proteins derived from the host cell. Subsequently, when the virus infects a new cell, it enters the cell by membrane fusion.

A common feature of all members of the *Herpesviridae* family is that they encode several enzymes that participate in nucleic acid metabolism. The best-characterized one is thymidine kinase (TK). The TK enzyme can phosphorylate nucleotides, resulting in incorporation into the genome. The discovery of the viral TK led to the development of a number of antiviral compounds that target this enzyme. The mechanism of action of these compounds is described below in "Antiviral Vaccines and Therapies."

Human Herpesviruses 1 and 2

VIROLOGY

The herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) are probably the best-known HHVs. The genomes are around 150 kb. HSV-1 is a common virus, and around 50 to 70% of a population can be infected. HSV-2 is less common, and approximately 5 to 20% of a population may be infected. The primary target cells for HSV-1 and HSV-2 are epithelial cells. Both viruses are neurotropic and require the presence of HSV TK to be able to replicate in neural cells.

A feature common to all herpesviruses is the ability to establish latency. Latency means that the cell is infected but for long periods the virus does not replicate. Latency occurs mainly at privileged sites, such as in nerve cells or neurons. Following infection of the primary target cell, one or more sensory neurons innervating the target cell area become infected. The virus is transported along the axon, and replication is initiated in the nerve cell nucleus. During latency, the genome is maintained episomally (separate from the chromosome) with a low level of transcription from the genome. The advantage of latency to the virus is that it effectively evades the host immune response. Reactivation of latent virus, often brought on by stress, is also the basis for the clinical appearance of the recurrent infection.

EPIDEMIOLOGY AND CLINICAL FEATURES

HSV-1 and HSV-2 infections have a worldwide distribution. HSV-1 is mainly transmitted in early childhood by contact with saliva from an infected person. If acquired later in life, viral transmission often occurs through kisses or contact with contaminated saliva or skin surfaces. The primary HSV-1 infection is often asymptomatic but can in some cases cause stomatitis. Stomatitis often appears as small blisters filled with liquid that are present bilaterally in the epithelium of the oral mucosa. The primary infections heal within 2 to 3 weeks. After the primary infection, HSV-1 is transported retrograde along the neural axons and lies latent in sensory nerve cells that innervate the oral cavity. These often stem from one of the trigeminal ganglia, and subsequently, during reactivation, the virus is transported along the axon and infects the epithelial cells present at the nerve end. Replication takes place in the local epithelial cells, and a visible lesion always appears unilaterally at the same site. Recurrences often appear two to four times per year. Prior to each episode, the patient can experience what is called prodromal symptoms, which include itching and discomfort at the involved area. When the blisters appear, they heal within a week. Thus, clinical symptoms of a primary HSV-1 infection can involve the whole oral cavity, whereas the clinical symptoms during a reactivated HSV-1 infection are unilateral. The mechanisms responsible for reactivation are not fully understood, although different factors have been associated with reactivation, such as exposure to UV light, other infections, or immunosuppression.

HSV-2 is a sexually transmitted disease (STD), and symptoms occur around the genital tract. A primary HSV-2 infection presents as blisters filled with liquid in, on, or around the genital organs. Similar to HSV-1, HSV-2 symptoms can also recur. During these recurrences, the viral infection is effectively transmitted. Due to sexual habits, oral HSV-2 infections may appear.

Mainly HSV-1, but also HSV-2, infections can cause severe complications such as encephalitis with a fatal outcome. This is rare, however, and these complications occur mainly in patients with a suppressed immune system, caused either by therapy (immunosuppression after transplantation) or by infection by another agent, such as HIV-1.

The presence of HSV-1 or HSV-2 is determined by detecting either the virus or the viral genome in scrapings from blisters or lesions. The virus is detected either directly by immunohistochemistry or by first culturing the virus in cell lines. The viral genome can be detected in body fluids or in other types of samples by the PCR method. Detection of specific immunoglobulin M (IgM) antibodies is sometimes used as a diagnostic tool. There is, however, a delay in the appearance of antibodies; hence, direct detection of the virus or the genome is preferred.

Varicella-Zoster Virus

VIROLOGY

Varicella-zoster virus (VZV) (also known as HHV-3) is a close relative of HSV-1 and -2. The genome of VZV is around 120 kb. Like all members of the *Herpesviridae*, VZV has an icosahedral capsid and a lipid envelope. VZV also latently infects sensory neurons. VZV is highly contagious and is transmitted through aerosols, by direct contact, and even through the air over shorter distances.

EPIDEMIOLOGY AND CLINICAL FEATURES

VZV is a childhood disease that occurs worldwide. Primary VZV infection causes the disease known as varicella or chicken pox. The incubation period lasts for 2 to 3 weeks, during which the person is highly infectious. During the incubation period, the virus first replicates in the regional lymph nodes of the throat, followed by infection and replication in the liver and in the spleen. Following this, a second viremia (viral particles in the bloodstream) spreads the virus to the skin and mucous membranes. The first symptoms to appear are flulike, with fever and respiratory tract involvement. During this period, the infected person is highly contagious. A few days later, the typical cutaneous symptoms appear in the form of an exanthema that develops into blisters. The blisters develop during the first week and then slowly dry out. Clearance of the blisters is often accompanied by an irritating itch. The symptomatic period usually lasts for 1 to 2 weeks. During the symptomatic period, latency of VZV in dorsal ganglia is established. Occasionally, blisters in the oral mucosa are seen during the acute phase of the disease.

Reactivation of VZV is termed herpes zoster or shingles. The reactivation is, similar to HSV-1, characterized by the appearance of blisters along the area innervated by one nerve, a dermatome, in which the virus has established a latent infection. The recurrences generally appear in persons over 60 years of age, although it can occur in younger individuals. A zoster neuralgia that is very painful can appear. The location is usually on the trunk but can also be on the facial area.

VZV is often diagnosed clinically. However, as with HSV, the presence of VZV can be detected by immunohistochemistry, tissue culture, or PCR.

Cytomegalovirus and Epstein-Barr Virus

VIROLOGY, EPIDEMIOLOGY, AND CLINICAL FEATURES

Cytomegalovirus (CMV; also known as HHV-5) infection is very common, and around 50 to 80% of a population can be asymptomatic carriers. Most immunocompetent subjects that become infected with CMV have asymptomatic infections. However, a primary CMV infection during the early phase of pregnancy can lead to severe complications for the fetus. The major problem with CMV is the reactivation in immunosuppressed patients, in which the infection can be life threatening. In transplant recipients and AIDS patients, CMV infection is closely monitored due to the immune suppression.

CMV has an extremely wide tropism and can infect almost any organ. CMV productively infects several types of leukocytes and endothelial cells, and the reservoir for latent CMV infection seems to be macrophages. Since these cells are present in most secretions, CMV infection can be transmitted by several routes and CMV can often be detected in saliva. This may be one reason why CMV has been related to various oral symptoms and diseases in which an inflammatory infiltrate is present. For example, CMV has been detected in periodontal pockets in patients with different forms of periodontitis. However, it cannot be excluded that the CMV present in the periodontal pocket derived from infected macrophages or leukocytes. Thus, there is yet no conclusive evidence that CMV plays a role in periodontitis. In addition, emerging data suggest a role for CMV in many inflammatory diseases including arteriosclerosis. CMV infections can be treated by the antiviral compound ganciclovir.

Epstein-Barr virus (EBV; also known as HHV-4) is also a very common virus, with 50 to 80% of populations infected. Importantly, EBV is one of the few viruses that have a clear relationship to the development of cancer. Most people become infected with EBV early in childhood and do not develop any symptoms of the primary infection. A primary disease later in life is more likely to be symptomatic, with fever and swollen adenoid glands, also called infectious mononucleosis or kissing disease. EBV infection is mainly transmitted by saliva but can also be transmitted in blood. The target cell for EBV is the B lymphocyte. In vitro, the transforming properties of EBV are evident, and EBV is widely used to immortalize B cells. Burkitt's lymphoma, seen mainly in Africa, was the first oncological disease to be associated with EBV. Other oncological diseases with an association to EBV are nasopharyngeal cancer and certain types of lymphomas. No specific antiviral therapy has yet been developed for EBV infections. However, several types of antiviral compounds, such as acyclovir, have been tested in the clinic without conclusive results.

Also of note is that EBV has been implicated in the etiology of periodontitis. However, since EBV latently and productively infects B cells and periodontitis is characterized by a lymphocyte infiltrate, this association may be accidental, and a direct cause-and-effect relationship is not yet clear.

Human Herpesviruses 6A/B, 7, and 8

HHV-6 is a recently described herpesvirus and seems to be a close relative of CMV. HHV-6 infection is very common and can be transmitted through saliva, causing an early childhood disease known as exanthema subitum, or the sixth disease. Most infections are subclinical, however. Severe disease as a consequence of primary HHV-6 infection is rare. New data suggest that HHV-6A and HHV-6B may be distinct viruses.

HHV-7, which is related to HHV-6 and CMV, was identified in the early 1990s. No unequivocal causal relationship between HHV-7 and any severe human disease has been documented yet. The most common association with disease for HHV-7 seems to be febrile periods in childhood. It is also possible that HHV-7 can cause complications in transplant patients due to immune suppression. HHV-7 can be detected in saliva, and the target cell seems to be $CD4^+$ T cells. Recent reports suggest that HHV-6 and -7 may be involved in more severe skin diseases such as drug-induced hypersensitivity syndrome (DIHS) and drug reaction with eosinophilia and systemic symptoms (DRESS).

Of these three recently discovered HHVs, HHV-8 has been found to be the possible cause for the oncological disease Kaposi's sarcoma. Kaposi's sarcoma, a tumor involving the blood vessels in the skin, has been observed mainly in Africa and in AIDS patients. HHV-8 seems to be transmitted by saliva and sexually.

Human Papillomaviruses

VIROLOGY

Human papillomaviruses (HPV) are members of the family *Papovaviridae* and are named for the most common clinical symptoms, papillomas and warts. HPV has a double-stranded DNA genome and an icosahedral capsid and lacks a lipid envelope. There are over 150 HPV types, some

of which seem to constitute the natural viral flora of the skin. The primary target cells for HPV are the epithelial cells in the dermis. HPV is also a virus that has been firmly associated with development of human cancer. Two HPV proteins have been found to have oncogenic properties in cell lines since they are capable of independently transforming cells in vitro.

EPIDEMIOLOGY AND CLINICAL FEATURES

Many HPV types seem to be natural inhabitants of the skin and have not been associated with any clinical symptoms. The most common symptom caused by HPV is warts on the skin or on the oral or genital mucosa. Most warts caused by HPV are harmless, but a few HPV types have been associated with development of cancer. In particular, HPV-16 and HPV-18 have been associated with invasive cervical cancer. HPV-16 has been detected in up to 50% of cervical cancers. However, infection by HPV-16 does not guarantee development of cancer, which complicates various screening measures to prevent development of cervical carcinoma.

The genital disease associated with HPV is genital warts, termed condyloma acuminatum. Genital HPV infection is a sexually transmitted disease. Genital HPV infection is more common in those who have many sexual partners. Diagnosis is generally performed by clinical inspection, by cytology, and by PCR-based techniques.

As previously mentioned, HPV can cause warts at many sites. In the oral cavity, some clinical symptoms have been associated with HPV. Focal epithelial hyperplasia has been associated with HPV-13 and HPV-32, two HPV types that seem to be restricted to the oral cavity. Other forms of oral warts, such as condyloma acuminatum and verruca vulgaris, are associated with infections with genital types of HPV. HPV-16 and HPV-18 have been suggested to be associated with malignancies in the nasopharynx and also in the oral cavity. However, these correlations are yet not as clear as those of HPV with cervical carcinoma.

Two vaccines have been introduced for the prevention of cervical cancer. The vaccines should be administered before the sexual debut, since there is no clear evidence for the success of postexposure vaccination in preventing cancer. The vaccines effectively prevent infections with the so-called high-risk types HPV-16 and -18 and the low-risk types HPV-6 and -11. The vaccines therefore most likely also prevent cancers caused by these viruses.

VIRUSES PRESENT IN THE ORAL CAVITY

The following text will discuss viruses that are present in the oral cavity but that do not replicate in oral tissues. This is not always a clear distinction, and some of these viruses have been proposed to participate in pathologies found in the oral cavity. However, the major reason for inclusion here is that these viruses are in fact present in the oral cavity and the diseases that they cause in other organs are often life threatening. Thus, those in the dental profession should be familiar with these infections since the high number of patients treated and the type of procedures performed may increase the risk of becoming infected or transmitting the infection.

Hepatitis B Virus

VIROLOGY

Hepatitis B virus (HBV) was discovered in the mid-1960s by Baruch S. Blumberg as he was looking for new coagulation factors. He noticed in an immune precipitation assay that a precipitate was formed between wells filled with sera from hemophiliacs and Australian aborigines. He designated this new antigen as the Australia antigen. Further studies revealed that the presence of the Australia antigen was related to different forms of hepatitis and in particular with parenterally transmitted hepatitis, also known as "serum" hepatitis. During the 1970s, it became clear that the Australia antigen was the envelope, or surface, protein of a virus that became known as HBV. In 1980, the complete genome of HBV was sequenced and revealed that HBV was the first member of a new family of viruses, the *Hepadnaviridae* (from the Latin *hepar*, meaning liver; *dna* for the DNA genome; and *-viridae* for virus). The partially double-stranded genome is circular and approximately 3.2 kb long. Partially double-stranded means that only a small part of the genome is single stranded.

The virus has an icosahedral nucleocapsid consisting of 180 to 240 copies of a single protein, the hepatitis B core antigen (HBcAg). The mature capsid contains the partially double-stranded DNA genome and a DNA polymerase. The DNA polymerase of HBV has a unique property in that it can synthesize a new DNA strand from an RNA template, thus having RT activity. This enzymatic activity is found only among the *Hepadnaviridae* and *Retroviridae* families.

The nucleocapsid is surrounded by a lipid bilayer into which the three viral envelope proteins have been inserted. These proteins are called the small (S), medium (M), and large (L) forms of the hepatitis B surface antigen (HBsAg). The mature extracellular and infectious HBV virion thus contains the viral genome, HBcAg, the viral polymerase, and a lipid layer containing HBsAg.

The small genome is organized in an effective way. The genome encodes a total of seven viral proteins (S-HBsAg, M-HBsAg, L-HBsAg, HBcAg, HBeAg, the polymerase, and the X protein). Theoretically, if only one reading frame within the genome were used, then the entire coding capacity would be approximately 1,070 amino acids. However, to increase the coding capacity, the HBV genome has overlapping reading frames. This means that, for example, the same gene segment codes for more than one protein using two or three different reading frames. By this approach, the coding capacity of the genome is drastically increased. As a consequence, a mutation at one nucleotide position may affect the sequence of more than one protein. This is most likely the basis for the genetic stability of the HBV genome.

HBV has a rather complicated replication cycle, and all steps are not fully understood. In brief, one or more of the HBsAg proteins binds to a yet-unknown receptor on the liver cell, or hepatocyte. The virus is then internalized by cellular endocytosis, the endosome and the viral envelope rupture, and the capsid is released in the cytoplasm. The capsid containing the genome disintegrates on the nuclear membrane, causing the genome and the polymerase to be released into the nucleus. The partially

double-stranded DNA genome is then converted to a covalently closed circular DNA (cccDNA). Replication of the genome starts with the synthesis of large RNA transcripts from the cccDNA. The transcripts are exported to the cytoplasm and used as templates for protein synthesis, except for one, which functions as the pregenome. The pregenome and the viral polymerase are encapsidated by HBcAg in the cytoplasm, and reverse transcription takes place within the capsid. This is distinct from the replication cycle of retroviruses, whereby the reverse transcription occurs in the cell cytoplasm after dissociation of the viral capsid. The viral capsid becomes enveloped by budding through HBsAg-containing areas of the endoplasmic reticulum membrane, and the mature virus is secreted into the bloodstream.

The HBV genome encodes five structural proteins: S-, M-, and L-HBsAg, HBcAg, and the polymerase. Two proteins, HBeAg and the X protein (HBx), are nonstructural proteins. The functions of these are not fully understood. HBeAg is a protein that is secreted into the bloodstream during periods of high-level viral replication. It has been proposed that HBeAg can pass from the circulation of the infected mother across the placenta to the developing fetus. Thus, if HBeAg is present during the development of the neonatal immune system, HBeAg will be perceived as a self-protein. The child will, therefore, have a selective defect in the T-cell response to HBV. The HBx protein has been proposed to function as a regulator of host cell gene transcription by acting as a transactivator that can turn on various cellular genes.

The HBV genome, or more likely parts thereof, can be integrated into the host cell genome. HBV infection is correlated with the development of liver cancer, or hepatocellular carcinoma (HCC). The mechanisms of HCC development are not understood, but integrated HBV sequences are often found in cancerous tissues.

EPIDEMIOLOGY AND CLINICAL FEATURES

To understand HBV epidemiology, it is important to know the different phases of the HBV infection. HBV is a surprisingly common virus, with more than 2 billion humans infected. HBV can cause acute and chronic infections. HBV replication and virion secretion are very effective. During periods of high levels of replication, the levels of HBV can reach 10^{10} to 10^{12} virus particles per milliliter of blood. Under such circumstances, one microliter (10^{-6} liters) of blood contains 10,000 to 1,000,000 infectious virus particles, during which time the infected person is highly contagious. This explains why HBV is such a common infection despite being a bloodborne virus. Hence, HBV is effectively transmitted by unprotected sexual contacts, sharing of hygienic products, and biting and fighting among both children and adults.

In acute HBV infection, the period of high-level HBV replication can last for up to 2 months but is generally shorter. In chronic HBV infection, periods of high-level viral replication can last for more than 30 years. Consequently, the major sources for new infections are individuals with chronic HBV infection and high levels of viral replication. Keep in mind that despite the high levels of viral replication, these persons do not display any obvious clinical symptoms of the disease.

Age is the most important factor determining whether the infection will be acute or chronic. A neonate infected by vertical transmission,

meaning transmission from an infected mother to her child at birth, has a 95% chance of developing a chronic HBV infection. In contrast, an adult with a normal immune system has a 5% chance of developing a chronic infection. Thus, the ability of HBV to establish a chronic infection is to a large extent determined by the maturity and the functionality of the immune system.

HBV has a global distribution and is transmitted trough contact with contaminated blood or other human fluids such as saliva, vaginal and seminal fluids, and breast milk; contaminated blood products; or contaminated medical devices. The prevalence of HBV infection varies greatly in different parts of the world. In developed countries such as those in Europe and North America (where vaccination is readily available), the prevalence of HBV infection in the general population ranges between 0.1% and 1%. In many parts of the world, including Africa, Asia, and South America, prevalence rates range between 1 and 15%. In certain areas of Asia, prevalence rates of up to 20% may occur. The main mode of transmission in Asia causing chronic infections is vertical transmission, which, as previously explained, is the most effective means to establish a chronic HBV infection.

In developed countries, HBV is transmitted mainly through sexual contacts and through sharing of contaminated needles and syringes among intravenous drug users (IVDUs). Among IVDUs, the incidence of HBV infection ranges from 20 to 50%, depending on the country.

HBV can cause a wide spectrum of clinical symptoms, with the most common being no symptoms at all, i.e., a subclinical or asymptomatic infection. The incubation period for acute HBV infection ranges from 2 to 6 months. Often the symptom debut can be pinpointed to a specific day. The classical symptom is icterus, meaning a yellow tone of the skin or the sclera of the eyes, caused by the leakage of bilirubin from damaged liver cells to the circulation. Patients who develop chronic HBV infection generally do not display any symptoms within the first years of infection. Instead, they have a high risk of developing severe liver disease after 5 to 20 years. Such patients also have the highest risk for developing HCC. The clinical symptoms of HBV infection, which reflect the killing of infected hepatocytes, are not caused directly by the virus. Instead, these symptoms are caused by the host immune system trying desperately to kill all the virus-producing factories, i.e., infected hepatocytes.

Today the serological diagnosis of HBV infection is an excellent clinical tool. Using a single blood sample, the phase of the infection, namely, acute, chronic replicating (i.e., highly infectious), chronic nonreplicating, or a cleared HBV infection can readily be determined (Table 3). The presence of HBsAg in serum always signals that the person is infected by HBV,

TABLE 3 Serological markers of HBV infection

Phase (infectivity under normal circumstances)	Marker detected in a serum sample				
	HBsAg	HBeAg/HBV DNA	IgM anti-HBc	IgG anti-HBc	IgG anti-HBs
Acute (highly infectious)	+++	+++	+++	+	+/–
Chronic, replicating (highly infectious)	+++	+++	–	+++	–
Chronic, nonreplicating (low infectivity)	+++	–	–	+++	–
Past infection (not infectious)	–	–	–	++	++

but it does not tell us the phase of the infection or if the patient is highly infectious. To determine infectivity, serum samples are tested for the presence of HBeAg or the presence of viral DNA. The level of virus replication and its infectivity can now be easily determined by a quantitative PCR. To determine whether the infection is acute or chronic, the presence of IgM antibodies to HBcAg (IgM anti-HBc) is tested. If these are present, the patient has an acute HBV infection, and if the patient is an adult, the infection is likely to clear within a couple of months. Patients who have cleared HBV infections have no HBsAg in serum but rather have IgG antibodies to HBsAg (anti-HBs) and anti-HBc. The markers described in Table 3 are those most widely used to diagnose HBV infections.

As a final remark, one could ask whether we ever can clear an HBV infection. The answer is, unfortunately, most likely not, as revealed by some interesting studies. For example, there are patients with cleared HBV infections from 20 years ago and who for many years have had no HBsAg in serum but had anti-HBs and anti-HBc. These patients became immune suppressed, and shortly thereafter the HBV infection reactivated. In another scenario, similar types of patients who died from an accident had their livers transplanted to HBV-negative recipients. Within a couple of weeks to months after transplantation, the recipients, who were put on aggressive immune suppressive therapy, developed an HBV infection. It is believed that immune suppression allows small foci of HBV-infected cells in the transplanted liver to start to replicate and spread. Thus, it would appear that once HBV infected, always HBV infected.

HBV THERAPY AND PREVENTION

In recent years, good progress in HBV therapy has been made. New antiviral compounds, originally developed to treat HIV-1 infection, are now routinely used to treat HBV. These compounds are RT inhibitors that act against the RT of both viruses. These suppress the viral replication but do not cure the infection. Thus, therapy is most likely lifelong. The first compound to be introduced in the late 1990s was lamivudine, or 3TC, which was found to have activity against HBV. However, therapy-resistant viral variants emerge within 6 to 12 months of therapy. More-recent compounds such as entecavir and tenofovir are less prone to generate resistant viruses and effectively suppress viral replication for years. The effectiveness of the therapy is monitored by quantifying the viral load in serum by a quantitative PCR. It is likely that a suppression of the viral replication will reduce both infectivity and the risk for development of liver cancer.

The first country to introduce general HBV vaccination of newborns was Taiwan in 1985. Now, more than 20 years later, the effectiveness of the vaccine regimen can be estimated. In 1985, 20% of the Taiwanese population was chronic carriers of HBV. In the vaccinated children born after 1985, the prevalence is now 2%. Thus, the effectiveness of the vaccine regimen is at least 90%. This suggests that the HBV vaccine is highly effective in preventing HBV infection and subsequently HBV-related liver cancer.

Hepatitis D Virus

Hepatitis D virus (HDV), or hepatitis delta virus, is a satellite virus to HBV. This means that HDV cannot replicate in persons who are not infected by HBV. The reason for this is that HDV uses the envelope

from HBV. Hence, HDV is composed of the HDV genome (small circular single-stranded RNA) and HDV-encoded capsid, and its envelope is derived from HBV. Infection with HDV can occur simultaneously with HBV (coinfection) or as a superinfection with chronic or carrier HBV infection. HDV is spread mainly among IVDUs and increases the severity of hepatitis in HBV-infected individuals. HDV infection often leads to liver failure in acute infections, or liver cirrhosis, which can progress to liver cancer, in chronic infections. Infection by HDV is effectively prevented by HBV vaccination.

Hepatitis C Virus

VIROLOGY

The hepatitis C virus (HCV) belongs to the *Flaviviridae* family. That means that HCV is enveloped and has a single, positive-sense RNA strand as the genome. As previously described, the HCV genome is targeted to ribosomes by the IRES and directly translated when released from the capsid. Following production of the viral RNA-dependent RNA polymerase, a negative RNA strand is synthesized, which is the template for the synthesis of new viral genomes. Much of what is known about the life cycle of HCV has been discovered using the so-called replicon system or the HCV cell culture system. This system is based on the ability of a single viral variant to infect a limited number of hepatoma cell lines in vitro. This has contributed to the identification of the receptors involved in HCV entry: CD81, Occludin, Claudin-1, and SRB-1. A key feature of HCV is the variability of the HCV genome. The molecular basis for this is that the viral RNA polymerase lacks proofreading ability. This means that any incorrect bases that were incorporated during genome synthesis are not removed. The effects of such mutations span the range from being lethal to being beneficial for the virus. If the mutations are beneficial, for example, helping the virus to escape the immune system or antiviral compounds, they can be selected for in the next generation of viruses. Certain regions of the genome accumulate mutations. In particular, one of the two envelope proteins has regions that seem to allow for a large set of different mutations. It is believed that the main reason for this localized variability is to allow the virus to escape the host immune response. Studies involving chimpanzees have shown that immunization with the envelope proteins from one strain does not protect against infection with another HCV strain. From this it is clear that vaccine development to prevent HCV infections will encounter the same problems of strain specificity as for HIV (discussed below). However, on the bright side, more-recent studies have shown that a previous HCV infection may confer some protection against a reinfection developing to chronicity. Thus, a vaccine might not protect against infection but may well protect against disease (i.e., chronicity).

HCV has, as does HBV, a very high replication rate. This, in combination with the high genetic variability, means that a person infected by HCV carries not only one single variant of the virus, but multiple, closely related variants of the virus termed quasispecies.

By comparing HCV strains from different parts of the world, at least six major variants have been described. The most common variant, genotype 1, accounts for around 50% of all HCV infections worldwide. There

are some regional differences with respect to the presence of the different genotypes. For example, genotypes 1, 2, and 3 are the dominating HCV variants in Europe and in the United States, whereas genotype 4 is the dominant genotype in Egypt.

EPIDEMIOLOGY AND CLINICAL FEATURES

HCV shares many of the transmission routes used by HBV, such as contact with contaminated blood, blood products, and medical devices. However, HCV is not transmitted effectively either by sexual or by vertical transmission. The reason for this is not known but may be due to the fact that the levels of HCV in the circulation are around 1,000-fold lower than for HBV. As a consequence, the levels of HCV in secretions outside the bloodstream are generally low. In contrast to HBV, HCV infection is not as dependent on the maturity of the immune system, since over 70% of all those infected develop a chronic infection. One reason for this may relate to the extreme genetic variability of the HCV genome. Another factor may be the host interferon (IFN) response, which can be predicted by determining polymorphisms in the interleukin-28B (IL-28B) gene.

Unlike the acute infection, chronic HCV infection does lead to an increased risk for developing HCC. Severe liver disease as a consequence of a chronic HCV infection is a slow process. It often takes more than 20 years from infection to develop a severe liver disease, although alcohol consumption accelerates the process.

HCV is effectively transmitted in various hospital settings, such as in wards where many patients are given intravenous injections and where the hygienic standards are suboptimal. Hence, HCV infection can be nosocomial. Several reports have described outbreaks of HCV that may have been due to the presence of a single HCV-infected patient in the ward. It is believed that HCV infections can be transmitted from patient to patient by hospital staff through the use of multidose vials. This is very important to remember within the dental profession and dental clinics in light of the extensive use and reuse of devices and materials. However, most epidemiological data suggest that HCV transmissions are rare at dental clinics in developed countries. This may not be true for developing countries.

The prevalence of HCV infection in developed countries is generally less than 1%. In particular, the screening of blood donors at blood banks for antibodies to HCV has drastically reduced the frequency of transfusion-associated HCV infections. In developing countries, the prevalence of HCV can reach up to 5% of the general population. There are many reasons for this increased prevalence, including a contribution by contaminated blood products and medical devices, suboptimal hygienic standards resulting in nosocomial transmission, or the practice of traditional medicine.

HCV infection is diagnosed by detecting HCV-specific antibodies and the viral RNA genome in a serum sample. There is no specific tool to distinguish acute from chronic infections. In contrast to HBV infection, is it highly likely that the HCV infection can be cleared. The rather unstable RNA genome has no ability to integrate into the host cell genome, nor does there appear to be a latent phase. Thus, continuous replication is most likely needed to ensure survival of the virus, which also means that these cells will be recognized by the immune system.

Human Immunodeficiency Virus

VIROLOGY

HIV was discovered in the early 1980s after the observation that several young homosexual men had died as a consequence of severe immunodeficiency. The virus was isolated from peripheral blood and was found to grow rapidly in tissue culture using stimulated fresh T cells or T-cell lines. HIV-1 almost exclusively infects cells expressing the CD4 cell surface marker, which is present on T helper (Th) cells, macrophages, dendritic cells, and certain types of neural glia cells.

HIV-1 and its close but less pathogenic relative found in West Africa, HIV-2, belong to the genus *Lentivirus* within the *Retroviridae* family. The characteristic feature of this family is that all viruses have the RT enzyme. The life cycle of HIV is rather complex but can be illustrated as follows. The virus binds to its receptor, the CD4 molecule, and to one of its coreceptors, CCR5 or CXCR4. Following binding, the viral envelope fuses with the cell membrane and releases the capsid containing two RNA strands and the RT enzyme. The key feature of retroviral infection is that the viral RNA genome has to be reverse transcribed to DNA and integrated into the host genome prior to replication of the viral genome. In the cell cytoplasm, the RT enzyme converts the RNA strands to double-stranded DNA, which is transported to the cell nucleus and integrated in the host genome. Following integration, replication and transcription of the viral genome occur. New viral proteins are synthesized, and new viral particles are formed. Some of the infected cells will develop into memory T cells and migrate to lymph nodes. There, the memory T cells will lie dormant until the correct infection that will activate them comes along, at which time they will start to produce HIV-1. This is how HIV-1 infection can remain for decades in latently infected cells. In addition, these latently infected cells are extremely difficult to reach by antiviral therapies.

An important aspect of the HIV-1 replication cycle is that the RT enzyme lacks proofreading capabilities. Thus, each time an HIV-1 RNA genome is reverse transcribed to DNA, a number of mutations are introduced. As is the case for HCV, these mutations can be beneficial for the virus and help HIV-1 escape the host immune system or acquire resistance to antiviral compounds. This is the molecular basis for the evolution of viral quasispecies.

The genetic variability of HIV-1 is one of the most problematic features to overcome both for the immune system and in vaccine development. The high variability of HIV-1 hampers the development of a universal vaccine against HIV-1, at least with the techniques and strategies that are used today.

EPIDEMIOLOGY AND CLINICAL FEATURES

HIV-1 infection has now spread throughout the world. Mathematical calculations suggest that the HIV-1 epidemic may have started in Africa around 1930. The changing infrastructure, habits, and increased movement of people over great distances are most likely the reasons for the global spread of HIV-1 during the late 1970s and early 1980s. It is generally believed that HIV-1, originating from a chimpanzee virus or a similar ancestor, was introduced to humans on several occasions throughout

history through the hunting of chimpanzees. The earliest evidence for HIV-1 infection of a human has been found in samples derived in the 1950s.

HIV-1 is mainly an STD but is also effectively transmitted by contaminated blood and blood products and poorly sterilized medical instruments. Many types of medical procedures can transmit HIV-1 infection if the correct precautions are not taken.

There are several phases in HIV-1 infection (Fig. 8). Within 2 to 4 weeks of becoming HIV-1 infected, around 50% of patients develop a clinical syndrome called primary HIV-1 infection (PHI). The symptoms of the primary HIV-1 infection are often characterized by fever, rash on the thorax, and swollen lymph nodes. These symptoms last around 1 to 3 weeks. PHI is diagnosed by the simultaneous presence of HIV-1 in peripheral blood and absence of HIV-1-specific antibodies. If no laboratory testing is performed, the PHI can easily be misdiagnosed as some more harmless infection. During PHI, there is a high rate of viral replication and a strong activation of the host immune response. Paradoxically, the activation of $CD4^+$ Th cells facilitates the establishment of the HIV-1 infection, since HIV-1 replicates very effectively in activated $CD4^+$ T cells. During or after the PHI, antibodies to HIV-1 develop and can be easily detected by laboratory tests.

PHI is followed by an asymptomatic phase of the infection that can last for many years and during which the virus is still replicating. A particular group of HIV-1-infected patients has been recognized, who without any treatment remain asymptomatic for up to 10 to 15 years, with a generally very low level of HIV-1 replication,. These have been referred to as long-term asymptomatics, long-term nonprogressors, or elite controllers. The vast majority of HIV-1-infected patients will eventually develop a gradually progressing immune deficiency. This can be monitored by laboratory tests whereby the number of $CD4^+$ T cells in the peripheral blood is determined.

FIGURE 8 The different clinical phases of HIV-1 infection and changes of $CD4^+$ T-cell counts and HIV-1 RNA levels in peripheral blood.
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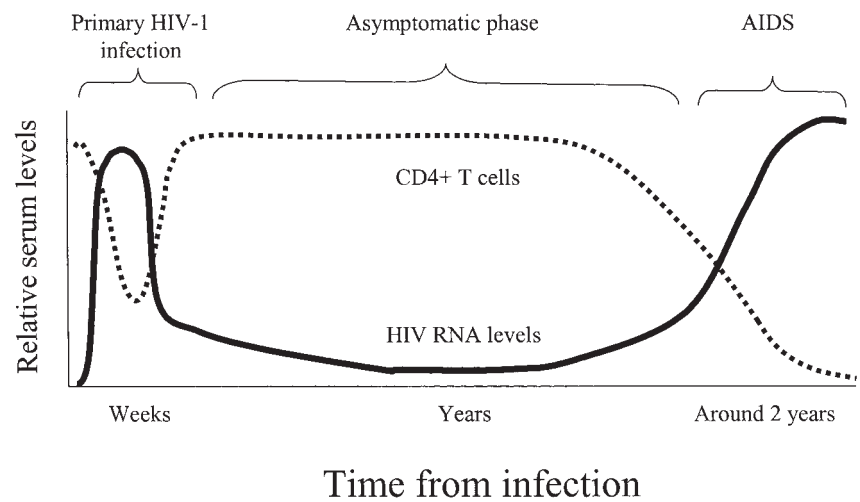


As the immune deficiency progresses, the patient becomes susceptible to infections and in particular opportunistic infections. An opportunistic infection is one in which the patient develops a symptomatic disease caused by a microbe present in the normal microbiota or by an exogenous organism that is usually nonpathogenic. In the presence of an intact immune system, these microbes are, in general, unable to cause disease. The onset of opportunistic lung infections such as that caused by *Pneumocystis jirovecii* is one of the criteria for diagnosis of AIDS.

If the HIV-1 infection is left untreated and allowed to develop into AIDS, it will inevitably lead to death. This rather dark perspective has now been radically changed by the introduction of effective antiviral compounds (discussed below).

As with all other conditions and infections that lead to suppression of immune function, HIV-induced immune deficiency will also have oral manifestations. Studies have shown that HIV-1-infected patients with an asymptomatic disease and a functional immune system usually do not differ from uninfected individuals in their oral health status. However, as the immune deficiency progresses, symptoms in the oral cavity start to appear. The most common condition seen in AIDS patients is stomatitis caused by the fungus *Candida albicans*. In fact, oral candidiasis can be used diagnostically to discover otherwise asymptomatic HIV-1 infections.

Another condition associated with HIV-1 infection is necrotizing ulcerative gingivitis (NUG). The clinical appearance of NUG is characterized by swelling and necrosis of the smooth gingival epithelium surrounding the teeth. Early on, it was believed that NUG was caused directly by HIV-1. However, it is now well documented that NUG is a consequence of the severe immune deficiency. In fact, HIV-1-related NUG closely resembles the gingival necrosis seen in patients treated with immune-suppressive drugs. NUG is effectively treated by an intense regimen of oral hygiene.

VIRAL IMMUNE RESPONSES

Viruses and the Innate Immune System

Viruses are obligate intracellular parasites. The host has therefore developed intracellular defense mechanisms and specific cell types to fight the viral infection. Inside most human cells, systems that react to a perceived assault are present. Such events are often referred to as danger signals, i.e., they activate the response mechanisms of the cell. One such danger signal is the presence of double-stranded RNA, which is not normally found in cells. When an RNA virus infects a cell, double-stranded RNA is present during replication of the viral genome. The danger signal generated by double-stranded RNA leads to activation of IFNs that in turn activate several antiviral pathways such as Toll-like receptor 3 or 7, RIG-I, MDA-5, or RNA-dependent protein kinases (PKR) and oligoadenylate synthetase. These pathways may directly interfere in different steps in the viral replication cycle to block the formation of new viral particles. Alternatively, a cascade of intracellular events can cause the cell to undergo programmed cell death, or apoptosis. Both pathways lead to the shutdown of the virus-producing factories. These intracellular antiviral systems are a part of innate immunity.

Several factors prevent infection by viruses. The most important is of course the first line of defense, i.e., intact skin and mucosal surfaces. If these barriers are broken, there are components of the innate immune system that initially combat most infections. Humoral components associated with innate immunity include various enzymes at mucosal surfaces, the complement system, and natural antibodies. The cell types most commonly associated with innate immunity are phagocytic cells such as macrophages, dendritic cells, and natural killer (NK) cells. The major role for macrophages and dendritic cells are as scavengers that engulf and degrade foreign substances. Through this mechanism, they can also signal to cells in the adaptive immune system. The NK cell is of particular importance in the defense against viral infections. The NK cell response to an infection is almost immediate and results in the release of several antiviral cytokines and cytotoxic substances. Such NK cell activation most likely assists in the activation of the specific immune system. One well-characterized mechanism for the activation of NK cells is that which occurs when the infecting virus blocks the surface presentation of major histocompatibility (MHC), or human leukocyte antigen (HLA), class I molecules. The NK cell is actively inhibited by the presence of class I molecules, so if the virus somehow blocks the display of these molecules, the NK cell will be activated and kill that particular cell.

Viruses and the Adaptive Immune System

Three major cell types make up the adaptive immune system: B cells, $CD4^+$ Th cells, and $CD8^+$ cytotoxic T cells (CTLs). The function of B cells is to produce specific antibodies that are reactive against the invading virus. If these antibodies can block the attachment of the virus to its cellular receptor or mediate uptake of the virus by macrophages or dendritic cells, they are called neutralizing antibodies. Thus, B cells and their associated antibodies are restricted mainly to acting directly on viruses or viral proteins outside the cell. The production of neutralizing antibodies is most likely of key importance in the protection against viral infections. Thus, a major aim in vaccine development to protect against viral infections is to induce B cells to produce antibodies that can neutralize a particular virus. Most often, such neutralizing antibodies are directed against surface-exposed regions of the extracellular virus, i.e., the structures that mediate binding to the cellular receptor. Another important role for B cells in the defense against viral infections is to act as antigen-presenting cells (APCs). A B cell can, through its surface-bound immunoglobulins, bind a virus particle or a viral protein. The particle, or protein, is taken up by the B cell, degraded into short peptides, and presented on the surface using MHC/HLA class II molecules. These peptide-containing class II molecules are then recognized by specific Th cells, which thus become activated. Activated Th cells subsequently induce the antigen-presenting B cell to differentiate into a highly efficient antibody-producing cell, called the plasma cell.

T cells are key mediators of the defense against viral infections. The two types of T cells, Th cells and CTLs, are activated by two distinct, but similar, pathways. As described, the Th cell is activated by short peptides derived from viral proteins that are presented by MHC/HLA class

II molecules on the surface of professional APCs such as B cells, macrophages, and different types of dendritic cells. The professional APC takes up the virus or the viral protein and degrades the protein(s) into peptides in the phagosome. These peptides, usually 10 to 15 amino acids long, contain target sequences by which they bind to the MHC/HLA class II molecules present in the phagosome. The peptide-MHC complex is then transported to the surface of the APC and thus becomes accessible to the Th cell. The T-cell receptor (TCR) of a Th cell specific for that particular viral peptide will recognize the peptide in the context of the MHC/HLA class II molecule on the surface of the APC. The TCR binds to the class II-bound peptide in a manner by which a number of surface molecules of the APC bind to surface molecules of the Th cell. This leads to activation signals in the Th cells whereby the Th cell becomes activated and starts to produce factors called cytokines. Some of these factors, such as IL-2, will induce cell division of the specific Th cell, leading to a clonal expansion, i.e., multiplication of that particular antiviral Th cell. Other factors, such as IFN-γ, can directly activate PKR and oligoadenylate synthetase to start the antiviral cascades in the virus-infected cells nearby, thus blocking virus replication. The key role for Th cells is, however, to support the maturation and differentiation of specific B cells and CTLs. Some of the activated antiviral Th cells will differentiate into long-lived memory cells. These are partly responsible for the protection against reinfection with the same virus that is often seen. If the same virus is encountered a second time, say a few years later, the memory B and T cells are rapidly activated and will clear the infection before it has established and begun to cause symptoms.

B cells and Th cells participate equally well in the immune response against bacterial and viral infections. In contrast, the activity of CTLs is exclusively directed against intracellular pathogens. After the virus has entered the cell and replication and synthesis of viral proteins have started, another intracellular antiviral event takes place (Fig. 9). Most viral proteins synthesized in the infected cell are used to build new viral particles or perform important functions in the viral life cycle. However, some viral proteins are degraded within the proteosome into peptides and shuttled by a peptide transporter system through the endoplasmic reticulum membrane, to associate with MHC/HLA class I molecules. Class I molecules containing viral peptides are transported to the surface of the infected cell, where they can be recognized by specific CTLs. The TCR of the CTL will bind to the class I-peptide complex, which activates the CTL. After activation, one cytotoxic mechanism of CTL is the production of a protein complex known as the perforin complex, which punches a number of holes in the cell membrane of the infected cell. This leads to the killing of the virus-infected cell and thus to destruction of one of the viral factories.

Viral Evasion Strategies

When all of these innate and adaptive immune system effectors are working perfectly, the infected host generally clears the viral infection. However, several viruses are able to establish chronic infections and remain in the host for extended periods. Exactly how viruses are able to establish chronic infections is largely unknown, but some mechanisms, which may

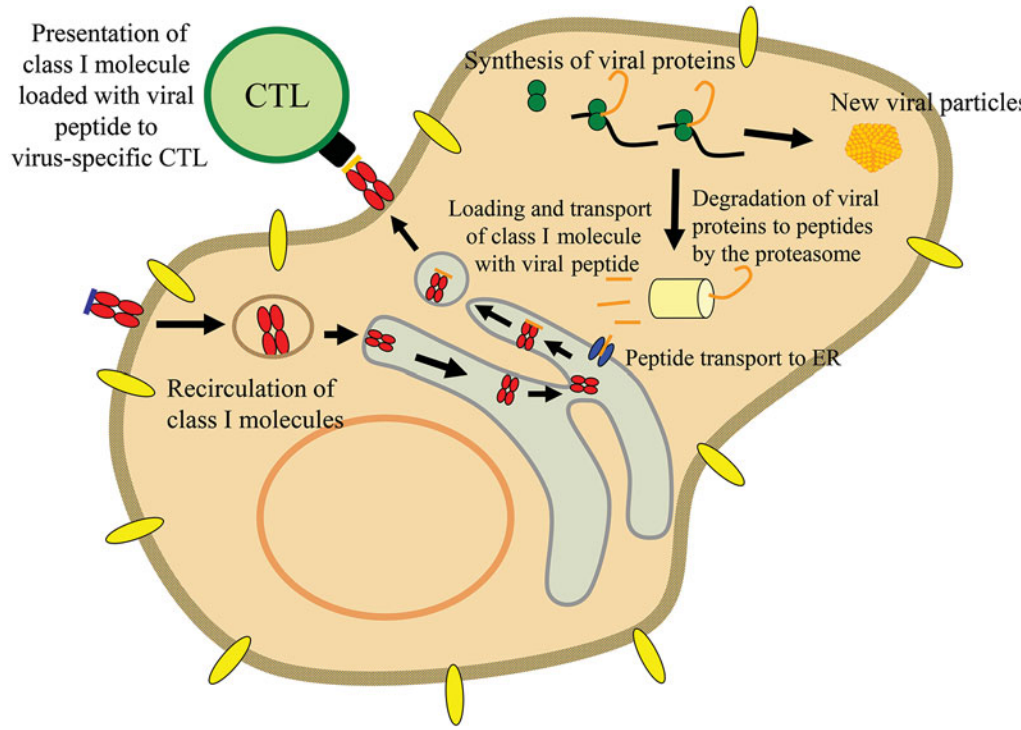


FIGURE 9 Mechanism of recognition of a virus-infected cell by the MHC/HLA class I antigen-presenting pathway. doi:10.1128/9781555818906.ch16.f9

be widely utilized, have been identified. Strategies include blocking the activation of the host immune response or avoiding an activated immune response. The following text will discuss four rather well-known strategies used by viruses to escape the host immune system.

1. Immune escape by inhibiting antigen presentation. The best-known example of inhibition of antigen presentation has been found among the *Herpesviridae*. For example, CMV produces four proteins called unique short region proteins that downregulate the surface expression of MHC/HLA class I molecules on CMV-infected cells. Thus, no peptides representing the viral proteins produced by the infected cell will be presented by class I molecules. Subsequently, there is no target for CTLs to recognize on the infected cell. Well, stop now—what about the NK cells that become activated by the absence of class I molecules, the reader now thinks. Correct, but like you, CMV has also thought about that and produces a homologue of HLA class I molecules that inhibit the activation of the NK cell. Thus, CMV effectively escapes the host immune system by blocking antigen presentation.
2. Immune escape by genetic variations in the viral genome. This is probably one of the better-known ways by which viruses escape the immune system. Both B and T cells specifically recognize viral proteins by recognizing regions of amino acid sequence (epitopes). Thus, if the virus accumulates mutations within the epitopes that are recognized by the B or T cells, the virus will be resistant to existing neutralizing antibodies and CTLs. Eventually, new specific

B cells or CTLs are generated to the new sequence, but at that time a different mutation may be introduced into the genome, so these cells are only antiviral for a short period. The best-known examples of viruses that use this strategy are HIV-1 and HCV.

3. Immune escape by using decoys or induction of immunological tolerance. The third way for viruses to avoid the host immune system is to produce viral proteins in order to swamp the immune response, or to produce proteins that reduce the activity of the antiviral immune response. A virus that actively pursues both these strategies is HBV. During HBV infection, the infected hepatocytes produce and secrete into the circulation a large excess of HBsAg. These amounts are much more than what is needed for the synthesis of new viral particles. The excess HBsAg proteins serve two functions. First, they bind (or block) host antibodies directed against HBsAg, preventing these antibodies from neutralizing infectious virus. This is the decoy function of HBsAg. Second, the constant presence of high levels of HBsAg in the circulation leads to inactivation of HBsAg-specific T cells. This approach thus leads to the induction of an immunologically tolerant state in the virus-specific T cells, and they are unable to fight the infection. As previously mentioned, another HBV protein called HBeAg is also active in inhibiting the T-cell response to HBV. A proposed mode of action is that HBeAg passes through the placenta of the infected mother and enters the circulation of the fetus. If HbeAg is present during the development of the fetal immune system, it is not recognized as a foreign antigen, so T cells specific for HBeAg are deleted, since they are regarded as reactive towards a self protein. When the child is infected by the mother's HBV strain at birth, a selective T-cell defect is present in the response to HBcAg and HBeAg (partly encoded by the same gene of the HBV genome). Thus, the newborn child is unable to mount an effective immune response against HBV, and the child can become chronically infected. This is supported by the observation that newborns of mothers infected by a mutant variant of HBV that is unable to produce HBeAg more often develop a severe liver disease that leads to clearance of the infection. Thus, the function of HBeAg is to induce an immunological tolerance to both HBeAg and HBcAg.
4. Inhibition of antiviral signaling. Several viruses have developed strategies to inhibit the innate IFN response. For example, the NS3/4A protease of HCV can cleave several host proteins, which effectively prevents downstream signaling events. Here the NS3/4A can inactivate a molecule involved in the RIG-I response pathway, which blocks the IFN response and allows HCV to maintain replication in the infected cell.

ANTIVIRAL VACCINES AND THERAPIES

History of Viral Vaccines

The first recognized vaccine to be developed in the Western world was actually a viral vaccine. In the 1790s, Jenner tested the idea that women who milked cows (milkmaids) were resistant to infection with smallpox

(variola). He noted that the milkmaids often developed a mild disease with blisters on their hands and concluded that these blisters may contain something that protects them from smallpox. He proceeded to isolate the liquid from the blisters from the women's hands and used this to inoculate a young boy, the eight-year-old James Phipps. He was then repeatedly exposed to smallpox and was found to be resistant to smallpox infection. This was the first step in the development of the smallpox vaccine.

The basis for the protection was that the milkmaids had been inoculated by a virus that was closely related to smallpox, known as cowpox virus or vaccinia virus (*vacca* is Latin for cow). Vaccinia virus is able to infect human cells but causes only a mild disease. However, the replication of vaccinia virus in human cells leads to the activation of B and T cells that also react with the vaccinia virus proteins. Since vaccinia and variola viruses are closely related, the sequences of vaccinia and variola virus proteins are also quite similar. Consequently, most of the B and T cells that recognize vaccinia virus proteins also recognize the proteins from variola virus. Thus, a previous exposure to vaccinia virus primes an immune response that is then protective against variola virus. These findings started the human vaccine era as we know it today.

We have now, 200 years later, been able to eradicate smallpox infections in humans using the cowpox vaccine. How was this possible? Well, four very important criteria were fulfilled.

1. Humans were the only host for variola virus. If humans are the only reservoir for a virus, then vaccination of all humans will effectively eradicate the virus, since no other susceptible host is available.
2. Variola virus caused only acute infections. Variola virus caused an acute infection that led either to the death of the host or to a complete clearance of the infection. Thus, when the infection was over, the host was either dead or resistant to reinfection. If a virus causes both acute and chronic infections or there are asymptomatic carriers, then there will be reservoirs of infected hosts for a long time. If the vaccine is not 100% effective, then there will always be a small number of subjects that carry the virus.
3. The cowpox vaccine was effective, cheap, and easy to produce, allowing for mass vaccination. The cowpox vaccine was quite easy to produce in large quantities and easy to administer. Almost everyone who got the vaccine developed an immune response that protected against infection with variola virus. Thus, those who were not effectively vaccinated were infected by variola virus and either died or became immune to reinfection. This was the perfect setting to be able to eradicate variola.
4. Smallpox virus has a stable genome. The smallpox viral genome does not vary. The genome of the smallpox virus does not differ substantially regardless of the part of the world from which it is isolated. Thus, one single vaccine could be used globally to induce protective immune responses.

Smallpox was proclaimed eradicated by the World Health Organization around 200 years after Jenner performed the first vaccination. Small amounts of the virus have been kept in well-guarded facilities in the United States and in the former Soviet Union. These stocks are kept in case a new

smallpox epidemic should occur. Whether we should destroy these virus stores has been widely debated over the past decades. If the reservoir of smallpox should fall into the wrong hands, this would constitute a serious threat of bioterrorism.

Antiviral Immunoglobulin Preparations and Vaccines

Viral infections can be prevented by either passive or active immunizations. Passive immunization means the transfer or injection of immune components, in general immunoglobulins (antibodies) that in the host can protect against one or more infections. The disadvantage with passive immunization is that no immunological memory develops. Thus, if protection is to be maintained, the antibody supply needs to be restored by new injections every 3 to 6 months. Today, immunoglobulin preparations are available for many diseases including HAV and HBV infections and rabies.

Active immunizations introduce an inactivated form, or parts, of the virus, after which the host actively develops immunity to the viral components. By this approach, an immunological memory is generated. This is the same approach as that used by Jenner with the vaccinia virus, and it was therefore termed vaccination by Louis Pasteur, in honor of Jenner's work.

Viral vaccines can be divided into different groups depending on how the vaccine is produced. The traditional way of making a viral vaccine is to use the whole viral particle of a nonpathogenic virus strain (attenuated vaccine) or to kill the pathogenic virus and use this as the vaccine (inactivated vaccine). Examples of attenuated whole-virus vaccines are vaccinia virus (a nonpathogenic strain), the orally administered attenuated poliovirus vaccine developed by Albert Sabin, and the combined measles, mumps, and rubella vaccine. Examples of inactivated whole-virus vaccines are the injectable poliovirus vaccine developed by Jonas Salk, the recent HAV vaccine, and the original plasma-derived HBV vaccine.

As shown in Table 4, there is a great variability in the effectiveness of the different forms of vaccines in how they activate the host immune response. The most potent vaccines to date are the attenuated whole-virus vaccines, as they allow a low level of viral replication. This means that the very potent danger signals are activated, which helps stimulate the immune response. In addition, since viral replication occurs, there is effective priming of CTLs. So, why are not all vaccines produced in this way? The answer is simple: either it is too dangerous (entailing the risk of provoking full-fledged disease) or it is not possible to do. The reasons it

TABLE 4 Types of viral vaccines and the immune responses they prime

	Immune responses primed[a]		
Vaccine type	**B cells (antibodies)**	**Th cells**	**CTLs**
Whole virus, attenuated	+++	+++	+++
Whole virus, inactivated	++	++	–
Subcomponent	++	++	–
Peptides	+	+	±
Genetic vaccine	+	+	+++

[a]The effectiveness by which different cell populations are activated ranges from not at all (–) to highly activated (+++).

cannot be achieved include the inability to grow the virus in cell culture or to isolate sufficient amounts of the virus from infected individuals or animals. If the virus can be grown in different cell culture systems but there is a risk that the attenuated virus may cause disease, the virus can be inactivated. This means that the virus present in the vaccine is unable to infect and replicate; in other words, it is dead (assuming that a virus can be called a living organism in the first place). This is achieved by different chemical compounds, such as formalin, or by different forms of irradiation. Unfortunately, these types of vaccines do not prime as broad and long-lived immune responses as do the attenuated vaccines, although they are generally very safe.

Techniques developed during the past 20 years have enabled the production of vaccines by recombinant DNA technology. By this process, the gene for one or more viral proteins can be put into bacteria, which will then produce large amounts of the viral protein. The protein is then purified and used as the vaccine. The first human vaccine produced in such a way was the HBV vaccine. The previous plasma-derived HBV vaccine was produced by purifying HBsAg (and virus particles) from blood plasma of HBV-infected individuals. The purified vaccine was then extensively inactivated. The plasma-derived HBV vaccine was highly effective, and no cases of hepatitis have ever resulted from vaccination. However, since human plasma may contain unknown, deleterious agents such as prions, the possibility of a risk of transmitting other agents remained. Subsequently, a new vaccine was developed, using a recombinant protein.

Synthetic peptides have been used in several clinical trials, but no commercial human vaccine has yet been introduced. The major problem with peptides is that they are not always immunogenic in everyone due to immune system heterogeneity.

A new type of vaccines is a group called genetic vaccines. In this case, a viral gene is introduced into a modified bacterial plasmid or into a modified viral vector. The plasmid, or viral vector, contains a eucaryotic promoter, which allows expression of the viral gene if the plasmid is introduced into a human cell. The plasmid, or viral vector, DNA is purified and injected into the host to be vaccinated. Some of the plasmid is taken up by the host cells and is transported into the nucleus, or if a viral RNA is released this can be processed in the cytoplasm. In the nucleus, the eucaryotic promoter of the plasmid is activated, mRNA corresponding to the viral protein is produced, and viral proteins are generated in the cytoplasm of the cell. In this way, only the gene for the viral protein is injected and the host cells produce the viral protein themselves. This method mimics the production of viral proteins during a natural infection and has been found highly effective when priming of CTLs is desired. However, there are still a lot of hurdles that have to be overcome before DNA-based, or other genetic vaccines, become widely used.

Antiviral Compounds and Therapies

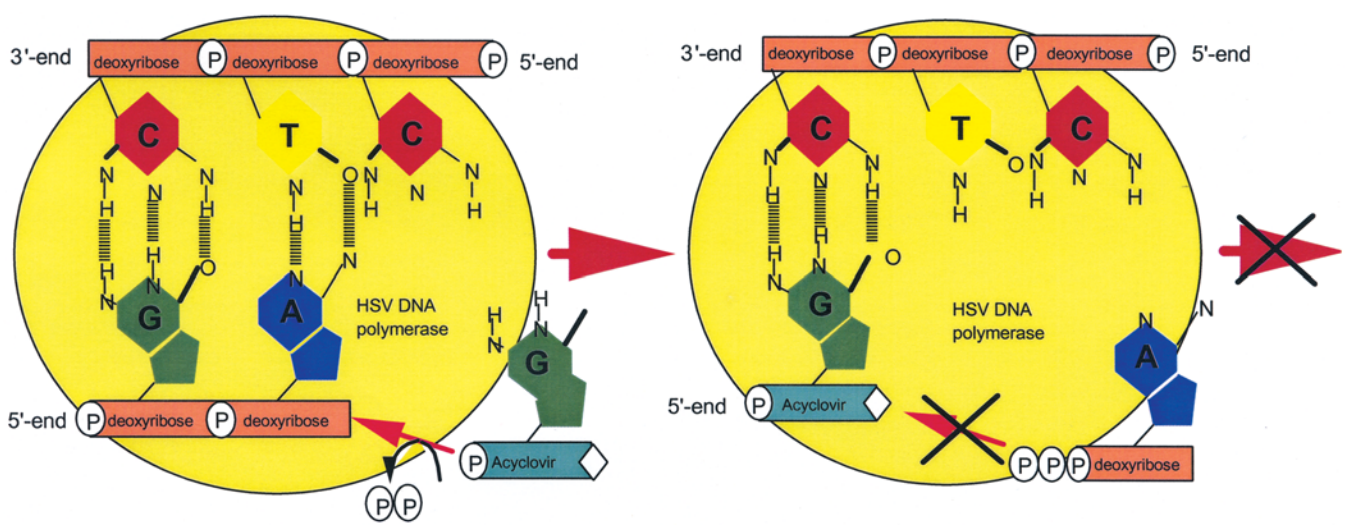
During the past 20 years, great progress has been made in the ability to treat viral infections. The major problem associated with the development of antiviral compounds is that the virus is an intracellular parasite and uses the host cell machinery for many steps in its life cycle. Thus, many compounds that are antiviral are also toxic for human cells. Despite this, several strategies have been used to block steps in the viral life cycle by

using compounds that have a higher specificity for viral enzymes. A classical example of an antiviral compound is the drug acyclovir, which is widely used to treat infections caused by HSV.

Acyclovir uses our knowledge about the HSV life cycle to specifically act against HSV. Acyclovir is what is known as a nucleoside analogue. Thus, the compound is very similar to deoxyguanidine, used in normal DNA synthesis, but lacks an active hydroxy group in the 3′-end of the molecule. However, the compound is unphosphorylated and needs to undergo a first phosphorylation before it can undergo further phosphorylations by cellular kinases prior to incorporation into the DNA strand (Fig. 10). The first phosphorylation of acyclovir is inefficiently carried out by cellular kinases but is very effectively done by the kinase encoded by HSV, a thymidine kinase (HSV-TK). When acyclovir is phosphorylated by HSV-TK, acyclovir becomes further phosphorylated and then is incorporated into the viral genome. Since acyclovir lacks the 3′-OH group, no more bases can be added to the new DNA strand, and the replication is terminated. This explains the specificity of acyclovir for HSV-infected cells, since these are the only cells that can activate the substance.

Since the development of acyclovir, several similar substances that are active against different viruses and different viral enzymes have been developed. In particular, the fight against HIV-1 has generated many new antiviral compounds and uses a wide range of targets. Examples of these are inhibitors of the RT enzyme, inhibitors of the viral protease, inhibitors of the integrase, and inhibitors of viral fusion. The infections that today are effectively treated using different regimens of specific antiviral therapies are HIV-1, HBV, HCV, HSV, and CMV. Current therapy of HIV-1 infections consists of a combination of at least three compounds and is often referred to as highly active antiretroviral therapy. Unfortunately, there is enough plasticity in the viral genome to allow for mutations within the viral target enzymes, which renders the virus resistant to one or all compounds. Viral variability is correlated with the number of replication cycles, since each replication generates mutations within the viral

FIGURE 10 Mechanism of action of the antiviral compound acyclovir.
doi:10.1128/9781555818906.ch16.f10



genome. It is therefore crucial that the patient take the different antiviral compounds at regular intervals, so that HIV-1 replication is continuously kept to a minimum. If the patient ignores this strict schedule, the virus may replicate, with mutations and development of resistant virus as a result. HIV-1 strains that are resistant to one or more antiviral compounds have now started to spread in the Western world, where antiviral therapy of HIV-1 is common. Another problem is the ability of HIV-1 to reside as a latent infection in certain cell types; i.e., the virus does not replicate its genome until the cell is activated. It is thought that in order to eradicate the HIV-1 present in one infected patient, an effective combination therapy would have to be maintained for several decades. This is of course almost impossible, since the drugs cause rather severe side effects and the risk of developing resistant virus is high. The general aim at present is therefore to combine the antiviral compounds with some type of specific immune therapy such as a vaccination. By this approach, it is believed that the antiviral compound will suppress the viral replication and that the vaccination will activate the T cells that can also kill latently infected cells. The paradox is, however, that the vaccine is designed to activate T cells, and HIV-1 replicates most efficiently in activated T cells. There is no doubt that the antiviral therapies for HIV-1 have completely changed the prognosis of the infection. It is possible that, even though we do not know this yet as a fact, a person who becomes HIV-1 infected at 20 years of age may well live to 60 years of age without development of AIDS—an almost normal life span. In addition, successful therapy reduces the risk of HIV-1 transmission.

Today, HBV is effectively treated using some of the same RT inhibitors used to treat HIV. However, therapy is life long and therefore needs improvement. Other ways to design antiviral therapies is to use different types of immune modulators, such as cytokines. The most widely used cytokine is IFN-α. The mode of action of IFN-α is not fully understood and may differ from virus to virus. However, one major action is the activation of the PKR system. Through this pathway, the viral RNA is degraded and the cell undergoes apoptosis. This is believed to be a major antiviral effect of IFN-α. There are most likely some additional antiviral effects exerted by IFN-α. For example, it has been shown that IFN-α effectively helps to activate the correct type of T cells that help to fight the viral infection. Pegylated IFN-α is today sometimes used to treat chronic infections caused by HBV and is still the cornerstone of HCV therapy. Around 50% of all those chronically infected by HCV can be cured by a combination therapy containing IFN-α. New directly acting antivirals are currently in clinical trials for HCV, and the hope is that HCV therapy may not need the addition of pegylated IFN-α. This is certainly desired, since IFN has severe side effects.

Another compound for which the mechanism of action is even less well understood is ribavirin. Ribavirin is a nucleoside analogue. It has been shown that ribavirin therapy of chronic HCV infections does not have any effect on the viral replication. However, when ribavirin is used in combination with IFN-α, the effect on the viral replication is better than that obtained when using IFN-α alone. It has been suggested that ribavirin acts as an immune modulator or as a mutagen that forces the virus into an error catastrophe.

KEY POINTS

Viruses are obligate intracellular organisms, since they have no metabolism of their own.

All viruses currently known contain a viral genome surrounded by a protein shell. Some viruses may also have a lipid envelope surrounding the protein shell (nucleocapsid) that contains the viral genome. The viral genome comes in many shapes and sizes, ranging from around 2,000 bases to around 200,000 bases. The viral genome can be composed of either RNA or DNA and can be linear or circular or consist of several chromosomes (segmented).

Viral genomes contain coding and noncoding regions. The coding regions encode all viral proteins, and the noncoding regions interact with the cellular machinery.

The viral proteins are either structural proteins (present in the extracellular virus) or nonstructural proteins (present only in the infected cell).

The most widely used criteria for the classification of viruses are the type of genome, the presence or absence of a lipid envelope, and the structure of the viral nucleocapsid (icosahedral or helical).

The viral life cycle can be summarized in the following steps: (i) attachment of the virus to the cell; (ii) entry of the virus into the cell by fusion, endocytosis, or translocation; (iii) uncoating and release of the viral genome; (iv) transcription or replication of the viral genome; (v) synthesis of viral proteins; (vi) assembly of new viral particles; and (vii) release of infectious virus by lysis of the cell or by nonlytic pathways.

The members of the *Herpesviridae* family are enveloped viruses with DNA genomes. The most important members of the *Herpesviridae* are HSV-1 (which causes oral herpes, a recurrent infection), HSV-2 (which causes genital herpes, an STD), VZV (which causes chicken pox as a primary infection and herpes zoster [shingles] as a recurrent infection), and CMV and EBV (which cause severe disease in immunosuppressed patients).

HPV is a DNA virus lacking a lipid envelope. HPV can cause oral warts or papillomas. HBV-6, -11, -16, and -18 have been associated with oral and/or cervical carcinoma. HPV vaccines can prevent infection with the types most commonly associated with carcinoma.

HBV is an enveloped virus with a DNA genome. HBV is transmitted by contaminated blood and by sexual contact. Infection is often asymptomatic for long periods, and HBV causes both acute and chronic infections. Chronic HBV infection increases the risk for developing liver cancer. The presence of HBsAg in serum signals ongoing infection. An effective vaccine is available.

HCV is an enveloped virus with an RNA genome. During each replication cycle, new mutations are introduced into the HCV genome. HCV is transmitted by contaminated blood and blood products. HCV causes a chronic infection in >70% of all who become infected, and chronic infection increases the risk for developing liver cancer. Chronic HCV infections can be treated with a combination of IFN-α and ribavirin. Recently introduced small-molecule enzyme inhibitors will also improve treatment. There is no vaccine currently available to prevent HCV infection.

HIV-1 is a member of the *Retroviridae* family. HIV-1 is an enveloped virus with an RNA genome and the enzyme RT, which can synthesize DNA from an RNA template. The HIV-1 genome is integrated in the human genome as a part of the viral replication cycle. HIV-1 infection can be asymptomatic for several years. HIV-1 infection is an STD and leads to the depletion of $CD4^+$ T cell. AIDS is a syndrome of opportunistic diseases that develops due to the immune deficiency caused by the HIV-1 infection. The mean survival time of a patient who has developed AIDS and who is left untreated is 2 years. No effective vaccine is available to prevent HIV-1 infection due in part to the high genetic variability of the viral genome. Today, effective combination antiviral therapies increase the life span of HIV-1-infected patients.

The most important cell types of the innate immune system in fighting viral infections are macrophages, dendritic cells, and NK/NKT cells.

The cells of the adaptive immune system that fight viral infections are B cells, Th cells, and cytotoxic T cells. Antibodies from B cells can neutralize viruses when they bind to the surface of the virus particle. Th cells recognize viral peptides through the class II presenting pathway. CTLs recognize and kill virus-infected cells through viral peptides presented by the class I pathway. Viruses can escape the host immune system by inhibiting antigen presentation, changing their genome through mutations, producing decoys and inducing immunological tolerance and immune dysregulation, and blocking innate immune responses.

Jenner performed the first vaccination against smallpox in 1790. It was possible to eradicate smallpox because an efficient vaccine was readily available; humans were the only host; smallpox caused only acute infections that either resolved or killed the host, and thus, there were no reservoirs of the virus; and the virus was genetically stable.

Passive immunization (injection of antibodies) does not generate immunological memory. In contrast, active immunization generates immunological memory. Viral vaccines can be divided into the following categories: attenuated whole-virus, inactivated whole-virus, subcomponent, peptide, and genetic vaccines.

Antiviral compounds can block or inhibit specific steps in the viral life cycle and act indirectly by activating or modulating the host immune system. The antiviral drug acyclovir is given as a prodrug that is phosphorylated, i.e., activated, by a viral enzyme and is therefore active only in virus-infected cells. Future antiviral therapies will most likely be combination therapies consisting of several antiviral compounds.

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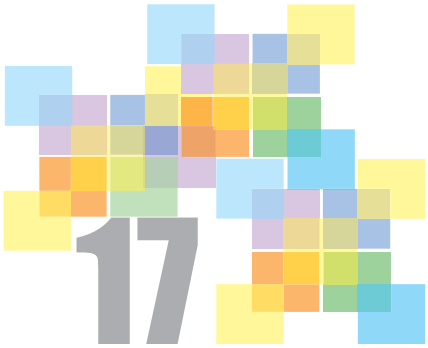

Fungi and Fungal Infections of the Oral Cavity

Richard D. Cannon and Norman A. Firth

INTRODUCTION

Fungi are normal, albeit minor, members of the oral microbial flora. The fungus that can be isolated most frequently from the human oral cavity is *Candida albicans*. This organism is a harmless commensal in approximately 20 to 40% of healthy individuals. Fungi can and do, however, cause oral mucosal diseases, particularly in immunocompromised individuals. The most common oral fungal infection is candidiasis, which has a variety of presentations, but several other fungi can cause oral lesions. There are fundamental structural and metabolic differences between fungi and other oral microorganisms, such as bacteria and viruses, and these differences affect the pathogenesis of the diseases they cause and the treatment of infected individuals. This chapter describes the biology and virulence of fungi, the major oral clinical conditions caused by fungi, and the treatment of people with oral fungal infections.

BIOLOGY

Fungi (singular: fungus) are a large group of microorganisms that constitute a kingdom within the domain *Eukarya*. As such, fungi are distinct from members of the animal and plant kingdoms and from bacteria and archaea. Fungi are ubiquitous microorganisms with important roles in the degradation of organic material, as food sources, and as key components in the food and biotechnology industries. Of the approximately 100,000 recognized fungal species, only about 150 are pathogenic for humans, and of these, a handful cause oral and perioral lesions.

Fungi are vegetative organisms that do not synthesize chlorophyll, are nonmotile, and have a basic structure consisting of individual cells or chains of cylindrical cells (a hypha [plural: hyphae]). Fungi are eukaryotic and share some structural and metabolic features of animal cells: they have DNA contained in a membrane-bound nucleus, possess organelles such as mitochondria and centrioles, and have 80S ribosomes (as opposed to the 70S ribosomes in bacteria). They also have features that distinguish them from animal cells: they almost always possess a rigid cell wall surrounding a plasma membrane that contains the sterol ergosterol rather than the cholesterol found in mammalian cell membranes.

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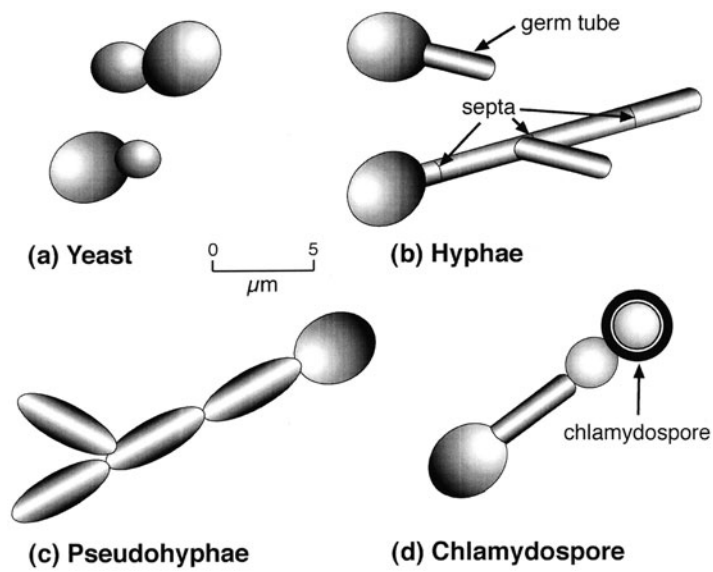
The cell walls of fungi are distinct from gram-positive or gram-negative bacterial cell walls. Fungal cell walls generally contain an outer amorphous layer of glycoproteins and inner layers, or enrichments, of polysaccharides such as glucans (polymers of glucose) and chitin (a polymer of *N*-acetylglucosamine), which confer rigidity and determine cell morphology. *Cryptococcus neoformans* possesses a distinctive capsule composed predominantly of the polysaccharide glucuronoxylomannan. In filamentous fungi, growth is limited mostly to the hyphal tip; in yeast, there is general wall expansion over the entire surface during the cell growth cycle. The molecules on the outer surface of fungi are important, as they are often involved in adherence and in interactions with the host defenses. The similarities and differences between fungi and mammalian cells and between fungi and other microorganisms are important for the development of specific antifungal drugs that are not toxic to humans and so are free from side effects.

Morphology

Fungal cells have a diameter of approximately 3 to 6 μm, and in general they are larger than bacteria and smaller than mammalian cells. Fungi have distinctive and varied cellular morphologies (Fig. 1). Those that exist predominantly in the unicellular state are usually ovoid and termed yeasts; examples are *C. albicans* and *C. neoformans*. Fungi that grow as hyphae are commonly called molds. The initial emergence of a hypha from a *C. albicans* yeast cell is referred to as a germ tube, even though *C. albicans* does not produce spores and does not germinate. The ability of yeast cells to produce germ tubes when incubated in serum is referred to as a *germ tube test* and is a property used for the presumptive identification of *C. albicans* cells. Hyphae consist of chains of individual cylindrical cells, each containing a nucleus and divided from adjacent cells by walls called septa. The presence and structure of septa are important in the taxonomy of fungi. Fungal hyphae can branch and form a mycelial mat.

FIGURE 1 Growth morphologies of *C. albicans*. doi:10.1128/9781555818906.ch17.f1



Some fungi exist in more than one cellular morphological form and are termed dimorphic or polymorphic, depending on the number of forms in which they grow. Dimorphic fungi include *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Sporothrix schenckii*. *C. albicans* is often referred to as a dimorphic fungus, as it exists mostly in either the yeast or hyphal morphological form. Strictly speaking, however, it is polymorphic, as it can also form sporelike chlamydospores on certain media, and grows as pseudohyphae, which differ from true hyphae in that they have constrictions at the septa and appear as chains of elongated yeast cells (Fig. 1). Fungal dimorphism has been extensively studied at the molecular genetic level, as it is believed to be involved in the pathogenesis of fungal diseases.

Macroscopically, yeast colonies on agar plates tend to be smooth with well-defined edges, whereas mold colonies are furry with individual hyphal threads visible at the edge of the colony. Mold colonies are sometimes pigmented toward the center (green, for example, for *Aspergillus* colonies) due to the maturation of spores. Some fungi produce conidia (singular: conidium), which are asexual spores supported on a hypha.

Replication

Fungal replication can be either sexual or asexual, and some fungi can undertake both. Fungi that lack a known sexual state are sometimes referred to as Deuteromycota (Fungi Imperfecti) and are usually members of the phyla Ascomycota and Basidiomycota. *C. albicans* possesses several genes homologous to those involved in sexual replication in other fungi, including the yeast *Saccharomyces cerevisiae*. Although genetically manipulated *C. albicans* can be shown to mate in vitro, there is currently no definitive evidence that *C. albicans* undergoes sexual reproduction in vivo. In the asexual replication of yeast, the mother cell produces a bud, which enlarges to form a daughter cell. The nucleus replicates by mitosis and migrates to the neck of the bud, and one replicated nucleus migrates into the daughter cell along with representative organelles. A cell wall septum is laid down at the neck between the mother and daughter cells, and the cells separate, leaving a bud scar on the mother cell wall and a birth scar on the daughter cell.

Filamentous fungi can replicate asexually or sexually. Both types of replication can involve the production of spores or conidia. Spores are often released from aerial fruiting bodies to be spread by air or water and are infectious agents. Sexual reproduction usually involves the fusion of either unicellular gametes or fusion of specialized hyphal elements.

PATHOGENESIS

Acquisition

Fungi are prevalent in the environment as well as being associated with humans and animals. Domestic animals are an important source of dermatophytic fungi such as *Microsporum canis*, which is associated with cats and dogs and can cause tinea corporis (ringworm) in humans. Other fungi are associated with birds; *C. neoformans*, for example, is carried by pigeons and can be disseminated through bird droppings. *C. neoformans*

has also been associated in the environment with two species of eucalyptus trees, the distribution of which correlates with the occurrence of cryptococcosis caused by *C. neoformans* var. *gattii*. The fungus *Aspergillus* is commonly found in rotting vegetation but has also been isolated from some foods, including pepper and spices. Certain fungi are endemic in the environment in particular geographic areas. *H. capsulatum* is commonly found in North and South America, the Philippines, Indonesia, Turkey, Israel, Italy, Switzerland, Africa, Australia, and Asia. *P. brasiliensis* appears to be associated with subtropical forest areas with high annual rainfall.

Fungi can therefore be readily acquired by humans from the environment. The route of entry often depends on the nature of the infectious agent (spores, conidia, or yeast) and the nature of the environmental reservoir. It can be via the lungs for fungal spores, through the mouth for *Candida*, and by direct contact with the skin for dermatophytes. *C. albicans* has been isolated from primates, domesticated and other mammals, marsupials, and birds, but transmission to humans is usually from other colonized humans. In the clinical setting, transfer can occur via the hands of health care workers. Fungi can colonize the mouth and become a component of the oral microbiota. Filamentous fungi can rarely be cultured from the oral cavity, but yeast can be cultured from the saliva of approximately 40% of healthy individuals. The yeast most commonly isolated from saliva is *C. albicans*. Other yeasts that may be present include *Candida tropicalis*, *Candida dubliniensis*, *Candida glabrata*, *Candida parapsilosis*, and *Candida krusei*. Baker's yeast, *S. cerevisiae*, can sometimes be isolated from the mouth, as can *Rhodotorula* species, which grow as distinctive red colonies on Sabouraud's agar. *C. dubliniensis*, which is phenotypically very similar to *C. albicans*, is a relatively recently described species found both in healthy individuals and in individuals with HIV infection. *C. dubliniensis*, like *C. albicans*, forms germ tubes in serum and chlamydospores on cornmeal agar, but, unlike *C. albicans*, it cannot grow at the elevated temperature of 45°C. *C. dubliniensis* can be further distinguished from *C. albicans* by specific DNA probes or PCR amplification of specific DNA sequences. Yeasts can colonize a variety of surfaces in the oral cavity and are often found associated with the dorsum of the tongue, the buccal (cheek) mucosa, and denture prostheses. *C. albicans* can also adhere to oral bacteria and salivary pellicles on oral surfaces and has been detected in dental plaque. *C. albicans* is a harmless commensal in a significant proportion of the population, but if the host immune system is impaired, *C. albicans* can cause disease. A number of factors predispose humans to oral candidiasis (Table 1). Fungal colonization of the oral cavity can lead to colonization of the gastrointestinal (GI) tract, which can act as a reservoir for the infection of other body sites such as the vagina or for dissemination via the blood.

TABLE 1 Factors predisposing to oral candidiasis

Age: being very young or old
HIV infection
Mucosal trauma/irritation, including denture wearing
Drug therapy
Antibiotics
Corticosteroids (local or systemic)
Immunosuppressives
Cytotoxics
Malnutrition
Iron deficiency
Vitamin B_{12} deficiency
Undiagnosed or poorly controlled diabetes mellitus
Leukemia
Agranulocytosis
Xerostomia
High carbohydrate diet

Virulence

Features associated with the growth and metabolism of fungi that cause damage to the host are termed virulence factors. Features that are not directly involved in host damage but are prerequisite for pathogenesis are termed virulence-associated factors. Such factors are involved in fungal adhesion and tissue damage and penetration, along with interaction with, and evasion of, the immune system.

Fungal adhesion is required for host colonization and usually involves cell surface adhesins that recognize and bind specific host receptor molecules. Adhesins are often glycoproteins, and the interaction with host receptors can be protein-protein or lectin-like (protein-carbohydrate). Several multifunctional *C. albicans* adhesins belong to the agglutinin-like sequence (ALS) gene family. Tissue penetration is more easily achieved by fungi growing as hyphae than by yeasts; thus, the dimorphic transition from yeast to hyphal growth in *C. albicans* is proposed to be a virulence factor for that fungus. Fungi secrete hydrolytic enzymes, such as proteinases and phospholipases, which can cause tissue damage and facilitate tissue penetration. Proteinases can also destroy immunoglobulins (Igs) and so help fungi evade innate and acquired host defenses. *C. albicans* possesses a family of secreted aspartic proteinases with at least 10 members (Sap1p through Sap10p). Different Sap isozymes are secreted at different stages of *Candida* infections and are thought to be involved in nutrient acquisition, adhesion, tissue penetration, immune evasion, cell injury after phagocytosis, activation of the blood clotting cascade, and enhanced vascular permeability. *C. albicans* also secretes phospholipases and has at least eight genes with homology to lipases. The ability of some fungi to bind complement proteins is thought to enable them to avoid destruction by the immune system. The carbohydrate capsule of *C. neoformans* inhibits phagocytosis by macrophages and results in reduced killing by the immune system. Several fungi, including *C. neoformans* and *Aspergillus fumigatus*, produce the pigment melanin, which is associated with the cell wall. The *C. neoformans* melanin is thought to protect cells against oxidants and UV light and to interfere with cell-mediated immunity.

The identification of new fungal virulence factors and the elucidation of fungal pathogenesis may provide novel drug targets or alternative treatment strategies. The specific inhibition of fungal adhesion may, for example, prevent colonization and hence preclude the initiation of disease. Drugs that inhibit proteinases may prevent the spread of fungal infections. The sequencing and annotation of the genomes of several pathogenic fungi, including *C. albicans*, *A. fumigatus*, and *C. neoformans*, are now complete, and it is hoped that this information will help identify new drug targets. It is debatable, however, whether the inhibition of single virulence-associated factors would be sufficient to cure people with fungal infections.

HOST DEFENSES AGAINST FUNGAL INFECTION

Host defenses against fungal infections involve both nonspecific and specific defense mechanisms.

Nonspecific Defense Mechanisms

Natural, nonspecific defense mechanisms are, in general, very effective in preventing oral fungal infections. These innate defense mechanisms include the barrier function of intact mucosae, saliva flow, and antimicrobial components of saliva such as histatins, which are effective against *C. albicans*. Most oral surfaces are colonized by bacteria against which fungi have to compete for nutrients. Following fungal colonization of mucosae or invasion of tissues, a nonspecific inflammatory response is often

elicited. Most fungi activate complement by the alternative pathway, become coated with C3 fragments, and attach to polymorphonuclear leukocytes, monocytes, and macrophages. The main leukocyte contributing to the containment and destruction of fungi is the neutrophil, but other components of the nonspecific response include eosinophils, basophils, platelets, and natural killer cells. Macrophages live longer than neutrophils and can persist at sites of infection. An early response to *H. capsulatum* infection is phagocytosis by macrophages, but the fungus can resist lysosomal killing.

Specific Defense Mechanisms

The specific immune response to fungal infections involves humoral and cell-mediated immunity. Antibodies are usually produced in response to fungi, and in the oral cavity the predominant Ig is secretory IgA. The main function of secretory IgA is to agglutinate microorganisms and prevent them from adhering to oral surfaces. Although IgA responses to fungi can be detected, they are not always effective in preventing colonization. Cell-mediated immunity plays an important role in preventing several fungal infections of humans. Individuals with primary or acquired T-cell immunodeficiencies—AIDS patients, for example, who have depleted functional $CD4^+$ T cells—are very susceptible to deep-seated or superficial fungal infections such as aspergillosis or oropharyngeal candidiasis.

ANTIFUNGAL THERAPY

To treat people with oral fungal infections, it is often necessary to first grow and identify the fungus responsible for the clinical symptoms.

Growth and Identification of Fungi

Fungi can be cultured from clinical specimens on Sabouraud's agar. Methods for sampling oral sites include wiping with sterile swabs, using phosphate-buffered saline in oral rinses, and the plating of saliva samples. Antibiotics such as chloramphenicol and gentamicin can be included in the agar to inhibit the growth of bacteria from the samples. Inoculated agar plates are generally incubated aerobically at 30°C for 48 h. There are several methods for identifying fungi. Microscopic examination of clinical samples can be informative. Samples can be obtained by scraping mucosal surfaces with a wooden spatula, or tongue depressor, and transferring the material to a clean glass slide for wet-mount microscopy. Alternatively, a biopsy whereby the sample is stained before microscopic examination may be indicated for some lesions (Table 2). This can reveal fungal cellular morphology: the presence of yeasts or hyphae, spores, conidia, or capsules and the size of these features (Fig. 2). Colony morphology of fungi is also distinctive. Fungal colonies can have a smooth surface or, for some molds, a furry appearance. The colony color, size, edge, elevation, and consistency are also informative. To identify fungi by growth features, specific agars may be required. Not all fungi, for example, will produce their characteristic spores on media used in primary culture from clinical specimens. Growth media containing various carbon and/or nitrogen sources can be used to determine fungal growth requirements and also determine fermentation patterns, which can distinguish species. Certain distinctive growth morphologies are used in the presumptive identification

TABLE 2 Staining methods to visualize fungi in clinical samples

Periodic acid-Schiff
Potassium hydroxide (KOH)
Grocott-Gomori methenamine silver
Gridley's method
Calcofluor white

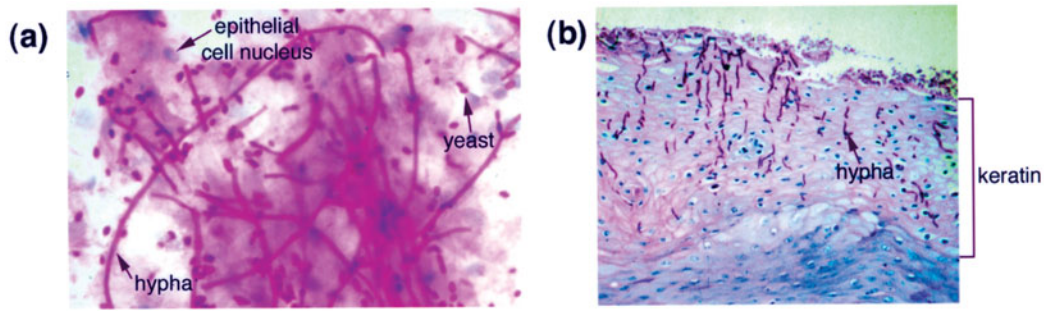


FIGURE 2 Histopathology of oral candidiasis. (a) Smear taken from pseudomembranous candidiasis lesion and stained with PAS; (b) section of pseudomembranous candidiasis lesion stained with PAS. doi:10.1128/9781555818906.ch17.f2

of fungi. *C. albicans*, for example, will produce characteristic germ tubes and then hyphae if incubated in fetal calf, bovine, rabbit, or human serum for 3 h at 37°C.

Identification of fungi by growth characteristics is time-consuming, as it usually requires both primary and secondary culture on specific media, which may take several days. Some chromogenic agars containing antibiotics can be used to identify yeast directly from clinical samples, as particular species grow on these agars as colonies with distinctive colors. The number of fungal species that can be reliably identified by this method is, however, limited.

Techniques have been developed to utilize the differences in the nucleic acid sequences of different fungal species in fungal identification. Hybridization of nucleic acids with labeled nucleic acid probes specific for a particular fungus can be used to detect that fungus in clinical samples. Usually there is amplification of the nucleic acid from the sample before hybridization. Alternatively, the ability to amplify a particular DNA sequence from a sample by using species-specific primers and PCR can be used to indicate the presence of a fungal species in the sample. These nucleic acid-based techniques can be relatively rapid and sensitive but may have the problem of false positives due to nucleic acid contamination, and they are presently carried out in laboratories rather than in the clinical setting. An increasingly common way to identify fungi is by PCR amplification and DNA sequencing of ribosomal RNA genes. Regions of these genes that are common between fungal species allow amplification of the RNA genes from fungi of interest, and sequencing the variable DNA between these conserved regions allows fungal identification. Typing of fungal strains within a species can be carried out by multilocus sequencing typing, whereby portions of a panel of housekeeping genes are sequenced and variations in the sequences between strains are used to distinguish strains. The advent of high-throughput next-generation DNA sequencing opens the possibility of identifying multiple fungal species and strains from primary clinical samples.

Principles of Antifungal Chemotherapy

Once the fungus present in significant numbers in a clinical sample is identified, it is possible to devise a treatment plan involving the application of appropriate antifungal agents. Most effective antifungal drugs

target features unique to the fungus and not present in mammalian cells. As fungi are eukaryotic, like their host, there are fewer potential specific drug targets than for bacteria. Most currently available antifungal drugs target membrane sterols (polyenes) and their biosynthesis (azoles), nucleic acid replication (flucytosine [5FC]), and cell wall biosynthesis (echinocandins). Polyenes insert themselves into fungal plasma membranes, associate with the sterol ergosterol, and form pores, which allow leakage of mono- and divalent ions and cytoplasmic components of low molecular mass. This leads to cell death, and so polyenes are fungicidal. Polyene drugs present problems with host toxicity and are generally less soluble than the less toxic triazole drugs. Azoles such as imidazoles and the more recent triazole derivatives target fungal cytochrome P450 enzymes and inhibit sterol biosynthesis by preventing 14α-demethylation of lanosterol. 5FC is a pyrimidine analog that is taken up by fungal cells and deaminated to 5-fluorouracil, which causes aberrant RNA synthesis and inhibits DNA synthesis. 5FC is fungicidal, but certain fungi, including a proportion of *C. albicans* strains, are intrinsically resistant to 5FC. 5FC is usually administered in combination with other antifungals, such as amphotericin B (a polyene), in the treatment of patients with systemic cryptococcosis.

The cell walls of fungi contain components that are not present in mammalian cells and thus represent obvious antifungal targets. Inhibitors of chitin biosynthesis (nikkomycins) are available, but they are not effective against *C. albicans*. Glucan biosynthesis has proved difficult to target, but the echinocandin inhibitors caspofungin, micafungin, and anidulafungin, which inhibit β-1,3-glucan synthase, are now available for intravenous use for esophageal candidiasis. Echinocandins are, in general, not effective against *C. neoformans* and some filamentous fungi.

Fungal drug resistance has emerged as a problem in the treatment of certain patients. HIV-positive individuals and AIDS patients not receiving highly active antiretroviral therapy, for example, frequently suffer from oropharyngeal candidiasis, and long-term treatment with triazole antifungals such as fluconazole sometimes fails because of the presence of resistant *Candida* strains. *C. glabrata* and *C. krusei* appear to be intrinsically resistant to fluconazole (primary resistance), and fluconazole resistance in *C. albicans* can be induced by exposure to the drug (secondary resistance). The susceptibility of fungi to antifungal drugs can be determined by broth dilution or agar diffusion (Etest) methods. These techniques give a value (minimum growth inhibitory concentration) for the fungal susceptibility to the drug, and there is generally a correlation between the MIC value and the clinical outcome of treatment with the drug. Drug resistance is most frequently seen with the azole and triazole antifungals. Fluconazole resistance in *C. albicans* can be due to overexpression of the drug target, mutations in the drug target, and most commonly for high-level resistance, overexpression of energy-dependent drug efflux pumps. Azole drugs are fungistatic, and use of these drugs can contribute to the recurrence of oral fungal infections.

CLINICAL CONDITIONS

The most prevalent fungus in the oral cavity and the primary etiologic agent in oral candidiasis is the yeast *C. albicans*. The name of this fungus is interesting because both parts of the binomial mean white (*Candida* is

a Latin adjective meaning white; *albicans* is the present participle of the Latin verb *albico*, being white). The name therefore doubly reflects the growth characteristics of the fungus; it grows as creamy white colonies on Sabouraud's agar, and it can also result in characteristic white lesions of the oral mucosae. In pseudomembranous and hyperplastic candidiasis, there can be many small white lesions scattered over the mucosae, giving a speckled appearance. These speckled lesions could explain the common term for candidiasis, "thrush," which is thought to be derived from the Scandinavian word torsk, which is used for both the disease and the bird. Oral thrush has been recognized clinically for centuries, but the causative agent proved elusive. In his book *Epidemics*, Hippocrates in the 4th century B.C.E. described two cases of oral aphthae, which were probably thrush. The first reference to the word thrush is from the writings of Samuel Pepys in 1665. In 1890, Zopf named the thrush fungus *Monilia albicans*, from which moniliasis, the early name for candidiasis, is derived. Although Berkhout proposed, in 1923, the genus *Candida* to accommodate *Monilia*, it was not until 1954 at the Eighth Botanical Congress in Paris that the generic name *Candida* was finally accepted.

Candida infections are common in patients with T-cell-type deficiencies but are uncommon in patients with B-cell deficiencies. It has been postulated that susceptibility to oral mucosal *Candida* infections is related more to altered T-cell function than to defects in humoral immunity, whereas prevention of systemic infection is mediated by specific serum antibodies as well as oral epithelial effector cells (granulocytes and macrophages).

Candidiasis

In 1966 Lehner published a classification of oral *Candida* infections that was accepted for many years. Lehner's classification recognized two major subdivisions: acute infections (which included pseudomembranous and atrophic candidiasis) and chronic infections (including atrophic and hyperplastic candidiasis). The emergence of *Candida* infections in immunocompromised hosts such as HIV/AIDS patients and organ transplant recipients has resulted in several changes to the currently accepted classification. In particular, the simple temporal relationship is no longer considered valid, as the pseudomembranous form can be long-lasting in the immunocompromised and other groups, for instance, long-term users of corticosteroid inhalers (asthmatics) in whom alterations in local immunity occur intraorally. Pseudomembranous candidiasis can therefore be considered to be acute or chronic. The term "atrophic" is a histopathological rather than clinical term; currently, "erythematous" is favored, as it describes the red nature of such lesions. This redness may be due to atrophy, but it may also be due to increased vascularity and inflammation. The clinical conditions angular cheilitis, denture stomatitis (chronic erythematous candidiasis), and median rhomboid glossitis may have a mixed bacterial and fungal etiology and therefore are considered to be *Candida*-associated lesions. The current classification of oral and perioral *Candida* infections is given in Table 3.

PSEUDOMEMBRANOUS CANDIDIASIS

Pseudomembranous candidiasis, colloquially known as thrush, most frequently affects infants, the elderly, and the terminally ill. It is uncommon

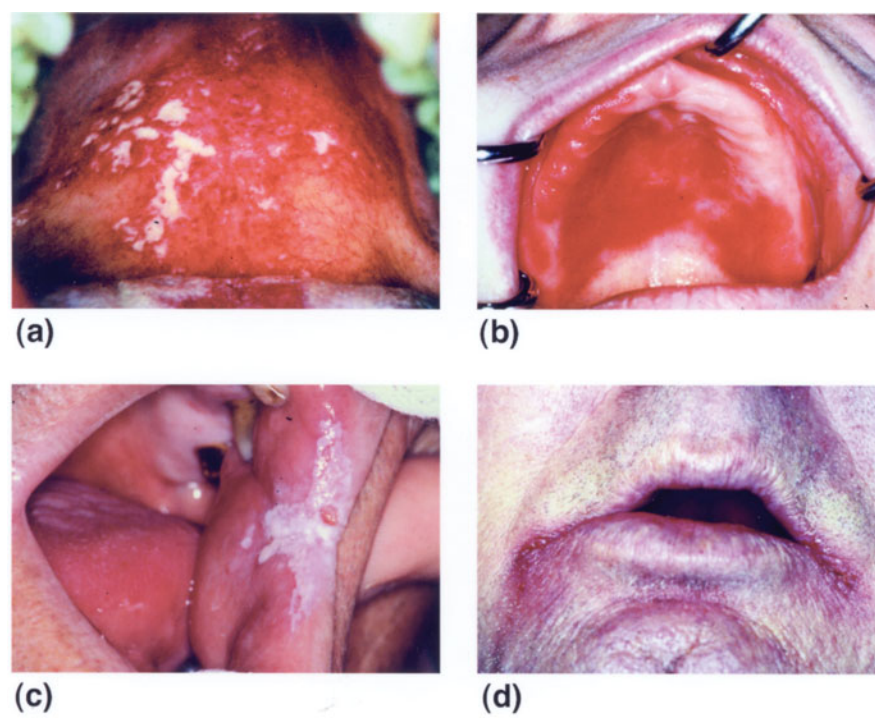
TABLE 3 Classification of *Candida* infections confined to oral and perioral tissues

***Candida* infection**	**Clinical presentation**
Acute pseudomembranous	Multiple removable white plaques
Acute erythematous	Generalized redness of tissue
Chronic plaque-like/nodular	Fixed white plaques on commissures
Chronic erythematous	Generalized redness of tissue on fitting surface of upper denture
Chronic pseudomembranous	Multiple removable white plaques
Candida-associated angular cheilitis	Bilateral cracks, angles of mouth
Median rhomboid glossitis	Fixed red/white lesion, dorsum of tongue

but may be an indicator of an underlying serious medical condition such as diabetes, leukemia, other malignancy, or HIV/AIDS. In addition, drug therapy with corticosteroid inhalers (as a preventive measure for asthmatics) may be associated with the development of pseudomembranous candidiasis.

The clinical appearance displays characteristic nonadherent creamy white plaques, patches, or flecks that are easily wiped off with a blunt instrument such as a wooden spatula or a mouth mirror (Fig. 3a). Scraping may produce bleeding and generally reveals an erythematous mucosa. The commonly affected sites are the soft palate, oropharynx, tongue, buccal mucosa, and gingiva (gum). Generally, pain is not a feature. When

FIGURE 3 Oral candidiasis. (a) Pseudomembranous candidiasis lesions on the palate; (b) chronic erythematous candidiasis on the palatal mucosa of an edentulous, full-denture-wearing patient; (c) plaquelike/nodular candidiasis at the commissure of upper and lower lips (mucosal surfaces); (d) angular cheilitis at the commissures of the mouth, involving skin. doi:10.1128/9781555818906.ch17.f3



observed under a microscope, the plaques consist of a mesh of *Candida* hyphae, entangled with desquamated epithelial cells, fibrin, keratin, necrotic debris, and bacteria (Fig. 2). The clinical diagnosis can be confirmed by smears (stained with periodic acid-Schiff [PAS] stain), oral rinse, or culture. Smears are useful for a quick confirmation of diagnosis if hyphae are observed. Cultures are useful in determining which species is involved and to which antifungal agents the strain is susceptible. Biopsy is not necessary. Treatment is discussed below.

ERYTHEMATOUS CANDIDIASIS

Erythematous candidiasis may be acute or chronic depending on duration. The acute form has also been called acute atrophic candidiasis or antibiotic sore mouth. As the latter term suggests, it is frequently associated with a preceding systemic course of broad-spectrum antibiotics or with topical antibiotic therapy. It may also be associated with the use of corticosteroid inhalers. This is the only form of candidiasis that is painful. The diagnosis can be confirmed by microscopic examination of smears or oral rinses, or by culture.

CHRONIC ERYTHEMATOUS CANDIDIASIS

Chronic erythematous candidiasis occurs on the palatal mucosa beneath full or partial maxillary dentures (Fig. 3b). There is a sharp demarcation between the affected and unaffected tissues. Occasionally, the edentulous mandible may be involved. Usually, patients are unaware of the condition. This form of candidiasis is more frequent among those who do not remove their dentures at night and wearers of old dentures. The diagnosis can be confirmed by smears obtained from both mucosal surfaces (palate and dorsum of tongue) and from the fitting surface of the denture. Oral rinse or culture may be employed, but as denture wearers have a higher *Candida* carriage rate than nondenture wearers, interpretation of positive results needs to be judged in relation to clinical findings and the site from which the sample was obtained. Hematological investigations to assess any underlying systemic predisposing factors, e.g., iron, vitamin B_{12}, or folate deficiency or undiagnosed or poorly controlled diabetes mellitus, are important. In addition to antifungal treatment (see below), patients should be instructed to remove their dentures at night and, after cleaning them, to soak them in either 2% chlorhexidine gluconate or 1% sodium hypochlorite overnight. If the dentures are old, unstable, or unretentive, the patient should be encouraged to have new dentures made.

PLAQUELIKE/NODULAR CANDIDIASIS

Plaquelike/nodular candidiasis (also called chronic hyperplastic candidiasis or candidal leukoplakia) is characterized by irregular white plaques that cannot be removed by scraping (Fig. 3c). Lesions are generally bilateral and occur on the buccal mucosa near the commissures at the level of the occlusal plane. Local factors such as tobacco smoking, denture wearing, and occlusal friction imply a multifactorial etiology. Often, biopsy is indicated to confirm the diagnosis because there may be worrying clinical signs (for example, induration, ulceration, etc.). The presence of *Candida* can be confirmed by microscopic examination of smears or by culture of swab or oral rinse samples. Hematological investigations

are also important to assess any underlying systemic predisposing factors as described above. The frequency of epithelial dysplasia in plaquelike/nodular candidiasis is four or five times higher than that estimated for other oral leukoplakias, and 9 to 40% of lesions develop into oral cancer compared with 2 to 6% for leukoplakias in general. Frequently, treatment with antifungal agents alone (see below) does not result in complete resolution. Addressing the other contributory factors may help. Clinical review is necessary, and complete removal by surgical means (scalpel or laser) should be considered.

ANGULAR CHEILITIS

Angular cheilitis presents as erythema and crusting and cracking in the commissural regions of the lips (Fig. 3d). This *Candida*-associated lesion frequently has a bacterial component. Associated factors include the deficiency states (iron, folate, or vitamin B_{12}), diabetes mellitus, HIV/AIDS, skin creasing due to age, poor dentures with reduced vertical dimension, and pooling of saliva in the affected areas. An intraoral *Candida* infection may also be present. Hematological investigations are appropriate, and the diagnosis may be confirmed by microscopic examination of lesional and intraoral smears or by culture of swabs or oral rinse. Treatment of both lesional tissue and the asymptomatic oral cavity is required.

MEDIAN RHOMBOID GLOSSITIS

Another *Candida*-associated lesion is median rhomboid glossitis, which often presents as a somewhat diamond-shaped lesion on the dorsum of the tongue near the junction of the anterior two-thirds and posterior one-third. An oral swab may confirm the mixed etiological microbiota. A biopsy is not necessary unless worrying clinical signs are present.

OTHER *CANDIDA* INFECTIONS

Other *Candida* infections occur rarely, usually in patients with underlying medical conditions (Table 4). These infections include cheilocandidiasis, mucocutaneous candidiasis, multifocal candidiasis, and *Candida* endocrinopathy syndrome.

TREATMENT

Treatment of people with oral *Candida* infections is usually topical (Table 5). Two useful polyenes, nystatin and amphotericin B, were discovered in the 1950s and are still of use today. Clinically, fungal polyene resistance is rare. Nystatin is highly toxic if it is administered parenterally and therefore is not suitable for systemic *Candida* infections. It is not absorbed from the GI tract, and therefore it is used for topical application intraorally. Unfortunately, it has an unpleasant taste, so preparations for oral use contain flavoring agents. Nystatin comes in a number of forms, including a cream, an ointment, tablets, a suspension, a gel, a pessary, and a pastille.

Amphotericin B also is not absorbed very well from the GI tract and is generally used topically; it comes in formulations similar to those of nystatin. Amphotericin B can be given intravenously for the treatment of systemic candidiasis. Both antifungals are fungicidal and have been used successfully in the treatment of the forms of oral candidiasis described

TABLE 4 Medical conditions in which oral, perioral, and generalized or systemic candidiasis occurs

Candida endocrinopathy syndrome
Diffuse chronic mucocutaneous candidiasis
Familial chronic mucocutaneous candidiasis
DiGeorge syndrome
AIDS

TABLE 5 Treatment of candidiasis in the immunocompetent[a] host

Candida infection	Topical treatment	Other considerations
Pseudomembranous	10-mg amphotericin B lozenges sucked 4 times daily (qid), 10–28 days	If topical steroid-related, rinse mouth after inhaling and/or use volumatic spacer
Erythematous	10-mg amphotericin B lozenges sucked qid, 10–28 days	If topical steroid-related, rinse mouth after inhaling and/or use volumatic spacer
Plaquelike/nodular	10-mg amphotericin B lozenges sucked qid, 10–28 days, + miconazole oral gel[b] applied to lesions	Biopsy may be indicated if clinical appearance is "worrying"
Denture-associated *Candida* lesions	Miconazole oral gel[b] applied to fitting surface of denture + 10-mg amphotericin B lozenges sucked qid, 10–28 days	Remove denture(s) and soak overnight in 2% chlorhexidine gluconate or 1% hypochlorite
Angular cheilitis	Miconazole oral gel[b] applied to lesions + 10-mg amphotericin B lozenges sucked qid, 10–28 days	Remove denture(s) if present and soak overnight in 2% chlorhexidine gluconate or 1% hypochlorite
Median rhomboid glossitis	10-mg amphotericin B lozenges sucked qid, 10–28 days	Biopsy may be indicated if clinical appearance is "worrying"

[a]Systemic therapy with fluconazole or itraconazole is indicated for patients with immunosuppression.

[b]Miconazole oral gel potentiates the anticoagulant effect of warfarin with potentially fatal consequences and therefore must never be prescribed for patients taking warfarin.

above. They can be used together, for example, nystatin ointment applied to the fitting surface of the denture and amphotericin B lozenges in the treatment of denture-associated chronic erythematous candidiasis, or nystatin ointment applied to the affected commissures and amphotericin B lozenges in the treatment of angular cheilitis. Not all forms of these agents are available in all countries. The use of antiseptic mouth rinses (e.g., chlorhexidine gluconate) for oral and denture hygiene should be considered, and for patients with xerostomia (dry mouth), sialagogues (e.g., pilocarpine) to stimulate salivary flow may be useful adjuncts.

More recently, imidazoles and triazoles have been used to treat oral candidiasis. They include miconazole, ketoconazole, clotrimazole, fluconazole, and itraconazole. Miconazole is not absorbed from the GI tract and is mainly used topically. It is reported to have a bacteriostatic effect in addition to being fungicidal and therefore is useful in the treatment of angular cheilitis. An important adverse reaction occurs if miconazole is absorbed topically in sufficient amounts in patients taking warfarin, an anticoagulant drug widely used to "thin the blood." Miconazole potentiates this effect, and the resultant internal hemorrhage is potentially fatal. Topical preparations of clotrimazole (oral troches) and itraconazole (solution) can also be used for oral candidiasis.

Ketoconazole was the first of the imidazoles developed that was systemically absorbed after oral administration. It is useful in the treatment of chronic mucocutaneous candidiasis and oral candidiasis in immunocompromised patients. It can have side effects such as nausea, cutaneous rash, pruritus, and hepatotoxicity. As alteration of liver function can occur, monitoring of liver enzymes is essential. The triazoles fluconazole and itraconazole, like the imidazoles, inhibit fungal biosynthesis of ergosterol, which is required for plasma membrane function. Fluconazole has been shown in a number of studies to be effective in treating HIV/AIDS-related oral candidiasis such as oropharyngeal candidiasis. Fluconazole-resistant strains of *Candida* have emerged, however, following the prophylactic use of fluconazole in HIV/AIDS patients.

Extended-spectrum triazoles also inhibit the cytochrome P450-dependent conversion of lanosterol to ergosterol. The resultant accumulation of toxic 14-methylsterols and depletion of membrane-associated ergosterol cause inhibition of cell growth. Antifungals in this class include voriconazole and posaconazole. Voriconazole is available in both intravenous and oral formulations, whereas posaconazole is available in oral formulation only.

Clinical trials have shown the echinocandin caspofungin to be as effective as amphotericin B for the treatment of patients with invasive candidiasis or candidemia, with significantly fewer adverse effects. Caspofungin, micafungin, and anidulafungin are available in intravenous formulations only.

Aspergillosis

Oral aspergillosis is a rare infection consisting of three forms. It may be saprophytic, in which there is fungal growth without invasion into viable tissue. It may be allergic—that is, a hypersensitivity reaction to fungal hyphae develops—or it may be invasive, in which case viable tissue is invaded by the fungus, resulting in severe necrosis. The saprophytic and allergic types have relatively low morbidity and mortality and affect the immunocompetent host. The invasive form occurs in immunocompromised individuals and has significant morbidity and mortality. Oral sites of involvement include soft palate, tongue, and gingiva. Lesions on the soft palate have generally been associated with upper respiratory tract involvement. The gingival lesions are seen in patients with hematological malignancy. Palatal lesions consist of oral ulceration surrounded by a margin of black tissue. The gingival lesions are painful, violaceous, and ultimately ulcerated with tissue necrosis.

Diagnosis can be made from cultures; however, this can take about a week. As the lesions are often clinically worrying in their appearance, biopsy is frequently performed and a pathology report is issued approximately a day later, i.e., before the results of culture are known. Once the diagnosis is established, aggressive systemic antifungal treatment (e.g., intravenous amphotericin B) can be commenced.

Cryptococcosis

Cryptococcosis involves the oral cavity on rare occasions. The disease is caused by *C. neoformans*, and immunocompromised patients are at risk of disseminated disease. Oral lesions may present as ulceration or as nodules. Sites of involvement include the tongue, palate, gingiva, and tooth socket following extraction. The differential diagnosis includes squamous cell carcinoma, tuberculosis, and traumatic ulcer. Diagnosis is confirmed by biopsy. Systemic treatment with amphotericin B, fluconazole, or itraconazole is indicated.

Histoplasmosis

Histoplasmosis is endemic in the United States in the Mississippi and Ohio River valleys, where 70 to 80% of adults have been infected (usually subclinically). Three forms of histoplasmosis are recognized: acute, chronic pulmonary, and progressive disseminated. Oral histoplasmosis may occur as either pulmonary or disseminated histoplasmosis or as a primary lesion in an otherwise healthy person. Oral histoplasmosis may be seen among

patients with HIV/AIDS and may rarely be the initial manifestation of the disease. Oral lesions may present as single or multiple indurated ulcers or as nodular lesions. The palate, tongue, buccal mucosa, gingiva, and lips are the usual sites of involvement. Diagnosis can be established from histopathological examination of a biopsy specimen, culture, direct examination of smears, or complement fixation tests. Treatment with amphotericin B, ketoconazole, or itraconazole is effective.

Blastomycosis

Blastomycosis is a chronic fungal infection caused by *B. dermatitidis*. This condition is found mainly in North America and occasionally in Africa. Pulmonary involvement is common, and the patient may present with symptoms similar to those of tuberculosis, that is, low-grade fever, weight loss, cough, and purulent sputum. The most common extrapulmonary affected organ is the skin. Oral lesions may occur and are generally due to dissemination from pulmonary disease. Proliferative or ulcerated lesions may occur on the hard palate, gingiva, tongue, or lips. Bone involvement (maxilla or mandible) may occur. Males are more commonly affected than females. Diagnosis is based on culture, although organisms may be seen histologically on tissue obtained from a biopsy. Amphotericin B is the mainstay of treatment. Surgical removal and debridement may be required.

Paracoccidioidomycosis

Paracoccidioidomycosis (South American blastomycosis) is a chronic disease caused by *P. brasiliensis*. The disease is endemic in Brazil but also occurs in Argentina, Venezuela, Bolivia, Peru, Uruguay, Mexico, and Costa Rica. Adult males from rural farming areas are most commonly affected, with lesions involving the mouth and pharynx. Oral lesions, which may be granular and present with ulceration, can result from either primary or secondary infection. Sites of involvement include the gingiva, palate, lips, and buccal mucosa. Gingival involvement may lead to tooth mobility. Palatal involvement may lead to perforation. Cervical lymphadenopathy can be the first sign of disease noted by the patient. Other forms of the disease include the pulmonary form, mucocutaneous-lymphangitic form, and disseminated disease. Often lesions are biopsied because of the worrying clinical appearance. Biopsy specimens show granulomas, multinucleated giant cells, and a mixed inflammatory cell infiltrate. Microabscesses and giant cells may contain fungal hyphae. Fungi can be more readily visualized on PAS-stained tissue sections. Smears can be taken and stained with either PAS or potassium hydroxide (KOH). Treatment is with either oral ketoconazole or intravenous amphotericin B.

Mucormycosis

Mucormycosis is rare but of significance, as it is frequently associated with a fatal outcome. It is an acute opportunistic infection usually involving debilitated individuals. The disease is caused by fungi of the family Mucoraceae, mainly *Rhizopus* and *Mucor*, and rarely other species. Predisposing factors leading to debilitation include poorly controlled diabetes mellitus with ketoacidosis, hematological malignancies, burns, malnutrition, uremia, liver cirrhosis, HIV/AIDS, organ transplantation, oncological chemotherapy, and other causes of immunosuppression. The recognized forms of the disease are rhinocerebral, pulmonary, gastrointestinal, and

disseminated. The rhinocerebral form is characterized by low-grade fever, general malaise, headache, sinus-associated pain, bloody nasal discharge, periorbital nasal or ocular swelling and edema, ptosis, and muscle paresis. Tissue necrosis may cause palatal ulceration and perforation. The differential diagnosis for rhinocerebral mucormycosis includes tertiary syphilis, tuberculosis, or malignant neoplasm (squamous cell carcinoma of the maxillary antrum or palatal mucosa). Biopsy and smears are appropriate laboratory tests, and computed tomography is useful in delineating the extent of the lesion. Treatment includes surgical debridement, systemic antifungal therapy, and management of predisposing factors.

KEY POINTS

Biology of fungi

Fungi are eukaryotic; they are structurally and metabolically distinct from bacteria. They are similar to mammalian cells but usually possess a cell wall.

Morphology. Fungi generally grow as yeast or hyphae. Those that can grow in both morphologies are termed dimorphic. *C. albicans* usually grows as yeast or hyphae but can grow as pseudohyphae and form chlamydospores.

Replication. Fungi can replicate sexually or asexually. Some fungi produce spores or conidia.

Pathogenesis of fungal infections

Acquisition of fungi from the environment. Fungi are prevalent in the environment, and several can colonize the human oral cavity. The fungi most commonly isolated from the oral cavity are *Candida* species.

Fungal virulence. Fungal factors associated with host damage include adherence, hydrolytic enzymes such as proteinases and phospholipases, evasion of the immune system, and melanin production.

Host defenses against fungal infection

Nonspecific defense mechanisms. The physical barrier or mucosae, salivary flow, salivary components, microbial competition, and phagocytes provide defenses against fungal colonization and invasion.

Specific defense mechanisms. Secretory IgA can prevent adhesion of fungi to oral surfaces. The cell-mediated immune response is important in preventing oral fungal infections.

Antifungal therapy

Growth and identification of fungi. Fungi can be identified by cellular and colony morphology, growth requirements, and specific DNA-based detection methods.

Principles of antifungal chemotherapy. Fungi present fewer specific drug targets than do bacteria. Currently, DNA synthesis is targeted by 5FC, plasma membrane integrity is targeted by polyenes, sterol biosynthesis is targeted by azoles, and glucan synthesis is targeted by candins. 5FC and polyenes are fungicidal, and azoles are fungistatic. There is a clinical problem of azole resistance in oropharyngeal candidiasis.

Clinical conditions

Candidiasis. Oral candidiasis is the most common oral fungal infection. Presentations of oral candidiasis include pseudomembranous, erythematous, and plaquelike/nodular candidiasis, angular cheilitis, and median rhomboid glossitis. Treatment involves amphotericin B lozenges and miconazole oral gel.

Aspergillosis. Oral aspergillosis may be saprophytic, allergic, or invasive and can affect the soft palate, tongue, and gingiva. Treatment is intravenous amphotericin B.

Cryptococcosis. Cryptococcosis rarely involves the oral cavity. Lesions present as ulcerations or nodules on the tongue, palate, gingiva, or tooth socket. Systemic treatment with amphotericin B or itraconazole is indicated.

Histoplasmosis. Histoplasmosis is endemic in the United States and presents as acute, chronic pulmonary, or progressive disseminated disease. Treatment with amphotericin B, ketoconazole, or itraconazole is effective.

Blastomycosis. Blastomycosis occurs mainly in North America and occasionally in Africa. Proliferative or ulcerated oral lesions may occur on the hard palate, gingiva, tongue, or lips. Amphotericin B is the mainstay of treatment. Surgical debridement may be considered.

Paracoccidioidomycosis. Paracoccidioidomycosis is endemic in Central and South America. Oral lesions, which may be granular and present with ulceration, can result from either primary or secondary infection. Treatment is with either amphotericin B or an azole such as ketoconazole.

Mucormycosis. Mucormycosis is rare but of significance because it is frequently associated with a fatal outcome. The recognized forms of the disease are rhinocerebral, pulmonary, gastrointestinal, and disseminated. Treatment includes surgical debridement, systemic antifungal therapy, and management of predisposing factors.

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Endodontic Microbiology

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INTRODUCTION

Endodontics relates to the study and treatment of diseases of the dental pulp tissue, located within the coronal pulp chamber and extending into the root canal. Dental pulp largely consists of undifferentiated connective tissue together with various associated immune cells. It is highly vascularized and nerve rich. Blood vessels and nerves enter through one or more apical foramina at the apex of the tooth. Under normal healthy conditions, intact enamel and dentin protect the pulp, acting as a physical barrier to injury and microbial intrusion and, as in other protected connective tissues, the enclosed vital dental pulp is sterile. Infections of the pulp are almost always secondary to breaches in this barrier resulting from caries, iatrogenic causes, or in some rare cases, traumatic occlusion. Endodontic infections contrast markedly with dental caries and periodontal diseases, which are directly associated with dental biofilms and may develop independently of any trauma. Bacteria associated with endodontic disease are usually part of the normal oral microbiota. However, when they gain access to the normally sterile pulp tissue, these bacteria can cause pathologic inflammatory responses. For this reason they are called "opportunistic pathogens." In this sense, most pulpal and periradicular (i.e., surrounding the root) inflammatory diseases are the result of opportunistic infections with endogenous oral bacteria.

HISTORY

The association of microbes and root canal infections dates back several centuries. In the 17th century, Antonie van Leeuwenhoek, using a handcrafted microscope, observed and described microorganisms, which he called "animalcules," in tissue material removed from the root canals of decayed teeth. However, little progress was made until 1894, when W. D. Miller, an American dentist working in Robert Koch's laboratory in Berlin, Germany, was able to culture bacteria from endodontic infections and describe their association with apical periodontitis. Although forms of root canal therapy have been found in early Egyptian skulls, it was not until the 18th century that preservation of infected and decaying teeth by treatment of the pulp was first documented by Pierre Fauchard. The

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technique involved cauterizing the exposed dental pulp and then filling the root canals with lead. More commonly, however, members of both the dental and medical professions believed that a tooth with an infected root canal was a "dead tooth" constituting a "focus of infection." This "focal infection theory" held that local infections, such as pyorrhea (periodontal disease) and dental abscesses, were responsible for many chronic diseases including arthritis, heart disease, and others for which there was no satisfactory etiological explanation at the time. William Hunter, a renowned London physician, was a prominent advocate of this idea. In 1910, he lectured at the McGill University Medical School on the role of sepsis and antisepsis in medicine. He claimed that many chronic diseases were cured following removal of this "focus of infection" by extraction of infected teeth, with no attempt to save the condemned teeth through root canal therapy.

The focal infection theory led to a stage in the history of dentistry in which the recommended treatment for endodontic and periodontal disease was invariably tooth extraction. This epidemic of mass extractions to cure many systemic diseases continued throughout the first half of the 20th century and well into the 1950s. Practitioners who advocated this treatment became known as "hundred percenters" because they would often extract all the teeth, regardless of the extent of disease.

Fortunately for the patients, by the 1930s, investigators had already begun to question the anecdotal evidence that formed the basis of the focal infection theory and initiated the modern phase of endodontic therapy. Research provided evidence that inflammatory lesions associated with teeth were manifestations of localized bacterial infections (see Chapter 19, however, for a discussion of current concepts of systemic manifestations of oral infections). On the basis of these studies, which indicated that endodontic infections were preventable, dentists began to apply the aseptic techniques used in surgery to reduce bacterial contamination of the operative field. In the context of endodontics, this meant isolating the tooth being treated with a rubber dam and disinfecting the area to be treated with antimicrobial agents. A corollary of this procedure involved culturing infected root canals for bacteria in much the same way physicians took cultures to identify bacteria associated with infectious diseases. Dr. Louis Grossman, considered by many to be the father of modern endodontics, demonstrated that root canals could be debrided to the point where culture samples of the debrided root canal could no longer detect viable bacteria. In 1965, experiments by Kakehashi et al. showed that pulpal exposure in germ-free rats did not lead to inflammation (Fig. 1). Conversely, similar pulp exposure in the presence of normal oral microbiota in conventional rats invariably led to pulpal inflammation and pulpal necrosis (Fig. 2). Taken together, these findings established the bacteriological etiology of endodontic inflammation. In germ-free rats, healing of the exposure occurred with the deposition of secondary dentin. Similar observations have been made in humans when pulp exposed to the contaminated oral environment rapidly resulted in inflammation of the pulp or periapical tissues. The association between localized bacterial infection and endodontic disease was therefore confirmed.

Today, although there are no comprehensive studies showing a direct cause-and-effect relationship between endodontic disease and systemic

FIGURE 1 Dental pulp 7 days after pulpal exposure in germ-free rats. The dental pulp remains viable even though food impaction is evident in the exposure site. Reprinted from S. Kakehashi, S. H. Stanley, and R. Fitzgerald, *Oral Surg. Oral Med. Oral Pathol.* **20:**342–349, 1965, with permission. doi:10.1128/9781555818906.ch18.f1

FIGURE 2 Pulp exposure in a conventional rat after 14 days, showing inflammation of pulp. Reprinted from S. Kakehashi, S. H. Stanley, and R. Fitzgerald, *Oral Surg. Oral Med. Oral Pathol.* **20:**342–349, 1965, with permission. doi:10.1128/9781555818906.ch18.f2

disease, endodontic disease is recognized as being a contributory factor to the total infectious burden of the host. It is reasonable to assume that the more inflammation and infections that an individual has, the higher the potential for systemic effects. Patients, especially those already medically compromised, are at risk for complications from oral facial infections, including infections that spread directly to other areas of the head and neck or to other parts of the body through bacteremia. Although rare, such complications can be life threatening. The reverse may also be true, since endodontic disease can be affected by systemic conditions and environmental factors. These include conditions such as diabetes, viral diseases, genetic diseases, gene polymorphisms, and acquired habits such as smoking.

SOURCES OF INFECTION

Initial infection of the pulp generally occurs when the protective enamel and dentin layers are compromised. This may be a result of extension of carious or periodontal lesions, damage resulting from dental treatments, daily wear and tear resulting from the mechanical processes of mastication, or systemically from another source within the oral cavity.

Invasion of the pulp as a result of direct extension of dental caries is the most common source of endodontic infection (Fig. 3). Results of studies indicating the presence of cariogenic bacteria, e.g., streptococci and lactobacilli, in the dentinal tubules of advancing carious lesions suggest that these may be the first organisms present in the pulp after a carious exposure. Initial invasion of the dentinal tubules produces dissolution of some of the minerals of the dentin, allowing colonization of the organic matrix by anaerobic organisms whose metabolism is generally based on proteolytic activity.

Cracks in the enamel or loss of the cementum that protects the underlying dentin and other mechanical exposures are frequent routes of pulpal infection. The inflammation that occurs during restorative procedures is associated with the exposure of pulpal connective tissue to microorganisms. In addition to this direct contact with bacteria, the mechanical pressures exerted during tooth preparation may force bacteria, or their by-products, into the tubules or the pulp in the absence of frank exposure. This phenomenon could account for the painful flare-ups (pulpal inflammation) that can occur after initial tooth preparation and restorative procedures have been completed. Independently of dental treatment, daily occlusal trauma also can lead to pulpal inflammation and the subsequent infection of the pulp by oral bacteria.

In theory, some pulpal and periradicular infections may occur through the process of anachoresis. Anachoresis is the seeding of microbes to areas of inflammation during bacteremia. It is well established that bacteremia can occur as a result of surgical and nonsurgical trauma in the oral cavity. Even tooth brushing, flossing, and mastication in patients with periodontal disease can result in bacteremia. These bacteria may circulate briefly in the bloodstream and relocate to remote tissues. Although there is some experimental evidence with animal models to support this concept, it has not been demonstrated in humans.

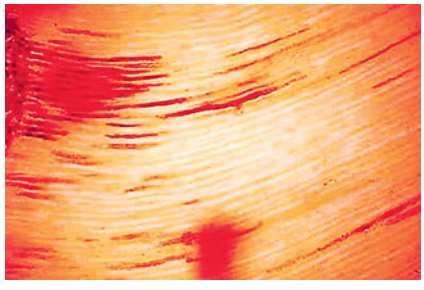

FIGURE 3 Section through a tooth with dental caries (left), showing penetration of bacteria into the dentinal tubules. Courtesy of Henry Trowbridge. doi:10.1128/9781555818906.ch18.f3

It is possible for the microorganisms in dental plaque associated with periodontal disease to infect the root canal system through the apical foramen or accessory canals along the lateral surfaces of the tooth. Another source of infection of root canal systems is the forcing of bacteria into the apical foramina of neighboring teeth during surgical procedures. In addition, once the dental pulp is necrotic, some investigators believe that bacteria may be transported from the gingival sulcus through exposed dentinal tubules (dead tracts) or lateral/furcation canals to the pulp chamber. However, this idea is questioned by other scientists, who have shown that the dentinal tubules in such cases are sclerotic and bacteria therefore cannot be found within them.

MICROBIOLOGY OF ROOT CANAL INFECTIONS

Today we know that in addition to bacteria, endodontic infections may be caused by fungi, *Archaea* (primitive bacteria resistant to high temperature and other extreme environmental conditions), viruses, and prions. Prior to 1970, commonly used microbiological techniques were unable to detect many of the species now known to be associated with endodontic infection. Most laboratory media and incubators did not support the growth of strictly anaerobic or very fastidious bacteria. Thus, cultured microorganisms included only those able to survive in limited quantities of oxygen. This accounts for the so-called "negative cultures" that were used to assess the success of debridement during treatment during this time. Obviously, such cultures could detect only a small portion of the microbiota potentially still present in the root canal. Another source of sampling error involved the difficulty in obtaining a representative sample that was not contaminated with normal oral microbiota. In addition, microbial samples were often incubated in a broth medium before being streaked onto solid medium to obtain individual isolates. This procedure leads to selection for rapidly multiplying bacteria rather than the full spectrum of those that may have been present originally. Thus, it is not surprising that the most frequently cultured species were the oral streptococci among the gram-positive bacteria and only the hardiest of the gram-negative bacteria. Spirochetes were often observed in bacterial smears, but at the time, these strictly anaerobic organisms could not be grown in the laboratory. It is believed that even now a high percentage of the normal oral microbiota is as yet uncultivable. The development of more sophisticated serologic and molecular biologic methods for identifying bacteria, even those that are uncultivable, has led to a number of recent studies to analyze and semiquantify the prevalence of various species in different stages of endodontic infection. The disadvantage of these studies is that they cannot distinguish between viable and dead bacteria.

Depending on the species, culturing and immunological methods require 10^2 to 10^5 cells for detection; DNA-DNA hybridization assays require 10^3 to 10^4 cells. More-recent studies have utilized molecular biological methods. The PCR assay and various modifications thereof can detect as few as 10 bacterial cells of a target species at the DNA level. Checkerboard DNA analysis and 16S ribosomal cloning have been used extensively to analyze the microbiota found in endodontic infections.

For ease of diagnosis and treatment, it would be ideal to establish a cause-and-effect relationship between one or several particular species and an associated pathology. Although a number of studies seeking to find relationships between specific species and/or complexes for defined clinical conditions (pulpal and periradicular infections) have been published, no clear associations have emerged. These investigations have demonstrated that polymicrobial endodontic infections have extensive individual variability. Pulpal infections usually feature complexes of anaerobic bacteria, including *Fusobacterium*, *Prevotella*, *Parvimonas* (formerly *Peptostreptococcus*), and some newly discovered genera such as *Olsenella* and *Dialister*. Figure 4 shows a chart compiled by Siqueira and Rocas showing the various phyla and species associated with infected pulp in the relative proportions detected in numerous studies. Many of these were first assigned through conventional microbial culturing techniques that may not be generally appropriate for cultivating more difficult bacteria. Newly recognized genera thought to be relevant to colonization of root canals and identified by molecular methods include *Catonella*, *Granulicatella*, *Abiotrophia*, *Gemella*, and *Eubacterium*. *Porphyromonas endodontalis* is more frequently detected than *Porphyromonas gingivalis* in pulpal infections. Most of these organisms seem to be found in asymptomatic lesions as well as in symptomatic lesions, arguing against there being specific groups of organisms associated with different clinical symptoms. However, symptomatic lesions appear to harbor less microbial

FIGURE 4 Chart showing the prevalent phyla, genera, and species found in root canal infections. Reprinted from J. Siqueira and I. Rocas, Microbiology and treatment of endodontic infections, chapt. 15, p. 572. *In* K. Hargreaves and S. Cohen (ed.), *Pathways of the Pulp*, 10th ed., 2010. Mosby, St. Louis, MO. (Used with permission.)
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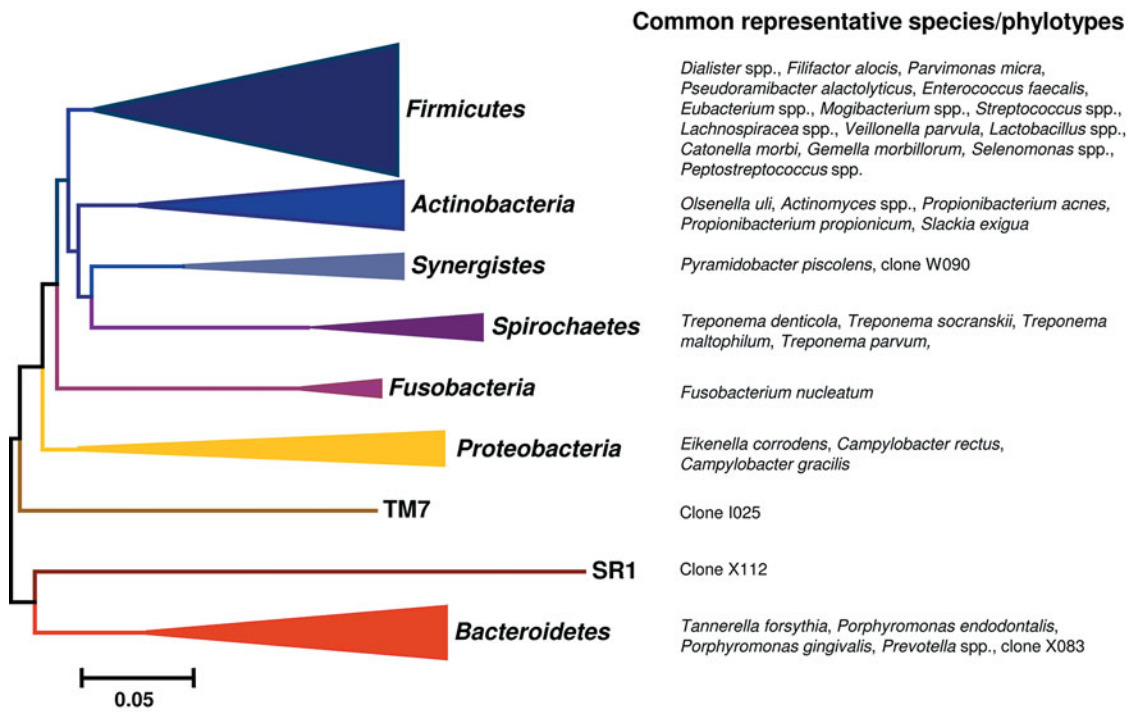

diversity, perhaps reflecting environmental selection. Such lesions typically show higher levels of *Fusobacterium*, *Dialister*, *Prevotella*, and *Eubacterium*, suggesting that a microbiota made up predominantly of mixed gram-negative organisms is the main target for anti-infection strategies. As stated previously, the takeaway lesson is that endodontic infections and their sequelae are usually associated with a complex microbiota. Although endodontic infections are dominated by bacteria, fungi, especially *Candida albicans*, have often been found, and filamentous fungi, such as *Aspergillus*, have also been detected.

Pathogenesis of Endodontic Infections

Once the pulp becomes necrotic, the root canal system is a sanctuary for microorganisms. Because the necrotic pulp is without vascular circulation, the host's immune system is unable to arrest the infection. Bacteria may be present as planktonic (free-living) cells, as bacterial aggregates, and as biofilms. Aggregates of bacteria and biofilms have been shown to provide the greatest resistance to disinfecting and antimicrobial agents. So-called "sulfur granules" are often found in surgical biopsy specimens of apical lesions. However, this is a misnomer: these granules actually consist of large clumps of several species of bacteria, often including *Actinomyces*. Biofilms in the root canal are similar to those found in dental plaque, except that these bacterial communities attach to the dentin surface of a root canal. As indicated in Fig. 4, these pulp biofilms are similar in microbial composition to the microbiota found in plaque biofilms. Common features of biofilms provide resistance to antimicrobial agents, such as embedding of the bacteria in an extracellular polymeric substance that provides resistance to penetration by the antimicrobial agent. The phenomenon of quorum sensing may allow the growth rate of the microbes to increase or decrease depending on nutrient availability. A biofilm may also adopt a resistant phenotype that can become the dominant phenotype able to persist for extended periods without nutrients.

The virulence properties of many of these biofilm microorganisms are similar to those found in other opportunistic infections. Bacteria exert their effects in both the pulpal and the periradicular tissues of the tooth by virtue of virulence factors such as endotoxins and other cellular components. They produce spreading factors such as collagenase, hyaluronidase, and other enzymes that not only break down connective tissues but often are cytotoxic. Exposure of pulp to microorganisms and their products results in an inflammatory response, as pulp cells respond through innate immune activation such as production of chemokines, which attract neutrophils and mononuclear cells. There are high levels of interleukin-8 (CXCL8) in infected pulp. Lipopolysaccharide (endotoxin) stimulates pulpal fibroblasts to produce interleukin-8, and this is modulated by neuropeptides, e.g., substance P. Left untreated, pulpal inflammation will progress to the apical regions and ultimately lead to periapical alveolar bone destruction. As indicated above, endodontic infection is usually initiated in the pulp chamber, from where it spreads to the root canal system. The end stage of the inflammatory process is characterized by pulp necrosis. The necrotic tissue in the canals remains infected and itself becomes the reservoir for the infectious agents. In the absence of treatment, consisting principally of chemomechanical debridement (i.e.,

cleaning the canal with instruments and appropriate irrigating solutions), the bacteria and the accompanying inflammation will spread to the periradicular tissues, resulting in either acute or chronic inflammation. An acute apical abscess and cellulitis are often accompanied by excruciating pain linked to the confined area in which the inflammatory mediators and exudate affect the sensitive nerve endings. Most often, the exudates are suppurative (purulent), i.e., containing pus, consisting of breakdown products of host cells, connective tissue elements, blood elements, microorganisms, and microbial by-products. Pain relief can be achieved by providing drainage through the pulp chamber or through incision for drainage from the apical area. If a chronic apical abscess develops, a sinus tract (fistula) that provides a pathway of drainage from the area of inflammation to an epithelial surface may develop. Occasionally, these are seen in cases where treatment has failed and abscesses develop at the root apex.

Control of infection and repair depend upon migration of mononuclear cells to the periapical site. However, dentin constituents can provoke uncontrolled activation of neutrophils, leading to further tissue damage and the formation of a cyst or granuloma. Hence, higher levels of chemokines in cysts may be an indicator for progression of the lesions.

Over the years, many investigators have reported that cultures from failed cases yield a facultative gram-positive coccus, *Enterococcus faecalis*. This organism often represents over 75% of the cultured microbiota in these cases. This organism, as its name implies, is an enteric commensal that is often found in food products and other sources. It can gain access to the pulp chamber during therapy, between visits when a temporary filling has been placed, or through infiltration into the permanent restoration. This organism is resistant to high temperature, high pH (up to pH 11), and a number of other hostile environmental conditions, and it often persists in the root canal. Recent studies have shown that many other significant species of bacteria are present in the canals in failed cases, which are not as easy to cultivate as *E. faecalis*.

HIV has been detected in the vital pulp of patients with HIV infection. Herpesviruses have also been found in endodontic infections and in abscesses and cellulitis. Herpesvirus-infected cells can release proinflammatory cytokines. The Epstein-Barr virus has been associated with irreversible pulpitis and periradicular inflammation. Both Epstein-Barr virus and human cytomegalovirus have been found in patients with symptomatic apical periodontitis and apical lesions larger than 5 mm in diameter. What role viruses play in endodontic infections is not known, but it has been speculated that viruses may compromise the host immune response. The presence of viruses in clinical samples does not necessarily mean that they are of etiologic significance in the pathogenesis of endodontic infections.

ENDODONTIC TREATMENT

In the treatment of vital teeth, endodontists have employed what was called “one-visit” endodontics. In those cases, since there was no active infection beyond the contamination associated with exposure of the pulp to the oral cavity, clinicians often extirpated the pulp, enlarged the canal,

and sealed (obturated) it in the same visit. Note that, except for location, this approach is identical to the treatment of wounds. In the case of fresh wounds, they are cleansed, the tissue tabs are removed, and if amenable to closure, the wounds are sutured (sealed) at the same time. One-visit endodontics, even in cases of necrotic pulps or chronic pulpitis, evolved as an extension of the one-visit concept for vital teeth.

The key issue in such approaches is to practice thorough chemomechanical debridement of the root canal system. If access to the root canal system and complete debridement can be accomplished, then the root canals may be obturated. However, many additional factors determine whether the canals can be sealed in a single visit. These constraints may include tooth anatomy, presence of calcifications in the canal, access to the tooth and its canals, patient management, drainage from the canals, and degree of discomfort. Under these circumstances, an antimicrobial agent is placed in the canals and the patient is scheduled for further treatment. Endodontic treatment has a very high success rate. Cases that do not heal may be managed by retreatment or by surgery to give a success rate of over 95%.

MICROBIOLOGICAL CONSIDERATIONS FOR OBTURATION

Few treatments are more dramatic than the pain relief that is achieved when a patient with a dental-alveolar abscess has the tooth opened for drainage. Through the 1970s, opening the tooth, prescribing antibiotics, and leaving it open for several days before continuing debridement was standard therapy. The major clinical concern was limiting further bacterial contamination that would occur if the canal were left open for drainage. Considering that saliva contains more than 10^8 bacteria per milliliter and that chewing could force bacteria and food into the open canal system, this was believed to be a potentially serious problem. Hence, if the tooth is left open for drainage, the patient should be scheduled to have the tooth closed within the next 24 to 48 hours to prevent gross contamination with opportunistic microbiota. Today, teeth are rarely left open because the faster the canal is completely debrided and sealed, the faster the infection will be controlled. Drainage usually stops during the debridement phase. Nonsteroidal anti-inflammatory drugs are prescribed for patients for pain relief unless there is a clinical contraindication. For cases with systemic signs and symptoms of infection or extensive swelling and cellulitis, prescriptions of adjunctive antibiotics are recommended. It should be kept in mind that just as in the treatment of abscesses in other areas of the body, including the gastrointestinal tract, the antibiotic is considered an adjunct to the surgical drainage that is deemed essential for successful treatment. Effective chemomechanical debridement of the root canal system and incision for drainage of periradicular swelling or cellulitis remove the reservoir of infection and allow the patient's immune system to begin healing. Endodontic success can only be measured after a sufficient time to allow apical healing. In some patients, this can be measured in months; for others it may take much longer. Thus, determination of when and how much drainage is necessary for a specific case depends almost totally on clinical acumen developed in practice and truly reflects part of the art of the practice of dentistry.

ANTIMICROBIAL AGENTS USED IN ENDODONTICS

Sodium hypochlorite (NaOCl or NaClO) has remained the most popular agent to irrigate the canal system during chemomechanical debridement. NaOCl is an excellent antimicrobial agent and dissolves necrotic tissue. EDTA is often used to demineralize the smear layer produced by the instruments. Chlorhexidine (CHX) is another agent often used to irrigate the canal system. It binds with dentin for prolonged antimicrobial efficacy, a property known as "substantivity." Although EDTA and CHX are good agents for debridement and disinfection, they do not dissolve necrotic tissue like NaOCl. Numerous other agents are being evaluated for efficacy along with improved delivery systems. In the past, positive-pressure needle irrigation with the solutions has been the standard. Recent studies have evaluated the use of negative pressure and sonic and ultrasonic procedures to improve the delivery of root canal irrigants. Ideally, the irrigants are delivered to the root end without being forced into the periradicular tissues. In addition, the use of photodynamic energy including lasers and UV light has been shown to increase the clinician's ability to disinfect the root canal system.

Calcium hydroxide has remained the antimicrobial agent of choice as a temporary root canal-filling material between appointments. It is usually prepared as a water paste. Since calcium hydroxide has a pH of about 12.5 but has low solubility, the concentrations of hydroxyl ions are low. Because of its low solubility, it does not penetrate far into dentinal tubules or anastomoses. To increase antimicrobial efficacy, some investigators have mixed calcium hydroxide with CHX or camphorated paramonochlorophenol. Calcium hydroxide appears to be well tolerated by host tissues and has been used for years for pulp capping, in which it stimulates the formation of reparative dentin over an exposed pulp. Calcium hydroxide also inactivates bacterial endotoxins. Endotoxins are found within infected canals and cause inflammatory reactions and bone resorption in the periradicular tissues.

Research for an improved intracanal medication includes the use of nanoparticles. Nanoparticles of chitosin, zinc oxide, and polylactic coglycolic acid nanoparticles encapsulated with photoactive drugs are under investigation.

SUMMARY

It is clear that microbial infection is the cause of endodontic pathogenesis. It is equally clear that as in the other major infections of the oral cavity, i.e., dental caries and periodontal diseases, the bacterial microbiota involved is exceedingly complex. Indeed, the spectrum of microbial species found in root canals and apical infections is similar to that associated with the periodontal diseases and includes not only bacteria but also fungi, *Archaea*, and viruses. The question remains whether there are specific microbes, mixtures of microbes, or even specific virulence factors that make the root canal system or periradicular tissues more likely to experience severe symptoms. It is evident that just as successful treatment of wound infection requires careful debridement before closure, so too success in endodontics requires meticulous attention to chemomechanical debridement of the root canal system before sealing.

KEY POINTS

Root canal infections are usually caused by colonization and invasion of endodontic tissues by opportunistic normal oral flora. Endodontic infections are polymicrobial and dominated by several species of bacteria but may contain fungi, *Archaea*, viruses, and possibly prions. Molecular methods, especially PCR, have demonstrated that each patient's endodontic infection has a complex community of microbes different from those microbes in infections of other individuals.

The infected root canal system with necrotic tissue is a reservoir of infection that the host immune system is not able to resolve because of lack of vascular circulation.

Treatment of endodontic infections is directed at debriding the canal thoroughly before sealing it. This treatment is analogous to the treatment of wound infections, i.e., debridement followed by closure.

Antibiotics are considered a pharmacodynamic adjunct to effective chemomechanical debridement of the root canal system and incision for drainage of facial swellings and cellulitis of endodontic origin.

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Systemic Disease and the Oral Microbiota

Jingyuan Fan, Massimo Costalonga, Karen F. Ross, and Mark C. Herzberg

INTRODUCTION

The oral microbiota comprises species that span the continuum from commensal to pathogenic. The pathogens are best known for their ability to cause infections that are normally limited to the oral cavity, including dental caries and periodontitis, the oral vesicular lesions caused by certain human herpesviruses (such as herpes simplex virus type 1 [HSV-1]), and the erosive mucosal lesions caused by *Candida albicans*. However, when these organisms or their components enter the circulation or connective tissues, they may increase the risk for systemic disease. A strong causal association has been suggested for oral bacteria in systemic diseases, including infective endocarditis and disseminated intravascular coagulation (DIC), and these organisms have also been associated with aspiration pneumoniae, septic shock, and brain abscesses. All are life-threatening diseases. Oral microorganisms have also been associated with pathogenic mechanisms in nephritis, rheumatoid arthritis, and Behçet's disease. As shown in some epidemiological studies, and supported by animal models, oral infections such as periodontal diseases have been associated with the occurrence of such diverse diseases as atherosclerosis and preterm low-birth-weight infants. Discovered only in the late 1960s, enterovirus 71 causes infectious vesicular eruptions of the perioral epithelium, hands, and feet as well as the oral and genital mucosas and can cause a serious encephalitis in infants and children and the immunocompromised. More recently, human papillomavirus (HPV) has become recognized as the primary etiological agent in head and neck cancer, an example of local infection causing regional and systemic disease of high morbidity and mortality. Similarly, other oral viruses such as human herpesvirus 8 (HHV-8) may be causative of other cancers, while HIV-1 can be transmitted through breached oral mucosa to cause AIDS. Molecular, animal, and human studies of infective endocarditis serve as an excellent proof-of-principle model to illustrate the entry of oral microbes into systemic compartments, subsequent dissemination, manifestations of virulence, and host responses. Understanding other systemic infections of oral origins must reflect the virulence characteristics of the causative microbiota. This chapter explores the underlying biological plausibility that oral microbes may contribute more generally to systemic disease.

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ROUTES FROM ORAL TO SYSTEMIC COMPARTMENTS

Breaches in the Oral Mucosa

Oral microbes reside in saliva and dental plaque and on and within the oral mucous membranes and epithelium of the gingival crevice. Oral microorganisms enter systemic compartments when dental procedures or injuries, frank trauma, or infections of the oral soft tissues create a breach. For example, in healthy individuals, certain microbes from dental plaque may invade the gingival epithelium and promote ulcerative lesions in the gingival crevicular epithelium. Similarly, ulcerative lesions form on the mucous membranes during herpesvirus infections in otherwise healthy people or in association with unremarkable infectious agents in immunocompromised individuals. Through these breaches in mucosal integrity, oral microbes enter the blood, resulting in polymicrobial bacteremias, fungemia, and viremia.

Transport and Translocation of Microbes

Oral microbes can also enter the systemic compartment when processed and transported by the mucosal immune system. Characterized by protective mechanisms in saliva, mucosal epithelial cells, dendritic cell lineages, B and T cells, and other lymphoid elements, the mucosal immune system provides surveillance against ingested environmental pathogens and is prominent in the oral cavity and the intestines. Interaction between oral microbes and the intestinal mucosa is a normal activity. Continuously swallowed at 10^5 cells per milliliter of saliva per minute, oral microbes interface with the intestines, or "gut." In the gut, particulate and soluble antigens are processed in the organized lymphoid tissues or Peyer's patches, which can result in stimulation or suppression of a systemic antigen-specific immune response. Proximal to the Peyer's patches, the M cells can translocate whole bacteria from the intestinal lumen to specialized phagocytic cells on the systemic side. These bacteria can then be processed, regulating an antigen-specific systemic immune response. Some of the features of the Peyer's patches and M cells are found in the oropharyngeal tonsils. The contiguous and more external oral mucosa, however, responds to surface bacteria and soluble antigens and protects itself through innate and adaptive immune mechanisms but lacks organized lymphoid tissues. Translocation of microbes would occur primarily though breaches in the epithelium. The mucosal immune system therefore facilitates entry of whole or processed microbes into the systemic compartment as part of a mechanism to foster systemic defense.

THE POTENTIAL OF COMMENSAL BACTERIA TO BEHAVE AS PATHOGENS

Several hundred species of bacteria reside in the oral cavity. Most are benign, causing no known disease in the mouth and surrounding tissues and coexisting peacefully with the host as commensals. When these bacteria enter the blood and other systemic compartments, the constitutively expressed genes may be sufficient to engender pathogenicity, for example, with an oral commensal organism like *Streptococcus sanguinis* (formerly *S. sanguis*). In 1948, the eminent microbiologist Theodor

Rosebury, working at Columbia University, identified oral streptoccci as "endogenous pathogens," able to cause disease in a nonnative environment. Whether oral bacteria rely solely on constitutively expressed genes to cause disease in nonnative compartments is less clear. Indeed, oral streptococci can express certain genes only when infecting intravascular surfaces, and the products of these in vivo-expressed genes may contribute to systemic disease.

Microbial virulence can change as new environmental stressors influence the pattern of expressed genes. Indeed, upon transition to a new environment, survival may require the regulated expression of genes. In some cases, expression of environmentally regulated genes may increase pathogenic potential or cause a harmless commensal to behave as a pathogen. To ensure survival in a foreign environment, benign commensal bacteria may be genetically programmed to become virulent.

Conventional wisdom holds that most bacteremias, viremias, and fungemias originating from oral foci are cleared uneventfully by the reticuloendothelial system, suggesting that their virulence in systemic compartments is insufficient to overcome the antimicrobial defenses of a healthy host. To survive in a stressful nonoral environment, the oral bacteria typically locate and colonize a relatively hospitable niche in an abnormal or injured anatomic site or an immunocompromised host. Consequently, oral microorganisms often cause systemic opportunistic infections by infecting abnormal anatomic sites, such as damaged heart valves, or by avoiding immune recognition and clearance in immunodeficient individuals. Hence, the association of oral microbiota with systemic diseases would often require the coincidence of transient bacteremias and underlying anatomical abnormalities and immunodeficiency. Alternatively, oral microbial infections of healthy cells and tissues in the systemic compartment could cause subclinical pathology or superimpose secondarily on other subclinical disease. Oral bacteria, such as the *Streptococcus* species *S. sanguinis*, *S. oralis*, *S. mutans*, and *S. salivarius*, have been isolated from foreign anatomic locations in systemic infections. Recent evidence suggests that these oral streptococci can also serve as accessory pathogens, increasing the virulence of known pathogens with which they can interact in heterotypic microbial communities. Given these scenarios, several mechanisms may enable oral bacteria to become virulent and cause disease upon infection of systemic organs and tissues.

Microbial Chameleons: Changing Gene Expression in Response to Environmental Signals

To adapt to changing ecological niches, bacteria have evolved the capacity to change the profiles of expressed genes. Environmentally regulated genes are modulated by intracellular signaling pathways that are activated when bacteria sense environmental signals via biochemically specific receptors. Altered expression of some genes may increase the virulence and pathogenicity of bacteria.

Gene expression in oral bacteria is regulated by many factors including changes in pH, oxygen level and oxidants such as hydrogen peroxide, ion concentrations, carbohydrate source, osmolarity, temperature, and access to protein substrates (Fig. 1). When oral bacteria gain access to the blood or other tissues, the new environment is dramatically different from

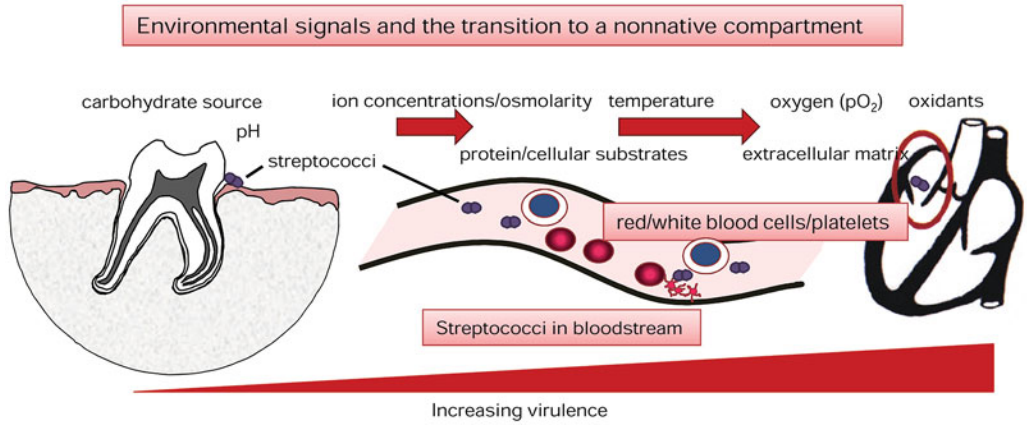

FIGURE 1 Environmental regulation of bacterial gene expression. Changes in environmental stressors such as temperature, pH, ion concentration, osmolarity, oxygen tension, and metabolic substrate can regulate bacterial gene expression. The example shown is for the oral streptococci; similar processes can occur in other oral organisms. doi:10.1128/9781555818906.ch19.f1

that of the oral cavity. For example, most sites in the mouth are slightly acidic, with a pH near 6. In dental plaque, the pH can dip below 5.0. However, in blood and other tissue fluids, pH is near neutral, about 7.4. At pH 6.5 to 7.0, oral streptococci release a histone-like protein (HlpA) into the medium. Release is not observed at or below pH 6.0. This is significant, as HlpA can complex with soluble lipoteichoic acid. When recognized by specific antibodies, the resulting immune complex may promote inflammation and increase the pathogenicity of the bacteria.

Other genes regulated by changes in environmental pH may also change oral bacterial virulence. When the pH increases from 6.2 to 7.3, *Streptococcus gordonii* upregulates expression of *msrA*, which encodes the enzyme methionine sulfoxide reductase. Methionine sulfoxide reductase protects organisms against oxidative damage and has potential roles in bacterial multiplication, stress resistance, and survival. Hydrogen peroxide produced by competing bacteria or host cells, including platelets at sites of intravascular injury, signals for change in gene expression patterns and increases the fitness of resistant species while reducing the competitiveness of sensitive organisms. The spectrum of expressed proteases also changes as oral streptococci transition to a more alkaline environment and respond to host proteins. As the pH elevates from 5.5 to 7.5, oral streptococci increase expression of thrombin-like activity. Since thrombin promotes polymerization of fibrinogen into fibrin and triggers platelet aggregation, oral streptococci exposed to an alkaline shift in pH may become increasingly thrombogenic and peroxide production may favor the growth of the thrombogenic species. Thrombogenicity of oral streptococci may shield the bacteria from phagocytes and facilitate infection and lesion formation on damaged heart valves during infective endocarditis and manifestations of disseminated intravascular coagulation.

Still other environmental factors change bacterial gene expression. For example, during infective endocarditis, extracellular matrix proteins become available to oral streptococci that have infected damaged heart valves. When exposed to the extracellular matrix proteins laminin and

collagen, oral streptococci express new or modified proteins and proteinases. A laminin-binding protein from *S. gordonii* is upregulated in the presence of laminin, increasing bacterial adhesion to the injured valve surface, whereas expression of dipeptidyl peptidases may degrade extracellular matrix and contribute to valve injury. As a regulated virulence factor, the streptococcal laminin-binding protein is a major serum antigen in patients that distinguishes infective endocarditis from noninfected valvulopathies. On its cell wall, *S. sanguinis* expresses a platelet aggregation-associated protein (PAAP), which enables the bacteria to induce platelet aggregation. When isolated from *S. sanguinis* cells, PAAP cannot induce platelet aggregation. In contrast, PAAP released from *S. sanguinis* cells grown with type I collagen can induce platelet aggregation, and hence *S. sanguinis* may become more thrombogenic under certain conditions. Since platelet aggregation is pivotal to the development of valvular vegetations in infective endocarditis, altered expression of PAAP may reflect environmentally regulated pathogenicity.

How can we learn if specific genes are expressed in selective environments? Several approaches have been developed to identify genes expressed in vivo but not in vitro (see chapters 7 and 8). Using in vivo expression technology, a promoterless reporter gene is randomly inserted into the streptococcal genome to create a mutational library. Expression of the upstream reporter in vivo but not in vitro identifies environmentally induced active genes. Alternatively, signature-tagged mutagenesis uses comparative hybridization to isolate mutants unable to survive in specified environmental conditions. An in vivo expression technology mutational library of *S. gordonii* has been used in an animal model of infective endocarditis. In experimental endocarditis, genes found to be induced by *S. gordonii* infecting heart valves include the above-mentioned *msrA*. Upregulation of *msrA* during infective endocarditis is consistent with a shift in environmental pH from acidic to neutral.

Whether oral bacteria express a unique set of genes in response to changes in pH or whether other environmental stressors induce a similar set of genes remains unclear. Certainly, the expression of some virulence genes appears to be regulated in a general response to stressors. How might that work? In bacteria, two-component systems are a common stimulus-response coupling mechanism. Environmental stress signals are sensed by a receptor or sensor histidine kinase (Fig. 2). Most sensor histidine kinases are membrane proteins with an N-terminal transmembrane region and an independent C-terminal autokinase domain. The extracellular or periplasmic domain of a sensor histidine kinase can bind directly to extracellular signals. Binding of a specific extracellular stress signal or, alternatively, conformational perturbation by the stressor results in autophosphorylation of a conserved histidine residue on the kinase. By autokinase transfer of high-energy phosphate from the receptor histidine kinase to an aspartate residue in the N-terminal receiver domain of the cognate response regulator protein, the signal is transduced into the cell (Fig. 2). Activated by phosphorylation, the response regulator C-terminal effector domain functions as a transcriptional regulator to turn on or turn off specifically targeted genes and trigger the corresponding response pathway. Many sensor kinases also have phosphatase activity and can dephosphorylate regulators, particularly in absence of the signal. In this

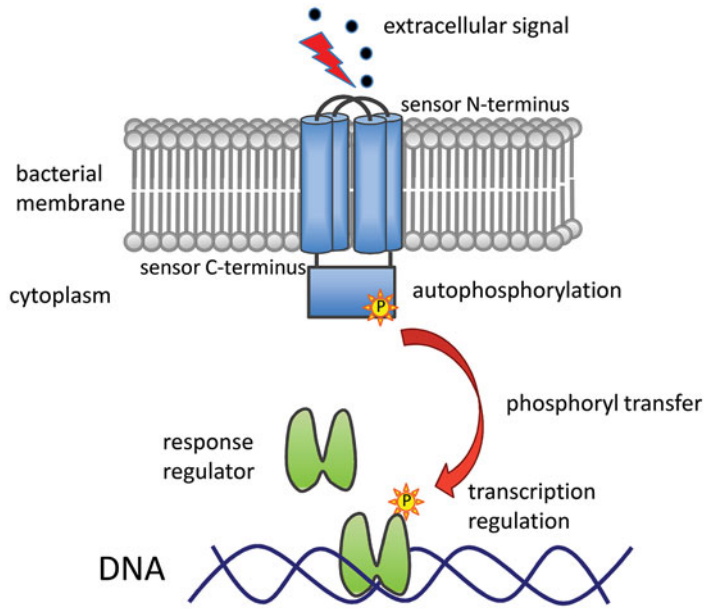

FIGURE 2 The typical two-component system. A transmembrane protein sensor binds to an extracellular signal via its N-terminal region, resulting in autophosphorylation of a histidine in the C-terminal kinase. The N terminus of the specific cytosolic response regulator is the target of phosphoryl transfer. This activates the regulator's C-terminal domain, and the specific target gene is activated or deactivated.
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way, a sensor and its mated regulator can coordinate their behaviors during signal transduction, and stressors that activate common response regulators are likely to regulate expression of a similar set of genes.

Another response to environmental signals involves changes in DNA topology, which makes the promoter region of target genes accessible to transcriptional regulators. As a result, environmental stresses can activate previously inactive virulence genes. *Escherichia coli* regulates pilus expression by a "flip-flopping" on-off genetic switch. The promoter for the pilus gene is a spontaneously invertible segment of the chromosome. Under colonizing conditions, the promoter segment flips to produce piliated organisms, which can stick to the mannose molecules on the surface of epithelial cells, increasing virulence.

Clearly, altered expression of genes modifies the behaviors of bacteria to cope with environmental conditions. Differential gene expression can be anticipated to be widespread among the oral bacteria. For example, the anaerobic periodontal pathogen *Porphyromonas gingivalis* regulates expression of its major fimbrial adhesin in response to temperature. Moreover, the organism regulates expression (both up and down) of almost 500 proteins when exposed to host epithelial cells. As we learn to model environmentally specific stressors and identify specific and globally regulated genes, we may be able to explain better how oral microbes behave as endogenous pathogens.

HOST DEFENSES

Microorganisms have evolved many mechanisms to adapt to changing environmental conditions, but the host is not defenseless. Since the oral cavity sustains large infectious and antigenic loads, the mucous membranes have evolved mechanisms to distinguish between pathogenic and commensal microorganisms and food molecules. The squamous epithelial cells and resident intraepithelial and lamina propria immune cells form

a sophisticated system of pathogen recognition, barrier protection, and immune response.

When microorganisms enter the mouth, they encounter a number of nonspecific defense mechanisms that help prevent them from binding to and penetrating the oral mucosa (see Chapters 2, 3, and 10). Saliva has a flushing effect, physically removing microbes to the gut by swallowing. Saliva contains a supply of nonimmune and immune factors, including mucin glycoproteins and salivary agglutinin, which bind and clear bacteria more efficiently by swallowing. A mucinous salivary film coats the oral soft and hard tissue surfaces. Constituents of the film may form a protective coating, one aspect of which is the blockade of epithelial cell receptors for bacterial adhesion and invasion.

Saliva contains antimicrobial peptides such as histatins, lysozyme, and lactoferrin. Other antimicrobial peptides are released into the saliva by epithelial cells and neutrophils. The epithelial- and neutrophil-derived antimicrobial peptides are present in higher concentrations on the mucosal surfaces, where they may be most active. Examples of these innate immune molecules include the defensins, which protect against harmful colonization by a wide variety of microorganisms. In the oral cavity, keratinocytes also express an antimicrobial protein, calprotectin, which appears to protect the oropharyngeal mucosa from invasion by pathogens such as *C. albicans*, *Listeria monocytogenes*, and *P. gingivalis*.

The mucosal epithelium also protects the underlying connective tissues against microbial entry by forming a tight and strong physical barrier. The keratins cornify as cells mature, making an abrasion-resistant epithelium. As the superficial cells bind microbes, the keratin-rich mucosal surface cells are shed by desquamation and replaced by cells from underlying layers. Bathed in antimicrobial saliva, these cells also express intrinsic innate antimicrobial mechanisms to protect against invading bacteria and fungi. Nonetheless, superficial mucosal keratinocytes contain oral bacteria that have invaded the cells. By shedding the mature superficial cells, the mucosa limits colonization and invasion by pathogenic microorganisms. Mucosal epithelial cells also form intercellular attachments, which further restrict access of oral microbes to intercellular spaces between epithelial cells and then into the connective tissues. Keratinocytes attach to one another with specialized intercellular junctions, which prevent the passage of water, solutes, and surface microbes between the oral cavity and interior tissue compartments. Healthy mucosal epithelium tends to protect against intercellular passage and transcytosis of microbes, limiting the ability of invasive pathogens to penetrate deeper into the tissue.

If the infectious agent manages to penetrate the mucosal surface and invade the epithelium, the damaged cells secrete proinflammatory cyokines such as interleukin 1 (IL-1), IL-6, IL-8, and macrophage chemoattractant protein 1 (MCP-1/CCL-2). These cytokines recruit phagocytic cells to the site of bacterial invasion, and IL-1α upregulates the production of calprotectin by neighboring epithelial cells, augmenting resistance to microbial invasion. Blood monocytes, neutrophils, tissue macrophages, dendritic cells, and Langerhans cells internalize (phagocytose), kill, and digest whole microorganisms, protecting the underlying tissues by creating a phagocytic barrier against further microbial invasion. These phagocytes

of the innate immune system react to a wide variety of pathogens without requiring prior sensitization.

How do mucosal keratinocytes recognize infectious agents? Microbes express certain highly conserved molecular structures that are distinct from self (the host). The innate immune system recognizes these pathogen-associated molecular patterns (PAMPs). For example, the general structure of lipopolysaccharide (LPS) is common to all gram-negative bacteria. Recognition of LPS by a host cell receptor would allow any gram-negative bacterium to be detected. In addition to LPS, common PAMPs include peptidoglycan, lipoteichoic acid, mannans, bacterial DNA, double-stranded RNA, and glucans. These patterns are recognized by host cell pattern recognition receptors (PRRs) expressed by the phagocytic cells and other cells of the innate immune system. PRRs include CD14, mannose receptor, C-type lectin endocytic receptor, known as DEC205, and molecules of the Toll-like receptor (TLR) family. These PRRs are expressed on the surface of cells and within endosomes. Other PRRs such as nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) and the retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) are expressed within the cytosol and detect PAMPs on viruses and other invasive microorganisms. Binding of these receptors with a PAMP directly induces the expression of inflammatory cytokines and chemokines, especially IL-1, IL-6, and tumor necrosis factor (TNF), MCP-1, and macrophage inflammatory protein 1α (MIP 1α), to alert the host to the presence of infection. These soluble signals then recruit additional phagocytic cells followed by antigen-specific memory lymphocytes to sustain the innate immune response.

Gingival keratinocytes express proinflammatory cytokines in response to LPS and other PAMPs by signaling through TLRs. The TLRs are upregulated by gamma interferon, another cytokine, increasing the response to PAMPs. Unlike leukocytes and dermal keratinocytes, gingival keratinocytes do not appear to express CD14, nor does saliva appear to contain sufficient LPS-binding protein to serve as a required cofactor for CD14-mediated signaling and cytokine expression. Hence, gingival keratinocytes probably respond to microorganisms through TLRs and not CD14.

The innate and adaptive immune systems interact and cooperate to produce an effective response against foreign invaders, as is summarized in Fig. 3.

FIGURE 3 Levels of protection against systemic infection by oral microbes. A local innate immune response helps contain the infection and delivers the antigen to local lymph nodes, leading to initiation of adaptive immunity and clearance of infection.
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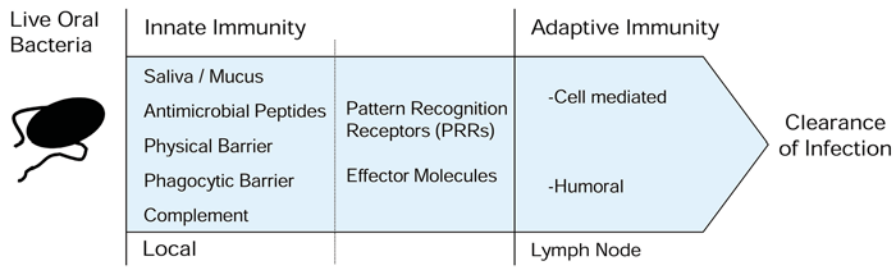

SYSTEMIC DISEASES ASSOCIATED WITH ORAL MICROBES

Infective Endocarditis

Perhaps the best-known example of a systemic infection that can be caused by oral bacteria is the life-threatening infective endocarditis. More than 15,000 new cases are diagnosed annually in the United States. This is a disease of injured or abnormal heart valves, which are typically asymptomatic until blood-borne infection occurs. The oral streptococci are the most common etiological agents. A mixture of oral pathogens and commensal bacteria enter the circulating blood through lesions in the oral tissues during infections such as periodontitis and periapical abscesses. These polymicrobial bacteremias may also occur spontaneously during innocent activities such as vigorous oral hygiene or can result from invasive dental procedures. After entry, the polymicrobial bolus disseminates in the circulating blood, and in the blood, even organisms that are harmless in the oral cavity can behave as pathogens and infect susceptible heart valves.

The valves and endocardial tissues at risk of infection are abnormal, generally because of earlier disease, injury, or developmental abnormality. Conditions creating risk of infective endocarditis include unrepaired cyanotic congenital heart diseases, prior heart valve surgery with residual defects, or cardiac transplantation resulting in valvulopathy. As a consequence of these conditions, the valvular subendothelium is exposed. Any exposure of subendothelium results in the deposition of fibrin and platelets and binding of tissue factor, which eventually lead to the formation of an aseptic thrombus on the heart valve as a normal wound-healing process (Fig. 4).

This aseptic thrombus is critical for bacterial infection. Bacteria bind the adherent platelets and exposed connective tissues during polymicrobial bacteremias. Overrepresented in polymicrobial dental plaque, *S. sanguinis* is the most common cause of infective endocarditis. In this new environment, *S. sanguinis* can adhere to the aseptic thrombus as well as extracellular matrix proteins on the exposed subendothelium (Fig. 4). On the other hand, certain *S. sanguinis* strains express PAAP, which can directly activate platelets to aggregate. Near the forming septic valvular thrombus, damaged endothelial cells and activated monocytes express tissue factor in response to LPS from gram-negative bacteria, which are also present in the blood during polymicrobial bacteremias. Tissue factor activates the coagulation cascade, which promotes the polymerization of clot-forming fibrin. The mass of microorganisms, platelets, and fibrin on diseased or injured heart valves or other endocardial surfaces constitutes a vegetation, which is characteristic of infective endocarditis. Protected from the immune system within the vegetation, the infections expand as the bacterial colonies grow. In this nonnative environment protected from the immune system, the streptococci change their modus operandi from that of harmless oral commensals to that of intravascular pathogenic microorganisms by environmental regulation of gene expression.

The vegetation itself is significant to the outcome of infective endocarditis. Shielded within the septic thrombus, the colonizing bacteria resist the action of the innate and adaptive immune systems and antibiotic therapy. Some infections may resolve if the infecting bacteria are sensitive to platelet microbicidal protein, an innate antibacterial defense protein

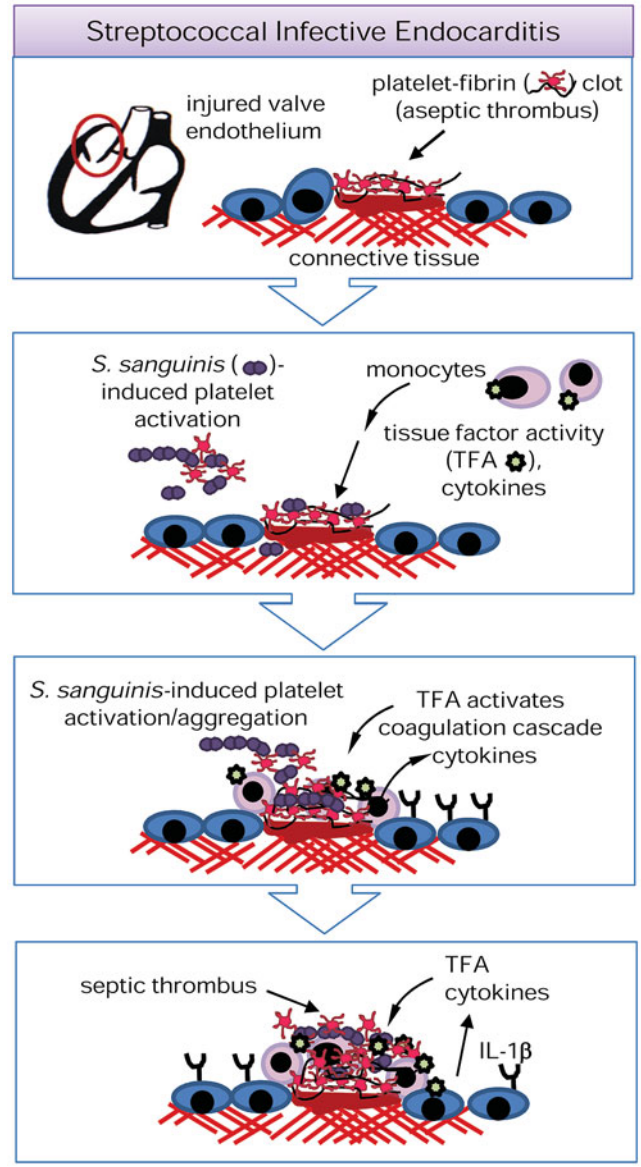

FIGURE 4 *S. sanguinis*-platelet interaction in infective endocarditis. Circulating platelets adhere to exposed connective tissue on damaged heart valves and form an aseptic thrombus. During transient polymicrobial bacteremia, *S. sanguinis* binds to circulating platelets, to platelets in the aseptic thrombus, and to exposed extracellular matrix. *S. sanguinis* directly interacts with platelets through PAAP, which activates and induces additional platelets to form aggregates in the circulating blood and on the heart valve. Aggregation requires the cross-linking of platelets to one another by fibrinogen molecules. The fibrinogen molecules are polymerized into fibrin by thrombin, forming an insoluble thrombus or platelet clot, which requires activation of the coagulation cascade by tissue factor activity (TFA). TFA is upregulated on monocytes that accumulate on the exterior of the septic thrombus as an inflammatory response is initiated and on neighboring endothelial cells. Monocytes and endothelium cells also release cytokines, such as IL-1β and TNF, which further amplify the inflammatory features of the septic vegetation. IL-1β can bind to specific receptors on endothelial cells and signal TFA and inflammatory responses in the cells. doi:10.1128/9781555818906.ch19.f4

released by activated platelets. However, as expected, most pathogens recovered from patients with infective endocarditis are resistant to platelet microbicidal protein. In spite of treatment with antibiotics, the mortality rates for infective endocarditis remain as high as 20 to 25 percent, with death resulting primarily from complications like congestive heart failure and neurologic events. Consequently, health care professionals are advised by the American Heart Association and other agencies internationally to reduce the risk of infective endocarditis among dental patients at high risk. For these patients, specific antibiotic prophylaxis regimens are prescribed before performing invasive procedures that induce bacteremia. The protocol is revised periodically as more becomes known. Check with the American Heart Association for the current recommendations.

Disseminated Intravascular Coagulation

In immunocompromised individuals including pediatric cancer patients, alpha-hemolytic oral (viridans group) streptococci cause frequent bacteremias. In up to 25% of pediatric cases, bacteremias result in viridans group streptococcal shock syndrome with mortality rates of 40 to 100% in different patient cohorts, perhaps caused by elaboration of streptococcal superantigens. A frequent and severe complication of streptococcal shock syndrome with sepsis is disseminated intravascular coagulation (DIC). In DIC, the processes of coagulation and fibrinolysis are dysregulated, and the result is widespread clotting with resultant bleeding. DIC associated with alpha-hemolytic streptococci is a major cause of mortality in pharmacologically immunosuppressed individuals awaiting organ transplantation and in older adults after surgical procedures. As with infective endocarditis, oral streptococci must gain access into the systemic compartment to cause DIC. In DIC in the immunocompromised, the most common portal of entry is the painful, erosive lesions of the oral and oropharyngeal mucous membranes known as mucositis. After translocating through the mucous membranes, the oral microbes can cause release of tissue factor, which triggers disseminated activation of the coagulation cascade. Fibrin becomes deposited in capillary beds and in small blood vessels, occluding the flow of blood to the major organs and tissues. If occlusion is prolonged, tissue and organ ischemia and infarction can result. With alpha-hemolytic streptococci, DIC is usually not accompanied by febrile illness, and the clinical outcome is often fatal. DIC and infective endocarditis illustrate the potential of otherwise benign oral, commensal streptococci to cause life-threatening disease in susceptible individuals.

Sequelae of Oral Viral Infections

The oral cavity harbors numerous viruses, and viral infection is typically asymptomatic. Most prominent are the herpesviruses (see Chapter 16). Primary infection with HSV-1 can cause a minor febrile illness in infants, marked by erythematous mucosal and perioral vesicles or blisters. HSV-1 travels along neurons from the oral epithelium and establishes latent infection in sensory ganglia. Reactivation of the latent infection is marked by the reappearance of the oral vesicular lesions, which contain infectious virus. Periodic viremias occur, and the virus has been linked epidemiologically and in laboratory models to systemic sequelae including atherosclerosis.

Other oral herpesviruses of interest include HHV-2, which is typically harbored in the gentiourinary tract and can be acquired as a sexually transmitted disease to take residence in the oral cavity. HHV-2 is strongly associated with occurrences of head and neck cancer. Other consequential oral viruses include Epstein-Barr virus (HHV-4), which causes mononucleosis; human cytomegalovirus (HHV-5), which can cause severe retinal and neurological disease in infants and the immunocompromised in addition to a mononucleosis-like syndrome; and Kaposi's sarcoma-associated herpesvirus (HHV-8), which causes Kaposi's sarcoma in HIV-1-positive patients and Castleman's disease.

With a pathogenic profile similar to that of some herpesviruses, the recently discovered (ca. 1969) enterovirus 71 also causes oral and perioral lesions in the young and an encephalitis-like disease. This emerging oral disease is easy to misdiagnose but is marked by vesicular lesions also on the hands and feet.

In recent years, there has been growing awareness of the role of HPV as an etiological agent in head and neck cancer. The carcinogenic action of HPV is independent of other well-established risk factors such as tobacco and alcohol use. Like HHV-2, HPV is likely acquired as a sexually transmitted disease from genitourinary foci. Hence, with the increased sexual activity among teenagers and young adults noted since the 1990s, there is a parallel increase in oral infections and HPV-positive head and neck cancers in nonsmokers.

Other Effects

Oral infections have come under scrutiny as possible risk factors for systemic conditions such as cardiovascular disease, cerebrovascular events, respiratory disease, and preterm low-birth-weight infants. Several epidemiological studies suggest that there is an association between dental health and cardiovascular disease. Specific pathogens in dental plaque, however, are more strongly associated with subclinical atherosclerosis. People with poorer dental health are more likely to have cardiovascular disease. This association is apparent in both retrospective and case-control studies and is specific for periodontal disease, not dental caries. Although epidemiological evidence can be confounded by the presence of similar risk factors for periodontitis and cardiovascular disease, the strength of evidence in support of a relationship is increased by findings of causal relationships in animal models. Similar epidemiological evidence also exists for an association between periodontal disease and preterm delivery of low-birth-weight infants, and periodontal bacteria have been identified in gestational tissues in these cases. Periodontal bacteria and phagocytosis-resistant aggregates shed from biofilms can be aspirated into the lungs. This can result in pneumonia, particularly in the institutionalized elderly who have a compromised ability to clear local infections.

OTHER POSSIBLE ASSOCIATIONS BETWEEN ORAL MICROBES AND SYSTEMIC DISEASE

Heat Shock Proteins

Molecular mimicry is among the most intriguing mechanisms whereby commensal bacteria may contribute to systemic disease. Bacteria, fungi, and mammalian hosts express the heat shock protein (HSP) families.

These proteins are highly conserved, showing nearly identical primary amino acid sequences across species. In humans, some heat shock proteins are cryptic, hidden from the view of the adaptive immune system. For example, HSP60 resides in mitochondria and is normally shielded from host immune surveillance. Consequently, the mammalian immune system considers HSP60 to be foreign. Upon exposure to HSP60, an immune response is initiated. Microbes that express HSP60 homologues, therefore, may trigger an autoimmune response upon infection.

HSPs are found in microorganisms and host cells during normal growth. Cellular expression increases in response to heat shock and other stressors. HSP60, HSP70, and HSP90 are the most significant HSP families. During heat shock and other stresses, newly synthesized proteins tend to unfold, complex, and become insoluble. After protein synthesis and translocation of proteins into intracellular compartments, HSPs bind or chaperone new unfolded or partially folded proteins. Chaperoned, newly synthesized proteins retain their native conformation and are protected against complexing into unwanted, insoluble globular aggregates. Molecular chaperones, therefore, form a wax paper-like protein layer to ensure that proteins destined for export will be able to assume a fully functional shape.

The structural similarity between HSPs of prokaryotic and eukaryotic origins is remarkable. Indeed, the host adaptive immune system may not distinguish between host and foreign HSP antigens. Human HSP60 expressed during normal growth is highly homologous to bacterial HSPs such as *Mycobacterium* HSP65, *S. sanguinis* HSP65, and *Helicobacter pylori* HSP60. Antibodies elicited by a bacterial HSP will generally cross-react with the others and with the human orthologue. During bacteremia, HSP concentrations increase in the blood. The presence of small amounts of bacterial HSPs induces low titers of anti-HSP antibodies. Anti-HSP antibodies apparently produced against Gram-negative bacteria can be detected in the serum and gingival crevicular fluid of dental patients. Antibodies to bacterial HSPs cross-react and block human HSP60 when accessible.

Oral bacterial HSP antigens eliciting antibodies that react with self or foreign HSPs will form immune complexes. Immune complexes will then activate the complement cascade. Anti-HSP antibodies in the blood and gingival crevicular fluid are associated with inflammation in the periodontal tissues. By reacting with cross-reactive self-antigens, therefore, immune complexes can form and deposit in different tissues of the body and contribute to systemic inflammation. The accumulation of immune complexes and activation of the complement system suggest a role for HSPs in some systemic diseases, including rheumatoid conditions and Behçet's disease, which is a syndrome of immune-mediated ulcers and eruptions of the mucous membranes of the eye, genitals, and oral cavity of young adult males.

In summary, HSP-specific antibodies produced in response to a localized increase in oral bacterial HSPs may gain access to the circulation. Antibodies against oral microbial HSPs can cross-react with self HSP antigens. Immune complexes form, activating the complement system. If deposited in the arterial wall (atherogenesis), joints (arthritis), or the mucous membranes (Behçet's disease), HSP mimicry can contribute to systemic disease.

Autorecognition Induced by Oral Microorganisms

In the epithelium of inductive sites such as the tonsils or the junctional epithelium, oral microorganisms interact with immune cells to activate antigen-specific T cells. Microbes can express antigens that mimic endogenous or "self" structural proteins. When presented to the mucosal or systemic immune system, these molecular mimics stimulate and activate naturally anergic T cells. Now specific for the self proteins, these activated T cells recirculate between the blood and the lymphoid organs and also through peripheral tissues. In the peripheral tissues, activated T cells can encounter these same self antigens and initiate autoimmune recognition.

S. sanguinis, for instance, can enter the blood periodically through breaches in the dentogingival junction or the oral mucosa. Some *S. sanguinis* strains express a collagen-like epitope within the PAAP. The PAAP epitope is partially homologous to the arthritogenic epitope of type II collagen. In rodents, primates, and patients with rheumatoid arthritis, type II collagen is a candidate antigen in autorecognition. *S. sanguinis* does not appear to activate naïve type II collagen-specific T cells but can stimulate primed type II collagen-specific T cells in vitro. In a murine arthritis model, *S. sanguinis* infection exacerbates arthritis in type II collagen-primed mice. $PAAP^+$ *S. sanguinis*, therefore, can activate memory T cells specific for type II collagen in vitro. In susceptible mice, $PAAP^+$ *S. sanguinis* exacerbates type II collagen-induced autoimmune arthritis.

On the other hand, mucosal exposure of $PAAP^+$ *S. sanguinis* to arthritis-susceptible neonatal mice (strain DBA/1J) inhibited the development of autoimmune arthritis in the adult. These results suggest that commensal bacteria can induce oral tolerance early in life and that the same bacteria can trigger autorecognition when memory autoreactive T-cell clones are already present. To extrapolate from these conclusions to rheumatoid arthritis-susceptible individuals, early colonization of newborns and infants by $PAAP^+$ *S. sanguinis* strains may be protective. Colonization or infection later in development could exacerbate arthritic episodes.

The oral microbial environment can also interfere with the adaptive immune response by activating a large number of T cells independently of their specificities. Some streptococcal molecules have superantigen-like function. The result is a polyclonal activation of $CD4^+$ T cells. Superantigens subvert the ordered T-cell response by nonspecifically activating T cells. The lymph nodes clog with the high density of proliferating T cells, which lack the specificities needed to direct B cells toward an efficient antibody response. The large ulcerative lesions seen in the oral mucosa of patients with Behçet's disease appear to result from local polyclonal activation of T cells by oral streptococcal superantigen-like molecules. Some nonspecifically activated T cells activate specific B cells. When superantigens activate T cells that are specific for local self antigens or HSPs, the activated T-helper cells can direct the production of autoantibodies and cytotoxic T cells. If directed against HSPs, autoantibodies and cytotoxic T cells may destroy HSP-expressing epithelial cells. The presence of bacterial superantigens may augment the self-directed immune response to destroy the epithelial layer, creating the painful ulcer seen in the oral cavity of patients with Behçet's disease.

Inflammation: a Link between Local Dental Disease and Systemic Pathology?

Periodontal disease is characterized by repeated episodes of inflammation associated with the presence in the periodontal pocket of cultivable microorganisms (such as *P. gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, and *Treponema denticola*) and uncultivable (or poorly cultivable) microorganisms (such as *Filifactor alocis*, *Dialister pneumosintes*, *Megasphaera* spp., and *Desulfobulbus* spp.). Many bacteria associated with periodontal disease are gram negative, and LPS can diffuse into ulcerated gingival tissue in the gingival sulcus or periodontal pocket. Macrophages are among the early immune cells that react to this local presence of bacteria. LPS is an especially potent activator of monocytes/macrophages, which respond by secreting soluble mediators of inflammation, i.e., TNF and IL-1β. When activated, macrophages also release prostaglandin E_2 (PGE_2), an arachidonic acid metabolite, which promotes vasodilation of the local microvasculature, adding to the inflammation. Locally, IL-1β and TNF increase cellular adhesion molecule expression by endothelial cells, which facilitates binding of neutrophils to the vessel wall, a process required for their extravasation into the affected tissues. When local concentrations of IL-1β and TNF are high, these soluble mediators can enter the circulatory system. IL-1β and TNF induce the liver to produce acute reactants, including C-reactive protein (CRP) (Fig. 5). Since CRP is not produced in the gingiva, its presence in the gingival crevicular fluid is a convenient marker of endovascular or systemic inflammation.

CRP binds specifically to C-polysaccharide, a membrane component of bacteria and fungi, and acts as an opsonin, promoting phagocytosis by macrophages. CRP also binds to damaged cells, and complexed CRP can activate complement. When stimulated by CRP, macrophages produce tissue factor, an initiator of coagulation. Elevated serum CRP is a normal occurrence during the body's response to infection and inflammation. Levels return to normal as the infection is cleared, so serum CRP can be used as a marker of infection or inflammation. In healthy subjects at risk for cardiovascular disease, those with high serum CRP levels were more likely to develop cardiovascular disease. Interestingly, it has been reported that periodontal patients have higher serum CRP levels than periodontally healthy patients.

There is increasing evidence that inflammatory events contribute to atherogenesis and cardiovascular disease. Binding or ligation of TLRs by PAMPs from *P. gingivalis* and virtually all microbes activates several transcription factors, including NF-κB. Activated NF-κB upregulates expression of a spectrum of genes associated with inflammation. The TLRs are expressed on immune surveillance cell types including monocytes. Monocytes that have infiltrated the blood vessel intima and taken up lipids (cholesterol) play a critical role in the formation of atherosclerotic plaques. Activated by IL-1β and TNF, these monocytes may be induced to ingest more lipid and to continue to secrete inflammatory cytokines. IL-1β can also promote smooth muscle cell proliferation, which may be responsible for thickening of the vessel wall associated with the atherosclerotic lesion.

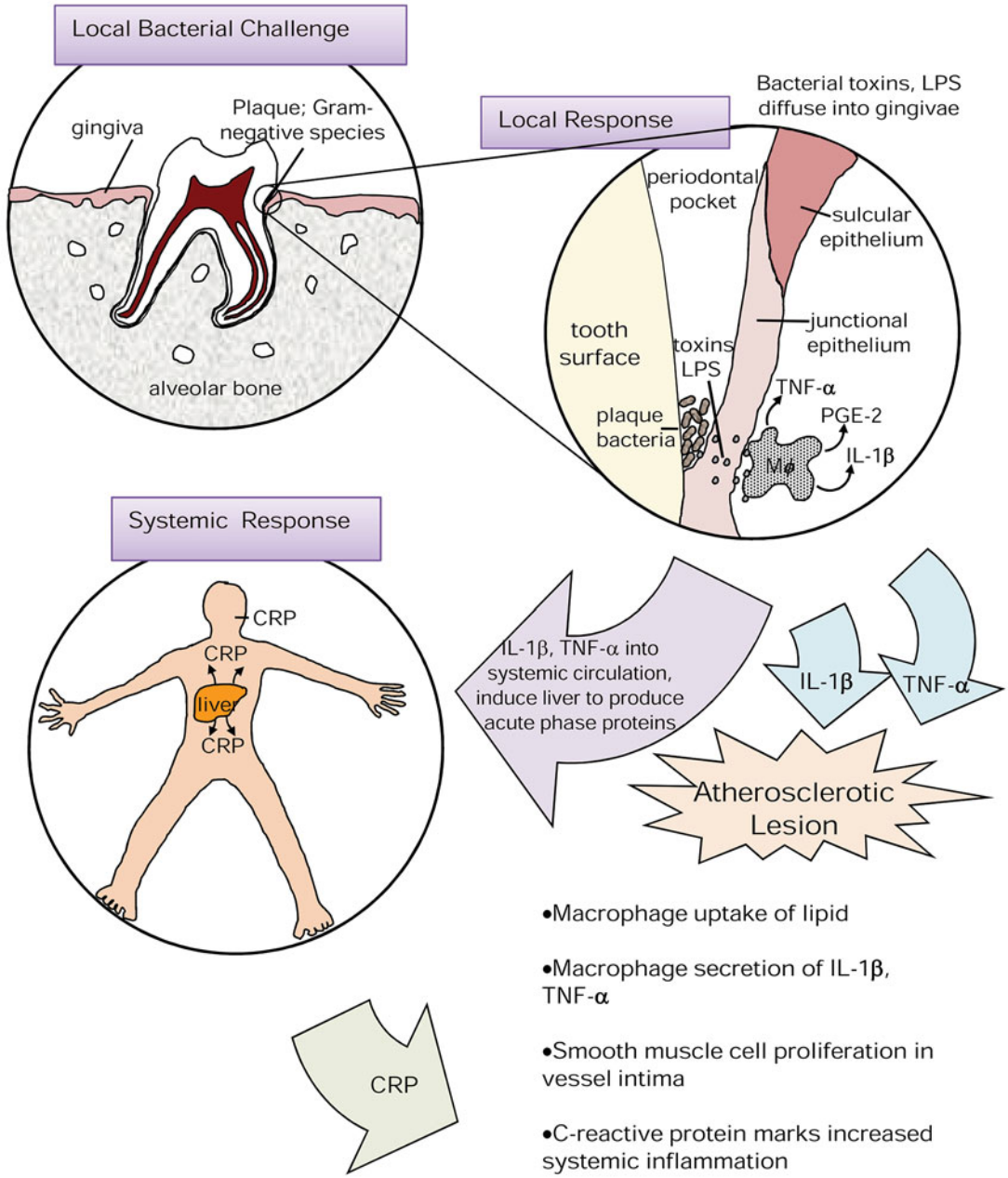

FIGURE 5 Oral microbes produce local inflammation to influence systemic pathology. In the gingival sulcus, bacteria and their PAMPs such as LPS affect underlying tissues, causing local inflammation. High levels of the inflammatory mediators TNF-α and IL-1β may enter the circulation and induce the liver to produce acute-phase reactants, such as CRP. IL-1β and TNF-α may also act on monocytes in existing atherosclerotic plaques, causing exacerbation of existing disease.
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Periodontal microorganisms may contribute to the proinflammatory infectious load created by other chronic infectious organisms such as *Chlamydia pneumoniae* and cytomegalovirus, which are often associated with atherosclerotic plaques. These organisms are endemic in otherwise healthy populations, potentially adding to the systemic pathogen burden. Among the endemic members of the periodontal microbiota, *P. gingivalis* DNA has been detected in specimens of human atherosclerotic plaques. Known to invade, ligate, and signal endothelial cells in culture through TLRs to induce expression of proinflammatory cytokines, *P. gingivalis* may also challenge the health and integrity of the endothelial lining of blood vessels by modulating the expression of cellular adhesion proteins. Indeed, proteinases produced by *P. gingivalis* (gingipains) can directly digest the adhesive structures on mucosal epithelial cells. Furthermore, *P. gingivalis* can induce epithelial cells to express matrix metalloproteinases, degrading the extracellular matrix, which is the foundation to which many cells attach. If it gains entry to the subendothelial myointima, *P. gingivalis* may also induce the formation of lipid-filled foam cells from macrophages. The formation of foam cells in vitro simulates a key step in atherogenesis. Finally, like *S. sanguinis* and certain other microorganisms that have been localized to atherosclerotic plaques from humans, *P. gingivalis* induces human platelets to aggregate in vitro. Hence, *P. gingivalis* and other microbes that chronically enter the blood circulation during mucosal infections may express similar pathogenic characteristics. To determine how, and to what extent, each infection and associated microorganisms contribute to cardiovascular disease outcomes is a difficult challenge for scientists. When present simultaneously or intermittently in the blood over time, however, the systemic pathogen burden represents the aggregate risk of atherosclerosis and thrombosis formed by the redundant pathogenicity of microorganisms such as *C. pneumoniae*, cytomegalovirus, *S. sanguinis*, and *P. gingivalis*.

Epidemiological studies have also suggested that women with periodontal disease gave birth to preterm low-birth-weight babies. Similar to our understanding of the contribution of periodontal health to cardiovascular disease, the modeling of the proinflammatory load of plaque bacteria in animals supports the hypothetical relationship. Soluble inflammatory mediators, such as PGE_2, could be the molecular link from a local infection (periodontitis) to a distal event, parturition. Uterine levels of PGE_2 normally rise as gestation nears completion. If some threshold PGE_2 level triggers the onset of labor, it is possible that the combined PGE_2 from a distant infection and uterine tissue may induce premature labor and delivery of a low-birth-weight baby. Periodontal bacteria such as *Fusobacterium nucleatum* and *P. gingivalis* that gain systemic access may also locate at and invade placental cells and tissues. Disruption of cytokine networks in the gestational tissues may also contribute to premature labor.

Thus, the microbes associated with periodontal disease may induce local monocytes to express inflammatory mediators. These mediators enter the systemic circulation and may exacerbate existing pathology at distant sites, such as atherosclerotic plaques, or trigger abnormal vascular function in specific tissues. For example, PGE_2 generated at the site of periodontal infection could enter the systemic circulation to increase uterine levels to trigger premature labor. Alternatively, or concurrently,

periodontal bacteria may spread systemically to cardiac or placental tissues and exert their pathogenic influence at these systemic sites. The associations between periodontal disease and both cardiovascular disease and preterm low-birth-weight infants are subjects of current investigations. Reconciling the disparate results and conclusions of epidemiological studies is a persistent challenge.

KEY POINTS

Commensal oral bacteria can become pathogenic when they gain access to environments other than the oral cavity. A breach in the integrity of the soft tissues of the oral cavity can be caused by dental procedures, injuries, or infections. The new environmental conditions that are found systemically induce changes in bacterial gene expression, which may increase virulence.

Oral streptococci that are benign in the oral cavity are often causative agents in infective endocarditis. In the systemic compartment, the streptococci locate on damaged or developmentally abnormal heart valves. The valvular endothelium can then slough, leaving the underlying connective tissues exposed. Circulating platelets are activated to repair the wound, and the platelets adhere to and spread on the exposed connective tissues. The streptococci then induce the platelets to bind fibrinogen, aggregate, and form a thrombus or platelet clot. The mass of microorganisms, platelets, and fibrin on diseased or injured heart valves or other endocardial surfaces constitutes a vegetation, which is characteristic of infective endocarditis.

Cross-reactivity of antibodies to bacterial HSPs with self HSPs may contribute to systemic diseases such as rheumatoid arthritis and Behçet's disease.

Bacterial mimicry of self peptides such as a collagen fragment by PAAP epitope of *S. sanguinis* may lead to autorecognition and autoimmune arthritis and related diseases.

Local intravascular inflammation caused by oral bacteria may be implicated in the progression of systemic diseases such as atherosclerosis. Periodontal bacteria such as *P. gingivalis* may contribute more directly to atherosclerosis by invading artery endothelial cells and inducing inflammation. *P. gingivalis* can also induce the formation of lipid-filled foam cells from macrophages, a key step in atherosclerosis. Spread of periodontal bacteria such as *F. nucleatum* and *P. gingivalis* to gestational tissues may disrupt local homeostasis and lead to preterm delivery of low-birth-weight infants.

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SECTION III

CONTROL OF ORAL DISEASES

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Immunological Intervention against Oral Diseases

Kohtaro Fujihashi, Michael W. Russell, and George Hajishengallis

INTRODUCTION AND HISTORICAL BACKGROUND

By the 1970s, there was adequate evidence that dental caries had a specific bacterial etiology and that saliva contained specific antibodies of a distinct type. These developments provided a scientific basis for the development of a caries vaccine and date back to 1924 with the first documented isolation of *Streptococcus mutans* from human carious lesions by J. K. Clarke. *S. mutans* was later divided into several species collectively known as "mutans streptococci," which include *Streptococcus sobrinus*, and the infectious and transmissible nature of dental caries was firmly established in the 1960s, in great part due to pioneering studies by R. J. Fitzgerald and P. H. Keyes. At about the same time that the importance of *S. mutans* was reinstated, J. F. Heremans isolated and characterized immunoglobulin A (IgA), and T. B. Tomasi, a few years later, identified the secretory form of IgA (S-IgA) in external secretions, including saliva. Soon thereafter, several research groups took up the challenge of developing a vaccine against mutans streptococci, as they reasoned that it was possible to prevent dental caries by inducing salivary S-IgA antibodies against the causative bacterial agents.

In addition to these major discoveries regarding caries etiology and S-IgA, other important factors that facilitated the undertaking of caries vaccine development included the development of quantitative caries models in experimental animals, the identification of critical virulence proteins of mutans streptococci, and the emerging understanding of the mechanisms operating in the mucosal immune system by which secretory S-IgA antibodies are elicited in salivary and other secretions (Table 1). Besides their significance in establishing proof-of-principle for induction of caries immunity, the pioneering caries vaccination studies led to the development of several concepts in mucosal immunity. In fact, the first ever demonstration that oral immunization of human subjects could lead to the generation of S-IgA antibodies in secretions was performed using *S. mutans*. Other early studies involved a different approach to caries immunization. These studies reported that protection against caries in monkey models could be mediated by serum IgG antibody elicited by systemic immunization with *S. mutans*. Overall, systemic or mucosal

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TABLE 1 Historical milestones in caries immunity

- "Mutans streptococci" as the principal cariogenic organisms
- S-IgA as the major salivary form of antibody
- Serotypes (now separate species) of mutans streptococci; cell wall antigens
- Glucosyltransferases and sucrose in glucan formation
- Development of animal models: rodents and primates
- Identification of surface proteins on mutans streptococci; adhesins; AgI/II
- Origins of salivary IgA within the integrated mucosal immune system
- Mother-infant transmission of mutans streptococci

immunization studies in rodents or monkeys, using mutans streptococcal antigen preparations, demonstrated that it was possible to induce protective immunity that could suppress the formation of carious lesions in the presence of a high-sucrose diet. Initial fears that immunization with whole mutans streptococci or ill-defined complex antigen preparations could elicit heart-reactive antibodies or serological cross-reactivity with human tissues were not adequately substantiated or explained. Furthermore, the identification of specific target antigens, particularly glucosyltransferases, glucan-binding proteins, and the AgI/II family of adhesins, has afforded the opportunity of developing vaccines that are demonstrably free of potential adverse consequences.

Since the first essential condition for vaccination against any infectious disease is to define the causative agent, the concept of vaccination against periodontal disease was inevitably slower to emerge. Indeed, such notions began to develop only after specific microorganisms, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* (in chronic and severe periodontitis) and *Aggregatibacter actinomycetemcomitans* (in localized aggressive periodontitis), were implicated as putative etiologic agents. However, there are additional challenges with regard to the development of vaccines against periodontitis. First, despite the importance of the above-mentioned species, additional bacteria seem to be involved in periodontal pathogenesis, and moreover, a significant percentage of the periodontal bacterial species are as yet uncultivable. It has also become evident that, in contrast to dental caries, which arise from the direct assault of bacterial products on a tissue, periodontal disease results as much from the undesirable side effects of the host immune response as from direct toxic effects of the causative organisms. This is conclusively demonstrated by the finding that severe combined immunodeficient mice, which lack both T and B cells and cannot mount adaptive immune responses, develop substantially less *P. gingivalis*-induced bone loss than immunocompetent mice. Moreover, mice genetically deficient in anti-inflammatory molecules, such as interleukin-10 (IL-10), are particularly susceptible to periodontitis as a result of impaired regulation of the inflammatory response. Therefore, although periodontitis is initiated by certain bacterial species, the key to success in developing a vaccine against this disease is an understanding of the immunopathological mechanisms that result in inflammatory damage to the periodontium while generally failing to control the pathogens.

Dental caries and periodontal disease account for the great majority of oral diseases and are discussed in more detail below. However, other

oral infections may also be amenable to immunological intervention by means of a vaccine. Oropharyngeal candidiasis is one potential candidate, but vaccination efforts are not as advanced as those for caries or even periodontal disease. Moreover, recent advances in the development of vaccines against genital herpesvirus and the release of two vaccines against carcinogenic types of human genital papillomavirus suggest that similar approaches may be effective against their oral equivalents.

THE MUCOSAL IMMUNE SYSTEM FROM AN ORAL PERSPECTIVE

Mucosal tissue encounters with foreign antigens and pathogens can result in the induction of mucosal and serum antibody responses and T-cell-mediated immunity. The mucosal immune system is anatomically and functionally divided into inductive and effector sites. Inductive sites are organized tissues in which foreign antigens are encountered and selectively taken up for initiation of immune responses. The more diffuse collections of B and T lymphocytes, differentiated plasma cells, antigen-presenting cells (APC; dendritic cells, macrophages, and major histocompatibility complex class II-positive B cells), as well as eosinophils, basophils, and mast cells in the lamina propria or the exocrine gland interstitium form the effector sites of mucosal immunity. The mucosal inductive sites, where S-IgA responses are initiated, include the Peyer's patches of the gut-associated lymphoid tissues (GALT) and similarly organized follicular lymphoid tissues in the large intestine and possibly in the bronchial tract, as well as the tonsils and adenoid in the pharynx (Waldeyer's ring) in humans (Fig. 1), which are the equivalent of the nasopharynx-associated lymphoid tissues (NALT) in rodents. The homing of activated B and T lymphocytes from GALT to effector tissues such as the lamina propria of the gastrointestinal tract, or from NALT to the nasal passages and the upper respiratory tract, forms the basis of what was initially perceived as the "common" mucosal immune system, a system that interconnects the inductive with the effector sites. However, over the years, evidence has accumulated that the mucosal system is not uniform but that particular effector sites are selectively supplied with mucosal B and T cells induced in a limited set of inductive sites. In the case of the salivary glands, the inductive sites appear to lie largely (but not exclusively) in the Waldeyer's ring in humans or the NALT in rodents, and to a lesser extent in the GALT.

These mucosal inductive tissues are covered by a specialized epithelium that contains M cells, which differ from the surrounding epithelial cells in that they possess a microfold apical membrane that can ingest antigenic materials and pass them through, by a transcytotic vesicular mechanism, to the underlying areas containing APC, B-cell zones with germinal centers, and adjacent T-cell areas. When stimulated by APC presenting processed antigenic peptides and antigen-sensitized T cells, the B cells in these mucosal inductive sites preferentially switch to express IgA as well as chemokine and other "homing" receptors. They then emigrate through the draining lymphatics into the circulation and home to mucosal effector sites while differentiating into polymeric IgA-secreting cells (Fig. 1). At the mucosal effector sites, the terminally differentiated plasma cells secrete large quantities of polymeric IgA close to the basolateral surfaces of the

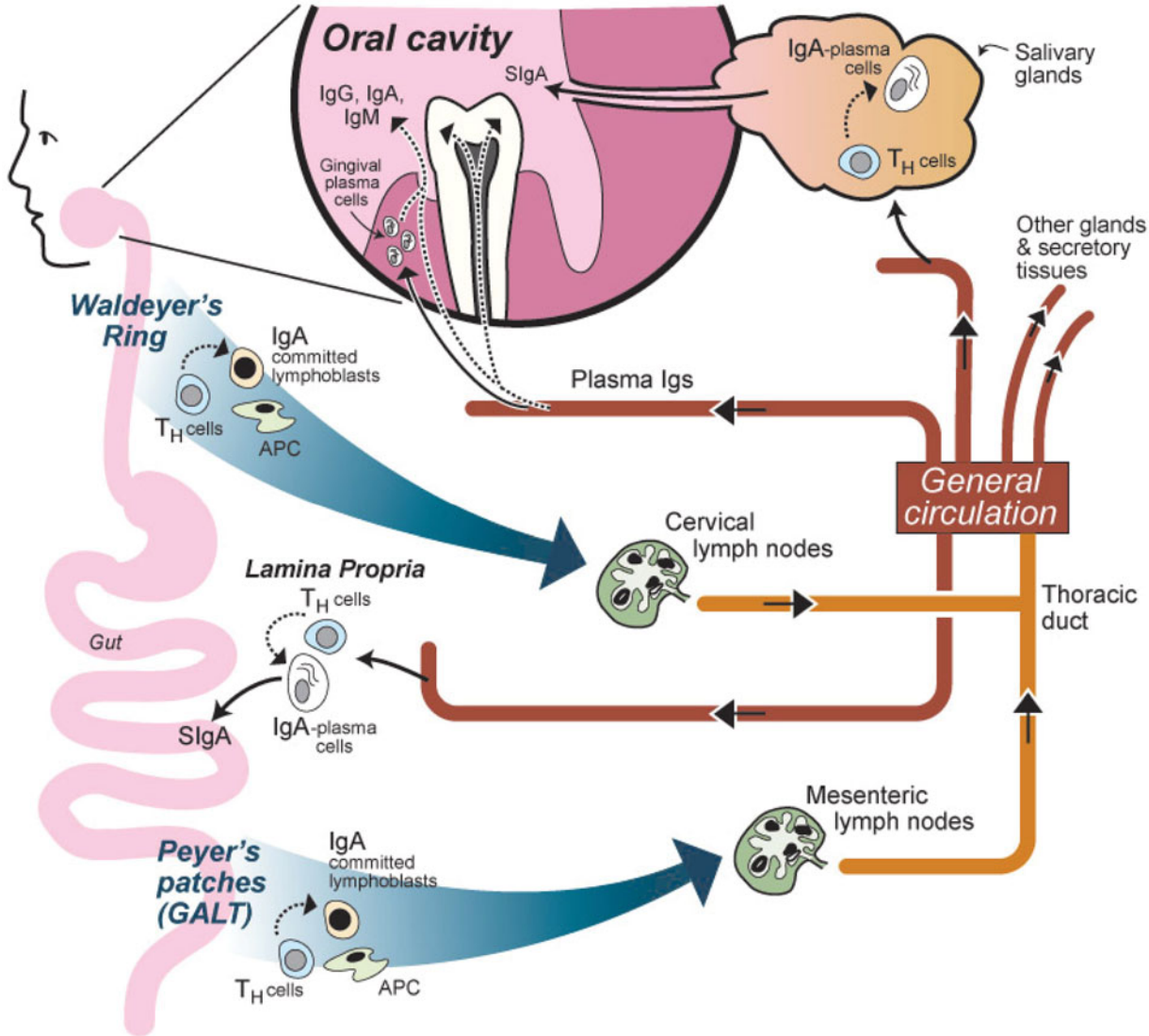

FIGURE 1 The secretory immune system from an oral perspective. Naïve mucosal B and T cells are stimulated by antigens that are processed by APC in central mucosal inductive sites, such as the Peyer's patches in the small intestine (GALT) or the pharyngeal Waldeyer's ring in humans (nasal lymphoid tissue in rodents). This results in the generation of specific IgA-committed B cells, which along with T cells migrate through the lymphatics, enter the circulation, and home to remote mucosal effector sites, including the salivary glands. In the salivary glands, the antigen-sensitized and IgA-committed B cells terminally differentiate into plasma cells that produce pIgA antibodies, which subsequently appear in the oral cavity as S-IgA antibodies. Small quantities of IgM, IgG, and IgA can transude from the circulation through the gingival crevices into the oral cavity. In advanced periodontitis, B cells localize in periodontal tissues and upon terminal differentiation into plasma cells secrete IgG, IgM, and monomeric IgA antibodies that enter the oral cavity through the gingival crevice. From Hajishengallis and Russell, Molecular approaches to vaccination against oral infections, *In* A. Rogers (ed.), *Molecular Oral Microbiology*, Caister Academic Press, Norfolk, United Kingdom, 2008. Used by permission.
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epithelia under which they reside. Cells in single-layered mucosal epithelia usually express a basolateral membrane receptor known as polymeric Ig receptor (pIgR), which binds J-chain-containing pIgA and transports it through vesicular transport to the apical surface. During transcytosis, the pIgR becomes covalently attached through disulfide exchange to the pIgA and subsequently is proteolytically cleaved from its transmembrane segment, forming the secretory component of S-IgA, which is thereby released into the lumen.

As the mucosal immune system is by far the largest component of the entire immune system, the production of IgA far exceeds the production of all other antibody isotypes. Daily production of S-IgA in adult humans has been estimated to amount to 3 to 8 grams, and the salivary output is in the range of 0.1 to 0.2 g/day. Not surprisingly, therefore, S-IgA antibodies constitute the main Ig isotype found in human saliva.

MUCOSAL VACCINATION ROUTES AND ADJUVANTS

Vaccines intended to induce salivary IgA antibody responses will need to target mucosal inductive tissues, preferably those located in the pharynx or intestinal tract. This presents several problems. The gastrointestinal tract is a hostile environment for many putative vaccine proteins owing to denaturation and degradation by stomach acid and digestive enzymes. The efficient uptake of vaccine proteins may also be problematic, so it is desirable to target the vaccine to the M cells for enhanced uptake. Micro- or nanoparticles are more readily taken up than soluble molecules, and a variety of particulate formulations, including polymer particles and liposomes, have been devised with various degrees of success. Besides enhancing the uptake, these approaches may also afford a degree of protection against stomach acid and digestive enzymes. A more challenging problem is that soluble antigens, especially purified proteins, have the tendency to induce a state of specific unresponsiveness rather than active immunity when applied to mucosal surfaces of the intestine, a phenomenon known as oral or mucosal tolerance. In part, this is because purified vaccine antigens generally fail to induce appropriate activation signals in APC, such as dendritic cells. These activation signals are readily induced by noxious microbial molecules, or intact pathogens, and are essential for bridging innate immune recognition to activation of adaptive immunity. An effective vaccine, therefore, would need to mimic these pathogenic stimuli in an appropriate manner, i.e., without concomitant toxicity. This can be accomplished through the use of safe and effective adjuvants in vaccine formulations.

Mucosal adjuvants and live attenuated viral or bacterial delivery systems are generally required for induction of effective mucosal immune responses. Adjuvants offer the advantage of eliciting mucosal as well as systemic immune responses. Some of the most effective strategies include the use of heat-labile enterotoxins and derivatives thereof, either as adjuvants or as coupled delivery agents. Two bacterial enterotoxins (cholera toxin [CT] and *Escherichia coli* heat-labile enterotoxin toxin [LT-I]) and their nontoxic mutants (mCT and mLT-I) are well-established mucosal adjuvants for the induction of both mucosal and systemic immunity to coadministered protein antigens. Mucosal administration of vaccine

together with CT or nontoxic mCTs induces $CD4^+$ Th2-type cells with characteristic plasma IgG and IgA as well as mucosal S-IgA antibody responses. Also effective as mucosal adjuvants are the type II *E. coli* heat-labile enterotoxins (LT-II), the B pentameric subunits of which uniquely stimulate Toll-like receptor (TLR) signaling. In addition, numerous studies have used recombinant *Salmonella* (and other bacteria) as oral delivery vehicles, and in most instances, this is an effective way to induce S-IgA antibody responses. For example, a recombinant attenuated *Salmonella* vector expressing the Tox C fragment of tetanus toxin elicits strong serum IgG and mucosal S-IgA anti-tetanus toxin antibody responses. Furthermore, splenic and Peyer's patch $CD4^+$ T cells from mice orally immunized with the Tox C-expressing *Salmonella* vector, when restimulated in vitro with tetanus toxoid, selectively induce Th1-type cytokines gamma interferon (IFN-γ) and IL-2 as well as the regulatory cytokine IL-10, implying the establishment of T-cell memory.

The discovery of TLRs has not only resulted in a resurgent interest in innate immunity but additionally suggested novel approaches to adjuvant development. The property of TLRs to function as adjuvant receptors is based on their ability to recognize and respond to conserved pathogen-associated molecular patterns. Pathogen-associated molecular patterns, which are primary targets of innate immune receptors, effectively alert the immune system and stimulate APC. At the molecular level, TLR-stimulated APC provide costimulatory signals for T-cell activation, ultimately leading to induction of cell-mediated or humoral immunity. In this regard, several adjuvants that have been shown to help poorly immunogenic proteins to induce an immune response are substances of microbial origin that activate TLRs. For example, monophosphoryl lipid A is a chemically modified form of the lipid A from *Salmonella* lipopolysaccharide that activates TLR4.

Although intranasal immunization effectively induces antigen-specific immunity in mucosal and systemic compartments, there is concern that some nasal immunization strategies involve the risk of antigen trafficking into the olfactory tissues and the central nervous system. To overcome this potential problem, sublingual immunization may be a safe alternative. Sublingual administration of antigens involves a noninvasive route, which has the advantage of requiring lower doses of antigen than the oral route, because it avoids exposure of the antigen to stomach acid and proteolytic enzymes in the gut. Several recent studies have used the sublingual route for vaccine delivery and have demonstrated the feasibility and effectiveness of inducing protective immunity.

RATIONALE FOR VACCINATION AGAINST DENTAL CARIES

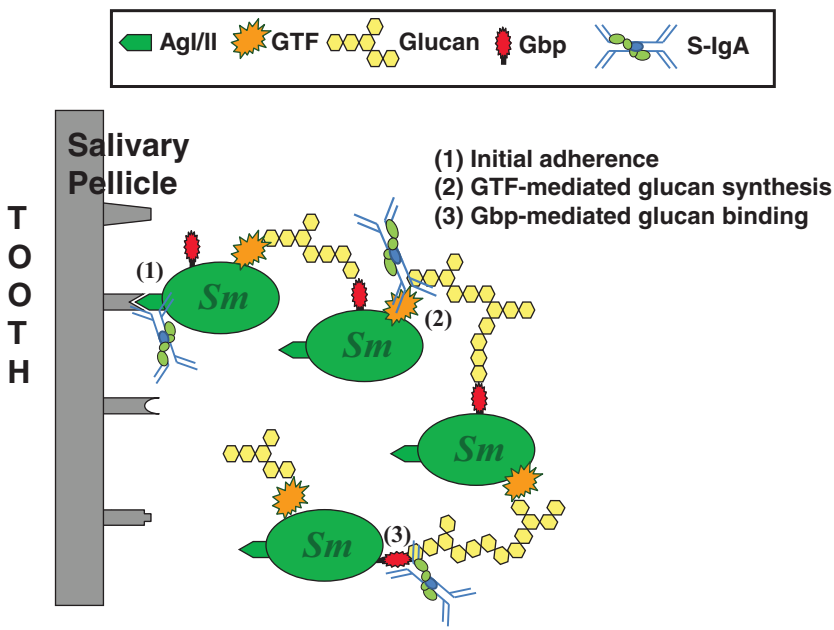
Dental caries is the result of localized demineralization of tooth enamel by, primarily, lactic acid secreted by acidogenic oral bacteria that ferment dietary sugars. However, there is a strong requirement for tooth colonization and biofilm formation for the bacterial acid production to reach sufficiently destructive levels. The causative bacteria are predominantly the mutans streptococci, mainly *S. mutans* and *S. sobrinus*. These organisms are thought to colonize tooth surfaces through a sucrose-independent initial adherence to the salivary pellicle that coats dental enamel, followed

by a sucrose-dependent process that consolidates interbacterial adherence and allows microbial accumulation and biofilm formation (Fig. 2). Studies on the molecular and functional characterization of key virulence factors in in vitro and in vivo quantitative models of dental caries have been crucial for the selection of appropriate caries vaccine targets. These include the family of AgI/II salivary adhesins, secreted or cell-associated glucosyltransferase (GTF) enzymes that produce adhesive glucans, and a group of glucan-binding proteins (Gbps). At least in principle, immunization to induce S-IgA antibodies against these virulence factors can interfere with the initial adherence and subsequent sucrose-dependent cell-to-cell accumulation of mutans streptococci (Fig. 2).

S-IgA represents the predominant isotype of antibody found in human saliva and thus the one to which oral microorganisms are mostly exposed. The S-IgA isotype is relatively resistant to proteolysis (and hence appropriate to function in the harsh environment of the oral cavity) and displays strong antiadherence and neutralizing activities (e.g., against microbial enzymes or toxins). Thus, the primary rational objective for caries vaccine development has been to induce production of salivary S-IgA antibodies that can block the sucrose-independent or sucrose-dependent colonization mechanisms of mutans streptococci.

As pointed out above, mucosal immunization strategies induce the production of both S-IgA antibodies in secretions and IgG (and monomeric IgA) antibodies in the circulation. Although S-IgA antibodies in

FIGURE 2 Tooth colonization by *S. mutans* and potential interference by S-IgA antibodies. *S. mutans* (*Sm*) uses its AgI/II adhesin to bind a receptor on the salivary pellicle that coats the tooth enamel (1). Subsequent production of extracellular glucans by GTF (2) and cell-to-cell adherence facilitated by the Gbps (3) allow the accumulation of *S. mutans* cells to a critical density at which the biofilm becomes pathogenic, i.e., secreting lactic acid at levels that can erode the tooth enamel. S-IgA antibodies can interfere with each of these steps (1 to 3) and suppress mutans streptococcal colonization. doi:10.1128/9781555818906.ch20.f2



saliva represent the primary defense mechanism against tooth colonization by mutans streptococci, circulating IgG antibodies may also be protective against caries at the gingival margin of the tooth, since the gingival crevicular fluid contains IgG, which is mainly derived by transudation from plasma (Fig. 1). IgG antibody can protect against caries by mediating opsonophagocytosis and killing of mutans streptococci by crevicular neutrophils. This mechanism may be particularly relevant during tooth eruption, when inflammation is common.

Naturally occurring S-IgA or IgG antibodies in saliva against mutans streptococci have been correlated positively or negatively with past experience of dental caries (as measured by indices of decayed, missing, and filled teeth). Therefore, the findings have been variously interpreted to suggest that antibodies can confer protection, or conversely, that they reflect previous exposure to mutans streptococci. In this regard, most of these studies have been cross-sectional in nature and conducted with adults, where high levels of salivary antibodies are more likely to reflect cumulative caries experience and antigenic challenge. Moreover, naturally occurring antibodies are not necessarily induced against the appropriate antigenic targets (i.e., potentially protective immunogens) and are unlikely to be present at sufficient levels during the establishment of mutans streptococci in young children. Additional confounding factors that complicate the interpretation of such correlative studies include uncontrolled variables such as diet and fluoride exposure. On the other hand, preventative active immunization of rodents or monkeys has provided proof-of-concept evidence for protective immunity against dental caries induced by mutans streptococci in the presence of a high-sucrose diet.

SALIVARY ADHESINS AS IMMUNOGENS AGAINST DENTAL CARIES

Mutans streptococci are not among the primary colonizers of the early dental biofilm. However, they can gain initial access to the tooth surfaces by means of AgI/II adhesins that recognize receptors on the salivary pellicle-coated enamel (such as the salivary glycoprotein gp340) or, indirectly, on the surface of early colonizing bacteria. *S. mutans* AgI/II has been implicated as a virulence factor in the rat caries model, in which wild-type *S. mutans* induces more carious lesions than an isogenic mutant lacking AgI/II.

The gene encoding the 167-kDa AgI/II adhesin (also variously known as antigen B, P1, PAc, SpaP, or SR) is highly conserved between serotypes of mutans streptococci and is homologous with genes from other streptococcal species encoding surface proteins. AgI/II proteins are characterized by alanine- and proline-rich repeat regions (A and P regions, respectively) in the N-terminal and middle thirds of the molecule, respectively. The precise functions of individual proteins of the AgI/II family vary, presumably reflecting species-specific adaptations for colonization at different sites.

Recent crystallographic analysis of *S. mutans* AgI/II has revealed that the adhesin displays an extended fibrillar structure, in which the A and P regions interact intimately with each other (Fig. 3). AgI/II appears to interact with its receptor in the salivary pellicle (gp340 salivary agglutinin) via two binding sites, one located distally to the cell wall and comprising A and P regions (specifically the A3 and P1 repeats) and another in the

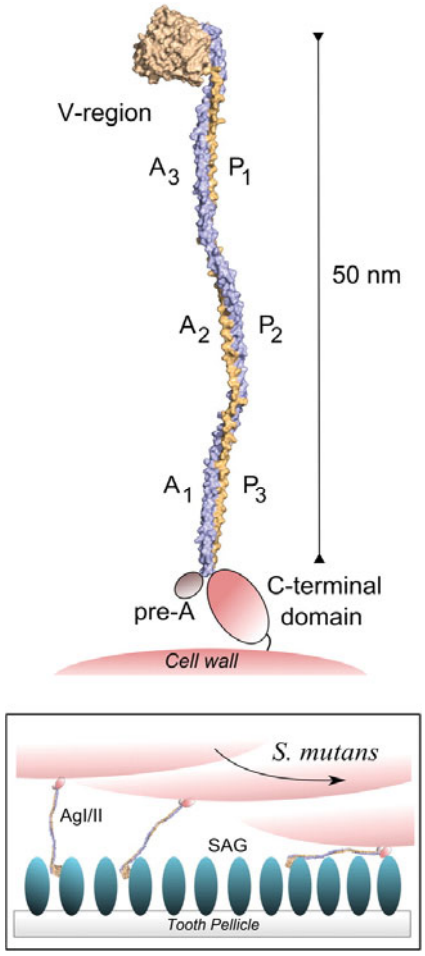


FIGURE 3 Model of the AgI/II structure and its interaction with its salivary receptor. AgI/II is approximately 167 kDa and contains >1,500 amino acid residues. The A region typically consists of three tandem repeats of 82 residues rich in alanine, and the P region usually has three tandem repeats of 39 residues rich in proline. The crystal structure of the *S. mutans* AgI/II has revealed that this adhesin displays an extended fibrillar structure, in which the A and P regions interact intimately with each other. Structural/functional studies suggest that the A and P regions interact to form a binding domain (specifically comprising the A3 and P1 repeats) that recognizes a receptor (salivary agglutinin [SAG]) deposited in the dental pellicle. A second AgI/II binding site for the SAG receptor is localized in the C-terminal domain close to the cell wall. The C-terminal domain consists of a typical gram-positive wall anchor and membrane-spanning segment by which it is linked to the cell wall peptidoglycan. From Larson et al., *Proc. Natl. Acad. Sci. USA* **107**:5983–5988, 2010. Used by permission. doi:10.1128/9781555818906.ch20.f3

C-terminal domain close to the cell wall (Fig. 3). Importantly, human S-IgA antibodies to AgI/II have been shown to inhibit the binding of isolated AgI/II and of AgI/II-bearing *S. mutans* cells to in vitro models of salivary pellicle-coated enamel. The antiadherence activity of S-IgA is significantly more potent than that of monomeric IgA or IgG antibodies. Nevertheless, IgG antibody to AgI/II can inhibit the saliva-mediated interbacterial adherence between *S. mutans* and early colonizers of the salivary pellicle. Therefore, antibodies to AgI/II have the potential to block both the direct and indirect anchoring of mutans streptococci to the tooth surface. *S. mutans* AgI/II and *S. sobrinus* SpaA (an AgI/II-family protein adhesin) cross-react serologically, owing to an overall 66% amino acid sequence identity. They may thus act as immunogens that confer cross-protection following immunization with either antigen.

A potential limitation for a protective role of S-IgA antibodies arises from the ability of bacterial IgA1 proteases to cleave human IgA1 into intact Fab and Fc fragments. These enzymes are produced by several species of oral streptococci including *S. gordonii*, *S. oralis*, and *S. mitis* (though *not* mutans streptococci) and may therefore be found in saliva. Cleavage of S-IgA1 by IgA1 protease counteracts the *S. mutans* adherence-inhibiting effect of S-IgA1 antibodies (Table 2).

TABLE 2 Potential immunological intervention against prototypical cariogenic or periodontopathic organisms and candidate immunogens

Virulence function	Anti-*S. mutans* strategies	Anti-*P. gingivalis* strategies
Colonization	Inhibition of binding to salivary pellicle or to early colonizers Immunogen: AgI/II Inhibition of glucan synthesis or glucan-mediated biofilm formation Immunogens: GTF, Gbp	Inhibition of binding to salivary pellicle, to early colonizers, or to gingival epithelium Immunogen: fimbriae
Bacterial persistence (resistance to killing)	Opsonization and phagocytosis Immunogen: AgI/II	Opsonization and phagocytosis Immunogens: fimbriae, gingipains
Invasion	Inhibition of entry into dentinal tubules Immunogen: AgI/II	Inhibition of invasion into epithelial cells Immunogen: fimbriae
Immune evasion	Neutralization of IgA1 protease activity in the oral cavity (hypothetical)	Inhibition of degradation of complement, cytokines, and innate immune receptors Immunogen: gingipains
Direct bacterially induced tissue destruction	Inhibition of metabolism, sugar uptake, acid production (hypothetical) Immunogens: not determined	Inhibition of gingipain-mediated tissue degradation Immunogen: gingipains

The ability of vaccines to induce immunity to dental caries in experimental animals is revealed by decreased colonization of tooth surfaces by implanted mutans streptococci and by reduction of subsequent carious lesion formation. The protective potential of purified AgI/II has been demonstrated by systemic or mucosal (intranasal) immunization of rhesus monkeys and rats in the presence of adjuvants. Similar protective effects have also been obtained using defined segments of it. For instance, intranasal vaccination of mice with cholera toxin subunit B (CTB) coupled to a 19-residue peptide (amino acids 301 to 319) from the N-terminal region of AgI/II resulted in strong inhibition of *S. mutans* colonization, although protection against caries was not addressed. However, a 42-kDa segment covering the A-repeat region of AgI/II (designated the saliva-binding region [SBR]), coupled to CTB and coadministered intranasally with an adjuvant dose of CT, has been shown to confer protection against caries in rats.

The SBR has additionally been shown to protect against oral colonization of *S. mutans* and caries formation in mice when delivered intranasally by an attenuated *Salmonella* vector. AgI/II contains at least two saliva-binding sites, and SBR may not be the only protective immunogenic region of AgI/II. Indeed, intact AgI/II immunogen confers improved anticaries protection compared to SBR, although the salivary IgA anti-SBR responses induced by the two immunogens are quite comparable. In this regard, topical application of monoclonal antibodies recognizing epitopes toward the C-terminal part of AgI/II prevents tooth colonization by *S. mutans* in nonhuman primates and results in significant caries reduction. Topical application of the same monoclonal antibodies prevents colonization of exogenously implanted strains of *S. mutans* or natural recolonization by indigenous *S. mutans* following oral prophylactic treatment, but a clinical trial in humans was inconclusive.

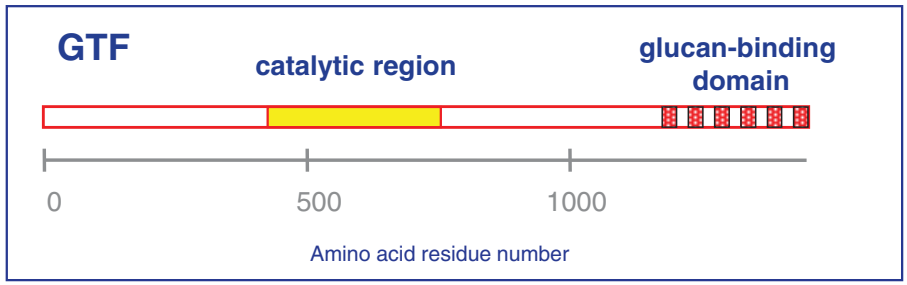
The mechanisms of caries protection through AgI/II-based vaccination may not be restricted to the ability of S-IgA antibodies to inhibit access of mutans streptococci to the tooth surfaces. For instance, gingival crevicular fluid-derived IgG antibodies to AgI/II can promote opsonophagocytosis and killing of cariogenic streptococci by neutrophils. Interestingly, AgI/II binds collagen type I and thereby helps *S. mutans* and *S. sobrinus* to invade root dentinal tubules. Therefore, IgG antibodies to AgI/II derived from the dental pulp might block dentinal tubule invasion by mutans streptococci and help to arrest developing lesions (Table 2).

CARIES IMMUNIZATION USING GTF AND GLUCAN-BINDING PROTEINS

The capacity of mutans streptococci for cell-to-cell adherence and development of cohesive and pathogenic biofilms depends heavily on the expression of GTFs. These enzymes (140 to 160 kDa) produce extracellular adhesive glucans that vary in chain length, content of α-1,3 and α-1,6 glucosyl linkages, and degree of branching and solubility. The role of GTF in cariogenicity has been established in animal caries models, in which isogenic mutants of *S. mutans* lacking one or more *gtf* genes became attenuated in terms of tooth colonization and induction of carious lesions. *S. mutans* expresses three genes for GTF: *gtfB*, responsible for insoluble glucan synthesis; *gtfC*, for soluble and insoluble glucan synthesis; and *gtfD*, for soluble glucan synthesis. *S. sobrinus* expresses *gtfI* and *gtfS*, encoding enzymes that produce insoluble and soluble glucans, respectively. Epitope mapping studies have indicated that the catalytic region is located in the N-terminal one-third of GTF, whereas a glucan-binding region is located in the C-terminal one-third (Fig. 4). Also important for mutans streptococcal virulence is the expression of distinct proteins that bind the glucans synthesized by GTF, the glucan-binding proteins (Gbps), and thereby mediate interstreptococcal adherence. *S. mutans* expresses three cell wall-associated Gbps (GbpA, GbpB, and GbpC), and *S. sobrinus* produces five Gbps (1 to 5).

It becomes evident, therefore, that antibody-mediated interference with the enzymatic or glucan-binding activities of the GTFs or Gbps may

FIGURE 4 Schematic representation of mutans streptococcal glucosyltransferases. GTFs are approximately 150 kDa and contain >1,300 amino acid residues. Epitope mapping, functional studies, and modeling based on amino acid sequences indicate that the catalytic regions of GTFs are located within the N-terminal halves of the molecules and that glucan-binding domains occur as a series of repeats at the C termini. doi:10.1128/9781555818906.ch20.f4



be a reasonable preventative approach against the establishment of cariogenic mutans streptococcal biofilms. In this regard, human salivary IgA antibodies inhibit the enzymatic activity of GTF. Moreover, IgG antibodies raised in animals against the catalytic or the glucan-binding region of GTFs inhibit glucan synthesis. Similar results have been obtained using IgG antibodies against synthetic peptides corresponding to specific sequences within the catalytic or glucan-binding region of GTFs. GTFs from *S. mutans* or *S. sobrinus* have been demonstrated to induce cross-neutralizing antibody responses, which are protective against experimental caries induced by infection with either species. In contrast, there is no significant antigenic cross-reactivity between *S. mutans* and *S. sobrinus* Gbps.

Oral immunization of rats with soluble or liposome-encapsulated intact GTF results in induction of salivary IgA antibodies that interfere with mutans streptococcal colonization and caries activity. Immunization of rats by subcutaneous injection (in the vicinity of salivary glands) of peptide sequences corresponding to either the catalytic or the glucan-binding domains of GTF suppresses *S. mutans*- or *S. sobrinus*-induced caries, presumably through inhibition of glucan synthesis. In similar vaccination studies using multiple antigenic peptide constructs that include epitopes from both the catalytic and glucan-binding regions of GTF, serum IgG and salivary IgA antibody responses and protection against caries were found to be higher than when a simple mixture of both peptides was used as immunogen. These antibodies also inhibit glucan formation by GTF in vitro. Additional diepitopic peptide constructs containing GbpB peptides together with sequences from the catalytic (CAT) or glucan-binding domains of GTF have been used to immunize rats. The most immunogenic construct is the GbpB-CAT combination, which elicits significant levels of serum IgG and salivary IgA antibodies to both components and induces protective immunity against *S. mutans*- or *S. sobrinus*-induced caries.

Few caries immunization studies using subunit vaccines have been performed in adult human volunteers. One such study involved oral immunization with GTF together with aluminum phosphate adjuvant. The vaccine induced a modest and short-lived salivary IgA response, which, however, succeeded in delaying the recolonization of indigenous mutans streptococci following dental prophylaxis, compared to nonimmunized control subjects. Salivary IgA responses to GTF have also been induced by intranasal immunization using liposome-encapsulated GTF alone or in the presence of monophosphoryl lipid A adjuvant.

TARGETING BOTH SUCROSE-INDEPENDENT AND SUCROSE-DEPENDENT COLONIZATION

Chimeric polypeptide immunogens comprising the SBR of AgI/II and a glucan-binding segment of GTF have been constructed with the objective of inducing immunity that might interfere with both the initial adherence of *S. mutans* to salivary pellicle-coated tooth surfaces and the ensuing sucrose-dependent accumulation of this cariogenic pathogen. One such chimeric construct has been shown to induce higher anti-SBR and anti-glucan-binding region antibody responses than its constituent parts, resulting in significant inhibition of *S. mutans* colonization. Consistent

with the proposed roles of AgI/II and GTF in sucrose-independent and sucrose-dependent adherence, respectively, rabbit IgG antibodies raised against a similar chimeric protein (i.e., comprising the AgI/II SBR and the GTF glucan-binding region) inhibit both the initial and the subsequent glucan-mediated adherence of *S. mutans* to a model tooth surface in vitro.

An interesting new development pursued particularly in China has focused on the use of DNA vaccines, which are intended to induce the synthesis of protein antigens in the recipient's tissues and thereby induce immune responses. For example, immunization of rats subcutaneously with a DNA vaccine encoding both the glucan-binding region of GTF and an AgI/II segment including both the SBR and the P region elicits serum and salivary antibody responses to both components and confers protection against caries. This DNA vaccine has been modified to include the catalytic region sequences of *S. sobrinus gtfI*, and mice immunized intranasally with this construct generated serum IgG and salivary IgA antibodies to all three encoded antigens and were protected against caries induced by *S. sobrinus* or *S. mutans*. The increased protection against *S. mutans*-induced caries was attributed to the high degree of homology between the amino acid sequences of *S. mutans* and *S. sobrinus* GTF.

From the studies discussed above, it becomes apparent that it is technically feasible to develop a vaccine against dental caries. Proof-of-principle evidence has been demonstrated in rodent and primate models that immunization elicits antibodies in serum and saliva against key antigenic targets of mutans streptococci, leading to reduced colonization and diminished development of dental caries. Mechanistic studies in vitro have moreover illustrated plausible mechanisms of action of these antibodies in inhibiting key steps in caries pathogenesis. However, these pivotal findings have not yet been translated into human clinical trials.

SAFETY CONSIDERATIONS AND PROSPECTS FOR A CARIES VACCINE

The first demonstration that oral immunization of human subjects with a bacterial suspension induces the production of S-IgA antibodies in mucosal secretions such as saliva was performed using whole *S. mutans* cells. Although this study was pivotal for establishing the concept of the "common" mucosal immune system, there were fears over the safety of immunization with whole mutans streptococci. However, the identification of important target antigens, particularly GTFs, Gbps, and the AgI/II family of adhesins, has led to the development of subunit vaccines free of potential adverse effects. In this regard, no molecularly defined antigen of mutans streptococci has been shown to cross-react serologically with human tissues or to induce heart-reactive antibody responses. It should be noted, however, that no vaccine is absolutely safe, as there is always a risk involved in any medical intervention.

Although these studies were useful for establishing safety and immunogenicity, they provided only limited information with regard to the preventative potential of caries vaccination. Indeed, adults are not the target population for a preventative vaccine, since mutans streptococcal colonization is already established in adults. Considering the natural history of tooth colonization by mutans streptococci and the ontogeny of

the salivary antibody response in young children, the most appropriate target population for a caries vaccine consists of infants at approximately 12 months of age. Although newborns do not have S-IgA in their saliva, their ability to elicit S-IgA antibody responses increases rapidly, and by 1 to 2 years of age their salivary IgA concentrations are comparable to those of adults. Colonization of the tooth surfaces by mutans streptococci occurs after the first year of age, i.e., at a time when infants are already immunologically competent with regard to eliciting S-IgA antibodies. Therefore, it should be possible to immunize young children of this age with appropriate streptococcal antigens to prevent or delay subsequent tooth colonization by mutans streptococci. One or more booster mucosal immunizations may be required, including one close to the time of the eruption of the permanent dentition, which opens up new niches for streptococcal colonization.

Passive immunization by the application of preformed antibodies against mutans streptococci may afford greater margins of safety. The generation of antibodies in milk by immunizing cows is an old concept that has been proposed for use against dental caries, but with little success so far. Promising results have been obtained using chicken egg yolk antibodies to GTF or Gpbs or using transgenic tobacco plants producing human S-IgA specific for AgI/II.

No caries immunization studies have yet been performed with young children, although such trials are essential for determining the feasibility and utility of a preventative caries vaccine. But is there a need for vaccination against dental caries? The incidence of dental caries has declined considerably in western nations over the past several decades, especially among the more affluent sectors of the population, owing mostly to the use of fluoride, improved access to professional dental care, and public awareness and education regarding better diet and oral hygiene habits. However, after many years of declining caries rates, childhood caries has risen in recent years, and it remains highly prevalent among underserved populations within both affluent and developing nations. Thus, a safe and effective vaccine could have significant impact among those groups having limited access to professional dental care, water fluoridation, or routine oral hygiene due to economic, behavioral, or cultural barriers. However, the need for a caries vaccine is a complex issue that involves both ethical and economic factors. There is general unwillingness to invest in a vaccine against a non-life-threatening disease. However, there is a link between *S. mutans* and fatal infectious endocarditis. Moreover, a caries vaccine could have a substantial impact in improving the quality of life (less morbidity, pain, suffering, and consequential effects) and reducing the significant financial costs associated with caries treatment.

VACCINE DEVELOPMENT AGAINST PERIODONTAL DISEASE

Periodontitis is an infection-driven chronic inflammatory disease that affects the integrity of the tooth-supporting tissues. Periodontal tissue degradation results in a large part from untoward inflammatory reactions arising from complex interactions between the host immune system and subgingival bacterial challenge. A unique feature of the periodontium

compared with other mucosal sites such as the gastrointestinal and respiratory tracts is that it consists of both mucosal and bone tissue. Thus, it is clear that the cellular and molecular interplay between mucosal and osteoimmune systems plays a critical role in the regulation of bone homeostasis. In this regard, receptor activator of NF-κB ligand (RANKL), a key differentiation factor for osteoclasts, is expressed by activated T and B cells in the inflamed periodontium. After almost 20 years of research seeking to understand the role of adaptive immunity in periodontitis, it is now clear that the disease cannot be adequately described in simple terms of the dichotomy between T helper types, i.e., Th1 versus Th2, which prevailed in the 1990s. However, the recent discovery of the Th17 subset, as well as of regulatory T-cell subsets, contributes to a more nuanced understanding of the role of adaptive immunity in periodontal disease.

The bacteria associated with periodontitis exist in a complex multispecies biofilm; however, it is thought that a more limited number of species are the major agents of human periodontal disease, for example, *P. gingivalis*. This pathogen, through its demonstrated ability to undermine critical innate host defenses, appears to promote the survival and virulence of the entire biofilm. Therefore, the concept of immunization against *P. gingivalis* as a means to control periodontal disease has a scientific basis, despite the many challenges associated with a periodontitis vaccine.

To control infectious diseases, most vaccine development efforts are aimed at conferring protection by antibody-mediated blockade of colonization factors, by neutralization of virulence enzymes or other factors involved in microbial evasion of immunity, or by opsonophagocytosis and killing of pathogens. All these approaches share the feature of decreasing the bacterial challenge rather than modulating the host response. Table 2 shows how these concepts apply to anti-*P. gingivalis* immunity in relation to similar vaccination strategies against *S. mutans* discussed above. However, what constitutes protective immunity against periodontitis has not yet been adequately elucidated, and at least some aspects of natural or vaccine-induced host responses may result in more harm than good. For example, opsonophagocytic modes of defense against bacterial infections always involve the collateral release of inflammatory mediators, tissue-destructive enzymes, and toxic substances such as reactive oxygen intermediates from the activated phagocytes, as well as the generation of split products of complement activation, which have chemoattractant and cell-activating properties. If the pathogens are eliminated by these mechanisms, then the inflammation is resolved and tissue healing ensues. In such cases, some initial tissue destruction would be a small price to pay. If the inflammatory response, however, does not eliminate the pathogenic insult, then the stage is set for persistent chronic inflammation. In addition, since activated lymphocytes are involved in RANKL-mediated activation of osteoclasts, a periodontal vaccine might have the undesired effect of stimulating osteoclastogenesis! Moreover, immunization might result in the formation of antibody-antigen complexes which can interact with Fc receptors on phagocytic cells and induce inflammation, including the generation of cytokines involved in bone resorption.

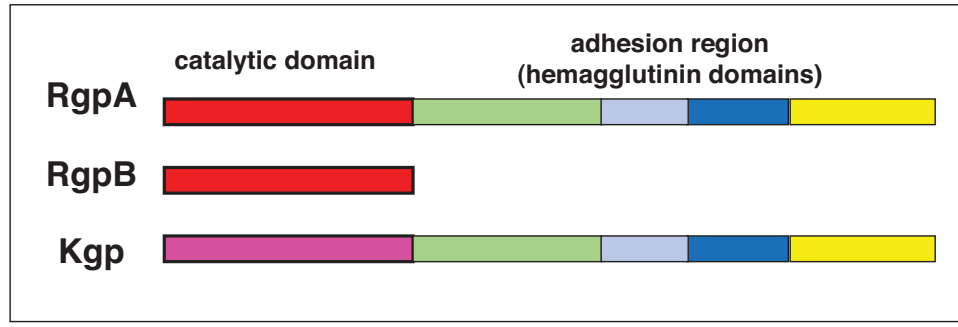
Nevertheless, a protective vaccine may be a feasible pursuit if appropriate immunogens and vaccination strategies that do not induce adverse

immune reactions can be identified. Our limited understanding of the immune regulatory mechanisms operating in periodontal disease pathogenesis means that the success of a candidate vaccine would have to be determined empirically. In contrast to caries vaccines, which are implicitly designed for prophylactic use, it is possible that periodontal vaccines could be more appropriate for therapeutic application in individuals already affected by the disease. This has the potential advantage that the principal target etiological agent(s) can be identified in each case. However, most experimental work in animal models so far has been designed to test periodontal vaccines in a prophylactic setting.

PROOF-OF-CONCEPT IMMUNIZATION AGAINST PERIODONTAL PATHOGENS IN RODENT MODELS

Despite the concerns discussed above, studies in rodents and nonhuman primates suggest the feasibility of developing a vaccine for the control of periodontal disease. The concept that vaccination against periodontal pathogens can confer protection against bone loss was first demonstrated in rodents immunized with whole bacterial cells or broken-cell preparations of *P. gingivalis* or *Eikenella corrodens*. However, due to the undesirable reactogenicity of these vaccines, subsequent efforts in animal models have focused on defined microbial molecules or genetically engineered subunits thereof. Subunit vaccine approaches have so far concentrated mainly on *P. gingivalis* virulence proteins, particularly its cysteine proteinases (RgpA, RgpB, and Kgp gingipains) (Fig. 5) as well as the fimbriae of *P. gingivalis* (Fig. 6) and of *A. actinomycetemcomitans*. Vaccination with defined subunit immunogens requires the use of appropriate adjuvants more than does immunization with whole bacterial cells, which contain intrinsic adjuvant substances (e.g., lipopolysaccharide). Some studies have used Freund's complete or incomplete adjuvant or CT, while others have used adjuvants already approved for human use, such as monophosphoryl lipid A, either alone or supplemented with trehalose dicorynomycolate.

FIGURE 5 Schematic representation of *P. gingivalis* gingipains RgpA, RgpB, and Kgp. The gingipains are a complex family of cysteine proteases, some of which contain hemagglutinin domains. RgpA and Kgp contain 1,706 and 1,723 amino acid residues, respectively (~190 kDa), and RgpB contains 736 residues (80 kDa). The catalytic domain of RgpA is largely homologous to RgpB, which lacks the hemagglutinin (adhesin) domain. The hemagglutinin domains of RgpA and Kgp are highly homologous. doi:10.1128/9781555818906.ch20.f5



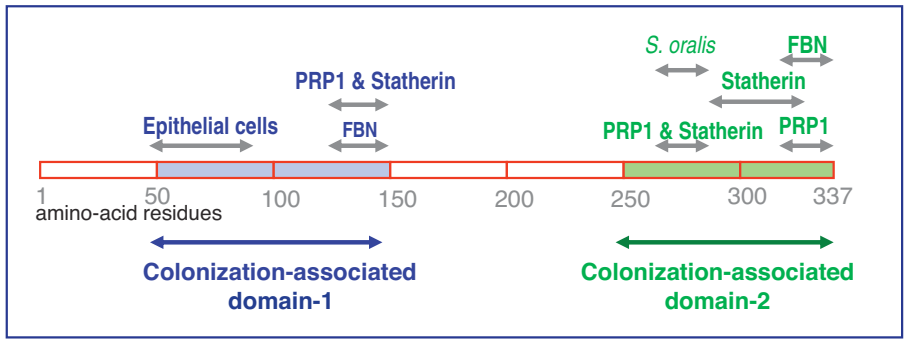


FIGURE 6 Schematic representation of *P. gingivalis* major fimbrial protein FimA. *P. gingivalis* FimA (37 kDa; 337 amino acid residues) contains multiple adhesion epitopes located at the N-terminal end and C-terminal one-third (colonization-associated domains 1 and 2, respectively). The adhesion epitopes mediate binding to salivary proteins (proline-rich protein 1 [PRP1] and statherin), to extracellular matrix proteins (fibronectin [FBN]), to epithelial cells, and to the early colonizing mitis group streptococci. doi:10.1128/9781555818906.ch20.f6

Subcutaneous immunization of rats with the hemoglobin-binding domain of gingipain from *P. gingivalis* results in specific IgG antibodies and modest protection against bone loss. The same immunogen administered with Freund's complete adjuvant potentiates the antibody responses but, strikingly, does not protect against bone loss. This paradoxical finding may have been due to inflammatory responses induced by the adjuvant leading to bone resorption. This study underscores the importance of selecting appropriate adjuvants that can enhance specific protective immunity without contributing to immunopathology. Rats immunized with a mixture of gingipains (RgpA and Kgp) in Freund's incomplete adjuvant generate high-titer serum IgG2a antibody responses to the gingipains, resulting in protection against *P. gingivalis*-induced periodontal bone loss and suppression of colonization by the pathogen.

Immunization by mucosal routes represents a more convenient and generally safer approach than systemic immunization and is the only method that can effectively generate S-IgA antibodies for protection against mucosal pathogens. However, many studies either have not addressed protection against disease (e.g., periodontal bone loss) or have failed to demonstrate it, although some have reported in vitro measures of potential efficacy. For example, intranasal immunization with recombinant hemagglutinin B from *P. gingivalis*, in the presence of monophosphoryl lipid A adjuvant, has been found to induce considerably higher serum IgG and salivary IgA antibody responses than immunization without adjuvant. Systemic or intranasal immunization of mice with a synthetic peptide (plus adjuvant) derived from the sequence of *A. actinomycetemcomitans* fimbriae resulted in preferential induction of specific serum IgG or salivary IgA antibodies, respectively. IgG antibodies to the peptide inhibit the adherence of fimbriated *A. actinomycetemcomitans* strains to experimental salivary pellicle.

Another approach to immunization against *P. gingivalis*-induced periodontal disease has exploited *S. gordonii* vectors that express cloned segments of the major fimbriae (FimA) of *P. gingivalis* (Fig. 6). Oral immunization of rats with these recombinant bacteria induces specific salivary

IgA and serum IgG antibody responses and protection against subsequent *P. gingivalis*-induced periodontal bone loss. CT or its derivatives have also been explored as mucosal adjuvants for vaccines against *P. gingivalis*. For example, intranasal immunization with *P. gingivalis* fimbriae plus CT adjuvant elicits S-IgA antibody responses in the submandibular glands. These antibodies inhibit attachment of *P. gingivalis* to epithelial cells and reduce inflammatory cytokine production by these cells.

Because of concerns about the safety of CT as an intranasal adjuvant, other approaches to enhancing intranasal immunization have been explored in the context of periodontal vaccines. For example, a 25-kDa antigenic region of *P. gingivalis* hemagglutinin A has been genetically fused to the *E. coli* maltose-binding protein. Mice immunized intranasally with this construct develop salivary S-IgA and serum IgG and IgA antibody responses leading to reduction of alveolar bone loss. The sublingual route of immunization may offer a safer alternative. Mice vaccinated sublingually with the 40-kDa outer membrane protein of *P. gingivalis* (along with another experimental adjuvant system) develop salivary S-IgA and serum IgG and IgA antibodies and protection against *P. gingivalis*-induced bone loss. Other studies have explored the use of novel adjuvant systems such as CpG oligodeoxynucleotides, which can stimulate TLR9, to induce immunity against *P. gingivalis*. Thus, it is clear that there are several promising ideas that suggest it should be possible to develop vaccines against periodontal disease. However, much more needs to be done to define appropriate antigens, adjuvant formulations, and immunization routes, in order to develop vaccine candidates that will be effective not only in inducing immune responses against the pathogens but also in suppressing the disease.

IMMUNIZATION OF NONHUMAN PRIMATES AGAINST PERIODONTAL DISEASE

Vaccination studies in monkeys are particularly valuable, as these animals are closer to humans than the more convenient and relatively inexpensive rodent models. However, studies using macaques to investigate immunity to periodontal disease have so far met with limited success. Immunization of monkeys with purified *P. gingivalis* cysteine proteinase in Freund's incomplete adjuvant elicited specific antibody responses but failed to suppress the emergence of this pathogen in the oral flora. Furthermore, there were no significant differences in periodontal bone loss between immunized and control animals. However, vaccination of monkeys with formalin-killed *P. gingivalis* elicited specific IgG responses and conferred significant protection against bone loss, although the inhibitory effect against *P. gingivalis* colonization was less pronounced. In another study, vaccination of monkeys resulted in decreased bone loss compared to nonimmunized controls, which correlated with dramatically suppressed levels of prostaglandin E2 in the gingival crevicular fluid. This study suggests that suppression of inflammatory mediators by immunization, perhaps owing to reduced pathogenic challenge as a result of antibody-dependent inhibition of colonization, constitutes a potentially protective mechanism against periodontitis. However, more studies are needed to define vaccination strategies and the immune response mechanisms that may be effective in controlling periodontal disease in primates.

CONCLUSIONS

Proof-of-principle evidence suggests that the development of a caries vaccine is a feasible objective. Indeed, immunization of rodents and primates resulting in antibodies in serum and saliva against key antigenic targets of mutans streptococci has been shown to suppress colonization by these organisms and to diminish the development of caries lesions. The key antigens include AgI/II, GTFs, and Gbps of *S. mutans* and *S. sobrinus*. However, translation of promising findings from animal models into human trials involves important ethical and economic issues. A safe, effective, and affordable vaccine could have significant impact, especially among underserved population groups that do not have ready access to modern professional dental care, and reduce the significant financial costs of treating caries.

The development of a vaccine for the control of periodontal disease presents more formidable challenges. Proof-of-principle studies in rodents show that immunization with functional domains of *P. gingivalis* gingipains and certain other protein antigens is a promising approach to control infection and inhibit periodontal bone loss. However, studies in nonhuman primates so far are largely inconclusive. Whether a gingipain-based vaccine will have a significant protective impact on periodontal disease in humans remains to be determined. Whereas specific immunity to known periodontal pathogens has been strongly implicated in protection against periodontitis, it has also become clear that the immune system itself plays an important role in the immunopathogenesis of periodontal disease. Currently, there is need for better understanding of the immune regulatory mechanisms operating in periodontal disease and for applying this knowledge in designing vaccines, including the selection of adjuvants. Besides enhancing specific immunity to target periodontal pathogens, vaccines should also elicit appropriate noninflammatory modes of immune response that mitigate tissue damage, resolve inflammation, and promote tissue healing. The challenge in periodontal vaccine development remains to fine-tune the host response to maximize protection and minimize its destructive aspects.

KEY POINTS

- Proof-of-principle has been demonstrated in rodent and primate caries models that immunization can elicit antibodies in serum and saliva against key antigenic targets of mutans streptococci and can suppress colonization by these organisms with subsequently diminished development of caries lesions.
- A safe, effective, and affordable caries vaccine could have significant impact among underserved populations without ready access to modern professional dental care, both within western nations and elsewhere.
- Whereas specific immunity to periodontal pathogens has been strongly implicated in protection against periodontitis, it has also become clear that the immune system itself plays an important role in the immunopathogenesis of this disease.
- The greater complexity of pathogenic mechanisms in periodontal disease compared to caries and the involvement of multiple putative periodontal pathogens complicate the development of a periodontitis vaccine. The challenge remains to fine-tune the periodontal host response to maximize protection and minimize its destructive aspects.

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Antibiotics and the Treatment of Infectious Diseases

Donald J. LeBlanc

ANTIBIOTICS: A CLASS OF THERAPEUTIC AGENT

Chemotherapeutic agents are chemical compounds used to treat diseases. A subgroup of these compounds is comprised of antimicrobial agents, which are used in the treatment of infectious diseases. The focus of this chapter will be antibiotics, which are either toxic to, or growth inhibiting for, bacteria. Antibiotics represent a separate class of antimicrobial agent, distinguished from antifungals (see Chapter 17), which are used to treat yeast and fungal infections, and antivirals (see Chapter 16), which aid in the treatment of viral infections. The distinction between antibiotics and antivirals is particularly important, because antibiotics are never effective against viral infections, whereas some antibiotics may also be effective in the treatment of certain fungal and, occasionally, parasitic infections.

Prior to the 20th century, people often died not of old age but in many instances in their 30s and 40s, if not in infancy, and more often than not from an infectious disease for which there was no treatment or cure. Paul Ehrlich, known as the "Father of Chemotherapy," began in 1910 a search for so-called magic bullets that would target specific causes of infectious diseases, with the introduction of Salvarsan, an arsenical, for the treatment of syphilis, and 2 years later, to treat trypanosomiasis (African sleeping sickness). Over the next 2 decades, Ehrlich and others employed arsenicals and a variety of dyes in the treatment of various infectious diseases. None of these agents was particularly effective or specific for infectious agents, and all were highly toxic to the patient. One early agent, optochin, a quinine derivative, was used in the treatment of *Streptococcus pneumoniae* infections as early as 1911. This was the first anti-infectious agent truly specific for bacterial infections; however, it was effective against *S. pneumoniae* only, and it was quickly realized that this bacterium became resistant to optochin too readily to warrant further use of it as a therapeutic agent. Today, optochin is used primarily to distinguish *S. pneumoniae* from other species of *Streptococcus*.

Alexander Fleming, a Scottish physician working in London, conducted research in bacteriology early in his career. An experiment he conducted in 1928 involved the streaking of several blood agar plates with a culture of *Staphylococcus aureus* that had been obtained from an infected patient. Almost immediately after streaking the plates, he went on

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vacation, leaving them on a benchtop. Upon returning to the lab a couple of weeks later, Fleming noticed that on the surface of one of the plates, in addition to numerous colonies of *S. aureus*, a mold had formed a colony on the agar surface in one area of the plate. He also noted that the area of the agar surface immediately surrounding the mold colony was devoid of any bacterial growth. He surmised from this that the mold had produced an antibacterial substance that it excreted and that this substance had diffused into the medium, inhibiting the growth of the *S. aureus* cells in that area of the plate. Fleming identified the mold as *Penicillium notatum* and named the antibacterial compound that he presumed it had produced "penicillin." In 1929, Sir Alexander Fleming published the first paper on penicillin. Unfortunately, Fleming was never able to purify penicillin or to produce sufficient quantities of it to conduct experiments on its therapeutic efficacy. It was not until 10 years later that Howard Florey, an Australian pathologist who had studied medicine at Oxford, working with Ernst Chain, a German-born British biochemist, purified penicillin and developed a method for its production in large quantities. Florey was invited to the United States to demonstrate his methods of penicillin purification and mass production, and by 1941, tons of penicillin were being produced on a monthly basis by U.S., Canadian, and British pharmaceutical companies. The first clinical trials to test the efficacy of penicillin were conducted in Boston, MA, in 1942, and beginning in early 1944 penicillin proved to be vital to the military during the remainder of World War II, since infections obtained during battle, or following surgery necessitated by battle injuries, were now effectively avoidable or treatable. After Fleming's discovery of penicillin but before Florey and Chain's purification and development of methods for its mass production and clinical application, Gerhard Domagk, a German pathologist and bacteriologist, discovered and tested the curing effects of a red dye, Prontosil, in mice and successfully applied it to cure his own daughter of a serious *S. pneumoniae* infection. Domagk patented the use of Prontosil as an anti-infective in 1932. Three years later, in 1935, French scientists discovered that the dye was converted in the body to sulfanilamide, the first of the sulfa drugs, which was actually the active antibacterial compound. The German pharmaceutical company L. G. Farben, which had hired Domagk following his early work on Prontosil, released it as the antibacterial agent Streptozon in 1936.

If one defines an antibiotic as a chemotherapeutic agent that is either toxic or growth inhibiting for bacteria, then optochin would have to be considered the first such agent. However, the first two commercially marketed antibacterial agents were Prontosil and penicillin, and of the two, penicillin is the active antibacterial agent, whereas the red dye Prontosil possesses no antibacterial activity but must be converted by patient enzymatic activity to sulfanilamide, which is the antibacterial compound. Thus, most microbiologists would trace the origin of the modern antibiotic era either to the discovery of penicillin in 1928 or to its purification and mass production in 1939. In addition, the vast majority of antibiotics discovered and marketed in the 20th century were, like penicillin, natural products of microbial metabolism (fungal, actinomycete, or bacterial) that possessed killing or inhibitory activity against bacteria, e.g., other

penicillins, cephalosporins, tetracyclines, aminoglycosides such as streptomycin, macrolides such as erythromycin, and glycopeptides such as vancomycin. Representatives of most classes of antibiotics in clinical use today are still either natural products or semisynthetic derivatives of these products. In fact, there are only four classes of totally synthetic antibiotic used clinically: the fluoroquinolones (e.g., ciprofloxacin), which were first introduced in the 1980s; the oxazolidinones, the first representative of which, linezolid, was approved for clinical use in the United States in 2000; lipopeptides, with daptomycin being the first to achieve approval in 2004; and the sulfa drugs. One other antibacterial drug, trimethoprim, is also totally synthetic. The vast majority of these antibacterial agents are used exclusively in the treatment of bacterial infection. Exceptions are the sulfa drugs, tetracyclines, and trimethoprim, which also have been used to treat infections caused by some protozoan parasites. One other antibacterial agent, metronidazole, which has no antimicrobial activity until converted to an active form by a reducing enzyme made only by anaerobic microbes and thus is considered a prodrug, is also useful for the treatment of some infections caused by anaerobic protozoans that produce such enzymes.

There are several ways by which antibiotics may be classified, the three most common being (i) the type of antimicrobial activity, i.e., cidal (lethal) or static (inhibiting growth without killing); (ii) their antimicrobial spectrum, usually either narrow or broad; and (iii) their mechanism of action, i.e., the cellular target of inhibition. The first classification can be misleading because a particular antibiotic may be cidal for some bacterial species or strains but have only static activity against others. In addition, an antibiotic may be bactericidal for a particular species at one concentration but bacteriostatic for the same species at lower concentrations. The spectrum of an antibiotic may be very narrow, i.e., effective against only one or a few species of bacteria, or only gram-positive bacteria, or only gram-negative bacteria. A broad-spectrum antibiotic is one that is effective against a variety of gram-positive and gram-negative bacterial species. One of the most useful methods of antibiotic classification is based on the bacterial target(s) of inhibition, i.e., a step in cell wall biosynthesis, a component of the protein synthetic machinery, nucleic acid synthesis, cell membrane function, or a step in intermediary metabolism, e.g., the so-called antimetabolites. The more common antibiotic targets and examples of the types of clinically useful antibiotics that inhibit them are listed in Table 1. The mechanisms by which antibiotics inhibit these targets will be the topics of the next several sections of this chapter.

Inhibitors of Cell Wall Synthesis

The pathway of bacterial peptidoglycan (PG) (cell wall) synthesis, the key enzymes that catalyze the various steps in its synthesis, and antibiotics known to inhibit one or more of these steps are illustrated in Fig. 1. Most of the enzymes involved in PG synthesis are unique to this pathway and are thus ideal antibiotic targets, since mammalian cells do not have cell walls. Many cell wall-inhibitory agents are specific for bacteria and, ideally, nontoxic to the human patient. Inhibitors of cell wall synthesis function only against growing bacterial cells that are actively incorporating

TABLE 1 Common targets of clinically useful antibiotics

Targets	Antibiotics
Protein synthesis	
30S ribosomal subunit	Aminoglycosides, tetracyclines, glycylcyclines
50S ribosomal subunit	Macrolides, chloramphenicol, oxazolidinones
$tRNA^{ile}$	Mupirocin
Elongation factor G	Fusidic acid
Nucleic acid synthesis	
DNA gyrase & topoisomerase IV	Nalidixic acid, fluoroquinolones
RNA polymerase β subunit	Rifampin
DNA	Metronidazole (prodrug)
Cell wall (peptidoglycan) synthesis	
Transpeptidases	β-Lactams (penicillins, cephalosporins, etc.)
Lipid II	Bacitracin
D-Ala-D-Ala	Glycopeptides (vancomycin, teicoplanin)
MurA	Phosphomycin
Antimetabolites	
Dihydrofolate reductase	Trimethoprim
Dihydropteroate synthetase	Sulfonamides
Mycolic acid synthesis	Isoniazid
Membranes	Polymyxins, daptomycin

[a]Adapted and updated from D. T. Moir, K. J. Shaw, R. S. Hare, and G. F. Vovis, *Antimicrob. Agents Chemother.* **45:**439–446, 1999.

precursors into the PG layer. The backbone of PG (also referred to as murein) consists of two sugar moieties, *N*-acetylglucosamine (Glc-NAc) and *N*-acetyl muramic acid (Mur-NAc). These precursors are synthesized in the bacterial cytoplasm, as are the amino acids that make up a pentapeptide molecule that is attached to Mur-NAc. The first reaction that occurs in the cytoplasm is catalyzed by a bifunctional enzyme, GlmU, which acetylates glucosamine-1-phosphate and subsequently adds UDP to it to form UDP-Glc-NAc. Subsequent steps are catalyzed by a series of enzymes referred to as MurA to MurF. Mur-Nac is synthesized from UDP-Glc-NAc via the activities of two enzymes, MurA and MurB, resulting in the uridylated product UDP-Mur-Nac. MurA is inactivated by fosfomycin, an antibiotic recommended for the treatment of uncomplicated urinary tract infections (acute cystitis) in women caused by susceptible strains of *Escherichia coli* and *Enterococcus faecalis*.

The next series of cytoplasmic steps involves the synthesis of UDP-muramyl tripeptide by the sequential addition to UDP-Mur-Nac of L-Ala, D-Glu, and *meso*-DAP (diaminopimelic acid) catalyzed by MurC, MurD, and MurE, respectively. DAP is replaced by lysine in a number of gram-positive bacterial species. Subsequently, a pentapeptide is formed by the addition to the UDP-muramyl tripeptide of the dipeptide D-Ala-D-Ala, catalyzed by MurF. Two enzymes are involved in the formation of D-Ala-D-Ala. First, an alanine racemase converts the naturally occurring L-Ala to D-Ala, and the dipeptide is synthesized by D-alanyl-D-alanine ligase. The racemase and ligase are both inhibited by D-cycloserine, but toxicity

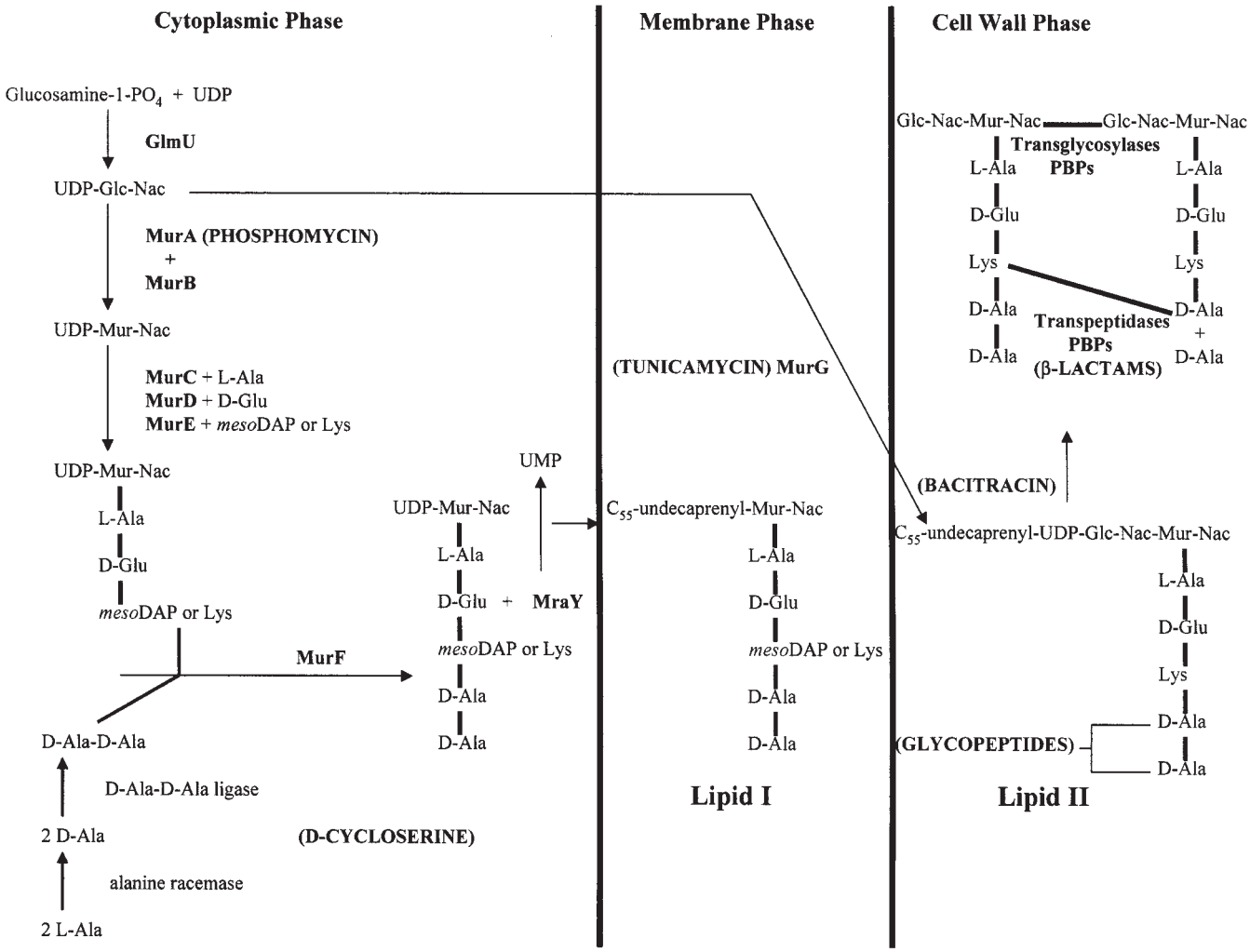


FIGURE 1 Peptidoglycan synthesis. The synthesis of cell wall precursor UDP-Mur-Nac-pentapeptide occurs in the bacterial cytoplasm. Precursor molecules are transported to the cytoplasmic membrane by the attachment to it of C_{55}-undecaprenyl in the formation of lipid I. Subsequently, lipid I is transported to the exterior side (periplasmic space in gram-negative bacteria) of the cytoplasmic membrane, and the disaccharide (UDP-Glc-Nac-Mur-Nac)-pentapeptide, designated lipid II, is formed. Subsequently, the C_{55}-undecaprenyl moiety is released for subsequent cycles, and the cell wall continues to grow on the exterior of the cytoplasmic membrane due to the transglycosylase and transpeptidase activities of PBPs. Enzymes catalyzing the PG synthetic steps are in bold letters, and antibiotics that target the various steps in PG synthesis are indicated in bold capital letters in parentheses. See the text for further details on PG synthesis and antibiotics that inhibit it. Adapted from I. Chopra, L. Hesse, and A. J. O'Neill, *J. Appl. Microbiol.* **92**(Suppl.):4S–15S, 2002. doi:10.1128/9781555818906.ch21.f1

issues have precluded the clinical use of this agent except as a second-line antibiotic in the treatment of tuberculosis.

The next steps in PG synthesis consist of the formation of two cytoplasmic membrane-bound lipid intermediates, lipid I and lipid II. The formation of lipid I involves MraY, an enzyme that catalyzes the removal of UDP from the muramyl pentapeptide and its replacement by the membrane component, C_{55}-undecaprenyl-phosphate. MurG catalyzes the addition of

GlcNAc to lipid I using UDP-GlcNAc as a cosubstrate, yielding the lipid-disaccharide-pentapeptide lipid II and resulting in the translocation of the disaccharide-pentapeptide from the inside of the cytoplasmic membrane to the outside, but still anchored to it by C_{55}-undecaprenyl-phosphate. In gram-negative bacteria, which have an outer membrane, lipid II is located in the periplasmic space. The antibiotic tunicamycin is an inhibitor of MurG, but it is too toxic for clinical use, due to its inhibition of a step in eukaryote glycoprotein synthesis.

Once exposed to the outer surface of the cytoplasmic membrane, the disaccharide pentapeptide subunits of the growing PG become substrates for two types of enzymatic activities, transglycosylases and transpeptidases, which form covalent linkages between adjacent disaccharides (GlcNAc-Mur-NAc) and pentapeptides, respectively, ultimately producing the mature, rigid PG layer. The transpeptidases form peptide bonds between the third amino acid in one pentapeptide (usually *meso*-DAP or lysine) and the fourth amino acid (D-Ala) in an adjacent pentapeptide, accompanied by the release of the second D-Ala. The joining of two disaccharide subunits, via transglycosylase activity, is accompanied by the hydrolysis of the pyrophosphate linkage, which results in release of the C_{55}-lipid phosphate for subsequent transport of disaccharide-pentapeptide subunits to the cytoplasmic membrane. Several antibiotics interfere with release of the C_{55}-lipid phosphate from lipid II, preventing further rounds of lipid I and lipid II synthesis and thus PG maturation. Only one such antibiotic, bacitracin, which prevents the recycling of the C_{55}-lipid phosphate moiety of lipid II, is of clinical significance. However, because of toxicity, it is used topically only.

The transglycosylase and transpeptidase activities associated with PG synthesis reside in a group of cytoplasmic membrane-associated proteins referred to collectively as penicillin-binding proteins, or PBPs. Bacteria synthesize a variety of PBPs and may contain as many as eight or more. The lower-molecular-weight PBPs tend to possess transpeptidase activity only, whereas those with higher molecular weights may be bifunctional, expressing both transpeptidase and transglycosylase activities. The best-known class of antibiotics, i.e., the β-lactams, bind to the PBPs and block their transpeptidase activity. The β-lactams include the penicillins (hence the name "penicillin-binding proteins"), the cephalosporins, the carbapenems, and the monobactams, and as the name implies, all contain the four-membered β-lactam ring, illustrated in Fig. 2.

Normally, the cross-linking transpeptidases form one DAP-D-Ala or Lys-D-Ala peptide bond and cleave one D-Ala-D-Ala peptide bond, releasing a free D-Ala. However, β-lactam antibiotic structures resemble the D-Ala-D-Ala terminus of the pentapeptide and thus bind to the PBP instead of the normal substrate. An acyl enzyme intermediate, in which the β-lactam ring is opened, is formed. Thus, the enzyme is not free to add further cross-links to the maturating PG, and the cell eventually lyses. In many bacterial species, inhibition of the cross-linking reaction also triggers the release of endogenous enzymes referred to as autolysins, which degrade existing mature PG, thus speeding up lysis and enhancing the lethality of β-lactam antibiotics.

The first β-lactams to be used clinically were the natural penicillins, penicillin G (acid labile) and penicillin V (acid stable), which have activity

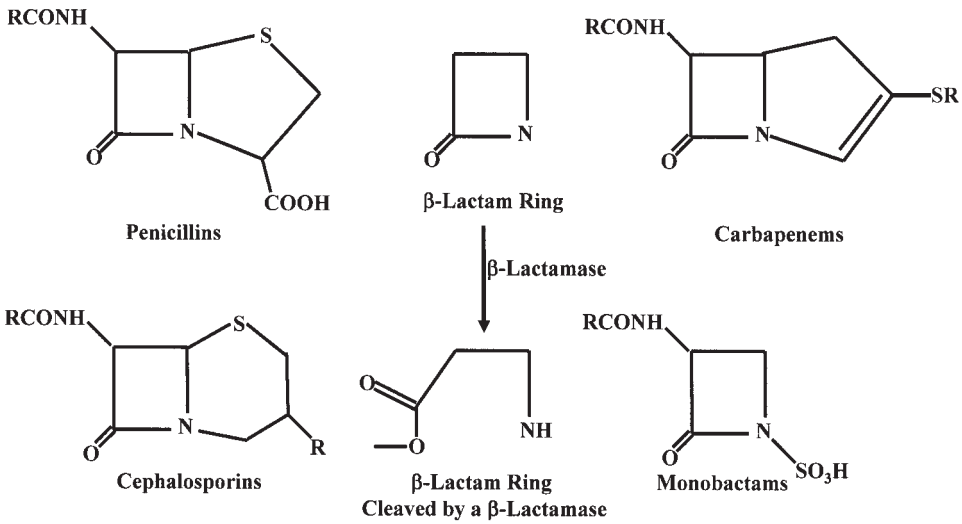


FIGURE 2 β-Lactam antibiotics. Shown are the core structures of the four major classes of β-lactams: penicillins, cephalosporins, carbapenems, and monobactams. Cleavage of the β-lactam ring by β-lactamases is illustrated in the center of the figure. doi:10.1128/9781555818906.ch21.f2

against the staphylococci, streptococci, enterococci, some gram-negative cocci, and spirochetes. The aminopenicillins amoxicillin and ampicillin are effective against the same bacterial species as the natural penicillins, but due to their ability to penetrate the outer membrane they also possess activity against many gram-negative bacterial species and were shown to be effective in the treatment of urinary tract infections. As *S. aureus* became resistant to the penicillins, due to the production of β-lactamase activity (see "R Factors and Other Antibiotic Resistance Plasmids" and "The Many Mechanisms of Antibiotic Resistance," below), the antistaphylococcal or β-lactamase-resistant penicillins, e.g., methicillin, were developed. The last of the penicillins to be introduced were the so-called broad-spectrum penicillins, also referred to as the antipseudomonal penicillins. These included piperacillin, mezlocillin, and ticarcillin, which exhibited activity against a broad spectrum of gram-negative bacilli, including *Pseudomonas* species, a number of anaerobic species, and enterococci. Penicillins G and V and the aminopenicillins have seen considerable use in the practice of dentistry, primarily because of their activity against the streptococci and many oral anaerobes. Amoxicillin has been used extensively for subacute endocarditis prophylaxis. However, with the emergence of oral streptococci resistant to all β-lactam antibiotics due to mutations in genes encoding PBPs and increases in the numbers of oral anaerobes expressing β-lactamase activities, the penicillins should be used with caution.

Next among the β-lactam antibiotics were the cephalosporins, which are derivatives of cephalosporin C, produced by the fungal genus *Cephalosporium*. The cephalosporins, four generations of which have been introduced into clinical practice, are active against most organisms that are susceptible to penicillins and, in general, are useful alternatives for patients that are allergic to penicillin. Approximately 8 to 10% of individuals who are treated with one penicillin will become allergic to all penicillins. Like the penicillins, the cephalosporins are relatively nontoxic, and fewer

individuals become hypersensitive. The "first-generation" cephalosporins, e.g., cefazolin, cephalothin, and cephalexin, are active against staphylococci and streptococci, but not enterococci, and most gram-negative pathogens. Most importantly, when first introduced, they were effective against β-lactamase-producing bacterial strains and became widely used prophylactically prior to surgery. As with the penicillins, resistance to the cephalosporins began to emerge, due to a large extent to a class of β-lactamases referred to as cephalosporinases.

The "second generation" of cephalosporins, such as cefamandole, cefuroxime, cefaclor, cephamycin, and cefoxitin, were unaffected by cephalosporinases produced by gram-negative rods and had some activity against anaerobic species but were much less effective against gram-positive bacteria than the first-generation cephalosporin molecules. Like the first-generation cephalosporins, they have been used prophylactically prior to surgery and found a use in the treatment of mixed aerobic-anaerobic infections.

"Third-generation" cephalosporins, e.g., cefotaxime, ceftazidime, ceftriaxone, ceftizoxime, and cefoperazone, exhibited a broader activity against gram-negative rods, especially members of the *Enterobacteriaceae* and *Pseudomonas* species, but had reduced activity against gram-negative cocci and very little gram-positive antibacterial activity. They were also useful for treatment of sexually transmitted diseases (STDs) such as gonorrhea (*Neisseria gonorrhoeae*) and syphilis (*Treponema pallidum*), as well as Lyme disease (*Borrelia burgdorferi*), and they were the only cephalosporins that could cross the blood-brain barrier.

The only "fourth-generation" cephalosporin approved for use in the United States, cefepime, is resistant to inactivation by a large array of β-lactamases produced by gram-negative bacteria and can readily pass through outer membrane porins.

Two newer classes of β-lactam antibiotics include the monobactams and carbapenems. The monobactam aztreonam has activity against a broad spectrum of gram-negative bacteria, including *Pseudomonas* species, but virtually no activity against gram-positive bacteria. It is also useful for treatment of β-lactam-sensitive patients, since it is the least likely of the β-lactam antibiotics to trigger a hypersensitivity response. The carbapenems imipenem and meropenem, which exhibit the broadest spectrum of activity of all the β-lactams, are effective against gram-positive and gram-negative bacteria.

Another group of β-lactams are the so-called β-lactamase inhibitors, e.g., clavulanic acid, sulbactam, and tazobactam. These compounds are not antibiotics as such, but they bind to β-lactamases almost permanently (very slowly released), preventing the binding of the β-lactamases to true β-lactam antibiotics such as penicillins, if present in the same environment. Combinations of β-lactamase inhibitors and β-lactamase-susceptible β-lactams currently are used therapeutically. The first of these combinations was Augmentin (amoxicillin plus clavulanic acid), which was followed by Unasyn (ampicillin plus sulbactam), Timentin (ticarcillin plus clavulanic acid), and Zosyn (piperacillin plus tazobactam).

The glycopeptide antibiotics (Fig. 3) vancomycin and teicoplanin are naturally occurring secondary metabolites of the bacteria *Amycolatopsis orientalis* and *Actinoplanes teichomyceticus*, respectively. These

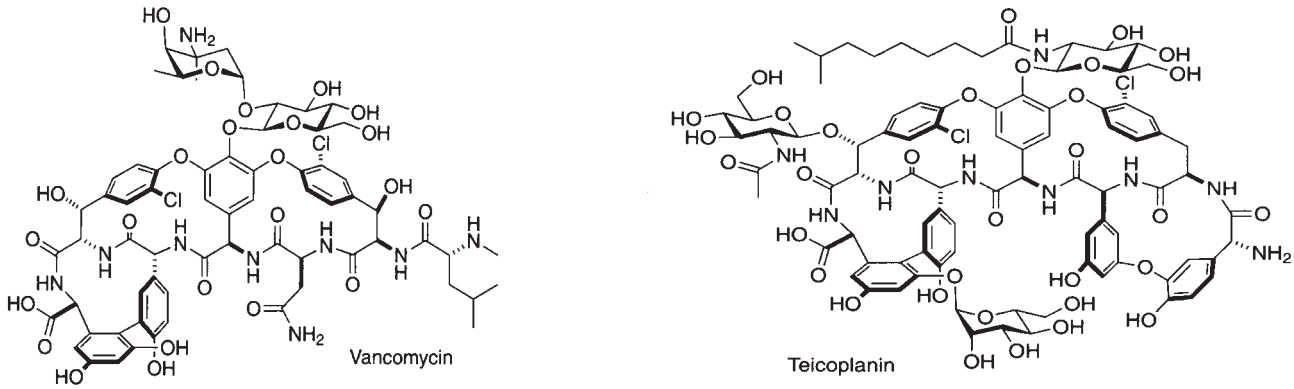


FIGURE 3 Glycopeptide antibiotics: structures of vancomycin and teicoplanin. Adapted from C. Walsh, *Antibiotics: Actions, Origins, Resistance* (ASM Press, Washington, DC, 2003). doi:10.1128/9781555818906.ch21.f3

antibiotics bind to the D-Ala-D-Ala terminus of the UDP-muramyl pentapeptide after transfer out of the cytoplasm, while they are still lipid linked. When bound to the murein, the glycopeptides block the binding of PBPs, inhibiting transpeptidation and transglycosylation reactions. Vancomycin is the only glycopeptide antibiotic licensed for use in the United States, whereas teicoplanin is used extensively in Europe. The glycopeptides are active only against gram-positive bacteria, being too large and too complex to penetrate the outer membranes of gram-negative cells. Vancomycin is administered intravenously for the treatment of infections caused by gram-positive cocci, such as species of *Enterococcus* and *Staphylococcus*, that are resistant to all other clinically useful antibiotics. It is administered orally in the treatment of antibiotic-associated colitis caused by *Clostridium difficile*.

Inhibitors of Translation

The protein biosynthetic process, i.e., translation, as well as some inhibitors of the process, is depicted in Fig. 4. The platform on which proteins are synthesized, i.e., on which mRNA cistrons are translated into polypeptides, is the ribosome. Bacterial 70S ribosomes are composed of two large complexes designated the 30S and 50S ribosomal subunits, each of which contains RNA and proteins. The 30S subunit consists of 16S rRNA and approximately 20 proteins, whereas the 50S subunit consists of 5S and 23S rRNA plus approximately 30 proteins.

Translation begins with the formation of an initiation complex consisting of mRNA, a 30S ribosomal subunit, and a charged fMet-tRNA^{fMet} and the binding and release of three initiation factors, IF1, IF2, and IF3. Formation of an initiation complex involves the binding of a 30S ribosomal subunit to the ribosomal binding site on mRNA, approximately 5 bases upstream of the start codon (usually AUG) of a gene (cistron). Premature binding of the 50S ribosomal subunit to the 30S subunit is prevented by the presence of IF1 and IF3. A charged fMet-tRNA^{fMet}, following activation by IF2 and GTP, then binds to the start codon on the mRNA via base pairing to the anticodon on the tRNA. In a step involving the hydrolysis of GTP, the three initiation factors dissociate from the 30S

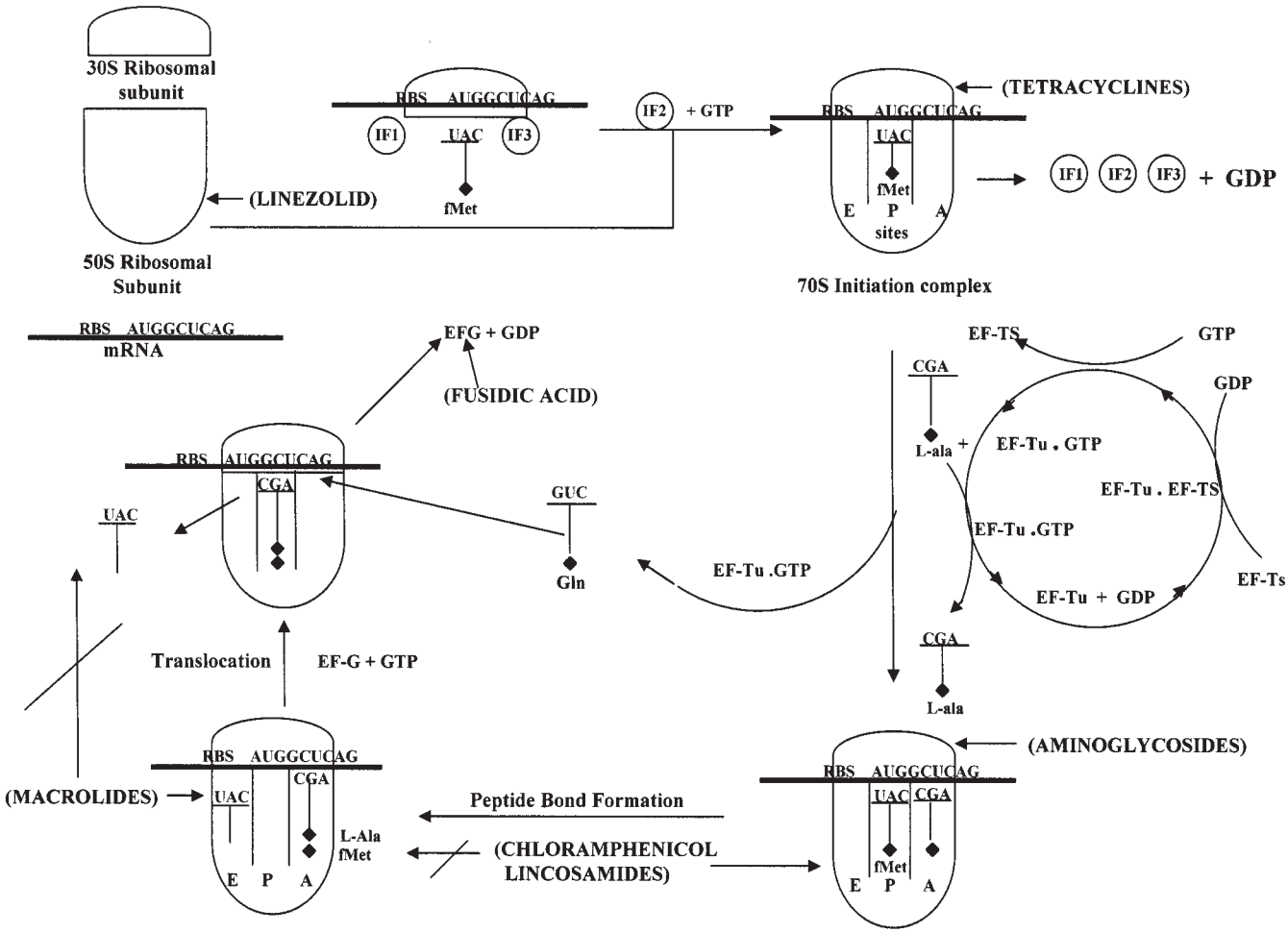


FIGURE 4 Translation and inhibition by antibiotics. Shown is the formation of the 70S ribosomal initiation complex, containing an mRNA molecule and fMet-tRNA, followed by subsequent steps in the cycle by which each additional amino acid is added to the growing polypeptide chain via the formation of a peptide bond between it and its predecessor. Specific antibiotics or antibiotic classes inhibiting specific steps in translation are shown in bold capital letters in parentheses. See the text for further details. Adapted from I. Chopra, L. Hesse, and A. J. O'Neill, *J. Appl. Microbiol.* **92**(Suppl.):4S–15S, 2002, and from J. W. Lengeler, G. Drews, and H. G. Schlegel, *Biology of the Prokaryotes* (Blackwell Science, Thieme, New York, NY, 1999).
doi:10.1128/9781555818906.ch21.f4

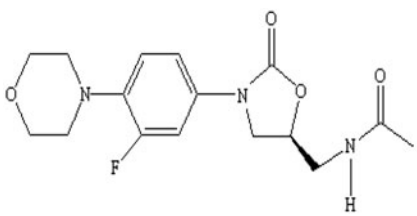


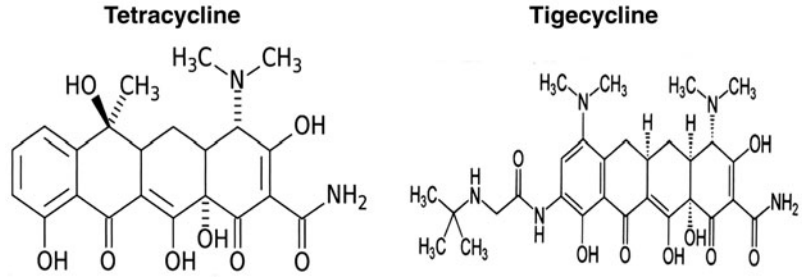
FIGURE 5 Structure of linezolid (Zyvox), an oxazolidinone.
doi:10.1128/9781555818906.ch21.f5

initiation complex, which now binds to a 50S ribosomal subunit, producing a 70S initiation complex in which the charged $tRNA^{fMet}$ is situated at the P (peptide) site, which is flanked on one side by the empty A (amino acid acceptor) site for binding of the next aminoacyl-charged tRNA encoded by the mRNA, and on the other side by the empty E (peptide exit) site. Linezolid (trade name, Zyvox) (Fig. 5) is the first member of the totally synthetic oxazolidinones granted FDA approval (in 2000) and represents the first new class of antibiotics introduced into clinical practice in more than 30 years. Linezolid inhibits bacterial protein synthesis at the initiation stage by binding to the 50S ribosomal subunit and is thought to block the formation of the 70S initiation complex. It is used to treat

infections caused by multidrug-resistant (MDR) gram-positive pathogens such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), and penicillin-resistant *S. pneumoniae*.

Translation continues via a series of elongation cycles, each of which results in the addition of a single amino acid to the growing polypeptide chain. Each cycle begins with the binding of an EF-Tu * GTP-activated and charged tRNA to the A site, the specific aminoacyl-tRNA bound being determined by the codon at that site. Tetracyclines (Fig. 6) bind to the 16S rRNA in the 30S ribosomal subunit and interfere with the binding of aminoacylated tRNA molecules to the ribosomal A site. They pass through porins on the outer membranes of gram-negative bacteria and through cytoplasmic membranes of all bacteria by energy-dependent, carrier-mediated processes. Their selective action against bacteria is based on greater uptake by bacteria than by human cells. Tetracycline use is contraindicated in pregnant women and children under the age of 8 years due to interference with bone development and to the brown staining of teeth in fetuses and children. These antibiotics are usually administered orally, with doxycycline and minocycline being more completely absorbed than tetracycline, oxytetracycline, or chlortetracycline. Because of the widespread existence of resistant strains, use of the tetracyclines currently is limited to the treatment of Lyme disease (*B. burgdorferi*), the STDs chlamydia and gonorrhea, and such dermatological conditions as acne (*Propionibacterium acnes*; syn. *Corynebacterium acnes*) and rosacea (reddening of the skin of the face). Tetracyclines are used, either systemically or locally (incorporated in a variety of fibers), as an adjunct to scaling and root planing in the treatment of periodontal disease. Whether or not the effectiveness of tetracyclines in this regard is due to their antibacterial activity or to their ability to reduce inflammation and inhibit collagenase activity, or both, has not been definitively established. Tigecycline (Fig. 6) (trade name, Tygacil) is the first of the glycylcycline class of antibiotics to receive approval by the FDA (in 2005) and is administered intravenously. It is structurally related to minocycline but has an expanded spectrum and is active against tetracycline-resistant bacteria, whether the resistance is due to efflux or ribosomal protection. Its spectrum includes several gram-positive and gram-negative species, a number of anaerobes, and atypical bacteria such as *Chlamydia pneumoniae*, *Legionella pneumophila*, and *Mycoplasma pneumoniae*. *Pseudomonas aeruginosa* is naturally resistant to tigecycline via a variety of efflux mechanisms.

FIGURE 6 Structures of tetracycline and tigecycline (a glycylcycline).
doi:10.1128/9781555818906.ch21.f6

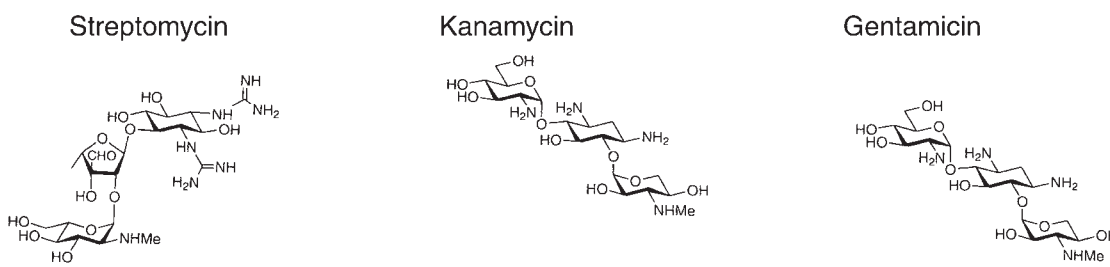


Mupirocin is a topical antibiotic used to treat skin infections, e.g., impetigo caused by staphylococci and streptococci. Mupirocin inhibits $tRNA^{ile}$ synthase, thus halting protein synthesis at an elongation cycle that calls for the incorporation of isoleucine into the growing peptide chain.

Activation of the tRNA molecules requires the hydrolysis of GTP to GDP + P_i, resulting in the formation of an EF-Tu * GDP complex. Ribosomal protein S12 is responsible for ensuring that the correct aminoacyl-tRNA has been bound to the A site. Streptomycin, an aminoglycoside (Fig. 7), interferes with this process and contributes to translational misreading. All aminoglycosides (amikacin, gentamicin, kanamycin, neomycin, and tobramycin) bind irreversibly to sites in the 16S rRNA in the 30S ribosomal subunit. This binding blocks the joining of the 50S subunit to the 30S subunit, thereby preventing initiation. These antibiotics also bind to the 30S ribosomal subunit of the 70S ribosome, causing distortion of the A site and ultimate irreversible inhibition of protein synthesis. These antibiotics, all of which (except semisynthetic derivatives such as amikacin) are produced by various species of actinomycetes found in the soil, pass through the outer membranes of gram-negative bacteria due to the disruption of membrane cross-bridges. They are actively transported through bacterial cytoplasmic membranes by mechanisms dependent on electron transport. Aminoglycosides are inactive against anaerobes and are used primarily to treat infections caused by gram-negative bacteria. They are occasionally used to treat gram-positive bacterial infections, in conjunction with a cell wall inhibitor such as a β-lactam or glycopeptide (see "Antibiotic combinations" below). All of the aminoglycosides are potentially nephrotoxic and ototoxic and have relatively narrow therapeutic indexes, i.e., antibacterial concentration to toxic concentration ratios.

Prior to each successive elongation cycle, with the addition of the next amino acid encoded by the mRNA, EF-Tu * GTP is regenerated from the EF-Tu * GDP complex by EF-Ts (thermostable) and GTP. The next step in an elongation cycle involves the formation of a peptide bond between the α-amino group of the amino acid just added to the A site and the activated carboxyl group of either fMet or the last amino acid that was added to a growing peptide chain. Formation of each peptide bond is catalyzed by peptidyl transferase activity of the 50S ribosomal subunit. Chloramphenicol and the lincosamides bind to 23S rRNA in the 50S ribosomal subunit and inhibit peptidyl transferase activity. Chloramphenicol, a very-broad-spectrum antibiotic, penetrates the cytoplasmic

FIGURE 7 Aminoglycosides. Shown are the structures of streptomycin, kanamycin, and gentamicin. Adapted from C. Walsh, *Antibiotics: Actions, Origins, Resistance* (ASM Press, Washington, DC, 2003). doi:10.1128/9781555818906.ch21.f7



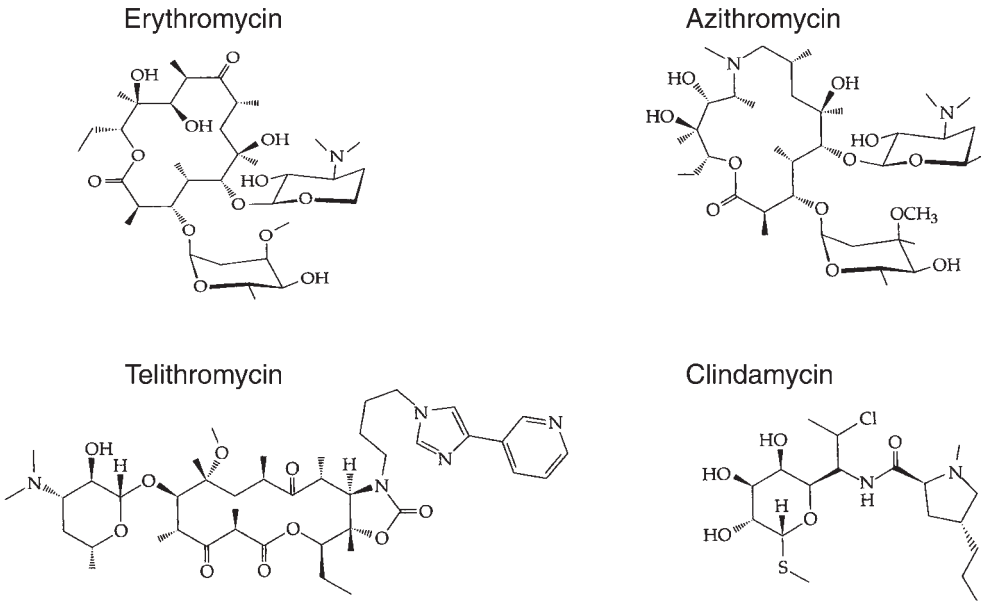


FIGURE 8 Macrolide and lincosamide antibiotics. Shown are the structures of the macrolides erythromycin, azithromycin, and telithromycin and the lincosamide clindamycin. Adapted from B. A. Wilson, A. A. Salyers, D. D. Whitt, and M. E. Winkler, *Bacterial Pathogenesis: a Molecular Approach*, 3rd ed. (ASM Press, Washington, DC, 2011). doi:10.1128/9781555818906.ch21.f8

membrane via an energy-dependent influx mechanism. However, it is used in developed countries with extreme caution and only if deemed absolutely necessary, due to rare but fatal cases of aplastic anemia. In underdeveloped countries, chloramphenicol is usually the least expensive and most widely available of antibiotics. Clinically, clindamycin (Fig. 8) is the most commonly used member of the lincosamides. It exhibits good activity against gram-positive and gram-negative anaerobes and is particularly effective against *Bacteroides* spp. It is also effective against gram-positive aerobes and is often used in the treatment of osteomyelitis because of its tendency to concentrate in bone. Clindamycin is a first-line antibiotic for the treatment of serious odontogenic infections, primarily because of its activity against such anaerobic genera, in addition to *Bacteroides*, as *Porphyromonas*, *Prevotella*, *Fusobacterium*, and *Peptostreptococcus*. It is also a first-line antibiotic in patients who are allergic to penicillins and a key alternative for prophylaxis in such patients.

Peptide bond formation is followed by the translocation of the ribosomal complex to the next codon on the mRNA molecule, which is mediated by elongation factor EF-G with the necessary energy supplied by the hydrolysis of GTP. Translocation of the ribosomal complex results in movement of the growing peptide chain to the P site, the release of the uncharged prior tRNA molecule, as well as EF-G, through the E site and a now-empty A site for initiation of the next elongation cycle that will result in the addition of the next amino acid to the growing peptide chain. Macrolides bind to 23S rRNA in the 50S ribosomal subunit and block peptide translocation and release of tRNA after peptide bond formation. They also inhibit the formation of the 50S ribosomal subunit. Erythromycin (Fig. 8) is the best known and still the most widely used of the macrolides.

It has activity against gram-positive cocci and is an important alternative to penicillin in allergic patients for the treatment of streptococcal infections. It also is used to treat infections of the upper and lower respiratory tract, skin, and soft tissues, as well as urinary tract infections, STDs such as syphilis and gonorrhea, and intestinal parasitic infections. The macrolides are relatively nontoxic but cause nausea and vomiting after oral administration in some patients. Such gastrointestinal disturbances tend to be less common with the newer macrolides, clarithromycin and azithromycin (Fig. 8). Azithromycin is particularly effective for the treatment of such STDs as chlamydial infections and gonorrhea, because it can be administered in a single dose, or at most two closely spaced oral doses, thus minimizing the problem of patient noncompliance. In addition to STDs, these two antibiotics are used extensively in the treatment of respiratory tract infections, as well as peptic ulcers caused by *Helicobacter pylori*. Azithromycin and clarithromycin have dental indications, primarily as an option for penicillin-allergic patients requiring prophylaxis for bacterial endocarditis.

Like azithromycin and clarithromycin, the ketolide telithromycin (Fig. 8) is a structural analog of erythromycin. Telithromycin has been approved for the treatment of community-acquired pneumonia. However, instances of severe hepatotoxicity have been reported, which will likely limit its use.

Synercid is a combination of two streptogramins, quinupristin and dalfopristin, which inhibit protein synthesis by the same mechanism as the macrolides and lincosamides. It was approved by the FDA in 1999 for the treatment of methicillin-susceptible *S. aureus*, *Streptococcus pyogenes*, and VRE. The streptogramin combination is used because each alone is bacteriostatic, whereas the combination is bactericidal.

Fusidic acid binds to EF-G, forming a stable complex with EF-G, GDP, and the ribosome. EF-G is not released for subsequent rounds of translocation and GTP hydrolysis. Fusidic acid is active against gram-positive but not gram-negative bacteria. One of the few indications for fusidic acid is infection due to penicillin-resistant *Staphylococcus*, especially osteomyelitis, since fusidic acid is concentrated in bone. However, a second antistaphylococcal agent is also required to prevent the development of resistance. Recently, fusidic acid, formulated as a cream, has shown promise for the treatment of impetigo in children 12 years old or younger.

The elongation cycles continue until a translocation step results in movement of the ribosomal complex to a stop codon on the mRNA, at which point release factor RF3, plus either RF1 or RF2, depending on the stop codon, will bind at the empty A site, forming a termination complex. The 50S-mediated peptidyl transferase activity then releases the polypeptide chain, which causes dissociation of the mRNA, release factors, and the 70S ribosome. Initiation factors IF1 and IF3, at the expense of GTP hydrolysis, cause the dissociation of the 70S ribosome into its 50S and 30S subunits.

Inhibitors of Transcription and Replication

The rifamycins bind to the β-subunit of DNA-dependent RNA polymerase and are the only antibiotics that block transcription, specifically the initiation of mRNA synthesis. Rifamycins are natural products of bacterial

(*Streptomyces mediterranei*) fermentation, but only semisynthetic derivatives, such as rifampin, rifapentine, and rifamide, are used clinically. They exhibit a broad spectrum of activity, which includes gram-positive bacteria, some gram-negative species, primarily cocci, some anaerobes, and *Chlamydia* spp. The rifamycins are seldom used as a single antimicrobial agent, due to high frequencies of mutation to resistance. The most important use of rifampin is in the treatment of tuberculosis, but always in combination with at least two other drugs. Rifampin may often color urine, feces, saliva, sputum, sweat, and tears an orange-red and can permanently stain contact lenses. Long-term treatment may cause a flulike syndrome, and some patients develop hepatotoxicity and thrombocytopenia.

All inhibitors of bacterial DNA replication interfere with the activities of one or both of the type II topoisomerases, either DNA gyrase only or DNA gyrase and topoisomerase IV. DNA gyrase, a heterotetramer comprised of two GyrA and two GyrB subunits, is the only bacterial enzyme able to introduce negative superhelical turns into DNA and is required for both initiation and continuation of DNA replication. At the termination of replication, topoisomerase IV catalyzes the separation (decatenation) of the two daughter DNA molecules, in order to permit their segregation to daughter cells during cell division. Topoisomerase IV, like DNA gyrase, is also a heterotetramer, consisting of two ParC and two ParE subunits. GyrB and ParE each have ATPase activity, providing energy for the activities of gyrase and topoisomerase IV, respectively.

The aminocoumarins, such as novobiocin and coumermycin, fermentation products of *Streptomyces* species, were the first topoisomerase inhibitors to be studied. They compete with ATP for binding to GyrB and are active against gram-positive, but not gram-negative, bacteria. Because of numerous toxic side effects and high frequencies of mutation to resistance, the aminocoumarins are not clinically useful. The quinolones (Fig. 9), on the other hand, have become major players in the arsenal of antimicrobial agents.

Quinolones bind to DNA-gyrase and DNA-topoisomerase IV complexes, stabilizing DNA strand breaks created by these enzymes, such that religation cannot occur, resulting in the accumulation of replication intermediates and the eventual death of the cell. Although the quinolones act against both enzymes, the major target in gram-negative bacteria is GyrA, whereas in gram-positive bacteria it tends to be ParC. The first of this family of synthetic agents, nalidixic acid, introduced in 1962, is active only against the *Enterobacteriaceae* and is used only for the treatment of urinary tract infections. Bacteria also exhibit high frequencies of mutations leading to resistance to nalidixic acid.

The addition of a fluoride atom to quinolones produced increased potency and an expanded spectrum, resulting in the introduction of a whole new family of fluoroquinolones into clinical practice, such as norfloxacin in 1986, ciprofloxacin in 1987, levofloxacin in 1996, gatifloxacin and moxifloxacin in 1999, and gemifloxacin in 2003. Not only do the fluoroquinolones have greater activity against the *Enterobacteriaceae*, but they are also active against other gram-negative, as well as gram-positive bacteria. Some of the fluoroquinolones also have activity against *P. aeruginosa*, *Mycoplasma*, *Chlamydia*, and *Mycobacterium* species. Approximately 5% of patients experience gastrointestinal problems, but serious

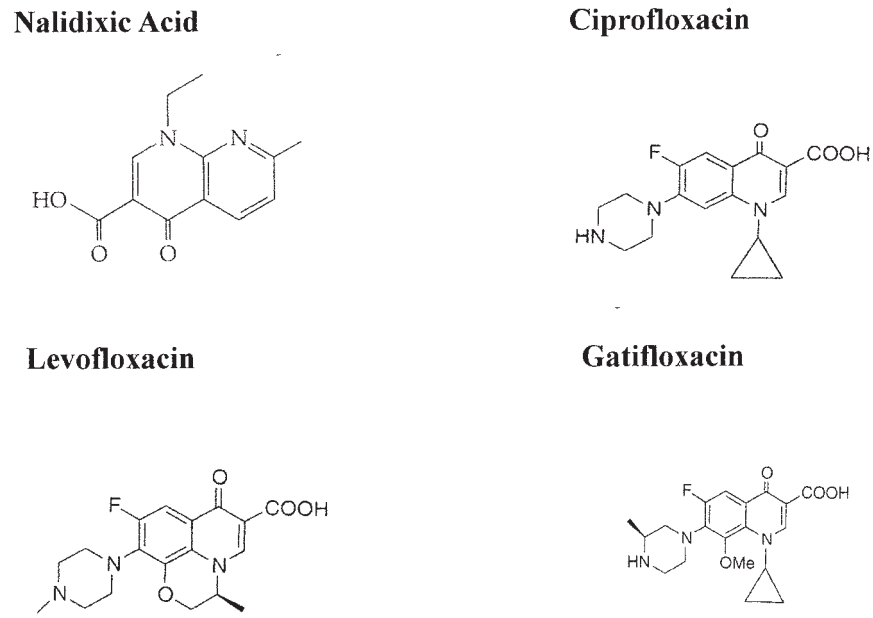


FIGURE 9 Quinolones. Shown are the structures of the quinolone nalidixic acid (adapted from B. A. Wilson, A. A. Salyers, D. D. Whitt, and M. E. Winkler, *Bacterial Pathogenesis: a Molecular Approach*, 3rd ed., ASM Press, Washington, DC, 2011) and the fluoroquinolones ciprofloxacin, levofloxacin, and gatifloxacin (adapted from C. Walsh, *Antibiotics: Actions, Origins, Resistance*, ASM Press, Washington, DC, 2003). doi:10.1128/9781555818906.ch21.f9

side effects are very uncommon with the fluoroquinolones. However, possible inhibition of cartilage development has precluded their licensing for use in children.

Miscellaneous Antibiotics

Sulfonamides are similar in structure to *para*-aminobenzoic acid (PABA), a precursor of folic acid (Fig. 10), and function as competitive inhibitors of dihydropteroate synthetase (DHPS), which catalyzes the synthesis of dihydropteroic acid from PABA and pteridine. The sulfa drug currently most commonly prescribed is sulfamethoxazole, which, although generally used in combination with trimethoprim (see below), is active against many gram-positive and gram-negative bacteria, as well as *Chlamydia* spp., *Plasmodium falciparum*, *Pneumocystis jirovecii* (formerly *P. carinii*), and *Toxoplasma gondii*. Trimethoprim has a pyrimidine-like structure resembling the amino-hydroxy-pyrimidine moiety of folic acid and functions as a competitive inhibitor of dihydrofolate reductase (DHFR). It may still be used as a single antimicrobial agent in the treatment of urinary tract infections and traveler's diarrhea, but its primary application is in combination with sulfamethoxazole.

Metronidazole (Flagyl), a member of the nitroimidazole class of antimicrobials, is an antibacterial and antiparasitic agent that is used to treat infections caused by anaerobes and some microaerophiles. It is effective against *Bacteroides* species and *H. pylori* and such parasites as *Trichomonas vaginalis*, *Giardia lamblia*, and *Entamoeba coli*. Metronidazole itself has no antibacterial activity, but under anaerobic conditions the

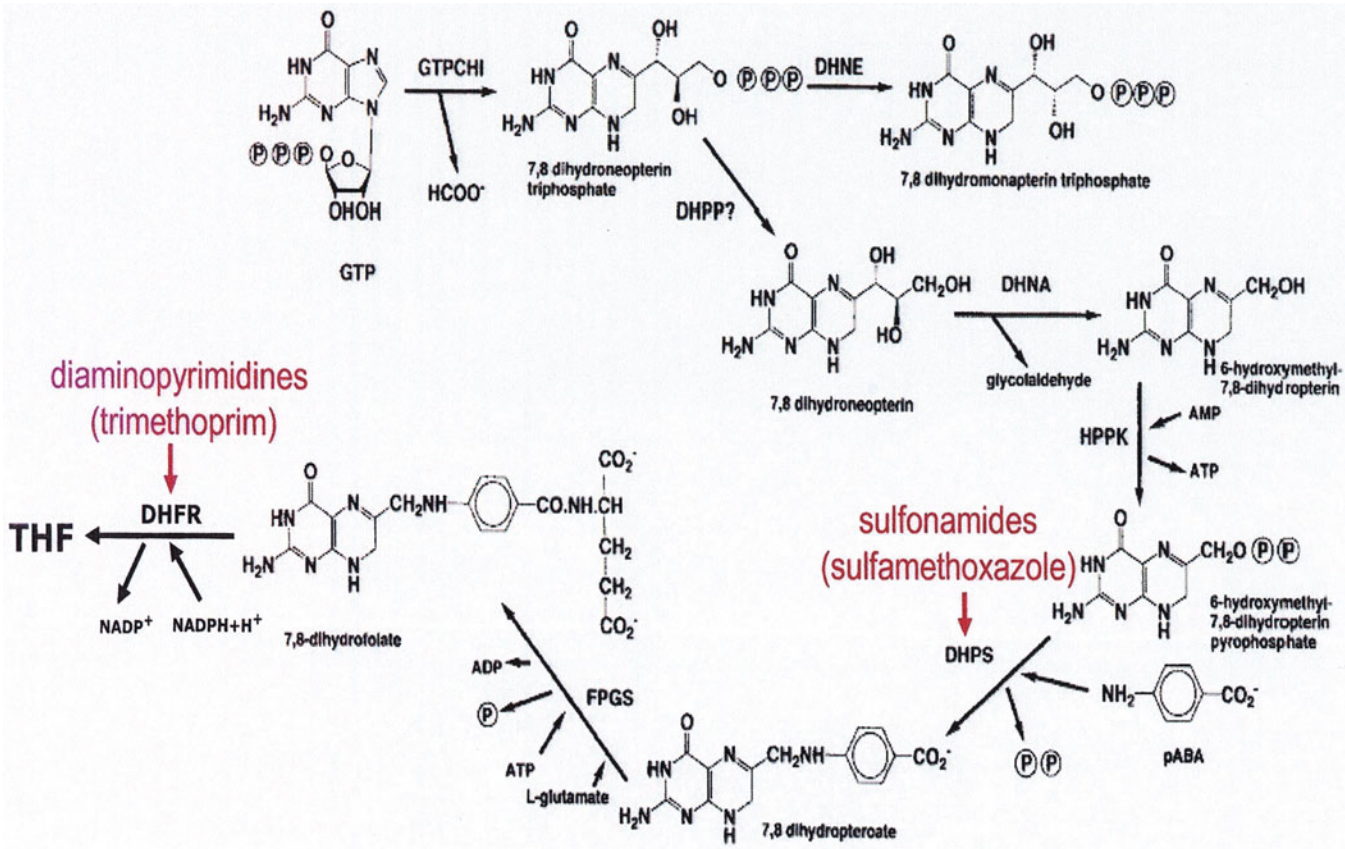


FIGURE 10 The folate biosynthetic pathway. Enzymatic steps involved in the bacterial synthesis of tetrahydrofolic acid (THF) are illustrated, as are two antibiotics, sulfamethoxazole and trimethoprim, that inhibit two key enzymes involved in THF synthesis, dihydropteroate synthetase (DHPS) and dihydrofolate reductase (DHFR), respectively. Reprinted with permission from A. Bermingham and J. P. Derrick, *Bioessays* **24:**637–648, 2002. doi:10.1128/9781555818906.ch21.f10

nitro group is reduced to produce radicals that cause DNA damage. Nitro group reduction requires enzymes that function at very low redox potential, as are present only in anaerobes and some microaerophiles.

Polypeptide antibiotics such as polymyxins and gramicidins appear to interact with phospholipids in cytoplasmic membranes, resulting in the disruption of the membrane and leakage of cellular contents. They are used topically for treatment of severe infections caused by certain gram-negative bacteria, systemic use being precluded by their toxicity.

Daptomycin (Fig. 11), the first lipopeptide approved for clinical use (in 2003), has cidal activity against gram-positive bacteria. Its mechanism of action involves a calcium-dependent binding to, and insertion of its lipophilic tail into, the cytoplasmic membrane, resulting in oligomerization and channel formation. This causes leakage of potassium and other ions and, consequently, cellular depolarization. Multiple failures in DNA, RNA, and protein synthesis follow, leading to death in the absence of lysis. Daptomycin is approved for the treatment of complicated skin and soft tissue infections caused by MRSA, VRE, glycopeptide-intermediate and -resistant *S. aureus*, and penicillin-resistant *S. pneumoniae*.

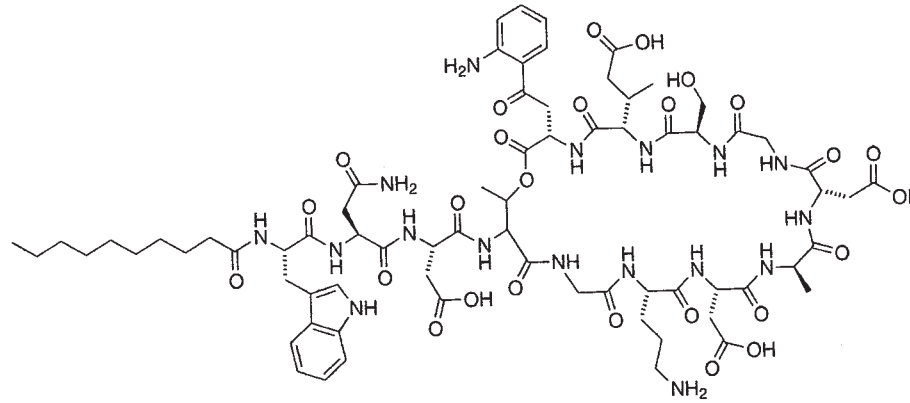


FIGURE 11 Daptomycin structure. Adapted from C. Walsh, *Antibiotics: Actions, Origins, Resistance* (ASM Press, Washington, DC, 2003).
doi:10.1128/9781555818906.ch21.f11

Treatment of Tuberculosis

Due largely to the long generation time of *Mycobacterium tuberculosis*, the location of the organism in infected individuals, and the extended latencies associated with tuberculosis, treatment of patients with single antibiotics results in the emergence of strains resistant to those antibiotics. Therefore, treatment must be initiated with multiple antimicrobial agents. Such treatment may involve three or more of such first-line drugs as isoniazid (INH), rifampin, pyrazinamide (PZA), ethambutol, and streptomycin or of such second-line antibiotics as *p*-aminosalicylic acid, ethionamide, D-cycloserine, fluoroquinolones, and the aminoglycosides kanamycin and amikacin, as well as other, lesser-known drugs. The first-line agents have higher efficacies and lower toxicities than the second-line agents. INH and ethionamide are prodrugs that, once activated via oxidation, inhibit an enzyme involved in mycolic acid synthesis. PZA is also a prodrug that must be activated, in this case by enzymes unique to *M. tuberculosis*, prior to inhibition of its target, which is unknown. The target(s) of ethambutol would appear to be several arabinosyltransferases involved in the synthesis of arabinans, which are unique to the cell walls of mycobacteria. *p*-Aminosalicylic acid is an inhibitor of folic acid synthesis.

Antibiotic Combinations

Occasionally, the combined activity of two antibiotics is greater than the activity expected from the effect of each individual antibiotic, a phenomenon referred to as synergism. The classic example of synergism is the simultaneous use of a β-lactam antibiotic, such as penicillin, and an aminoglycoside, such as streptomycin. The synergistic effect is thought to derive from an enhancement of cell permeability to the aminoglycoside due to the inhibition of cell wall synthesis by the β-lactam. The combined use of trimethoprim and sulfamethoxazole, also known as cotrimoxazole, is also thought to be synergistic. Combination treatment need not involve antibiotic synergism. For example, such combinations may be used to prevent or delay the emergence of resistant microorganisms, as in the combined use of isoniazid, rifampin, and ethambutol for the treatment of tuberculosis; to overcome polymicrobial infections, such

as intra-abdominal abscesses or endodontal infections in which the different bacteria involved may be susceptible to different antibiotics; or to treat particularly serious infections at a stage prior to the identification of the infectious agent(s).

There are some instances in which the combined effect of two antibiotics is less than that of either antibiotic alone. Such antagonism is typically associated with the combined use of a bacteriostatic, e.g., chloramphenicol, and a bactericidal antibiotic, e.g., streptomycin. Macrolides, lincosamides, and chloramphenicol are antagonistic, due to their overlapping binding sites on the ribosome.

Measurements of Antibiotic Potency

The strength or potency of any antibiotic against bacteria varies from species to species, and even from strain to strain within the same species. The degree of potency of an antibiotic is measured in terms of the minimal concentration of that agent required to completely inhibit the growth of a bacterial strain in question, and is termed the minimal inhibitory concentration (MIC), usually measured in μg/ml. MICs can be measured precisely by setting up a series of antibiotic solutions, usually in twofold increments, made up in a growth medium determined by the particular species being examined, and adding to these solutions a predetermined number of bacterial cells, e.g., 10^5, per solution, followed by incubation of the inoculated solutions at a temperature and for a period of time, generally 24 to 48 hours, also determined by the species under study. If, in a particular test, the bacteria were able to grow in solutions containing 1, 2, 4, and 8 μg/ml of the antibiotic but did not grow in solutions containing 16, 32, 64, or 128 μg/ml, then the MIC of that antibiotic for that particular bacterial isolate would be reported as 16 μg/ml (Fig. 12). The MIC provides an indication of the concentration of antibiotic required to prevent the growth of the test organism but does not indicate the concentration of the antibiotic required to kill the strain, i.e., the minimal bactericidal concentration (MBC), or if the antibiotic is cidal at any concentration. If the experiment illustrated in Fig. 12 is carried one step further, the MBC can be determined. In this case, aliquots of the cultures from which the MIC was read are subcultured on petri dishes containing agar-based media devoid of any antibiotic. The plates are then incubated for 24 to 48 hours and examined for the presence of bacterial colonies. In this case, the lowest concentration of antibiotic in the original solution that did not produce any colonies on the antibiotic-free plates would be designated the MBC, 32 μg/ml. Methods of MIC determination have been standardized and updated over time for all clinically available antibiotics and for all relevant bacterial species. These standards are published by the National Committee for Clinical Laboratory Standards (NCCLS), so that results obtained in different laboratories can be compared.

Often, it is only necessary to know whether or not the organism(s) likely causing a particular infection is susceptible or resistant to one or more of the antibiotics normally used to treat those infections. In such cases, particularly in the hospital setting, a quantitative disk diffusion test is employed. A disk impregnated with a standard amount of antibiotic is placed on a petri dish containing an agar-based medium (usually Mueller-Hinton agar) that has been inoculated with a standard concentration of

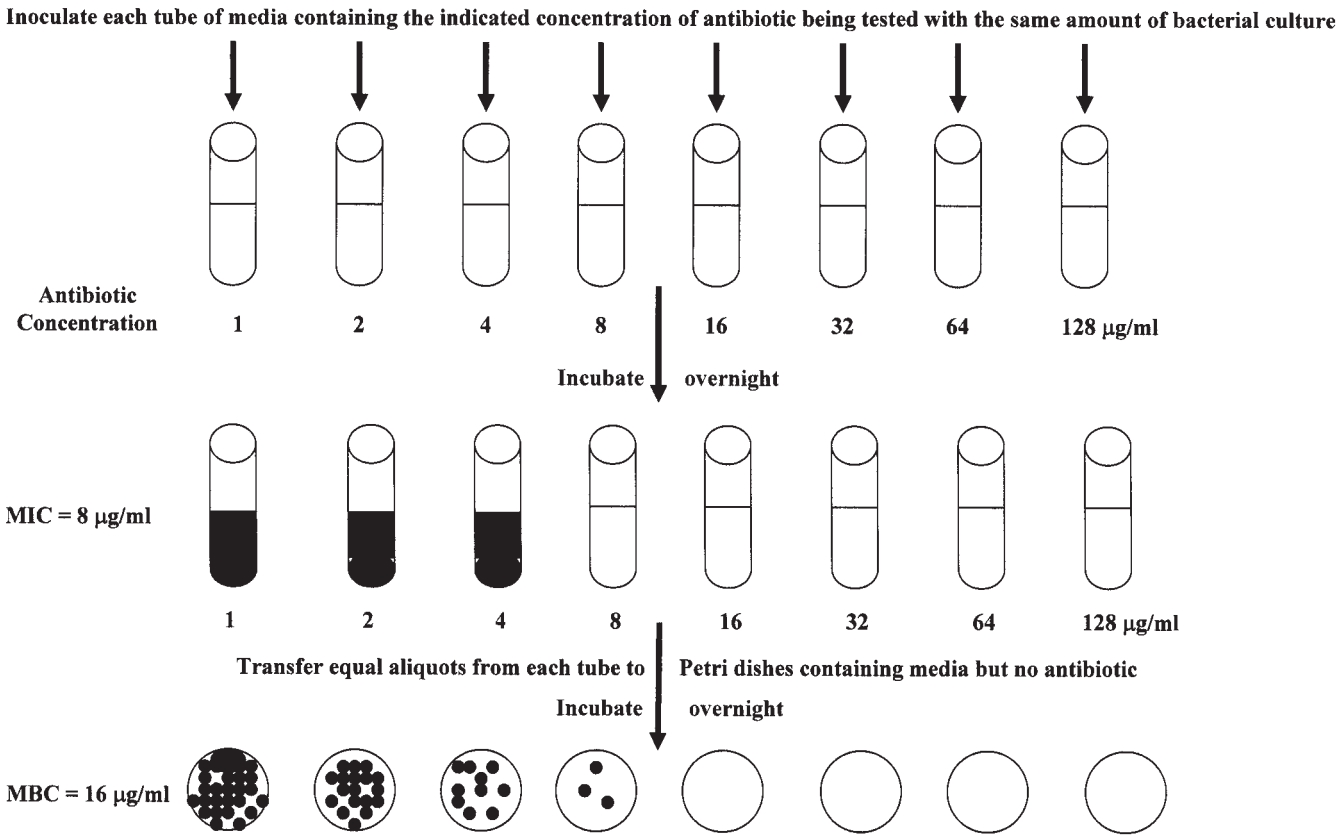


FIGURE 12 Determination of MIC and MBC of an antibiotic. A series of sterile test tubes (or wells in a microtiter plate) are set up, each containing a broth medium that will permit the growth of the bacterial pathogen of interest, as well as twofold dilutions of the antibiotic of which the MIC is to be determined. Each tube is inoculated with a specific number of bacteria, e.g., 10^5, in an overnight culture. All tubes are incubated overnight or longer, depending on the bacterial species. Each tube is then examined for growth (increased turbidity, e.g., optical density at a specific wavelength in a spectrophotometer). The concentration of antibiotic that completely inhibits the growth of the bacterium, in the instance illustrated, 8 μg/ml, is recorded as the MIC. In order to determine what concentration of the antibiotic is necessary to kill the bacterium, if the antibiotic is bactericidal, an aliquot, e.g., 0.1 ml, of each culture used to determine the MIC is spotted onto a petri dish containing an agar-based medium but lacking antibiotic and is spread over the entire surface of the medium. All petri dishes are incubated overnight or longer and then examined for the presence of bacterial colonies. That plate on which no colonies can be detected is used to determine the MBC of the antibiotic under study. In the case illustrated, the first plate in the series that contained no colonies had been inoculated with medium from the tube containing 16 μg/ml of antibiotic, which is thus determined to be the MBC. doi:10.1128/9781555818906.ch21.f12

the bacterial isolate to be tested. The plate is incubated for a specified period of time and then examined for zones of inhibition of bacterial growth around the disk. If the strain is resistant to the antibiotic on the disk, it will have grown up to, or very close to, the edge of the disk. If it is susceptible to the antibiotic, there will be a clear zone around the disk within which growth of the strain has been inhibited. Based on previously determined results published in tables, the diameter of the zone will be interpreted as an indication of a susceptible, intermediate, or resistant bacterial strain.

ANTIBIOTIC RESISTANCE

Resistance to Antibiotics Follows Their Introduction into Clinical Practice

Howard Florey, Ernst Chain, and Norman Heatley, working in England and then in the United States, had devised by 1939 methods for the production of large quantities of penicillin. Penicillin was shipped to Massachusetts General Hospital for the treatment of more than 200 badly burned survivors of a major fire in Boston, most of whom were saved from fatal infections. Toward the end of the war, sufficient quantities were available to treat Allied troops injured in battle, which for the first time lowered significantly the numbers of military casualties due to wound infections. Shortly thereafter, penicillin became readily available to the general public and, until the mid-1950s, without prescription. Individuals began to use the antibiotic for numerous ailments, both appropriate and inappropriate, and often at inappropriate doses. Strains of *S. aureus* resistant to high levels of penicillin were isolated from patients as early as 1942, and by 1946, in one particular hospital approximately 14% of *S. aureus* strains isolated from patients were resistant. Today, greater than 90% of *S. aureus* isolates from hospitals and the community are resistant to penicillin. Similar stories have played out over and over such that the introduction of every new antibiotic, or class of antibiotic, into clinical practice has been followed by the emergence of bacterial pathogens exhibiting resistance to clinically relevant levels of that antibiotic, often within 1 to 3 years of its introduction (Table 2).

R Factors and Other Antibiotic Resistance Plasmids

Beginning in the mid-1930s, sulfonamides were the only drugs available for the treatment of dysentery. However, by 1950, virtually 100%

TABLE 2 Introduction of antibiotics into clinical practice followed by emergence of resistance in key bacterial species

Antibiotic	Yr introduced	Emergence of resistance in:	Resistance first reported in:
Sulfonamides	1935	*Shigella dysenteriae*	1940s
Penicillin	1941	*Staphylococcus aureus*	1942
Penicillin	1941	*Streptococcus pneumoniae*	1970
Streptomycin	1943	*Enterobacteriaceae*	1940s
Tetracycline	1948	Numerous genera and species	1950s
Erythromycin	1952	*Streptococcus* species	1960s
Vancomycin	1956	*Enterococcus faecalis*	1988
Vancomycin	1956	*Staphylococcus aureus*	2002
Trimethoprim	1956	*Salmonella enterica* serovar Typhimurium	1959
Methicillin	1960	*Staphylococcus aureus*	1961
Ampicillin	1963	*Escherichia coli*	1963
Ampicillin	1963	*Haemophilus influenzae*	1974
Ampicillin	1963	*Neisseria gonorrhoeae*	1976
Gentamicin	1964	*Enterococcus faecalis*	1983
Fluoroquinolones	1984	*Enterobacteriaceae*	1990s
Linezolid	2000	*Enterococcus* species	2001
Daptomycin	2003	MRSA	2003
Tigecycline	2005	*Enterobacteriaceae*	2010

of *Shigella* strains isolated from such patients in Japan were resistant to these drugs. Fortunately, also by 1950, the antibiotics streptomycin, tetracycline, and chloramphenicol had become available for the treatment of these *Shigella* infections. Due in large measure to their experience with the sulfonamides, the Japanese began to monitor *Shigella* isolates for their susceptibility to these three antibiotics as well as sulfonamides. Strains resistant to the antibiotics began to emerge at very low frequencies, with five isolates resistant to streptomycin and only two resistant to tetracycline, of nearly 5,000 isolates examined in 1953, and resistant strains continued to appear at low frequencies through 1956. Beginning in 1957, however, not only did the number of isolates resistant to one of the three antibiotics increase, but strains resistant to two and even all three of these antibiotics emerged, and frequencies of isolation of such multiply resistant strains also increased over the next 3 years. These isolates were also resistant to sulfonamides. Assuming that resistance to each of these antimicrobial agents was encoded by a different gene and that the frequency of mutation to resistance for each gene could be as high as 1 per 10^6 bacteria per generation, Japanese investigators calculated that the highest frequency at which a relevant mutation could have occurred in each of the four genes in the same bacterial cell was one in 10^{24} bacteria. Clearly, such multiple antimicrobial resistance could not be explained on the basis of mutation. These investigators had also observed that multiply resistant and totally susceptible *Shigella* strains could be isolated from the same patient and that both *Shigella* and *E. coli* strains exhibiting the same multiple-resistance phenotype could also be obtained from the same patient. They postulated that the resistance genes might be transferred together between these two species in the intestinal tracts of patients and demonstrated such transfer in the laboratory by mixed incubation of susceptible *E. coli* and multiresistant *Shigella* strains, and vice versa. They also demonstrated a requirement for cell-to-cell contact between donor and recipient strains, as had been demonstrated for the F factor. The investigators proposed a new term, "R factor," for a genetic element able to transfer antibiotic resistance from one bacterial cell to another by conjugation. Subsequently, it was shown that R factors were conjugative plasmids that encoded one or more antibiotic resistance genes. Throughout the 1960s and into the early 1970s, it appeared that resistance to antibiotics among clinical isolates of the *Enterobacteriaceae* was almost exclusively plasmid mediated, either by large conjugative plasmids (R factors) or by smaller nontransmissible plasmids that were often mobilizable by conjugative plasmids present in the same cells.

Penicillin resistance in *S. aureus* was shown to be mediated by a large, nonconjugative plasmid, and the gene responsible for penicillin resistance of *S. aureus* coded for the production of an enzyme, penicillinase (later designated β-lactamase), which inactivates penicillin by cleavage of its β-lactam ring (Fig. 2). The macrolide erythromycin became available in 1952 for treatment of infections caused by gram-positive bacteria, and by the 1960s strains of *S. aureus* and *S. pyogenes* resistant to this antibiotic were being isolated from patients. Resistance to trimethoprim, a totally synthetic antibiotic, was first observed in England among isolates of *Salmonella enterica* serovar Typhimurium in 1959, just 3 years after its introduction (Table 2). These resistance traits were also shown to be plasmid mediated, at least among the early isolates.

Acquired Antibiotic Resistance

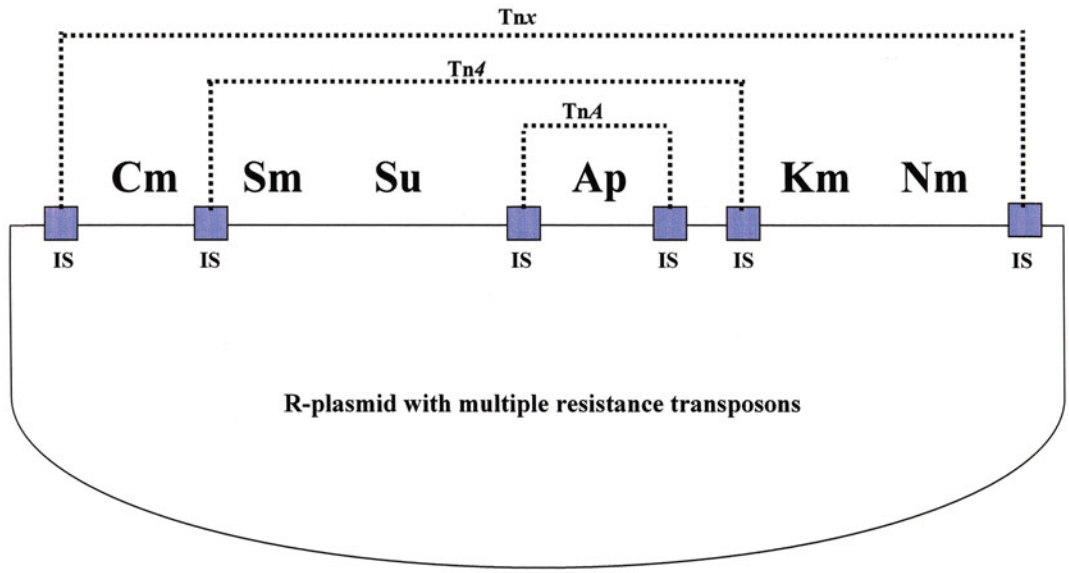
Pathogenic bacterial species that were becoming resistant to antibiotics were said to have acquired these resistance traits, since initially, these species had been shown to be intrinsically susceptible to those antibiotics, and their resistance traits were due to specific genes carried on plasmids as opposed to mutations in chromosomal genes. These plasmid genes coded for proteins that rendered the host bacterium resistant by one of four basic mechanisms, i.e., by enzymatic detoxification of the antibiotic; by alteration of the antibiotic target site; by altered uptake or retention of the antibiotic; or via a bypass of the antibiotic target. Examples of enzymatic detoxification include the hydrolysis of the β-lactam ring of penicillins and other β-lactam antibiotics by β-lactamases, the acetylation of chloramphenicol by a chloramphenicol-specific acetyltransferase, and the phosphorylation, adenylylation, or acetylation of different residues on an aminoglycoside molecule. The earliest example of alteration of an antibiotic target site was the methylation of an adenine residue of 23S rRNA encoded by an *erm* gene that results in resistance to macrolides such as erythromycin, lincosamides such as lincomycin or clindamycin, and streptogramin B type antibiotics such as virginiamycin, the so-called macrolide-lincosamide-streptogramin B (MLS) resistance phenotype. The classic example of altered uptake or retention of an antibiotic is active efflux from the cell of tetracycline, encoded by some *tet* genes, e.g., *tetA* and *tetB*, common to many gram-negative bacteria, or the gram-positive bacterial determinant *tetK*. The sulfonamides and trimethoprim inhibit specific enzymes in the folate pathway, and acquired resistance to these antimicrobials involves bypassing these sensitive steps via plasmid-mediated production of sulfonamide-resistant DHPS or trimethoprim-resistant DHFR.

Transposons and Other Mobile Genetic Elements Carry Antibiotic Resistance Genes

The aminopenicillin ampicillin, with a broader gram-negative bacterial spectrum than penicillin G, was introduced into clinical practice in 1963, and in that same year *E. coli* strains resistant to this antibiotic began to emerge. Antibiotic resistance transposons, i.e., individual resistance genes flanked by insertion element (IS) sequences (see Chapter 7) were being discovered and analyzed by several laboratories in the early 1970s. One such transposon, designated Tn*3*, encodes TEM β-lactamase activity that destroys penicillin and ampicillin. Initially, Tn*3* was shown to be present on a variety of different plasmids isolated not only from *E. coli* but also from several different species of *Enterobacteriaceae*. Ampicillin is also effective for the treatment of infections caused by *Haemophilus influenzae* and is safe to use on children. However, in 1974, three infants died from bacterial meningitis following treatment with ampicillin. All had been infected by ampicillin-resistant strains of *H. influenzae*. Subsequently, it was shown that the strains harbored plasmids that carried approximately one-third of Tn*3* but that the rest of each plasmid was unrelated to any known *Enterobacteriaceae* plasmid. One scenario to explain how this may have occurred went as follows. A plasmid carrying Tn*3* had been transferred from a bacterial species, e.g., *E. coli*, to a strain of *H. influenzae*, but the

introduced plasmid could not replicate in the new host. However, the incoming plasmid survived long enough for the transposon to relocate on an indigenous *H. influenzae* plasmid, creating a new R plasmid and broadening the spectrum of species now resistant to penicillins. Two years after the emergence of ampicillin resistance in *H. influenzae*, strains of penicillin-resistant *N. gonorrhoeae* began to appear. In this case, resistant isolates were shown to harbor a small, β-lactamase-encoding plasmid with greater than 90% homology to a plasmid isolated from a strain of *Haemophilus parainfluenzae*, each containing approximately 40% of Tn*3*. Subsequently, it was shown that small β-lactamase-encoding plasmids could be mobilized from strain to strain by a conjugative plasmid indigenous to *N. gonorrhoeae*. Thus, although the first penicillin-resistant strains of *N. gonorrhoeae* may have received this trait from an unrelated bacterial species, possibly via conjugation or transformation, penicillin resistance was now being spread among *N. gonorrhoeae* strains via conjugative mobilization. From the early 1970s through the mid-1980s, transposons encoding a variety of antibiotic resistance traits were discovered and studied in gram-positive as well as gram-negative bacterial species. It was also observed that transposons often had a tendency to insert in a plasmid adjacent to other transposons, such that resistance regions of R plasmids evolved by the successive addition of individual transposons, often forming new transposons encoding multiple resistance traits, as illustrated in Fig. 13.

FIGURE 13 Evolution of a multiple antibiotic resistance plasmid by acquisition of transposons. The plasmid illustrated encodes resistance to six different antibiotics: chloramphenicol (Cm), streptomycin (Sm), sulfonamides (Su), ampicillin (Ap), kanamycin (Km), and neomycin (Nm). The acquisition of the genes encoding these traits apparently occurred via the insertion of three different transposons: Tn*A*, a well-studied β-lactamase-encoding transposon; a second transposon, Tn*4*, encoding resistance to Sm and Su; and yet a third transposon, Tn*X*, encoding resistance to Cm, Km, and Nm, that inserted on either side of the two other transposons, or into which Tn*A* and Tn*4* inserted. doi:10.1128/9781555818906.ch21.f13



Throughout the 1970s and into the 1980s, it became a popular belief that all acquired antibiotic resistance was plasmid mediated and that the dissemination of resistance plasmids among bacterial strains was by the transfer of a conjugative plasmid, or via the mobilization by such a plasmid of a nonconjugative plasmid, and maybe occasionally via plasmid transformation. Furthermore, multiple-resistance plasmids, whether conjugative or not, evolved by the acquisition of transposons. There was, however, a growing body of anecdotal "data" to suggest the existence of bacterial strains that expressed acquired resistance phenotypes but from which no plasmid DNA could be isolated. In some instances, these traits could be transferred from one strain to another, by a process that required cell-to-cell contact, but with no evidence for the presence of plasmid DNA in either donor or transconjugant strains. In 1982, an explanation for such results was forthcoming with the first published description of a conjugative transposon, Tn*916*. This transposon, 18 kb in size, not only carried a *tetM* determinant of tetracycline resistance but also encoded all the information required for its excision from the host chromosome, circularization, conjugative transfer, and reinsertion into the chromosome of its new host, all in the absence of plasmid DNA. Conjugative transposons of various sizes and encoding multiple-resistance traits have since been described, and such transposons are known to be capable of inserting into any type of bacterial replicon, chromosome, phage, or plasmid. Unlike previously described tetracycline resistance determinants, *tetM* did not encode resistance by an efflux mechanism, but rather the product of *tetM* was shown to protect the ribosome from the inhibitory activity of not only tetracycline but also the newer tetracycline, minocycline, resistance to which is not provided by the efflux-encoding genes. In addition, whereas each tetracycline efflux-mediating gene was found exclusively in gram-positive or gram-negative species, but never both, ribosomal protection determinants *tetM* and, later, several others were detected and shown to express in gram-positive and gram-negative bacteria, as well as the cell wall-free *Mycoplasma* species.

Vancomycin has been available for the treatment of serious nosocomial infections caused by gram-positive bacteria since 1956, and no resistance was encountered until 1988, when isolates of VRE began to appear in France, soon to be followed in the United States. The mechanism of resistance involved alteration of the target of vancomycin, the D-Ala-D-Ala portion of UDP-muramyl pentapeptide. The terminal D-Ala residue in resistant isolates is replaced by D-lactate. While vancomycin binds to D-Ala-D-Ala, it cannot bind to D-Ala-D-Lac. In 1989, resistance to vancomycin was shown to be mediated by a conjugative plasmid isolated from a strain of *E. faecalis*, and in 1993, the genes responsible for resistance were located on a transposon, Tn*1546* (illustrated in Fig. 14), carried on a conjugative plasmid. The genes encoding enzymes directly involved in vancomycin resistance are *vanH*, *vanA*, *vanX*, and *vanY*. A dehydrogenase that converts pyruvate to D-lactate is encoded by *vanH*. A ligase encoded by *vanA* joins D-Lac to a D-Ala residue to form D-Ala-D-Lac, which is added to UDP-muramyl tripeptide, in place of D-Ala-D-Ala. The *vanX* gene encodes a carboxypeptidase, which hydrolyzes any D-Ala-D-Ala dipeptides that have been formed, and *vanY* encodes a carboxypeptidase

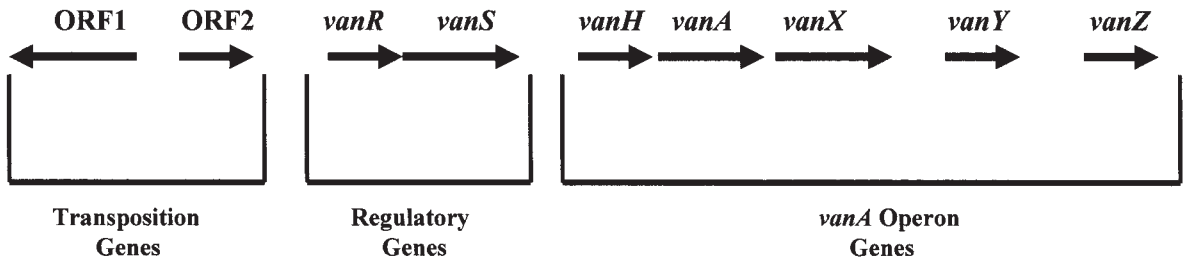


FIGURE 14 Organization of genes on VanA transposon Tn*1546*. Depicted are the genes encoded by the transposon. ORF1 and ORF2, which are not part of the *vanA* operon, encode enzymes involved in the transposition process. *vanR* and *vanS* encode the two-component regulatory system by which the expression of the operon genes are induced in the presence of vancomycin. *vanH*, *vanA*, *vanX*, *vanY*, and *vanZ* encode enzymes involved in the replacement of D-Ala-D-Ala with D-Ala-D-Lac in the cell wall precursor pentapeptide, as described in greater detail in the text. Adapted from M. Arthur, F. Depardieu, G. Gerbaud, M. Galimand, R. Leclercq, and P. Courvalin, *J. Bacteriol.* **179:**97–106, 1997. doi:10.1128/9781555818906.ch21.f14

to hydrolyze any pentapeptides that contain D-Ala-D-Ala. The function of *vanZ* remains unknown. The *van*S and *vanR* genes encode proteins of a two-component regulatory system through which expression of the *van* genes is induced in the presence of vancomycin. Open reading frame 1 (ORF1) and ORF2 are genes that encode proteins involved in transposition. Transposons encoding similar vancomycin resistance operons, mediating the so-called VanB, VanD, and VanG phenotypes, have also been described. More recently, in 1995, a >25-kb conjugative transposon, Tn*5482*, encoding a VanB phenotype was identified in a strain of *Enterococcus faecium*.

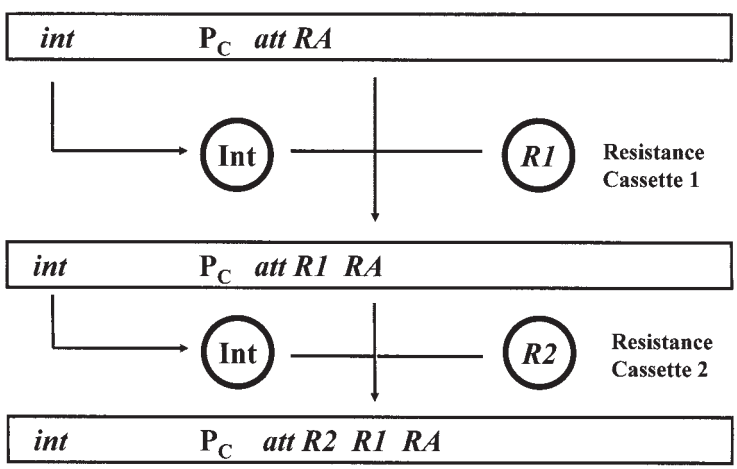
Vancomycin has been the drug of choice for the treatment of MRSA since the 1960s. A strain of *S. aureus* resistant to intermediate concentrations of vancomycin (4 to 8 μg/ml), termed vancomycin-intermediate *S. aureus* (VISA), was isolated from a patient in Japan in 1996, and similar strains were appearing throughout the world shortly thereafter. The VISA strains were shown to produce three to five times as much PBP2 or PBP2′ as wild-type *S. aureus* and to accumulate intracellularly three to eight times as much murein monomer precursors as wild-type strains. Microscopically, the cell walls of these strains were much thicker than normal. Infections caused by VISA strains were still treatable by higher concentrations of vancomycin. The real fear, for some time, was that *S. aureus* might obtain a vancomycin resistance operon from a resistant enterococcal strain, and in fact, transfer of a plasmid carrying a *vanA* transposon from a strain of *E. faecalis* to *S. aureus* was demonstrated in the laboratory in 1992. However, it was not until 2002 that the first clinical isolate of *S. aureus* harboring a conjugative plasmid that encoded the VanA phenotype was obtained, in Michigan. The plasmid in that isolate had clearly come from an *Enterococcus* species. A similar strain of *S. aureus*, with a 120-kb VanA-encoding plasmid, was isolated from a patient in Pennsylvania later in the same year, and in 2004, another isolate was obtained from a patient in New York. The MIC of vancomycin against these resistant *S. aureus* (VRSA) strains is ≥32 μg/ml. Currently, the isolation of nine VRSA strains has been reported; all but the two mentioned above were from patients in Michigan, and all nine strains were MRSA. The transposon,

Tn*1546*, had relocated onto a resident staphylococcal plasmid in five of the isolates from Michigan. Thus far, there have been no reports of isolation of VRSA from elsewhere in the United States or the rest of the world.

The gene responsible for β-lactamase production by *S. aureus* isolates obtained from patients in the 1940s was designated *blaZ*. The gene is part of a transposable element found on a large plasmid that often encodes resistance to other antibiotics, such as gentamicin and erythromycin, and is present in an overwhelming majority of *S. aureus* strains isolated from patients in hospitals, as well as in the community. Methicillin, the first of the semisynthetic penicillins, and which is resistant to the activity of the β-lactamase encoded by *blaZ*, was introduced into clinical practice in 1961. As was true with penicillin-resistant *S. aureus*, MRSA began to appear almost immediately, first in the hospital setting, followed much later (2000) in the community. Methicillin resistance is encoded by the *mecA* gene, which is part of a chromosome-borne mobile genetic element found only in MRSA. Although methicillin resistance is common in hospitals and in the community, only a few clones of *S. aureus* carry it, transfer of *mecA* being relatively rare. The *mecA* gene encodes a penicillin binding protein, PBP2a (also called PBP2′), which has a very low affinity for all β-lactam antibiotics. PBP2a is not found in methicillin-susceptible *S. aureus*. Methicillin resistance equals resistance to all β-lactam antibiotics, including cephalosporins.

A new type of genetic element, the integron, was identified in the late 1980s. There are two types of integrons, the so-called superintegrons, which can carry more than 100 genes, and the resistance integrons, which can carry up to 10 different genes encoding resistance to antibiotics or disinfectants. All integrons are composed of three essential elements, illustrated in Fig. 15. The first of these elements is a gene (*int*) encoding an integrase, which mediates the insertion, by a *recA*-independent mechanism,

FIGURE 15 Building of an integron. The top box depicts a simple integron composed of an integrase gene, *int*, a strong constitutively expressed promoter, P_C, an attachment site for insertion of resistance gene cassettes just downstream of the promoter, and one resistance gene, *RA*. The two bottom boxes depict the addition of new cassettes to the integron, catalyzed by the integrase, Int. Expression of the three resistance cassettes depicted in the bottom box is controlled by the single promoter. Adapted from D. Mazel, *ASM News* **70**:520–525, 2004. doi:10.1128/9781555818906.ch21.f15



of circularized segments of DNA designated gene cassettes at the second essential integron element, the attachment site (*att*). Gene cassettes are ORFs without promoters. However, they are inserted within an integron downstream of the third essential element of the integron, a strong promoter, designated P_c, which controls the expression of all the ORFs in a given integron. Integrons may be located on plasmids or in the host chromosome, and some are located within transposons. Initially, integrons were identified only in gram-negative bacterial species, but more recently they have been detected in such gram-positive bacterial genera as *Corynebacterium*, *Mycobacterium*, and *Enterococcus*. It has been discovered that the R-factor-mediated resistance to sulfonamides, tetracycline, streptomycin, and chloramphenicol in one of the multiply resistant *Shigella dysenteriae* strains isolated in Japan in the 1950s was encoded by an integron.

The Role of Mutations in Antibiotic Resistance

Resistance to fluoroquinolones in most bacterial species has been traced to mutations that result in amino acid changes in either DNA gyrase or topoisomerase IV. Such mutations are usually identified in the *gyrA* gene of DNA gyrase or in the *parC* gene of topoisomerase IV, although occasionally mutations contributing to resistance have been identified in the *gyrB* or *parE* genes. Low-level resistance can, for the most part, be traced to one or more mutations in the genes encoding the primary target of the fluoroquinolones to which resistance is exhibited, usually gyrase in gram-negative bacteria and topoisomerase IV in gram-positive bacteria. Mutations resulting in fluoroquinolone resistance are located in a specific region of the gene referred to as the quinolone resistance determining region, or QRDR. Mutations in the primary target of a fluoroquinolone lower the sensitivity of that target to the antibiotic such that subsequent mutations, resulting in higher levels of resistance, may be in either *gyrA* and/or *parC* or, to a lesser extent, in *gyrB* or *parE*.

Penicillin-resistant strains of *S. pneumoniae* were isolated for the first time only in the late 1970s, in South Africa and in the United States. Coincident with the isolation of penicillin-resistant *S. pneumoniae* in South Africa, resistant viridans group streptococci, such as *Streptococcus mitis* and *Streptococcus sanguinis*, were being isolated from the nasopharynxes of the same children. Resistance to penicillin in *S. pneumoniae* and in the viridans group of streptococci has been traced to the presence of multiple mutations in the region of the genes associated with the binding affinity for penicillin of the larger PBPs, such that their affinities for these antibiotics are lowered considerably. Once such strains emerged, their resistance could easily have been disseminated among several members of the viridans group of streptococci or strains of *S. pneumoniae*, due to the ability of these species to achieve a state of natural competence for transformation. That the exchange of PBP-encoding genes has actually occurred in nature was clearly demonstrated in two types of studies. First, the genes encoding PBP2b from several penicillin-resistant isolates of *S. pneumoniae* were shown to have a mosaic structure with large blocks of DNA sequence clearly derived from *S. mitis*. These mosaic genes likely arose via localized recombinational events between an incoming gene and its resident chromosomal homolog in the transformed recipient strain. In another study, PBP2b-encoding genes from penicillin-resistant isolates of *Streptococcus oralis* were shown to be greater than 99% identical in

sequence to the homolog from a penicillin-resistant strain of *S. pneumoniae*. Thus, although it very likely required several years for a few strains of viridans group streptococci, and/or *S. pneumoniae*, to accumulate a sufficient number of mutations in a PBP-encoding gene to render the bacterium resistant, such resistance has been and continues to be broadly disseminated among these streptococcal species via transformation.

That a scenario similar to the one just described for penicillin-resistant, naturally transformable streptococci might also have occurred in the case of resistance to the fluoroquinolones among these bacterial species has been demonstrated in the laboratory. It has been possible to transfer to *S. pneumoniae* resistance to fluoroquinolones with DNA obtained from resistant strains of *S. oralis*, *S. mitis*, *S. sanguinis*, and *Streptococcus constellatus* containing mutations in *parC*. Similarly, DNA from an *S. pneumoniae* transformant could be used to transform *S. mitis* to resistance. Even more significantly, high-level fluoroquinolone resistance was transferred to *S. pneumoniae* with DNA obtained from a strain of *S. mitis* that had mutations in both *gyrA* and *parC*. Since these two genes are separated by a considerable distance on the chromosome, such a transformation required two independent events in a single transformation and occurred at a very low frequency, but it did occur and likely could occur in nature.

Linezolid, an oxazolidinone, was approved by the FDA for the treatment of infections caused by gram-positive bacteria in 2000. As mentioned earlier in this chapter, linezolid represented the first new class of antibiotics to be introduced into clinical practice in more than 30 years. Although its bacterial target, the 50S ribosomal subunit, was not new, its mechanism of action, inhibition of formation of the translational initiation complex, was. In addition, all enterococcal and staphylococcal strains in a panel of clinical isolates tested were susceptible to this antibiotic, and frequencies of spontaneous mutation to resistance were extremely low, less than 1 in $>10^{10}$ bacteria for all relevant species tested. All of the above data suggested that the emergence of resistance to linezolid in the clinical setting would be rare. However, during clinical trials, prior to its approval by the FDA, linezolid-resistant enterococci were isolated from 2 of 169 patients with VRE infections who had been treated with the antibiotic in a compassionate-use program. SENTRY, a worldwide (North and South America, Europe, Asia, Australia, and South Africa) antimicrobial surveillance program that monitors the susceptibility of major bacterial pathogens to relevant antibiotics, reported that of 9,833 gram-positive clinical isolates obtained from January 2001 to June 2002, 8 were resistant to linezolid. Only three of the eight patients from which the resistant isolates had been obtained had received linezolid. The appearance of linezolid-resistant enterococci was reported for the first time in several countries from 2002 to 2004. Among all isolates studied, resistance was attributed to a mutation in residue 257 in domain V of 23S rRNA, which results in a change from guanine to uridine, or to thymidine in the DNA. Most bacterial species have multiple 23S rRNA genes, and results of one study have demonstrated a correlation between the level of resistance to linezolid and the percentage of 23S rRNA genes that contain the G257T mutation.

Daptomycin was approved for treatment of gram-positive bacterial infections in 2005. Although the emergence of resistance was expected to be and remains rare, resistant isolates of *S. aureus* and *E. faecalis* were obtained from patients being treated during the clinical trial stages. The first

report of resistance after approval involved the isolation of a daptomycin-resistant strain of MRSA in 2005, with no mechanism determined. Although the exact mechanism of daptomycin resistance remains unknown, it is thought to involve alterations in the cytoplasmic membrane, more specifically in membrane potential, due to mutation.

Resistance to metronidazole among anaerobic and microaerophilic bacteria can usually be attributed to mutations that result in the alteration or loss of the reductase or hydrogenase, or a cofactor such as flavodoxin, involved in the conversion of the prodrug to an active antibacterial compound within the cell. However, in some clinical isolates of *Bacteroides* species, resistance is due to the activity of the product of one of at least four known genes designated *ninA*, *ninB*, etc. The actual mechanism of resistance is not known. However, many *nin* genes have been shown to be carried on plasmids, some of which are transmissible.

The genes *rpoA*, *-B*, *-C*, and *-D* encode the α, β, β′, and σ subunits of RNA polymerase. Clinical isolates, as well as laboratory-derived rifampin-resistant strains of several bacterial species, all contain mutations in *rpoB*. Single mutations in *rpoB* result in high-level resistance, which would certainly explain the high frequencies at which isolates with clinically relevant levels of resistance to rifampin are obtained. Resistance to other first-line drugs used in the treatment of tuberculosis also occurs via mutation. The activation of INH (isonicotinic acid hydrazide) requires KatG, a catalase-peroxidase enzyme encoded by the gene *katG*. Approximately 70% of INH-resistant strains of *M. tuberculosis* have been found to have mutations in *katG*. The tuberculosis prodrug PZA (pyrazinamide) is activated by an amidase encoded by the gene *pncA*. Clinical isolates of *M. tuberculosis* resistant to PZA almost always have missense, termination, insertion, and/or deletion mutations in *pncA*. Enzymes for the synthesis of arabinogalactan by *M. tuberculosis* are encoded on the *emb* operon, which consists of *embC*, *embA*, and *embB*. The last gene encodes the enzyme arabinosyl transferase, which is the enzyme specifically inhibited by ethambutol (EMB). EMB-resistant clinical isolates of *M. tuberculosis* have been shown to have amino acid substitutions in arabinosyl transferase that can be correlated with EMB resistance.

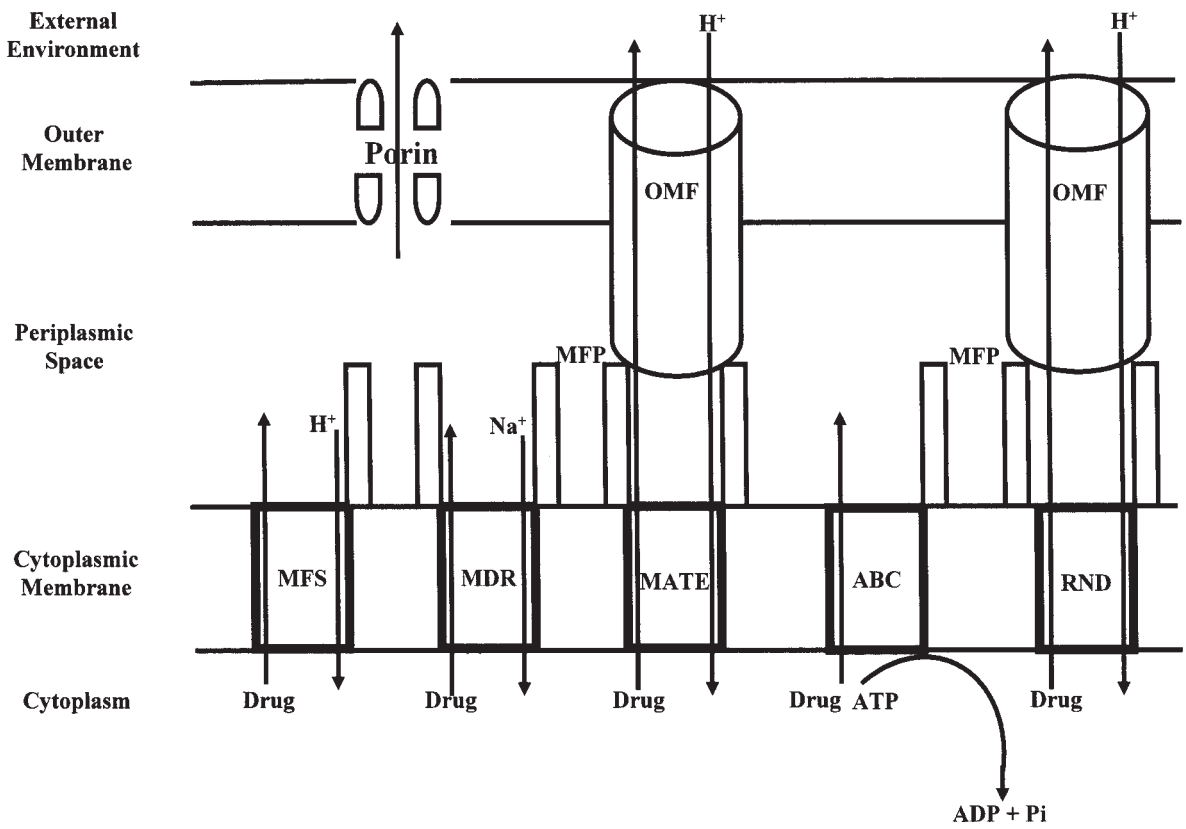
Although many sulfonamide- or trimethoprim-resistant clinical isolates of *Enterobacteriaceae* were shown early on to harbor plasmids carrying genes that encoded dihydropteroate synthase (DHPS) or dihydrofolate reductase (DHFR) enzymes, respectively, that expressed wild-type activities, they were not inhibited by the respective antimicrobial agent. However, a large number of clinical isolates resistant to one or the other of these two agents have since been shown to contain mutations that have altered the chromosomal gene and/or its regulation. For example, sulfonamide-resistant mutants of *S. pneumoniae* were shown to contain mutations in the *sulA* gene resulting in two amino acid duplications in DHPS. Trimethoprim-resistant mutants of *H. influenzae* were shown to overproduce an altered chromosomally determined DHFR due to changes in the −35 promoter sequence and mutations in the gene for DHFR that resulted in two amino acid changes in the DHFR protein. Similarly, trimethoprim-resistant *E. coli* clinical isolates were shown to contain up-mutations in the −35 promoter sequence and several mutations in the DHFR gene.

Efflux Pumps: Association with Acquired and Intrinsic Resistance and Mutation to Resistance

One mechanism of resistance to some antibiotics, as already mentioned with regard to tetracycline, involves the acquisition of genes encoding proteins able to pump the antibiotic out of the cell. In addition, virtually all bacterial species encode on their chromosomes one to several transport systems that are, or can become, involved in efflux from the cell of single to multiple antibiotics, thus contributing to their antibiotic resistance phenotypes. To date, five families of bacterial efflux transporters, or pumps, have been described, and their arrangements on the surfaces of bacterial cells are illustrated in Fig. 16.

The ATP-binding cassette (ABC) superfamily of efflux pump proteins, i.e., the chromosomally encoded ABC transporters, for which the energy for transport is derived from ATP, are primarily involved in the transfer in and/or out of the bacterial cell of sugars, polysaccharides, amino acids, proteins, ions, and iron complexes. Several ABC transporters, mostly putative, but some previously characterized, have been identified

FIGURE 16 Classes of efflux pumps mediating drug resistance. The different classes of efflux pumps are explained in detail in the text. Gram-positive bacteria require only the cytoplasmic membrane component for transport to the environment, whereas gram-negative bacteria also employ periplasmic membrane fusion proteins (MFP) and outer membrane factor (OMF), also referred to as outer membrane protein (OMP), or a porin. Adapted from X.-Z. Li and H. Nikaido, *Drugs* **64:**159–204, 2004, and from K. Poole, *Curr. Pharm. Biotechnol.* **3:**77–98, 2002.
doi:10.1128/9781555818906.ch21.f16



in every bacterial species whose genome has been sequenced. However, the MacAB system of *E. coli* is the only one that has actually been shown to mediate the efflux of antibiotics in a gram-negative bacterium. This *macAB*-encoded system, composed of the periplasmic membrane fusion protein (MFP), MacA, and an integral cytoplasmic membrane protein, MacB, transports macrolides into the periplasmic space, where they are then transferred out of the cell through the multifunctional outer membrane channel, TolC.

The major facilitator superfamily (MFS) of transporters includes many intrinsically encoded symporters and antiporters of sugars, anions, and various metabolites, which generally function as single-component protein pumps. The transport energy for the MFS pumps is derived from the cell's electrochemical gradient, i.e., proton motive force (PMF). Examples of single component MF transporters are the fluoroquinolone efflux pumps NorA of *S. aureus* and PmrA of *S. pneumoniae*. Also included in this family are the macrolide efflux pumps MefA (first identified in *S. pyogenes*) and MefE (found first in *S. pneumoniae*), which mediate resistance to macrolides but not to lincosamides or streptogramins. The *mefA* and *mefE* genes are both encoded by conjugative transposons, and in some strains of *S. pyogenes*, the *mefA*-encoding transposon is located within the genome of a lysogenic bacteriophage. Thus, dissemination of these macrolide resistance elements can occur via conjugation, transformation, or transduction (at least among strains of *S. pyogenes*). The EmrAB-TolC pump of *E. coli* consists of the MF protein EmrB and the MFP EmrA, which act in conjunction with TolC to transport nalidixic acid and hydrophobic toxic compounds out of the cell. The plasmid- or transposon-mediated tetracycline resistance efflux pumps, such as TetL and TetK of gram-positive bacteria and TetA (initially described in Tn*10*) and TetB of gram-negative bacteria, are also members of the MF family of transporters.

The multidrug and toxic compound extrusion family comprises a group of electrochemical gradient-dependent transport proteins similar to the MF proteins. However, the energy source for transport, in these cases, is usually an Na^+ gradient rather than the H^+ gradient. Examples are the chromosomally encoded NorM and YdhE pumps of *Vibrio parahaemolyticus* and *E. coli*, respectively, which mediate resistance to aminoglycosides and fluoroquinolones, as well as cationic dyes.

A fourth family of efflux pumps, the small MDR transporters, like the MF transporters, are driven by the PMF. However, these pumps transport only antiseptics and disinfectants and include the Smr protein of *S. aureus* and the EmrE protein of *E. coli*.

The resistance-nodulation-cell division (RND) family of efflux pumps, which are usually chromosomally encoded, play a major role in the intrinsic resistance to antibiotics of several gram-negative bacterial species, such as *P. aeruginosa*, *Acinetobacter baumannii*, *H. influenzae*, many species of *Enterobacteriaceae*, and the anaerobic periodontal pathogen *Porphyromonas gingivalis* (Table 3). The RND pumps are usually complexes of proteins consisting of a PMF-driven cytoplasmic membrane transporter, a periplasmic membrane fusion protein, and an outer membrane channel and mediate the efflux of multiple classes of antibiotics. The constitutively expressed MexAB-OprM pump of *P. aeruginosa* renders this bacterium

TABLE 3 RND family multidrug efflux pumps in representative gram-negative species[a]

Bacterial species	Efflux system component			Substrates[b]
	MFP	RND	OMP	
Acinetobacter baumannii	AdeA	AdeB	AdeC	AG, Ct, Tc, Em, Cm, Tp, FQ
	AdeI	AdeJ	AdeK	BL, Tc, Cm SG, Rf
Enterobacter aerogenes	AcrA	AcrB	TolC	Ac, Cm, FQ, Mc, Nv, SDS, Tc
Escherichia coli	AcrA	AcrB	TolC	Ac, BL, BS, Cm, Cv, Eb, FA, ML, Nv, OS, Rf, SDS
	AcrA	AcrD	TolC	AG, Dc, Fu, Nv
	AcrE	AcrF	TolC	Similar to AcrA-AcrB-TolC
	MdtA	MdtBC	TolC	Dc, Nv
	YhiU	YhiV	TolC	Dc
Haemophilus influenzae	AcrA	AcrB	TolC	Ac, Cv, Eb, Em, Nv, Rf, SDS
Porphyromonas gingivalis	XepA	XepB	XepC	Ac, Eb, Pu, Rf, SDS
Pseudomonas aeruginosa	MexA	MexB	OprM	Ac, AG, BL, Cm, Cv, Eb, ML, Nv, OS, SDS, SF, Tc, Tp, Tr
	MexC	MexD	OprJ	Cm, CP, FQ, Tc, Tr
	MexE	MexF	OprN	Cm, FQ
	MexX	MexY	OprM	AG, ML, Tc
	MexH	MexI	OpmD	Vanadium
	MexJ	MexK	OprM	Em, Tc, Tr
Serratia marcescens	?	MexF-like	?	FQ
Salmonella enterica serovar Typhimurium	AcrA	AcrB	TolC	BL, FQ

[a]Adapted and updated from X.-Z. Li and H. Nikaido, *Drugs* **64:**159–204, 2004.

[b]Abbreviations: Ac, acriflavin; AG, aminoglycosides; BL, β-lactams; BS, bile salts; Cm, chloramphenicol; CP, cephalosporins; Ct, cefotaxime; Cv, crystal violet; Dc, deoxycholate; Eb, ethidium bromide; Em, erythromycin; FA, fatty acids; FQ, fluoroquinolones; Fu, fusidic acid; Mc, mitomycin; ML, macrolides; Nv, novobiocin; OS, organic solvents; Pu, puromycin; Rf, rifampicin; SF, sulfonamides; SDS, sodium dodecyl sulfate; Tc, tetracycline; Tp, trimethoprim; Tr, triclosan.

resistant to members of such antibiotic classes as the β-lactams, aminoglycosides, macrolides, sulfonamides, and tetracyclines; to chloramphenicol, novobiocin, and trimethoprim; to toxic dyes such as acridine orange, ethidium bromide, and crystal violet; to several organic solvents; and to the biocide triclosan, found in many household products such as antibacterial soaps and toothpastes. At least five other chromosomally encoded RND efflux systems have been identified in *P. aeruginosa*, making this opportunistic pathogen one of the most highly antibiotic resistant of all bacteria.

Tigecycline has been used since 2005 for the treatment of infections caused by MDR *A. baumannii*. However, 67% of MDR *A. baumannii* isolates were shown to exhibit tigecycline resistance in the results of a study published in 2007. The emergence of resistance to this antibiotic in a bacterial species normally susceptible to it was not observed until 2010. In one study, AcrAB-mediated resistance to tigecycline was observed in a clinical isolate of *Enterobacter cloacae* obtained from a patient who had been treated with ciprofloxacin. Also reported in 2010 was the isolation of a tigecycline-resistant strain of *Klebsiella pneumoniae* from a patient being treated with this antibiotic. The mechanism of resistance, in this instance, was not determined.

Oral Microbial Resistance

Much of the early work on antibiotic resistance in members of the oral microbial flora concentrated on the oral streptococci. One of the earliest reports of antibiotic resistance in oral bacteria described, in 1974, isolates

of *Streptococcus mutans* from endocarditis patients that were resistant to penicillin (one isolate), vancomycin (five isolates), chloramphenicol (nine isolates), and tetracycline (three isolates). Another report in 1980 described the effects of tetracycline therapy of periodontal patients on the resistance of the viridans group streptococci to this antibiotic. The results of this study showed that the proportion of resistant streptococci isolated from the gingivae of patients usually increased dramatically during treatment but in most patients decreased to pretreatment levels within a few months following the cessation of treatment. This report was one of the first to demonstrate the low proportion of viridans group streptococci that harbored plasmids (<14%), and in no instance was the resistance to tetracycline shown to be plasmid mediated. In 1992, the results of a study in France indicated that approximately 5% of viridans group streptococci expressed high-level resistance (MICs, >2,000 μg/ml) to aminoglycosides. Some of the isolates obtained in the latter study were able to transfer their resistance to the aminoglycosides kanamycin and streptomycin, as well as their MLS and tetracycline resistance phenotypes, *en bloc* to susceptible streptococcal species via mating on solid surfaces. In 1984, an *S. sanguinis* isolate from a patient with bacteremia was shown to have a vancomycin MIC of >128 μg/ml. Unfortunately, the mechanisms of antibiotic resistance expressed were not determined in any of the above-mentioned studies. However, in 1984 several resistant strains of viridans group streptococci were shown to carry *tetM*, and five of these strains contained a Tn*916*-like conjugative element. Subsequently, *tetO* was cloned and characterized from a strain of *S. mutans* (1988), and in 1990 clinical isolates of *Streptococcus milleri* were shown to encode the streptomycin resistance determinant *aadE* (seven strains), the kanamycin resistance gene *aph3A* (eight strains), and *ermB*, *tetM*, and *tetO* (11, 14, and 3 strains, respectively).

Numerous tetracycline resistance determinants have been identified in a variety of oral bacterial species: *tetB* in isolates of *Aggregatibacter actinomycetemcomitans* and *Treponema denticola*; *tetL*, normally considered a gram-positive bacterial gene, in *Eikenella corrodens*, *Neisseria sicca*, *Fusobacterium nucleatum*, and *Veillonella parvula*; *tetQ* in *Prevotella* species, *V. parvula*, and *Capnocytophaga* species; and *tetM* in a variety of gram-positive and gram-negative species. More recently, the relatively new determinant *tetW* was detected in isolates of oral species belonging to the genera *Streptomyces*, *Lactobacillus*, *Actinomyces*, *Veillonella*, *Streptococcus*, *Neisseria*, and *Prevotella*.

Since the late 1980s, a number of oral bacterial species, both gram-positive and gram-negative, have been shown to produce β-lactamases. The presence on plasmids of genes encoding β-lactamase activity has been demonstrated in isolates of *E. corrodens* and *Neisseria perflava-Neisseria sicca*, but the location of β-lactamase-encoding genes in such oral microorganisms as *F. nucleatum*, *Prevotella* species, *Tannerella forsythia*, *Porphyromonas* species, and *Veillonella* species has not been established. In addition, as mentioned earlier, PBP-mediated resistance to β-lactam antibiotics has been disseminated among the viridans group streptococci, as well as *S. pneumoniae*, by transformation among strains able to achieve a state of natural competence.

It seems obvious at this time that at least some species of oral bacteria have either developed or acquired resistance to virtually all clinically useful antibiotics, as is true of species normally occupying other niches. Although the vast majority of these oral species are considered to be commensal and not generally associated with infectious diseases, these bacteria clearly can and often do serve as reservoirs of antibiotic resistance, ready to transfer their traits by one or more genetic mechanisms to pathogenic species that share or pass through the oral environment.

ANTIBIOTIC RESISTANCE IN THE 21ST CENTURY

Genetic Elements in Resistance Spread

We have seen that bacterial pathogens have evolved and/or acquired numerous mechanisms by which they may resist the lethal or inhibitory effects of antibiotics. Strains of virtually every species of bacteria that inhabit humans, animals, or even plants, pathogenic or commensal, have been shown to express resistance to one or more and often several different antibiotics and antibiotic classes. New resistance traits and new species expressing older traits have been and continue to be selected by our appropriate and inappropriate use of antibiotics following their dissemination on a variety of genetic elements that may be transferred via several different mechanisms. The types of genetic elements associated with antibiotic resistance and the mechanisms by which they may be disseminated, of which we are currently aware, are summarized in Table 4. Genes encoding antibiotic resistance may be carried on conjugative or nonconjugative plasmids and transposons or within integrons. The resistance gene repertoire of a transposon or integron may expand by the insertion of new resistance transposons or by the addition of new resistance gene cassettes, respectively. Transposons and integrons may be located on any type of replicon, chromosomal, plasmid, or bacteriophage, and the resistance traits encoded by these replicons may be transferred

TABLE 4 Characteristics of genetic elements involved in resistance gene dissemination

Genetic element	Characteristics
Transmissible plasmid	Double-stranded circular, autonomously replicating molecule able to mediate its own transfer from cell to cell involving direct contact, or mobilizing for transfer of other genetic elements
Conjugative transposon	Nonreplicating genetic element of a discrete size able to integrate into bacterial replicons, or to excise from such replicons in the form of a circular transfer intermediate; encodes genes essential for conjugative transfer
Mobilizable plasmid	Circular, double-stranded replicating molecule encoding genes that permit the use of a conjugative plasmid to facilitate its transfer from cell to cell via conjugation
Transposon	Nonreplicating element of a discrete size able to move from one segment of a replicon to another, or from one replicon to another replicon in the same cell
Gene cassette	Circular, nonreplicating segment of DNA encoding an open reading frame without promoters; inserts into integrons
Integron	Integrated segment of DNA encoding an integrase, a promoter, and an insertion site (*att*) for the integration and expression of gene cassettes
Transducing bacteriophage	Bacterial virus able to package non-phage encoded DNA, which may be transferred to cells of the same or very closely related species by transduction
Bacterial chromosome	Major replicon of bacterial cells that may encode resistance to antibiotics and which may subsequently be transferred by transformation to species able to achieve a state of natural competence

from bacterial strain to bacterial strain via conjugation, transformation, or transduction. Even resistance traits that have evolved by one or more mutational events within a gene may be disseminated by one of these transfer mechanisms, especially by the transformation of those species of bacteria able to achieve a natural state of competence.

The Many Mechanisms of Antibiotic Resistance

Historically, resistance to penicillin began with the plasmid-mediated synthesis, by strains of *S. aureus*, of an enzyme, penicillinase, that cleaved the β-lactam ring of penicillin, rendering it ineffective as an antimicrobial. Subsequently, a large variety of β-lactam antibiotics were developed and introduced into clinical practice, many so as to increase the spectrum of antibacterial activity, but also to provide newer varieties, such as the cephalosporins, carbapenems, and monobactams, that would be resistant to the existing β-lactamases. However, the introduction of each new type of β-lactam antibiotic was soon followed by the emergence of newer β-lactamases that could destroy not only the newer β-lactams but also, in many instances, their predecessors. At least two types of mechanisms have been elucidated by which the newer β-lactamases have evolved. First, mutations in plasmid-mediated β-lactamase-encoding genes have resulted in enzymes with increased affinity for some of the newer β-lactams. Second, an inducible chromosomal gene, *ampC*, common among many gram-negative bacterial species, has very high intrinsic affinity for later-generation cephalosporins but is not induced in their presence. However, mutations have occurred in the regulatory genes that control their expression, resulting in derepressed (constitutive) production of higher levels of the enzyme and, consequently, resistance to those newer cephalosporins. Regardless of the mechanism by which they have evolved, almost 900 different β-lactamases have been identified in gram-positive and gram-negative bacteria. Particularly troubling have been the extended-spectrum beta-lactamases, enzymes encoded by plasmids in various species of *Enterobacteriaceae* that confer resistance to most beta-lactam antibiotics, including penicillins, cephalosporins, and the monobactam aztreonam. Community-acquired extended-spectrum beta-lactamase-producing *Enterobacteriaceae* are prevalent worldwide. Carbapenems are the best antimicrobial agent for infections caused by such organisms. β-Lactamases with activity against virtually all β-lactam antibiotics have been identified and characterized in the last few years. A genetic element is responsible for the dissemination of one such enzyme, the so-called New Delhi metallo-β-lactamase 1. This element was first identified in 2008 in a strain of *K. pneumoniae* isolated from a patient in New Delhi. The enzyme inactivates all penicillins, cephalosporins, carbapenems, and monobactams. Infections caused by *Enterobacteriaceae* carrying this element have since been documented, in addition to India, in Pakistan, Bangladesh, Brazil, Canada, Japan, the United Kingdom, and the United States. An enzyme with a similar activity spectrum, *K. pneumoniae* carbapenemase has been identified in several *K. pneumoniae* clinical isolates in the United States.

In addition, *S. aureus* also found a way to overcome the activity of the β-lactam methicillin (becoming MRSA), via the acquisition of a gene, *mecA*, encoding a new PBP with a low affinity for β-lactam antibiotics,

including methicillin. Hospital-acquired MRSA first appeared in 1961, and these infections have been on the increase ever since. The first community outbreak occurred in 1980, and community-acquired infections of MRSA also continue to increase. Several species of streptococci apparently accumulated, over a period of <60 years since the introduction of penicillin into clinical practice, a sufficient number of mutations in the gene encoding PBP2a to reduce its affinity to penicillins such that strains producing it are resistant to all β-lactams. These mutated genes were subsequently broadly disseminated among a variety of streptococcal species that are naturally transformable, thus speeding up the process of evolution. Somewhat puzzling, but certainly welcome, is the fact that *S. pyogenes* has remained exquisitely susceptible to penicillins after all these years of selective pressure. However, resistance to antibiotics, such as a macrolide, used to treat group A streptococcal infections in patients who cannot take a β-lactam antibiotic has been on the increase. Other bacterial species, such as *P. aeruginosa*, have found ways to avoid the lethal activity of these antibiotics by keeping them out of the cell via efflux pumps.

Resistance to the tetracyclines is probably the most common antibiotic resistance phenotype known, and representatives of virtually every bacterial species examined have been shown to be resistant to this class of antibiotics. The most common mechanism of tetracycline resistance involves the transport of the antibiotic out of the cell at such a rate that the ribosomes, although sensitive to tetracyclines, never come into contact with them. Several genes encoding these tetracycline-specific efflux pumps have been identified in gram-negative (*tetA* and *tetB* being the most common) and gram-positive (*tetK* and *tetL*) bacteria. Another mechanism of tetracycline resistance involves the production of a protein that protects the ribosome from the antibiotics. Several genes, such as *tetM*, *tetO*, *tetQ*, and *tetS*, have been detected in gram-positive and gram-negative species, as well as such cell wall-free bacteria as *Mycoplasma* species. Bacterial strains expressing one of these genes are also resistant to minocycline.

Resistance of most bacterial species to the fluoroquinolones generally involves an accumulation of one or more mutations in the QRDR of *gyrA* and/or *parC*, with in some instances an occasional mutation in *gyrB* or *parE*. Overexpression of the chromosomally encoded efflux pump gene *norA* or *pmrA* by *S. aureus* or *S. pneumoniae*, respectively, may also lead to low-level fluoroquinolone resistance. The intrinsic resistance of *P. aeruginosa* to fluoroquinolones can be attributed to the activity of at least four RND efflux pumps, especially the MexAB-OprM system. Quinolone resistance mediated by a transmissible plasmid was described for the first time in 1998. Since then, strains of *Enterobacteriaceae* species have been isolated that harbor a plasmid carrying the quinolone resistance gene, *qnr*, encoding a protein that protects DNA gyrase from inhibition by fluoroquinolones.

For every antibiotic in clinical practice, there are bacterial species that produce one or more efflux pumps providing resistance to that antibiotic. Most such pumps, with the exception of the Tet, Mef, and Cml proteins, mediating resistance to tetracycline, macrolides, and chloramphenicol, respectively, are not antibiotic specific but rather mediate resistance to several different antibiotics or antibiotic classes, as well as other types of compounds that are toxic to the bacteria that express them. On the other

hand, most antibiotics to which resistance is provided by efflux pumps have, like the β-lactams, also selected for other mechanisms of resistance. These mechanisms include enzymes that catalyze the acetylation of aminoglycosides and chloramphenicol; the adenylylation or phosphorylation of aminoglycosides; the methylation of a specific adenine residue in 23S rRNA resulting in the MLS resistance phenotype; the synthesis of an antibiotic-resistant enzyme catalyzing an intermediate step in the synthesis of tetrahydrofolic acid, such as dihydropteroate synthetase (sulfonamide resistance) or dihydrofolate reductase (trimethoprim resistance); or the replacement of D-Ala with D-Lac as the terminal residue in the murein pentapeptide precursors of gram-positive bacterial cell walls, resulting in resistance to glycopeptides such as vancomycin. And, of course, there are the single mutations, or accumulations of mutations, that continue to result in resistance of bacterial species to members of whole classes of antibiotics, such as the fluoroquinolones.

The emergence in the 1990s of MDR tuberculosis, i.e., caused by isolates resistant to two of the first-line drugs used to treat the disease, INH and rifampin, posed a threat to its control in the United States and worldwide. Treatment of patients with MDR tuberculosis necessitated the use of second-line drugs, which are less effective, more toxic, and much more expensive than the first-line drugs. Early in the 21st century, extensively resistant strains of *M. tuberculosis*, now resistant to INH, rifampin, the fluoroquinolones, and the second-line drugs amikacin, kanamycin, or capreomycin, began to emerge. Tuberculosis is one of the leading causes of death from infectious diseases worldwide. In 2006, estimates placed the number of individuals infected with *M. tuberculosis* at 2 billion (one-third of the world population), with approximately 9 million becoming ill from tuberculosis, and approximately 2 million dying as a result. The last number can only increase with increases in the numbers of MDR and extensively resistant strains causing these infections.

Why So Much Resistance?

Contributions to the selection and maintenance of antibiotic-resistant bacteria have been numerous and from many different sources. What should be clear is the fact that the use of any antibiotic, be it appropriate, inappropriate, or downright abusive, will have an effect on any bacterium with which it comes in contact. Whether used in the practice of human or veterinary medicine, in dentistry, in animal husbandry, agriculture, or aquaculture, antibiotics will inhibit the growth of or kill not only the intended bacterial target but also all other susceptible species present and may also select for the growth of any resistant strains of bacteria that happen to be in the same environment, be they pathogenic or commensal, and these may in time pass their resistance trait(s) to other commensal and/or pathogenic species, which in turn may be disseminated among other humans, animals, or plants in which they can grow or even thrive. Thus, all applications of antibiotics over the past 70 plus years have contributed to the current state in which we find ourselves. So, where have these contributions come from, and are they all necessary?

The medical and dental communities have certainly made their share of contributions to the current state of antibiotic resistance. However, no convincing argument can be made against the use of antibiotics to

treat serious or potentially life-threatening bacterial infections with the appropriate antibiotic(s), and any risk of antibiotic resistance selection is far outweighed by clear-cut documentation of decreases in morbidity and mortality due to these practices throughout the modern antibiotic era. In addition, many though not all types of surgery, both necessary and elective, are safer or even feasible because of the availability of antibiotics for prophylactic purposes. On the other hand, there are clearly many examples of inappropriate or excessive use of these life-saving agents by both physicians and dentists. For example, the vast majority of cases of diarrhea are caused by infectious agents other than bacteria, e.g., viruses or protozoan parasites, and most cases, including those caused by bacteria, clear up on their own without antibiotic treatment. The same is true for the majority of respiratory infections and earaches. Yet, some physicians prescribe antibiotics for almost any ailment, just to make the patient or parent feel that something is being done or because the patient or parent demands a prescription for an antibiotic. One might question the need to use antibiotics as adjuncts to scaling and scraping in the treatment of periodontal disease, especially since it generally is not known if the causative organism(s) in individual cases is even susceptible to the antibiotics of choice, generally tetracycline or metronidazole. Furthermore, the need for prophylactic regimens involving the use of amoxicillin, clindamycin, cephalosporins, or macrolides for dental procedures in patients with cardiac conditions has never been scientifically documented.

Commercial contributions to antibiotic resistance have been many. Antibiotics have been used in subtherapeutic amounts as growth promotants in the poultry and swine industries since the 1950s. The actual effects of antibiotics on growth promotion have diminished over the years, but their inclusion in feeds for this purpose has continued. In addition, more antibiotics are used today than ever for the treatment and prophylaxis of animal infections, due in part to less than ideal animal husbandry practices, such as the raising of these food animals under extremely crowded conditions. There are no clear-cut data to show that the selection of antibiotic-resistant bacteria due to the use of these agents as growth promotants, or in animal husbandry, has actually resulted in specific untreatable human infections. However, data accumulated since the 1960s have demonstrated that these practices do, in fact, select for resistant gram-positive and gram-negative bacteria in animals and that the very same genes encoding the resistance traits, or the genetic elements on which they are carried, exist in bacterial species isolated from both humans and animals. The ability of resistant bacteria isolated from either humans or animals to transfer these traits to each other has been demonstrated in the laboratory over and over again. Although there is little definitive evidence to confirm the direction in which such transfer can occur, or actually has occurred, in nature, the data that do exist at all levels would lead any reasonably intelligent and logically thinking individual to conclude that the dissemination of antibiotic-resistant bacteria, and/or the genes and genetic elements on which they are found, has occurred in nature in both directions. Other commercial applications of antibiotics include their use in agriculture and aquaculture for the prevention and treatment of bacterial infections. Some antibiotics are available over the counter for the treatment of domestic pets and have been used on occasion

for the self-treatment of perceived infections in their human owners. The direct promotion by the pharmaceutical industry of antibiotics on television and in the printed media has certainly contributed to the demands by patients that their physicians prescribe many of these antibiotics for treatment of undiagnosed ailments.

Other contributions to antibiotic resistance can be attributed to human behavioral patterns and various socioeconomic conditions. A major contributor has been the issue of patient noncompliance, particularly failure to take the full prescribed regimen or lowering of the recommended dose. An antibiotic is prescribed at a particular dose and for a specific period of time because the results of clinical trials have indicated that that is what is required to completely eradicate the infecting bacterium. Very often, a patient will feel better after a few days on the antibiotic and may stop taking it and save the rest for another time, but the infection may recur because the full regimen would have been required to completely eliminate the cause. Reducing the dose, e.g., by cutting the pill in half, may result in too low a concentration of the antibiotic to eliminate the infecting pathogen. Both of these practices, failure to take a full regimen and lowering of the prescribed dose, can foster the development or selection of resistant bacterial strains. The sequential rather than simultaneous use of a multiple-antibiotic regimen is another example of patient noncompliance. Such behavior, often typical of individuals who cannot afford the prescribed treatment regimen, is a major reason for the emergence of MDR tuberculosis and extensively resistant tuberculosis strains.

Many antibiotics are available over the counter in a number of Central and South American, Asian, and African developing countries. This has led to self treatment for any number of undiagnosed ailments, all of which have undoubtedly resulted in the selection and propagation of numerous antibiotic-resistant bacteria, both pathogenic and commensal. Antibiotics can often be obtained on the black market or online without prescriptions. Because such antibiotics are often manufactured in countries that do not have properly regulated safety and quality control measures, there is no way to know the quality or effectiveness of these drugs.

FUTURE PROSPECTS

Will the Modern Antibiotic Era Soon Be Over?

Antimicrobial agents for the treatment of infectious diseases caused by bacteria have been available since the sulfa drugs in the 1930s. Newer antibacterials have been introduced continuously since, more recently with such novel drugs as linezolid, daptomycin, and tigecycline, to treat infections caused by bacterial strains that have become resistant to the older agents. However, the rate at which new antimicrobial agents have been approved by the FDA and marketed by the pharmaceutical industry has been in steady decline for the past 30 years. Since the 5-year period from 1983 to 1987, when 16 new antibiotics were approved, the number has decreased in every subsequent 5-year period, such that only seven new chemical entities were approved for marketing between 2003 and 2007, and only two new antibiotics were approved between 2008 and 2012.

Much of this decline in the introduction of new antibiotics can be correlated with a decline in antibiotic discovery research by the pharmaceutical industry, beginning around 1989, when approximately one-half of the major pharmaceutical companies in the United States and Japan either halted or significantly decreased their antibiotic discovery efforts. In addition, throughout the 1990s a series of mergers and acquisitions resulted in fewer pharmaceutical companies, such that today five of the largest pharmaceutical companies, Pfizer, GlaxoSmithKline, Novartis, Bristol-Myers Squibb, and Aventis, were once 32 different companies. Between 2001 and 2002, three companies that had continued to pursue active anti-infectious disease research programs, Bristol-Meyers Squibb, Eli Lilly, and Wyeth, halted these programs altogether, and both Abbott and Glaxo-SmithKline downsized their efforts considerably. And in 2011, Pfizer, the largest pharmaceutical company in the world, announced that it would terminate all antibacterial research by the end of the year.

In order to understand the steady decline in the numbers of new drugs that have been brought to market over the past 30 years, it is necessary to review some of the key steps in the evolution of the regulatory environment associated with the discovery, development, and marketing of new antibiotics. In the 1930s, when the sulfa drugs were the only antimicrobials available, their use was essentially unregulated. The Food, Drug & Cosmetics Act was passed by the U.S. Congress in 1938. Included in this act was the requirement that new drugs be shown to be safe, and the newly formed agency was granted the authority to inspect factories manufacturing drugs. The ability to produce large quantities of penicillin, made possible by Florey and associates, made the necessary clinical trials possible in the early 1940s. In 1945, Congress passed the penicillin amendment, which mandated that the FDA was to test and certify both the safety and efficacy of all penicillin products. This was later extended to all antibiotics. Under the Kefauver-Harris Drug Amendment, passed in 1962, drug manufacturers were now required to prove the efficacy and safety of new drugs before they could market them. Then, in 1977, the Bioresearch Monitoring Program was introduced, which stipulated that the drug companies must ensure the quality and integrity of any data submitted to the FDA and provide protection of human subjects in clinical trials. All of the above regulations were introduced for the protection of the consumer, and a significant majority would agree that they were necessary. However, they all added to the total cost of drug production.

The evolution of drug regulation was accompanied by an evolution of the procedures applied by the pharmaceutical industry to the discovery, development, and marketing of new drugs. The specific steps in these processes, as they apply particularly to antibiotics, are summarized as follows.

The first step in antibiotic discovery initially involved the testing for antibacterial activity of natural products and derivates of natural products and later involved the testing of synthetic chemical compound libraries occasionally exceeding a million chemical entities. It is then necessary to identify and purify those compounds having antibacterial activity and to develop methods for high product yield for testing such attributes as potency, bacterial spectrum, solubility, and stability. The surviving

compounds are then tested in animals for safety, dosage required to eliminate target bacterial species, toxicity, ADME, and PK/PD. The acronym ADME stands for absorption, distribution, metabolism, and excretion, and PK stands for pharmacokinetics and PD for pharmacodynamics of the compound being tested. Absorption refers to the ability of the antibacterial compound to reach the particular tissue or organ infected. To do so, it must be taken into the bloodstream. If the compound is soluble in a nontoxic solvent, it can be taken orally, but it must survive the stomach and be able to permeate the intestinal wall for entry into the bloodstream. Such a compound is considered bioavailable. If not soluble, it may still be useful if administered intravenously or as an inhalant, which would limit its applicability. Once in the bloodstream, the compound's distribution, i.e., the tissues and organs to which is it delivered, is determined. This can be dependent on the size of the compound, whether or not it binds to proteins in the blood and would not be available in the tissues and organs reached, or whether or not it forms a complex with other compounds in the target tissues or organs and thus is no longer active. Irreversible binding to blood proteins has resulted in the demise of many promising antibacterial compounds. Nearly all compounds are subject to metabolism by the body; i.e., they are broken down. It is necessary to know what they are broken down to, e.g., inactive or even toxic derivatives, and how quickly they are broken down and by what organ (usually the liver). The test compound must eventually be removed by the body. Most often, excretion is through the kidneys in urine, or in the feces. PK is the study of the time course of compound concentrations, or levels, in the body, and PD is the study of the relationship between compound levels and their antibacterial effects. The combined results of PK and PD help to determine eventual dosages required for the treatment of targeted infections. Should results of any number of the above tests suggest the need for improvement of the compound, a team of chemists then makes the necessary changes. However, it would have to be demonstrated that each new derivative of the compound was indeed improved in the parameters for which the changes were made, and if so, it would then be subjected to the rest of the battery of discovery-associated tests. These rounds of chemical modification and retesting would go on until a compound with attributes that fulfilled all discovery parameters was obtained.

The earlier steps in antibiotic discovery have changed drastically in the modern era of genomics and with the availability of other 21st century tools. Today, the discovery team might identify an essential gene in one of its major target pathogenic species and then assess the potential spectrum of that gene by analyzing the genome sequences of many bacterial strains and species and validating the essentiality of the gene in each. An assay that measures activity of the essential gene product would then be developed, followed by the further development of a high-throughput screen, which combines the activity assay, an easy method for detection and possibly quantitation of the loss of activity, robotics for setting up and running thousands of assays in microtiter plates with between 96 and >1,000 wells requiring minimal amounts of the test compound(s), and computer programs for collection, analysis, and presentation of the results. The need for whole-cell inhibitory assays

has not been eliminated because the best candidate molecules, from the standpoint of target inhibition, must then be shown in most instances to enter the bacterial cell and in all cases to actually inhibit the growth of, or kill, the bacteria via inhibition of the intended target activity. Relatively newer fields such as proteomics and metabolomics and major improvements in such tools as spectrometry and crystallography are now applied to the antibiotic discovery process by teams of biological and physical scientists. All of these newer and/or improved methods also add to the costs of discovery research.

Patents are applied for to secure rights to the most promising antibiotic candidates that emerge from the discovery process. The clock then generally starts with regard to exclusivity, and the process of antibiotic development begins. The first step in this process involves submission to the FDA of an application for an investigational new drug (IND) exemption. If this is approved, the pharmaceutical company begins conducting a series of clinical trials, in a very specific order. Phase I trials take 1 to 2 years and usually involve between 100 and 300 healthy volunteers. The purpose of these studies is to assess the overall safety of the compound; determine the best route of administration; estimate dose range, tolerance, toxicity, and effect on selected organ systems; and conduct ADME and PK/PD studies for comparison of the results to those obtained in animals. Next, phase II clinical trials are conducted. These also may run for 1 to 2 years, but they involve between 300 and 1,000 subjects with infections for which the antibiotic is intended. With phase II trials, the major focus is on therapeutic efficacy and continued monitoring of safety. Finally, phase III clinical trials are conducted to compare the therapeutic efficacy of the study drug with other marketed antibiotics. The monitoring of safety continues in these studies, and optimal dosage is verified. If the compound is intended for more than one indication, a separate set of phase II and phase III trials must be conducted for each type of infection. Patents on new drugs are valid for 20 years, but the time spent in the development phase results in a shorter period of market exclusivity. A major obstacle to the development of new antibiotics in this regard has been the continued tightening of FDA regulations since the turn of the century. Currently, new antibiotics must be shown to be superior to any antibiotic(s) for which replacement is intended. These requirements entail more expensive and longer clinical trials before the company can begin the marketing process.

The final steps that the drug company must take comprise the antibiotic marketing process. Upon completion of phase III clinical trials, a new drug application (NDA) is submitted to the FDA. The FDA reviews all of the data submitted, and if they are acceptable, a letter of approval is issued. This is then followed by a meeting between the FDA and company representatives to examine all promotional materials, any proposed advertisements, and the package insert, which contains information on allowable uses of the antibiotic as well as potential known side effects. The package insert is then distributed to prescribers and pharmacists. The antibiotic can now be marketed, and its use is restricted to specific indications covered in the clinical trials and dosages recommended in the package insert. The company continues with phase IV clinical trials to enhance its efficacy and safety database.

The cost of antibiotic discovery and development has increased considerably over the past 4 decades. In the 1970s, a compound could go from detection of its antibacterial activity to the initiation of marketing for a total cost of $50 million to $60 million. The cost of the same process was up to nearly a quarter million dollars in the 1980s and as much as $400 million in the 1990s. Today, the cost from initial discovery to launch can be anywhere from $800 million to $1 billion. The reasons for these increased costs are numerous. Novel antibacterial compounds have become more difficult to discover. The vast majority of antibiotics marketed since the 1940s have been natural products of microbial (e.g., fungal and bacterial) secondary metabolism or derivatives of these products. The standard natural product sources are becoming tapped out, and drug companies are now testing large libraries of synthetic compounds for antibacterial activity. However, many of these compound libraries have been developed initially for therapeutic purposes other than the cure of bacterial infections, in many instances during searches for drugs to treat such chronic conditions as diabetes, high cholesterol, heart disease, or depression or different types of cancer. For the most part, it would seem unreasonable to expect a significant number of the compounds in such libraries to have many of the properties that make for a good antibiotic. In order to achieve reasonable market expectations, new antibiotics must be approved for multiple indications, each of which requires a separate series of clinical trials. Furthermore, since the turn of the century companies have been required to conduct active comparator-controlled, noninferiority trials for each indication. Partly because of increases in the cost to bring any new drug to market, the pharmaceutical industry has become enamored with the concept of "blockbuster drugs," such as Prozac and Lipitor, which have provided huge profits and allowed the recovery of their costs in a reasonable time frame. Blockbuster antibiotics yield far less than other types of therapeutic agents. Infectious diseases are generally acute and require a single prescription to obtain a cure. Many chronic diseases require lifetime treatment, prescriptions being perpetually renewed. Thus, blockbuster agents for the treatment of chronic diseases can yield as much as $1 to 1.5 billion a year, whereas blockbuster infectious disease agents, such as antibiotics, generally yield no more than $500 million a year, and most antibiotics never reach blockbuster status. Yet, the cost of taking an antibiotic from discovery to market can be the same as that of the same process for a drug used to treat a chronic disease.

What Can Be Done To Extend the Antibiotic Era?

The preservation of the effectiveness of antibiotics and the continued discovery, development, and marketing of newer agents to replace those whose effectiveness cannot be extended will require conscious and sustained effort from everyone.

With regard to the preservation of our current antibiotic armamentarium, educational efforts and global monitoring of antibiotic use and abuse are absolutely required if the antibiotic overuse and abuse seen worldwide is ever to be lessened significantly, if not eliminated. Two organizations, the Association for the Prudent Use of Antibiotics (APUA) and the World Health Organization (WHO), have taken on the burden of education and

monitoring with regard to antibiotic use worldwide. Hospital personnel must play an active role in the prevention of hospital-acquired infections as well as the selection and spread of antibiotic-resistant bacterial strains. Some hospitals have established antibiotic rotations to allow resistant strains to die out when possible, so that older antibiotics can regain their usefulness. Other approaches in hospitals have involved the installation and monitoring of more-effective infection control practices by all staff, and more recently, hospitals are being required to self-report incidences of hospital-acquired infections. Community practitioners must begin and continue to prescribe antibiotics only when appropriate and not acquiesce to inappropriate patient demands. Patients must return to a dependence on the experts regarding the use of antibiotics by not insisting on antibiotic prescriptions for treatment of ailments not caused by bacteria, by not self-prescribing, and by striving for 100% compliance when antibiotics are prescribed. Since hand washing has been shown to be the best method of infectious disease prevention, everyone should make it a habit to wash hands thoroughly and often.

The pharmaceutical industry is not likely to continue or reestablish antimicrobial research programs unless the anticipated returns from the sale of new antibiotics are not diminished significantly by the costs associated with the discovery and development of such agents. In this regard, government regulatory agencies must establish reasonable industry guidelines and rules for the acceptability of new antibiotics. Why is it necessary that a new agent be superior to that which it is intended to replace when a major reason for development of the new agent is the decrease in effectiveness of its predecessor due to the emergence and dissemination of pathogens resistant to it? Would not a new effective, even if not superior, agent be preferable to none at all? The public, too, must become more reasonable when it comes to risks associated with the use of new antibiotics. There are no drugs on the market that can be taken with absolutely no risk. At least some potential side effects, a proportion of those being actually quite serious, are associated with all marketed drugs. The funding of more discovery research by government agencies might help to lessen the risks and costs of antimicrobial research to the drug industry. Along the same vein, the establishment of "small biotech-big Pharma" partnerships or similar arrangements between academic institutions and pharmaceutical companies might prove more productive and less costly.

Finally, research and development of vaccines by the pharmaceutical industry may be part of the solution in the battle against the development of and selection for antibiotic-resistant bacteria. A vaccine covering diphtheria, pertussis (whooping cough), and tetanus (DPT) has kept the incidence of these diseases substantially under control in developed countries for many years. More recent additions include vaccines against *H. influenzae* type b (Hib) and *Neisseria meningitidis*, the multivalent (7-, 9-, and 23-valent) *S. pneumoniae* vaccines, and the 9-valent pneumococcal-group C meningococcal (Prc9-MenC) vaccines. Vaccines to prevent infections by *E. coli*, group B *Streptococcus*, species of *Klebsiella*, and group A *N. meningitidis* are in development, and vaccines against *E. faecalis*, *Francisella tularensis*, *S. pyogenes*, and *Moraxella catarrhalis* are in various stages of discovery research.

KEY POINTS

The modern antibiotic era is generally considered to have begun in the mid-1940s, when sufficient quantities of the β-lactam antibiotic penicillin became available to help prevent and successfully treat previously fatal bacterial infections among Allied troops. Numerous derivatives of penicillin, four generations of cephalosporins, and the newer β-lactams, the carbapenems and monobactams, soon followed, each new version extending the spectrum of susceptible pathogens covered by the previous members of the class, or overcoming resistance to some or all of the earlier agents. At the same time, new classes of antibiotics inhibiting different bacterial targets, such as the aminoglycosides, tetracyclines, macrolides, fluoroquinolones, and most recently, the oxazolidinone linezolid (2000), the lipopeptide daptomycin (2003), and the glycylcycline tigecycline (2005), were being added to the antibacterial armamentarium.

Despite the large number of antibiotics available, these agents actually target a very limited number of essential bacterial functions, i.e., the synthesis of cell walls; macromolecular biosynthetic processes, such as replication, transcription, or translation; or steps in intermediary metabolism, such as the folic acid pathway. An occasional agent will target a different function, such as the cell membrane (polymyxins and daptomycin) or DNA itself (activated metronidazole).

Each new antibiotic introduced into clinical practice has been followed, often very rapidly, by the emergence of resistance. Such resistance may involve the following: acquisition of a gene that encodes an enzyme able to destroy or inactivate the antibiotic, or one that allows the cell to bypass the normal target of the antibiotic, e.g., PBPs with lower affinity for β-lactams, or dihydrofolate reductases with decreased affinity for trimethoprim; mutations in a gene encoding, or regulating the expression of, an antibiotic target; or acquisition or activation of a chromosomally determined efflux pump that excretes the antibiotic from the cell and away from the antibiotic target. Thus, for every antibiotic available, there is at least one and usually two or three mechanisms available to bacteria by which they may overcome its inhibitory or lethal activity. Because genes mediating antibiotic resistance may be located within transposons or integrons, they may be present on plasmids, the host chromosome, or even bacteriophage DNA. Antibiotic resistance traits may be transferred from strain to strain in a given species and even between different species and genera of bacteria by plasmid- or transposon-mediated conjugation, conjugative mobilization, transformation, and occasionally transduction.

Once resistance to any antibiotic emerges, a strain carrying it will be selected, or its transfer to new strains and species may be facilitated and subsequent newly resistant strains selected, by the appropriate and inappropriate use of that antibiotic in human and animal medicine, as a growth promoter in animal feeds, or for the protection of crops and marine farms. Because many bacterial species have become resistant to multiple antibiotics, the use of any one of them to which it is resistant will select for its entire resistance phenotype. The presence of so many antibiotics, and so much of each, in the environment has produced strains of enterococci and staphylococci that are resistant to all available antibiotics and *S. pneumoniae* strains resistant to most clinically useful antibiotics. New untreatable pathogens are sure to arise in the future.

With regard to the discovery and/or development of new antibiotics or classes of antibiotic, it is generally assumed that the low-hanging fruit have all been picked. Discovery of chemical compounds that are inhibitory to bacterial cells without an accompanying toxicity to humans has become more difficult with time and considerably more expensive. Many pharmaceutical companies have abandoned the search for new antibiotics because the monetary rewards do not seem sufficient to warrant the expense of their development. With regard to those companies that have remained in the hunt, all of the available modern technologies are being applied to the discovery and development of new antibiotics, such as high-throughput screens to test millions of compounds in short periods of time (weeks to no more than months); the availability of the genome sequences of numerous pathogenic species to identify and validate the essentiality of potentially new antimicrobial targets; sequencing, proteomic, and metabolomic technologies to aid in the identification of the functions of genes of unknown function; crystallography to elucidate the structures and mechanisms of action of potential new targets and to aid in the design of chemical entities that will inhibit them; and the latest methods of chemical synthesis, such as combinatorial chemistry, by which many forms of a primary structure can be made. We must keep in mind, however, that the introduction of every new antibiotic or antibiotic class into clinical use has been followed by the emergence of bacterial pathogens that are resistant to it. It can also be stated with some degree of certainty that every new antimicrobial agent, no matter how novel the agent or its target, introduced in the future will also be followed by the appearance of resistant strains of at least some pathogenic species. For, as remarked by Nobel Laureate Paul Berg, "Mother Nature always bats last."

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Infection Control in Dentistry

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INTRODUCTION

Dental professionals are exposed to a wide variety of microorganisms in the blood and saliva of patients. These microorganisms may cause infectious diseases such as the common cold, pneumonia, tuberculosis, herpes, hepatitis B and C, and AIDS. The use of effective infection control procedures and universal precautions in the dental office and the dental laboratory prevents cross-contamination that could put dentists, dental office staff, dental technicians, and patients at risk for infection.

Infection control in dentistry, as in other health professions, involves application of a risk management decision-making process, including risk identification, assessment, or analysis, and implementation of design and procedures for risk control. "Cross-infection" is typically defined as the transmission of infectious agents among patients and staff within the clinical environment. Recent research findings suggest that oral microorganisms, in addition to causing certain systemic infections such as endocarditis, may contribute to the development of chronic inflammatory diseases including cardiovascular diseases. This highlights the need to place more emphasis on controlling the risks of cross-infection in the practice of dentistry. Moreover, the consequences of dealing with lapses of infection control after the fact are dire, both economically and in public relations terms.

Infection control is an important component of the overall risk management process that should be in place in dental operatories and clinics of all sizes. For this purpose, the concept of "risk" has several possible definitions, each of which has relevance to the practice of dentistry:

- Chance of loss or injury; hazard; danger; peril
- The possibility of suffering harm or loss
- A factor, element, or course involving uncertain danger or hazard
- The danger or probability of loss to a dental malpractice insurer

The primary goal of all infection control measures is to minimize the risk to dental patients and staff of infection caused by exposure to infectious material in the course of dental treatment. At-risk populations include patients, dental professionals, and support staff, all of whom

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include individuals with different levels of susceptibility to infection. The challenge to the dental professional is to implement infection control procedures and practices that best protect everyone involved without compromising the quality of care.

In medicine, especially as practiced in hospitals, greater attention is given to the problem of infections of nosocomial (hospital-acquired) and iatrogenic (therapy-related) origins, especially those involving antibiotic-resistant bacterial strains. Respiratory and mucosal infections due to hospital-acquired bacterial and viral pathogens are a significant cause of mortality, especially in immunocompromised patients. Many of these infections are extremely difficult to treat due to the high incidence of multiple antibiotic resistance. The most frequently isolated bacterial agents in these infections include *Enterococcus* spp., *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. For example, many nosocomial *S. aureus* infections involve strains resistant to the class of antibiotics most frequently used to treat staphylococcal infections. These strains, designated methicillin-resistant *S. aureus* (MRSA), are typically resistant to all useful antibiotics with the exception of vancomycin. The prevalence of MRSA strains in nosocomial *S. aureus* infections rose dramatically from approximately 30% to nearly 50% in the decade prior to 2004. This rate has dropped somewhat in the last few years, presumably due to targeted surveillance and increased awareness of infection control procedures. Transmissible vancomycin resistance is common among enterococci, and MRSA strains with high-level vancomycin resistance have been isolated in several U.S. hospitals. Although the prevalence of antibiotic-resistant bacterial strains is likely lower in the community than in hospital settings, the trend toward prevalence of pathogens resistant to multiple antibiotics is an especially serious public health issue that is likely to have increasing implications for the dental profession, especially in designing appropriate treatment for patients with a higher susceptibility to infection.

For several reasons, infection control in hospitals is a much more complex process than is infection control in the typical dental practice setting. Patient care in the hospital setting, including dental care, usually involves large numbers of staff with differing levels of training and understanding of infection control. Hospitalized patients are much more likely to be susceptible to infection. Moreover, like hospitalized patients, dental patients receiving treatment in a hospital setting are at higher risk for exposure to antibiotic-resistant bacterial pathogens than dental patients treated in the private practice setting. Despite these differences, the principles on which infection control practices are based are essentially identical in the two situations.

The goal of this chapter is to identify risks for the transmission of infection in dental treatment settings, define strategies for managing these risks based on scientific evidence, and provide protocols for risk management. It is crucial for dental practitioners to realize that in addition to dealing with real risks of cross-infection during dental treatment, they must also address risk perception. Perception of risk has played a significant and not always positive role in shaping public health policy and has at times dramatically affected the relationship between the health professions and the public. As new potential risks are identified, policy makers, patients, and health care practitioners are prone to either under- or

overreact based on fear. Universal precautions (see below) have been developed to address this issue. As scientific evidence becomes available on the actual risk of infection from newly identified potential threats, it is important that risk management strategies be revised and that health professionals and the public be educated. Perception of risk continues to play a key role in the dialogue surrounding risk management of issues such as control of biofilms in dental water lines and transmission of various relatively newly recognized infectious agents including HIV, prion-related encephalopathies, and severe acute respiratory syndrome (SARS). Some of these infectious agents (HIV, for example) have become relatively more manageable than they were 10 years ago, while the actual potential risk of some other agents may be less than originally perceived.

Many of the current discussions and procedures for infection control are found on websites of professional organizations and governmental agencies, in particular the American Dental Association (ADA) and the Centers for Disease Control and Prevention (CDC) (see Table 1). These sources are updated regularly as new information in the field is assessed and procedures for minimizing the risk of iatrogenic infections in dentistry evolve.

TABLE 1 Electronic resources for infection control information

Organization	Publication	Last update	Web address or contact
American Dental Association	Oral Health Topics: Infection Control (includes both dentist and patient versions)	Updated regularly	http://www.ada.org/2697.aspx
American Dental Association	Statement on Infection Control in Dentistry	2004	http://www.ada.org/1857.aspx
American Dental Association	Statement on Dental Unit Water Lines	2012	http://www.ada.org/1856.aspx
American Dental Association	Statement on Treatment of Patients with Infectious Diseases	2012	http://www.ada.org/1858.aspx
U.S. Centers for Disease Control	Recommended Infection-Control Practices for Dentistry	*MMWR Morb. Mortal. Wkly. Rep.* **52**(RR17)**:**1–61, 2003	http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5217a1.htm
U.S. Centers for Disease Control	Centers for Disease Control, Oral Health Resources Index	2012	http://www.cdc.gov/oralhealth/infectioncontrol/index.htm
U.S. Centers for Disease Control	Guideline for Hand Hygiene in Health-Care Settings	*MMWR Morb. Mortal. Wkly. Rep.* **51**(RR16)**:**1–44	http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5116a1.htm
U.S. Centers for Disease Control	Bloodborne Pathogens in Dental Settings	*MMWR Morb. Mortal. Wkly. Rep.* **52**(RR17), 2003	http://www.cdc.gov/ncidod/hip/BLOOD/dental.htm
U.S. Centers for Disease Control	Prevention of 2009 H1N1 Influenza Transmission in Dental Health Care Settings	2009	http://www.cdc.gov/OralHealth/infectioncontrol/factsheets/2009_h1n1.htm
U.S. Centers for Disease Control	Influenza Vaccination Information for Health Care Workers	2011	http://www.cdc.gov/flu/healthcareworkers.htm
AIDS Education Training Center	National HIV/AIDS Clinicians' Consultation Center	2010	http://www.nccc.ucsf.edu/ HIV Consultation Service (M-F, 9am–8pm EST): 1-800-933-3413 Clinicians' Post-exposure Prophylaxis Hotline (24/7): 1-800-448-4911
U.S. Centers for Disease Control	Disinfection & Sterilization in Healthcare Facilities, 2008	2008	http://www.cdc.gov/hicpac/pdf/guidelines/Disinfection_Nov_2008.pdf
U.S. Food and Drug Administration	Sterilants and High Level Disinfectants	2009	http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/ReprocessingofSingle-UseDevices/ucm133514.htm

INTRODUCTION TO RISK CONTROL

Quality Assurance Is the Promise of Performance

The commitment to act for the benefit of the patient and the public is the cornerstone of virtually all professional codes of ethics, including the ADA's Principles of Ethics and Code of Professional Conduct. Practice of appropriate infection control procedures is an important part of overall assurance of quality care. Outcome data from quality assurance assessments provide the evidence that the dentist is meeting his or her ethical and legal obligations to patients and society.

Cross-Infection Control Is Essentially a Set of Management Strategies for Risk Control

Risk control in dentistry uses a single-tier approach whereby all patients are treated as though they are a potential source of infectious pathogens. This single-tier approach is known as "universal precautions." It applies the concept of risk to the procedure, not to the patient. Whereas all patients are treated equally in terms of the quality assurance mechanisms that constitute infection control, the importance of medical history for determining best patient care must not be overlooked. However, this is not a substitute for proper infection control. The universal precautions system of infectious disease control assumes that all body fluids are infectious and requires every employee exposed to direct contact with body fluids to be protected as though such body fluids were infected with hepatitis B virus (HBV), hepatitis C (HCV), or HIV. Universal precautions are intended to prevent parenteral mucous membrane and nonintact skin exposures of health care workers to blood-borne pathogens. Specifically, dental health care workers should consider blood, saliva, and gingival fluid from all patients to be infectious.

What Is Risk Management?

For the purposes of dental practice, cross-infection control equals risk management. The control of cross-infection involves a particular application of a risk management decision-making process, with risk identification, assessment, or analysis, and implementation of risk control procedures. Although quantitative data on the actual risks associated with many specific procedures are somewhat limited, the design of infection control protocols takes into account several important factors, including the following:

- Known risks and hazards of specific dental procedures (for example, dental surgery in patients with implanted medical devices)
- Known risks and hazards of other procedures (for example, injections)
- Regulatory requirements of governments and professional societies
- Perception of risk in the community served
- Perception of risk within the dental profession

Several concepts in this list require further explanation. The distinction between "hazard" and "risk" is important. Hazard is the potential harm (including the number of people exposed and the severity of consequences) that may be suffered due to some particular incident or procedure. Risk is the quantification of a hazard in terms of probability of

the occurrence of harm. Risk assessment also considers the likelihood of transmission of the infectious agent and the severity of the outcome of being infected. Perceptions of risk are influenced by many factors other than scientifically determined risk. These determinants of perceived risk tend to involve beliefs and feelings that are most easily understood from a sociological or psychological perspective. A common example of perceived risk is that riding in an automobile is safer than flying in an airplane. The statistics on passenger injury or death in airplane and automobile crashes do not support this perception, but it is nevertheless widespread. Any profession that deals with the public must address both real risks and the public's perception of risks associated with the profession.

In designing effective risk management strategies for infection control in dentistry, it is necessary to address actual hazards and risks as well as to acknowledge the importance of perceived risks to the community and its confidence in the profession. A good example of this has been the public perception of a risk of HIV transmission via dental procedures. Indeed, even the dental profession perceived the risk of treating HIV patients as high and was initially resistant to the treatment of these patients until the facts became clear that there was an extremely low risk of HIV transmission by this route, especially compared with the much higher risk of transmission of HBV or HCV.

CROSS-INFECTION RISKS IN DENTISTRY

Possible sources of infection in the dental care setting include the following:

- Patients with infectious disease (e.g., influenza, measles, or tuberculosis)
- Patients in the prodromal or convalescent stage of infection (e.g., herpes simplex virus)
- Healthy (or asymptomatic) carriers of disease-causing organisms (e.g., *Streptococcus pyogenes*, *Neisseria meningitidis*, and *Haemophilus influenzae*)
- Environmental sources: airborne organisms or biofilms in water lines or on equipment or instruments

Routes of Spread of Infection

There are several possible routes of transmission of infectious agents. Certain types of infectious agents are more likely to be transmitted by one route than another, so it is important to understand the significance and possible consequences of a failure of infection control procedures in any of the following transmission routes. The major potential risks include contamination of wounds during surgery and contamination of sterilized instruments during storage.

EXAMPLES OF CROSS-INFECTION ROUTES: AIRBORNE AND MATERIALS HANDLING

Examples of dust-borne routes

- *S. aureus* from skin scales
- *Clostridium tetani* from environmental dust
- These and other organisms released from solid surfaces

- Source: skin scales, wound dressings, solid surfaces
- Risk: contamination of wounds during surgery; contamination of sterilized instruments during storage

Examples of airborne routes

- Large droplets >100 μm fall to the ground within 2 m of the source and contribute to surface contamination and dust spread.
- Droplet nuclei <100 μm, usually 5 to 10 μm, remain in the air for hours and can be inhaled into lungs. This was an important factor that made past incidents involving release of weaponized anthrax spores so highly infectious and difficult to remove from contaminated locations.
- Sources: speaking, sneezing, all intraoral procedures. Massive increase when using ultrasonic scaling, air-rotor, and air/water syringe.

MICROORGANISMS SPREAD BY AEROSOL

Viruses: HBV, Epstein-Barr virus, varicella-zoster virus, rubella virus, measles virus, and respiratory viruses (e.g., influenza virus, rhinovirus, and adenovirus)

Bacteria: *Mycobacterium tuberculosis* (tuberculosis), *Bordetella pertussis* (whooping cough), *Legionella pneumophila* (Legionnaires' disease), *N. meningitidis* (bacterial meningitis), *Streptococcus pneumoniae* (bacterial pneumonia), and *S. pyogenes* (strep throat, etc.)

PREVENTION OF AEROSOL TRANSMISSION

Aerosol transmission may be prevented in the following ways:

1. Elimination or limitation of organisms at source:
   - Postpone elective treatment during infective period.
2. Interruption of transmission:
   - Use rubber dam and high-speed suction.
   - Use hand instruments when possible.
   - Flush ultrasonic scalers and air/water syringes for 2 min at the start of the practice day and for 30 s between patients.
   - Have adequate ventilation (air exchanges) in surgery. Typical standards for adequate ventilation are in the range of 5 to 8 liters/s/occupant, or approximately 6 air changes per hour.
3. Protection of the potential recipient:
   - Use masks and protective eyewear.
   - Vaccinate when possible, e.g., HBV, *Mycobacterium bovis* BCG, rubella, and influenza.

EXAMPLES OF CONTACT ROUTES OF CROSS-INFECTION

Person to person

- Person: direct spread from person to person by hands and clothes or fomites (towels, etc.)

FIGURE 1 Examples of single-use supplies and instruments versus those that need to be sterilized or disinfected. Single-use products minimize the possibility of cross-contamination. The bulk containers should be disinfected between patients, unless the material is dispensed into a disposable container (disclosing solution in cups, for example). (A) Examples of multiple-use and corresponding disposable single-use devices, including dental floss (bulk versus single-use containers); tongue retractor/saliva ejectors; impression trays; scalpel handles; and single-use matrix band and retainer versus reusable retainer with disposable band. (B) Examples of multiple-use and corresponding disposable supplies: bulk cement liquid, powder and measuring scoop versus single-use capsule; multiuse acid etchant in syringe versus single-use plastic ampoule; bulk plaque-disclosing solution versus tablet or single dose dispensed into a cup; bulk versus single-dose gingival retraction cord.
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- Risk: viruses, e.g., HBV, HCV, herpes simplex virus, respiratory syncytial virus, SARS, gram-negative bacilli, and *S. aureus*
- Prevention: handwashing, gloves, and protective clothing

Equipment

- Dental instruments, chairs and units, and impression materials
- Risk: bacterial and viral infectious agents
- Prevention:
  1. Sterilization of instruments (see below)
  2. Use of disposable products and individual dose packaging of dental materials and supplies (Fig. 1). The relatively high cost of disposables is balanced by the assurance of sterility and reduced costs of autoclave operation.
  3. Disinfection of dental materials in contact with patients (impressions, for example)
  4. Environmental hygiene: use of barriers (Fig. 2), spray-wipe-spray disinfectants
  5. Defining zones in the dental operatory (contaminated areas and clean areas)
  6. Correct disposal of infected waste

Fluids

- Dental water supplies, disinfectants, and detergents
- Risk: HBV, HCV, gram-negative bacilli including *Pseudomonas* and *Acinetobacter*
- Prevention: flushing water supply lines and using sterile water and biocide in water

FIGURE 2 Example of the correct use of barriers and personal protective equipment. The patient, operator, and assistant are all wearing protective eyewear. The operator and assistant are wearing gloves, masks, and gowns. Note the two disposable cups that are available to cover the dental handpiece when a bur is in place.
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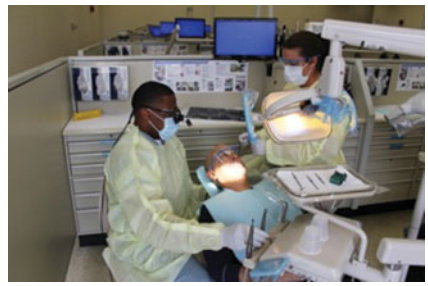

EXAMPLES OF PARENTERAL SPREAD OF CROSS-INFECTION

- Source of inoculation: blood, saliva, and secretions. Inoculation injury may be via eye, breach in intact skin, mucous membranes, or other sharps injury. The level of inoculation and infective dose of the particular organism determine the actual risk to the operator. For example, a needlestick injury with a hollow needle injecting a significant volume of blood with a high titer of HBV or HBC is a high risk.
- Risk: viral infections including HBV, HCV, and HIV. Relative risk following inoculation injury: HBV (30%), HCV (10%), and HIV (0.3%).
- Prevention: adequate training and effective sharps policy including safe disposal of sharps, safe cleaning and sterilization of sharps, safe resheathing of needles; hepatitis B vaccination; wearing gloves, dressing wounds, and removing or covering dental burs when they are in dental handpieces.

Management of Recently Identified Infection Control Risks

VACCINATION OF HEALTH CARE WORKERS

One of the more effective preventative measures for infection control in health care settings is vaccination of health care workers against specific communicable diseases. While this would seem to be an obvious strategy given the education and training of health care professionals, it is an area in which there is room for major improvement. Most health care professionals (as well as most of the population) receive the standard childhood vaccination series (measles, mumps, and rubella [MMR], diphtheria, pertussis, and tetanus [DPT], polio, HBV vaccine, etc.), and older practioners have likely received smallpox and poliovirus vaccines as well. In recent years, annual vaccination against viral influenza has become the recommended standard. Flu vaccination (which is typically effective only against the current variant of the virus) was originally recommended for members of specific high-risk groups including the elderly and young children. Current recommendations are that all persons aged 6 months and older should be vaccinated annually. The actual vaccination rates are much lower (<45% for the overall U.S. population in 2010–2011), with the elderly being vaccinated at much higher rates than other age groups.

From the standpoint of infection control, placing patients at risk by failure of health care workers to be immunized against preventable communicable diseases cannot be professionally or ethically justified. Nevertheless, the CDC reports that fewer than 65% of U.S. health care workers received flu vaccines during the 2010–2011 flu season, a percentage that has risen only very slowly over the previous decade. While there are no available data specific to the dental profession in North America, there is no reason to assume that the levels of vaccination in this group are significantly different. This suggests that there is a serious issue of risk perception regarding the influenza vaccine. There appear to be several factors contributing to this low level of influenza vaccination in the health care

professions including the variable year-to-year effectiveness of the vaccine, unsubstantiated claims of harmful effects of this and other vaccines, and persistent antivaccine efforts in some segments of the population. These issues need to be directly addressed by the dental community at the preprofessional, graduate, and postgraduate education levels.

EMERGING INFECTIOUS DISEASES

Within the past 2 decades, several new or newly recognized human infectious diseases have emerged. Some cases, such as HIV infection, became extremely serious public health concerns. Others, such as SARS and transmissible spongiform encephalopathies (TSEs) were essentially contained after causing some serious public health problems. While some of these life-threatening diseases have limited effective treatment options, others (HIV, for example) have become relatively more manageable than they were 10 years ago. These factors, combined with uncertainty surrounding epidemiology and relative infectivity, have contributed to the public's concern that they may be at risk of infection during dental treatment. With all of these diseases, there has been emphasis on both identifying potentially infected persons within the population and designing specific infection control methodologies to minimize the risks of transmission.

The AIDS pandemic, in particular, has resulted in revisions of accepted infection control practices, especially with regard to practices that help minimize exposure to blood. Prior to this, the use of gloves and special care with instruments were suggested during treatment of known carriers of HBV. The subsequent acceptance of universal precautions and increased attention to infection control in medicine and dentistry were a direct result of recognition of the infectious nature of HIV during its latent, asymptomatic phase. The mandated use of gloves and other protective equipment, the increased emphasis on proper use of sharps, and increased management of potentially infected waste have served to greatly limit the likelihood of HIV transmission in dental practice. Assuming rigorous procedural adherence to universal precautions, available evidence indicates a very low risk for both acquisition and transmission of HIV infection among dental health care workers and patients. In fact, the increased use of barrier controls and related procedures has most likely resulted in reduced risk for transmission of other pathogens that are much more infectious than HIV.

It is important to note that there is very limited evidence available to date to suggest that dental procedures conducted with rigorous attention to infection control standards present a risk to the public or to the practitioner. There are, of course, issues of understanding of and compliance with proper infection control procedures, but numerous studies indicate that with respect to changes mandated since the discovery of HIV, infection control in dentistry has improved markedly in recent years. Even so, infection control standards are constantly evolving in response to new information on specific infectious agents and newly available technologies.

Regularly updated training and education of dental personnel in infection control theory and procedure are especially necessary in regard to diseases such as HIV/AIDS, for which new knowledge and information regularly become available. One useful resource for clinicians is the

National HIV/AIDS Clinicians' Consultation Center in San Francisco, CA (Table 1; see also http://www.ucsf.edu/hivcntr/). This service provides open access to extensive and up-to-date training and educational materials. In addition, the service offers health care providers HIV clinical and drug information through individualized telephone consultation, as well as a telephone hotline for managing occupational exposure to HIV, HBV, and HCV (Table 1).

Two other recently identified emerging infectious diseases have been in the public eye. The acute respiratory disease SARS, which may have originated from civets in China, appears to have been at least temporarily contained after significant outbreaks in Asia and Canada. In those cases, isolation and quarantine of infected individuals and their contacts have effectively limited the spread of this highly contagious viral disease, which appears to resolve within a few weeks. Infection control measures concentrated on the isolation of potentially infective foci and barrier protection of health care workers. To date, there are no published reports of implications of SARS for the practice of dentistry beyond the current standard of universal precautions. Standard practice already indicates postponing dental treatment for contagious individuals, whatever the disease.

The TSE diseases (commonly referred to as "mad cow" disease) present a somewhat different situation. A human TSE has been associated with eating cattle products infected with a prion (transmissible variant protein) that causes the bovine form of TSE. Although human TSE cases of bovine origin have not been identified in North America at the time of writing, bovine TSE has been detected in a very small number of cows in North America, and game animals (elk and deer) in several states are known to suffer from a "chronic wasting disease" that is closely related to bovine TSE. While there have been no reports of a connection between human degenerative neurological disease and bovine or game animal TSEs in North America, continued surveillance remains important to assess the possible risks.

To date, there have been no significant changes in infection control measures in North America in response to the possible threat posed by prion-associated diseases. In this context, the response of the European dental community may prove instructive. An outbreak of bovine TSE in England began in the 1980s. The disease was first observed in 1996 in humans and was found to be associated with the consumption of infected bovine tissue. There have not been large numbers of deaths to date, but the numbers will likely continue to rise due to the long incubation period of TSEs.

One of the more serious issues in dealing with TSEs from a health care standpoint is that infective prion particles can survive standard sterilization and disinfection techniques. For this reason, British practice standards recommend disposable products or, in instances when decontamination is necessary, extremely high levels of disinfection. For TSE decontamination, vacuum steam sterilization (134 to 137°C for 18 min) followed by six successive cycles of 3 min each at 134 to 137°C is recommended. The recommended chemical disinfectant is 20,000 ppm of chlorine for 1 h, 2 M sodium hydroxide for 1 h or (for histological samples) 96% formic acid. It is suggested that all confirmed and suspected TSE patients receive dental treatment in a hospital setting.

In summary, the risk to public health posed by dental transmission of TSE appears to be small compared to that for hospital surgery in similar scenarios. The risks of transmission of infection from dental instruments are very low, provided that optimal standards of infection control and decontamination are maintained. In all surgical procedures, the key consideration in minimizing any risk of transmission is ensuring the efficacy of instrument decontamination, with emphasis on cleaning the instruments, even though current methods cannot remove such risks completely.

BIOFILMS IN DENTAL WATER LINES

Biofilms in dental unit water lines (DUWLs) and the potential of biofilm-derived organisms to cause nosocomial infections in dental patients and dental staff have been the subject of continuing study and considerable controversy in recent years. The unique feature of DUWLs is the capacity for rapid development of a biofilm on the dental water supply lines combined with the generation of potentially contaminated aerosols to which patients and dental staff are regularly exposed. Dental equipment such as retracting shutoff valves, antiretracting valves that tend to fail, or water lines that are inaccessible contribute to a situation in which virtually every standard dental unit contains water with higher numbers of bacteria than the source water for the system. Typical DUWL biofilms are composed primarily of environmental bacteria and other microorganisms derived from the source system, either tap or bottled water. The majority of the organisms in the DUWL biofilm are harmless environmental species, but some dental units may harbor opportunistic respiratory pathogens, such as *P. aeruginosa*, *Mycobacterium* spp., and *Legionella* spp. The presence of organisms apparently derived from the oral cavity has been reported (Table 2). This is assumed to be due to backflow from older dental apparatus.

Risk assessment analysis suggests a generally low level of hazard from biofilm organisms contaminating DUWLs on the respiratory health of both the dental team and patients. However, definitive case-controlled clinical studies to quantitate these risks have not been done. Nevertheless, to be in general compliance with public health standards for drinking water, the ADA suggests (and many jurisdictions mandate) that water from DUWLs contain fewer than 200 CFU/ml. This standard is relatively easily maintained in the reservoirs of DUWL systems by periodic disinfection and flushing. Higher levels of DUWL organisms are typically found upon initial flushing of lines following a period of inactivity (e.g., early morning).

Several studies have suggested that exposure to *Legionella* spp. from DUWL may pose a health risk to dental personnel due to the opportunistic nature of this organism, its apparent ability to thrive in the DUWL environment, and the chronic exposure of these workers to DUWL aerosols. In recent studies, *Legionella* species were detected in over one-half of DUWL samples, although *L. pneumophila* was present in less than 10%. Furthermore, over one-third of dental personnel have been found to possess serum antibodies to *L. pneumophila*. Together, these studies suggest that chronic exposure to elevated levels of *Legionella* spp. may be a potential health risk for dental personnel and immunocompromised patients.

Current recommendations by the ADA and CDC are summarized below and are regularly updated on the websites listed in Table 1. At

TABLE 2 Microbes that may be present in dental unit water lines[a]

Bacterium (genus)	Most likely source
Achromotobacter	Water
Acinetobacter	Water
Actinomyces	Oral
Alcaligenes	Water
Bacillus	Water
Bacteroides	Oral
Flavobacterium	Water
Fusobacterium	Oral
Klebsiella	Water
Lactobacillus	Oral
Legionella	Water
Micrococcus	Water
Mycobacterium	Water
Nocardia	Oral
Ochromobacterium	Water
Pasteurella	Water
Peptostreptococcus	Oral
Pseudomonas	Water
Serratia	Water
Sphingomonas	Water
Staphylococcus	Oral
Streptococcus	Oral
Xanthomonas	Water
Fungi	
Penicillium	Water
Cladosporium	Water
Alternaria	Water
Scopulariopsis	Water
Protozoa	
Acanthamoeba	Water

[a]Compiled from various studies, not quantified.

present, commercially available options for improving dental unit water quality are rather limited. They include the use of the following strategies:

- Independent DUWL water reservoirs
- Chemical treatment regimens for DUWL
- Daily draining and air purging regimens

Procedural strategies for maintaining DUWL quality and minimizing risk of infection include the following:

- After each patient visit, discharge water and air for a minimum of 20 to 30 s from any dental device connected to the dental water system that enters a patient's mouth (e.g., handpieces, ultrasonic scalers, or air/water syringes).
- Consult with dental water line manufacturers to (i) determine suitable methods and equipment to obtain the recommended water quality and (ii) determine appropriate methods for monitoring the water to ensure quality is maintained.
- Consult with the dental unit manufacturer regarding the need for periodic maintenance of antiretraction mechanisms.

In summary, the quality of DUWLs is of considerable importance since patients and dental staff are regularly exposed to water and aerosols generated from this system. The significance of such exposure to patients and the dental staff is not well understood, and there is presently no convincing evidence of a public health problem from exposure to dental unit water. However, the goal of infection control is to minimize the risk from exposure to potential pathogens and to create a safe working environment in which to treat patients, and it is relatively simple to minimize the bacterial load in DUWLs. Several infection control methods and prevention strategies designed to reduce the impact of the biofilm on dental water contamination are currently available and suitable for use in general practice. These include periodic flushing and disinfection regimens and isolated sterile water reservoirs. In-line, replaceable point-of-use filtration systems were developed in recent years, but these have proven generally impractical and are not currently marketed in the United States. The bacterial load in dental unit water can be kept at or below recommended guidelines for drinking water (fewer than 200 CFU/ml) by using a combination of readily available measures and strict adherence to maintenance protocols.

PRACTICAL APPLICATION OF INFECTION CONTROL MEASURES IN GENERAL DENTISTRY

Definitions of Terms

Sterilization is the process by which all living cells, viable spores, viruses, and viroids are either destroyed or removed from an object or habitat. A sterile object is totally free of viable microorganisms, spores, and other infectious agents. When sterilization is achieved by a chemical agent, the chemical is called a sterilant.

Disinfection is the killing, inhibition, or removal of microorganisms that may cause disease. Disinfecting agents, usually chemical, can be used on inanimate objects or on skin and mucosal membrane prior to medical intervention. A disinfectant does not necessarily sterilize an object because viable spores and a few microorganisms may remain.

Sanitization is closely related to disinfection. In sanitization, the microbial population is reduced to levels that are considered safe by public health standards. The inanimate object is usually cleaned as well as partially disinfected. For example, sanitizers are used to clean eating utensils in restaurants.

Problems Posed for Prevention of Cross-Infection in General Dental Practice

There are several characteristics of dental technique and the dental operatory itself that contribute to the overall risk of transmission of infection during the course of standard dental procedures. Even the smaller dental practices treat high numbers of patients, resulting in a rapid turnover of patients. It is not uncommon for a single practitioner to schedule more than 25 patients per day. A wide variety and number of instruments are typically used, including high-speed dental drills and sonic scaling devices that generate aerosols. Many invasive minor surgical procedures result in breaches of epithelial tissue with associated bleeding. Depending on the

oral health of the patient, dental hygiene treatment can also result in various degrees of bleeding.

The practical (as well as the financial) burden of preventing transmission of infection rests with the practitioner. Overall success in minimizing risk to both dental practitioners and patients relies on the design and rigorous implementation of infection control policies and procedures that must be a seamless part of normal daily procedures involving all members of the staff. These procedures can be divided into categories as listed below:

A. Personal protection for staff
B. Physical design and written infection control policies, etc.
C. Rigorous implementation of infection control policies
D. Interactions with patients during treatment: importance of medical histories

Note that the risk to be assessed is associated with the dental procedure, not the patient; i.e., the procedure on a given patient may constitute a high or low risk of cross-infection (see "Universal Precautions" below).

A. Personal protection for dental staff:
- Vaccinations as required, including HBV
- Glasses with side shields (for both practitioner and patient)
- Masks changed between patients
- Routine use of gloves and suitable protective clothing
- Training of staff and regular review of procedures
- Recording of accidents and incidents and regular quality assurance review

B. Physical design and written infection control policies, etc.:
- Design the operatory to allow zoning and ease of handwashing and disinfection of surfaces
- Choose equipment that is easily cleaned and disinfected
- Disinfect water lines and exposed surfaces, including floors, regularly

C. Rigorous implementation of infection control policies:
- Sanitization (cleaning) of instruments
- Sterilization of instruments
- Disinfection of impressions
- Use of disposable and single-use dental products (Fig. 1)
- Disinfection or covering of surfaces (barriers) (Fig. 2)
- Adequate high-speed evacuation system
- Adequate room ventilation
- Safe disposal of waste (biological as well as sharps waste)

D. Interactions with patients during treatment: importance of medical histories. While maintaining universal precautions such that infection control is driven by the dental procedures, it is necessary to take individual patient health issues into account when designing treatment. This is to ensure that patients at higher risk for

nosocomial infections due to dental procedures are protected at a level that is appropriate for their situation. For instance, antibiotic prophylaxis to reduce the risk of endocarditis or bacteremia may be indicated for certain classes of patients, including those with implanted and prosthetic medical devices and those with a history of certain cardiac valvular diseases or disorders.

CONCLUSION

The threat posed by cross-infection in dentistry can be minimized by the application of good clinical dental practices; involving the whole dental team in safe practices; working in a safe, efficient environment; an assessment of the risk involved in the operative procedure on a given patient; and continuous reappraisal of the measures necessary to prevent cross-contamination and infection.

Universal Precautions

The following paragraphs provide an example of the specific procedures and behaviors required to implement universal precautions in the dental care setting. Similar procedures should be followed in smaller clinics and practices, with appropriate adaptation for the likely lack of the large centralized sterilization and dispensing facilities of an academic dental clinic.

HANDWASHING

Because health care workers can easily pick up and transmit organisms from patients as well as other contaminated environmental surfaces, handwashing is an essential first step in the practice of universal precautions. Most resident microorganisms found in the superficial layers of the skin are not highly virulent but may be responsible for some skin infections. Ordinary handwashing will remove organisms from skin; however, it may not remove them from around rings and under long fingernails. Make sure handwashing is effective by keeping nails cut short and well manicured. Rings, watches, and bracelets should be removed while treating patients. Handwashing or decontamination should take place before putting on gloves and at the intervals listed below:

1. After glove removal
2. Whenever hands or other skin surfaces are contaminated with blood or body fluids containing visible evidence of blood
3. Whenever hands or other skin surfaces inadvertently come in contact with contaminated surfaces or objects

Gloves should be changed when visibly damaged, as well as between patients and before leaving the operatory.

Ordinary handwashing techniques

Ordinary handwashing should take place before examinations, restorative procedures, or suture removal. No special soap is required. Ensure that the mask, safety glasses, radiographs or computer images are in place before washing hands and donning gloves.

1. Lather hands well with soap and water; rub vigorously together for at least 10 s so that all surfaces are scrubbed. If hands are visibly soiled, rub the lather over them for longer than 10 s or wash and rinse hands two or three times.

2. Rinse hands thoroughly under a stream of cool or tepid water.
3. Blot hands dry with disposable paper towels.
4. Shut off the faucet using a clean paper towel to avoid contaminating hands with the faucet handle. (When exiting the restroom, use a clean paper towel to grab onto the door handle and open the door.)
5. Apply hand lotion periodically throughout the day as needed.

Surgical scrub

A special antimicrobial product is needed anytime a surgical scrub is required.

1. Scrub hands and arms to the elbows with an antimicrobial liquid product for several minutes.
2. Rinse thoroughly to remove soap.
3. Dry hands on sterile towel.

Waterless hand products

In addition to handwashing, hand care is very important. Constant handwashing removes moisture from the skin. Depending on local regulations and practice, waterless hand rinse products may be acceptable for use in the dental care setting. Alcohol-based rinse products are not a replacement for soap and water but are an acceptable alternative for hands that are visibly clean. These alcohol gel solutions can effectively destroy transient skin microbes, and they have the advantages of leaving the hands dry for gloving and containing emollients for skin care. Use emollient hand cream or lotion between washings. Avoid using products that contain petroleum or other oil emollients. These formulations weaken latex gloves. In addition, moisturizing products should be applied sparingly between glovings to avoid excessive buildup of moisture under the gloves.

PROTECTIVE BARRIERS

Gloves

Wearing gloves protects health care workers by providing an extra barrier against the entry of microorganisms through any breaks in the skin. Gloves also protect patients from becoming infected with microorganisms that may be present on the hands of the health care worker. Wearing gloves, however, is no substitute for handwashing. Wearing gloves and handwashing may not provide enough protection if weeping dermatitis or open sores are present. Health care workers with these conditions must refrain from direct patient contact and handling patient care equipment until the condition has cleared up or should place a waterproof dressing over minor cuts and abrasions before donning gloves.

Gloves must be worn whenever hands are put into a patient's mouth or if a health care worker touches instruments, equipment, or surfaces that may be contaminated. A new pair of gloves should be used for every patient contact. A new pair of gloves should be worn during all cleaning and decontaminating procedures. Gloves used before, during, or after contact should not be worn outside the treatment areas.

The selection of gloves should be consistent with the procedure being performed. Three types of gloves are available:

1. Disposable examination gloves are used for procedures involving contact with the oral mucous membrane. These gloves are made of latex, nitrile, or vinyl. There are no reported differences in the effectiveness of intact gloves of different types when used as a barrier against contact with blood.
2. General-purpose utility gloves are used when cleaning instruments, equipment, and contaminated surfaces. Rubber household gloves are suitable and can be decontaminated with iodophor and reused.
3. Sterile disposable gloves are used when sterility is necessary during either restorative or surgical procedures.

Neither sterile surgical nor examination gloves are designed for reuse. Washing these gloves may damage the gloves and actually cause "wicking," increasing the flow of liquid through undetectable holes in the gloves. General-purpose utility gloves may be decontaminated and reused if intact. If a glove is torn, punctured, or becomes compromised, remove it immediately and dispose of it properly. Health care workers who are regularly exposed to latex products are at increased risk for latex hypersensitivity. Latex-free gloves are available as an alternative, and there is a strong trend among health care professionanls toward the universal use of nitrile gloves and elimination of latex products. Chemicals used in latex glove manufacturing include mercaptobenzothiazoles, thiurams, carbamates, guanidines, amine compounds, and phenolic compounds. Individuals vary in the type and degree of sensitivity to these materials. The following represent the types of reactions commonly experienced by individuals (both practitioners and patients) who are hypersensitive to latex:

Irritant contact dermatitis. This is the most prevalent dermal problem noted on the hands of health care workers. Contact dermatitis typically results from direct contact with chemical irritants. Frequent handwashing and improper drying technique of the hands may also contribute to the condition. Irritant contact dermatitis will usually appear as red, chapped, or dry skin. Occasionally, fissures and vesicles will develop.

Delayed (type IV) hypersensitivity. This condition is an allergic contact dermatitis typically resulting from residue of the chemicals used in glove manufacturing as well as chemicals from handwashing products. The condition will usually appear as a red rash that forms on the back of the hand. Fissures and fluid-filled vesicles may also develop as part of the condition. The rash will continue to develop for up to 48 h after initial contact with the allergen.

Immediate (type I) hypersensitivity. This condition is typically the most serious dermal reaction. Type I hypersensitivity is an immunoglobulin E-mediated response that, when triggered by contact with latex gloves or other products containing latex, will begin affecting an exposed sensitive

individual in 30 min or less. Symptoms can include redness, itching, swelling, asthma, conjunctivitis, and rhinitis. In extreme cases, anaphylaxis may occur shortly after the initial exposure, leading to respiratory distress, low blood pressure, and potentially death.

Masks and glasses

There can be extensive spatter of blood and saliva during many dental procedures. Spattered material may enter the practitioner's eyes, nose, and mouth, where mucous membranes can provide easy entrance for microorganisms. Health care workers must wear a mask with protective glasses with side shields or a face shield. These protective barriers must be used for facial protection whenever blood or fluids contaminated by blood may be spattered (i.e., during all patient treatments, while cleaning instruments, and while disposing of contaminated fluids). The face mask should be handled by touching the periphery (outside edges) only and should not contact the mouth while being worn.

A new face mask must be used for every patient. Always replace a face mask when it becomes wet during a single treatment. A wet mask may tend to collapse against the face and may not provide a barrier to microorganisms. In addition, face masks should not be pulled down around the chin or neck when not covering the mouth.

Protective glasses and side shields or a face shield must be worn during all dental procedures. Protective eyewear must be decontaminated with detergent and water between patients. If glasses need to be put down before they have been decontaminated, they should be placed on a disposable towel out of the way. Glasses should not be handled with unprotected hands until they have been decontaminated. Nondisposable eye-protective equipment should be washed with soap and water between patients and disinfected with a tuberculocidal "hospital disinfectant" that is registered with the U.S. Environmental Protection Agency.

Gowns

Impermeable gowns that cover clothing should be worn at all times during patient care and subsequently laundered or disposed of properly.

USE AND DISPOSAL OF NEEDLES AND OTHER SHARP INSTRUMENTS

Many dental instruments or items are sharp and can easily pierce or cut skin. These instruments include needles, scalpels, explorers, scalers, rotating burs, endodontic files, orthodontic wires, rotating pumice, and stone wheels. Contaminated sharp objects should be disposed of in a puncture-resistant leakproof sharps container. Sharps should not be disposed of in the regular trash. Disposable needles should not be recapped by hand, and needles should not be bent, broken, or otherwise manipulated by hand.

Important: One exception to the rule against recapping is aspirating syringes, which are not fully disposable and are regularly used in the dental practice setting. If the disposable needle is removed from the syringe without recapping it, injury can occur. Aspirating syringes should be recapped using the one-handed "scoop" technique or a resheathing device. A syringe should not be recapped by using both hands or by any other technique that involves moving the point of a used needle toward the body.

Infection Control Checklist

Before patient treatment:

- Ensure equipment is sterilized or disinfected.
- Put disposable cover in place.
- Place instruments on bracket table.
- Set out materials and mixing instruments.

During patient treatment:

- Treat all patients as potentially infectious.
- Wear new gloves for each patient and discard punctured gloves; wash hands.
- Wear masks and protective eyewear and clothing.
- Use a rubber dam when appropriate and high-speed aspiration; ensure good ventilation.
- Only sheath needles by using a device.

After patient treatment:

- Dispose of sharps and segregate clinical waste.
- Clean and sterilize all instruments.
- Clean and disinfect all contaminated areas.
- Clean and disinfect impressions and dental appliances before sending them to the laboratory.
- Prepare the surgery for the next patient.

At the end of each session:

- Dispose of all clinical waste.
- Clean and disinfect all work surfaces.
- Disinfect the aspirator, tubing, and spittoon.
- Clean the chair and the unit.

Sterilization of Instruments

The aim of sterilization of instruments and subsequent storage for use is to deliver sterile instruments to the chairside, thereby preventing the risk of cross-infection from patient to patient and between dental health care workers and patient by contaminated instruments. Several methods are available for sterilization of dental instruments, including the steam autoclave, the chemical vapor sterilizer ("chemiclave"), and the dry-heat oven. Of these, the autoclave has become the professional standard because of its reliability and relatively rapid cycling time, and its use is mandated in many states. Recommended operating conditions for the steam autoclave for heat-sensitive instruments are 121 to 124°C at 1.1 to 1.25 bar pressure for a minimum of 15 min, or 134 to 137°C at 2.1 to 2.3 bar pressure for a minimum of 3 min (ADA, 1996). These recommended parameters vary slightly, depending on the particular manufacturer's instructions.

Soiled instruments should be cleaned before sterilization, since the presence of blood and other debris can adversely affect steam penetration and thereby prevent the sterilization temperature from being reached on all surfaces. Appropriate hand- and eye-protective equipment should

be used when handling contaminated instruments. It is generally recommended to use a two-stage presterilization procedure including soaking in a detergent solution followed by thorough cleaning. Appropriate steam-permeable wrapping material should be used to maintain sterility of instruments until use.

Quality assurance of the sterilization process is an absolute necessity in dentistry. In the case of the autoclave, regular monitoring is necessary to ensure that the instrument is operating at a temperature and pressure that will kill all microbes. While these instruments typically have pressure- and temperature-monitoring equipment, these numerical recordings are potentially subject to mechanical malfunction. Similarly, chemical indicators such as autoclave tape, which changes color upon reaching a certain temperature, are suitable for routine use but do not directly demonstrate autoclave performance.

Since it is impractical to test sterility by direct culturing of all instruments, a standardized direct method of functional performance testing using biological indicators has been developed. Biological indicators, generally spores of *Bacillus stearothermophilus* or *Bacillus subtilis*, depending on the type of sterilizer, provide the best assurance of sterility by challenging the sterilizer with quantifiable, highly resistant spores. Many states now mandate the use of these spore tests, which are available in several forms. Paper spore strips impregnated with 10^4 to 10^5 bacterial spores per strip are designed to be incubated in culture medium following sterilization treatment. This type of spore strip system is typically managed by a commercial or institutional sterilization-monitoring service. A simpler method of spore testing is the spore test ampoule such as Attest (3M Healthcare, St. Paul, MN), which is designed to be placed in the center of an autoclave load and incubated on removal. The ampoule contains a glass vial of culture medium with a color indicator. After sterilization, the vial is "crushed" to join the growth media with the processed spore strip. The ampoule is incubated for 48 h for a visual change readout. A color change to yellow indicates surviving spores and a positive result. This system, combined with a mini-incubator, can be used in an individual practice. A drawback to all spore test systems is the delay of 12 to 48 h before test results can be read, and longer if an off-site monitoring service is used. However, the spore test system remains the current standard of practice for quality assurance of sterilization.

The information listed above can provide a guide for implementation of a standardized sterilization protocol for the individual dental practice. Specific written protocols should be in place as an integral part of a standardized and documented infection control program. The protocol should include written procedures on how to operate the instrument, how to carry out a daily test, what information must be recorded, and what to do if there is a failed sterilization cycle. The responsible user or administrator must ensure that all operators are fully trained, daily tests are carried out correctly, all faults are recorded, the autoclave/sterilizer is properly maintained, and adequate sterilization records are kept.

The advantages and drawbacks of sterilization procedures available to the dental community are given in Table 3. In the autoclave, steam is heated above the boiling point of water by applying pressure. Steam then denatures proteins, ultimately leading to cell death. Autoclaves have a

TABLE 3 Advantages and disadvantages of sterilization methods

Method	Advantages	Disadvantages
Steam	Quick and easy	May leave instruments wet, causing them to rust
	Allows for sterile packaging	Requires packaging
	Very reliable	Damages plastics
	Penetrates fabric- and paper-wrapped packs	May dull certain sharp items
Chemical vapor	Quick	Requires good ventilation
	Can be used with packaged items	Cannot handle large loads
	Penetrates paper-wrapped packs	Does not penetrate fabric-wrapped packs
	Very reliable	Damages certain plastics
	Does not rust instruments	Replacing special solution increases cost
	Leaves instruments dry	
Dry heat	Inexpensive and easy	Is slow, requires longer processing time
	Very reliable	Requires careful loading
	Leaves instruments dry	Damages plastics
	Does not rust instruments	Melts or destroys some metal or solder joints
	Requires little maintenance	Chars fabric
Ethylene oxide	Reliable	Requires long processing time
	Requires relatively low temperatures	Units are expensive
		Toxicity of ethylene oxide gas
		Requires additional 24 h for the gas to dissipate from porous material
Chemical disinfectant/sterilant	Inexpensive initially	Has a limited life
	Can sterilize items that would be damaged by heat	Cannot be checked for effectiveness
		Protective clothing required during use
		Toxic fumes require special ventilation
		Cannot be used with packaged items
		Must be rinsed off with sterile water
		May corrode instruments

large capacity; however, items damaged by water or that will rust or corrode should not be sterilized by this method. Dry-heat ovens also kill by protein denaturation; however, in the absence of water, this process is less efficient. Hence, killing by hot air requires at least 160°C for over an hour. The chemiclave uses a mixture of chemicals, often primarily formaldehyde, that are volatilized by heating. The unit operates under pressures of 20 to 25 psi, which produces a temperature of 130°C and allows sterilization in 20 to 30 min. Chemical sterilants are generally alkylating agents that can denature proteins without the presence of water. Chemicals such as ethylene oxide, formaldehyde, and β-propiolactone can all be used for sterilization; however, they are very toxic to humans.

Disinfecting agents (Table 4) are useful when sterilization is not practicable or cannot be achieved, such as large areas of floor or benches or large pieces of equipment. They are also useful for skin and mucosal surfaces. Disinfectants can be protein denaturants (e.g., alcohols and phenolics), oxidizing agents (e.g., halogens), or active against lipid bilayer membranes (e.g., quaternary ammonium compounds). UV light can also be used to disinfect benchtops.

Methods for the correct disposal of biohazardous waste are given in Table 5. In the event that infection control procedures break down and exposure to HBV- or HIV-infected material is suspected, postexposure management is outlined in Tables 6 and 7.

TABLE 4 Surface disinfectants

Category	Advantage	Disadvantage
Synthetic phenolics (<3% [vol/vol], approx. 1 h)	EPA registered	Prepare fresh daily
	Broad spectrum activity	May degrade plastic and etch glass with prolonged exposure
	Tuberculocidal	
	Can be used on metal, glass, rubber, and plastic	Difficult to rinse off certain materials
Quaternary ammonium compounds (concn and effective time vary with product and manufacturer)	EPA registered	Not a tuberculocidal or virucidal (hydrophilic viruses)
	Bactericidal against gram-positive bacteria	Inactivated by organic matter, soaps, and hard water
	Low tissue toxicity	Variable activity against gram-negative bacteria
Quaternary ammonium compounds/alcohol (concn and effective time vary with product and manufacturer)	EPA registered	Inactivated by organic matter, soaps, and hard water
	Broad spectrum activity	Film accumulation
	Tuberculocidal	
	Pleasant odor	
	Low tissue toxicity	
Iodophors (450 ppm, 10 min on precleaned surface; otherwise up to several hours)	EPA registered	Prepare fresh daily
	Broad spectrum activity	May discolor some surfaces
	Tuberculocidal	Inactivated by hard water
Hypochlorites (600–6,000 ppm [1:10–1:100 diluted bleach]); fungi: up to 1 h.	EPA registered	Prepare fresh daily for most
	Broad spectrum activity	May degrade plastic and rubber
	Tuberculocidal	Corrosive to metal
	Economical	Reduces activity by organic matter
		Skin and eye irritant
Alcohols (70% ethyl or 70% isopropyl; 20 min required for *Cryptosporidium*, much less for other organisms)	EPA registered	May degrade some plastics, rubber, and metal
	Rapidly bactericidal	Reduces activity by organic matter
	Tuberculocidal	Rapid evaporating
	Only slightly irritating	Not recommended for environmental surface cleaning
	Economical	

TABLE 5 Disposal of infected waste[a]

Waste	In the office	Out of the office
Needles	"Sharps" container	Dispose of every 90 days to an approved landfill or commercial medical waste hauler
Scalpels		
Broken glass		
Disposable syringes		
Intravenous tubing with needle attached		
Orthodontic wire	"Sharps" container	Dispose of with other office waste, at any time
Liquid blood	Flush into sewer system	
Items dripping with blood or saliva	Options:	
	(i) Flush into sewer system	
	(ii) Incinerate	
Blood/saliva-soaked items	Regulated waste container	Dispose of every 90 days (same as sharps)
Pathological waste/unfixed tissue	Autoclave	Label as "Decontaminated Medical Waste" and dispose of with other office waste
Teeth	Options[b]:	Dispose of every 90 days (same as sharps)
	(i) Return to patient	
	(ii) Regulated waste container	
Items stained with blood	Office waste container	Dispose of with other office waste in the dumpster or garbage can
Items moist with saliva		
Gloves, masks, etc.		
Patients' disposable items not soaked or caked with blood or saliva		

[a]This table summarizes the handling, discarding, and disposal of medical waste from the dental office in compliance with the Medical Waste Regulatory Act and the Bloodborne Pathogens Standard.

[b]Note that autoclaving of extracted teeth containing dental amalgam restorations is not appropriate.

TABLE 6 Hepatitis B virus postexposure management

	Treatment when source is found to be:		
Status of exposed worker[a]	**HBsAg positive**	**HbsAg negative**	**Unknown or not tested**
Unvaccinated	1. Initiate HBV vaccine AND 2. Worker should receive a single dose of HBIG[b] as soon as possible and within 24 h, if possible.	Initiate HBV vaccine	Initiate HBV vaccine
Previously vaccinated[c]			
Known responder	Test exposed worker for anti-HBs: 1. If adequate,[c] no treatment 2. If inadequate, HBV booster dose	No treatment	No treatment
Known nonresponder	1. Worker should receive two doses of HBIG; second dose is administered 1 month after the first. OR 2. Worker should receive one dose HBIG plus one dose hepatitis B vaccine.	No treatment	If known high-risk source, may treat worker as if source were HBsAg positive
Response unknown	Test exposed worker for anti-HBs: 1. If inadequate, one dose of HBIG plus HBV booster dose 2. If adequate, no treatment	No treatment	Test exposed worker for anti-HBs: 1. If inadequate, HBV booster dose 2. If adequate, no treatment

[a]Once an exposure has occurred, the blood of the individual from whom exposure occurred should be tested for hepatitis B surface antigen (HBsAg) and antibody to HIV antibody. The information given in this table is based on recommendations from the CDC: Hepatitis B virus: a comprehensive strategy for eliminating transmission in the United States through universal childhood vaccination: recommendations of the Immunization Practices Advisory Committee (ACIP). *MMWR Morb. Mortal. Wkly Rep.* **55**(RR-16)**:**1–33, 2005.

[b]HBIG, HBV immunoglobulin.

[c]Exposed worker has already been vaccinated against hepatitis B.

[d]Adequate anti-HBs is >10 mIU.

TABLE 7 Public Health Service recommendations for chemoprophylaxis after occupational exposure to HIV, by type of exposure and source material[a]

Type of exposure	Source material[b]	Antiretroviral prophylaxis[c]	Antiretroviral regimen[d]
Percutaneous	Blood[e]		
	Highest risk	Recommend	ZDV plus 3TC plus IDV
	Increased risk	Recommend	ZDV plus 3TC plus IDV[f]
	No increased risk	Offer	ZDV plus 3TC
	Fluid containing visible blood or other potentially infectious fluid,[g] or tissue	Offer	ZDV plus 3TC
	Other body fluid (e.g., urine)	Not offer	
Mucous membrane	Blood	Offer	ZDV plus 3TC plus IDV[f]
	Fluid containing visible blood or other potentially infectious fluid,[g] or tissue	Offer	ZDV plus 3TC
	Other body fluid (e.g., urine)	Not offer	
Skin increased risk[h]	Blood	Offer	ZDV plus 3TC plus IDV[f]
	Fluid containing visible blood or other potentially infectious fluid,[g] or tissue	Offer	ZDV plus 3TC
	Other body fluid (e.g., urine)	Not offer	

[a]The information in this table is based on recommendations from Centers for Disease Control and Prevention: Updated U.S. Public Health Service Guidelines for the Management of Occupational Exposures to HIV and Recommendations for Postexposure Prophylaxis, *MMWR Morb. Mortal. Wkly. Rep*. **54**(RR09)**:**1–17, 2005.

[b]Any exposure to concentrated HIV (e.g., in a research laboratory or production facility) is treated as percutaneous exposure to blood at the highest risk.

[c]Recommend: postexposure prophylaxis (PEP) should be recommended to the exposed worker with counseling. Offer: PEP should be offered to the exposed worker with counseling. Not offer: PEP should not be offered because these are not occupational exposures to HIV.

[d]Regimens: ZDV, zidovudine, 200 mg, three times a day; 3TC, lamivudine, 150 mg two times a day; IDV, indinavir, 800 mg three times a day (if IDV is not available, saquinavir may be used, 600 mg three times a day). Prophylaxis is given for 4 weeks.

[e]Highest risk: *Both* larger volume of blood (e.g., deep injury with large diameter hollow needle previously in source patient's vein or artery, especially involving an injection of source patient's blood) *and* blood containing a high titer of HIV (e.g., source with acute retroviral illness or end stage of AIDS; viral load measurement may be considered, but its use in relation to PEP has not been evaluated). Increased risk: *Either* exposure to larger volume of blood *or* blood with a high titer of HIV (e.g., solid suture needle injury from source patient with asymptomatic HIV infection).

[f]Possible toxicity of additional drug may not be warranted.

[g]Includes semen; vaginal secretions; and cerebrospinal, synovial, pleural, peritoneal, and amniotic fluids.

[h]For skin, risk is increased for exposures involving a high titer of HIV, prolonged contact, an extensive area, or an area in which the skin integrity is visibly compromised. For skin exposures without increased risk, the risk for drug toxicity outweighs the benefit of PEP.

KEY POINTS

Dental professionals are exposed to a wide variety of microorganisms in the blood and saliva of patients. These microorganisms may cause infectious diseases such as the common cold, influenza, pneumonia, tuberculosis, herpes, hepatitis, transmissible spongiform encephalopathy, and AIDS. The use of effective infection control procedures and universal precautions in the dental office and the dental laboratory will prevent cross-contamination that could put dentists, dental office staff, dental technicians, and patients at risk for infection.

Infection control in dentistry, as in other health professions, involves application of a risk management decision-making process, including risk identification, assessment, or analysis, and implementation of design and procedures for risk control. Cross-infection is typically defined as the transmission of infectious agents among patients and staff within the clinical environment.

Risk control in dentistry uses a single-tier approach, whereby all patients are treated as though they are a potential source of infectious pathogens. This single-tier approach is known as universal precautions. It applies the concept of risk to the procedure, not the patient. Emphasis should be placed on consistent adherence to recommended infection control strategies, including hand hygiene, the use of protective barriers, appropriate methods of sterilization or disinfection, and vaccination when possible.

Possible sources of infection in the dental care setting include patients with infectious disease (e.g., influenza, measles, or tuberculosis); patients in the prodromal or convalescent stage of infection (e.g., herpes simplex virus); healthy (or asymptomatic) carriers of disease-causing organisms (e.g., *S. pyogenes*, *N. meningitidis*, and *H. influenzae*); and environmental sources such as airborne organisms or biofilms in waterlines or on equipment or instruments.

Routes of spread include airborne spread such as through dust or aerosols; contact, including person to person or with contaminated equipment or fluids; and parenteral, through accidental puncture with needles, and so forth.

Sterilization is the process by which all living cells, viable spores, viruses, and viroids are either destroyed or removed from an object or habitat. A sterile object is totally free of viable microorganisms, spores, and other infectious agents.

Sterilization can be achieved by physical or chemical means. Physical methods include the steam autoclave, the hot-air oven, and the chemiclave. Chemical sterilants include ethylene oxide and formaldehyde.

Disinfection is the killing, inhibition, or removal of microorganisms that may cause disease. Disinfecting agents, usually chemical, can be used on inanimate objects or on skin and mucosal membrane prior to medical intervention. A disinfectant does not necessarily sterilize an object because viable spores and a few microorganisms may remain.

Sanitization is closely related to disinfection. In sanitization, the microbial population is reduced to levels that are considered safe by public health standards. The inanimate object is usually cleaned as well as partially disinfected. For example, sanitizers are used to clean eating utensils in restaurants.

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FURTHER READING

Molinari, J. A., and J. A. Harte. 2010. *Cottone's Practical Infection Control in Dentistry.* Lippincott Williams & Wilkins, Baltimore, MD. This comprehensive text is also available in a digital online version.

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