Oral Microbiology at a Glance

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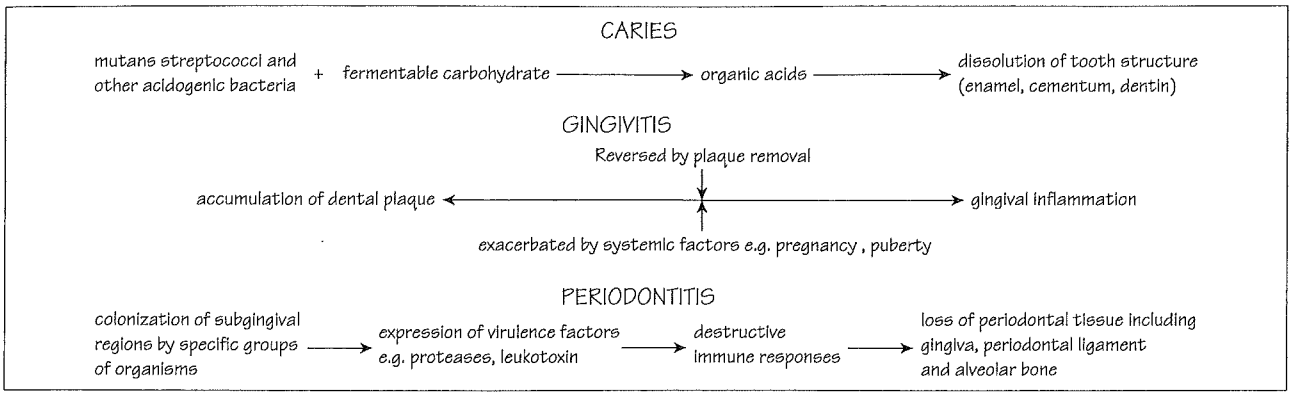

Figure 1.1 Etiology of the major bacterial diseases in the oral cavity

Table 1.1 Important oral diseases, their manifestations and the major microorganisms involved.

Disease	Description	Microorganisms implicated
Caries	Decay (loss) of tooth enamel (dental caries) or dentin (dentinal caries), or root dentin (root aries)	*Streptococcus, Lactobacillus, Actinomyces* (root caries)
Gingivitis	Redness and swelling (inflammation) of the gingival tissues (gums)	*Actinomyces, Fusobacterium, Bacteroides, Prevotella*
Periodontitis	Inflammation and either rapid (aggressive, either generalized or localized) or slower (chronic) destruction of the tissues supporting the tooth	*Aggregatibacter* (localized), *Porphyromonas, Treponema, Tannerella, Fusobacterium, Prevotella*
Implantitis	Infection and destruction of tissues surrounding a dental titanium implant	*Staphylococcus, Pseudomonas, Porphyromonas, Prevotella*
Pulpitis	Infection of the pulp, inflammation around the apex of the root, leading to abscess formation (periapical granuloma)	*Fusobacterium, Dialister, Peptostreptococcus, Porphyromonas*
Halitosis	Oral malodor	*Fusobacterium, Porphyromonas, Prevotella, Treponema, Eubacterium*
Pharyngitis	Redness and inflammation of the pharynx	Group A *Streptococcus, Neisseria, Haemophilus*, Coxsackie A virus
Tonsillitis	Infection and inflammation of the tonsils	Group A *Streptococcus, Haemophilus*
Leukoplakia	White patches on the buccal mucosal epithelium or tongue	*Candida*, human papilloma virus (HPV)
Stomatitis	Reddening and inflammation of the oral mucosa	*Candida albicans, Candida tropicalis, other Candida species*
Actinomycosis	Hard swelling (cyst) within the gums	*Actinomyces israelii*
Cold sores	Surface (superficial) red, dry lesions close to the lips	Herpes simplex virus (HSV)

The oral cavity is the most complex and the most accessible microbial ecosystem of the human body. The teeth, gingivae (gums), tongue, throat and buccal mucosa (cheeks) all provide different surfaces for microbial colonization. The constant production of saliva and intermittent provision of sugars and amino acids from ingested food provides nutrients for microbial growth. The human oral cavity is home to about 700 identified species of bacteria. This number will probably turn out to be closer to 1000 in the future, when all taxa and phyla have been recorded. It is also home to at least 30 species of fungi (mainly of the genus *Candida*), several species of protozoa (which graze on the bacteria for food), and various intracellular viruses. Generalizing, in a single subject it is usual to find between 20–50 species of bacteria at healthy oral sites. At diseased sites there is a tendency for higher numbers of different species to be present, perhaps 200 or more. These facts underline two main features in the field of oral microbiology. There are a number of different micro-environments within the oral cavity and the ecology of these is complex and diverse. Second, microorganisms do not exist as single species; rather they are almost always present in communities.

Commensals and pathogens

The organisms present in the oral cavity are a mixture of commensals and pathogens. A commensal microorganism is defined as one that lives on or within a host but does not cause any apparent disease. However, this terminology may be misleading, as many commensal bacteria can, under certain conditions, be associated with human disease. Subjects whose immune systems are not working optimally, i.e. immunocompromised, are especially susceptible to infections by microbes that are commensal in healthy individuals. For these reasons, commensals are nowadays often referred to as opportunistic pathogens.

Many of the cultivated bacteria present in the mouth probably contribute to oral diseases to a greater or lesser extent, because these diseases are almost always associated with polymicrobial infections (see Figure 1.1). Monospecies infections are rare; however, localized aggressive periodontitis (LAP) is predominantly associated with *Aggregatibacter actinomycetemcomitans*, while *Actinomyces israelii* can cause oral cysts (see Table 1.1). Overt pathogens are organisms that usually cause disease when present, unless the host has protective immunity. There are very few organisms in the oral cavity and nasopharynx that can be considered overt pathogens. *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus pneumoniae* (Pneumococcus), *Neisseria meningitidis* (Meningococccus) and *Haemophilus influenzae* all reside within the nasopharynx and have the potential to cause life-threatening diseases. It is important to note, however, that even in such cases these bacteria may also be carried by subjects with no overt signs of disease. This is termed the carrier state. Vaccination of children against meningococcus (MenC) or *H. influenzae* (Hib) has been very effective in protecting against disease. In addition, the immunization programs have led to reductions in the numbers of carriers of these bacteria in the human population. One of the problems with this kind of approach is that removal of one species of bacteria from a population creates a vacant niche for arrival of other organisms. This might result in replacement by a similar species of different serotype that is not covered by the vaccine. This occurs in children immunized using the 7-serotype (heptavalent) pneumococcal conjugate vaccine. Alternatively, a different bacterium may become resident, such as *Staphylococcus aureus* on the nasal mucosa of subjects immunized against Pneumococcus.

Oral diseases

Almost every member of the human population is afflicted at some stage of their lives with an oral disease (see Table 1.1). The incidence of dental caries has declined generally in the developed world, due in part to fluoride in the water supply, in toothpaste, or taken in tablet form. However there are many groups within societies that are still seriously afflicted with caries. Polymicrobial infections of the gingivae and sub-gingival regions (periodontitis, implantitis and pulpitis) are major conditions requiring clinical intervention. These diseases impose a significant burden on the heath care system. Halitosis is often caused by bacteria on the tongue processing proteins into volatile sulfur compounds. Pharyngitis and tonsillitis are common diseases in children and are caused by bacteria or by viruses (see Table 1.1). Osteonecrosis of the jaw is associated with the use of bisphosphonates particularly in cancer patients with multiple myeloma. Fungal infections, most frequently by the yeast *Candida albicans*, are associated with reduced salivary flow, ill-fitting dentures, hormonal changes, or compromised immune function. Viral infections of the oral mucosa include HPV, EBV and HSV.

2 In the beginning

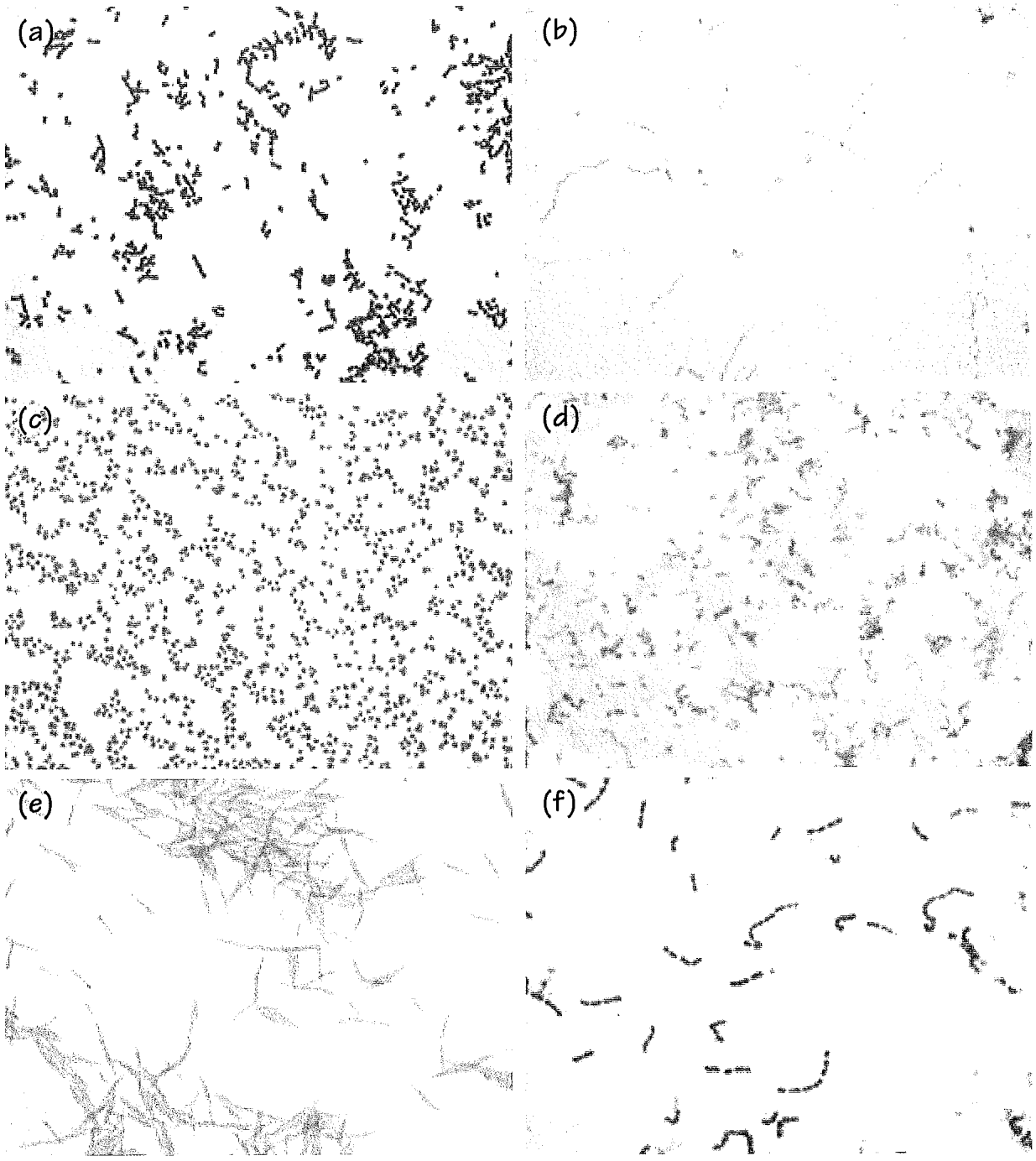

Figure 2.1 *Gram stains of important oral bacteria observed by microscopy: (a) Streptococcus gordonii; (b) Treponema denticola; (c) Neisseria sp.; (d) Porphyromonas gingivalis; (e) Fusobacterium nucleatum; (f) Peptostreptococcus micros (Parvimonas micra). Gram-positive bacteria stain purple while Gram-negative bacteria stain pink.*

Evidence that humankind has long practiced dentistry comes from archeological findings. The ancient Egyptians, Greeks and Romans produced carefully shaped ivory or bone teeth, sometimes held together with metal wire. In the Middle Ages dentistry was practised by barber-surgeons. Toothache was cured by extraction, with no anesthetic, except perhaps alcohol. By 1800 the scientific study of dentistry was beginning to emerge. The first use of ether as an anesthetic for tooth extraction was in 1846. The growth of dentistry and dental surgery led to the opening of schools, the first in Baltimore (1839) and then a school at the London Dental Hospital, Soho Square, in 1859.

The first microbes

The beginnings of microbiology as a subject lie with oral microbiology. Antoine van Leeuwenhoek was the first recorded scientist to discover microorganisms. He wrote in 1676 about the white stuff between his teeth that, when examined under his extraordinary microscopes with hand-made lenses, contained "animalcules" or little animals. Some of the microbes that he recorded in his drawings may be recognized today as classic bacterial rods, cocci, fusiforms and spirochetes (see Gram stains of these types of organisms in Figure 2.1). However, these detailed studies were not linked to oral disease at that time as tooth decay was thought to originate within the tooth (vital theory). Later, the chemical theory held that putrification of food produced a chemical that dissolved enamel. A microbial etiology for caries began to emerge in the 1840s when Erdl described microorganisms from carious lesions that were proposed to cause decay.

Microbial basis of infectious disease

In the late nineteenth century, Robert Koch discovered the infectious nature of anthrax, the bacterium causing tuberculosis, and the vibrio bacterium that caused cholera. Koch was the first to grow bacterial colonies on gelatine, a solid medium later developed as agar. He described different colony morphologies and concluded that the smaller colonies comprised microbial cells that divided more slowly than those within the larger colonies. However, he is best known for Koch's postulates, which today are still the basis for proving that a disease is caused by a specific microorganism. The postulates are that the microorganism must be: found in all cases of the disease examined; prepared and maintained in pure culture; capable of producing the original infection in animal models; and retrievable from an infected host. The reason that these are introduced here is because many oral diseases are not caused by one microorganism, but by polymicrobial consortia of bacteria. In these cases one bacterium depends upon the presence of others to grow or to express virulence factors. In addition, the human oral microbiota is unique to humans; therefore few appropriate animal models are available to fulfill Koch's postulates for oral diseases. This means that Koch's postulates must be modified for the unique oral situation and combine information from studies of association, treatment, host responses and virulence factors.

Miller and the chemoparasitic theory of caries

WD Miller, an American microbiologist working in Berlin, laid the foundation of modern oral microbiology. Miller was able to observe oral bacteria within tissues, in particular streptococci penetrating the tubules of dentin. Miller's most significant contribution was to synthesize the work of Koch (isolation of pure cultures), Pasteur (fermentation of sugars to lactic acid) and Magitot (fermentation of sugars could dissolve teeth *in vitro*), and to expand the theories of Mills and Underwood, to formulate the chemoparasitic theory (1890). In essence correct, this theory states that caries is caused by acids produced by oral bacteria following fermentation of sugars. With the description of dental plaque independently by Black and by Williams in 1898, the key elements of our modern concept of the etiology of dental caries were in place. It is noteworthy, however, that it was not until the mid-1970s that there was a consensus view that periodontal disease (periodontitis) is caused by oral bacteria.

Oral and dental research

With the establishment of dental schools and oral biology/microbiology departments in the USA and UK, came research and new developments in dental microbiology. Dentistry started to become recognized as a science as well as a clinical profession. However, oral microbiology was relatively slow to develop in comparison to other microbiology fields. This was partly because many of the oral microorganisms are anaerobic and require special cultivation methods that were not available at that time. Oral microbiology research was reinvigorated in the USA in 1948, with the establishment of a research facility at the National Institutes of Health, Maryland, which led to the discovery of dental caries as an infectious disease (Chapter 3).

3 Caries as an infectious disease

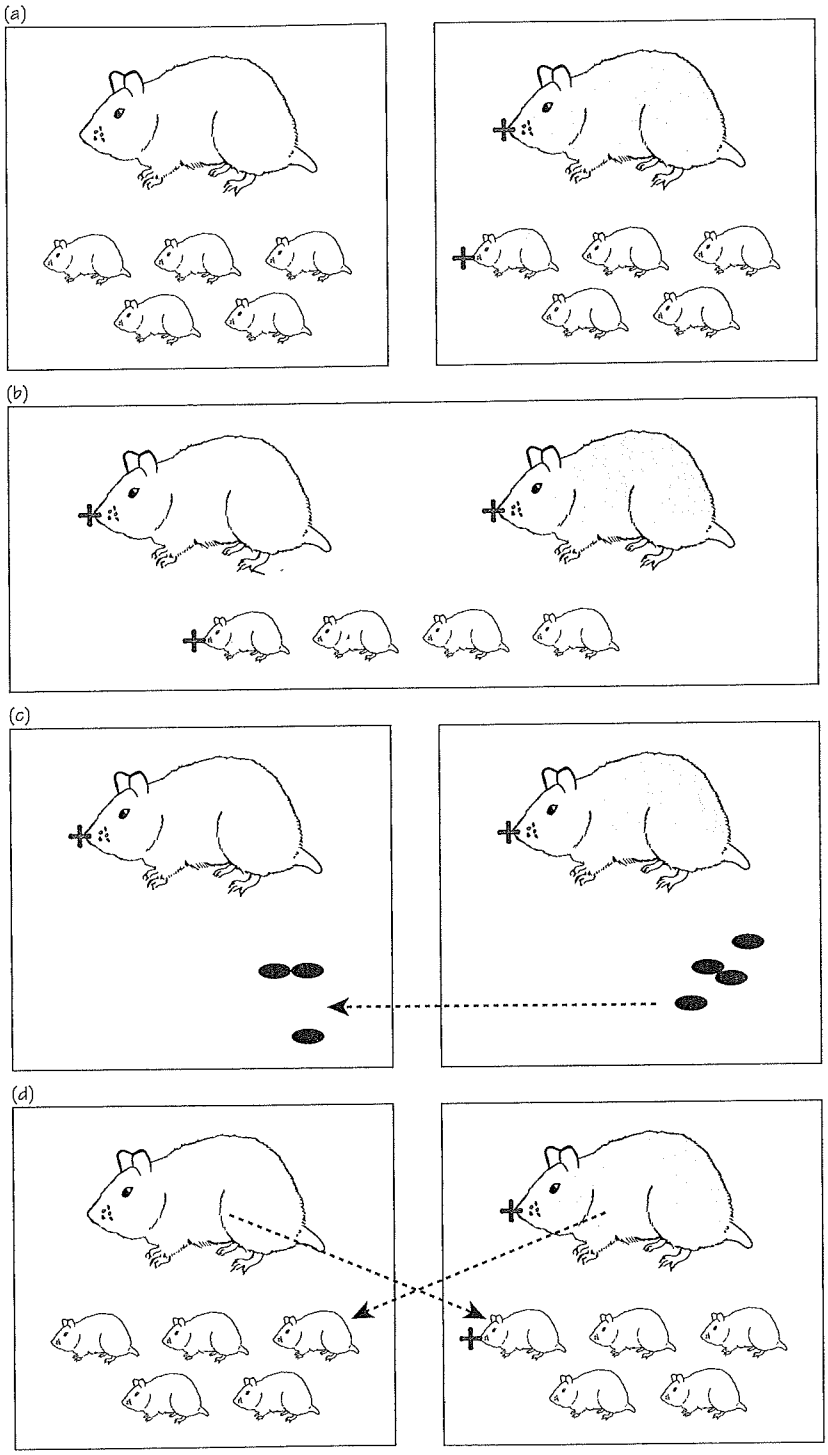

Figure 3.1 Experiments of Keyes and Fitzgerald in the 1950s to test whether caries was an infectious or genetically determined disease using golden or albino hamsters and their pups. + indicates the presence of caries. Redrawn with permission from Slots J, Taubman MA (eds) *Contemporary Oral Microbiology and Immunology* (1992), Mosby Year Book, St Louis.

In 1920s London there were several groups of microbiologists studying various aspects of human infections. Killian Clarke worked at the Royal Dental School, where research was funded only through charitable contributions. Clarke researched in collaboration with Fish at St Mary's Hospital, Paddington, where four years later Fleming discovered penicillin. Clarke described a spherical bacterium that formed chains of cells, isolated from dental caries lesions. He named this organism *Streptococcus* (streptus (Greek) flexible chain, and coccus (Greek) berry) *mutans* (different morphological forms which he believed were mutants). *S. mutans* produced lactic acid as a main by-product from glucose fermentation (homolactic). But there was a strong cohort of *Lactobacillus* microbiologists at the time, and Clarke's attempts to have *Streptococcus* named as a new genus were foiled. With the depression of the 1930s and the outbreak of the Second World War, this discovery was not followed up in the UK.

Dental caries as a transmissible disease

The National Institutes of Health, Bethesda, USA, established a dental research institute in 1948. Fitzgerald and Keyes had set up a germ-free animal unit, and noted that a line of golden hamsters developed tooth decay when fed a high sugar diet, while an albino line of hamsters did not (see Figure 3.1a). Either tooth decay was genetic, or the golden hamsters carried an agent that the albinos did not. To demonstrate that caries was due to an infectious agent, a series of novel and exacting experiments were undertaken. When the two lines of hamsters were caged together, all adult females and their pups developed dental caries (panel b). The fecal pellets from the golden hamsters, when transferred to the albinos, resulted in the albinos developing caries (c). Lastly, caries-free albino pups, when transferred to a golden hamster mother, went on to develop caries (d). These observations suggested that caries was a transmissible disease, and not a genetic condition.

Streptococcus mutans

By the late 1950s, Keyes and Fitzgerald began working on the nature of this transmissible factor. A *Streptococcus* was purified from carious lesions of hamsters (also from rats) that was strongly acidogenic (producing acid) and non-proteolytic. When these bacteria were fed to *Streptococcus* and caries-free hamsters, dental caries then developed in those animals on a high sugar diet. The *Streptococcus* could be recovered from the mouths of infected hamsters, and shown to cause caries when re-inoculated into germ-free animals. These observations fully satisfied Koch's postulates. Interestingly, it turned out that the albino hamsters were only caries free because they had been exposed to high concentrations of antibiotics in previous studies aimed at establishing germ-free animals. It was not until 1968 that it was accepted that the *Streptococcus* isolated from hamsters was the same *S. mutans* as that described by Clarke in 1924.

Mutans group streptococci

Following this pioneering work with hamsters and rats, many laboratories throughout the world, along with the NIH group, went on to isolate *S. mutans*-like bacteria from humans and monkeys. These bacteria were shown to induce dental caries in hamsters, rats, gerbils and monkeys. The organism *S. mutans* is now subdivided into species including *S. cricetus* (hamster), *S. rattus* (rat), *S. ferus*, *S. macacae* (monkey), *S. sobrinus*, and *S. downeii*. These are referred to as the mutans group streptococci.

Link between *S. mutans* and dental caries

Further studies with animals led to a temporal relationship being established between colonization by mutans group streptococci and subsequent attack on the teeth to generate white spot (early caries) lesions. Epidemiological studies in humans then began to seek correlations between numbers of *S. mutans* present on teeth and the development of dental caries. Numerous studies suggested positive correlations, and so led to the idea that *S. mutans* levels were a good indicator of active caries, and may indeed be predictive. Strip or dip culture methods were developed to monitor mutans group streptococci levels in saliva samples from children. High levels of mutans group streptococci often correlated well with caries activity in populations. However, these correlations were not perfect and often broke down at the level of individual patients. Today it is clear that there are individuals and population groups of high caries susceptibility with low levels of mutans group streptococci and vice versa. The reasons for these remain largely unexplained.

Immunity to caries

Because dental caries resulted from bacterial infection, this led to ideas that immunity could be induced. Immunization against dental caries in primates was reported in 1969. Since then many studies have demonstrated that serum antibodies, mainly immunoglobulin G (IgG), and salivary antibodies, mainly secretory immunoglobulin (S-IgA), are induced following vaccination of animals with *S. mutans* cells or purified *S. mutans* antigens. Development of a vaccine for humans has not yet occurred (see Chapter 21), and even if scientifically achievable may not be accepted for what is (most commonly) not a life-threatening disease.

4 General properties of saliva

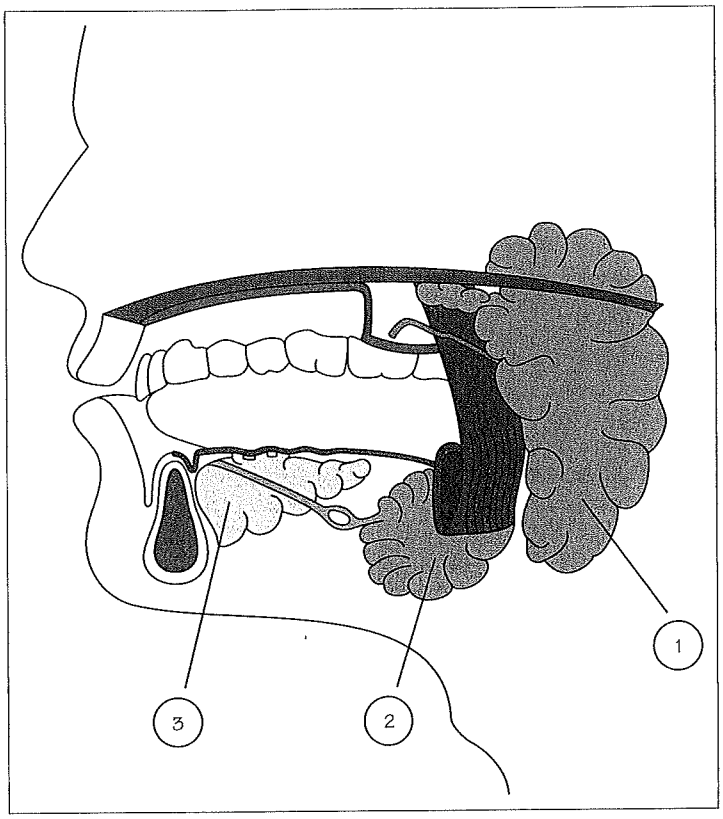

Figure 4.1 Positions of the major salivary glands (1 parotid; 2 sub-mandibular; 3 sub-lingual).

Table 4.1 Major functions of saliva.

1 Physico-mechanical flushing
2 Tissue coating: lubrication and permeability barrier
3 Modulation of the oral microbiota
4 Antacid and neutralization of deleterious materials
5 Regulation of calcium and phosphate equilibrium
6 Digestion

Table 4.2 Major components of salivary pellicle.

Albumin
Amylase
Lysozyme
Lactoferrin
Acidic proline-rich proteins
Proline-rich glycoproteins
Statherin
Mucin-glycoprotein (MG)1
MG2
Carbonic anhydrase
Secretory immunoglobulin A (S-IgA)

Around 0.5 to 1.5 liters of saliva are secreted into the mouth each day. Saliva is responsible for flushing the epithelial surfaces and for lubrication and protection of tissues, and an adequate flow of saliva is essential for the maintenance of both hard and soft tissue integrity. Saliva is hypotonic, with an average pH of around 6.7. Saliva contains both organic compounds (2–3 g/l protein, notably the enzyme amylase) and inorganic compounds including the electrolytes bicarbonate, chloride, potassium and sodium.

Most of the important physiological properties of saliva have been deduced from subjects who have deficiencies in saliva flow rate, saliva production, or in specific salivary components (Table 4.1). Salivary flow rate and composition can be affected by a range of infectious diseases, clinical conditions, e.g. wearing of dentures, clinical treatments, e.g. radiation therapies for oral cancer, or pharmaceutical drugs. The symptoms of dry mouth (xerostomia), due to deficiencies in salivary flow, are frequently accompanied by increased susceptibility to oral microbial diseases as is discussed in the following chapters.

Saliva production

There are three pairs of salivary glands in the human (see Figure 4.1). The parotid glands (shown as (1) in Figure 4.1), with the parotid ducts emanating in the cheeks, supply a fluid containing bicarbonate and phosphate ions, agglutinins (glycoproteins), α-amylase (degrades starch), proline-rich proteins and a range of other proteins and glycoproteins. Plasma cells, originating in the bone marrow, localize to the parotid glands and produce S-IgA which is also present in parotid secretions. The sub-mandibular glands (2) located beneath the floor of the mouth, produce about 70% of saliva in the oral cavity, and this contains mucous and serous (serum derived) components. The sub-lingual glands (3) located anterior to the sub-mandibular glands produce mainly mucous secretions. There are many other minor salivary glands located throughout the oral cavity. Von Ebner's glands are found in the papillae of the tongue, and they produce a serous secretion that is essential for taste. It is suggested that because whole saliva contains serous proteins (derived from gingival crevicular fluid), as well as body cells (from tissues in the mouth), it may in the future provide a means for more rapid diagnosis of conditions that currently require blood or tissue samples for analysis.

Protective role of saliva

There are between 1 million and 100 million bacteria present in 1 ml of saliva, depending upon oral hygiene, frequency of food consumption, and salivary flow rate. Saliva in the fluid phase acts principally to flush out bacteria from the mouth. Saliva contains agglutinins that aggregate bacteria, thus preventing adherence to surfaces, and the bacterial clumps are removed by swallowing or expectoration (Chapter 5). Saliva possesses a number of additional antimicrobial components that are discussed in the succeeding chapters.

Salivary pellicle

Saliva forms a coating on all the surfaces that are present in the mouth, and at the back of the throat. Teeth, gums, dentures, mouth guards, all get rapidly coated with salivary components. A thick mucus coating forms on the soft tissues. The mucus concentrates the many hundreds of different proteins, glycoproteins, glycolipids and lipids present in saliva so they form a protective film. The salivary films formed on hard surfaces, such as natural teeth, or dentures, are very thin (less than 1 micron) and are known as acquired pellicle (Table 4.2). Acquired pellicle on enamel forms around 30 seconds after eruption of the tooth or professional cleaning. The composition of the acquired pellicle differs according to the surface on which it is formed. Enamel is ionic in composition and so binds to charged molecules. Acrylic materials used in denture production can be hydrophobic and lack significant electrostatic charge, so bind to molecules that are uncharged.

Components that are released from bacterial and host cells can also be incorporated into pellicle. Transglutaminase from epithelial cells can be found in pellicle. GTF and FTF (Chapter 16) from bacteria can become incorporated into pellicle and remain enzymatically active, thus increasing the levels of the associated glucan and fructan polymers. In that regard, amylase in pellicle remains active and may continue to hydrolyze starch to glucose that can be used by oral streptococci for fermentation with resultant acid production.

The acquired pellicle provides receptors for bacterial adhesion. For example, the agglutinins that aggregate bacteria in suspension also promote adhesion when deposited in the enamel pellicle. Interestingly, some salivary molecules, such as the PRPs, will only bind bacteria when deposited on surfaces, as a conformational change in the protein occurs which exposes previously hidden binding sites (cryptitopes). Proteolytic activity by some organisms such as *P. gingivalis* can also expose cryptitopes. Fortunately, as bacteria and their hosts have co-evolved, the organisms that bind to immobilized salivary components on surfaces tend to be oral commensals. Unrestricted plaque build up, however, will irritate the gums and cause inflammation (gingivitis).

Saliva as a nutrient

Saliva can provide growth nutrients for bacteria. Various bacteria produce proteases that degrade salivary proteins into peptides and amino acids, which can be used by the bacteria when exogenous nutrients are limiting. Bacteria can also produce glycan hydrolases that cleave sugar residues from the salivary glycoproteins, so that the sugars can be used for bacterial growth. Again, through co-evolution the bacteria that readily colonize and grow in saliva are mostly harmless and may help exclude pathogens.

5 Salivary mucins and agglutinins

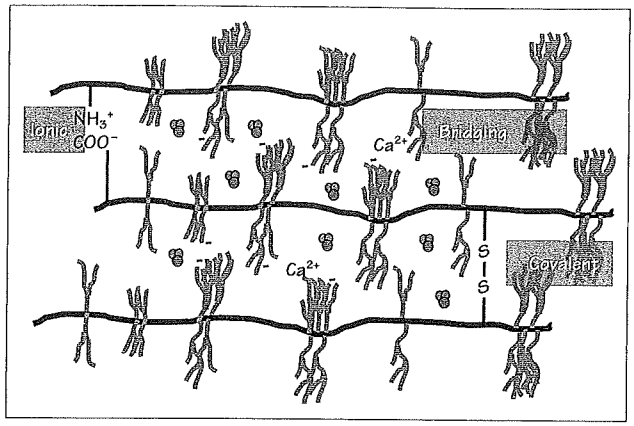

Figure 5.1 Mucins are the basis of saliva function. Alignment of mucin chains by ionic or covalent bonds, or by calcium ion bridging, traps water to form a hydrated gel.

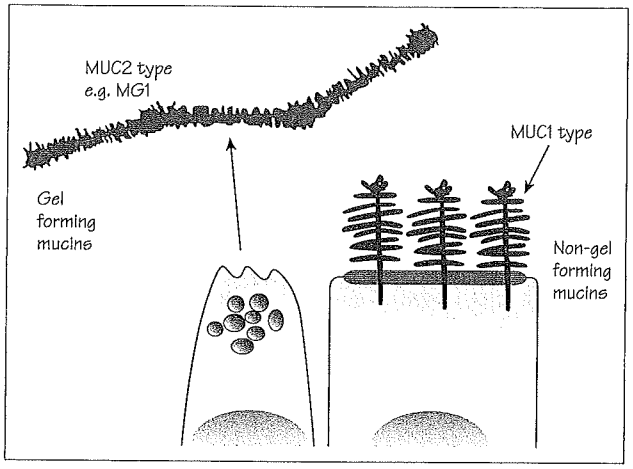

Figure 5.2 Gel forming and membrane bound mucins in the oral cavity.

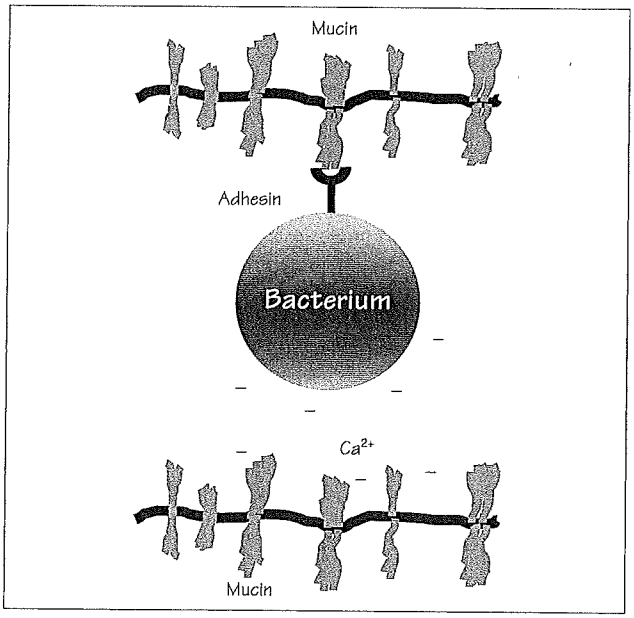

Figure 5.3 Depiction of mechanisms of bacterial agglutination by salivary mucins, involving direct interaction of bacterial surface proteins with mucin carbohydrate chains, and Ca^{2+} bridging of negative charges.

Table 5.1 Characteristics of mucins.

Large extracellular molecules with many sugar chains
Membrane bound or gel-forming
Mucin domains rich in serine, threonine, proline
Oligosaccharides (glycans) linked to hydroxy amino acids via N-acetyl-galactosamine
Site-specific, e.g. salivary mucins different from intestinal mucins

Mucins are present at all surfaces within the human body that are exposed to the environment. The outer layers of mucosal surfaces are comprised of epithelial cells. These can be highly keratinized (such as in the nose or insides of the cheeks), less keratinized (gums), or ciliated (respiratory tract). Salivary mucins are extremely large proteins carrying chains of sugar residues linked together. The permutations of sugar linkage, in linear or branched structures, are almost endless, hence the study of mucin biology is very complex. In saliva there are two main types of mucin, designated mucin glycoprotein 1 (MG1) and the smaller mucin glycoprotein 2 (MG2). MG1 is encoded by the *MUC5B* gene and MG2 is encoded by the *MUC7* gene. Mucins are produced by all salivary glands except (or in very low amounts) by the parotid gland.

Composition of mucins

Mucins are composed of an amino acid chain backbone (polypeptide) with chains of sugar residues attached to the amino acids serine, threonine, or asparagine (Table 5.1). The sugar chains (oligosaccharides) may be spread all over the molecule or localized to specific regions. The most frequently found saccharides are sialic acid (N-acetylneuraminic acid, NeuNAc), glucose (Glc), galactose (Gal), fucose (Fuc), N-acetyl-glucosamine (GlcNAc), N-acetyl-galactosamine (GalNAc), and mannose (Man). The oligosaccharide chains and the amino acids work in conjunction to determine the biochemical properties of mucins.

Properties of mucins

Mucins form complexes that are key to their functional properties. Charged carboxyl or amino groups present on amino acids can form ionic cross links with oppositely charged groups on adjacent polypeptide chains. Cysteine residues present in one chain can form disulfide bonds with cysteine residues in another chain. Both of these inter-chain interactions tend to align mucin chains with each other (Figure 5.1). Sialic acid residues, which are highly negatively charged, can then interact with sialic acid residues on aligned chains through bridging with Ca^{2+} ions. The sialic acid residues are very important to mucin function, as they are to other systems in the human body. The negatively charged residues act to keep the mucin chains apart, allowing water molecules to become trapped between them. Mucin function depends upon this trapping of water molecules, thus determining the degree of hydration of the mucin gel. Mucin has high viscosity when it is hydrated and gel-like, and MG1 is the main gel-forming mucin in saliva (Figure 5.2). The viscosity and elastic properties of saliva are attributed to the gel-forming mucins. These have multiple cysteine-rich domains that form disulfide bonds and thus generate multimeric complexes. The non gel-forming mucins provide a relatively close coating of epithelial cells, protecting the cell membrane from physical or biological damage. Some of them are membrane tethered (Figure 5.2). They lack the cysteine rich domains of the gel forming mucins.

Bacterial agglutination

Since bacteria usually have a net negative surface charge, they tend not to bind directly to negatively charged mucins. Instead, they may interact through bridging reactions involving divalent cations such as Ca^{2+} ions (see Figure 5.3). However, many bacteria express cell surface proteins (lectins) that specifically recognize oligosaccharides present on the salivary mucins. In this manner salivary mucins are bound by multiple bacteria and agglutination results. MG2 is thought to be more important than MG1 for agglutinating bacteria in saliva. Agglutination of some oral streptococci is lost after removal of the terminal sialic acid of the oligosaccharide side chains, indicating that the bacterial lectin has sialic acid specificity. Another high molecular weight agglutinin is the parotid glycoprotein gp340 (also known as salivary agglutinin, or DMBT1), a member of the scavenger receptor cysteine-rich protein family. gp340 agglutinates *S. mutans* cells through Ca^{2+}-dependent interaction with an alanine rich repetitive domain of the AgI/II family P1 protein on the streptococcal surface. gp340 also agglutinates group A streptococci and the N-terminal cysteine rich domain of gp340 interacts with HIV gp120 sequences and inhibits viral infection. Other salivary compounds that can aggregate bacteria include lysozyme, β_2-microglobin and S-IgA (Chapter 6). As mentioned in Chapter 4, agglutinins that are present in the acquired pellicle on enamel surfaces can act as receptors for bacterial adhesion and promote colonization.

6 Secretory immunoglobulin A

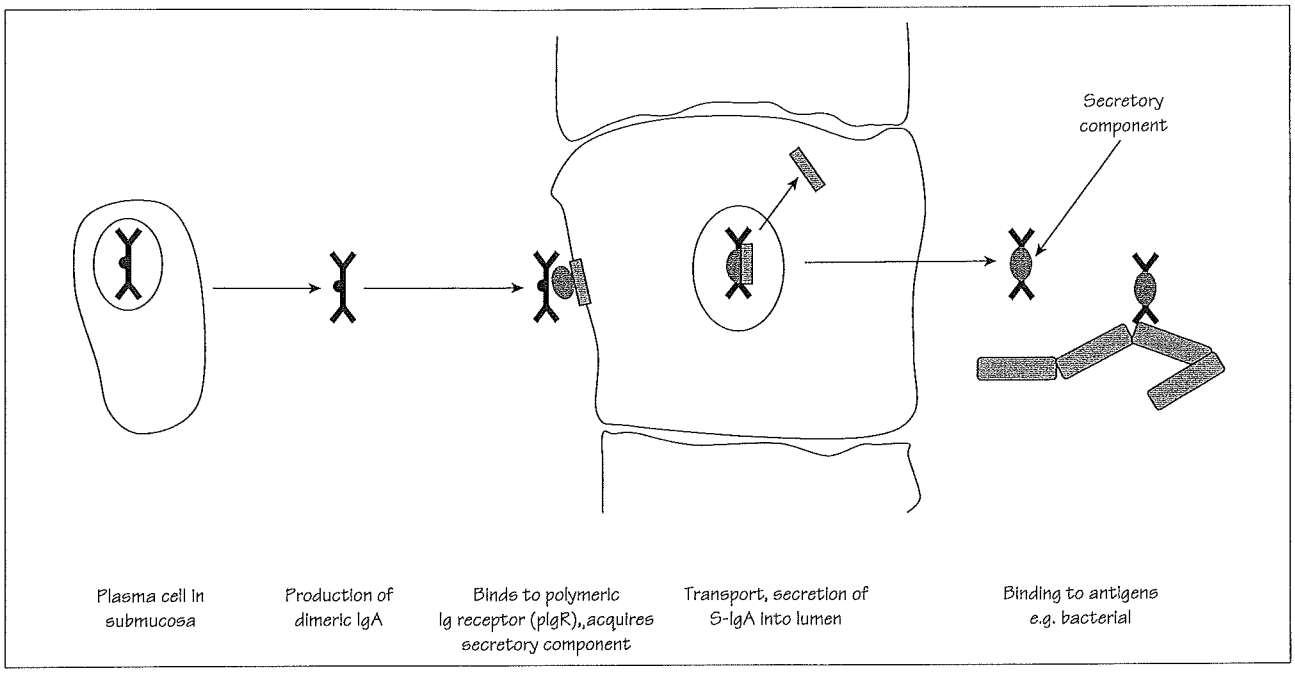

Figure 6.1 Steps in production and secretion of IgA at the mucosal surface.

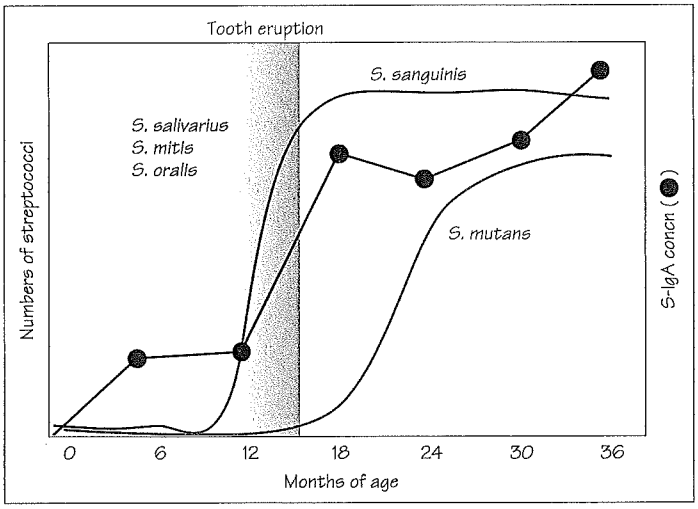

Figure 6.2 Graph depicting the time of acquisition of different streptococci in the mouth after birth, and the development of the secretory immune system after one year, approximately coincident with tooth eruption.

Table 6.1 Acquisition of oral immune functions.

Age (months)	Antibody (source and type)
0–6	Maternal IgG (serum)
	Maternal IgA (milk)
12–18	Infant IgG (teething)
18–36	Infant IgA (saliva)

The major immunoglobulin in the salivary secretions is immunoglobulin A (IgA). This molecule is secreted as a complex with a linking chain by cells that are found close to the parotid gland. The secreted form of IgA is called secretory IgA (or S-IgA). It is found at all mucosal sites, such as the gastrointestinal tract, respiratory tract and urogenital tract, and it is also present in tears and breast milk (in addition to saliva). There are two isoforms, or subclasses, of IgA designated IgA1 and IgA2 and saliva contains approximately equal proportions of each. IgA is also found in serum (where IgA1 is in higher concentration) and is made by bone marrow B cells. In the blood, IgA interacts with a receptor on immune effector cells designated CD89. This initiates inflammatory reactions and activates phagocytosis by macrophages. IgA1 recognizes protein antigens whereas IgA2 is directed against polysaccharides, LPS and LTA. IgA is found in secretions as S-IgA, which consists of two (dimeric) or four (tetrameric) IgA monomers held together by a joining chain (J chain) polypeptide, and by a secretory component (SC) polypeptide.

Production of S-IgA

Polymeric IgA (mainly the secretory dimer) is produced by plasma cells in the lamina propria which is found underlying mucosal surfaces. Polymeric IgA binds to the polymeric immunoglobulin (Ig) receptor protein (pIgR) present on the basolateral surface of epithelial cells and is taken up into the cells by endocytosis. The receptor-IgA complex traverses the cellular compartments and is secreted at the luminal (outside) surface of the epithelial cells still attached to the receptor. The pIgR receptor is then cleaved by protease and the dimeric IgA molecule, linked by the J chain, and containing a portion (SC) of pIgR, are free to diffuse into the secretions (Figure 6.1).

Functions of S-IgA

The primary function of S-IgA is immune exclusion. S-IgA is glycosylated and this anchors S-IgA to the mucus lining of the epithelial surface and thus inhibits attachment and tissue penetration by viruses, bacteria, and their released antigens such as LPS, toxins and environmental antigens. IgA antibodies do not activate complement, thus minimizing disruption of the epithelial barrier layer. Free in saliva, polymeric IgA effectively aggregates bacteria. Glycans on S-IgA are also able to non-specifically trap bacteria. S-IgA interacts with mucins and so-called scavenger proteins such as gp340 present in saliva to generate heterotypic complexes that trap bacteria and stimulate macrophage migration.

Inactivation of salivary defenses

The oral microbiota has evolved to grow and survive in the human mouth despite the salivary defenses, and these organisms have devised means to resist IgA and salivary defenses. For example, some *Streptococcus*, *Haemophilus* and *Neisseria* species produce proteases that specifically cleave S-IgA1, disrupting functions such as complexing and clumping. S-IgA1 fragments may also promote bacterial adherence and accumulation. IgA2 has a deletion in the hinge region that renders it resistant to these proteases. Other bacteria produce glycan hydrolysases that cleave sugar chains from mucins. This causes changes in mucin properties making them much less efficient, both in binding bacteria and in lubrication.

Development of S-IgA

At birth, a child has a poorly functioning immune system. Serum antibodies are mainly derived from the mother, and the child produces no S-IgA antibodies. It takes over a year to generate a significant salivary IgA titer (see Figure 6.2). Colostrum and breast milk are rich in S-IgA antibodies so it is thought that these provide protection against oral, oropharyngeal and GI tract infections in children. The main source of immune protection for a child up to 12 months is thus from the mother (Table 6.1).

The window of infectivity

After about six months of age, the first teeth begin to erupt. This is associated with a change in the oral microbiota to incorporate bacteria that adhere well and colonize the tooth surfaces. While *Streptococcus salivarius* and *Streptococcus oralis* are already colonizing the oral mucosal surfaces, when teeth start to appear bacteria such as *Streptococcus sanguinis* and *Streptococcus mutans* are provided with a hard surface to colonize (Figure 6.2). The main agent of dental caries, *S. mutans*, tends to appear later in the mouth than *S. sanguinis*, and follows an increase in IgA titer. This so-called window of infectivity (Figure 6.2) may be important for the oral health of the child. It has been found that the earlier children acquire *S. mutans* then the greater their risk of childhood caries.

Selective IgA deficiency

This is a genetic immunodeficiency in which the serum level of IgA is undetectable, although levels of the other main immunoglobulins IgG and IgM are normal. Although the amount of IgA produced is similar to the total of all other Igs combined, IgA deficiency is largely asymptomatic. This is most likely because IgM can compensate for IgA, and those individuals that are also defective in IgM have a higher caries incidence. IgA deficiency also causes a slight increased risk of oral, respiratory and gastrointestinal infections, and of developing autoimmune diseases in middle age.

7 Anti-microbial properties of saliva

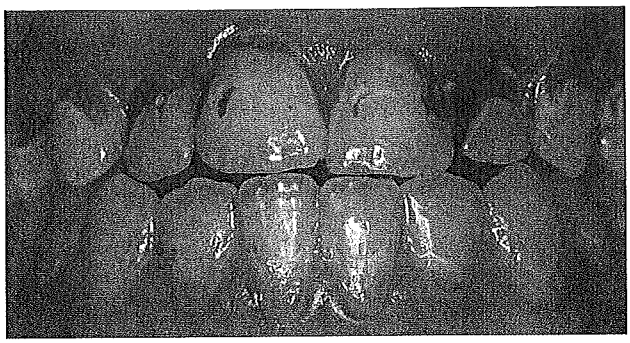

Figure 7.1 Rampant caries such as occurs in patients with xerostomia (reduced salivary flow).

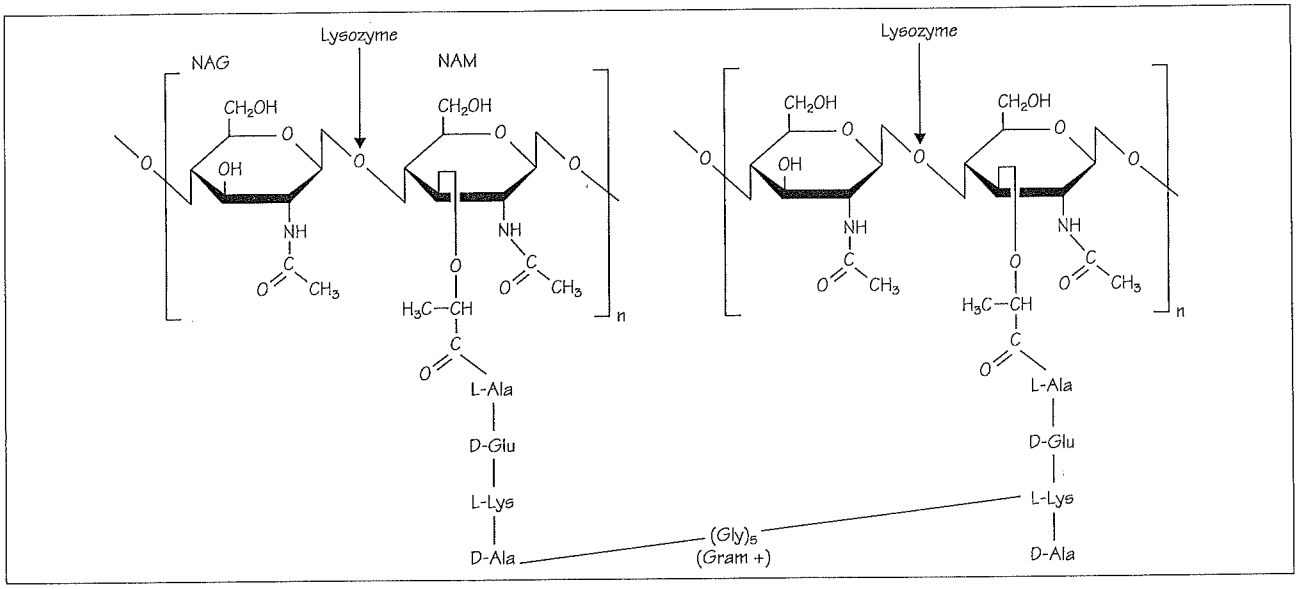

Figure 7.2 Action of lysozyme on Gram-positive bacterial peptidoglycan which consists of repeating units two joined amino sugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), with a pentapeptide coming off of the NAM. Lysozyme cleaves the β-(1,4)-glycosidic bonds.

Table 7.1 Properties of salivary histatins that inhibit oral bacteria and fungi.

Family of histidine rich peptides in saliva
Histatin 5 potent inhibitor of *Candida albicans*
Does *not* work like the HBDs
Binds to receptor, peptide enters the cells, cell cycle arrested, efflux of ATP, respiratory apparatus inhibited
Anaerobic cells are less susceptible

Impaired salivary flow is deleterious to oral health. For example, an inadequate salivary flow rate increases the incidence of dental caries (see Figure 7.1), for at least three reasons. First, there is greater bacterial retention in the mouth and more dental plaque forms; second, the acids produced by bacteria such as mutans group streptococci are inefficiently neutralized; and third, the enamel surface does not efficiently re-mineralize.

Agglutination, physical flushing of bacterial and salivary IgA are discussed in the preceding chapters; however, saliva also contains a number of anti-microbial compounds that can restrict plaque accumulation and kill bacteria and other microorganisms.

Anti-microbial components in saliva

(1) Lysozyme Lysozyme is a basic protein found in most secretions, including saliva, where it is present in high concentrations. Salivary lysozyme originates from both the salivary gland secretions and from gingival crevicular fluid (GCF). Lysozyme digests the cell walls of Gram-positive bacteria by breaking the β(1-4) bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan (Figure 7.2). Lysozyme can also activate autolysins in bacterial cell walls. Not surprisingly, many successful oral colonizers are relatively resistant to killing by lysozyme. Lysozyme can also bind and aggregate bacteria and facilitate clearance by swallowing or expectoration. In addition, lysozyme contains small amphipathic sequences in the C-terminal region that are capable of killing bacteria.

(2) Salivary peroxidase Peroxidase in saliva is derived from the salivary glands and PMNs, and catalyzes the oxidation of thiocyanate (SCN^-) to hypothiocyanite ($OSCN^-$) by hydrogen peroxide, which is produced by the aerobic metabolism of oral bacteria. At acid pH, $OSCN^-$ becomes protonated (and uncharged) and readily passes through bacterial membranes. Hypothiocyanite oxidizes SH groups in bacterial enzymes and inhibits bacterial metabolism. Reduction of hydrogen peroxide to water by peroxidase also prevents oxidative damage to the host soft tissues.

(3) Lactoferrin Lactoferrin is an iron binding glycoprotein produced from glandular acinar cells, epithelial cells and phagocytic cells. Lactoferrin inhibits bacterial growth by binding and sequestering Fe^{2+} ions, and in the apo (iron free) state can be toxic to bacteria and interfere with bacterial adhesion. A 25-residue N-terminal proteolytically derived peptide fragment termed lactoferricin also kills bacteria through depolarization of cytoplasmic membranes.

(4) Histatins Histatins are cationic histidine rich proteins that kill *Candida albicans* and some bacteria (Table 7.1). At least 12 histatins are present in saliva, resulting from truncations or proteolysis of the genetically distinct histatins 1 and 3. Histatin 5 (the N-terminal 24 amino acids of histatin 3) is a major salivary histatin and is very effective in killing yeast. Histatins bind to a *Candida* membrane receptor, then the peptide is taken up by the cells. This results in arrest of the cell cycle and the cells lose ATP by efflux. Histatins can also regulate hydroxyapatite crystal growth, inhibit bacterial cysteine proteinases and prevent bacterial coaggregation.

(5) Cystatins Cystatins are cysteine rich peptides that inhibit bacterial cysteine proteases. Cystatins also regulate inflammation by inhibiting host proteases and up-regulating cytokines. Von Ebner gland protein is another cysteine protease inhibitor.

(6) Chromogranin A Chromogranin A is produced by the sub-mandibular and sub-lingual glands, and is processed to release an N-terminal peptide, vasostatin-1, which is antibacterial and antifungal.

(7) Antiviral factors These include secretory leukocyte protease inhibitor (SLPI), gp340, Thrombospondin 1 (TSP1) and proline rich proteins (PRPs). SLPI is a serine protease inhibitor that can inhibit the infectivity of the HIV virus, and is also bactericidal and fungicidal. TSP1 is a high molecular weight extracellular matrix glycoprotein that is secreted by the sub-mandibular and sub-lingual glands. TSP1 can bind to regions of the HIV gp120 protein and prevent binding to CD4. Basic PRPs may also inhibit HIV infectivity by binding to gp120.

Salivary glands also produce β-defensins (Chapter 8), and whole saliva will contain serous proteins derived from the gingival crevicular fluid (GCF, Chapter 9) and compounds released by epithelial cells. Hence, whole saliva contains low levels of IgG, IgM and complement components from GCF, and calprotectin (a calcium binding anti-microbial protein) from epithelial cells.

8 Innate defenses

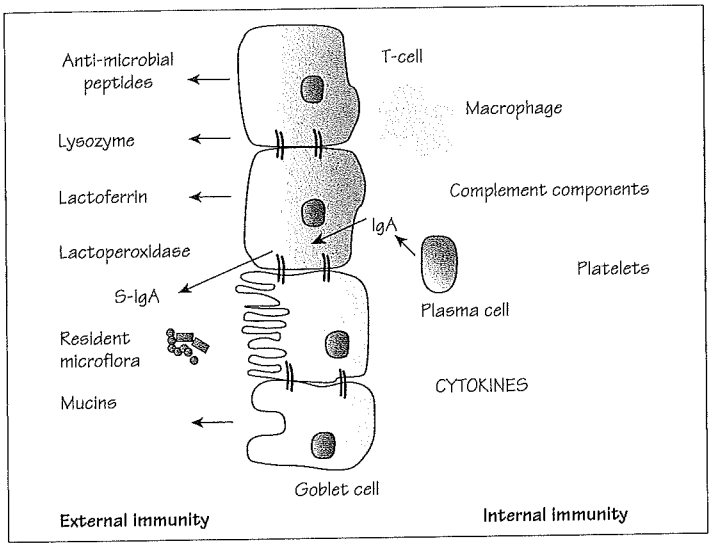

Figure 8.1 Major components of the innate defense system. Redrawn with permission from Henderson B, Wilson M, McNab R and Lax A (eds) *Cellular Microbiology* (1999), John Wiley & Sons, Chichester.

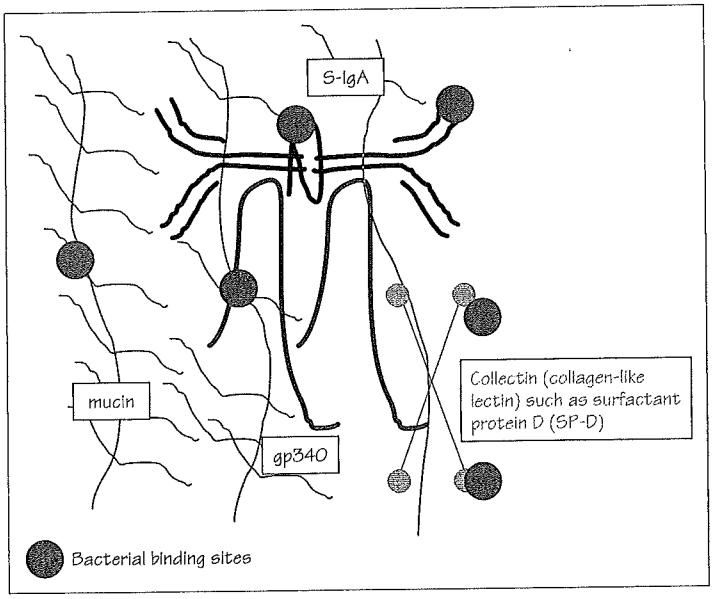

Figure 8.2 Multiple salivary molecules possess binding sites for bacteria that can facilitate entrapment of organisms and clearance.

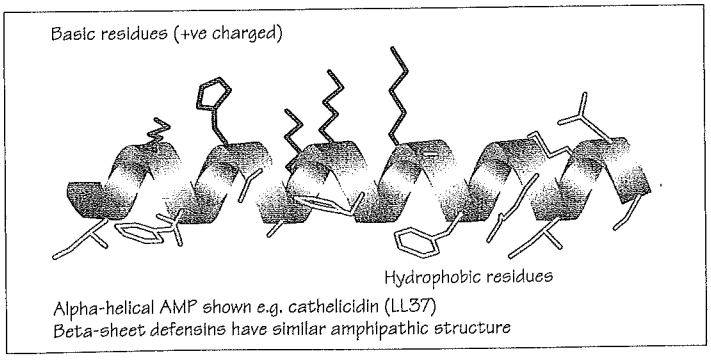

Figure 8.3 Amphipathic structure of anti-microbial peptides (AMPs).

Innate immunity is non-specific and does not require prior exposure to infectious agents, so it is "always on". In the oral cavity, the first layer of innate immunity comprises physical barriers such as the epithelium; phagocytic cells such as polymorphonuclear neutrophils (PMNs) that are recruited from the circulation into the gingival tissue and the subgingival region; and inhibitory actions of commensal microorganisms (Figure 8.1). In addition, the oral fluids possess anti-microbial factors as discussed in Chapter 7, many of which can bind and entrap microorganisms (Figure 8.2). The second line of innate immunity is inflammation that acts to localize infectious agents at sites of mucosal penetration. Inflammation also provides a link between innate immunity and acquired immunity.

Epithelial and phagocytic cells utilize pattern recognition receptors (PRRs) such as Toll-like receptors (TLRS) to bind and characterize microbe associated molecular patterns (MAMPs), repeating patterns of molecules not found on host cells. Examples of MAMPs include LPS of Gram-negative bacteria, LTA of Gram-positive bacteria, peptidoglycan, flagellin, pilin, unmethylated CpG islands of bacterial DNA, fungal mannans and viral double-stranded DNA. There are at least 11 TLRs that recognize distinct MAMPs. TLR binding to a MAMP signals epithelial cells and leukocytes to secrete proinflammatory cytokines and chemokines, and anti-microbial peptides through activation of the transcription factor NF-κB.

Within the cytoplasm of leukocytes and epithelial cells, the NOD (nucleotide binding oligomerization domain)-like receptor family recognize MAMPs derived from intracellular bacteria and also activate proinflammatory cytokines and anti-microbial peptides through NF-κB. NOD1 recognizes a specific peptidoglycan fragment containing diaminopimelic acid, more frequently found in Gram-negative bacteria. NOD2 recognizes a muramyl dipeptide (MDP) fragment derived from peptidoglycan which is common to both Gram-positive and Gram-negative bacteria. Other NOD-like receptors (NLRs) can detect dsRNA and dsDNA. Upon detection of specific MAMPs, some NLRs undergo conformational changes and assemble a molecular platform called the inflammasome. The inflammasome processes and activates pro-caspase 1 and the resultant active enzyme mediates proteolytic maturation of the inflammatory cytokines IL-1β and IL-18.

As both commensal and pathogenic organisms express MAMPs that will be recognized by TLRs and NLRs, activation of an inflammatory response may also require the participation of so-called "danger signals" released by dying or injured cells, which indicate the presence of pathogenic activity.

Epithelial cells and leukocytes produce nitric oxide which is a free radical that is toxic to bacteria by causing DNA damage and degrading of iron sulfur centers into iron ions and iron-nitrosyl compounds.

Anti-microbial peptides (AMPs)

These are natural antibiotics produced by insects, shellfish, frogs, toads, mammals, etc. There are many different classes, but most peptides contain between 20 and 40 amino acid residues. The sequences of these peptides are different but their properties are similar. They adopt amphiphilic (or amphipathic) architecture, one side being positively charged and the other side being hydrophobic (Figure 8.3). The positively charged side interacts directly with biological membranes, and this then helps the molecule enter the membrane and form pores which leads to membrane disruption. AMP activity depends principally upon the lipid composition of the membrane, and mammalian cells that express specific AMPs are rendered resistant to their action by virtue of a lower phospholipid composition. AMPs may be useful antibiotics in the future, particularly as AMPs are even active against methicillin resistant *Staphylococcus aureus* (MRSA) and work in seconds on susceptible bacteria. While AMPs have a broad spectrum of activity, some bacteria are resistant. For example, *Salmonella* is resistant to macrophage AMPs. Also, *S. aureus* cells produce modified membrane lipids or wall teichoic acids that resist binding of the positively charged AMPs. *P. gingivalis* becomes more resistant to AMPs following exposure to environmental stresses, including sublethal doses of β-defensins. Less pathogenic members of the oral microbiota such as some *F. nucleatum* strains are inherently resistant to the action of AMPs in the oral cavity. Biologically active AMPs cannot be chemically synthesized at present, so there is much interest in trying to find the minimal active portions and synthesize these commercially.

Human AMPs

Defensins, produced by humans and primates, are 15 to 20 amino acid residue peptides with six to eight conserved cysteines. The alpha-defensins in humans (six known to date) are produced by leukocytes, PMNs and intestinal Paneth cells. The beta-defensins are produced by epithelial cells and leukocytes. There are four major types (HBD1-4) and many other variants. HBD1 is expressed constitutively in oral epithelial cells, while HBD2 is induced by bacterial infection. Cathelicidin LL37 is produced by leukocytes, skin and respiratory epithelia. These proteins are all important in protecting against microbial infections, particularly when the acquired immune system is in the development stages. However, AMPs are not just anti-microbial, they cause histamine release from mast cells, interact with complement, are chemotactic for monocytes, enhance wound closure, stimulate antigen-presenting dendritic cells and stimulate cytokine production. Moreover, HBD3 induces expression of the costimulatory molecules CD80, CD86 and CD4 on monocytes and myeloid dendritic cells in a TLR-dependent manner.

9 Microbes in the oral cavity

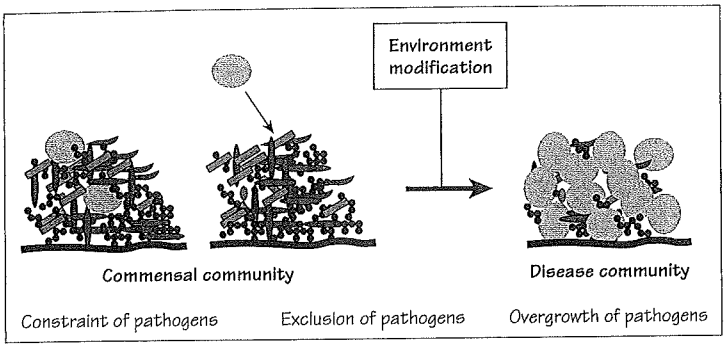

Figure 9.1 Depiction of commensal and pathogenic microbial communities. In commensal communities, pathogens (blue) can be present but constrained or excluded by the commensals. The right panel reflects the ecological plaque hypothesis whereby conditions change such that pathogens can become dominant.

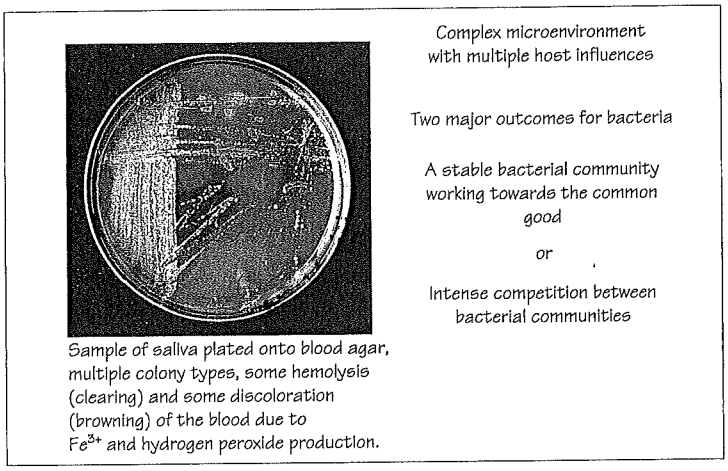

Figure 9.2 Opposing constraints in the colonization of the oral cavity.

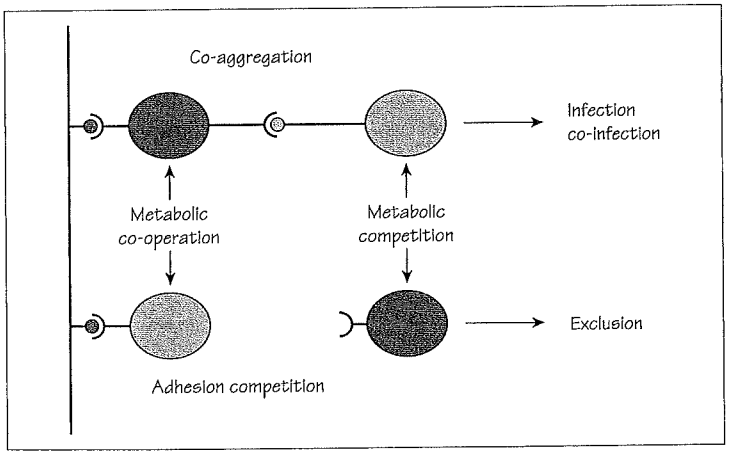

Figure 9.3 Synergistic and antagonistic interactions among oral bacterial colonizers. Commensal (blue) and potentially pathogenic (purple) bacteria might coexist in oral microbial communities. Provided that the pathogen can use available receptors for adhesion, either on the host surface or on the surfaces of antecedent bound bacteria, and provided that metabolic deficiencies or dependencies are fulfilled, the pathogen grows and survives in the microbial community. Depending upon host susceptibility this could potentially result in a polymicrobial infection typical of most oral diseases. In the lower half of the diagram, the combined effect of receptor unavailability and nutritional inadequacy, either because the necessary partner organisms are not present or because the environment is unfavorable, leads to exclusion of the potential pathogen from the community. Reproduced with permission from Jenkinson HF, Lamont RJ Oral microbial communities in sickness and in health. *Trends in Microbiology* (2005): 13, 589–595.

Table 9.1 Colonization or invasive disease?

Continuous interplay between microbes and host
Bacteria respond rapidly to host environmental changes
Microbial communities are self-limiting
Bacteria compete for adhesion receptors
Virulence factors prolong intracellular survival and promote tissue damage

Many of the bacteria present in the oral cavity are anaerobic (growing only in the absence of oxygen under reduced conditions) or facultatively anaerobic (able to grow under more oxidized conditions, but also anaerobically). The anaerobic organisms are able to grow and survive because of the presence of the facultative anaerobes and aerobic (oxygen-requiring) bacteria. These facultative and aerobic bacteria remove oxygen and oxidized compounds from the environment and thus generate conditions that are oxygen depleted and reduced. The bacteria that initially colonize the salivary pellicle on the tooth surface, tongue, palate and pharynx are mainly facultative anaerobes. Most of the bacteria within the genus *Streptococcus* fall into this category, as do many of the other microbial species associated with coronal plaque, including plaque formed within fissures of the tooth crown. In addition, the fungus *Candida albicans* is facultatively anaerobic and can tolerate low pH. Candida is able to colonize the buccal epithelium and the tongue, but can also be found in carious lesions where the pH is < 5.0.

Primary colonizers

Bacteria that first colonize salivary pellicle present on the tooth surface are designated primary colonizers, and are mainly streptococci. The major species include *Streptococcus oralis*, *mitis*, *sanguinis*, *parasanguinis* and *gordonii*. In addition, *Actinomyces*, *Veillonella*, *Gemella*, *Abiotrophia*, and *Granulicatella* are usually detected. These bacteria, except *Veillonella*, stain Gram-positive and are facultative anaerobes. Some of them are difficult to culture in the laboratory, e.g. *Gemella*, *Abiotrophia* and *Granulicatella*. Up to 80% of the organisms present within initial plaque formed on cleaned tooth surfaces are streptococci. Bacterial colonization of mucosal surfaces is less abundant than the tooth surfaces as epithelial cells are continually dying and sloughing off. Communities of bacteria can also survive and develop within epithelial cells without causing host cell death.

Beneficial effects of bacterial colonizers

Despite extensive microbial colonization of many mucosal surfaces, health is the normal state of the host. In the GI tract resident bacteria can be beneficial through a number of mechanisms: (1) provision of simplified carbohydrates, amino acids and vitamins; (2) prevention of overgrowth or colonization of pathogens by competition for niches or nutrients, or by inducing immune cross-reactivity; (3) stimulation of vascularisation and development of intestinal villi; (4) enhancement of the immune system development. These mechanisms are less well studied in the oral cavity; however, resident bacteria can stimulate host innate immune competence. Moreover, oral bacteria induce global changes in the host epithelial cell transcriptome (the pattern of expressed genes, Chapter 11), and these mRNA responses are distinctive for individual organisms.

Ecological plaque hypothesis

Oral disease occurs when the host microbe balance is disrupted at the cellular or molecular level (Table 9.1). Many oral diseases are polymicrobial in origin and both microbial and host factors contribute to the initiation and progression of disease (Figure 9.1). The ecological plaque hypothesis holds that shifts in the relative proportions of organisms can be the forerunner to the development of disease. These populational shifts can be caused by a change in environmental conditions, perhaps caused by dietary intake, or by local or systemic host immune status. As the bacterial community matures, the constituents can cooperate synergistically or be antagonistic to one another (Figure 9.2). The newly dominant bacteria then modify the micro-environment, thus sustaining their presence at the expense of other microorganisms (Figure 9.3).

Ecology and disease

(1) Caries The development of acidogenic plaque containing higher levels of mutans group streptococci able to tolerate lower pH, is promoted by the more frequent dietary intake of fermentable sugars. The rapid increase in the proportions of acid-tolerating bacteria then leads to exclusion of other more acid-sensitive microorganisms.

(2) Gingivitis Inflammation and reddening of the gums (gingivitis) is caused by build up of supragingival plaque at the gingival margins (the line between tooth and gum) and can be exacerbated by calculus (tartar) which is a tissue irritant. Pregnancy, diabetes mellitus and the onset of puberty increase the risk of gingivitis, all associated with hormonal changes that affect host susceptibility. Moreover, many of the oral Gram-negative anaerobes can also metabolize human hormones. The risk of gingivitis is also increased with misaligned teeth, rough edges of fillings and ill fitting or unclean dentures. The sudden onset of gingivitis in a normal, healthy person may be related to viral infection. The inflammation associated with gingivitis is reversible but may, over time, predispose to periodontitis.

(3) Periodontitis This is a long-term chronic condition. Left undisturbed, supragingival plaque will extend down below the gingival margin and become subgingival plaque. The subgingival region between the tooth root and the sulcular epithelium is known as the gingival crevice or sulcus (that deepens to the periodontal pocket in periodontitis). This environment is less oxygenated, which will favor the growth of the anaerobes that can cause periodontal tissue destruction and bone loss. Saliva does not penetrate this region well, and the fluid phase comprises gingival crevicular fluid (GCF). GCF is a serum exudate that contains most serum components, which also favors the growth of oral Gram-negative anaerobes. GCF flow increases with inflammation and will also contain components released from phagocytic cells and epithelial cells.

10 Molecular microbial taxonomy

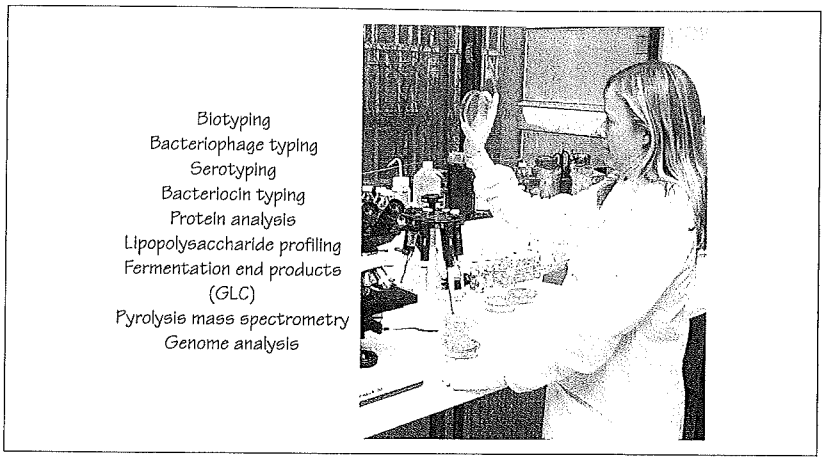

Figure 10.1 Techniques used in the identification and subtyping of pure cultures of bacteria.

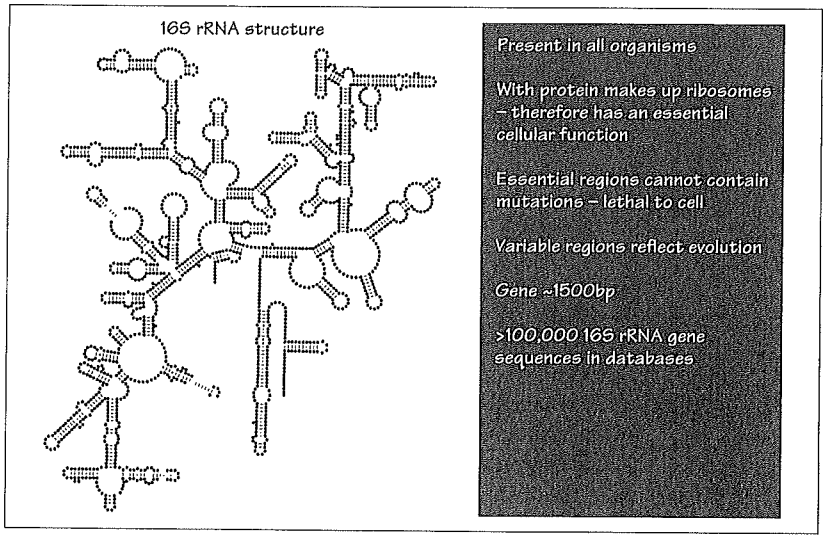

Figure 10.2 Features of 16S rRNA.

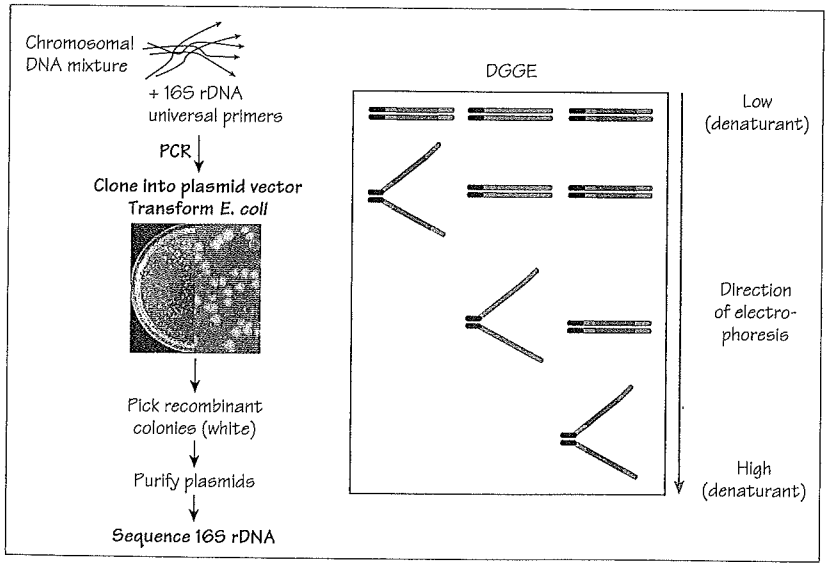

Figure 10.3 Generalized method for producing 16S rDNA sequence information by PCR-cloning (left panel). Right panel, diagrammatic representation of DGGE (Denaturing Gradient Gel Electrophoresis).

Oral microbiology has traditionally relied on the ability to cultivate bacteria from saliva and from dental plaque in order to define the species present. Originally, only a few species of bacteria were considered of importance in oral disease. However, as culture methods became more sophisticated, particularly with the development of anaerobic cabinets for cultivation of oxygen-sensitive organisms, it became evident that the complexity of microbial species present had been under-estimated. Then, with the development over the last ten years of non-culture molecular methods to identify bacteria, it has become apparent that laboratory cultivation of bacteria alone is inadequate for assessing the complexities of microbial populations. Nowadays, molecular techniques provide sensitive and accurate means to determine snapshots of the microorganisms present, dead or alive, in microbial communities at different oral sites. In addition, there are a number of techniques for the identification of species and subtyping of species into biologically relevant groups or clones (Figure 10.1). Metagenomics describes the study of microbial populations through analyses of the total genomes present. It is important to note, however, that these techniques do not necessarily permit a quantitative measure of the relative numbers of microbes within a community. Other analytical methods such as proteomics, which potentially identifies all proteins present within a sample, may be applied to provide a measure of the functional diversity within a population. A newer technique of metabolomics seeks to identify all the metabolites, by mass spectrometry or NMR spectroscopy, associated with a microbial population. These techniques all aim to provide genetic or biochemical fingerprints of microbial populations so as to relate these to the types of organisms present and, perhaps more importantly, attempt to define patterns associated with diseased sites as opposed to healthy sites.

Molecular taxonomy

As a result of utilizing molecular methods to determine the genomic components of oral microbial communities it is estimated that 40–50% of the oral microbial population is not yet cultivable. This is because the culture media available are not suitable for growing these organisms. In addition, it is likely many grow together as dependent partners or groups and so may be impossible to isolate in pure culture. Molecular methods of identification do not necessarily pick up such dependencies but provide vital information about the microbes present. Molecular taxonomy is generally based around differences in sequences of 16S ribosomal RNA genes (approximately 1500 base pairs) (Figure 10.2). These are present in all microorganisms and together with ribosomal proteins make up the smaller ribosomal sub-unit, essential for protein synthesis. The essential regions within 16S rRNA cannot contain mutations because these are lethal to the cell. However, the variable regions within the genes reflect evolution. There are now > 100,000 16S rRNA gene sequences in databases. Polymerase chain reaction (PCR) is used to amplify the 16S rRNA gene with universal primers that bind to the conserved (essential) regions. The sequence of the gene is then compared with the sequences present in the databases. A > 98% match with a sequence suggests the bacterium belongs to that species. A match of 97% or less indicates a new species or phylotype. To determine the species present in a population, total DNA is extracted, subjected to PCR, and then the rDNA products are cloned and sequenced (see Figure 10.3 left panel).

Denaturing gradient gel electrophoresis

This technique is becoming more popular as a means to identify components within complex microbial population. In this technique PCR amplification across a variable sequence within the rDNA is performed using a primer with a GC-clamp (see Figure 10.2 right panel). The amplicons (approx. 190 bp) are then separated by electrophoresis through a gel with a low to high denaturation gradient of urea and temperature. Those PCR fragments (termed amplimers) with lower GC content (overall fewer H bonds linking the DNA strands) will denature at lower temperature and become retarded within the gel, while those with higher GC content will run further. The GC clamp keeps the amplimer together as double stranded DNA. The separate gel bands are excised, cloned and then sequenced for identification.

DNA chips

With the advent of precise molecular printing techniques, whereby multiple species-specific 16S rRNA gene fragments or oligonucleotides can be imprinted onto membranes or glass slides, it is possible to obtain estimations of bacteria within a population. The analytical power of these kinds of techniques depends entirely upon the accuracy of the information on the DNA chip. In simplest form, plaque or periodontal samples are taken, the DNA extracted, and the 16S rRNA genes for all the bacteria present PCR amplified with fluorescently-labeled universal primers. The amplified material is then hybridized to the DNA from target bacteria present on the chip, and the signal measured from the fluorescent label. Semi-quantitative estimates of numbers of bacteria of interest may be obtained, but only for the 16S rRNA genes of those bacteria which are represented on the chip. More analytically complex, but fundamentally the same, techniques such as checkerboard hybridization, can give multiple comparative readouts of species in different samples.