

Bacterial interactions in dental biofilm

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Biofilms are masses of microorganisms that bind to and multiply on a solid surface, typically with a fluid bathing the microbes. The microorganisms that are not attached but are free-floating in an aqueous environment are termed planktonic cells. Traditionally, microbiology research has addressed results from planktonic bacterial cells. However, many recent studies have indicated that biofilms are the preferred form of growth of most microbes and particularly those of a pathogenic nature. Biofilms on animal hosts have significantly increased resistance to various antimicrobials compared to planktonic cells. These microbial communities form microcolonies that interact with each other using very sophisticated communication methods (i.e., quorum-sensing). The development of unique microbiological tools to detect and assess the various biofilms around us is a tremendously important focus of research in many laboratories. In the present review, we discuss the major biofilm mechanisms and the interactions among oral bacteria.

Introduction

Anton van Leeuwenhoek was the first scientist who observed microorganisms with a microscope in 1683.¹ One of the first samples he examined was his own dental plaque or biofilm. Ever since then, microorganisms began to attract researchers' interests. In the beginning, scientists mainly focused on single bacterial species performance in broth culture. This type of bacterial growth is termed planktonic growth. But later, scientists realized that most, if not all, bacteria live in biofilms instead of broth. Therefore, biofilm investigations drew scientists' attention, and this area of research has become popular in recent years. Biofilms are defined as orientated aggregations of microorganisms attached to each other or to a surface and enclosed in extracellular polymeric substance (EPS) produced by themselves.²⁻⁵ Furthermore, many biofilms are bathed by some flowing fluid (i.e., water flowing over moss-covered rocks in a stream or saliva flowing over dental plaque on a tooth surface). The components of mature biofilm are approximately 5–25% bacterial cells and 75–95% glycocalyx matrix.⁶

Biofilms can be found almost everywhere associated with moisture. It is well known that there are at least 10 times

more bacteria inhabiting the human body than the number of human cells. In nature, biofilms are found in industrial bioreactors, on rocks in streams, and in animal host environments such as in or on the otolaryngologic, vaginal and gastrointestinal tracts. Otolaryngologic biofilms are typically constituted by *Pseudomonas aeruginosa*-containing EPS-cellular towers that are separated by open passages that deliver nutrients and dispose of metabolic wastes. The classical mushroom-type biofilm structures formed by *P. aeruginosa* contain one pseudomonad strain in the stalks and a second different strain in the mushroom heads.⁷ The human gastrointestinal tract contains a rich and diverse microbiota along its length and the study of the gastrointestinal tract microbiome is a rapidly growing field. A large number of intestinal bacterial species on the intestinal mucosa surface form dense biofilms. There are a total of about 10¹⁴ intestinal bacterial species, mainly in the colon.⁸ In the gastrointestinal tract, most microorganisms colonize the large intestine. Gut biofilm is composed of both living and dead bacteria in the mucus layer lining mucosal surfaces or on food residues in the lumen of the gut. Bacteroides and bifidobacteria are the predominant bacterial species attaching to particulate matter in stool and were shown to be phenotypically similar to the nonadherent microbiota. Vaginal bacterial groups in healthy women are generally divided into resident bacteria, non-resident bacteria and occasionally resident bacteria. Microorganisms in the healthy vagina maintain balance with the host and the environment. This process is called ecological balance. Lactobacilli are dominant in the vaginal microorganisms of a healthy woman, although other bacteria can be present in lower numbers.⁹ Therefore, a reduction in the numbers of vaginal lactobacilli can lead to vaginal flora imbalance, decrease in vaginal cleanliness and abnormal pH, so that harmful microorganisms are increased and the large population of endogenous bacteria are decreased.

There are over 700 different bacterial species in the oral microflora.²⁻⁵ Those species colonize the teeth, tongue, oral mucosa, hard palate, carious lesions, periodontal pocket, et al. The distribution of microflora in the oral cavity is not random, most species prefer certain sites over others due to the particular local environment those sites provide, such as the anaerobic environment provided by the periodontal pocket.¹⁰ The majority of the microflora benefit our health while the minority are harmful, however, most research focuses only on those pathogenic species giving the public an illusion that bacteria are our "enemies." Actually, most bacteria are "friendly" commensals and even essential components to our health. Roberts and Darveau¹¹ proposed that dental biofilm and their products contribute to healthy periodontium.

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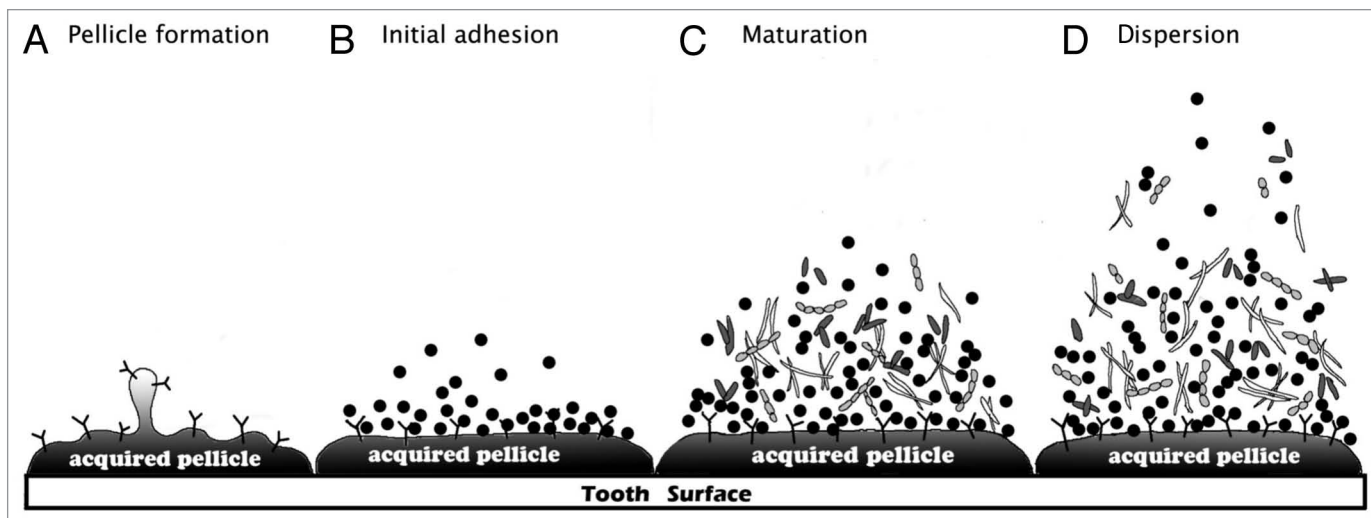


Figure 1. Oral biofilm formation. This diagram represents each step of oral biofilm formation. (A) Pellicle formation. The pellicle is a thin film derived from salivary glycoproteins attached to a clean tooth surface. (B) Initial adhesion. Pioneer bacteria in saliva recognize the binding proteins in acquired pellicle and attach to them. This adhesion is reversible. (C) Maturation. Different bacterial species coaggregate and mature biofilm forms. (D) Dispersion. Bacteria disperse from the biofilm surface and spread to colonize a new site.

Microflora can be found in both caries-free and periodontitis-free people and caries-affected and periodontitis-affected people, and many clinical studies reveal that the portion of certain bacterial species such as *Streptococcus mutans* or *Porphyromonas gingivalis*, respectively, is increased in patients with caries or periodontitis. Therefore, it seems that the competition that results between beneficial bacteria and virulent bacteria leads to either a healthy or sick status of human beings. Competition between members of the dental microflora are very complex and many antagonistic characteristics can be observed from competition for initial attachment on tooth surfaces or for later attachment to pioneer bacteria, competition from bacteriocins or hydrogen peroxide secreted and from facilitating the growth of some species which inhibit other species. To date only some of the details of these mechanisms are known. The present review will provide an overview of the formation of dental biofilm, the major mechanisms of oral bacterial interactions and inter-species bacterial interactions. Due to the large number of oral bacterial species, only the best characterized species are included in this review.

Oral Biofilm Formation

Acquired pellicle formation. Oral biofilm is unique among the various types of biofilms as it typically requires host salivary glycoproteins to attach to. The first step of oral biofilm formation is the attachment of acquired pellicle, which is a thin protein-containing film derived from salivary glycoproteins, to a clean tooth surface (Fig. 1). The mechanism of acquired pellicle formation is based on Gibbs law of free enthalpy in that more energy will be released if glycoproteins are attached to the tooth surface. Many interactions among the various glycoproteins, other salivary components, and the tooth surface are involved. The forces of those interactions can be roughly divided into three types: long-range forces (50–100 nm between the two interacting

components), medium-range forces (10–50 nm) and short-range forces (less than 5 nm). The long range forces include Coulomb interactions, van der Waals forces and dipole-dipole interactions; the medium range forces include hydrophobic interactions; and the short range forces include covalent bonds, electrostatic interactions, hydrogen bonds, ionic interactions and Lewis acid-base interactions.¹² With these forces, proteins are absorbed and rearranged, some conformational changes are undertaken,¹³ and the new pellicle forms are ready for pioneer bacteria adhesion.

Initial adhesion. Bacteria adhesion to the pellicle is the second step of biofilm formation. Some of the planktonic bacteria may recognize binding proteins in acquired pellicle, i.e., α -amylase and proline-rich glycoproteins/proteins and bind to the pellicle (Fig. 1). However, in this stage the attachment is reversible and those initially attached bacteria can detach from the pellicle easily. The early attachments are primarily based on electrostatic attractions or physical attachments,¹⁴ but later, chemical forces become predominant. As soon as the pioneer bacteria attach to the pellicle, they begin to excrete EPS, which helps the bacteria stay bound together and attach to the pellicle. The main surface attachment structures of bacteria are fimbriae and fibrils. Fimbriae are composed of fimbrellins; while fibrils are like fimbriae, but they are shorter and thinner than fimbriae with more or less density distributed on the cell surface.¹⁵ The forces present in the pioneer bacterial adhesion to the salivary acquired pellicle are hydrogen bonds, hydrophobic interactions, calcium bridges, van der Waals forces, acid-base interactions and electrostatic interactions.¹² *Actinomyces* spp, *Streptococcus* spp, *Haemophilus* spp, *Capnocytophaga* spp, *Veillonella* spp, and *Neisseria* are the main pioneer bacterial genera attaching to the tooth surface.^{16–18} Those early colonizations help those species occupy space and gain advantage in later competition with other species.¹⁹

Maturation. Since earlier reviews, such as Kolenbrander et al.,^{4,20} Hannig and Hannig,¹² and Hojo et al.⁵ already provided

very comprehensive and detailed summaries of biofilm maturation, in the present article we briefly discuss mature biofilm formation and coaggregations between bacteria. After the pioneer bacteria attach, those early colonizing bacteria provide specific binding sites either directly or through salivary glycoproteins binding to the pioneer organisms for subsequent bacterial colonization and promote the development of biofilm (Fig. 1). Later colonizing bacteria recognize polysaccharide or protein receptors on the pioneer bacterial cell surface and attach to them. Bacteria coaggregate, forming the typical corn cob forms, bristle brush forms, or other forms in mature oral biofilm. Subsequent attached bacterial species include *Fusobacterium nucleatum*, *Treponema* spp, *Tannerella forsythensis*, *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, et al.^{17,20} The microbial components of mature biofilm are quite different from the initial biofilm. A proportional shift occurs during biofilm development. The relative amount of Streptococci and Neisseria decrease, while the amount of Actinomyces, Corynebacterium, Fusobacterium and Veillonella increase.¹⁶ Bacterial aggregation is a very complex process. Mature biofilms typically contain many porous layers and water channels through the biofilm, providing the bacteria essential nutrients.

Aggregation is the foundation of bacterial interactions in biofilm formation. Generally, one bacterial species can coaggregate with many other bacterial species by cell-to-cell recognition. Paired aggregation tests among 300 oral bacteria species indicated more than 90% undergo coaggregation.²¹ However, aggregation is also specific because one bacterial strain cannot aggregate with any random bacterial strains, i.e., *S. mutans* aggregates with *F. nucleatum* but not with *P. gingivalis*. The fundamental mechanism of aggregation is polysaccharide recognition between bacteria. The polysaccharide recognition sites vary from one paired bacterial recognition to another paired bacterial recognition, because one bacterial cell has several different receptors which are complementary to different adhesions belonging to other bacterial species. Many aggregations are inhibited by lactose but not by sucrose. McIntire²² reported the aggregation between Streptococcus and Actinomyces was 90% inhibited by lactose and D-galactose, but was only 10% inhibited by maltose, sucrose, cellobiose, or other monosaccharides. Coaggregation bridges usually refer to a structure of one bacterial species with two or more different receptors which can be recognized by different adhesions of two or more different bacterial species. Those bacterial species could aggregate by attaching to the first bacterial species; i.e., *F. nucleatum* is one of the best known coaggregation bridge species that facilitates streptococcal and obligate anaerobes aggregation. However, on the other hand, if two bacterial cells recognize the same receptor, the two cells would also compete for the binding site.

Dispersion of biofilm cells. In mature biofilm, bacteria leave the biofilm by single cell detachment or a cluster of cells detaching (Fig. 1). The model of biofilm dispersion includes erosion, sloughing and seeding.²³ At least two possible reasons can be explained for the detachment. The first is due to limited nutrients present at the original site and bacteria have to locate a new site with more nutrients for growth. This nutrient-seeking reason

could be further divided into active dispersion and passive dispersion. Active dispersion is conducted by the particular bacterium themselves, while passive dispersion is conducted by other bacterial species which compete for the nutrient with this particular bacteria. The second reason is host defense such as fluid shear force of saliva, which tries to limit biofilm development. For more detailed information about dispersion, please refer to Kaplan's review.²³

Cooperation in Biofilm

Almost all bacteria in nature grow in biofilm communities, where bacteria work as a well organized society. Bacterial cells in biofilm acquire distinguishing characteristics compared to planktonic cells. Previous studies in our lab²⁴ demonstrated different protein expression in biofilm and planktonic *S. mutans*, in which fructan hydrolase, exo-beta-D-fructosidase, fructanase and cell division protein FtsG were enhanced in biofilm organisms compared to planktonic cells, but antigen I/II, glucosamine-fructose-6-phosphate aminotransferase I and chaperonin GroEL were repressed in biofilm cells. Some of our preliminary data indicate that in planktonic cells low concentrations of nicotine (0.25–0.5 mg/ml) enhanced *S. mutans* growth, while higher concentrations (2–4 mg/ml) of nicotine repressed *S. mutans* growth, but in biofilm both low and high concentrations of nicotine enhanced *S. mutans* growth (Huang, Li and Gregory, manuscript in preparation). For most bacterial species, biofilms are the preferred form of growth.²⁵ Biofilm provides many advantages to the reproduction, metabolism and defense of bacteria from other bacteria or the host.

Metabolic communication. The foundation of biofilm metabolic communication is coaggregation, which provides cells close access and makes communication more convenient between bacterial species.

EPS, as mentioned above, is the major component of biofilms. All biofilms contain EPS, although the type of EPS in biofilms varies from one biofilm to the other due to the bacterial growth status and the substrates for bacterial metabolism. Because almost all bacterial species in biofilm can undergo biosynthesis and degrade EPS, EPS becomes the communication medium between bacterial species. Other functions of EPS are to provide shelter to the bacteria as the cross-linked EPS can block harmful agents outside of the biofilm and trap nutrients from the environment for a local higher concentration of the nutrients and extracellular enzymes for better biosynthesis of substrate. EPS helps biofilm maintain biofilm structures. Although the biofilm structures change according to the bacterial species in the biofilm and the nutrients available in the environment, EPS adapts to a new confirmation and becomes compatible with the requirement of the biofilm. EPS also influences iron exchange within biofilms and controls the hydrophilic or hydrophobic characteristics of the biofilm itself.

Oxygen metabolism and exchange within the biofilm between different aerobic and obligate anaerobic species has a special significant role for the survival of obligate anaerobes. It is difficult for obligate anaerobe species to live in an aerobic environment

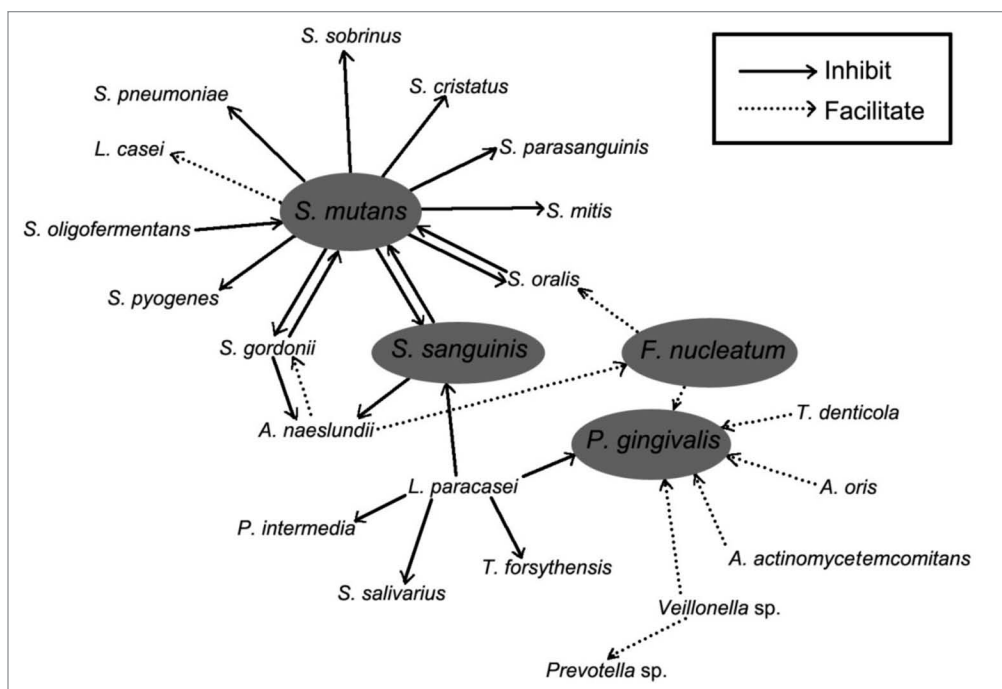


Figure 2. Bacterial interactions. Solid lines indicate inhibition while dashed lines indicate facilitation. The direction of the arrow indicates the direction of inhibition or facilitation.

without the cooperation of aerobic species. The aerobic species consume oxygen in the environment, resulting in a lower level of oxygen concentration and producing a local redox potential gradation which provides local anaerobic conditions. The local anaerobic environment is the foundation of obligate anaerobic species survival. *F. nucleatum* is an important bridge bacterium, which can aggregate with both aerobic and obligate anaerobic species, allowing the two species to live together (Fig. 1).^{26,27} Without *F. nucleatum*, the number of obligate anaerobic species would decrease sharply in the oral cavity.²⁸

Some bacterial species can help other species survive by depleting acid and improving the local environment. *F. nucleatum* and *Prevotella intermedia* can grow in a wide pH range from 5.0 to 7.0, but *P. gingivalis* is very susceptible to a pH lower than 6.5. However, *P. intermedia* and *F. nucleatum* can produce ammonia and organic acids by fermenting glutamate and aspartate,²⁹ providing a more neutral environment for *P. gingivalis* and other acid-sensitive bacterial species.

Other metabolic communications between oral bacterial species, such as short-chain fatty acids, exogenous quinines and vitamin K, were thoughtfully reviewed by Hojo and colleagues.⁵

Resistance to antibiotics. Antibiotic resistance has become a worldwide problem in public health. The resistance of Streptococci to penicillin, amoxicillin, trimethoprim-sulfamethoxazole and erythromycin was observed in children treated with antibiotics for otitis.³⁰ Biofilms can protect bacteria from a challenging environment with several host defense mechanisms directed towards bacteria or protect from applied antibiotics. The antigens of biofilm bacteria are hidden in the biofilm matrix and become less susceptible to the host immune

system. Physical injury of the biofilm can also be reduced by the biofilm matrix. The short distance between bacterial cells makes cell-to-cell metabolic communications more frequent.³¹ Scientists postulated a conception of “insurance hypothesis,” which stated a single species was more vulnerable by the environment than multiple species. If there are more species in the biofilm, and the structure of the biofilm is more complex and mature, the resistance to antibiotics is greater.³² The antibiotic resistance of bacterial cells in biofilm was reported to be 1,000 to 1,500 times greater than the resistance of planktonic cells³³ and has become a rising problem in recent years. Antibiotic resistance genes can be transferred between bacterial cells within biofilm resulting in a biofilm-wide resistance to the antibiotics.³⁴ The common carrier of resistance genes are plasmids, which are circular double-stranded DNA species presenting mainly in bacteria. The replication of plasmids is independent of chromosomal DNA replication and the number of plasmids in a cell varies widely. Plasmids with antibiotic resistance genes are gained by bacterial conjugation.

Sedlacek and Walker³⁵ compared the antibiotic effect between biofilm and planktonic cells. There were 19 subgingival species investigated: *Actinomyces naeslundii*, *Actinomyces meyeri*, *Bacteroides* species, *Bifidobacterium* species, *Campylobacter* species, *Eubacterium* species, *Fusobacterium alocis*, *F. nucleatum*, *Faecalibacterium prausnitzii*, *P. intermedia/nigrescens*, *Prevotella loeschii*, *Streptococcus intermedius*, *Streptococcus parasanguis*, *Streptococcus sanguinis* (earlier named *Streptococcus sanguis*), *Veillonella atypical* and *Veillonella parvula*. Both biofilm and planktonic cells were treated with tetracycline, amoxicillin and clindamycin with a concentration range from 0 to 2,048 µg/ml. Almost all biofilm cells displayed a higher minimum inhibitory

concentration (MIC) compared to their relative planktonic cells. *A. naeslundii*, *Campylobacter* species, *F. nucleatum*, *P. intermedia/nigrescens*, *P. loescheii*, *S. intermedius*, *S. parasanguis*, *S. sanguinis*, *V. atypical* and *V. parvula* demonstrated very significant (4–250-fold) antibiotic resistance increases between biofilm and planktonic cells.

In addition, antibiotic resistance varies from one antibiotic to another antibiotic. Sedlacek and Walker³⁵ found the concentrations to inhibit *F. nucleatum* growth are 256, 128 and 64 µg/ml for tetracycline, amoxicillin and clindamycin, respectively. Besides, antibiotic resistance varies from one bacterial species to another species. For example, the MIC of tetracycline on single species biofilm cells were 64 µg/ml for *A. meyeri*, but 512–2,048 µg/ml for *A. naeslundii*.³⁵

Competition in Biofilm

As a complex and diverse society, competition is highly prevalent among bacteria within biofilm. The bacterial species compete for nutrients, binding sites, and the chance to survive. Several major competitive mechanisms are widely adopted by bacteria, i.e., bacteriocin synthesis, quorum sensing and hydrogen peroxide excretion.

Bacteriocins. In biofilm, certain bacteria express specific or non-specific proteins (bacteriocins) which can affect other bacteria. Bacteriocins are different from traditional antibiotics in that they usually only act on its own strain or strains similar to the producing species.^{36–39}

Studies have shown that Streptococci possess the strongest ability of producing bacteriocins among all oral bacteria.³⁹ Bacteriocins produced by *S. mutans* can be differentiated into two types termed mutacins. The first type are lantibiotics. Lantibiotics are a class of antibiotic peptides containing a characteristic polycyclic thioether, the amino acids lanthionine or methylanthionine, as well as the unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid. The lantibiotics are synthesized by many Gram positive bacterial species. Lantibiotics contain mutacins I, II and III. The second type are non-lantibiotics containing mutacins IV and V. The inhibition spectrum of lantibiotics on Gram-positive bacteria is relatively wide, while the non-lantibiotics have a narrow antimicrobial spectrum, only acting on *S. sanguinis*.³⁷ Mutacins can enhance the competitiveness of *S. mutans* successful colonization and its ability to flourish in the biofilm.^{39,40} In addition, *Streptococcus salivarius* is an important oral probiotic, and its production of bacteriocins, such as salivaricin A (SalA), strongly inhibits *Streptococcus pyogenes*.³⁸ Therefore, the bacteriocins expressed by some strains greatly affect other strains living in biofilm.

Recently, Perry et al. found that when the concentration of competence-stimulating peptide (CSP; see below) is high in the micro-environment, mutacin V can be used as a precursor of intracellular auto-active bacteriocin or death effector, mediating *S. mutans* autolysis via a novel mechanism, which is vividly called intracellular action against self. Perry speculated that there may be some relationship between the autolysis of *S. mutans* and genetic transformation in the bacterium.

Bacteriocins have a broad prospect for application in the field of medicine. A new class of drugs can be developed which is targeted and contains only minor side effects, using its unique narrow-spectrum antibacterial properties, to avoid microbial flora and bacterial resistance and achieve the desired clinical effect under the condition of ensuring the balance of normal flora in the human body.

Quorum sensing (QS). Quorum sensing is the self-induced secretion of one or more agents (autoinducer, AI) in response to changes in bacterial density and the surrounding environment, i.e., the bacteria reach a certain threshold (or quorum of cell types, the number of colonies or bacteria), which initiates gene expression to regulate cell or group behavior. Different groups of bacteria have different effects of conditioning; many bacteria secrete the same inducer, thereby regulating the interaction between different types of bacterial behavior.

In biofilm, the process of bacteria producing signal molecules, transporting, sensing and controlling a series of acts is called a quorum sensing system. The majority of biofilm bacteria can sense the bacterial biofilm microenvironment through these systems to control their own physiological characteristics, however, planktonic bacteria do not exhibit these physiological functions.^{43,44} QS systems control a wide range of responses, including bacterial surface adhesion, extracellular matrix production, synthesis of biosurfactants, spore formation, competency, bioluminescence and virulence factor expression, etc.^{45,46} QS systems are highly specific and accurate, which are the basis of precise regulations of the different bacterial phenotypes. In complex microbial communities, this careful regulation is truly fascinating because each bacterium can also produce a variety of QS signaling molecules with a variety of signal systems (such as the *Vibrio harveyi* signaling system with three kinds of AI) but those signaling molecules mainly regulate this particular species instead of affecting other species.^{47,48} Different signaling molecules in various signaling pathways can also influence each other, constituting a sophisticated three-dimensional network of multi-level and large-scale signaling systems.⁴⁹

There are three types of recognized QS systems in bacteria: the first type is in Gram-positive bacteria and uses oligopeptides as signal molecules; the second type in Gram-negative bacteria uses an acyl-homoserine lactone as a signal molecule; and the third type is conserved in many Gram-negative and Gram-positive bacteria using AI-2 as a signal molecule.⁵⁰

Oligo-peptides do not enter the cell. They usually bind to membrane sensor proteins of the two-component signal transduction system, but some of them are usually transported to the cytoplasm before binding to their respective receptors.^{51,52} Gram-positive bacteria such as *Staphylococcus aureus* often use this complex QS system in which modified oligo-peptides serve as AIs.⁵³

The acyl homoserine lactones (AHL) are usually detected through binding or activating cytoplasmic receptor proteins, which dimerize when the AHL binds. The receptor proteins can also bind to promoter regions of target genes, and then activate or repress the transcription of genes.⁵⁴ AHL are produced and recognized uniquely. If one species produces an AHL, this special AHL usually can only be recognized by the same species.⁵³ AHL-type

AIs are detected by a sensor kinase, which is a membrane-bound two-component hybrid protein. The phosphoryl group is transferred from a sensor kinase to a histidine phosphotransfer protein and subsequently to a response regulator molecule. In addition, to the AHL-type AI, which is species-specific, an interspecies AI called AI-2 (a furonosyl borate ester) has been reported as a signal ligand for certain QS systems.⁵³

AI-2 is an important signal molecule in multi-species biofilms. AI-2 plays a decisive promoting role in biofilm formation and maturation. The *luxS* gene expressing AI-2 is conserved among many species of bacteria,⁵⁵ including *S. mutans*, *Streptococcus gordonii*, *Streptococcus oralis*, *P. gingivalis*, *A. actinomycetemcomitans* and other oral microorganisms.^{56,57} AI-2 can also mediate oral Streptococci and Actinomycetes to form mixed biofilm.⁵⁸ The formation of biofilm depends on AI-2 produced by oral Streptococci. For example, bacteria expressing a mutation in the *luxS* gene in oral Streptococci cannot express AI-2, resulting in a failure of communication between oral Streptococci and Actinomycetes. This failure leads to loose biofilm formation and significantly decreased bacterial density.⁵¹ In recent years, reports of interference of these bacterial QS systems and new ways of inhibiting biofilm formation, has opened new avenues for intractable infectious disease research.

Competence-stimulating peptide (CSP). QS, which is peptide based, has been shown to regulate genetic competence,⁵⁹ biofilm formation,⁶⁰ and acid tolerance⁶¹ of bacteria. Recent evidence has shown that competence-stimulating peptide (CSP) can induce alarmones which are intracellular signal molecules and are produced due to harsh environmental factors, and can convey sophisticated messages in a population including the induction of altruistic cellular suicide under stressful conditions. CSP is a small molecule which induces bacteria into competence and then the bacteria can obtain exogenous DNA and change genetic information.⁶²

S. mutans usually grows on enamel surfaces in biofilms and it is often considered as the major etiological agent of dental caries in humans.⁴⁷ *Candida albicans* is also commonly found in human dental caries, where *S. mutans* interacts with *C. albicans*. *S. mutans* inhibits *C. albicans* ability to produce germ tubes in co-cultures even when *S. mutans* is physically separated from *C. albicans*. A recent study shows that the QS molecule CSP, secreted during *S. mutans* early growth stages, inhibits formation of *C. albicans* germ tubes.⁶³

In *S. mutans*, the QS systems consist of approximately five proteins, including a 21-amino-acid CSP. It has been reported that the *comC* gene encodes CSP, CSP is an inactive precursor at that point, but it is then cleaved and exported by the ABC transporter, ComAB.⁶⁴ In *S. mutans*, genetic competence and bacteriocin production are regulated by the ComED system which specifically senses and responds to CSP.⁶⁵ CSP is synthesized in the cell and released into the extracellular medium, mediating the QS system. When the bacterial density of biofilm increases, CSP molecules in the external environment reach a certain numerical threshold concentration and begin to regulate bacterial density. CSP can regulate physiological activities of *S. mutans*, including biofilm formation, generation of bacteriocins, stress response,

acid tolerance and genetic conversion, etc.^{37,44} Previous studies in our lab demonstrated that *spaP* is regulated by CSP because the *spaP* gene was abolished in *com* gene-deficient strains. However, direct addition of synthetic CSP to a low-density culture failed to repress *spaP* gene expression (Chen and Gregory, personal observation).

CSP signaling molecules are highly species-specific. A CSP produced by one bacterium rarely interferes with the activity of CSP produced by a different bacterium. Eckert et al. has developed a specific highly novel antibacterial agent using the species specific nature of CSP, called specifically targeted antimicrobial peptides (STAMPs). STAMPs contain two functional domains, the first domain is a targeting peptide domain and the second domain is an antimicrobial peptide domain. The antibacterial domain recognizes *S. mutans* and kills it, but there is no effect on other types of non-cariogenic streptococci. This confirms the specificity of the STAMP.

Bacterial Species Interactions in Biofilms

Due to limited growing space and nutrients, bacteria from different species compete with each other in biofilms (Fig. 2). Every species has its own stratagem to compete against others.

Streptococcus mutans. *S. mutans* is considered as one of the most important cariogenic bacteria.⁶⁷ It is a common bacterial species in human beings because it is present in almost everyone's oral cavity. However, in caries-free oral cavities the percentage of *S. mutans* is very low; while in caries-affected oral cavities the percentage of *S. mutans* is high. The shift of *S. mutans* proportion from low percentage to high percentage is associated with caries.⁶⁸

Kreth et al.¹⁹ reported dual species inhibitory activities of *S. mutans* on *S. gordonii*, *S. pyogenes*, *Streptococcus mitis* ATCC 903, *Streptococcus pneumoniae*, *Streptococcus cristatus* and *S. sanguinis* were significant; the inhibitions on *S. oralis*, *S. mitis* ATCC 33399 and *S. parasanguinis* were medium; and the inhibition on *S. sobrinus* was mild.

The numbers of *S. mutans* and *S. sanguinis* are negatively associated. If the level of *S. mutans* is high, the level of *S. sanguinis* is low; while if the level of *S. sanguinis* is high, the level of *S. mutans* is low. This possibly explains the reason why caries-active individuals have low numbers of *S. sanguinis* and caries-free subjects have higher numbers of *S. sanguinis*. In a dual-species *S. mutans* and *S. sanguinis* biofilm, if *S. mutans* colonized first, the growth of the later colonized *S. sanguinis* would be inhibited by *S. mutans*. However, if *S. sanguinis* colonized first, the growth of the later colonized *S. mutans* would be inhibited by *S. sanguinis*. Furthermore, if *S. mutans* and *S. sanguinis* colonized together at the same time, either species can inhibit the other. Therefore, the order of colonization determines the result of the competition.¹⁹ This result confirmed earlier research done by Kemp et al. in 1983.⁶⁹ Clinical studies also confirmed the competition between *S. gordonii* and *S. mutans* or between *S. sanguinis* and *S. mutans*.⁷⁰ Further research¹⁹ suggested the inhibition of *S. mutans* on *S. sanguinis* was caused by an excreted diffusible substance instead of nutrient deprivation. However, the amount of nutrients present plays a significant role in the competition. When nutrients were

rich, both *S. mutans* and *S. sanguinis* grew well, no matter which species colonized first.

One mechanism of *S. mutans* inhibition on *S. sanguinis* is the large amount of organic acid *S. mutans* produces. Although both *S. mutans* and *S. sanguinis* can metabolize glucose and produce lactate when incubated with excess glucose, *S. mutans* produces more acid than *S. sanguinis* due to the higher ATP-glucose phosphotransferase activity of *S. mutans* than *S. sanguinis*.⁷¹ Therefore, the lactic acid produced by *S. mutans* glucose metabolism is excreted into the environment and inhibits the growth of *S. sanguinis*, resulting in an increased ratio of *S. mutans* over *S. sanguinis*.⁷² Kreth et al.¹⁹ confirmed these results. When *S. mutans* and *S. sanguinis* were cultivated under acidic conditions (pH 5.5) at the same time, *S. mutans* grew better than *S. sanguinis*. Acidic conditions can repress or damage the ATP-glucose phosphotransferase activity of both strains, but the repression on *S. mutans* was less than on *S. sanguinis* (Fig. 3).⁷²

A second mechanism of *S. mutans* inhibition on *S. sanguinis* is the mutacin *S. mutans* excretes. Both *S. mutans* mutacins I and IV can inhibit the growth of *S. sanguinis*.⁶⁵ The mutacin inhibition order of *S. mutans* on *S. sanguinis* from the smallest to the largest was double-mutant strain (I-IV⁻), mutacin I-defective strain (I-IV⁺), mutacin IV-defective strain (I⁺IV⁻) and wild-type strain (I⁺IV⁺). The presence or absence of oxygen affects mutacin expression. Under aerobic conditions the mutacin IV structural gene was upregulated 5-fold compared with a non-anaerobic condition. But no significant difference was observed in mutacin I gene expression between the two oxygen conditions (Fig. 3).⁴⁰

A third mechanism is that *S. mutans* inhibits the ability of *S. sanguinis* to produce hydrogen peroxide (H₂O₂). The H₂O₂ production of *S. sanguinis* was 66% reduced when *S. sanguinis* was cultivated with *S. mutans* compared with cultivated alone (Fig. 3).¹⁹

***Streptococcus sanguinis*.** *S. sanguinis* is one of the early colonizers in biofilm and is considered a beneficial species in the oral biofilm ecosystem. It can produce H₂O₂ as a means of excreting excessive oxygen and serves as a non-specific antimicrobial agent, which can inhibit *S. mutans* and other anaerobic periodontal pathogens growth.⁷³ However, it is obtrusive to say *S. sanguinis* is 100% benign due to its association with bacterial endocarditis.⁷⁴

S. sanguinis competes with other bacteria by producing a bacteriocin of *S. sanguinis* (sanguicin) or excreting H₂O₂.⁷⁵ H₂O₂ can inhibit the glycolysis and protein synthesis of target bacteria. Baldeck and Marquis⁷⁶ reported both biofilm and planktonic *S. mutans* cells were sensitive to H₂O₂. Low concentrations (16.3 mmol/L) of H₂O₂ could strongly inhibit glycolysis and are primarily bacteriostatic. The most sensitive target was glyceraldehyde-3-phosphate dehydrogenase and a less sensitive target was the phosphoenolpyruvate:glucose phosphotransferase pathway. High concentrations (30 mmol/L) of H₂O₂ could inhibit protein synthesis, resulting in cell damage due to methionine oxidation or lack of cell repair mechanisms.^{76,77} A eukaryotic-type serine/threonine protein kinase (STPK) in the *S. mutans* cell-envelope is related to H₂O₂ antimicrobial resistance and a STPK-defective strain of *S. mutans* was observed to be more sensitive to H₂O₂.⁷⁸ Kreth et al.⁴⁰ observed the inhibition of *S. mutans* by *S. sanguinis*

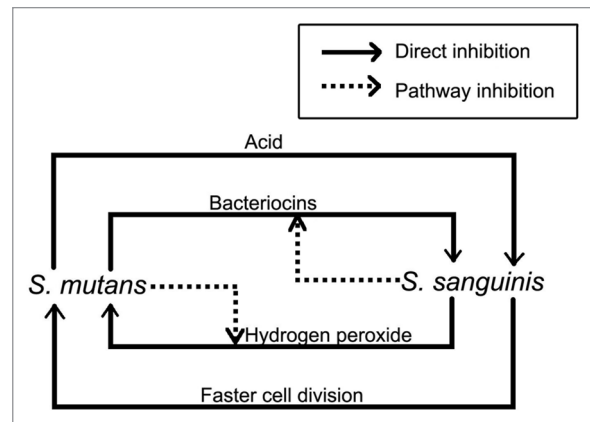


Figure 3. Interaction between *S. mutans* and *S. sanguinis*. Lines indicate inhibition pathways, and the direction of the arrow indicates the direction of inhibition or facilitation.

was reduced when grown with glucose. The reason of this reduction in inhibition is that glucose may repress the expression of pyruvate oxidase. Pyruvate oxidase is related to H₂O₂ formation, as H₂O₂, acetyl phosphate and carbon dioxide are synthesized from pyruvate by pyruvate oxidase. Therefore, glucose indirectly represses the excretion of H₂O₂ by *S. sanguinis*. The amount of oxygen in the environment also affects the competition between *S. sanguinis* and *S. mutans*. The inhibition of *S. mutans* by *S. sanguinis* is increased under aerobic conditions compared with anaerobic conditions.⁴⁰ *S. sanguinis* inhibits *S. mutans* production of mutacin. The mutacin production of *S. mutans* was 20% of when cultivated with *S. sanguinis* compared with cultivated alone.⁶⁵ Furthermore, the doubling time of *S. sanguinis* is shorter than the generation time of *S. mutans*, and this more rapid reproduction facilitates *S. sanguinis* competing against *S. mutans*.⁷⁹

Previously, we investigated the secretion of immunoglobulin A1 (IgA1) protease of *S. sanguinis* in dual-species culture with *S. mutans*. We found the IgA1 protease of *S. sanguinis* was enhanced when it was incubated with *S. mutans* or *Actinomyces viscosus*. The enhancing factors from *S. mutans* or *A. viscosus* are soluble and less than 14 kDa in size. Further research confirmed that *S. mutans* strain MT8148, TH16, ATCC 10449, KPSK2, V310, V318, Ingbritt and ATCC 25175, and *S. sobrinus* strain ATCC 27352 also enhanced IgA1 protease expression of *S. sanguinis*. These results suggested that the enhancing factors may serve an ecological role facilitating *S. mutans*, *A. viscosus* and *S. sobrinus* to evade anti-bacterial IgA antibodies in saliva and gain an opportunity to multiply.⁸⁰

***Streptococcus gordonii*.** *S. gordonii* is one of the pioneer bacteria attached to supragingival pellicle. Clinical studies confirmed competition between *S. gordonii* and *S. mutans*.⁷⁰ Kreth et al.⁴⁰ observed the inhibition of *S. mutans* by *S. gordonii*, but this inhibition was less than the inhibition of *S. mutans* by *S. sanguinis*. Like *S. sanguinis*, the inhibition of *S. mutans* by *S. gordonii* is stronger under aerobic conditions than under anaerobic conditions.⁴⁰ Interestingly, the inhibition was reduced with glucose. The mechanism of this reduction is similar to that of *S. sanguinis*, in that pyruvate oxidase is affected and the amount of H₂O₂

is reduced. This decrease in inhibition was more significant in *S. gordonii* than in *S. sanguinis*.⁴⁰

Furthermore, *S. gordonii* inhibits *A. naeslundii* growth by excreting H₂O₂. *S. gordonii* is usually detected together in dental biofilm with Veillonella species. Additional research reported *S. gordonii* can coaggregate with Veillonella species.²⁸

Streptococcus oralis. The doubling time of *S. oralis* is shorter than the generation time of *S. mutans*, and this more rapid reproduction facilitates *S. oralis* competing against *S. mutans*.⁷⁹ *S. oralis* is typically detected together with Veillonella species, while additional research reported *S. oralis* can coaggregate with Veillonella species.²⁸

Streptococcus oligofermentans. *S. oligofermentans* can produce H₂O₂ mainly by two methods. The first method is to metabolize the lactic acid produced by *S. mutans* into H₂O₂ by lactate oxidase, while the second method is to metabolize the peroxide to H₂O₂ by L-amino acid oxidase. The H₂O₂ produced by *S. oligofermentans* can cooperate with the H₂O₂ produced by *S. sanguinis* and increase inhibition of *S. mutans* growth.⁷³

Porphyromonas gingivalis. In a series of dual-species experiments, *P. gingivalis* was demonstrated to grow with *S. gordonii*, *Actinomyces oris*, Veillonella sp, *F. nucleatum* and *A. actinomycetemcomitans*, but not with *S. oralis*. In a three-species model,⁸¹ *P. gingivalis* was demonstrated to grow with dual-species of Veillonella sp and *A. actinomycetemcomitans*, and dual-species of Veillonella sp and *F. nucleatum*, but *P. gingivalis* does not grow with either dual-species of *S. oralis* and *A. actinomycetemcomitans* or dual-species of *S. oralis* and *F. nucleatum*. Therefore, *P. gingivalis* apparently cannot grow in the presence of *S. oralis*. However, in a three-species biofilm with *S. gordonii* and *S. oralis*, *P. gingivalis* grew well,⁸¹ this result indicated that *S. gordonii* facilitated *P. gingivalis* growth.

P. gingivalis is usually detected together with *Treponema denticola* in subgingival plaque. *T. denticola* can produce succinic acid which was identified in the phospholipids and lipids on the *P. gingivalis* cell envelope.⁸² However, *P. gingivalis* produces isobutyric acid which increases *T. denticola* growth.⁸³ Therefore, a cooperative synergistic growth relationship may exist between *P. gingivalis* and *T. denticola*.

Fusobacterium nucleatum. Fusobacteria are the predominate Gram-negative bacterial species in mature dental plaque. However, in the initial colonization, streptococci and actinomyces are the predominate species. The coaggregation of Fusobacteria with other bacterial species is a pivotal link between primary and secondary dental plaque colonization.^{20,84} *F. nucleatum* is a crucial microorganism in dental biofilms because of its coordinator role, which coaggregates with both late and early dental biofilm colonizers.²⁰

The coaggregation between *F. nucleatum* and other Gram-negative bacterial species are governed by lectin-carbohydrate receptor interactions. For example, the coaggregation between *P. gingivalis* and *F. nucleatum* is regulated by a galactoside *P. gingivalis* moiety and a *F. nucleatum* lectin, which can be inhibited by lactose. *P. gingivalis* capsular polysaccharides and lipopolysaccharides are involved through receptors which mediate coaggregation between oral bacterial species.⁸⁵ In contrast, although

coaggregation between *F. nucleatum* and many Gram-positive bacterial species has been observed, those coaggregations are rarely inhibited by sugars.^{26,86,87} Thus, intergeneric coaggregation between *F. nucleatum* and Gram-negative organisms is significantly distinct from *F. nucleatum* binding to Gram-positive bacteria.

Recent research demonstrates that *F. nucleatum* is difficult to grow in saliva without the presence of *A. naeslundii*. Interestingly *S. oralis* cells increased in the presence of *F. nucleatum*. Therefore, Fusobacteria require *A. naeslundii* for its own biofilm formation and improve the initial colonization of streptococci in certain dual-species biofilms.⁸⁸

Oral bacteria will confront oxygen stress in dental biofilm. The survival of oral anaerobic bacterial species depends on the specific tolerance of each species to oxygen and the bacterial interactions within the biofilm community. *F. nucleatum* supports *P. gingivalis* growth by providing a capnophilic environment when growing in an oxygenated and CO₂ depleted environment.⁸⁹ In a two stage chemostat system, the survival of obligate anaerobic microorganisms in an aerated environment was improved by the coaggregation with *F. nucleatum*.²⁸

Lactobacillus casei. *L. casei* is commonly detected in cariogenic biofilm. This strain can produce abundant lactic acid and has a wide pH range. However, the tooth surface adherence of this acid tolerant strain is weak. Wen et al. observed that the biofilm formation of *L. casei* alone was less than the biofilm formation of dual-species culture with *S. mutans*. The reason for this improvement was that *S. mutans* provided *L. casei* glucosyltransferase (GTF) enzymes, with which glucans were synthesized, and therefore bacterial adhesion was improved.

Lactobacillus paracasei. *L. paracasei* produces bacteriocins, which make pores in the cytoplasmic membranes of *P. gingivalis*, *P. intermedia*, *T. forsythensis*, *S. salivarius* and *S. sanguinis*, inhibiting the growth of those strains. This bacteriocin has a molecular weight of approximately 56 kDa with a composition of 68% carbohydrate and 32% protein. This protein has cationic amino acids present near the N-terminus, hydrophobic amino acids present in the central region and hydrophilic residues present at the C-terminus.^{90,91}

Actinomyces naeslundii. *A. naeslundii* is one of the pioneer bacteria in dental biofilm formation, and it is related to periodontal disease and root caries. *A. naeslundii* can protect many bacterial species from H₂O₂, because *A. naeslundii* consumes H₂O₂ by protein oxidation.⁹² *A. naeslundii* also upregulates the *spxB* gene (which encodes for pyruvate oxidase) of *S. gordonii*. Furthermore, *A. naeslundii* can provide *S. gordonii* arginine, which is related to the protein oxidation effect exhibited in *A. naeslundii*. The residual amount of *S. gordonii* intracellular arginine is limited due to the excretion of H₂O₂. Therefore, growth of *S. gordonii* was increased by aggregation with *A. naeslundii*. In the presence of *A. naeslundii*, *S. gordonii* can grow under arginine depletion conditions in which *S. gordonii* usually does not survive.^{93,94}

Veillonella. Veillonella species can produce vitamin K, which can be utilized by *Prevotella* spp and *Porphyromonas* spp for their growth.⁹⁵ Veillonella species utilize the organic acids produced

by *S. mutans* in a symbiotic manner that benefits the growth of both species.

Conclusions

For hundreds of years, scientists and dentists have been looking for efficient methods to control oral diseases. Since the oral ecosystem view has become more widely accepted and oral bacterial interactions have been more elucidated, in the future there may be medications to take advantage of inter-bacterial antagonism to control and prevent those diseases. However, future research must address the interactions within biofilm which are not fully

understood today. Because of the complex and variable nature of biofilm, this goal can only be fulfilled with future investigations focused on the model of oral ecosystems and relative factors, with advanced understanding of the mechanisms of bacterial biofilm interaction mechanisms.

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