

Molecular Principles of Adhesion and Biofilm Formation

Jens Kreth and Mark C. Herzberg

Abstract Oral bacteria are responsible for oral health and disease, including caries, periodontal disease, and endodontic infections. The development of oral diseases is intimately linked with the ability of oral bacteria to form and reside in an adherent multispecies consortium named biofilm. The oral biofilm provides a protective environment for the bacterial community and its formation is a genetically controlled process. In this chapter, we present a general overview of developmental mechanisms employed by individual members of the oral biofilm. The species composition of the oral biofilm and the oral microbiome is discussed historically and in the context of newly developed next-generation sequencing techniques. Furthermore, biofilm-specific regulatory mechanisms and phenotypic traits are explained to provide the reader with a comprehensive overview of oral biofilm formation and its role in health and disease.

1 Introduction

1.1 What Are Biofilms?

Microbial communities are commonly referred to as biofilms (Costerton et al. 1995). These communities are found associated with humans, generally on the skin or mucous membranes, but can also be found in natural (e.g., rivers and streams or soil) and artificial environments (e.g., on the surfaces of the places where we live and work). In general, biofilms connote the lifestyles of aggregated, sessile, or attached microbes in any environment and contrast free-floating, planktonic

J. Kreth (✉)

Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA
e-mail: Jens-Kreth@ouhsc.edu

M.C. Herzberg

Department of Diagnostic and Biological Sciences, School of Dentistry, Mucosal and Vaccine Research Center, Minneapolis VA Medical Center, University of Minnesota, Minneapolis, MN, USA

counterparts. The definition of biofilms has changed over time to include relevant new discoveries in biofilm research and to appreciate their structural and developmental complexity. An early definition as microbial aggregates attached to a living or nonliving surface embedded within extracellular polymeric substances (EPS) of bacterial origin has been extended to include aggregated cell masses floating in a liquid phase and cell aggregates in air–liquid interfaces.

One of the defining steps in biofilm development is the production of extracellular polysaccharides (EPS) (Flemming and Wingender 2010). In nature, what we term EPS actually consists of bacterial polysaccharides, proteins, nucleic acids, and lipids. The EPS contributes to the architecture of the biofilm community. In medically relevant biofilms, host-derived components play an important role in the initiation of biofilm development and should be considered a part of the EPS. For example, a conditioning saliva-derived film (the acquired salivary pellicle) is essential for the attachment of the initial oral biofilm forming bacteria (Hannig et al. 2005). Hall-Stoodley et al. therefore suggested that biofilm with EPS exists as “aggregated, microbial cells surrounded by a polymeric self-produced matrix, which may contain host components” (Hall-Stoodley et al. 2012).

1.2 Why Do Biofilms Form?

Human microbes of medical interest live predominantly in biofilms. The microorganisms dwelling in biofilm communities are estimated to cause about 80 % of infections (Costerton et al. 1999; Costerton 2001), suggesting that virulence is favored for the biofilm residents. Most human-associated microbial species are highly adapted to a specific body site; residing in a biofilm avoids dislocation to a less favorable environment. By residing in sessile communities, microorganisms are less likely to face eradication. For example, to optimize retention on selected oral surfaces, members of oral biofilms have developed mechanisms to optimize binding to specific cell and tissue sites (Zhang et al 2005). The successive integration of new members into the initial biofilm is also promoted by specific cell surface receptors to facilitate species–species aggregation (Jakubovics et al. 2014). To enable growth in the selected oral niche, the colonizing microflora can effectively metabolize salivary components.

The biofilm community also protects its ecological niche against invading, nonresident species that would otherwise overrun the space. In a process called colonization resistance, this community-based interference or antagonism prevents integration of *Pseudomonas aeruginosa*, for example, into human salivary microbial biofilms (He et al. 2011; van der Waaij et al. 1971). The protective biofilm environment also extends to the host–biofilm interface. The innate and adaptive arms of the immune system more effectively eliminate planktonic cells than microorganisms in biofilms. Several mechanisms can be in play. For example, in biofilms *Staphylococcus epidermidis* cells resist deposition of the antimicrobial complement component C3b and immunoglobulin G (IgG) on cell surfaces, thus

diminishing opsonization required for phagocyte-mediated killing (Kristian et al. 2008). Similarly, *Staphylococcus aureus* cells in biofilms resist macrophage phagocytosis by circumventing bacterial recognition pathways mediated by toll-like receptors TLR2 and TLR9 (Thurlow et al. 2011). Both TLRs usually recognize bacterial components (pathogen-associated molecular patterns, PAMPs), which are expressed on cells in the biofilm, but appear masked by the presence of the EPS. Interestingly, initial biofilm formation by *Pseudomonas aeruginosa* is facilitated by the presence of human neutrophils through *P. aeruginosa* attachment to neutrophil-derived actin and DNA (Walker et al. 2005), further illustrating that adaptation to the protective, anti-phagocytic biofilm environment sustains viability and long-term persistence.

The biofilm can likely modulate the host-immune response depending on the species composition. *Porphyromonas gingivalis*, a member of the subgingival biofilm community associated with the development of periodontal disease, can downregulate specific immune mediators. For example, the presence of *P. gingivalis* in a ten species in vitro biofilm model was required to downregulate proinflammatory interleukin- $\text{I}\beta$ and the NLRP3 inflammasome (Belibasakis et al. 2012), which are required for the effective elimination of bacteria by the host (Taxman et al. 2010). *P. gingivalis* is therefore suggested to use this strategy to manipulate the local inflammatory immune response and evade host surveillance with the ultimate benefit of survival at the host–biofilm interface (Bostanci and Belibasakis 2012).

Microorganisms residing in a biofilm community also enjoy greater resistance against antimicrobials. To combat infecting microbes in biofilms, conventional antibiotics are required at 10- to 1000-fold greater concentrations. Bacteria are also able to respond to the presence of antibiotics like methicillin by forming biofilms, as shown for *S. aureus* and several other species (Kaplan 2011), indicating a specific mechanistic behavior of bacterial cells encountering potentially life-threatening conditions. Similarly, microbes encounter daily challenges from the host innate immunity. Antimicrobial peptides are produced by the oral mucosa to target bacteria residing or passing through the oral cavity (Diamond et al. 2008; Gorr 2012). Found in saliva, antimicrobial peptides are less effective against biofilms than planktonic cells (Helmerhorst et al. 1999; Mazda et al. 2012; Wei et al. 2006).

1.2.1 Formation of Diffusion Barrier and Adsorbant Surface

Several factors influence biofilm susceptibility to antimicrobials as described in the following section. The EPS can form both a diffusion barrier and affinity matrix, partitioning the microbes from an antimicrobial compound or peptide. As an affinity matrix, the EPS actively binds antimicrobial substances to limit penetration into the biofilm. By slowing and limiting the diffusion of the antimicrobial, EPS reduces the effective local concentration reaching the viable cells in the biofilm community. The structure of the EPS affects the rate of diffusion of antimicrobials;

diffusion would be more limiting as the size of the antimicrobials increases. For example, fluorescent probes of varying molecular weights—surrogates for antimicrobial compounds—penetrated into a preformed *in vitro* biofilm consortium with different efficiencies. The diffusion limitation reflected molecular sieving, which could be predicted by the molecular weight of the fluorescent probes. The pore diameter for the particular biofilm EPS was estimated to be between 2.6 and 4.6 nm (Thurnheer et al. 2003). The pore size of EPS is expected to vary with the microbial species composition in the biofilm and the structure of the synthesized EPS, but little is known. In some conditions, diffusion limitation is not achieved by the EPS. For example, the antibiotics vancomycin and rifampin can effectively penetrate the biofilms of *S. epidermidis*, but fail to eradicate the biofilm-dwelling bacteria (Dunne et al. 1993), suggesting another mechanism responsible for the reduced susceptibility.

Biofilms, including the EPS and the compact colonies of cells, also limit diffusion of components required for the growth of the resident cells and removal of secreted metabolic end products. The EPS and cell colonies function generally as a constraint on diffusion and as a molecular sieve. As shown for *ex vivo* oral biofilms, oxygen availability is limited in deeper parts of the biofilm (von Ohle et al. 2010), repressing the respiratory activity of oral biofilm bacteria (Nguyen et al. 2002). The cells grow slower because of suboptimal conditions for metabolic activity. The microbial heterogeneity in segments of the biofilm community is both a cause and consequence of regional differences in metabolic activity. Heterogeneous metabolic activity in biofilms leads to reduced cellular content of RNA and proteins in some regions of the biofilm, while growth occurs elsewhere (Sternberg et al. 1999; Xu et al. 1998). Regions with slow growth may show greater antibiotic resistance. Mature subgingival *ex vivo* biofilms with limited nutrient supply are less susceptible to chlorhexidine and other antibiotics when compared to newly formed, metabolically active biofilms (Sedlacek and Walker 2007; Shen et al. 2011).

1.2.2 Biofilm-Specific Development of Genetic Resistance

During biofilm development, specific traits can be expressed that confer antibiotic resistance, which is not observed in planktonic cells. For example, a glucosyl-transferase (encoded by *ndvB*) in *P. aeruginosa* is responsible for the production of cyclic periplasmatic glucans. The expression of *ndvB* is specific for biofilm cells and seems to be absent in planktonic cells. Inactivation of *ndvB* leads to the loss of high-level, biofilm-specific antibiotic resistance (Mah et al. 2003). The cyclic glucans produced by NdvB can interact with antibiotics, thus sequestering antibiotics away from their cellular targets (Beaudoin et al. 2012; Mah et al. 2003). Similarly, during biofilm development, cells of *S. aureus* and *Salmonella enterica* serovar Typhimurium upregulate specific multidrug efflux pumps that transport antimicrobial compounds out the cell (He and Ahn 2011). *Candida albicans* also upregulates drug efflux pumps during biofilm formation, which may increase resistance to antifungal components during oral candidiasis (Ramage et al. 2002).

1.2.3 Emergence of Persister Cells

Bacterial persistence during antibiotic treatment can be attributed to persister cells (Bigger 1944). Persister cells are a small metabolically inactive, dormant subpopulation found in biofilm and planktonic cultures (Balaban 2011; Lewis 2010). Their minimal physiological activity facilitates extreme tolerance against antimicrobial treatments. In contrast, resistant bacteria acquire either a mutation or encode a specific gene conferring antibiotic resistance. The persistent state is not passed on to the offspring. Once the antibiotic challenge to the population is removed, the persister cells resume metabolic activity and repopulate the infected area, and new persister cells can appear.

The biofilm can also shield inhabitants from clearance by the immune system, contributing to the pathogenesis of chronic infections such as cystic fibrosis and tuberculosis (Allison et al. 2011). Immune cells and antibodies can attack the outermost surface of the biofilm, but bacterial cells within are protected. Antimicrobial therapy can also select for increased occurrence of persisters, as shown for *C. albicans* isolated from biofilms of oral candidiasis patients (Lafleur et al. 2010). For detailed information about the genetic regulation of persistence, see reference Lewis (2010). Persisting bacteria and fungi can cause recurrence of infection once antibiotic treatment concludes.

Biofilms, therefore, show greater resistance to antibiotics and immune defense mechanisms due to several mechanisms, which may have evolved to protect the community. The outermost layers of the biofilm form both an antibiotic diffusion barrier and adsorbent. Since human-associated biofilms are typically polymicrobial consortia, each resident species can create its own microenvironment. Bacterial species differ in their susceptibility to antibiotic treatment. Under selective pressure of antibiotic treatment, the less susceptible species will tend to survive. In concert with the selective advantages provided by formation and growth in biofilms, infections associated with biofilms tend to be treated ineffectively by antibiotics.

Growth of microbes in biofilm communities offers other advantages. During starvation conditions, survival of bacteria generally favors species residing in a community. Indeed, several species form biofilms to mitigate starvation conditions. Within biofilms, the lower metabolic activity might help cells to survive times of insufficient carbohydrates, nitrogen, and phosphorus in the oral biofilm. In general, microbes in biofilms are more resistant to stress (Coenye 2010), which can include nutrient deprivation, changes in oxygen tension, or extremes of pH and temperature.

In a biofilm community, resident cells enjoy intercellular communication. Microbial communication is crucial for concerted gene regulation as a response to environmental changes. The close proximity of cells in the biofilm seems to create an ideal environment to talk. A common form of communication between bacteria is the production of signaling molecules, which can be sensed by neighbors. The reduced diffusion in biofilms facilitates a localized critical increase of signaling molecules, which can trigger a corresponding response in the

microenvironment. Intercellular communication is important for general stress adaptation and community development. Intercellular communication is discussed in detail in Sects. 5 and 6.

1.3 Challenges for Biofilms in the Oral Environment

Among the human-associated bacterial communities, oral biofilms reside in an anatomical site that encounters diverse environmental challenges. These challenges include perturbations from the external environment and microbial growth control provided within the oral cavity by the innate arm of the immune system. The oral cavity is bathed in saliva, which contains innate immune effector molecules that originate in the salivary glands and in the mucosal epithelial cells that line the oral surfaces. The net effect of the challenges to biofilm communities will vary in the different niches found on the oral surfaces and within tissue folds and crevices. The oral mucosal epithelium is a continuously shedding and renewing tissue. Cells containing oral microorganisms are shed and replaced with new sterile cells, which rapidly bind and are invaded by oral microorganisms. The cycle of shedding, renewal, and reinfection repeats continuously.

Located in close proximity to the oral epithelium, teeth are non-shedding surfaces. Tooth eruption and entry into the oral cavity breach the covering mucosal epithelium. As the teeth erupt, the gingiva, a band of keratinized squamous mucosal epithelium, surrounds and attaches to the tooth surface and forms a penetration barrier. As the erupting tooth and the surrounding epithelium mature, a gingival crevice forms between the tooth and the attached gingiva. The gingival crevice is bathed with a specialized fluid called gingival crevice fluid, which arises as a serum transudate that percolates from the connective tissues through the thin crevicular epithelium. The oral surfaces are more generally bathed in saliva. The composition of bathing saliva varies in intraoral locales based on proximity to the different major and minor salivary glands. Oxygen tension, temperature, and humidity can also differ because of proximity to ambient air. For microbes, the mouth contains, therefore, an infinite number of microenvironments, each one contiguous with its neighbors. The distinct ecological determinants tend to select for survival and growth of certain microbial species while excluding others. Therefore, the bacterial composition differs for the subgingival and supragingival biofilms, which also differ from the species composition of the tongue.

Oral biofilms are also challenged frequently by sudden environmental changes due to host behavior. During host food intake, low nutrient availability for biofilms can be replaced by relative overabundance. Masticated foods and lactic acid released mainly by oral streptococci and actinomyces after carbohydrate fermentation cause sudden changes in pH. Additional physical and chemical stresses to the biofilm communities can be caused by sudden changes in temperature, osmolarity, and the mastication process during and after food ingestion. To accommodate to the stresses, the oral biofilm communities have become genetically diverse.

In individual subjects, the species richness is estimated to be 250 to 300 different species (Keijser et al. 2008; Zaura et al. 2009). The species diversity creates intrinsic competition for space and nutrients. Nonetheless, the oral biofilms maintain homeostasis as a vital part of overall human oral health.

2 Species Composition of the Human Oral Biofilm

The first ever description of bacteria by Antonie Philips van Leeuwenhoek was based on his own dental plaque samples. Visualized through his primitive compound microscope, the initial drawings reported in 1683 suggested that the oral biofilm is a multispecies consortium (Hall 1989). As technology has advanced in the last several decades, our knowledge of the species composition of oral biofilms has exploded in healthy subjects and others with oral diseases. Our understanding of disease development has increased and potential pathogenic species have been associated with caries and periodontal disease.

A new concept of disease development has been developed (Beighton 2005; Kleinberg 2002; Marsh 2003; Takahashi and Nyvad 2008; van Houte 1994). Today, the events leading to caries and periodontal disease are better explained by shifts in bacterial ecology and disturbances in biofilm homeostasis than the presence of a specific pathogen. The ecological plaque theory as defined by Marsh suggests that environmental pressures like low pH cause a shift in the bacterial ecology of the dental biofilm from health-promoting bacteria to primarily acidogenic, aciduric, and cariogenic species (Marsh 2003, 2009). Furthermore, the polymicrobial synergy and dysbiosis (PSD) model acknowledge that the presence and biofilm-specific activities of “keystone” species like *Porphyromonas gingivalis* and *Tannerella forsythus* are required to fully promote the pathogenic potential of the biofilm (Hajishengallis and Lamont 2012). Healthy microbial homeostasis is usually promoted by the interplay between host behavior, host defense mechanisms, and biofilm intrinsic mechanisms. Excessive intake of fermentable carbohydrates can perturb dental plaque homeostasis by lowering the pH. Higher proportions of conditional pathogens are selected, whereas under healthy conditions the composition of the dental plaque would not cause any problems for the host.

More than 300 years after the first visualization of the life-forms in dental plaque, new techniques and approaches are used to study oral microbial composition. Improvements in sensitivity and the development of high-throughput sequencing allow us to estimate that the oral biofilms contain up to 19,000 species/ phylotypes (Keijser et al. 2008). The complexity of oral biofilms is far greater than previously realized. Reflecting the complexity of these communities, the composition of oral biofilms is now called the microbiome. By use of informatics approaches and continued technological advances, new understanding of the oral microbiome in health and disease and breakthrough discoveries are to be expected.

2.1 *Composition of Human Oral Biofilms: Pre-microbiome Era*

The microbial composition of dental plaque has been characterized using culture-dependent and culture-independent approaches (Aas et al. 2005, 2008; Becker et al. 2002; Kroes et al. 1999; Kumar et al. 2003; Paster et al. 2001, 2006). The culture-independent method uses amplification of the species-specific 16S rRNA gene, followed by cloning of the respective amplicons and sequencing. When plaque was sampled from five subjects at nine different healthy intraoral sites using this method, 141 predominant species were detected (Aas et al. 2005). The genus *Streptococcus* is the most abundant, including the species *Streptococcus sanguinis*, *S. gordonii*, *S. mitis*, *S. oralis*, and *S. salivarius* (Aas et al. 2005). The streptococci constitute over 80 % of initial biofilm formers and 20 % of mature plaque biofilms (Rosan and Lamont 2000). Because of their interactions with other genera and species, the presence of certain species of streptococci specifies the initial species succession of successful biofilm formation as discussed below. Among the five subjects, several genera were identified to be in common including *Streptococcus*, *Veillonella*, *Granulicatella*, and *Gemella* (Aas et al. 2005). The nine intraoral plaque sites each showed distinct microflora. Interestingly, *Streptococcus mitis* was isolated from all intraoral sites and was present in all 5 individuals. Consistent with the absence of clinically detectable dental diseases, “red complex” species associated with periodontal disease were not found including *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* (Socransky et al. 1998). Similarly cariogenic species like *S. mutans*, *Lactobacillus* spp., and *Bifidobacterium* spp. were not isolated (Aas et al. 2005).

Using a more sensitive method in species detection, 16S rRNA amplification, cloning, and sequencing, the bacterial diversity of the oral cavity was determined in 10 healthy subjects (Bik et al. 2010). Overall, 247 species-level phylotypes were identified, including “red complex” species in three of the subjects. Periodontal pathogens seem to be present in subjects with periodontal health, although at low levels. All 10 subjects shared a core set of 15 bacterial genera, with subject-specific differences at the species and strain level (Bik et al. 2010). Several species were only found in 1 or 2 subjects, suggesting that the interindividual environment, behavior, and genetic background contribute to the species composition of the oral biofilm. These studies provided important contributions to our understanding of the overall composition of normal bacterial flora of the oral cavity.

To understand bacterial ecology during caries development, the microbial composition of healthy enamel in 30 subjects was compared with caries lesions of increasing severity in 30 subjects with childhood caries (Becker et al. 2002). The 23 most prominent bacterial species were investigated. As expected, cariogenic *S. mutans* was identified in relatively high abundance in subjects with clinical signs of caries development, and significantly fewer were recovered on sound enamel surfaces and in subjects without any sign of caries. *Actinomyces gerencseriae* and *Bifidobacterium* spp. were also more prevalent in caries than in healthy sites.

Conversely, *S. sanguinis*, *S. gordonii*, and *Actinomyces* serotype II were associated with intact, caries-free teeth and lower caries prevalence. Caries-associated species like *S. mutans* and *Lactobacillus* spp. increase in numbers during caries development (Torlakovic et al. 2012). With more sophisticated analytical methods, however, the species composition is further defined and includes other previously not detected species (Torlakovic et al. 2012). These data indicate that the species composition of oral biofilms varies from health to disease and fully support the ecological plaque theory.

2.2 Composition of Human Oral Biofilms: Microbiome Era

The Human Microbiome Project (HMP) Consortium seeks to provide a comprehensive overview of human microbial communities sampled on 18 sites of the healthy body (Consortium 2012a) (and <http://www.hmpdacc.org>). In contrast to the limited genus and species identification of methods in the pre-microbiome era, at least 300 bacterial reference genomes will be sequenced. The HMP uses a deep-sequencing approach and provides an in-depth analysis of the available data. New species can be identified and the overall metabolic pathways can be constructed when shared by the sampled body site and the inhabiting biofilm community. The sampling protocols and processing are precisely defined allowing for direct comparison of results from the samples from each subject (Consortium 2012a).

What have we learned about the oral biofilm community so far from the HMP? Nearly 5000 simultaneous samples were collected from 15 (male) to 18 (female) body sites from 242 healthy adult men and women. The human ecosystem is now known to be comprised of more than 10,000 microbial species; 81–99 % of the genera have been identified. With the first milestone announcement on June 13, 2012, several of the many concurrent publications report on the oral biofilm community (Consortium 2012b; Rho et al. 2012; Segata et al. 2012; Wu et al. 2012). When compared to the other sampled sites, the oral communities were among the most diverse (Huse et al. 2012). At the genus and species levels, tooth-associated communities were moderately distinct from other oral surfaces although some genera were widely present in most sites (Consortium 2012b; Huse et al. 2012). The unique composition of each site during health appeared stable. Genera with pathogenic members were well represented among this disease-free cohort (Segata et al. 2012). Although the microbial composition differed from site to site between buccal mucosa, supragingival plaque, and tongue dorsum, core metabolic processes were expressed in most environments (Consortium 2012b). In each environment, different consortia of species performed the core metabolic processes. Refinements in the analysis of available data sets, notwithstanding, core metabolic functions can be provided to the biofilm by previously unrecognized functional and ecological biodiversity in different oral sites and dramatically different distributions of oral species among individuals (Eren et al. 2014).

Clearly, the HMB project has given us a high-resolution overview of the species composition of the human oral biofilm with novel insights into the microbial collaborations needed for full metabolic capabilities of the community. The appreciation of the diversity at different oral sites begs the question of how colonization and metabolic activity reflects the health or disease potential of the biofilm in caries, for example. To address this question, it is feasible to determine the functional gene expression of dental plaque bacteria from different oral sites using RNA-seq. The actual metabolically active proportion of the consortium can be identified in terms of gene expression at any given oral site. RNA generation requires active metabolism and inactive or dead bacteria would be excluded from such an analysis. This meta-transcriptomic approach has been used only recently, but the actively transcribed gene sets and the resulting metabolic profile of the consortia were clearly defined (Benitez-Paez et al. 2014; Peterson et al. 2014). By ascribing functions to genes expressed in dental plaque and other oral communities, activities can be characterized. Further exciting discoveries will increase our understanding of oral biofilms at a different level.

3 First Steps in the Development of Biofilms

The crucial steps in the initial development of biofilms have been well documented. For example, the spatiotemporal pattern of early oral biofilm formation has been traced in the human host using specific removable appliances harboring dental enamel chips (Diaz et al. 2006). After 4 and 8 h of chip placement, oral streptococci were the predominant initially colonizing species. Indeed, the initial colonizers generally belong to the genus *Streptococcus*; some species are constant members representing a core group of initial biofilm formers (Diaz et al. 2006; Rosan and Lamont 2000).

Initial attachment is considered a reversible process. Initial or pioneer colonizers adhere and detach from the tooth surface, and the cycle repeats until permanent attachment is achieved. This irreversible attachment stage is typically mediated by high-affinity binding between proteins and polysaccharides on the bacterial cell surface to specific receptors on the tooth or cell. Within the bacteria, adhesion is controlled by a genetic program associated with a change in the expression of biofilm-related genes (Cowan et al. 1987; Hasty et al. 1992; Nobbs et al. 2009).

Biofilm development progresses with the formation of small micro-colonies of streptococci and a few non-streptococci (Diaz et al. 2006). This newly formed biofilm matures into a complex mature biofilm community by intrinsic growth and recruitment of other species into the developing biofilm. The growth and accrual of a complex community of microbial cells is accompanied by EPS production.

To discuss the molecular principles of biofilm formation, we will concentrate on oral streptococci as the best-studied oral bacterial species and initial biofilm former.

3.1 Attachment to the Tooth Surfaces

3.1.1 Salivary Pellicle Adhesion

The molecular principles of adhesion are based on successive steps. First, oral streptococci adhere to macromolecular complexes found on the saliva-coated tooth or mucosal cells. This salivary film is commonly called pellicle. Although oral streptococci are able to adhere directly to hydroxyapatite (Tanaka et al. 1996), the major mineral found in dental enamel, the initial attachment process involves the pellicle since the teeth and oral surfaces are consistently bathed with saliva and naked enamel occurs rarely in the oral cavity. All evidence indicates that the streptococcal surface interacts with salivary components including salivary proteins.

Salivary proteins solubilized in the liquid or gel fractions of saliva are able to attach avidly to the tooth surface via negatively charged residues and electrostatic interactions through the hydrophilic regions; when tightly bound to hydroxyapatite, hydrophobic domains buried within the three-dimensional structure of the salivary proteins can become exposed and facilitate additional interactions (Lamkin and Oppenheim 1993; Lindh 2002). This adsorbed complex of proteins and other macromolecules constitutes the acquired salivary pellicle, which immediately forms on the emerging tooth or after tooth cleaning by bathing in the constant flow of saliva. Components in saliva and in the pellicle known to interact with the adhering microorganisms include proline-rich proteins, alpha-amylase, secretory IgA, mucin glycoproteins, and glycoprotein (gp) 340 (for comprehensive reviews, see Jenkinson and Lamont 1997, Nobbs et al. 2009, Nobbs et al. 2011).

Binding to salivary pellicle proteins is mediated by protein–protein or protein–carbohydrate interactions between the pellicle surface and the streptococcal surface. Amylase is the most abundant salivary protein and is present in the salivary pellicle and dental plaque (Aguirre et al. 1987; Orstavik and Kraus 1973). Amylase can complex with sIgA in the salivary pellicle to form a binding receptor for certain oral streptococci (Gong et al. 2000). Several oral streptococci bind amylase alone. *S. gordonii* and *S. mitis* encode specific amylase binding proteins (Li et al. 2002a; Vorrasi et al. 2010). Amylase binding protein B, AbpB, contributes to biofilm formation based upon the inability of an AbpB mutant to colonize starch-fed rats (Tanzer et al. 2003). For initial biofilm formation, binding to amylase is of greater physiological significance than the physical attachment process. After streptococcal binding, amylase retains about 50 % of its enzymatic function to catalyze the hydrolysis of α -1,4-glucosidic linkages in dietary starch (Scannapieco et al. 1990). Ingestion of starch enzymatically produces glucose, maltose, and maltodextrins in close proximity to the streptococcal surface. These hydrolysis products of starch can be immediately transported into streptococci by carbohydrate-specific transporters. Adhesion is therefore linked to a metabolic advantage, which illustrates the complex nature of the oral biofilm.

Several other surface adhesins have been identified in oral streptococci mediating the binding to salivary components, including SsaB, FimA, Hsa, GspB, SspB, and SpaA (Holmes et al. 1998; Nobbs et al. 2011). *Streptococcus gordonii* adhesins Hsa and GspB recognize carbohydrate moieties of glycosylated salivary components. Both proteins mediate adhesion to the salivary pellicle through lectin-like recognition of sialic-acid-containing salivary mucin MG2 and salivary agglutinin. It is not surprising that oral streptococci have evolved several surface proteins with redundant function to ensure proper binding to their specific niche in the oral cavity. The oral cavity contains the only ecological niches allowing long-term survival of oral streptococci and these microorganisms must adhere or die. Interestingly, the genetic regulatory circuit of adhesin expression can compensate for imbalances in surface protein display, suggesting a fail-safe mechanism of adherence (unpublished data).

3.2 Binding to Cellular Components

Streptococcal surface proteins also bind the bacteria to host cellular components and salivary proteins that form a pellicle on mucosal epithelial cells. About 80 % of the oral cavity surface consists of soft tissue, providing a large area for bacterial attachment (Nobbs et al. 2011). Attachment to oral epithelial cells requires multiple *S. gordonii* adhesin proteins (Davies et al. 2009). Certain oral streptococci bind to mammalian fibronectin, a structural glycoprotein involved in cell–matrix interactions (Labat-Robert 2012; Nobbs et al. 2011), which facilitates adhesion to cells (Okahashi et al. 2010). *S. gordonii* Hsa mediates binding to sialic acid, which is found on the N-linked glycans of fibronectin and other glycoproteins. Binding to fibronectin is probably an important biological target evolutionarily since *S. gordonii* adhesins CshA and CshB also bind to fibronectin peptide domains present in the backbone. Similarly, the adhesins SspA and SspB also recognize fibronectin (Jakubovics et al. 2009).

Oral bacteria employ other host components as binding receptors including the integrin family of cell surface receptors responsible for cell–cell attachment (Nobbs et al. 2009, 2011). For example, the *S. mutans* multi-ligand antigen I/II family adhesin SpaP mediates interaction with $\alpha 5 \beta 1$ integrins (Engels-Deutsch et al. 2011), other host components, and bacterial cells of other species. Antigen I/II also mediates binding to fibronectin, collagen, salivary glycoproteins, glycoprotein 340, platelets, integrins, and *A. naeslundii* (Brady et al. 2010). To confer different binding capabilities, heterologous protein targets can bind distinct protein domains in the antigen I/II protein. For example, the alanine-rich repeat domain (A-region) found on the N-terminus binds to collagen type I. The C-terminal region is responsible for interactions with *P. gingivalis* (Brady et al. 2010), whereas the central variable domain (V-region) interacts with surface ligands of *Actinomyces naeslundii*. The A-region and the V-region each interact with salivary

glycoproteins. The proline-rich repeated domain (P-region) plays a role in antigen I/II protein folding and self-aggregation.

The periodontal pathogen *P. gingivalis* expresses cell surface fimbriae, encoded by *fimA*, which is critical for interactions with host cell membrane components. FimA mediates binding to epithelial cells through the interaction with extracellular matrix proteins like fibrinogen, vitronectin, and type I collagen (Murakami et al. 1996; Naito and Gibbons 1988; Nakamura et al. 1999). In a survey to connect the periodontal health status to the prevalence of specific *fimA* genotypes, a correlation was observed between *fimA* types II and IV and periodontitis patients. Type II had a prevalence of 75 % and is most likely a contributing factor to the etiology of periodontal disease (Amano et al. 2000; Amano 2003).

Interestingly, bacterial interactions with the host not only benefit colonization, but it also stimulates cell signaling and immune response. For example, *S. mutans* binding to epithelial surface receptors in the oral cavity induces the synthesis and release of proinflammatory cytokines like interleukin-6 and interleukin-8, which promote neutrophil chemotaxis and degranulation (Engels-Deutsch et al. 2003). Binding also induces expression of epithelial surface molecules such as ICAM-1 and VCAM-1 (Vernier-Georgenthum et al. 1998), which facilitate transendothelial migration of neutrophils during the inflammatory response. In contrast, *P. gingivalis* binding to cell surfaces dampens or interferes with the host-immune response and may also promote periodontal tissue destruction. *P. gingivalis* fimbriae trap ECM proteins like fibronectin and vitronectin, thereby interfering with integrin-mediated signal transduction. As a consequence, the periodontal pathogen appears to subvert the response to repair and heal compromised tissue (Scragg et al. 1999).

Therefore, surface adhesin proteins on oral bacteria do not only ensure binding to oral surfaces during biofilm development but also contribute to the virulence of the respective species and influence the capacity of the host to respond to the presence of the biofilm communities.

3.3 EPS Production

After initial attachment of bacterial cells, the next step in biofilm formation is defined by the production of EPS. The best investigated EPS in the oral cavity is glucans produced by streptococcal glucosyltransferases (Gtfs) from monomeric sugars. If dietary sucrose is available in excess, glucosyltransferase activity is intimately linked to caries development. Several oral species express Gtfs, but *S. mutans* appears to synthesize the majority of the glucans in oral biofilms. All using sucrose as substrate, three different Gtfs (GtfBCD) have been identified in *S. mutans*. GtfBCD each synthesize a chemically distinct glucan. GtfB synthesizes primarily insoluble α -1,3-linked glucans, GtfC produces a mixture of soluble (α -1,6-linked glucans) and insoluble glucans, and GtfD forms predominantly soluble glucans. Of special interest is the ability of the secreted enzymes to

associate with the salivary pellicle. GtfC and GtfD can both bind to the pellicle, GtfD through the association with alpha-amylase. The *S. gordonii* glucosyl-transferase GtfD also binds to alpha-amylase. GtfB remains bound to the streptococcal surface and is able to bind to surfaces of other bacterial species. On bacterial surfaces and the pellicle, the adsorbed Gtfs actively synthesize the respective glucan polymers, effectively rendering non-Gtf encoding species into glucan producers (Koo et al. 2013).

Adherence of streptococci to the preformed glucan polymers is facilitated by specific *S. mutans* glucan-binding proteins, mainly GbpC and GbpB. GbpC and GbpB bind cells of *S. mutans* to the mesh of glucan polymers and associated proteins forming an EPS superstructure important for morphogenesis and the 3D architecture of the oral biofilm. Subsequently, other species are able to bind to the glucans and increase the species richness of the biofilm.

The role of *S. mutans* in the oral biofilm has mainly been investigated in the context of caries development. The role of *S. mutans* in healthy conditions, however, has not been defined. *S. mutans* is found in low abundance in healthy subjects, and yet its ability to provide a mesh-like structure for attachment of several species might contribute to initial biofilm development for healthy communities. The transition from a healthy to a pathogenic cariogenic biofilm community would occur if excess dietary sucrose selects for increased abundance of *S. mutans*.

Another EPS component present in oral biofilms is extracellular DNA (eDNA), but its role in oral biofilm formation is less well studied. Several oral streptococci, including *S. mutans* (Klein et al. 2010), are able to release DNA into the extracellular environment through an autolytic process, which contributes to biofilm formation. When treated with DNase I to hydrolyze extracellular DNA, *S. mutans* biofilms grown in the presence of sucrose and starch showed a significant reduction in biomass. Growth in sucrose and starch upregulates the autolysin *lytT* gene, which correlates with presence of eDNA. Another mechanism of DNA release in *S. mutans* involves a newly identified auto-active bacteriocin, which induces cell death and release of eDNA from the bacteriocin producer (Perry et al. 2009).

Release of eDNA appears to be a highly conserved activity and other streptococci have evolved corresponding mechanisms. *S. gordonii* and *S. sanguinis* produce eDNA during aerobic growth closely associated with the production of H₂O₂ (Kreth et al. 2008). Both species release high molecular weight DNA of chromosomal origin (Kreth et al. 2009). Deletion of the gene encoding pyruvate oxidase dramatically reduced H₂O₂ production and eDNA release (Kreth et al. 2009). When H₂O₂ production is limited by growth in oxygen-limited conditions, eDNA is also reduced (Zheng et al. 2011a, b). Unlike *S. mutans*, *S. gordonii* and *S. sanguinis* only require H₂O₂ to induce the DNA release process. In *S. gordonii*, however, the major autolysin AtlS can also be involved in DNA release (Liu and Burne 2011). Deletion of AtlS in *S. gordonii* prevented autolysis and production of eDNA under aerobic conditions (Liu and Burne 2011). In contrast, under anaerobic conditions, eDNA release can be induced by H₂O₂ addition without any obvious bacterial cell lysis (Itzek et al. 2011). Streptococci may, therefore, have several mechanisms to trigger eDNA release in response to different internal and/or external stimuli.

The generation of eDNA as a consequence of aerobic growth in a H_2O_2 -dependent mechanism is consistent with the roles of *S. gordonii* and *S. sanguinis* as initial biofilm colonizers. Once the biofilm establishes and matures with *S. sanguinis* and *S. gordonii* as permanent residents, the oxygen tension inside the biofilm declines, and these species would cease to produce H_2O_2 and eDNA. At that time point in biofilm formation, the contribution of eDNA to the EPS matrix is complete.

Overall, the binding capacities of oral streptococci to the salivary pellicle, host cellular components, and other members of the oral biofilm community allow for the colonization of the oral cavity, spanning the hard surfaces of teeth and the oral mucosa. Oral biofilms are, therefore, bacterial habitats with complex host–biofilm interactions.

4 Bacterial Interactions Leading to Species Succession in Biofilms: Co-aggregation and Adhesion

Oral biofilm formation is driven by individual species that adhere initially to non-colonized surfaces. On these surfaces, the initial colonizers show clonal growth. Other species are recruited, often by co-aggregation, or serendipitously interact to form a mature biofilm community. Concurrently, co-aggregation of genetically distinct bacteria can occur in saliva and multispecies complexes can then intercalate into the maturing biofilm. Co-aggregation during biofilm formation follows a specific colonization pattern based on the compatible co-aggregation pairings between bacterial species. Co-aggregation pairs have been well investigated in vitro and in situ over the years leading to a model of spatiotemporal tooth colonization reviewed in numerous excellent articles.

In biofilms, different species reside and grow in close proximity. The localization of colonies of different species is rooted in the mutual benefits each derives on the metabolic and cell signal levels. Hence, co-aggregation of streptococci, occurring through interactions between lectin-like adhesin with receptor polysaccharide on the partner species, facilitates intergeneric cell–cell communication. Bringing heterologous species into close proximity also facilitates metabolic collaborations. For example, veillonellae are known to co-aggregate with streptococci and actinomyces. In biofilms, the three species create a complex metabolic relationship. *Veillonella* spp. are unable to metabolize carbohydrates but readily ferment organic acids like lactic acid. The common end product of streptococcal and actinomyces metabolism is lactic acid, which is used by *Veillonella* spp. for growth. At the same time removal of lactic acid prevents acidification. Acidification would inhibit the growth of the lactic acid producer itself.

The production of H_2O_2 by oral streptococci is another important factor in this ménage à trois. Some species are producers of H_2O_2 , whereas others are sensitive to the oxidative stress. Certain oral streptococci have the ability to produce H_2O_2 that selects for the integration of compatible species into the developing biofilm

community since new members must be able to cope with oxidative stress. *A. naeslundii* is inhibited by H_2O_2 produced by *S. gordonii*, but in co-aggregation cultures both species grow together in close proximity (Jakubovics et al. 2008a, b). *S. gordonii* in coculture grows more readily than *A. naeslundii*, becoming the dominant species at a ratio of about 9 to 1. Why do *S. gordonii* need *A. naeslundii* in the community? *A. naeslundii* provides the H_2O_2 -degrading enzyme catalase, which can reduce oxidative damage to surface proteins inflicted by H_2O_2 produced by *S. gordonii* (Jakubovics et al. 2008b). This example of interspecies cooperation compliments *S. gordonii*'s own mechanism to maintain the reduced state and function of the adhesins by using the oxidative repair activity of methionine sulfoxide reductase, MsrA (Lei et al. 2011). The low ratio of *A. naeslundii* to *S. gordonii* would, therefore, allow for sufficient production of H_2O_2 to inhibit susceptible species competing for the same niche. Like *A. naeslundii*, some strains of *Veillonella* also produce catalase, which would also protect *S. gordonii*. The interactions between streptococci, veillonellae, and actinomyces occur in vivo given the spatial proximity of the three species in human plaque samples (Valm et al. 2011), suggesting that the mutualisms actually occur in nature.

A striking and seemingly antagonistic relationship exists between *S. gordonii* and *P. gingivalis*. Both are aggregation partners and *P. gingivalis* can be found together during initial biofilm formation at 8 h (Diaz et al. 2006). During initial plaque formation, the environment shifts from oxygen-rich to microaerophilic. Yet, *P. gingivalis* is a strictly anaerobic species (Naito et al. 2008). During initial biofilm development, *P. gingivalis* is susceptible to oxidative stress resulting from the relatively high oxygen tension produced by inspired air and H_2O_2 produced by *S. gordonii*.

The initial interaction between oral streptococci and *P. gingivalis* is most likely mediated by *P. gingivalis* FimA and the streptococcal surface located glyceraldehyde-3-phosphate dehydrogenase (Maeda et al. 2004a, b, c). The binding increases in strength through the subsequent interaction between *S. gordonii* SspAB with the *P. gingivalis* short fimbrial protein subunit, Mfa (Demuth et al. 2001). During biofilm community development with *P. gingivalis*, several required *S. gordonii* genes were identified in a genetic screen (Kuboniwa et al. 2006). Among them is *spxB*, which encodes the pyruvate oxidase responsible for the production of H_2O_2 . Why is this *S. gordonii* gene important for the interaction with *P. gingivalis* when the gene product generates a toxic by-product? One important feature could be the metabolic function of the pyruvate oxidase. The metabolic activity of streptococcal cells appears to create an anaerobic environment by rapidly consuming oxygen. As a consequence, H_2O_2 production declines. It is tempting to speculate that the initial association of *P. gingivalis* with *S. gordonii* is not accompanied by growth of *P. gingivalis*; but once the anaerobic environment is created, *P. gingivalis* can proliferate.

The initial in vivo oral biofilm microbial community is not restricted to *P. gingivalis* and *S. gordonii* but includes other streptococci and species like *Veillonella* and *Actinomyces*. Over time, the complexity of the community

increases. The interplay between H_2O_2 and catalase production appears to be important in spatial and temporal development of the community.

Biofilm development through selective integration of species into existing microbial communities also reflects concurrent exclusion of certain other species. For example, *S. cristatus* downregulates the expression of the *P. gingivalis* *fimA* gene, which encodes the surface protein responsible for the attachment to other bacteria (Xie et al. 2000). *S. cristatus* communicates with *P. gingivalis* to exclude this species from the microenvironment. The downregulation of *fimA* requires initial contact of the two organisms and appears to depend on the presence of *S. cristatus* arginine deiminase (Lin et al. 2008). Hence, *S. cristatus* may have the ability to shape its own neighborhood by selecting its interacting partners. It is not hard to imagine how this ability could influence the acquisition of later successive colonizers in the developing biofilm.

5 Regulation of Adhesion and Biofilm Formation

When compared to planktonic conditions (when cells grow unattached to a surface), biofilm cells differentially regulate approximately 10 % of the bacterial genome, suggesting biofilm-specific regulation of gene expression (Rendueles and Ghigo 2012). For example, *S. mutans* UA159 cells in biofilms regulate about 12 % of its genome with about 139 genes activated and 104 genes repressed when compared to planktonic cells (Shemesh et al. 2007). Interestingly, most of the genes significantly downregulated were from cells in the depth of the biofilm close to the attachment surface. The selectively downregulated genes were largely involved in attachment, suggesting that those genes are involved in the transition from the planktonic to sessile phenotype, but that these genes are downregulated when no longer required for biofilm attachment or maturation (Shemesh et al. 2007). The initial step of biofilm formation and the subsequent maturation of the biofilm community are directed, therefore, by a genetically regulated developmental program.

The biofilm-specific regulation of genes seems to be specific for each species. During the transition into the biofilm phenotype, the periodontal pathogen *Aggregatibacter actinomycetemcomitans* does not downregulate any genes but upregulates about 355 open reading frames (ORF), including a large set of ribosomal genes and potential virulence genes. In contrast, *P. gingivalis* upregulated only 26 ORFs and downregulated 193 ORFs, most encoding “hypothetical” proteins (Frias-Lopez and Duran-Pinedo 2012).

The decision of planktonic cells to attach to a surface depends on environmental conditions (Petrova and Sauer 2012). Well-known environmental triggers include macrophages co-localized with planktonic bacteria and nutritional deficits (discussed above). Generally, the response to environmental signals is mediated by receptors complexed with response regulators (transcription factors). Typically these signaling systems are termed two-component systems (TCSs). Ca^{2+} is a common environmental cue and *S. mutans* regulates calcium-dependent biofilm

formation by signaling through the CiaX_{RH} three-component system. CiaX is a small, secreted peptide with a calcium-binding (SD) domain (He et al. 2008). Mutation of CiaX diminished *S. mutans* biofilm formation in vitro, probably because calcium is required to initiate biofilm developmental pathways.

Biofilm formation involves several other signal transduction systems. The TCS BfrAB is indispensable for *S. gordonii* single-species biofilm development (Zhang et al. 2004). By binding to a specific DNA consensus sequence, the response regulator BfrA directly regulates the expression of several genes, including the two ABC transporters encoded by the *bfrCD* and *bfrEFG* operons (Zhang et al. 2009). Interestingly, the DNA binding domain of *S. gordonii* BfrA is able to bind to the homologous operons identified in *S. sanguinis*, suggesting that BfrAB controls biofilm development in *S. sanguinis* as well. Two other genes controlled by BfrAB in *S. gordonii*, *bfrC* and *bfrG*, are also required for dual-species biofilm maturation with *P. gingivalis* (Kuboniwa et al. 2006). Hence, in vivo biofilm development requires coordinated signaling pathways to ensure adhesion and interaction with other species.

In general, TCSs appear to be pleiotropic regulators. Planktonic and biofilm phenotypes are influenced by more than one TCS, suggesting vigorous cross talk between individual TCSs to enable the biofilm lifestyle. For example, the TCSs VicRK, HdrRM, and CiaX_{RH} all affect *S. mutans* biofilm formation, competence, and virulence. The competence regulatory TCS ComED is also involved in biofilm development and is discussed later in Sect. 6.

The transition from planktonic to sessile cells leading to the irreversible attachment in several species has been associated with the level of the ubiquitous intracellular messenger signaling molecule bis-(3'-5')-cyclic di-GMP (c-di-GMP) (Jonas et al. 2009). The production of c-di-GMP is under the control of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs). c-di-GMP is synthesized from GTP by DGCs and degraded by PDEs (Mills et al. 2011). High c-di-GMP levels promote adhesion by the production of adhesive extracellular matrix components including fimbriae in *S. typhimurium* and *P. aeruginosa* (Kader et al. 2006; Kulasekara et al. 2005). Low intracellular levels of c-di-GMP promote motility behavior like swimming, swarming, and twitching motility (Simm et al. 2004). Inactivation of a DGC ortholog (*gcp*) in *S. mutans* also leads to impaired biofilm formation. Gcp encodes the only protein in *S. mutans* with DGC activity (Yan et al. 2010).

The oral streptococci need to adhere or become extinct. These species are not particularly invasive of soft tissues, and so to survive they clearly invest in mechanisms that facilitate adhesion to surfaces. Indeed these species may possess a fail-safe mechanism to ensure that the protein adhesins are always expressed sufficiently to withstand blockade or loss of one or more of these crucial surface proteins. For example, *S. gordonii* overexpresses two alternative, well-studied adhesins when one or more major adhesins (SspAB) are mutated and deleted (Zhang et al. 2005). Furthermore, when surface adhesins cannot be properly presented on the cell wall by deletion of the enzyme, Sortase A, virtually all of the protein adhesins are overexpressed (Nobbs et al. 2007a). Hence, the cells appear

to be genetically programmed to provide an ample abundance of surface adhesin proteins to compensate for any loss of expression or defect in processing, and the importance of adhesion and biofilm formation to the survival of these species is evident from the multiple mechanisms that sustain and regulate these functions.

6 Ecological Aspects of Oral Biofilm Development

6.1 Communication and the Consequences

Bacteria use small diffusible molecules or peptides as signals for cell-to-cell communication. Communication can be intra- and interspecies specific and aid in the coordination of bacterial population behavior as needed in biofilm formation and community development. During early biofilm formation on the tooth surface, the sparse colonization and small clusters of cell aggregates attached to the salivary pellicle influence the ability of members of the biofilm community to communicate with each other. Only cells in the immediate vicinity of a communicating cell can interact with diffusible signals or metabolites. The distance from the target cell receptors (i.e., TCSs) and concentration of the diffusible signaling molecules are crucial limiting parameters in cell-to-cell communication. For example, the importance of spatial clustering on the efficiency of diffusible signals has been suggested by mathematical modeling (Hense et al. 2007). Communication can also be influenced by intrinsic distortion in the biofilm. For example, the important *S. mutans* communication peptide CSP (see below), which regulates stress adaptation, biofilm formation, and competence development, is degraded in dual-species biofilms with *S. gordonii*. Degradation is mediated by challisin, a secreted protease. Since a challisin homologue is encoded in the genome of the early colonizer *S. sanguinis*, other oral streptococci might use similar mechanisms.

Metabolic cooperativity between members of the oral biofilm also requires communication. For example, *S. gordonii* amylase gene showed increased expression when cells grew in close proximity to *V. atypica* in a saliva-conditioned flow cell (Egland et al. 2004). *V. atypica* benefits from induction of amylase gene expression in *S. gordonii*. Amylase degrades starch to monomeric glucose, which can be fermented by *S. gordonii* to lactic acid, promoting growth of *V. atypica*. This cooperative interaction is only effective over short distances, suggesting the action of a diffusible substance (Egland et al. 2004).

Diffusible signals are also required for dual-species biofilm formation of *S. gordonii* with *P. gingivalis* and *S. oralis* with *A. naeslundii* (McNab et al. 2003; Rickard et al. 2006). Biofilm development for these pairs of species depends on the signal autoinducer 2 (AI-2). AI-2 is a cell-density-dependent signaling molecule produced by many bacterial species. Although AI-2 shows species-species structural differences, many species can sense and respond to AI-2. Therefore, AI-2 is considered as a nonspecific signaling molecule for

inter-bacterial communication (McNab and Lamont 2003). Accordingly, *S. gordonii* and *S. oralis* AI-2 mutants were not able to form a dual-species biofilm with *P. gingivalis* or *A. naeslundii* (McNab et al. 2003). In the natural setting of the oral biofilm, the metabolic signaling of AI-2 is likely to influence the response of multiple species and the structure of the developing oral biofilm.

6.2 Interspecies Antagonism

The oral biofilm is a competitive environment. Even during initial biofilm formation when colonization space is ample, competition exists. For example, the initial colonizers *S. sanguinis* and *S. gordonii* express specific cell surface adhesin molecules with similar binding specificities. Hence, they compete to bind similar sites in the salivary pellicle (Nobbs et al. 2007b). Yet, *S. sanguinis* has a greater natural prevalence in plaque and saliva, but fails to outcompete *S. gordonii* completely. *S. gordonii* apparently survives because it competes to adhere to the saliva-coated tooth more effectively with *S. sanguinis* than any other tested oral streptococci (Liljemark et al. 1979, 1981). The competitive advantage of *S. gordonii* was attributable to expression of the sialic-acid-binding protein Hsa based upon mutational analysis (Nobbs et al. 2007b). This surface adhesin enables *S. gordonii* to successfully compete for binding to the tooth surface with the genetically similar, but more abundant *S. sanguinis*. Hence, the efficiency of adherence to certain salivary components is crucial in the competitive oral environment.

Several oral bacteria can also express antimicrobial peptides (bacteriocins) to antagonize competing species. Interspecies antagonism of *S. sanguinis* or *S. gordonii* with *S. mutans* has been modeled with a specific deferred antagonism assay, and the outcome is determined by the sequence of colonization, nutritional availability, and environmental pressures (Kreth et al. 2005a, 2008). All three streptococcal species produce small chromosomally encoded bacteriocins (Deng et al. 2004; Fujimura and Nakamura 1979; Schlegel and Slade 1972). Bacteriocins of *S. mutans* have been well characterized (84, 99), and a recent review highlights the sophisticated regulation of their production (Merritt and Qi 2012; Qi et al. 2000, 2001). The production of certain bacteriocins by *S. mutans* is controlled in a cell-density-dependent manner by the ComCDE system that controls competence development and biofilm formation (Heng et al. 2007; Kreth et al. 2005b). One of the distinctive features of bacteriocins is the target range of susceptible bacteria. The bacteriocins produced by *S. mutans* are able to inhibit *S. sanguinis* and *S. gordonii* among other oral streptococci (Kreth et al. 2005a). Conversely, the bacteriocins produced by *S. gordonii* and *S. sanguinis* do not target *S. mutans*. Nonetheless, *S. gordonii* and *S. sanguinis* are able to inhibit the growth of *S. mutans* by generating an alternative antimicrobial compound, H₂O₂ (Kreth et al. 2005a), as described above in Sect. 4.

During initial colonization when the cell density is relatively low, H_2O_2 production might be ecologically advantageous relative to bacteriocin production, which requires high cell density to trigger bacteriocin gene expression. H_2O_2 production is oxygen dependent (Kreth et al. 2008), and the oxygen tension in saliva is sufficient to allow for aerobic respiration and hydrogen peroxide production during initial colonization (Marquis 1995). In addition, both *S. sanguinis* and *S. gordonii* are only weakly inhibited by their own H_2O_2 production. Once the biofilm reaches a critical thickness and cell density, diffusion becomes limited and the oxygen tension declines. The decline in oxygen tension might decrease H_2O_2 production to a non-inhibiting level. Under these biofilm conditions, *S. mutans* might use bacteriocins to inhibit *S. sanguinis* and *S. gordonii*, since bacteriocin production is oxygen independent (Kreth et al. 2008). In high-cell-density conditions, *S. mutans* becomes an aggressive competitor for *S. sanguinis* and *S. gordonii*, which may explain why it is able to initiate a shift in composition to the community to create a caries-promoting environment.

Bacteriocins target the membrane of the susceptible species and cause leakage of the cell contents (Oppegard et al. 2007). To effectively compete with *S. sanguinis* and *S. gordonii*, *S. mutans* coordinates bacteriocin production with another important cell function, competence development (see below) (Kreth et al. 2005b; van der Ploeg 2005). Competence is the natural ability of some oral streptococci to incorporate extracellular DNA from the environment (Cvitkovitch 2001). Under the control of the *S. mutans* competence system, mutacin IV is produced before inducing the expression of genes required for DNA uptake. In this way, *S. mutans* uses mutacin IV to kill the target organism and release the cellular contents, including DNA. Subsequently, *S. mutans* cells become competent and take up the released DNA (Kreth et al. 2005b). Although not beneficial for the lysed bacterial species, this interspecies genetic exchange tends to promote evolutionary survival of traits of the target species in the daughter cells of competent *S. mutans*.

6.3 Competence and Genetic Exchange

Competence-dependent genetic exchange requires availability of eDNA and the ability of the competent species to take up DNA from the environment. The development of competence is signaled by the accumulation of a species-specific competence peptide, which is produced and secreted into the environment (competence-stimulating peptide or CSP). CSP serves as a signal to induce competence in the producing population and serves as a form of intraspecies (or interspecies) communication (Johnsborg and Havarstein 2009).

Each streptococcal species produces a unique CSP (Whatmore et al. 1999). Competence development facilitates cell-density-dependent cell regulation but might also reflect other environmental conditions like flow rates and diffusion from the producing species. Competence development follows a basic architecture in most streptococci. Competence development is encoded in the *comCDE* operon,

including CSP and the TCS ComDE, which senses and provides transcriptional response to the environmental peptide. Secreted CSP binds to the membrane-spanning ComD sensor kinase (a receptor). Binding of CSP induces a conformational change in ComD, which activates the intracellular kinase domain and promotes subsequent ComD autophosphorylation (P-ComD). ComE is the response regulator and the recipient of the transferred phosphoryl group from P-ComD. P-ComE is able to bind to chromosomal DNA to an imperfect direct repeat (ComE-box) (Ween et al. 1999). This specific binding motif (ComE-box) is found in the promoter region of several genes considered to be early competence genes. The early genes include the *comCDE* operon itself and the *comAB* operon. The *comAB* operon encodes the CSP-specific transporter ComAB, which processes and exports the pre-peptide CSP as the CSP. The secreted CSP, therefore, signals an increase in its own expression and production, secretion, and sensing via ComDE (Claverys et al. 2006; Johnsborg et al. 2007).

Another early competence gene, the gene encoding the alternative sigma subunit ComX of the DNA-dependent RNA polymerase, is transcribed. ComX is essential for competence development and regulates other genes required for the synthesis of the DNA uptake system and recombination. ComX recognizes a unique DNA element named *cin*-box important for the activation of late competence genes (Lee and Morrison 1999).

Competence development in *S. sanguinis* and *S. gordonii* seems to follow this blueprint first described in *S. pneumoniae*. After CSP is added to non-competent cell cultures, *S. gordonii* develops transient competence peaking at 10 to 20 min (Vickerman et al. 2007). After 5 min of exposure, the initial responsive genes are *comCDE*, *comAB*, and the *comX* homologue *comR*. Regulated by ComR, late genes are expressed including genes encoding proteins required for uptake and DNA recombination. Competence declines about 30 to 40 min after CSP addition (Vickerman et al. 2007).

In *S. sanguinis*, the ComAB homologues are not encoded to transport and process CSP. The pre-peptide lacks the double glycine processing site in the N-terminal leader sequence as found in ComAB processed CSPs of other streptococci (Rodriguez et al. 2011; Xu et al. 2007). The usage of DNA microarray technology enabled the identification of other genes not initially identified in the competence developmental circuit. Addition of CSP to *S. sanguinis* induced about 122 genes and downregulated about 83, compared to *S. gordonii* with 162 induced and 89 downregulated genes. However, the number of genes belonging to the early competence genes was considerably lower in *S. sanguinis* with only five genes compared to 28 for *S. pneumoniae* and 35 for *S. gordonii*. The group of early genes in *S. sanguinis* contained *comDE*, *comX*, and two uncharacterized open reading frames, suggesting that this constitutes a minimal set of early competence genes (Rodriguez et al. 2011; Xu et al. 2007).

The high-cell-density oral biofilm seems to be a favorable environment for genetic exchange (Li et al. 2002b). Several active mechanisms are employed by oral bacteria to ensure the availability of eDNA. *S. gordonii* and *S. sanguinis* release homologous and heterologous DNA in a H₂O₂-dependent process, and *S. mutans* is

able to release its own DNA and DNA from closely related species in a bacteriocin-dependent process (Kreth et al. 2005b, 2008, 2009; Perry et al. 2009). The biofilm matrix contains eDNA, which might be a source for competent bacteria (Flemming and Wingender 2010). Oral bacteria are able to take up DNA during competence development, including the abundant oral biofilm genus *Streptococcus* and the periodontal pathogen *P. gingivalis*. Interestingly, a recent study with *P. gingivalis* demonstrated that natural competence is the dominant form of chromosomal DNA transfer in this perio-pathogen (Tribble et al. 2012). Indeed, eDNA is found in *P. gingivalis* biofilms, further indicating that this EPS compound might have multiple functions during biofilm development. Since competence development is density dependent, the high-cell-density conditions in biofilms favor genetic exchange.

S. mutans, *S. sanguinis*, and *S. gordonii* each show a competence-influenced biofilm phenotype (Bizzini et al. 2006; Li et al. 2002b; Zhu et al. 2011). Although the biofilm community seems to be better protected from outside environmental perturbation than planktonic cells, the biofilm cannot afford to be evolutionarily idle. Competence allows for a long-term adaptation due to the incorporation of new genetic traits via horizontal gene transfer (Roberts and Kreth 2014). Not only is horizontal gene transfer important in the evolution of bacterial genomes, it also allows bacteria to repair DNA damage via homologous recombination, a mechanism to swap and replace damaged segments of DNA with correct versions. Uptake of eDNA, therefore, appears to promote species diversity and adaptation using information stored in the DNA of any targeted bacterial species in the oral cavity.

S. sanguinis and *S. gordonii* control the expression of several other genes not involved in the uptake of DNA in a CSP-dependent manner. For example, contact with antibiotics can induce competence development. Consequently, it is now widely accepted that the competence system is part of a general stress response for oral streptococci to adapt to changing environmental conditions.

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