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INVITED MEDICAL REVIEW

The road to ruin: the formation of disease-associated oral biofilms

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The colonization of oral surfaces by micro-organisms occurs in a characteristic sequence of stages, each of which is potentially amenable to external intervention. The process begins with the adhesion of bacteria to host receptors on epithelial cells or in the salivary pellicle covering tooth surfaces. Interbacterial cell–cell binding interactions facilitate the attachment of new species and increase the diversity of the adherent microbial population. Microbial growth in oral biofilms is influenced by the exchange of chemical signals, metabolites and toxic products between neighbouring cells. Bacterial cells on tooth surfaces (dental plaque) produce extracellular polymers such as complex carbohydrates and nucleic acids. These large molecules form a protective matrix that contributes to the development of dental caries and, possibly, to periodontitis. The identification of key microbial factors underlying each step in the formation of oral biofilms will provide new opportunities for preventative or therapeutic measures aimed at controlling oral infectious diseases.

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Introduction

Bacteria like to live at surfaces. Whether it is the air–liquid interface at the top of a pond or the solid substratum of a human tooth, surfaces tend to concentrate nutrients and provide a stable habitat that allows the development of organized microbial communities. When it became clear that the behaviour of surface-associated bacteria cannot be predicted from observations made on micro-organisms cultured in rich laboratory broths, a new term for describing sessile microbial populations was introduced and the scientific topic of 'biofilm research' was born. Biofilms are defined as communities of microbial cells and associated extracellular polymeric substances that are present at an interface. In many cases, biofilm bacteria are up to 1000 times more resistant to antimicrobial agents than planktonic cells. Resistance is mediated by tough intercellular matrices, slow growing cells and the up-regulation of antimicrobial systems in biofilm cells (Zhang and Mah, 2008). In addition, mechanisms for the tight adhesion of bacteria to underlying substrata impede the efficient removal of biofilms by physical or chemical means.

In the human oral cavity, biofilms form on the surface of hard and soft tissues, and sometimes both (for example, subgingival dental plaque). Bacteria in oral biofilms that are exposed to the mouth are frequently removed, either by shedding of epithelial cells or by mechanical shear from the movement of tongue and cheeks or from toothbrushing. However, oral bacteria are remarkably well adapted to recolonizing host surfaces. Colonization of clean enamel surfaces begins within minutes, and after a few hours, extensive microbial deposition can be observed (Palmer *et al*, 2006). The development of oral biofilms depends on interactions between bacterial cell-surface adhesins and host receptors. Bacteria–bacteria interactions promote further colonization and lead to complex microbial communities, in many cases consisting of 50 or more different species of bacteria at a single location (Aas *et al*, 2005). Chemical communication between bacteria is critical for the stable coexistence of different species within oral biofilms. Maturation of biofilms is associated with the production of an extracellular matrix composed of polysaccharides and other macromolecules such as nucleic acids. The accumulation of oral biofilms on tooth surfaces (dental plaque) can lead to the development of dental caries or periodontitis, two of the most common diseases in humans. Interactions between different species of bacteria, and between bacteria and the host, are central to the development of oral biofilms. A

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detailed understanding of the key steps in the formation of these biofilms will provide new opportunities for intervention against oral diseases of microbial origin.

Adhesion, coadhesion and coaggregation

Adhesion of bacteria to the salivary pellicle represents the first step in the colonization of enamel surfaces in the mouth (Figure 1). Oral streptococci have been consistently shown to be the major primary colonizers of clean enamel surfaces, and these organisms constitute 60–80% of dental plaque bacteria within 4 to 8 h (Nyvad and Kilian, 1987, 1990; Diaz *et al*, 2006; Dige *et al*, 2009). Oral viridans streptococci express numerous protein and lipoprotein adhesins on the cell surface. For example, the genome of *Streptococcus sanguinis* encodes at least 93 polypeptides that are predicted to be anchored on the surface of cells and potentially may be involved in adhesion (Xu *et al*, 2007). This battery of cell-surface exposed molecules endows streptococci with an unusually broad capacity for binding human or bacterial receptors. For a comprehensive description of adhesion and colonization by streptococci, the reader is referred to an excellent recent review (Nobbs *et al*, 2009).

There is undoubtedly some functional redundancy between different cell-surface adhesins, and not all adhesins are necessarily present in every strain of a given streptococcal species. However, certain adhesin proteins are widely conserved in oral streptococci and these polypeptides are likely to play key roles in adhesion to oral surfaces. For example, the antigen I/II (AgI/II) family of proteins are expressed by commensal oral streptococci such as *S. gordonii*, *S. oralis* and *S. sanguinis*, by cariogenic mutans streptococci (*S. mutans* and *S. sobrinus*) and by extra-oral streptococci such as *S. pyogenes* (Jakubovics *et al*, 2005b; Zhang *et al*, 2006). Polypeptides of the AgI/II family are ~160–180 kDa and have a number of characteristic structural domains (Figure 2a). AgI/II proteins play a central role in binding to salivary agglutinin glycoprotein gp340 (Jakubovics *et al*, 2005a,b) (Figure 2b). When gp340 is integrated into the acquired enamel pellicle, binding promotes adhesion of streptococci. However, gp340 is also present in the fluid phase and here interactions with streptococcal AgI/II proteins result in aggregation of bacterial cells. Large bacterial aggregates do not adhere well to surfaces (Liljemark *et al*, 1981) and are removed from the mouth by swallowing. AgI/II proteins may also influence binding to soft tissues. *Streptococcus gordonii* produces two AgI/II polypeptides, SspA and SspB, and these have been shown to interact with extracellular matrix proteins, collagen and fibronectin (Heddle *et al*, 2003; Jakubovics *et al*, 2009).

There is strong evidence that adherent streptococci on oral surfaces recruit other bacteria to the biofilm. Streptococci interact with a number of isolated oral bacteria *in vitro* and are frequently observed in close association with non-streptococcal bacteria in dental plaque. Adhesive interactions between bacteria can be observed in the laboratory by vortex mixing dense suspensions of cells. Within 10 s, cell–cell adhesion results in the formation of large clumps, or coaggregates, which are visible to the naked eye. Coaggregation only occurs between compatible partner organisms, and is dependent on the presence of cell-surface adhesins on one cell type and cognate receptors on the other. Similar adhesion-receptor interactions may facilitate the binding of planktonic bacteria to immobilized cells on the tooth surface, and this process is termed coadhesion. Streptococcal AgI/II proteins are among a number of bacterial cell-surface molecules that have been shown to mediate coaggregation and coadhesion. Not all AgI/II proteins are equivalent in this regard. Thus, *S. gordonii* SspB protein mediates a strong coaggregation interaction with *Actinomyces oris* T14V (Figure 2c), whereas SspA polypeptide does not recognize this strain (Egland *et al*, 2001; Jakubovics *et al*, 2005b). Conversely, SspA and a number of other AgI/II proteins from different species of *Streptococcus* promote coaggregation with *Actinomyces naeslundii* PK606. By producing two distinct AgI/II proteins, *S. gordonii* increases its range of potential coaggregation partners. *Streptococcus gordonii* AgI/II proteins also bind to the periodontal pathogen *Porphyromonas gingivalis* and this interaction enables *P. gingivalis* to attach to pre-existing *S. gordonii* biofilms (Lamont *et al*, 2002). In addition, the AgI/II polypeptides of *S. gordonii* recognize receptors on the surface of *Candida albicans* hyphae (Bamford *et al*, 2009) (Figure 2d).

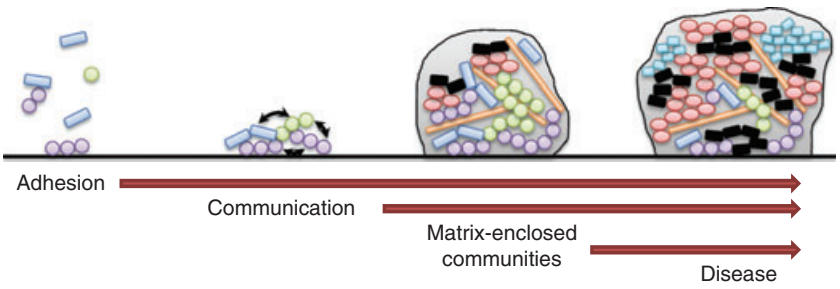

Figure 1 Stages in the formation of dental plaque. Colonization of tooth surfaces is initiated by bacterial adhesion to the salivary pellicle. Coaggregation and coadhesion facilitate the development of multispecies communities. The exchange of chemicals between neighbouring bacteria promotes co-operation or competition. In some cases, communication does not occur until a critical biomass is reached. Adherent bacteria produce a matrix of complex carbohydrates and/or extracellular nucleic acids, which helps to bind the biofilm together and to protect the encased cells. Dental caries or periodontitis arises from a shift in the microflora and an accumulation of pathogenic bacteria

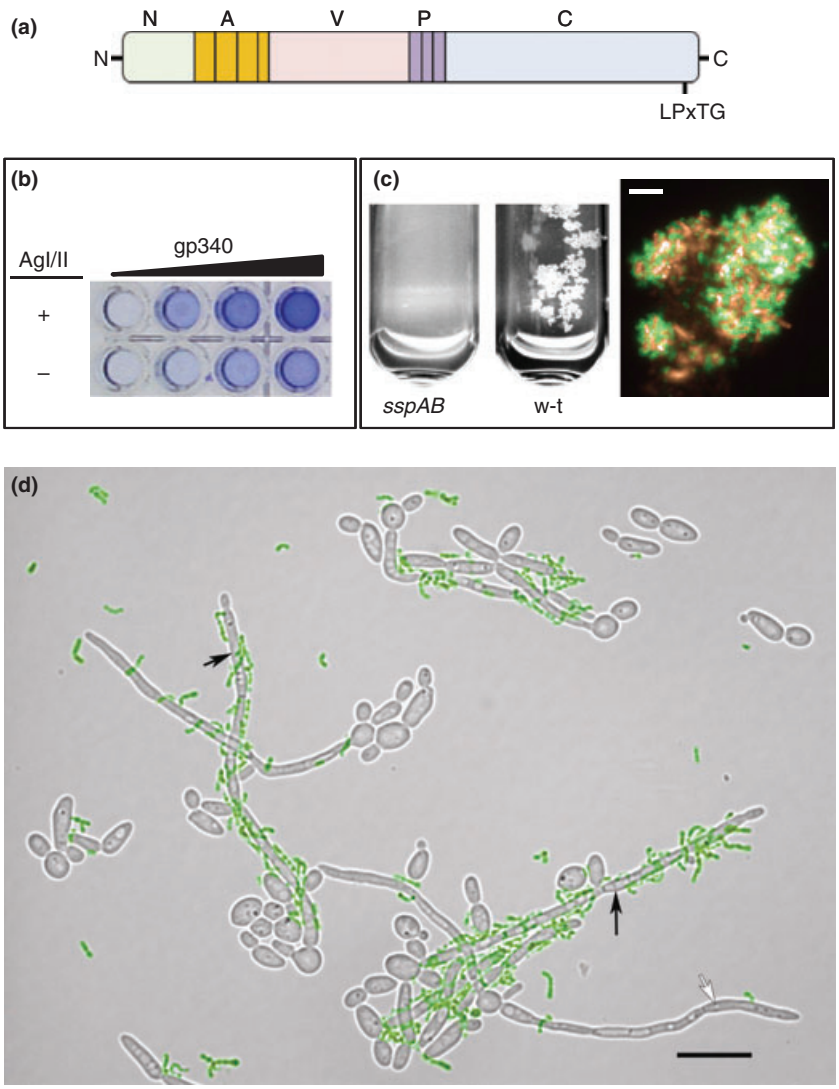

Figure 2 Adhesive interactions mediated by streptococcal cell-surface antigen I/II (AgI/II) proteins. (**a**) AgI/II polypeptides are ~160–180 kDa and are composed of the following structural domains: An *N*-terminal region containing a signal for secretion across the cell membrane, three to four repeated Alanine-rich sequences, a central variable region, approximately three repeated proline-rich elements and a C-terminal domain containing an LP × TG motif that is a substrate for sortase-mediated cross-linking to cell wall peptidoglycan. (**b**) Disruption of *sspA* and *sspB* genes, encoding AgI/II proteins in *S. gordonii*, reduces adhesion to immobilized salivary agglutinin glycoprotein gp340. Bacteria that are bound to gp340 coated on the surface of microtitre wells were visualized by staining with crystal violet. Note that the AgI/II mutant retains some capacity for gp340 binding because of a functionally redundant adhesin, Hsa. (**c**) AgI/II proteins also mediate coaggregation with *A. oris*. In a test tube, wild-type *S. gordonii* forms macroscopic coaggregates when mixed with *A. oris*, whereas an *sspAB* mutant does not. Microscopically, *S. gordonii* (green) and *A. oris* (orange) appear well dispersed throughout coaggregates. Bar = 10 μm. (**d**) *S. gordonii* AgI/II proteins are involved in adhesion to *C. albicans* hyphae. Some hyphae bear many receptors for streptococci (black arrows), whereas others appear nearly devoid of streptococcal binding (white arrow). Bar = 10 μm. Image D was kindly provided by L.C. Dutton and H.F. Jenkinson, University of Bristol

Coaggregation between oral bacteria has been studied in detail and over a thousand pairwise interactions have been investigated (Kolenbrander *et al*, 2006). *Fusobacterium nucleatum* is the most promiscuous coaggregation partner identified to date, and strains of *F. nucleatum* coaggregate with almost all other oral bacteria. *Fusobacterium nucleatum* is obligately anaerobic and is not usually found in nascent supragingival dental plaque. However, levels of *F. nucleatum* increase when plaque grows below the gum line and this organism can make up 20% or more of the bacteria in subgingival dental plaque (Suzuki *et al*, 2004). *Fusobacterium nucleatum* appears to act as bridging organism by binding to both early colonizers such as streptococci, and later colonizers including periodontal pathogens. Recently, a large (350 kDa) cell-surface protein of *F. nucleatum*, RadD, has been shown to mediate arginine-inhibitable coaggregation with *S. sanguinis* (Kaplan *et al*, 2009). Unlike the wild type, an *F. nucleatum radD* knockout mutant did not form a structured biofilm with *S. sanguinis*, indicating that, in this system, coaggregation interactions direct the spatial distribution of the partner organisms in biofilms.

In the early stages of biofilm formation, adhesion is the primary interaction between bacterial cells. Adhesin-receptor interactions hold the partner organisms in juxtaposition. Subsequent growth and further coadhesion of bacteria from saliva increase the local cell density and lead to the development of microenvironments within the biofilm. Within these close-knit microbial communities, metabolic products and signalling molecules produced by cells of one species influence neighbouring bacteria. Sometimes, the result is interspecies competition to the detriment of one or both organisms. However, many interactions between oral bacteria are mutually beneficial. It is becoming clear that communities of bacteria are better adapted to growth in saliva than single species of bacteria in isolation.

Interbacterial interactions

Historically, the analysis of microbial traits has begun with the isolation of bacteria in pure cultures. In dental plaque, however, most bacteria are in close proximity to cells of different species and interbacterial interactions have profound effects on the participating organisms.

To understand which genes and proteins are critical for the survival and growth of bacteria in oral biofilms, it is necessary to analyse mixed cultures of bacteria. Given that mature dental plaque may contain 100 or more different species of bacteria, the task of identifying relevant interspecies interactions is daunting. Nevertheless, some progress in this area has been made in recent years. The development of high-throughput techniques such as microarrays and next-generation sequencing promises to enhance significantly the pace of research on mixed-species populations.

Competition

Competitive interactions between oral bacteria can be demonstrated easily *in vitro*. For example, many oral streptococci including *S. mutans* and *S. salivarius* produce antimicrobial peptides (bacteriocins) that have bactericidal activity against a range of oral strains (van der Ploeg, 2005; Hyink *et al*, 2007). In *S. mutans*, production of bacteriocins is coordinated with the development of competence, a state in which the cells can take up and incorporate extracellular DNA (eDNA) from the environment (van der Ploeg, 2005; Kreth *et al*, 2006). Therefore, bacteriocin secretion potentially benefits the producing organism by providing DNA. The DNA may be incorporated into the chromosome and used as a source of new genetic information, or it may be broken down for energy. Alternatively, eDNA produced by the action of bacteriocins may remain in the biofilm and act as a stabilizing matrix molecule. In fact, eDNA is apparently so important to *S. mutans* that this organism has evolved a mechanism to release DNA from a proportion of its own population when other bacteria are not available. This apparently altruistic process involves the induction of an intracellular bacteriocin, CipB (mutacin V), in ~1% of the population in response to competence stimulating peptide (Perry *et al*, 2009). Disruption of the gene encoding CipB significantly reduced competence for genetic transformation in the population as a whole, indicating that CipB-dependent autolysis is important for the transfer of genetic material between *S. mutans* cells.

Most oral streptococci belong to the 'viridans' group, named from their production of a greenish tinge when cultured on blood agar. This arises from hydrogen peroxide (H_2O_2), which is secreted by streptococci and bleaches the haemoglobin in blood. H_2O_2 is non-polar and can cross bacterial cell membranes and oxidize intracellular macromolecules including lipids, DNA and proteins, causing stress or cell death. The streptococci that produce H_2O_2 are relatively resistant to its effects. By contrast, other oral bacteria are killed or prevented from growing by streptococcal H_2O_2 (Holmberg and Hallander, 1973; Jakubovics *et al*, 2008b). It is not clear whether sufficient concentrations of H_2O_2 for antimicrobial activity accumulate in supragingival dental plaque, where small molecules are continually washed out of the biofilm. However, antimicrobial activity of H_2O_2 may be important during the formation of subgingival dental plaque, which is contained within an enclosed space. The presence of an oxidizing agent will restrict the growth of anaerobic bacteria including periodontal pathogens such as *P. gingivalis*. Concordantly, levels of the peroxidogenic streptococcus, *S. sanguinis* are correlated with the absence of periodontal disease (Stingu *et al*, 2008; Colombo *et al*, 2009).

During the transition from oral health to disease, the microbial composition of dental plaque shifts from predominantly commensal bacteria to a population containing a large proportion of disease-associated organisms (Marsh, 2006). In the case of dental caries, the shift in population involves a reduction in the diversity of micro-organisms at active caries sites (Aas *et al*, 2008), because of the production of hydrogen ions by acidogenic bacteria. The drop in pH is essentially a competitive factor that favours the survival and growth of aciduric and acidogenic organisms such as mutans streptococci, lactobacilli and bifidobacteria. Although pH-driven competition is most easily seen in mature dental plaque associated with active caries lesions, the onset of competition probably arises much earlier. The early colonizing streptococci such as *S. sanguinis*, *S. gordonii*, *S. mitis* and *S. oralis*, produce acid from sugars, albeit generally to a lesser extent than *S. mutans* or *S. sobrinus*. It has been speculated that low levels of acid production by non-mutans streptococci prepare the ground for the incorporation of more acidogenic organisms into the biofilm (Takahashi and Nyvad, 2008).

Mutualism

Saliva is the primary nutrient for supragingival dental plaque bacteria. Dietary carbohydrates play an important role in the development of caries but do not have a major impact on health-associated dental plaque (Bowden and Li, 1997). *In vitro*, very few oral bacteria in monoculture can utilize saliva as the sole source of nutrients. Dental plaque as a whole, however, can degrade host mucins and other salivary components efficiently (Wickström and Svensäter, 2008; Wickström *et al*, 2009). In chemostat studies, microbial consortia composed of five different species were able to grow on hog gastric mucin, a model for human salivary mucins (Bradshaw *et al*, 1994). Growth was enhanced when additional oral bacteria with novel catabolic capabilities were introduced into the system. These data demonstrate that complex communities are better equipped for growth on saliva than individual species in isolation.

During the first few hours of dental plaque accumulation on cleaned enamel surfaces, bacterial cells can be observed in small communities, composed of two or three different species (Chalmers *et al*, 2008). Is there sufficient microbial diversity in these micro-communities to permit mutualistic growth of the interacting organisms? To address this question, a number of recent studies have investigated the abilities of two- or three-species partnerships of isolated micro-organisms to grow in saliva-fed biofilms (Periasamy *et al*, 2009; Periasamy and Kolenbrander, 2009a,b; 2010). Interestingly, many examples of mutualistic communities were identified, where mixed-species populations grew in

salivary biofilms, whereas the individual species in monoculture did not. For example, in anaerobic biofilms, growth of the periodontal pathogen *P. gingivalis* was promoted by pairing with all but one of six species tested (Periasamy and Kolenbrander, 2009b). Only *S. oralis* was incompatible with *P. gingivalis*. Even here, inclusion of *S. gordonii* in addition to *S. oralis* and *P. gingivalis* resulted in mutualistic growth of all three organisms. Clearly, there is a degree of specificity in mutualistic associations between oral bacteria. This was further demonstrated when interactions between *S. oralis* and *F. nucleatum* were investigated (Periasamy *et al*, 2009). *Streptococcus oralis* and *F. nucleatum* coaggregate, but this interaction does not lead to the growth of *F. nucleatum*. However, when *A. oris* (*A. naeslundii*), which coaggregates with both *S. oralis* and *F. nucleatum*, was introduced into the system, *F. nucleatum* grew. Such studies have shown that there are many productive interactions that occur between oral bacteria, but at present, it is difficult to predict which organisms will form mutually beneficial partnerships. The next challenge will be to identify the molecular basis of these interactions to inform the development of new measures for oral biofilm control.

There is evidence that both metabolic interactions and the exchange of signalling molecules (discussed below) contribute to mutualism in oral biofilms. Several nutritional interrelationships have been identified between oral bacteria including, for example, the production and utilization of lactic acid. Lactic acid is a metabolic waste product of streptococci and lactobacilli and, on the other hand, it is a major energy source for *Aggregatibacter actinomycetemcomitans* and veillonellae. *Aggregatibacter actinomycetemcomitans* can utilize lactate produced by oral streptococci for growth. Thus, in medium containing sucrose as the sole carbon source, *A. actinomycetemcomitans* did not grow in monoculture, but was able to grow in the presence of *S. gordonii* because of the streptococcus-mediated conversion of sucrose to lactate (Brown and Whiteley, 2007). Similarly, *Veillonella parvula* utilizes lactate produced by *Streptococcus salivarius* and, in doing so, stimulates glycolysis in the streptococcus (Hamilton and Ng, 1983). In biofilms grown in flowcells with saliva as the sole source of nutrients, *Veillonella* sp. participates in numerous mutualistic partnerships including with the lactate-producing organism *S. oralis*. Neither *S. oralis* nor *Veillonella* sp. grew in monoculture in this model. However, when inoculated together, both organisms increased 8-fold resulting in a nearly confluent biofilm (Periasamy and Kolenbrander, 2010). In addition, *Veillonella* sp. overcame the incompatibility of *P. gingivalis* and *S. oralis*. In three-species biofilms containing *Veillonella* sp., *P. gingivalis* and *S. oralis*, all organisms grew (Figure 3). Measurements of lactate concentration in the biofilm effluent demonstrated that *Veillonella* sp. consumed residual lactate in the saliva and also removed lactate produced by *S. oralis* (Periasamy and Kolenbrander, 2010). These data indicate that lactate consumption is likely to be one key driving force for mutualistic associations between oral biofilm bacteria.

Communication

Many interspecies interactions between oral bacteria lead to changes in gene expression in one or both of the partner organisms. The identification of genes that are regulated by neighbouring bacteria will help us to understand the microbial factors and processes that become important when bacteria form mixed-species communities. In the case of dual-species interactions between veillonellae and streptococci, gene regulation apparently affects carbohydrate storage in the streptococcus. Thus, the *amyB* gene of *S. gordonii*, encoding α-amylase, was up-regulated in batch culture with *V. atypica* (Egland *et al*, 2004). Placing a dialysis membrane between the streptococci and the veillonellae did not prevent *amyB* gene regulation, indicating that regulation occurs in response to a diffusible signal from *V. atypica*. Using a green fluorescent protein fusion construct, *amyB* expression was shown to be up-regulated in *S. gordonii* cells juxtaposed with *V. atypica* in flowcell biofilms (Egland *et al*, 2004). Therefore, even in a flowing system where signals are washed out of the biofilm, communication was possible at short range. The signal produced by *V. atypica* remains to be identified. However, it is likely to be closely linked to carbon metabolism as sensing is mediated by the *S. gordonii* carbon catabolism regulator CcpA (Johnson *et al*, 2009). Increased expression of *S. gordonii* α-amylase likely leads to mobilization of energy reserves, stored as intracellular polysaccharides, and thus increases the availability of lactic acid for *V. atypica*.

In some circumstances, it is the formation of structured communities, rather than the presence of another organism *per se*, that leads to gene regulation. For example, the expression of 23 genes was changed >3-fold in *S. gordonii* cells following coaggregation with *A. oris* (Jakubovics *et al*, 2008a). Coculture of *S. gordonii* with *A. oris* was not sufficient for gene regulation; changes in gene expression were observed only in cultures where coaggregation had been induced by vigorously mixing dense cell suspensions before diluting with growth medium. Nine of the 23 genes that were regulated in this system were involved in arginine metabolism. *Actinomyces oris* was shown to stabilize *S. gordonii* arginine biosynthesis and to enable the growth of *S. gordonii* in low (<0.1 mM) arginine. Coaggregation was essential for this effect. In cocultures without induced coaggregation, growth of *S. gordonii* was not observed until coaggregates had formed spontaneously >9 h after inoculation. At present, it remains unclear whether *S. gordonii* responds to cell–cell contact directly, or whether a quorum sensing system is involved.

There is strong evidence that oral bacteria sense and respond to high cell densities. Thus, autoaggregation of *F. nucleatum* led to changes in the expression of almost 100 genes, many of which encoded nutrient transporters or carbohydrate metabolism enzymes (Merritt *et al*, 2009). The artificial induction of high cell densities by centrifugation of cells caused a similar pattern of gene regulation. Centrifugation has also been employed to

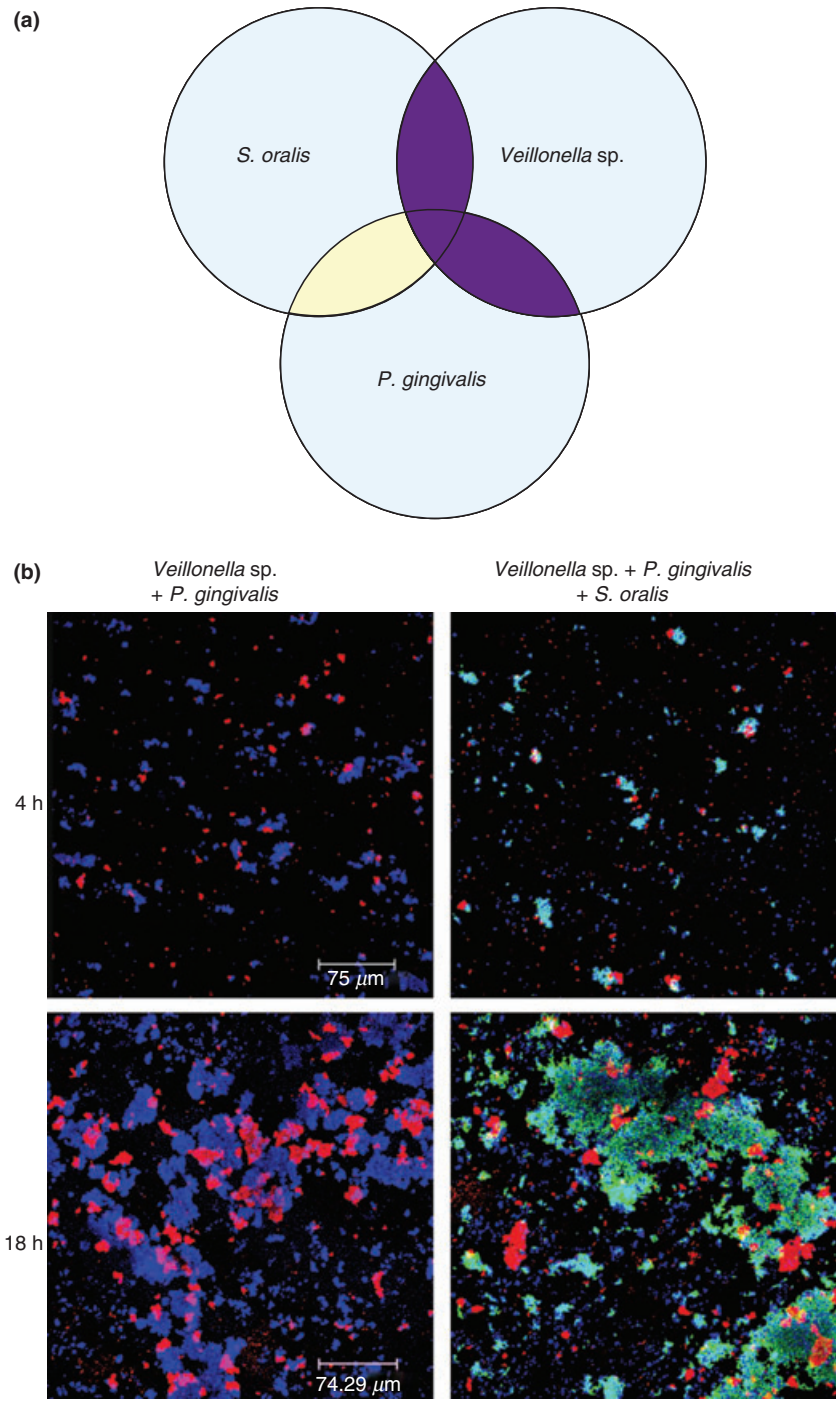

Figure 3 Mutualistic interactions between three species in saliva-fed biofilms. Monocultures of *S. oralis*, *Veillonella* sp. or *P. gingivalis* grew poorly in flowcell biofilms with saliva as the sole source of nutrients. (**a**) Cocultures of *S. oralis* and *Veillonella* sp. or of *P. gingivalis* and *Veillonella* sp. exhibited mutualism: within 18 h, the biomass of each partner in these interactions had increased significantly over the initial inoculum (indicated by purple colour). Mutualism was not observed in the dual-species pairing of *S. oralis* and *P. gingivalis* (yellow). However, including *Veillonella* sp. in addition to these organisms resulted in mutualistic growth of all three species. (**b**) Mutualistic interactions in biofilms observed by confocal microscopy. Species were differentiated by staining with fluorophore-conjugated antisera. In the left panels, *Veillonella* sp. appears blue and *P. gingivalis* is red. In the right hand panels, *S. oralis* appears blue, *Veillonella* sp. is green and *P. gingivalis* is red. Strong growth of each organism can be seen between 4 h and 18 h. Images in **b** were supplied by S. Periasamy

create high-density populations of *S. mutans*. In these cultures, two bacteriocins and at least six other genes were found to be cell density regulated (Merritt *et al*, 2007). Of note, a two component system, *hdrRM*, involved in the development of competence and bacteriocin production, was induced in densely packed cells. The coordinated production of bacteriocins and competence genes in dense cell populations represents an efficient mechanism for optimizing DNA acquisition from juxtaposed cells in microbial communities (Okinaga *et al*, 2010).

The species-specific quorum sensing regulators N-acyl homoserine lactones (autoinducer-1) have not been identified in oral bacteria. However, many oral microorganisms produce and/or respond to the interspecies signal autoinducer-2 (AI-2). Autoinducer-2 is the collective term given to a number of molecules that spontaneously form an equilibrium when 4,5-dihydroxy-2,3-pentanedione (DPD) is dissolved in water. Bacteria produce AI-2 during amino acid metabolism as a product of the enzyme encoded by the *luxS* gene. In cocultures of *S. oralis* and *A. oris* (*A. naeslundii*), AI-2 is

essential for mutualistic biofilm growth with saliva as the sole source of nutrients (Rickard *et al*, 2006). Neither *S. oralis* nor *A. oris* grew well in monoculture. When inoculated together into a flowcell, however, the organisms formed profuse biofilms. A *luxS* mutant of *S. oralis* was unable to grow in monoculture or in coculture with *A. oris*. Growth was restored by genetic complementation of the *luxS* mutation or by the addition of exogenous AI-2. Interestingly, AI-2 was only effective over a limited concentration range. The optimal concentration for mutualistic growth was 0.8 nM; concentrations in excess of 8 nM were significantly less effective (Rickard *et al*, 2006). These data suggest that bacteria have sensitive mechanisms to tune responses to the prevailing concentration of AI-2 surrounding the cell. Recently, techniques have been developed for the sensitive measurement of AI-2 in saliva (Campagna *et al*, 2009). Whole saliva from eight volunteers contained between 200 nM and 1000 nM AI-2, concentrations far in excess of those supporting mutualism between *S. oralis* and *A. oris*. It is likely that salivary AI-2 concentrations are much lower at the start of plaque accretion, immediately after the bulk of dental plaque has been removed by toothbrushing. However, currently it remains unknown whether the salivary AI-2 concentration is correlated with the extent, or the composition, of dental plaque.

The molecular basis of AI-2 sensing by oral bacteria is not well understood at present. The sole exception is *A. actinomycetemcomitans*, in which two distinct receptors for AI-2, LsrB and RbsB, have been identified (Shao *et al*, 2007a). In a nutrient-rich medium, the growth of *A. actinomycetemcomitans* in flowcell biofilms was dependent upon production and sensing of AI-2 (Shao *et al*, 2007b). Using a microtitre well biofilm model, *A. actinomycetemcomitans* cells were able to grow attached to surfaces, but growth did not begin until >24 h after inoculation (Periasamy and Kolenbrander, 2009a). It is possible that AI-2 gradually accumulates over time, and that growth is triggered only when a threshold AI-2 concentration is reached. Previous studies have shown that crude preparations of culture-free supernatant from oral bacteria stimulate the growth of other strains (Liljemark *et al*, 1997). Moreover, the development of natural dental plaque involves a rapid burst of growth following the accumulation of a threshold concentration of bacteria, approximately $2–6 \times 10^6$ cells mm^{-2} (Liljemark *et al*, 1997). Taken together, the above studies provide strong evidence that bacterial growth in dental plaque is dependent on cell density sensing.

The biofilm matrix

The secretion of large molecules by adherent bacteria, either by active secretion mechanisms or by cell lysis, leads to the development of a macromolecular scaffold surrounding the cells. This biofilm matrix helps to bind the cells to the surface and acts as an ion exchange resin to restrict the flow of charged or reactive molecules through the biofilm. A great deal of research has been directed towards the insoluble carbohydrate polymers produced by oral streptococci, because these appear to play a major role in the progression of dental caries (reviewed by Banas and Vickerman, 2003; Russell, 2009). More recently, eDNA has been recognized as an important constituent of biofilm matrices. At present, however, very little remains known about the function of microbial eDNA in oral biofilms.

The production of organic acids from sugars is central to the caries process. Many acidogenic oral bacteria, including mutans and non-mutans streptococci and lactobacilli, also convert sucrose into extracellular glucan or fructan polymers. The water solubility of these polymers is determined by the degree of branching. *Streptococcus mutans*, for example, synthesizes water-soluble glucans containing primarily α-1,6 linkages and a water-insoluble glucan, known as mutan, with largely α-1,3 linkages. These carbohydrates are produced by glucosyltransferase (GTF) enzymes, secreted proteins of approximately 140–175 kDa that hydrolyse sucrose into the monosaccharides fructose and glucose, and these enzymes polymerize the glucose to form glucans. In *S. mutans*, water-soluble glucans are synthesized by the product of the *gtfD* gene, whereas *gtfB* and *gtfC* encode enzymes responsible for insoluble glucan production (Banas and Vickerman, 2003). In addition, an inulin-type fructan is synthesized by fructosyltransferase (FTF), encoded by the *ftf* gene. The insoluble glucans are an important component of biofilm matrices and provide attachment sites for planktonic mutans streptococci. Soluble glucans, on the other hand, appear to be dispensable for sucrose-dependent adhesion of *S. mutans* (Yamashita *et al*, 1993). Nevertheless, studies of knockout mutants indicate that all GTFs and FTF are required for the cariogenicity of *S. mutans* in rat models (Munro *et al*, 1991; Yamashita *et al*, 1993).

There is some evidence that the production of exopolysaccharides by oral streptococci is up-regulated when cells are in biofilms. In *S. mutans* GS5, for example, the expression of *gtfB*, *gtfC* and *ftf* was, respectively, 22-fold, 15-fold and 12-fold increased in biofilms compared with planktonic cells (Shemesh *et al*, 2007b). However, up-regulation of *gtfB* and *ftf* genes was not observed in biofilm cells of *S. mutans* UA159 (Shemesh *et al*, 2007a), indicating that biofilm-mediated regulation of these genes is strain-specific. Further studies are needed using different strains of *S. mutans*, and with other glucan- and fructan-producing streptococci, to determine whether biofilm-mediated regulation of exopolysaccharide production is an important feature of biofilm development *in vivo*.

The primary nutrient source for subgingival dental plaque is gingival crevicular fluid, an exudate derived from serum. The concentration of simple sugars available to bacteria growing beneath the gum line is very low and consequently glucans and fructans are not major constituents of subgingival biofilms. So what is the biofilm matrix in subgingival dental plaque and is it important in disease? At present, there is very little evidence that directly addresses this question. Extracel-

lular proteins certainly appear to be involved in periodontitis: the cysteine proteases of *P. gingivalis*, for example, have multifarious effects on host immunity (Pathirana *et al*, 2010). However, most proteins are too small to form a structural biofilm scaffold without extensive polymerization. Perhaps a more likely candidate for the major subgingival biofilm matrix molecule is DNA. Extracellular DNA was first shown to be a key biofilm matrix component in *Pseudomonas aeruginosa* biofilms (Whitchurch *et al*, 2002). Degradation of eDNA by treatment with DNase I dissolved biofilms and released attached cells. Subsequently, eDNA has been shown to be an integral component of biofilm matrices in many different bacteria including Gram-positive organisms such as *Enterococcus faecalis*, Gram-negative organisms such as *Neisseria meningitides* and the fungus *C. albicans* (Guiton *et al*, 2009; Martins *et al*, 2009; Lappann *et al*, 2010). Oral streptococci release eDNA during planktonic growth by a mechanism that depends on endogenous production of H_2O_2, but apparently does not involve extensive cell lysis (Kreth *et al*, 2009). However, the function of eDNA in oral bacterial biofilms remains to be elucidated.

Treating biofilm-related oral diseases

The primary methods for controlling dental plaque-related diseases at present involve removing as much plaque as possible as often as possible, and protecting enamel with fluoride. These approaches are reasonably effective, yet dental caries and periodontitis remain among the most prevalent diseases in the Western World. Clearly, there is room for improvement. An attractive option is to interfere with plaque accumulation by controlling microbial adhesion, interbacterial communication or establishment of the biofilm matrix. Control measures may be targeted to specific bacteria or aimed more generally at reducing the total accumulation of plaque.

One approach that has received a great deal of attention involves preventing adhesion of *S. mutans* by targeting the AgI/II adhesin to reduce the incidence of caries. Early studies in rhesus monkeys using *S. mutans* AgI/II protein as a vaccine demonstrated the potential of this approach (Lehner *et al*, 1980). Vaccines have been improved by, for example, conjugation of AgI/II with the cholera toxin B subunit (Hajishengallis *et al*, 2005) or priming the immune system with DNA (Li *et al*, 2009). However, it has proved difficult to raise sufficient support from industry for large-scale clinical trials of a vaccine against a disease that is clearly not life-threatening. Nevertheless, there is still momentum for vaccine development within the scientific community (Taubman and Nash, 2006). An alternative to active immunization is to block adhesins with specific inhibitors such as peptides. A synthetic peptide, P1025, corresponding to a fragment of the *S. mutans* AgI/II, blocks adhesion to gp340 and has been shown to be effective in clinical trials (Younson and Kelly, 2004). Peptides targeting AgI/II proteins may also be useful in protection against periodontitis. An Ssp*B* adherence *r*egion (BAR) peptide has been identified that inhibits the recruitment of *P. gingivalis* to biofilms containing *S. gordonii* (Daep *et al*, 2008). The inhibition of interbacterial binding interactions is an exciting new approach to control the oral microflora. A great advantage of this type of strategy is that bacterial resistance would not be predicted to develop easily, as modification of an adhesin to reduce binding to an applied peptide would also impair adhesion to the natural substrate.

The recognition that interspecies communication is important during oral biofilm formation promises to open new avenues for controlling the accumulation of dental plaque. Natural and synthetic compounds have been developed to blockade homoserine lactone-mediated quorum sensing and control infections caused by *P. aeruginosa* (reviewed by Bjarnsholt and Givskov, 2007; Njoroge and Sperandio, 2009). Similarly, a number of substances have been reported to act as potent antagonists for AI-2 signalling, including the natural furanone fimbrolide, alkyl-DPD compounds and nucleoside analogues (Brackman *et al*, 2009; Lowery *et al*, 2009). It is not yet clear which points of the AI-2 signalling pathway are targeted by these compounds or whether they are active against AI-2-dependent communication between oral bacteria. However, in light of the apparent importance of AI-2 in the development of monospecies and dual-species oral bacterial biofilms, AI-2 antagonists (and possibly also AI-2 agonists) would be predicted to bring about major changes in the structure and microbial composition of dental plaque.

Removal of plaque bacteria from tooth surfaces may be facilitated by strategies that interfere with the biofilm matrix. Like AgI/II polypeptides, GTFs of mutans streptococci have been developed as vaccine candidates (Taubman and Nash, 2006). Peptides against catalytic and glucan-binding domains of GTFs inhibit their function, and a single molecule containing two copies of each peptide was effective at reducing colonization by mutans streptococci in a rodent model of caries (Taubman *et al*, 2001). Vaccines against GTFs aim to prevent the formation of a tenacious carbohydrate matrix. An alternative approach is to use enzymes to help degrade a preformed matrix. Bacteria produce such enzymes naturally to disperse from biofilms. The biofilm dispersing glycosidase produced by *A.* (*Actinobacillus*) *actinomycetemcomitans* has been identified (Kaplan *et al*, 2003). This enzyme, known as dispersin B, effectively dissolves matrices of biofilms formed by diverse micro-organisms including *Escherichia coli*, *Staphylococcus epidermidis*, *Pseudomonas fluorescens* and *Yersinia pestis* (Itoh *et al*, 2005). However, with the exception of *A. actinomycetemcomitans*, it is not yet known whether oral bacteria produce biofilm matrix molecules that are sensitive to dispersin B. In fact, given the diversity of extracellular carbohydrates that are produced by bacteria, it seems highly unlikely that dispersin B will be universally effective against oral biofilms. Nucleic acids, by contrast, have a much more uniform structure. If it is found that eDNA is critical for

the stability of oral biofilms, then the use of DNA degrading enzymes to help disrupt biofilms would become a very real prospect.

Summary

The fundamental steps in the development of oral biofilms are the adhesion of bacteria present in saliva to hard or soft tissues, followed by the growth of attached cells. Attachment and colonization are influenced by several other processes including coadhesion, cell–cell communication and the development of a biofilm matrix. Ultimately, disease arises from the production of acid by cariogenic bacteria (in the case of dental caries) or from the triggering of an immune response that leads to gingivitis and/or periodontitis. There is an urgent need for improved measures to control dental plaque-related diseases. The development of new treatments will be greatly enhanced by a detailed understanding, at the molecular level, of the processes that lead to the formation of dental plaque. An idea of the complexity of the microbiota of the human mouth can be gleaned from analysis of the gut microflora, which contains similar numbers of bacterial species to the oral cavity. A recent large-scale metagenomic analysis indicates that each individual carries $>500\,000$ prevalent bacterial or archaeal genes in the gut (Qin *et al*, 2010). Similar metagenomic analyses of the oral microflora are in progress. Such studies will greatly enhance the rate at which new genes are discovered and characterized. It is hoped that the subsequent translation of these discoveries into new clinically useful agents will, in due course, revolutionize our approach to controlling oral diseases.

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