Oral multispecies biofilm development and the key role of cell–cell distance

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Abstract | Growth of oral bacteria *in situ* requires adhesion to a surface because the constant flow of host secretions thwarts the ability of planktonic cells to grow before they are swallowed. Therefore, oral bacteria evolved to form biofilms on hard tooth surfaces and on soft epithelial tissues, which often contain multiple bacterial species. Because these biofilms are easy to study, they have become the paradigm of multispecies biofilms. In this Review we describe the factors involved in the formation of these biofilms, including the initial adherence to the oral tissues and teeth, cooperation between bacterial species in the biofilm, signalling between the bacteria and its role in pathogenesis, and the transfer of DNA between bacteria. In all these aspects distance between cells of different species is integral for oral biofilm growth.

Phylotype
A taxonomic unit typically defined by 99% similar 16S rRNA gene sequences; this is the molecular equivalent of a species and it allows inclusion of yet-to-be cultured organisms in a taxonomic framework.

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Most microbial ecosystems contain large numbers of genetically distinct microorganisms, and the human mouth is not different. The extent of microbial diversity depends partially on the adopted definition of a microbial species or phylotype and on the method of analysis applied[1–3]. Molecular analysis of oral microbial communities by cloning and sequencing the bacterial 16S rRNA genes present has indicated that the human mouth provides a habitat for approximately 700 species of bacteria, and that between 100 and 200 different species are present in the healthy mouth of any individual[4]. More exhaustive surveys using pyrosequencing technologies have suggested that the diversity of bacteria in an individual oral cavity may be slightly higher, around 500 species[5]. Each technique has limitations, and the increased sensitivity of new methods makes it more difficult to distinguish between the autochthonous (that is, naturally occurring) flora and the allochthonous (that is, transient) population. Nevertheless, it is clear that the oral cavity presents a diverse microbial ecosystem in which polymicrobial populations are the norm.

Accessibility of the oral microbial communities during half a century of traditional bacteriological investigation has made this community one of the best-described human microbial systems. Molecular sequencing methods confirm that the oral microbial flora comprises a high percentage of cultured members, and the as-yet-uncultured members are becoming amenable to the cultivation, which is necessary for metabolic characterization. Besides high diversity, the oral ecosystem is characterized by succession, natural disturbances (by oral hygiene), biogeographical nuances (for example, different conditions in the teeth compared with the tongue) and interaction with host tissue and secretions. Integration of our knowledge of these factors will enable definition and, eventually, prediction of the transitions from health to disease in the oral cavity, and will serve as a paradigm for other biofilm systems in general and mammalian microbiomes in particular.

This Review focuses on multispecies oral biofilms, the spatial distribution of the species in biofilms and the signalling between species with an emphasis on the role of the small molecule signal autoinducer 2 (AI2), although other communication signals will undoubtedly be discovered — for example, arginine deiminase[6]. We conclude that in biofilms, cell–cell distance is fundamental for intermicrobial communication processes.

Adherence in oral biofilms

Biogeography of the oral cavity. The mouth can be viewed as an island: a unique environment in the human body that is characterized by near-constant presence of liquid water (in saliva), by short-term but extreme temperature fluctuation, by an externally exposed hard surface (teeth) and by wide variation in carbon and nitrogen input, including a basal component (saliva) that is complex but contains only limited bacterial energy sources. Its microbial inhabitants are rarely found outside the oropharynx. However, examination at the millimeter or micrometer scale reveals that

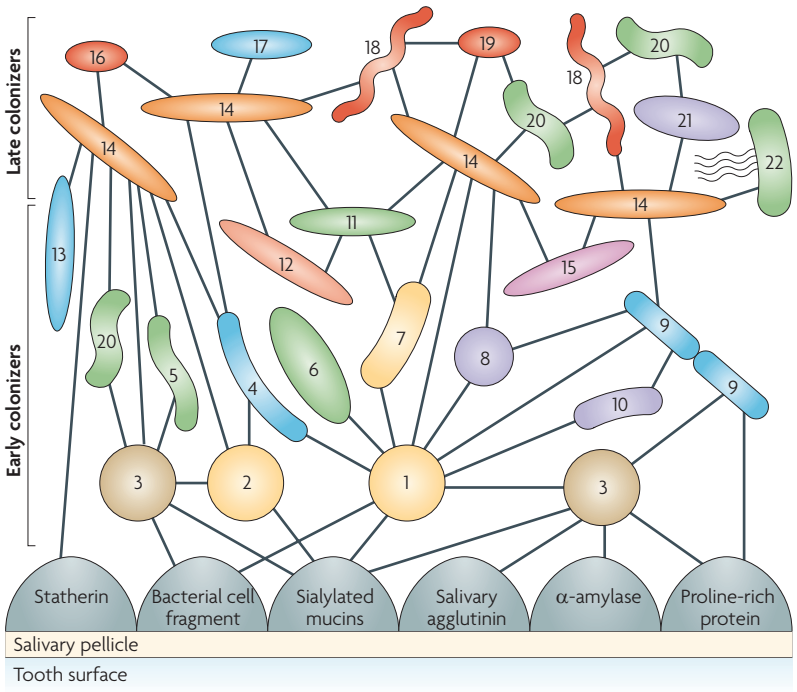


Figure 1 | **Oral bacterial colonization.** Spatiotemporal model of oral bacterial colonization, showing recognition of salivary pellicle receptors by initial colonizing bacteria and coaggregations between initial colonizers, fusobacteria and late colonizers of the tooth surface. Collectively, these interactions are proposed to represent development of dental plaque. Starting at the bottom, initial colonizers, *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus oralis* and *Streptococcus sanguinis*, bind to complementary salivary receptors (sialylated mucins, proline-rich protein, α-amylase, salivary agglutinin and bacterial cell fragments) in the acquired pellicle coating the tooth surface. Late colonizers bind to previously bound bacteria. Sequential binding results in the appearance of nascent surfaces that bridge with the next coaggregating partner cell. Coaggregation is different from aggregation that occurs between genetically identical cells and from agglutination of cells through interaction of cells with soluble molecules, for example, antibodies. Most coaggregations are between cells of different genera; *Fusobacterium nucleatum* strains, for example, coaggregate intergenerically with representatives of all oral bacterial species. However, intrageneric coaggregation among fusobacterial strains is only rarely observed. In sharp contrast, streptococci exhibit broad intrageneric coaggregation partnerships (for example, *S. gordonii* and *S. oralis*) as well as intraspecies partnerships (for example, *S. gordonii* DL1 and *S. gordonii* 38). Each bacterial strain exhibits specificity in partners. For example, some streptococci are capable of coaggregating with certain *Veillonella* spp., whereas other streptococci cannot coaggregate with those veillonellae but do coaggregate with a separate group of veillonellae[24]. Figure modified, with permission, from REF. 106 © American Society for Microbiology (2002).

Gingival crevicular fluid
Host-derived exudate into the sulcus.

Salivary pellicle
A layer of proteins and glycoproteins of salivary origin that permanently coats the surfaces of oral tissues.

Desquamating surface
A surface that sheds the outer layers.

Coadhesion
The adherence of a planktonic microorganism to a genetically distinct microbial cell that is immobilized on a surface.

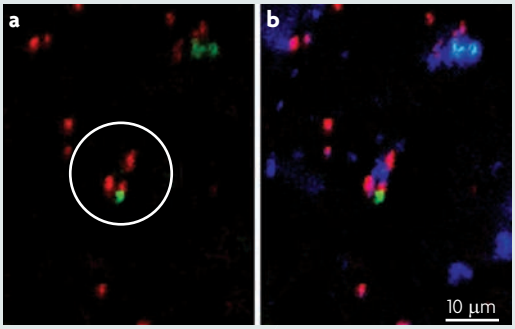
Coaggregation
The binding of two genetically distinct microorganisms suspended in the fluid phase that occurs by means of highly specific interactions between components on the respective cell surfaces.

the oral cavity presents a broad palette of environmental conditions and is therefore more similar to a temperate continent than to an island. Distinct microenvironments occur at secretion sites of saliva or gingival crevicular fluid and can be defined by the degree and nature of contact between epithelial and hard tissues. The composition of the biofilm community reflects its position on this oral continent[7] as well as changes in the local environment that are induced by intrinsic metabolism of the community itself. Tooth-associated oral biofilms can be roughly divided into supragingival biofilms (on exposed enamel surfaces) and subgingival biofilms (below the gumline and within the periodontal pocket or sulcus).

Importance of adherence to biofilm communities. Oral microorganisms that cannot adhere to a surface are transported by salivary flow out of the mouth and down the digestive tract. It is therefore not surprising that all oral bacteria possess mechanisms of adherence to solid surfaces coated with salivary pellicles such as teeth, to desquamating surfaces such as epithelial tissue or to bacteria that are already attached to the surface.

Adherence of microbial cells to immobilized bacteria is called coadhesion[8], and binding of bacteria in suspension is called coaggregation. Both are defined as specific cell–cell interactions between genetically distinct cells, for example between the oral bacteria *Capnocytophaga gingivalis* and *Actinomyces israelii* or between *Prevotella loescheii* and *Streptococcus sanguinis*. In fact, all of the roughly 1,000 oral bacterial strains that have been examined have at least one coaggregation partner, and highly specific partnerships exist. This specificity in partners, together with inhibition of coaggregation by certain sugars or by protease treatment of partners and the temporal order of appearance of species on a professionally cleaned tooth surface led to the proposal that the ability to coaggregate has a role in determining the succession of genera that colonize enamel[9] (FIG. 1). Coaggregation has been shown between isolated microorganisms from communities

Box 1 | **The enamel chip model and cell–cell recognition *in situ***



Similarly to the study of microbial ecology, retrievable substrata are used to obtain undisturbed biofilms. These are currently the only way to obtain spatially resolved data on undisturbed oral biofilms. For oral biofilms, the substratum that closely mimics the tooth surface is the tooth surface itself, in the form of a small piece of human enamel. These retrievable enamel substrata have been used for molecular phylogenic characterization of early plaque species[13] and for *in situ* characterization of spatiotemporal structure of multispecies communities[17,24,25].

Enamel is sparsely colonized by multispecies communities in the first 8 hours after cleaning[17,18], although communities composed of actinomyces, streptococci and veillonellae can be detected during this time[24,25]. However, in the absence of defined cultures of oral bacteria, it is difficult to obtain an understanding of physiological interactions in the community or the manner by which the communities are established. To isolate specific organisms, a spatially resolved isolation approach such as micromanipulation is required. Multiple fluorescently labelled antibodies can be used to label communities that contain specific bacteria, although antibodies are rarely species specific, and additional bacteria might be present. Part **a** of the figure is a community on an enamel chip composed of receptor polysaccharide (RPS)-bearing streptococci and veillonellae that is stained with quantum dot 655 (QD655)-conjugated RPS-specific antibodies (red) and QD525-conjugated R1-specific antibodies (which recognize veillonellae). Staining with 4′,6-diamidino-2-phenylindole (DAPI) (part **b**) reveals cells that were not labelled by antibody, one of which is located in the representative community.

This community was subsequently manipulated off the chip and brought into culture[20]. In addition to the antibody-targeted organisms, an antibody-unreactive streptococcus was isolated, which participated in RPS-mediated coaggregation with the RPS-bearing streptococcus. Once the organisms were cultured, it was possible to reassemble the community *in vitro*. Thus, a set of organisms known to occur together in initial plaque was shown to interact to form a functional community. It is likely that several different types of small interactive communities are established by cell–cell recognition and that such communities are the basic building blocks for oral biofilms *in vivo*. Access to native communities and the ability to reassemble community participants as flourishing entities are important features that establish oral biofilms as paradigms for multispecies biofilms. Image reproduced, with permission, from REF. 20 © American Society for Microbiology (2008).

outside the oral cavity, including the human urogenital tract, the human intestine and freshwater environments[10–12]. Thus, coaggregation and coadhesion may be important mechanisms for the growth and stability of multispecies biofilms in environments outside the oral cavity as well.

Building a biofilm — adhering to the surface. The predominant initial colonizers, streptococci and actinomyces[13–19], and their coaggregation partners have been a primary focus of investigation[20–32]. We propose that these initial colonizers exploit a set of characteristics that are well adapted for community formation and multispecies growth and that these initial communities are the cornerstones of biofilm regrowth after each oral hygiene procedure. Thus, the natural addition of species in oral biofilms provides a paradigm for biofilm investigations in all natural settings.

Supragingival dental plaque
Dental plaque that occurs on areas of the teeth that are not covered by gum tissue.

Subgingival dental plaque
Dental plaque on tooth surfaces below the level of the gums.

Streptococci in particular recognize receptors in the salivary pellicle (FIG. 1), which coats enamel immediately after the surface is cleaned. Most of the receptors, such as statherin, proline-rich proteins, salivary α-amylase, sialylated mucins and salivary agglutinin, originate from the host. Actinomyces bind to proline-rich proteins and statherin, a phosphate-containing protein. Fusobacteria bind to statherin but not to proline-rich proteins.

Building a biofilm — adhering to other bacteria. The mechanisms mediating coaggregation and coadhesion are probably identical, and in this Review we use the term coaggregation to describe this process. Bacteria of the genus *Fusobacterium* exhibit more partnerships than any other genus (FIG. 1). Because strains of *Fusobacterium nucleatum* coaggregate with initial, early and late colonizers, we have suggested that they are important 'bridge' organisms in the succession of genera in naturally developing dental plaque[9]. The ability of *F. nucleatum* to coaggregate is essential for this role in multispecies biofilm formation, as mutants that lack the 350 kDa cell surface protein RadD that mediates coaggregation with *S. sanguinis* could not form structured dual species biofilms with this partner organism[33].

Multispecies microbial communication. Transitions of species (FIG. 1) within populations that constitute supragingival dental plaque and subgingival dental plaque can pose problems for the development of tractable model systems to study communication within dynamic oral communities. The complexity of population structure in older supragingival and subgingival plaque makes the study of initial colonization a requirement for understanding the developmental process. A major advance occurred with the use of retrievable enamel chips in the human oral cavity[17] (BOX 1).

The processes of colonization and development in the oral biofilm are better described than for any other biofilm system in nature. A repeatable microbial succession takes place[13,14,16] that depends on interactions between organisms, and between organisms and their environment, indicating that the interaction is not random. Interspecies communication is an important aspect of biofilm development and is part of a burgeoning research area that has been addressed in several excellent opinion pieces[34–38]. At the moment, investigations of interspecies bacterial communication revolve around the proposed universal signalling molecule AI2 (REF. 39). AI2 has a central role in interspecies signalling and has been shown to play an important part in biofilm development[36]. In addition to communication between oral bacteria, interplay between the human host and the oral microbial community[40–42] contributes to the spatiotemporal reproducibility in dental plaque development. In this Review, we discuss communication in the context of spatial relationships: the proximity between communicating organisms. Cell–cell distance is a key driver that both determines and is determined by signalling and communication processes. Communication is used here to represent the exchange of molecules between genetically distinct cells that results in a change

Box 2 | **Movement of molecules through oral biofilms**

The mouth is an open environment, bathed in saliva that is constantly replenished. For bacteria to communicate effectively with one another in oral biofilms, they must produce molecules that accumulate in the local microenvironment to concentrations that are sufficient for signalling. The local concentration of a microbial product is determined by the rate of production and the rate of removal by reaction, uptake into neighbouring bacterial cells or diffusion out of the biofilm. Diffusion of molecules through model three-species oral biofilms has been determined using a range of fluorescently labelled macromolecules[104]. All molecules reached the centre of clusters within 3 minutes. Smaller molecules penetrated within 10 seconds, and the diffusion constant through biofilms of the largest molecule tested, immunoglobulin G (150 kDa), was 22% of that through pure water. Therefore, oral biofilms apparently provide little resistance to diffusion of extracellular molecules. Nevertheless, movement of molecules through biofilms can be impaired if the molecules interact with bacterial cell surfaces, as do cations for example. Local concentrations of signalling molecules that are sufficient to elicit responses can be attained if the molecules are produced at a high rate and if they trigger responses at low concentrations. The signalling molecule autoinducer 2 (AI2) triggers responses at low concentrations; picomolar concentrations of AI2 promote mutualistic biofilm growth of cocultures of *Streptococcus oralis* and *Actinomyces oris*[30]. In some cases, communication might occur in the absence of signalling molecules through direct cell–cell contact between genetically distinct cells.

in phenotype or gene expression in the receiver. For signals that do not easily move through biofilms (BOX 2), juxtaposition of cells is crucial for effective interbacterial communication.

Transitions of oral biofilms

Transitions in supragingival biofilms. Streptococci, the most abundant organisms in oral biofilms, ferment low-molecular-weight carbohydrates to acids such as acetate, formate and lactate. Salivary fermentable carbohydrate is found primarily in the form of complex glycoproteins that are a poor primary carbon source; scarce low-molecular-weight carbohydrates (for example, glucose) are rapidly removed by bacterial metabolism. Resting activity in these biofilms depends on syntrophic metabolism of salivary glycoprotein[26,43,44]. Although the innate buffering capacity and high turnover of saliva maintain the pH above 6.0 at the tooth surface, when low-molecular-weight carbohydrate becomes abundant while eating and drinking and bacterial metabolism peaks, the local pH can drop to near 5, the crucial pH at which enamel dissolution (leading to caries (tooth decay and cavities)) occurs. The crucial pH in dental plaques of each individual is modulated by many variables[45], including salivary concentrations of Ca^{2+}, PO_4^{3-} and OH^-, salivary production rate, dietary acid intake, host immune response and plaque community composition. When dietary carbohydrate is exhausted, pH rises and enamel dissolution ceases. Communities that contain aciduric (acid-generating acid-tolerant) bacteria, such as *Streptococcus mutans* and lactobacilli, are particularly cariogenic because they continue to grow at a pH lower than that at which other bacteria thrive[46]. Moreover, as pH drops, acid-tolerant bacteria outcompete other bacteria and thus constitute a higher proportion of the community. Interestingly, some streptococci metabolize arginine present in salivary oligopeptides: a metabolic pathway results in the production of ammonium ions, thereby increasing pH within the biofilm. These streptococci can succeed despite the absence of low-molecular-weight carbohydrates[47].

The role of a balanced microbial community (microbial homeostasis) is described in the ecological plaque hypothesis[48], which relates disease (in this case caries) to shifts in the overall community metabolism, subsequent modification of local environment, host factors and bidirectional transitions between flora of varying cariogenicity. Implicit in this hypothesis is that the community is crucial for the formation of caries — microorganisms that are typically not considered cariogenic can be shown to be associated with progression of the disease[49], and clinical evidence indicates that progression of even grossly cavitated carious lesions can be halted if oral hygiene is improved[50].

Transitions in subgingival biofilms — periodontal diseases. Below the gum line, gingival crevicular fluid seeps around the root surface to form a fluid phase that bears little similarity to saliva in its composition and is instead more akin to serum. Similarly, the microflora differs from that found above the gum. Subgingival anaerobic bacteria exposed to gingival crevicular fluid occupy a niche that is characterized by catabolism of amino acids from exogenous protein through secreted proteases, and the overall species diversity is higher than that of supragingival biofilms[4]. Some of these anaerobic bacteria are considered to be periodontopathogens (for example, *Porphyromonas gingivalis*) and cause periodontitis. Periodontopathogens are thought to misdirect host defence and increase tissue-destructive inflammation[51]. As with caries, the subgingival community undergoes a disease-related succession, and the high diversity in this community means that microbial succession and periodontal disease progression are even more complex than for caries. In healthy individuals, streptococci, actinomyces and veillonellae dominate, whereas periodontopathogens are present in relatively low numbers. Poor oral hygiene changes the environment to support increased periodontopathogen biomass[52], leading to gingival detachment and bone and tooth loss. Treatment of the disease causes shifts in proportions of organisms towards those characteristic of healthy sites[53].

Coaggregation versus coculture

Coaggregation: distance is crucial. Coaggregation is an excellent model for the study of gene regulation in response to intimate interbacterial interactions. Coaggregation between compatible partners is easily induced *in vitro* by vortex mixing of dense cell

Periodontitis
Inflammatory gum disease involving the destruction of the tissues surrounding the teeth, loss of attachment of the gums and the creation of a 'pocket' between the teeth and gums.

Box 3 | **Probing communication using microarrays**

Coaggregation provides an excellent model for investigating interactions between different bacteria for which distance is crucial. Comparison of gene expression in coaggregates in mixed-species communities with that in equivalent monocultures can identify processes that are important during multispecies biofilm formation, and could also give clues regarding the nature of molecules that mediate communication. Microarrays have been employed to identify responses of *Streptococcus gordonii* to coaggregation with *Actinomyces oris*[22]. As *S. gordonii* is known to coaggregate with *Porphyromonas gingivalis*[60,70,105] and many other oral bacterial species, it would be interesting to investigate which genes would be identified if the partner organism were changed to *P. gingivalis*, *Veillonella* spp. or *Fusobacterium nucleatum* to determine whether there are the universal or partner-specific responses to coaggregation. Similarly, it is not clear whether a single 'snapshot' is sufficient to reveal the extent of coaggregation-dependent gene regulation. In the case of the *S. gordonii–A. oris* microarray study, gene expression was assessed 3 hours after the formation of coaggregates. Would the same genes have been identified if the samples for microarray had been collected immediately after inducing coaggregation, or after 2 hours rather than 3 hours? Currently, five microarrays for oral bacteria are available, by application, from the National Institute of Dental and Craniofacial Research (NIDCR) Oral Microbial Microarray Initiative website . Applying these, along with custom-designed microarrays, to study gene expression in multispecies cultures will reveal much about how bacteria sense interactions with neighbouring cells.

suspensions of the partners. The cells remain coaggregated following dilution in growth medium, and extensive coaggregates remain when each organism multiplies. In a coculture in which the partner organisms are not vortexed and are not intimately associated initially, coaggregation occurs spontaneously after several hours. Gene expression in the coaggregating pair of *Streptococcus gordonii* strain DL1 and *Actinomyces oris* strain ATCC 43146 is different when the two species are cocultured and when they are coaggregated[22]; distance is crucial in this case. *S. gordonii* DL1 in coaggregates with *A. oris* ATCC 43146 had more than a threefold change in the expression level of several genes compared with coculture, indicating that there is a specific transcriptional response after cell–cell contact[22]. This included the *bfbCDARBGF* operon associated with β-glucoside utilization, which is involved in biofilm formation[54]. Coordinated regulation of genes involved in arginine biosynthesis and transport was observed, highlighting the importance of this biosynthetic pathway to this intergeneric pair. Arginine biosynthesis in *S. gordonii* DL1 grown in monoculture is inefficient, and this streptococcus cannot grow aerobically at arginine concentrations below 0.1 mM. However, in coaggregates with *A. oris* ATCC 43146, *S. gordonii* DL1 grew to high cell density in the absence of arginine. Importantly, in the same medium, *S. gordonii* DL1 failed to grow in coculture with *A. oris* ATCC 43146 until coaggregates slowly formed during a 9-hour coculture period. These data suggest that *A. oris* ATCC 43146 stabilizes aerobic growth of *S. gordonii* DL1 in low arginine conditions but only when cell–cell distances are short, as they are in coaggregates. In this system, *S. gordonii* apparently benefits from the high cell density environment of coaggregates. It is not clear whether the streptococci respond to a signal or a metabolite from *A. oris* ATCC 43146, or whether the role of *A. oris* ATCC 43146 in this interaction is simply to bind *S. gordonii* DL1 cells together and promote intraspecies quorum sensing. Aggregation of *S. gordonii* DL1 can be induced by lectins or salivary proteins, and high cell density cultures can be produced by centrifuging cultures. It would be interesting to determine the gene regulation response of *S. gordonii* DL1 to aggregation in monoculture. Of note, a specific transcriptional response after autoaggregation has recently been described in *F. nucleatum*[55].

Competition, cooperation and survival must be balanced in stable multispecies communities. Hydrogen peroxide, which is produced by many streptococci, can be used as a competitive advantage in oral bacterial communities. Hydrogen peroxide can cross bacterial cell membranes and oxidize cellular macromolecules, including DNA and proteins. As a result, carbonyl groups can be introduced into the side chains of arginine, proline, lysine and threonine residues and thereby target the protein for degradation[56]. Hydrogen peroxide limits the growth of streptococci in batch culture and on agar plates, and some streptococci, such as *Streptococcus pneumoniae*, are routinely cultured in catalase-containing media. Certain oral bacteria, including *A. oris* ATCC 43146, produce catalase and degrade hydrogen peroxide. In coaggregates, *A. oris* ATCC 43146 protects *S. gordonii* DL1 from self-inflicted oxidative damage[23]. Aerobic monocultures of *S. gordonii* DL1 exhibited extensive protein oxidation and were rapidly killed following growth (more than 99% of the cells died within 24 hours after entry into stationary phase), minimal protein oxidation and no loss of viability was observed in coaggregates. These results suggest that hydrogen peroxide may dominate competitive and cooperative interactions[57] that lead to multispecies natural communities in dental plaque. Additional analyses with *A. oris* ATCC 43146 and a streptococcal *spxB* mutant, which does not produce hydrogen peroxide, will provide insight into the potency of hydrogen peroxide in altering the development of the oral microbial community (BOX 3).

F. nucleatum coaggregates with *P. gingivalis*, and both species coaggregate with *S. gordonii*: a proteomics analysis of *P. gingivalis* in this three-member community revealed that 403 proteins were less abundant and 89 proteins were more abundant than in cultures of *P. gingivalis* alone and that there was an overall increase in the levels of proteins involved in protein synthesis[58]. A *P. gingivalis* mutant that lacked HmuR, a major haemin uptake protein that is regulated by cell–cell contact, had a decreased ability to partner with *S. gordonii* and *F. nucleatum* in three-member communities. An earlier study of *P. gingivalis* interactions with *S. gordonii* revealed that over 30 *P. gingivalis* genes were differentially regulated in response to cell–cell interaction[59]. Collectively, these studies represent just the beginning of an exciting foray into the potential role of small molecule signals and gene regulation in the development of multispecies communities.

Coculture. Coculture in a closed system can be sufficient for interspecies signal transduction, as has been shown with *Veillonella atypica* and *S. gordonii* V288 (REF. 60). In this system, the gene encoding α-amylase in *S. gordonii* V288, *amyB*, was upregulated in response to

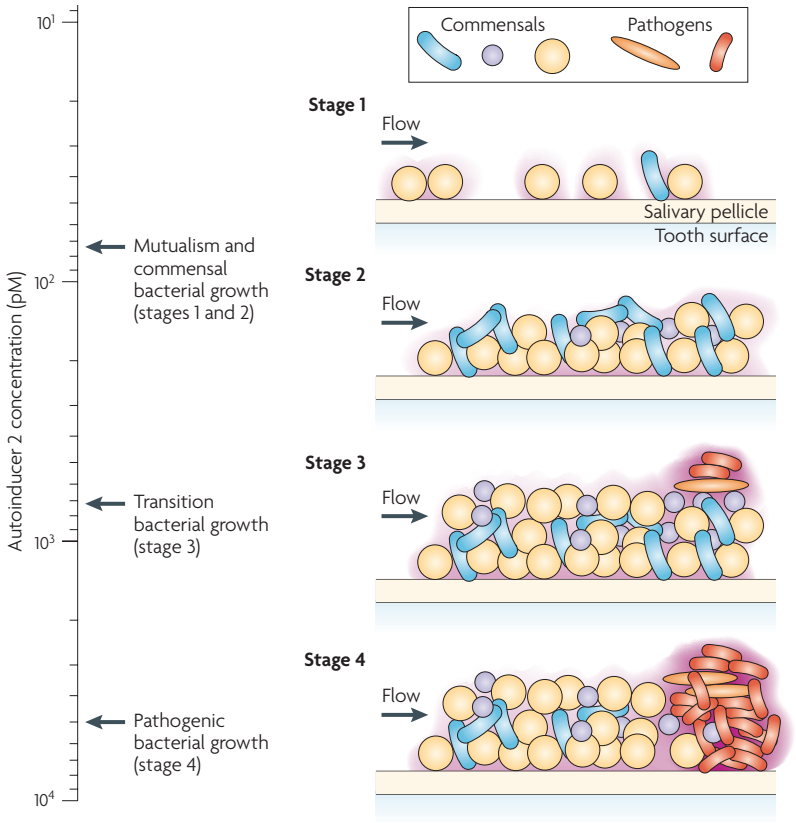


Figure 2 | **Relationship between succession of oral communities and autoinducer 2.** Dental plaque development is diagrammed with respect to time. The relative amount of autoinducer 2 (AI2) produced by commensal bacteria and pathogens is indicated. Commensals (depicted in stages 1 and 2) respond to the lowest AI2 concentrations (below 100 pM), which results in mutualism and bacterial growth. Initial colonizers such as streptococci (yellow) and actinomyces (blue) bind to the salivary pellicle (stage 1), which coats the enamel, and subsequently grow together with veillonellae (purple) as multispecies biofilm communities (stage 2). As the commensal bacterial biomass increases by cell division and by accretion, the AI2 concentration increases, which improves the communication among transition bacterial species such as fusobacteria (orange; stage 3). Finally, when the AI2 concentration is the highest (stage 4), the pathogens (red) are favoured for growth and join the developing biofilm communities. This model offers a simple way to conceptualize the importance of distance in crucial communication in each multispecies community producing AI2 and each community responding optimally to a particular local concentration of the signal.

V. atypica. In closed batch culture, gene regulation did not depend on coaggregation but occurred even when a dialysis membrane separated the organisms. However, intimate cell–cell contact was required to induce the *amyB* promoter in an open flowcell system, indicating that the putative signal was diffusible and accumulated in a closed space[60]. The *amyB* promoter region contains a 7-base pair inverted repeat that matches 11 of 14 positions in the consensus of the catabolite-response element, which is recognized by the CcpA family of transcription regulators. The streptococcal CcpA homologue (previously known as RegG) was shown to be required for the *V. atypica*-induced α-amylase expression in this interspecies interaction[61]. Wild-type *S. gordonii* exhibited a threefold increase in α-amylase activity in cocultures with veillonellae compared with monocultures, whereas no increase in α-amylase activity was observed in the *S. gordonii ccpA* mutant during coculture[61]. Thus, interspecies cell–cell contact was not necessary for interaction between this pair of oral bacteria.

Intergeneric and interkingdom signalling

During interkingdom signalling between the oral organisms *S. gordonii* and *Candida albicans*[62], no cell–cell contact is required. *Candida* spp., and in particular *C. albicans*, cause uncomfortable infections of the oral soft tissues[63]. Interactions with *S. gordonii* lead to numerous changes in *C. albicans*, including increased production of hyphae and the activation or repression of three mitogen-activated protein kinases involved in morphogenetic switching. Furthermore *S. gordonii* counteracts the inhibition of hyphal formation by *C. albicans* that is induced by the intercellular signalling molecule farnesol. These observations suggest that there is a complex interaction between streptococci and *Candida* spp. At least part of this interaction involves a diffusible interkingdom signalling molecule, as spent culture medium from *S. gordonii* can induce morphological changes in *C. albicans*[62].

Autoinducer 2 in spatiotemporal development of multispecies communities. The signalling molecule AI2 has been proposed to act as a universal intergeneric signalling molecule and has an important role in the formation of multispecies biofilms[39]. *Streptococcus oralis* 34 and *A. oris* T14V cannot grow as monoculture biofilms in flowing saliva, but together they grow luxuriantly[26]. These bacteria coaggregate, and this may enhance the efficiency of diffusible signal exchange between partners. AI2 is likely to be the signal that stimulates mutualistic growth as a *luxS* mutant of *S. oralis* 34, which cannot produce AI2, does not form dual-species biofilms[30]. Luxuriant growth is restored with the addition of 4,5-dihydroxy-2,3-pentanedione (DPD; the molecule that spontaneously converts into the various derivatives known collectively as AI2)[64].

Interestingly, maximal biomass of the *S. oralis* 34 *luxS* mutant–*A. oris* T14V biofilm was achieved at 0.8 nM DPD; at 800 nM DPD, biofilm biomass was equivalent to that seen without added DPD. Similarly, in monoculture systems of other oral streptococci virulence factor production and antibiotic resistance were optimal at ~ 1 nM DPD[65,66]. This concentration is about 100-fold below the level that is detectable by the bioluminescence assay in *Vibrio harveyi*[67], thereby suggesting that AI2 signalling in oral communities may operate at concentrations below those typical in other settings. In the flowing environment of the oral cavity, signal build-up and resulting distance over which a signal threshold can be reached may be small. When bacteria are brought together by coaggregation, the effective AI2 threshold concentration can be reached when the production rate of AI2 is lower than that necessary when the bacteria are further apart. Interplay between partner selection in coaggregation, AI2 production and washout rates, and threshold AI2 levels for a given response may be the basis for initiation and relative fitness of oral communities across the range of environmental conditions in the oral cavity.

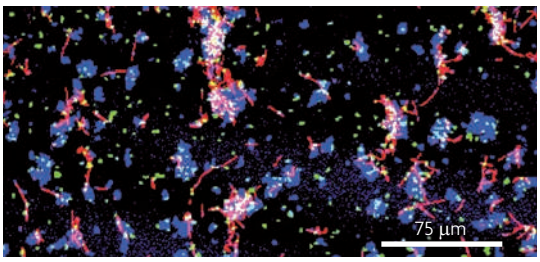


Figure 3 | **Mutualistic *Fusobacterium nucleatum–Aggregatibacter actinomycetemcomitans–Veillonella* sp. biofilm.** Confocal micrograph of mutualistic biofilm communities of *Fusobacterium nucleatum* (red), *Aggregatibacter actinomycetemcomitans* (green), and *Veillonella* sp. (blue) formed as multispecies networks grown in a flowcell for 18 hours on saliva as the sole nutritional source. Intimate interspecies cell contact is evident, and corncob arrangements of the slender fusobacterial cells with coccoid aggregatibacter and spherical veillonella cells are visible. Bacterial cells are stained with species-specific fluorophore-conjugated immunoglobulin G. Image reproduced, with permission, from REF. 26 © American Society for Microbiology (2001).

In a model based on these principles, it is likely that different concentrations of AI2 play a part in the communication between different species. A schematic of this hypothesis that is relevant to biofilm development in the human oral cavity is presented in FIG. 2. Many oral bacteria produce less AI2 compared with oral pathogens when grown as monoculture[68], suggesting that commensal bacteria such as *S. oralis* and *A. oris* respond to AI2 levels that are below those produced by species associated with the transition from a commensal community to a pathogenic community, for example *F. nucleatum*. Even though they are present in significantly high numbers in saliva, fusobacteria are infrequent in dental plaque during the first 8 hours of biofilm development[69], probably because environmental conditions during this period do not support their growth. However, fusobacteria produce high levels of AI2 (REF. 68), coaggregate with a wide range of early and late colonizers[70] and become the dominant Gram-negative species in biofilms associated with healthy gums and gingivitis plaque[71]. As biomass increases on the tooth surface and conditions arise that allow entry of fusobacteria, local AI2 concentration would increase, and the other transition and pathogenic species would begin to experience AI2 levels that would initiate signal-dependent changes in gene expression. By contrast, commensal bacteria are inhibited by the increasing concentration of AI2, as documented for the *S. oralis–A. oris* pair. Oral hygiene procedures such as brushing and flossing remove much of the biomass, the local AI2 concentration is markedly reduced for those that remain, and the process returns to stage 1, when commensals again dominate. Stages 1 and 2 are states of health, which can be maintained by routine and frequent oral hygiene procedures. Failure to conduct regular oral hygiene procedures leads to the community compositions represented in stages 3 and 4, which induce gingivitis and periodontal disease. These ideas provide a framework for AI2 as a modulator of these transitions.

Bacteria respond to AI2 by internalizing the molecule, so retention of community-generated AI2 and internalization of AI2 by the members of the community is essential for biofilm development. The internalization transport apparatus described in *Salmonella enterica* subspecies *enterica* serovar Typhimurium includes the AI2-binding protein LsrB, which is encoded in the *lsr* (LuxS-regulated) operon[72]. Although numerous oral species produce AI2, only *Aggregatibacter actinomycetemcomitans* is known to possess an LsrB homologue[73,74], which is required for biofilm formation in this species[73,74]. *A. actinomycetemcomitans* forms mutualistic communities with *Veillonella* sp. and *F. nucleatum*[28](FIG. 3), and, similarly to *S. oralis–A. oris* pair described earlier, the *A. actinomycetemcomitans, Veillonella* sp. and *F. nucleatum* three-membered community could be AI2 responsive. Given the widespread production and response to AI2 in oral biofilms and the lack of LsrB or LuxP (a related AI2 receptor, so far found only in *Vibrio* spp.) in almost all oral bacteria, it is likely that there are additional AI2 receptors in oral bacteria. In fact, the ribose-binding protein RbsB has been identified as an additional AI2 receptor in *A. actinomycetemcomitans*[73]. Progress in the role of AI2 in sustaining community growth will occur by integrating knowledge of LsrB and related AI2-binding proteins in oral species with investigations of sustainable model multispecies communities.

DNA transfer in biofilms

Biofilms provide an excellent environment for DNA exchange because cells are in close proximity and DNA can be trapped within the extracellular matrix. Indeed, horizontal gene transfer between oral streptococci in biofilm communities has been reported[75,76], and many genera of oral bacteria, including *Actinomyces*, *Bifidobacterium*, *Fusobacterium*, *Haemophilus*, *Peptostreptococcus*, *Streptococcus* and *Veillonella*[77], contain conjugative transposons that facilitate the DNA transfer between bacteria through conjugation (cell–cell mating). Analysis of the genomes of sequenced oral bacteria suggests that past horizontal gene transfer events account for between 5% and 45% of genes in different species[78]. *P. gingivalis* strain ATCC 33277 contains 13 regions of atypical nucleotide composition that are likely to have been acquired from other microorganisms[79]. In fact, a large degree of variation exists between strains of *P. gingivalis*, suggesting that this organism has undergone frequent genetic recombination events that have resulted in a panmictic population structure (that is, a multispecies population that has arisen through random exchange of DNA between individuals)[80]. The transfer of DNA between different strains of *P. gingivalis*, and between *P. gingivalis* and *Escherichia coli*, seems to occur by conjugation[81].

Oral streptococci are naturally competent, and it is possible that the DNA in the extracellular matrix is transmitted without direct cell–cell contact. Indeed, a conjugation-defective plasmid could be transferred from *Treponema denticola* to *S. gordonii* in biofilms[82]. Although competence-dependent transformation does not require a physical bridge between the partner cells, the spatial arrangement of cells in biofilms may have a key role in

Gingivitis
Minor and reversible inflammation of the gum tissue.

Competent
State of bacteria in which they can take up extracellular DNA from the environment.

Box 4 | **Communities as aetiological agents**

The relevance of Koch's postulates of microbial disease has been questioned recently, primarily resulting from our modern perspective on the difficulties associated with culture of organisms and on the genetics of bacterial pathogenicity. Some microbiologists might take issue with Koch's (paraphrased) 1st and 3rd postulates, which state that a given disease is caused by one biological agent and that the agent must be isolated, cultured and used to cause disease in a healthy host. For example, most oral microbiologists would agree that the mere presence of the cariogenic *Streptococcus mutans* or of the periodontopathogenic *Porphyromonas gingivalis* is not sufficient to cause either caries or periodontitis in most individuals. Conversely, the absence of *S. mutans* does not ensure caries-free dentition. Single organisms can be reduced in number with little change in outcome for the host because the vacated niche is filled by another bacterium (or group of bacteria) with similar functionality in pathogenesis. Thus, the relationship of various bacterial physiologies to one another, and the overall functionality (caries inducing and periodontitis inducing) created by the community, are important for disease development.

Substitution of 'the community' for 'the agent' would make Koch's first postulate applicable to oral polymicrobial diseases. Perhaps we will one day be able to isolate, grow and manipulate complex oral bacterial communities in the laboratory to the degree necessary to test fulfilment of the third postulate. As for Koch's postulate that requires the agent not be found in a non-pathogenic situation, periodontal diseases and caries are clear exceptions in which the determining factor is not the presence or absence of an agent, in this case the presence of single known periodontopathogens, but rather the interplay between host response and community composition (the proportion of each peridontopathogen and commensal bacterium within the community). The same community may display a different level of virulence in a different host, but the relative numbers of community members may vary in a manner established by host factors and by pathogenesis-associated niches.

this process as it provides a local source of DNA to competent cells. The development of competence in streptococci occurs in response to sensing a secreted signal molecule, competence-stimulating peptide (CSP), which is encoded by *comC*. The sequence of *comC* and its product, CSP, varies between streptococci, which respond only to the variant of CSP that they produce. Consequently, CSP-mediated communication is essentially species dependent or even strain dependent. Competence development in *S. mutans* is apparently far more efficient in monospecies biofilms than in planktonic cells, and it has been postulated that CSP acts as a quorum-sensing regulator in this organism[83].

In addition to their role in DNA uptake and incorporation, *S. mutans* competence genes also have key roles in releasing DNA, which stabilizes the architecture of biofilms[84]. In multispecies biofilms, CSP signalling by *S. mutans* is a target for competition by other oral streptococci. *Streptococcus salivarius* produces an extracellular product that interferes with *S. mutans* CSP and inhibits biofilm formation[85]. *S. sanguinis*, *Streptococcus mitis*, *S. oralis* and *S. gordonii* also interfere with *S. mutans* CSP. In the case of *S. gordonii*, the CSP-degrading activity was mediated by the extracellular protease challisin[86]. Degradation of CSP by oral streptococci may be a mechanism for self-protection, as high concentrations of *S. mutans* CSP trigger the production and secretion of bacteriocins, which kill neighbouring cells and release their DNA[87]. *S. mutans* is immune to its own extracellular bacteriocins, but the accumulation of CSP triggers the synthesis of an intracellular bacteriocin and subsequent autolysis in approximately 1% of the *S. mutans* population[88]. Therefore, in the absence of other species, CSP-mediated quorum sensing enables *S. mutans* to obtain extracellular DNA from a subpopulation of *S. mutans* cells. Extracellular DNA is an important component of monospecies and multispecies biofilms that are formed by many Gram-positive and Gram-negative bacteria[89–91] and has been reported in natural microbial populations such as soil ecosystems[92]. Thus, the release of DNA from microbial cells in oral biofilms can be beneficial for neighbouring bacteria in at least two regards: because it stabilizes the structural integrity of the biofilm and because it disseminates, for example, antibiotic resistance traits throughout the dental plaque population under conditions of stress. Dissemination of antibiotic resistance traits is of particular concern, as antibiotics are widely used for the treatment of periodontal disease.

Communities, not species

Culturable microflora of accessible human body sites have been described through traditional bacteriological approaches. Data from 16S rRNA gene cloning and sequencing studies indicate that the oral cavity has roughly 700 phylotypes of which slightly less than half have been cultivated[4]. In comparison the skin of the forearm bears 182 phylotypes, of which 80% are cultivated[93]. The power of molecular methods in identifying new phylotypes is shown by a study in which 28 of 56 fresh subgingival bacterial isolates (cultivated bacteria) were new phylotypes[94]. Paramount to microbial community structure is the niche (determined by the available nutrients) of the set of phylotypes comprising the community and that a similar niche might comprise a different set of phylotypes. This perspective requires advances in bacterial isolation[95] and domestication[96], such that organisms, in addition to sequences, can be studied. It is possible that physiological aspects associated with a particular long-cultivated organism will be found to be misleading in comparison to those of identical or closely related 'wild' isolates. In particular, clear-cut discrepancies in biofilm formation exist between clinical isolates and the common laboratory strain in *Bacillus subtilis*[97,98], *Staphylococcus aureus*[99], *A. actinomycetemcomitans*[100] and *F. nucleatum*[101]; in all but the last case, the molecular basis of the phenotypic difference has been defined. Polyphasic taxonomic approaches that combine molecular information with physiological data will yield profiles that will be useful for tracking and predicting rapid successions of community members in natural populations[24], which are obtained easily from oral biofilms, another aspect of the paradigmatic nature of these systems.

Oral diseases are influenced by microbial communities, not by single pathogens. Commensal microorganism–pathogen transitions are driven by a range of variables, many of which are controllable. The current nonspecific approaches to oral hygiene do much to prevent such transitions; this suggests that community-level changes, which are driven by niche alterations, are the key changes[48,102]. A revision of Koch's postulates, in which the community is the aetiological agent (BOX 4), may be useful. In this hypothesis, the downstream consequences of community metabolism (for example, acid production) are the important factors in genesis and

progression of the disease. Therefore, metabolic relationships within a community, created and maintained by different groups of phylotypes, could result in similar consequences. Spatial relationships between phylotypes, and between community and host, may markedly affect niche transitions[13,25]. Culturable or yet-to-be cultured organisms that are not well studied may drive transition or support stability.

Summary and future directions

Human oral biofilms exist as multispecies communities, which can be isolated and reconstructed *in vitro* in model saliva-based systems. Several three-species communities are mutualistic during growth on saliva[27,28,31,32], suggesting that communication occurs among them (FIG. 3). Signals such as AI2 affect community growth, and the mechanisms of community responses are prime targets for future discovery. One likely discovery will be the regulation of community genome expression (the collective expression of all community members) in oral niches through the uptake of community-generated AI2, especially by species that do not produce this molecule, such as that known for the relationship between two soil species: *Sinorhizobium meliloti* internalizes AI2 produced by *Erwinia carotovora*[103]. The advance of microfluidics research[19] will be of benefit to studies using saliva to investigate communities and processes associated with oral diseases. Ultimately, it may be possible to intervene in these processes and modify the overall structure and activity of associated microbial multispecies communities. Bacteria communicate with each other and the host to create functional communities. Investigation of bacterial communication modes by measuring gene expression in these communities in a spatiotemporally resolved manner will aid our understanding of the interbacterial interactions that are known to occur in the biofilm. The oral biofilm has been, and will remain, the paradigm system for these studies.

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Competing interests statement.
The authors declare no competing financial interests.

DATABASES

Entrez Genome Project: http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj
Aggregatibacter actinomycetemcomitans | *Candida albicans* | *Fusobacterium nucleatum* | *Porphyromonas gingivalis* | *Streptococcus gordonii* | *Streptococcus mutans* | *Streptococcus oralis* | *Streptococcus sanguinis*

FURTHER INFORMATION

Paul E. Kolenbrander's homepage: http://www.nidcr.nih.gov/Research/NIDCRLaboratories/OralImmunity/Kolenbrander.html
NIDCR Oral Microbial Microarray Initiative: http://www.nidcr.nih.gov/Research/DER/IntegrativeBiologyAndInfectiousDiseases/NOMMI.html

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