Cell-to-Cell Distance: Essential Currency of Oral Multispecies Biofilm Communities

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Abstract:

Growth of human oral bacteria *in situ* requires adhesion to a surface: the constant flow of host secretions thwarts planktonic cells' ability to double before they are swallowed. Oral bacteria coevolved with their host to form biofilms on hard tooth surfaces and on soft epithelial tissues. They exist in paradigmatic multispecies model communities that are easy to study: they are readily accessible as are their host's secretions. Single species growth solely on saliva is rare, but multispecies communities thrive on it as a sole nutritional source. Distance between cells of different species is the primary currency for oral biofilm growth.

Introduction:

In the early stages of microbial evolution on Earth, communities of genetically identical microorganisms may have been the norm. Nowadays, these monospecies populations remain only as ancient relics hidden in the depths of the Earth. Indeed, the first truly monospecies microbial community was not discovered until 2008, when extensive sequence analysis of samples from a South African mine 2.8 km below the surface yielded a single microbial genome of *Candidatus Desulforudis audaxviator* [1]. The vast majority of microbial ecosystems contain large numbers of genetically distinct microorganisms. The extent of microbial diversity depends partially on the adopted definition of a microbial species or phylotype and on the method of analysis applied [2-4]. Recent surveys using pyrosequencing technologies indicate that natural populations such as microbial communities at deep-sea hydrothermal vents consist of tens of thousands of

phylogenetically distinct microorganisms [5]. When applied to supragingival (above the gumline) dental plaque, similar methods indicate that between 19,000 and 26,000 different microbial phylotypes inhabit this environment [6]. Therefore, in terms of phylogenetic diversity, microbial communities in the oral cavity are typical of those found currently in other microbial ecosystems.

Accessibility coupled with a half-century of traditional bacteriological investigation has resulted in oral microbial communities becoming one of the best-described human microbial systems. Molecular methods confirm their high percentage of cultured members, and the as-yet-uncultured members are becoming amenable to the cultivation necessary for metabolic characterization. Besides high diversity, the oral ecosystem is characterized by succession, natural disturbances (oral hygiene), biogeographical nuances (e.g., teeth vs. tongue), and interaction with host tissue and secretions. Integration of these factors will enable definition and, eventually, prediction of the transitions from health to disease in the oral cavity, as well as serving as a paradigm for other biofilm systems in general and mammalian microbiomes in particular.

This review discusses multispecies biofilms with only limited mention of single-species investigations. The spatial distribution of the species in biofilms has profound consequences for the activity and stability of the community as a whole. Here, we describe how dental plaque biofilms develop and how oral microbial communities are sustained in health and disease. We emphasize the significance of the small molecule signal autoinducer-2 (AI-2), although signaling molecules such as arginine deiminase

(ArcA) have been reported [7] and other communication signals will undoubtedly also be discovered. Within biofilms, cell-to-cell distance is fundamental to inter-microbial communication processes.

I. Oral communities are multispecies

Biofilm biology began with the near-exclusive study of *Pseudomonas aeruginosa* in *in vitro* systems using standard bacteriological media but now encompasses a broad palette of organisms, culture conditions and model systems. A re-orientation has occurred. A dramatic increase in multispecies biofilm studies has accompanied the realization that intraspecies and interspecies relationships are integral to bacterial communities "in the wild". Relationships between bacterial cells in biofilms are by default spatiotemporal, and they are the genesis as well as the culmination of the biofilm community. The oral cavity provides paradigm biofilms for investigation of the driving forces and natural consequences of multispecies community organization and development.

Importance of adherence to biofilm communities. Oral microbes unable to adhere to a surface are transported by salivary flow out of the mouth and down the digestive tract. Not surprisingly, all oral bacteria possess mechanisms of adherence to salivary pellicle-coated solid surfaces such as teeth, to desquamating surfaces such as epithelial tissue, or to bacteria that are already surface-attached.

Adherence of microbial cells to immobilized bacteria is called coadhesion [8]. If the bacterial cells are in suspension, it is called coaggregation: both coadhesion and coaggregation are defined as specific cell-cell interactions between genetically distinct cells, for example between *Capnocytophaga gingivalis* and *Actinomyces israelii* or between *Prevotella loescheii* and *Streptococcus sanguinis*. In fact, all of the roughly 1000 oral bacterial strains we have examined have at least one coaggregation partner, and highly specific partnerships exist. This specificity in partners **[Box 1]**, together with inhibition of coaggregations by certain sugars or by protease treatment of partners, as well as the temporal order of appearance of species on a professionally cleaned tooth surface led to the proposal[9] that the ability to coaggregate plays a role in succession of genera that colonize enamel (**Fig. 1**).

Streptococci, especially, recognize receptors in the salivary pellicle (**Fig. 1**), which coats enamel immediately after the surface is cleaned. The host is the origin for most of the receptors, such as statherin, proline-rich proteins, salivary alpha-amylase, sialylated mucins, and salivary agglutinin. Actinomyces bind to proline-rich proteins and statherin, a phosphate-containing protein. Fusobacteria bind to statherin but not proline-rich proteins. The mechanisms mediating coaggregation and coadhesion appear to be identical, and we will use coaggregation to describe this phenomenon here. The genus *Fusobacterium* exhibits more partnerships than any other genus. Because strains of *F. 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. Mutants lacking the 350 kDa cell surface protein *radD* that mediates coaggregation with *S. sanguinis* were unable to form structured dual species biofilms with this partner organism [10]. Coaggregation has been demonstrated between isolated microorganisms from communities outside the oral cavity, including the human urogenital tract and intestine, and freshwater environments [11-13]. Thus, coaggregation and coadhesion may be important mechanisms for the growth and stability of multispecies biofilms in environments outside the oral cavity as well.

The predominant initial colonizers streptococci and actinomyces [14-17] and their coaggregation partners have been our primary focus [18-27]. We propose that these initial colonizers exploit a set of characteristics perfected 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 succession of species in oral biofilms provides a paradigm for biofilm investigations in all natural settings.

Multispecies microbial communication. By definition, non-random, repeatable microbial succession requires organization and communication. Communication is used here to represent the exchanges of signals, metabolites and other molecules that occur when genetically distinct cells develop into communities. Oral communities re-form after their disruption by tooth brushing and flossing. Inter-species communication is however an important and emerging discussion point that has been addressed in several excellent opinion pieces [28-32]. Microbe-to-microbe communication has been presented in the context of definitions used to characterize communication between eukaryotes and the

consequences on evolution of eukaryotic species [31]and in the context of a proposed universal signaling molecule AI- 2 [33]and its potential role in biofilm development [30]. Communication between the human host and the oral microbial community [34-36]as well as communication among the highly diverse microbial species contribute to the spatiotemporal reproducibility in dental plaque development. In this review, we discuss communication in the context of spatial relationships: the proximity of the communicating organisms to one another. Cell-cell distance is a key factor that both determines and is determined by signaling and communication processes. For signals that do not easily move through biofilms **[Box 2]**, juxtaposition of cells is critical for effective interbacterial communication.

II. Transitions of oral biofilms

Biogeography of the oral cavity. The mouth can be viewed as an island: a unique environment within the human body characterized by near-constant presence of liquid water (in saliva), by short-term yet extreme temperature fluctuation, by an externally exposed hard surface (teeth), and by wide variation in carbon/nitrogen input including a basal component (saliva) that is complex yet possessive of only limited bacterial energy sources. Its microbial inhabitants are rarely found outside the oropharynx. However, upon close examination (at the scale of mm or μm), it becomes apparent that the oral cavity presents a broad palette of environmental conditions and is therefore more akin to a temperate continent than to an island. Distinct microenvironments occur at secretion sites of saliva or of gingival crevicular fluid (GCF), and can be defined by the degree and

nature of contact between epithelial tissues and hard tissues. Biofilm community composition reflects position on this oral continent [37] as well as changes in the local environment 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 (within the periodontal pocket or sulcus).

Transitions in supragingival biofilms - feast and famine. Streptococci, the most-numerous organisms in oral biofilms, ferment low-molecular-weight (LMW) carbohydrates to acids such as acetate, formate and lactate. Salivary fermentable carbohydrate occurs primarily in the form of complex glycoprotein that is of limited value as a primary carbon source; the scarce low-molecular-weight carbohydrate (*e.g.*, glucose) is rapidly removed by bacterial metabolism. "Resting" activity in these biofilms depends on syntrophic metabolism of salivary glycoprotein [24, 38, 39]. The innate buffering capacity and high turnover of saliva maintain the pH above 6.0 at the tooth-surface. LMW carbohydrate becomes abundant while eating and drinking; bacterial metabolism peaks, and the local pH can drop to near 5: the so-called critical pH at which enamel dissolution (leading to caries) occurs. The critical pH of each individual is modulated by multiple variables [40]: salivary $Ca^{2+}/PO_4^{3-}/OH^-$ concentration, salivary production rate, dietary acid intake, host immune response, and by plaque community composition. Once 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 [41] and, as pH drops, acid-tolerant bacteria

out-compete others to become a higher proportion of the community. Interestingly, some streptococci metabolize arginine present in salivary oligopeptides: a metabolic pathway results in ammonium production and thereby elevates plaque pH. These streptococci can succeed despite absence of LMW carbohydrate [42]. The role of a balanced microbial community (microbial homeostasis) is described in the "ecological plaque hypothesis" [43], which relates disease (caries) to shifts in the overall community metabolism, subsequent modification of local environment, host factors, and bidirectional transitions between floras of varying cariogenicity. Implicit in this hypothesis is that the community is critical – organisms not typically considered cariogenic can be shown to be associated with progression of the disease [44] and clinical evidence indicates that progression of even grossly cavitated caries lesions can be halted if oral hygiene is improved [45].

Transitions in subgingival biofilms - periodontal diseases. Below the gum line, gingival crevicular fluid (exudate of host tissue into the sulcus) is the fluid phase; it bears little similarity to saliva, instead it is more akin to serum. The microflora likewise differ from that found supragingivally. Anaerobic bacteria occupy a niche characterized by catabolism of amino acids from exogenous protein via secreted proteases, and the overall species diversity is higher than for supragingival biofilms [46]. Some of these bacteria are considered to be periodontopathogens (e.g., *Porphyromonas gingivalis*) which are hypothesized to misdirect host defense and increase tissue-destructive inflammation [47]. 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; periodontopathogens are present in relatively low numbers. Poor oral hygiene changes the environment to support increased periodontopathogen biomass [48]; gingival detachment and bone and tooth loss follow. Treatment of the disease causes shifts in proportions of organisms towards those characteristic of healthy sites [49].

III. Initial colonization and the human enamel chip model

Clearly, transitions of species within populations that constitute supra- and sub-gingival plaque can pose problems for the development of tractable model systems to study oral communities. The complexity of population structure in older supra- and sub-gingival plaque makes 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 [16]. Sterilized retrievable enamel has been used for molecular phylogenic characterization of early plaque species [14] and for in situ characterization of spatiotemporal structure of mixed-species communities [15, 22, 23, 50, 51]. The small steps already taken by researchers towards understanding oral biofilms include determining the variety of species by culturing, the variety of phylotypes by molecular cloning techniques, and the succession of species with respect to host's age, host's gingival and periodontal health status, and time following the host's oral hygiene procedure.

Enamel is known to be sparsely colonized by multispecies communities in the first eight hours after cleaning [16, 17], and actinomyces, receptor polysaccharide (RPS)-bearing streptococci, and veillonellae are known to be in close contact in these initial communities [22, 23]. An understanding of physiological interactions within the community as well as the manner by which the communities are established is difficult to develop without having in culture precisely those organisms that comprise the community. To obtain these organisms, a spatially resolved isolation approach such as micromanipulation is required. However, micromanipulation requires time for the investigator to locate a prospective community and time for removal of the intact community. Photobleaching of fluorophores is a problem during long viewing periods, a constraint that can be overcome by using highly photostable quantum dots as the fluorophore in immunofluorescence identification of members within the multispecies community [19]. Quantum-dot-labeled primary antibodies against streptococcal receptor polysaccharide and against veillonellae were used to locate communities on enamel [18]. The labeled community might also contain members that are not antibody-labeled, i.e., are not seen (**Fig.2**). Indeed, in addition to the antibody-labeled veillonella and RPS-bearing streptococcus, an antibody-unreactive streptococcus was isolated. The streptococci (*S. gordonii*, non-targeted; *S. oralis* targeted by anti-RPS antibody) were coaggregation partners, consistent with a role for this cell-cell recognition in plaque formation. Because only these three species were present in this community, it is likely that small interactive communities such as these are established by cell-cell recognition and are the basic building blocks for oral biofilms in vivo. 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, indicating the significance of a critical absence of distance between cells. Access to actual communities and the ability to reassemble community participants as flourishing entities are important features that establish oral biofilms as paradigm systems.

IV. Coaggregation vs. coculture

Coaggregation: distance is critical. Coaggregation is an excellent model for the study of gene regulation in response to intimate interbacterial interactions. Coaggregation between compatible partners is easily induced by vortex mixing dense cell suspensions of the partners. With respect to gene regulation in the coaggregating pair of *S. gordonii* DL1 and *A. oris* ATCC 43146, two-species cocultured is vastly different from two-species coaggregated [20]. Distance is critical. Microarray analysis of *S. gordonii* DL1 genes regulated in coaggregates with *A. oris* ATCC 43146 revealed that the expression of only 23 genes changed >3-fold in coaggregates, suggesting a high degree of specificity of gene regulation in response to coaggregation [20]. Seven of the genes are the *bfbCDARBGF* operon associated with beta-glucoside utilization and identified by others to be involved in biofilm formation [52], which supports the idea that cell-cell surface interactions within coaggregates are useful models for studies of biofilms. Nine of the 23 genes were involved in arginine biosynthesis and transport, indicating the importance of this biosynthetic pathway to this intergeneric pair. In pure culture, *S. gordonii* DL1 arginine biosynthesis is inefficient; this streptococcus cannot grow aerobically at arginine

concentrations below 0.1mM. In coaggregates with *A. oris*, streptococci grew to high cell density, but, importantly, they failed to grow in coculture with actinomyces until coaggregates slowly formed during a 9-hr incubation period of coculturing. These data suggest that actinomyces stabilize streptococcal growth in low arginine but only when cell-to-cell distances are very short, as they are in coaggregates. The close proximity provided by cell-cell contact confers the ability to grow aerobically even in conditions of arginine limitation [20]. Clearly, the signal appears not to be diffusible; coaggregation is required; coculture in a closed system is insufficient. Interspecies juxtaposition, even in this closed system, is essential for communication.

Competition, cooperation and survival must be balanced in multispecies communities. Hydrogen peroxide is produced by many streptococci, and within oral bacterial communities, can be used as a competitive edge. Hydrogen peroxide can cross bacterial cell membranes and effectuate the oxidation of macromolecules including DNA and proteins. Detrimental carbonyl groups can be introduced into the side chains of arginine, proline, lysine, and threonine: this oxidation is irreversible, and carbonylated proteins are targeted for degradation [53]. Hydrogen peroxide is produced by pyruvate oxidase, SpxB, and in the microarray analysis of gene regulation by cell-cell contact in coaggregates of *S. gordonii* and *A. oris*, *spxB*, one of the 23 genes, was upregulated [20]. A connection between *spxB* and arginine biosynthesis was considered through possible carbonylation of amino acids [21]. Whereas monocultures of *S. gordonii* exhibited extensive protein oxidation and were rapidly killed following growth (>99% of cells died within 24 h after entry into stationary phase), in coaggregates minimal protein oxidation and no loss of

viability was observed. Therefore, *A. oris* protects *S. gordonii* from self-inflicted oxidative damage. This protective effect is presumably accomplished by the catalase of *A. oris* that also protects the actinomyces when cell densities of the species are low. Curiously, when allowed to grow together overnight, the streptococci kill actinomyces; *A. oris* cell numbers in coaggregates are 90 % lower than as monocultures [21]. In vivo interactions between streptococci and actinomyces are influenced by external factors including enzymes from saliva and from other oral bacteria. Perhaps, in vivo salivary and bacterial peroxidases reduce the hydrogen peroxide to levels that are tolerated by *A. oris*. Thus, while benefits to streptococci are evident, advantages for actinomyces remain unknown. These results suggest that hydrogen peroxide is a potent molecule that may dominate competitive and cooperative interactions [54] that lead to multispecies natural communities in dental plaque. A second microarray analysis with a streptococcal *spxB* mutant and *A. oris* might give illumination to the potency of hydrogen peroxide in altering oral microbial community development **[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 versus *P. gingivalis* alone revealed 403 downregulated and 89 upregulated proteins and an overall increase in proteins involved in protein synthesis [55]. One of the community-regulated *P. gingivalis* proteins, HmuR, a major hemin uptake protein, was targeted, and a Δ*hmuR* mutant was less able than the parent to partner with *S. gordonii* and *F. nucleatum* in three-member communities. An earlier study of *P. gingivalis* interactions with *S. gordonii* revealed over 30 porphyromonad genes were differentially regulated [56].

Collectively, these studies represent just the beginning of an exciting foray into the potential role of small molecule signals such as AI-2 in the development of multispecies communities.

Coculture. Coculture in a closed system can be sufficient for interspecies signal transduction, as has been shown in the coculturing of *Veillonella* sp. and *S. gordonii* V288 [57]. In this system, *S. gordonii* α-amylase was up-regulated in response to *Veillonella* sp. In closed batch culture, gene regulation was not dependent on coaggregation but occurred even when a dialysis membrane separated the organisms. However, intimate cell-cell contact was required to induce a streptococcal α-amylase (*amyB*) promoter-directed GFP expression in an open flowcell system, indicating that the putative signal was diffusible and accumulated in a closed space [57]. The *amyB* promoter region contains a 7-bp inverted repeat that matches 11 of 14 positions in the consensus of the catabolite-response element (CRE), and this site is recognized by the CcpA family of transcription regulators. The streptococcal CcpA homolog (previously called RegG) was shown to be required for the veillonellae-induced amylase expression in this interspecies interaction [58]. Wild type *S. gordonii* exhibited 3-fold higher amylase activity in cocultures with veillonellae than as monocultures, whereas no regulation of α-amylase was observed in the *ccpA* mutant during coculture [58]. Thus, interspecies cell-cell contact was not necessary for interaction between this pair of oral bacteria.

Cell-cell contact was also shown to be unnecessary for interkingdom signaling between oral organisms, specifically between *S. gordonii* and *Candida albicans* [59]. *Candida* spp.,

and particularly *C. albicans*, cause uncomfortable infections of the oral soft tissues primarily in very young, elderly or immunocompromised individuals [60]. It has long been known that treatment with antibacterial agents predisposes to overgrowth of *C. albicans* in the mouth [61], yet very little is understood about how the oral bacteria modulate colonisation by *Candida* spp. However, many species of oral bacteria coaggregate with *C. albicans* [62], and a recent study [59] provides evidence that interactions between oral bacteria and *Candida* spp. involve cross-talk between bacterial and fungal cell-cell communication systems. Two-species *S. gordonii*/*C. albicans* biofilms contained more biomass than the sum of the equivalent monospecies biofilms, indicating that interactions between these microorganisms are synergistic. Interactions with *S. gordonii* led to a number of changes in *C. albicans*, including increased production of hyphae and the activation or repression of three MAP kinases involved in morphogenetic switching. *S. gordonii* also interfered with the ability of the *C. albicans* intercellular signalling molecule farnesol to inhibit hyphal formation. *S. gordonii* adhered to *C. albicans* hyphae *via* interactions between streptococcal antigen I/II proteins and an unidentified *C. albicans* receptor. However, cell-cell contact was not essential for the *S. gordonii*-induced modulation of fungal hyphae formation, as shown by the production of hyphae in response to *S. gordonii* spent culture medium [59]. Therefore, it appears that *S. gordonii* produces a diffusible interkingdom signalling molecule that induces responses in *C. albicans* and interferes with *C. albicans* cell-cell communication.

V. DNA transfer in biofilms

A prime example of a microbial process where distance is critical and cell-cell contact is required is the transfer of DNA between cells by mating (conjugation). Genes encoded by conjugative transposons have been found in many genera of oral bacteria, including (but not limited to) *Actinomyces*, *Bifidobacterium*, *Fusobacterium*, *Haemophilus*, *Peptostreptococcus*, *Streptococcus* and *Veillonella* [63]. Analysis of sequenced oral bacterial genomes suggests that past horizontal gene transfer events account for between 5% and 45% of genes in different species [64]. For example, analysis of *P. gingivalis* strain ATCC 33277 revealed 13 regions of atypical nucleotide composition that are likely to have been acquired from foreign organisms [65]. A large degree of variation exists between strains of *P. gingivalis* suggesting that this organism has undergone frequent genetic recombination events resulting in a panmictic population structure, i.e. a multispecies population that has arisen through random exchange of DNA between individuals [66]. Extensive strain variation is characteristic of pathogens that are also present in the indigenous microflora, such as *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*. However, unlike these organisms, *P. gingivalis* does not undergo natural genetic transformation. It therefore seems likely that *P. gingivalis* has acquired foreign DNA through conjugation in oral biofilms. Conjugative transfer of DNA between different strains of *P. gingivalis* has been demonstrated in the laboratory [67], but has yet to be shown in biofilms.

Biofilms would appear to provide an excellent environment for DNA exchange since cells are in close proximity to one another, and DNA can be trapped within the extracellular matrix. There are several reports of horizontal gene transfer between oral

streptococci in biofilm communities. For example, conjugative elements harboring antibiotic resistance genes were transferred between streptococci in microcosm dental plaque biofilms [68] and in the mouths of volunteers [69]. Oral streptococci are naturally transformable, and it is possible that in these experiments extracellular-matrix DNA was transmitted without direct cell-cell contact. Indeed, a conjugation-defective plasmid could be transferred from *T. denticola* to *S. gordonii* in biofilms [70]. Although competence-dependent transformation does not require a physical bridge between the partner cells, the spatial arrangement of cells within biofilms may play a key role in this process by providing 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 the *comC* gene. The sequence of *comC*, and of its product CSP, varies between streptococci. Streptococci respond only to the variant of CSP that they produce. Consequently, CSP-mediated communication is essentially species- 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 [71].

In addition to their role in DNA uptake and incorporation, *S. mutans* competence genes also play key roles in releasing DNA, which stabilizes the architecture of biofilms [72]. In multispecies biofilms, CSP signalling by *S. mutans* is a target for competition by other oral streptococci. *S. salivarius* produces an extracellular product that interferes with *S. mutans* CSP and inhibits biofilm formation [73]. *S. sanguinis*, *S. mitis*, *S. oralis* and *S. gordonii* also interfere with *S. mutans* CSP. In the case of *S. gordonii*, the CSP-degrading

activity was identified as the extracellular protease challisin [74]. Degradation of CSP by oral streptococci may be a mechanism for self-protection, since high concentrations of *S. mutans* CSP trigger the production and secretion of bacteriocins that kill neighbouring cells and release their DNA [75]. *S. mutans* is immune to its own extracellular bacteriocins. However, the accumulation of CSP triggers the synthesis of an intracellular bacteriocin, and subsequent autolysis, in approximately 1% of the *S. mutans* population [76]. 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 mixed-species biofilms formed by many Gram-positive and Gram-negative bacteria [77, 78] and has been reported in natural microbial populations such as soil ecosystems [79]. Thus, the release of DNA from microbial cells in oral biofilms can be beneficial for neighboring bacteria in at least two regards: by stabilizing the structural integrity of the biofilm and, under conditions of stress, by disseminating resistance traits throughout the dental plaque population. The latter is of particular relevance for antibiotic treatment of periodontal disease, since there is concern that antibiotic interventions may select for widespread resistance among the dental plaque microflora.

VI. Mutualism and commensalism

Saliva as the sole nutritional source for multispecies growth. Flowcells viewed with confocal microscopy have been used to explore mutualistic growth of oral communities on saliva [24]. *Aggregatibacter actinomycetemcomitans*, *Veillonella* sp., and *F. nucleatum*

are unable to grow on saliva as monospecies biofilms, but pairwise and all together, they grow very well [80], indicating multispecies cooperation and mutualism **(Fig. 3)**. Biofilm growth on a transferable solid-phase polystyrene peg (TSP) [81, 82]submerged in saliva can be measured without imaging by, instead, using quantitative real-time PCR (qPCR). Multispecies biofilms can be quantified using species/strain specific primers [18, 25]. The polystyrene peg fits into a well in a 96-well microtiter plate and is a closed system, marginally 'open' when the TSP is transferred from one well to fresh saliva in another well. *Veillonella* sp. PK1910 grows in TSP biofilms only when streptococci are present [18], and *F. nucleatum* ATCC 10953 requires *A. oris* ATCC 43146 for growth in communities that include *S. oralis* 34 [25]. Viewing the veillonellae-streptococci biofilms by CSLM on the TSP surface revealed interdigitation of species and close cell-cell contact, indicating that minimal distance between cells is critical for community growth on saliva [18]. Thus, although saliva is a complex nutritional source, cooperation among oral bacterial species permits it to be a beneficial growth substrate.

Role of autoinducer-2 (AI-2) in a flowing environment. AI-2 is produced by the enzyme encoded by *luxS*, and it has been proposed as a universal intergeneric signal molecule [33]. *S. oralis* 34 and *A. oris* T14V are unable to grow as monoculture biofilms in flowing saliva, but together they grow luxuriantly [24]. These bacteria coaggregate, and their mutualism suggests that signals might be exchanged intergenerically in coaggregates. If *S. oralis* 34 is replaced by its isogenic *luxS* mutant, then the dual-species biofilm with *A. oris* T14V fails to grow; this observation supports the idea that AI-2 might be a signal required for mutualistic growth [27]. AI-2 is an umbrella designation given to a collection

of molecules formed from spontaneous rearrangement of DPD [83, 84], and, in solution, the various forms of AI-2 are in equilibrium [84]. Although *S. oralis* 34 and *A. oris* T14V produced AI-2 when grown planktonically in commercial media, AI-2 levels were low, below the detection limit of the *Vibrio harveyi* bioluminescence-based assay [85] (<50nM), in saliva-grown cells [27]. These data indicate that AI-2 is active at extremely low concentrations in dual species *S. oralis*-*A. oris* biofilms.

Luxuriant interdigitated growth of two-species biofilms was restored when synthetic DPD was added to the *S. oralis* 34 *luxS* mutant-*A. oris* T14V pair: restoration also occurred with a genetically complemented *S. oralis* 34 *luxS* mutant-*A. oris* T14V pair [27]. Maximal biofilm growth of the *S. oralis* 34 *luxS* mutant-*A. oris* T14V pair was achieved at 0.8nM DPD; at lower or higher concentrations, biofilm biomass was reduced. At 800nM DPD, co-culture biofilm biomass was equivalent to that seen without added DPD, indicating that the optimal concentration for chemical complementation in the oral system is 100-fold below the level detectable by the bioluminescence assay.

Recent improvements in saliva-grown biofilms and in AI-2 detection may facilitate rapid advances in understanding the potential role of AI-2 as a universal signal in natural biofilms, such as dental plaque. It is possible that the failure of the *S. oralis*-*A. oris* biofilm to produce a detectable level of AI-2 arises from the low overall biomass (source of production) coupled with the dilution by flow. High cell densities of 1×10^9 cells/ml are attainable using a sorbarod device [86]: a cellulose-fiber cartridge in which the colonizable surface area is high when compared with the liquid volume. Such cell

densities compare favorably with those in planktonic broth cultures typically assayed for AI-2 by the bioluminescence assay. In the sorbarod biofilm model *S. oralis* 34 and *A. oris* T14V were in intimate contact on the fibers [26]. At these high cell densities, AI-2 was detected in nanomolar amounts by the bioluminescence assay. Recent advances in AI-2 detection methods now make it possible to detect AI-2 in biological samples containing concentrations as low as 5 nM [87] or even 230 pM [88], thereby greatly increasing the scope of AI-2 investigation in biofilm systems. For example, sensitive chemical-based detection methods would clarify the AI-2 contribution in cell-free conditioned medium from *A. actinomycetemcomitans* that complements a *luxS* mutation in *P. gingivalis* [89]. This communication between two periodontopathogens is a model system for exploring the role of AI-2 as a universal interspecies signaling molecule.

Local vs. global concentration of AI-2 concentration, equilibrium form, and proposal for relationship with spatiotemporal development of multispecies communities. Because the effective signal concentration of AI-2 differs across several orders of magnitude between the oral system and the *V. harveyi* bioassay, it is likely that different concentrations of AI-2 are optimal for communication between different species. A diagrammatic representation of this hypothesis relevant to biofilm development in the human oral cavity is presented in **Figure 4**. Dental plaque development is diagrammed with respect to time. The relative amount of AI-2 produced by commensal bacteria and pathogens is shown in the box at the bottom right of the figure. A survey of many pure culture supernatant fluids of oral strains has shown that, generally speaking, commensal bacteria produce less AI-2 compared to oral pathogens [90]. We propose that commensal bacteria

such as *S. oralis* and *A. oris* communicate optimally at AI-2 levels below those optimal for species such as *F. nucleatum* that might be associated with the transition from a commensal community to a pathogenic community. Although present in significantly high numbers in saliva, fusobacteria are infrequent in dental plaque during the first 8 h of development [91], suggesting that the microenvironment and possibly the AI-2 concentration are not optimal. Fusobacteria produce high amounts of AI-2 [90], coaggregate with a wide variety of early and late colonizers [92], and become the dominant gram-negative species in healthy and gingivitis plaque [93]. As biomass increases on the tooth surface, pathogenic and transition species flourish in the high AI-2 concentration. However, commensals are inhibited because the AI-2 level is too high, as was the case for the *S. oralis*-*A. oris* pair discussed earlier. Oral hygiene procedures such as brushing and flossing return the enamel surface to stage 1 where commensals again dominate. We propose that 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 might lead to stages 3 and 4 and towards gingivitis and periodontal disease. This model offers a simple way to conceptualize distance-critical communication within each multispecies community producing AI-2 and each community responding optimally to a particular local concentration of the signal.

In the flowing environment of the oral cavity, coaggregation among the community members is a way in which local signal concentration can be maintained. Coaggregation reduces the distance between cells of participating partners and facilitates signal exchange within each community. In the model presented in **Fig. 4**, the local AI-2

concentration increases within communities and encourages incorporation of species that function at higher signal concentration while discouraging growth of the pioneer species that function at lower signal concentration.

In this model, retention of community-generated AI-2 and internalization of AI-2 by the members of the community is essential for biofilm development. The internalization transport apparatus described in *Salmonella enterica* serovar Typhimurium includes the AI-2 binding protein LsrB, which is encoded in an operon *lsr* (for LuxS Regulated) [94]. Whereas numerous oral species produce AI-2, only A. *actinomycetemcomitans* is known to possess an LsrB homologue [95, 96], and the LsrB protein was required for biofilm formation of *A. actinomycetemcomitans* [95, 96]. *A. actinomycetemcomitans* forms mutualistic communities with *Veillonella* sp. and *F. nucleatum* [80], and, like the *S. oralis*-*A. oris* pair described earlier, the *A. actinomycetemcomitans, Veillonella* sp. and *F. nucleatum* three-membered community could be an AI-2-responsive community.

Although most oral species might produce AI-2, the equilibrium form of DPD to which a given species responds might be distinct from forms that are favored by other species in the community. The particular form of DPD stabilized within a multispecies community in the biofilm might be dependent on the respective concentrations of acid/base, oxygen level, redox level, type of nutrient or endproduct in this micro-environment. Within the dental plaque biofilm numerous micro-environments exist, each with its own acid/base concentration, redox potential, and nutrient level and type [97]. Some sites in a biofilm might support development of a certain cluster of species, whereas a nearby site could

support a completely distinct multispecies community because the site has an altered acid/base concentration, redox potential, and nutrient level and type. The development of these communities can be dependent upon the same universal inter-species signal, AI-2, which is produced by the initial occupants of the site. Not only could the AI-2 local concentration differ, but the equilibrium forms of AI-2 might constantly re-stabilize in coordination with multispecies community changes through growth and coaggregation. A multispecies community could alter its microenvironment in response to the AI-2 signaled changes in gene expression by the community members. Altered acid/base concentration, redox potential, and nutrient level and type at the site could be outcomes of gene expression, which in turn cause changes in the composition and proportion of community members as they rearrange and develop into mature biofilm communities. Thus, the optimal concentration and equilibrium forms of AI-2 for sustained community growth are hypothesized to direct distance-critical multispecies community development of oral dental plaque biofilms (**Figs. 1 and 4**).

VII. Communities, not species

Traditional bacteriological approaches have described culturable microflora of accessible human body sites. Data from 16S-rRNA gene cloning and sequencing studies indicate that the oral cavity has roughly 700 phylotypes of which slightly less than half are cultivated [46]. In comparison the skin of the forearm bears 182 phylotypes of which 80% are cultivated [98]. The power of molecular methods in identifying new phylotypes is demonstrated by a study in which 28 of 56 fresh subgingival bacterial isolates (*i.e.*,

cultivated bacteria) were new phylotypes [99]. The impact on oral microbial ecology of this phylotype diversity is difficult to assess, especially in the context of multispecies interactions. Paramount to microbial community structure is the niche (the metabolism) of the set of phylotypes comprising the community and that a similar niche might be composed of a different set of phylotypes. This perspective requires advances in bacterial isolation [100] and domestication [101] 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* [102, 103], *Staphylococcus aureus* [104], *A. actinomycetemcomitans* [105], and *F. nucleatum* [106]; 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 useful for tracking and predicting rapid successions of community members in natural populations [22]. Communities composed of 'wild' isolates can be 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-pathogen transitions are driven by a host of variables, many of which are controllable. The current non-specific approaches to oral hygiene do much to prevent such transitions; this suggests that community-level changes are the key changes, which are driven by niche alterations [43, 107]. A revision of Koch's postulates, whereby the

community is the etiological agent [**Box 4**], may be useful. In this hypothesis, the downstream consequences of community metabolism (*e.g.*, acid production) are the important factors in genesis and progression of the disease. Therefore, metabolic relationships within a community, created and maintained by different suites of phylotypes, could result in similar consequences. Spatial relationships between phylotypes, and between community and host, may dramatically effect niche transitions [14, 23]. Little-studied culturable, or soon-to-be cultured, organisms may drive transition or support stability.

VIII. Summary and Future Direction

Human oral biofilms exist as multispecies communities, which can be isolated and reconstructed in vitro in model saliva-based systems. Several species are mutualistic during growth on saliva, suggesting that communication occurs among them. Signals such as AI-2 affect community growth, and the mechanisms of community responses are prime targets for future discovery. One likely discovery will be regulation of 'community' genome expression (the collective expression of all community members) in oral niches through the uptake of 'community-generated' AI-2, especially by species that do not produce AI-2, such as that known for the relationship between two soil species, *Sinorhizobium meliloti* internalizes AI-2 produced by *Erwinia carotovora* [108]. The advance of microfluidics research will benefit 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. How do these bacteria communicate with one another to create functional communities? Is the host role one of action, reaction, or both? What are the specific niches important to the transition from health to disease, and can healthcare providers intercede in that progression in a more efficient manner than is currently in use? How are the microbes changing in an evolutionary sense and what does this mean for individual species as well as for the community? These questions can be best answered by investigation of gene expression in a spatiotemporally resolved manner, by examination of interbacterial interactions known to occur in the biofilm. The oral biofilm has been, and will remain, the paradigm system for these studies.

Boxes 1, 2, 3, and 4:

Box 1. Specificity of coaggregations

Many coaggregations are inhibited by simple sugars such as lactose, and these coaggregation partnerships are mediated by a lectin-like protein adhesin on one cell type and a complementary carbohydrate receptor on the partner cell type. Coaggregation is different from aggregation that occurs between genetically identical cells, and it is different 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 commonly in culture. 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 are unable to coaggregate with those veillonellae but do coaggregate with a separate group of veillonellae [22].

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 sufficient for signalling. The local concentration of a microbial product is determined by the rate of production and the rate of removal via 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 variety of fluorescently labelled macromolecules [109]. Molecules up to 200 μm in diameter reached the centre of cell clusters within 3 min; smaller molecules penetrated the centre of the biofilms within 10 s. For the largest molecule tested, immunoglobulin G (MW 150 kDa), the diffusion constant through biofilms was 22% of that through pure water. Therefore, oral biofilms apparently provide relatively little resistance to diffusion of extracellular molecules. Nevertheless, movement of molecules through biofilms may be impaired if the molecules interact with bacterial cell surfaces, as do cations for example. Local concentrations of signalling molecules 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 latter certainly applies to signaling molecule AI-2: picomolar concentrations of AI-2 promote mutualistic biofilm growth of *S. oralis*/*A. oris* cocultures [27]. In some cases, communication might occur in the absence of signaling molecules, through direct cell-to-cell contact between genetically distinct cells.

Box 3. Probing distance-critical communication using microarrays

In the post-genomic era, a powerful approach for analysing interspecies communication is provided by DNA microarrays. All species of oral bacteria are capable of

coaggregation, and coaggregation provides an excellent model for investigating distance-critical interactions between different bacteria. Gene expression in mixed-species communities (coaggregates) can be compared with that in equivalent monocultures. Potentially, genes identified in this way may reveal processes that are important during multispecies biofilm formation, and may also give clues regarding the nature of molecules that mediate communication. Microarrays have been employed, for example, to identify responses of *S. gordonii* to coaggregation with *A. oris* [20]. *S. gordonii* is known to coaggregate with *P. gingivalis* [57, 92, 110], as well as many other oral species. Which genes would be identified if the partner organism were changed to *P. gingivalis*, *Veillonella* sp. or *F. nucleatum*? Are there universal responses to coaggregation or is the pattern of gene regulation entirely dependent on the interacting partner? 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, would the same genes have been identified if the samples for microarray had been collected immediately after inducing coaggregation, or after 2 h rather than 3 h? Currently, five microarrays for oral bacteria are available, upon application, to research scientists from the NIDCR Oral Microbial Microarray Initiative (http://www.nidcr.nih.gov/Research/DER/IntegrativeBiologyAndInfectiousDiseases/NOMMI.htm). Applying these, along with custom-designed microarrays, to study gene expression in mixed-species cultures will reveal a great deal about how bacteria sense interactions with neighbouring cells.

Box 4. Communities as etiological agents

The relevance of "Koch's postulates" of microbial disease has been questioned of late, 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 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 insufficient to cause either caries or periodontitis in most individuals. Conversely, the absence of *S. mutans* does not insure caries-free dentition. The suite of organisms carried by healthy individuals is not necessarily greatly different to that of diseased individuals. 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, periodontitis-inducing) created by the community, are key. 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 fulfillment of the third postulate. As for Koch's postulate that requires the agent not be found in a non-pathogenic situation, the recent recognition of some diseases as polymicrobial in origin does seem to allow for an exception to his otherwise historically proven ideas.

Glossary

Salivary pellicle

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

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.

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.

Gingivitis

Minor and reversible inflammation of the gum tissue.

Periodontitis

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

Mutualism

An interaction between two or more microorganisms that has beneficial consequences for the partners involved.

Figure legends:

Fig. 1. 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. Each coaggregation depicted is known to occur in a pairwise test. Collectively, these interactions are proposed to represent development of dental plaque. Starting at the bottom, initial colonizers bind via adhesins (round-tipped black line symbols) to complementary salivary receptors (blue-green vertical round-topped columns) 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. Several kinds of coaggregations are shown as complementary sets of symbols of different shapes. One set is depicted in the box at the top. Proposed adhesins (symbols with a stem) represent cell-surface components that are heat inactivated (cell suspension heated to 85^{o}C for 30 min) and protease sensitive; their complementary receptors (symbols without a stem) are unaffected by heat or protease. Identical symbols represent components that are functionally similar but may not be structurally identical. Rectangular symbols represent lactose-inhibitable coaggregations. Other symbols represent components that have no known inhibitor. Communication among cells is favored by their close cell-cell contact. The bacterial species shown are

Aggregatibacter actinomycetemcomitans (formerly *Actinobacillus actinomycetemcomitans*), *Actinomyces israelii, Actinomyces naeslundii, Actinomyces oris, Capnocytophaga gingivalis, Capnocytophaga ochracea, Capnocytophaga sputigena, Eikenella corrodens, Eubacterium* spp., *Fusobacterium nucleatum, Haemophilus parainfluenzae, Porphyromonas gingivalis, Prevotella denticola, Prevotella intermedia, Prevotella loescheii, Propionibacterium acnes, Selenomonas flueggei, Streptococcus gordonii, Streptococcus mitis, Streptococcus oralis, Streptococcus sanguinis, Tannerella forsythia, Treponema denticola*, and *Veillonella* spp. (figure redrawn from Kolenbrander, et al. [111])

Fig. 2. Confocal micrograph of 8 h-dental plaque from the study of Chalmers, et al. [18]. (A) QD-based primary immunofluorescence reveals RPS-bearing streptococci reactive with QD655-conjugated-anti-RPS (red) juxtaposed with veillonellae reactive with QD525-conjugated-anti-R1 (green). A community representative of those selected for micromanipulation is circled. (B) Same field of view as in panel A but with DAPI-stained cells (blue) also shown. The general nucleic acid stain DAPI reveals antibody-unreactive cells, one of which is located in the representative community. DAPI was not used on micromanipulated samples. Bar, 10 μm.

Fig. 3. 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 h 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.

Fig. 4. Illustration of proposed relationship between succession of oral communities and the AI-2 concentrations to which they respond. Commensals (depicted in stages 1 and 2) respond to the lowest AI-2 concentrations (below 100 pM), which results in mutualism and bacterial growth. Initial colonizers such as streptococci (blue circles) and actinomyces (dark blue oblong shapes) bind to the salivary pellicle (stage 1), which coats the enamel, and subsequently grow together with veillonellae (yellow circles) as mixed-species biofilm communities (stage 2). As the commensal bacterial biomass increases by cell division and by accretion, the AI-2 concentration increases, which improves the communication among transition bacterial species such as fusobacteria (orange slender tapered rods; stage 3). Finally, when the AI-2 concentration is the highest (stage 4), the pathogens are favored for growth and join the developing biofilm communities.

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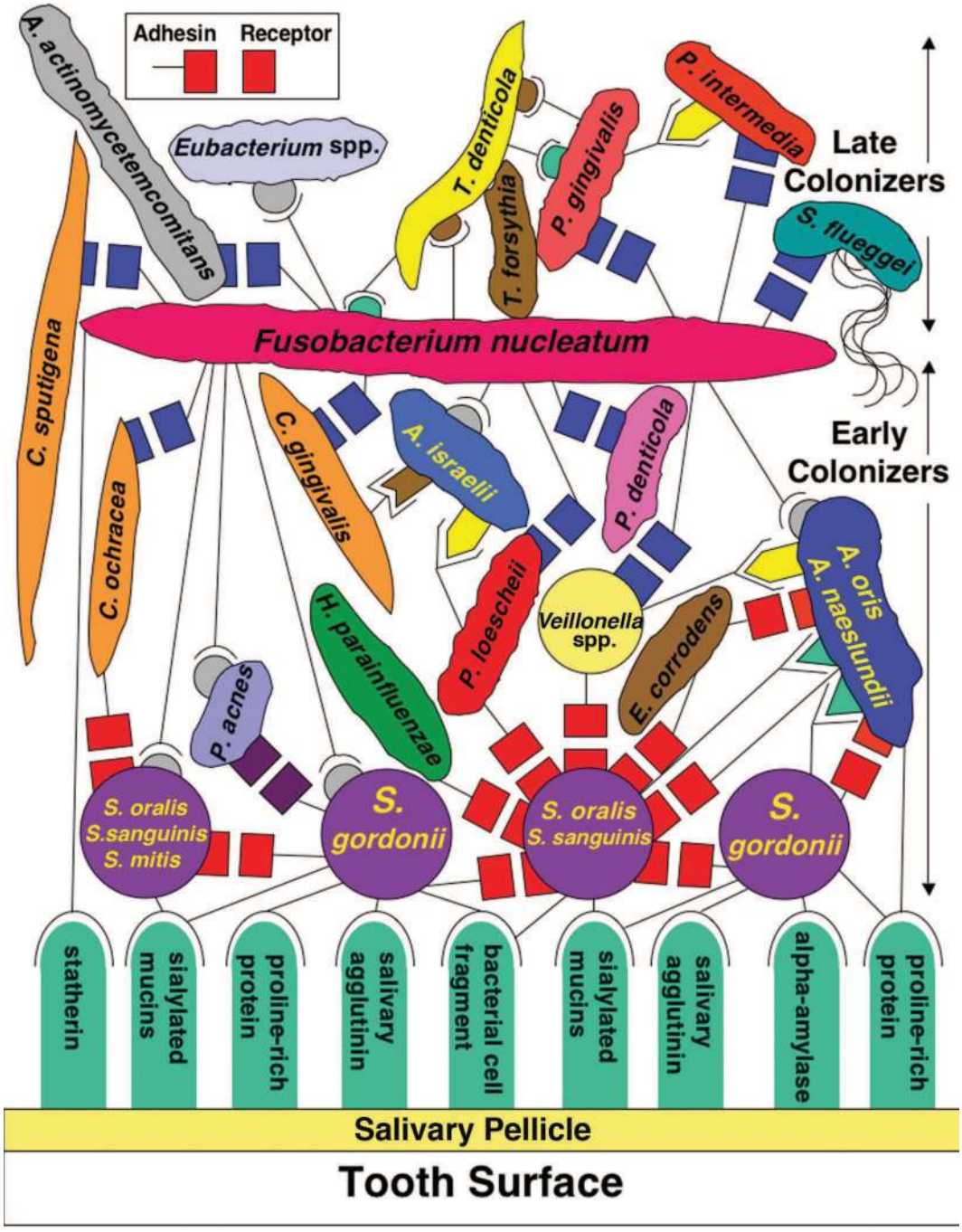
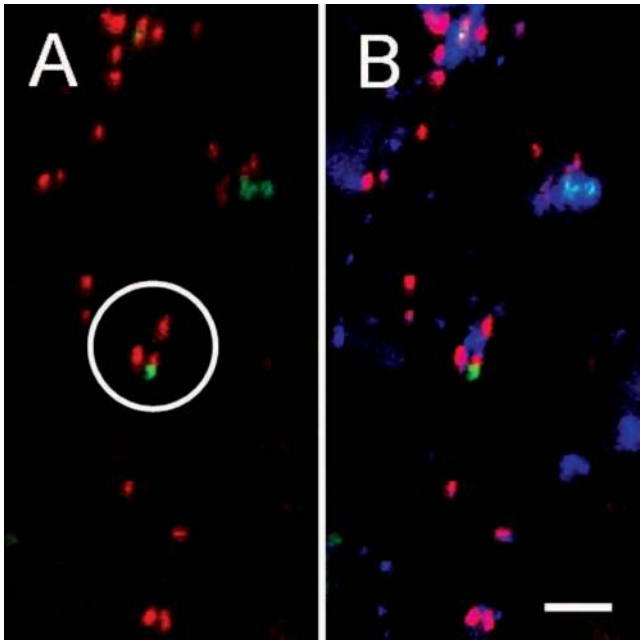
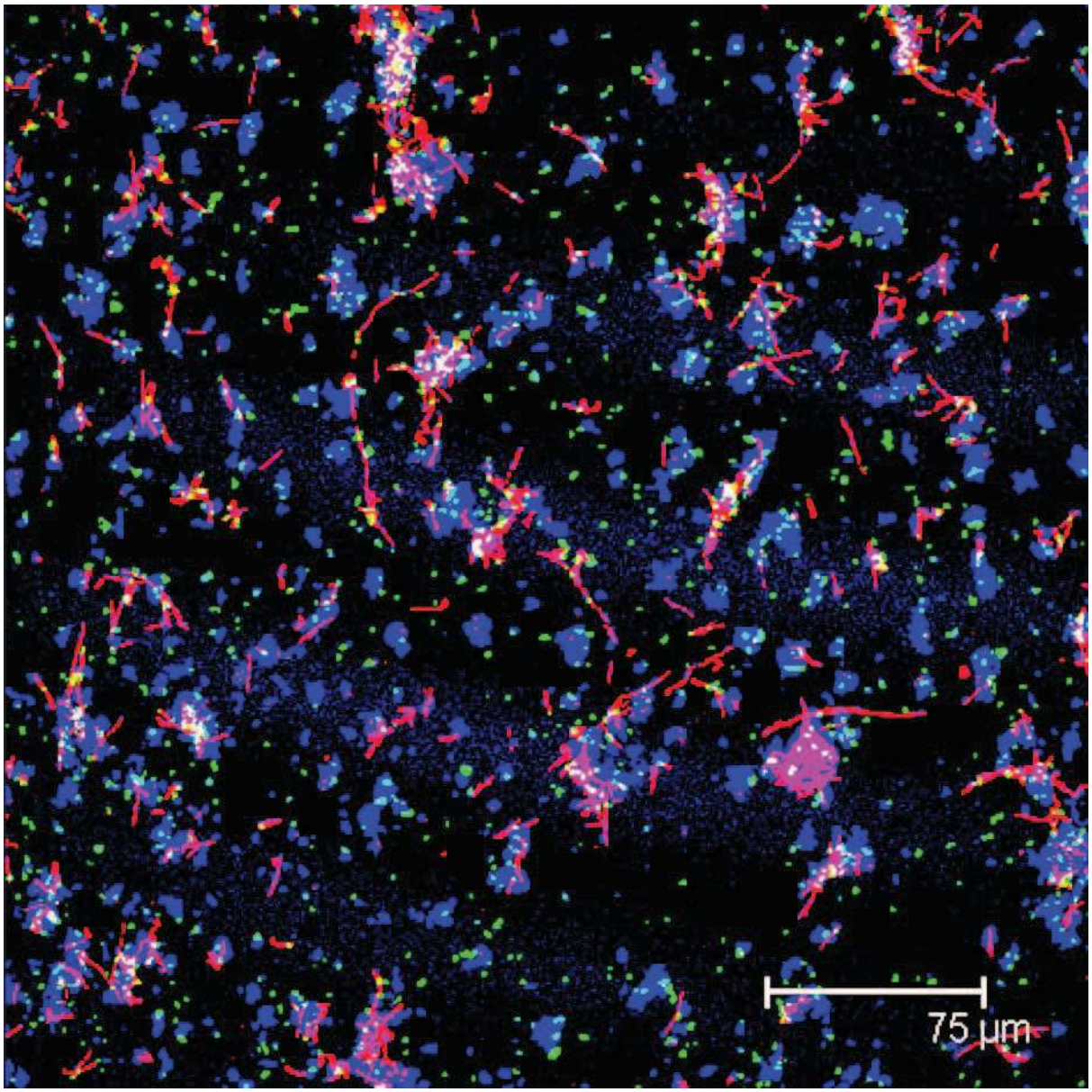
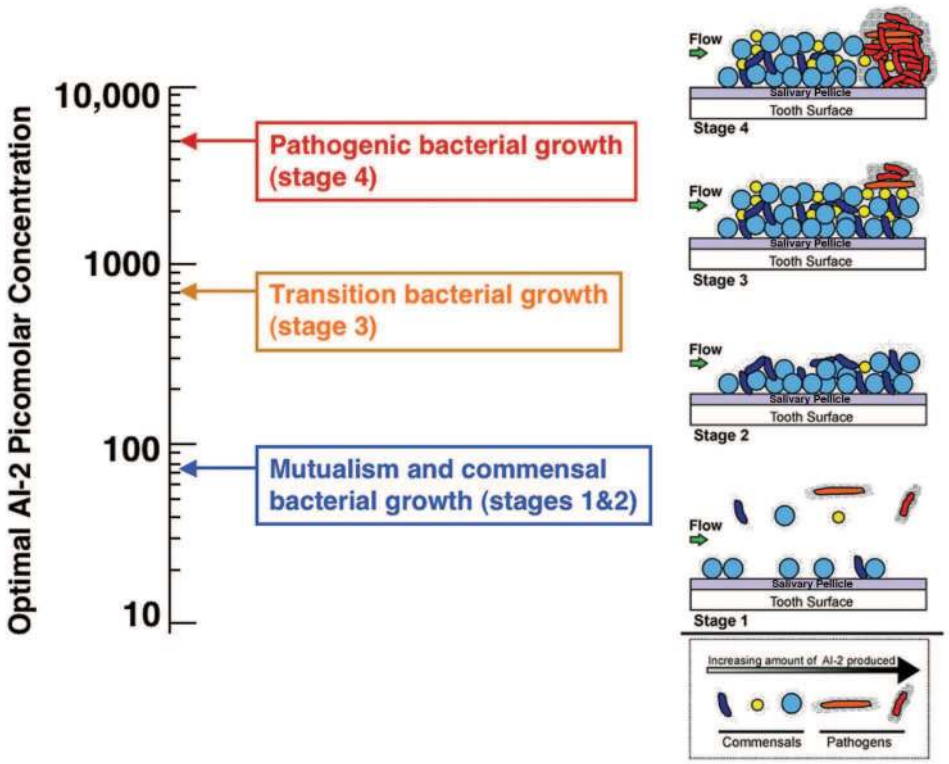

Adhesin
Receptor
A. actinomycetemcomitans
Eubacterium spp.
T. denticola
T. forsythia
P. gingivalis
P. intermedia
Late Colonizers
S. flueggei
Fusobacterium nucleatum
C. sputigena
C. ochracea
C. gingivalis
A. israelii
P. denticola
Early Colonizers
P. loescheii
Veillonella spp.
E. corrodens
A. oris
A. naeslundii
H. parainfluenzae
P. acnes
S. oralis S.sanguinis S. mitis
S. gordonii
S. oralis S. sanguinis
S. gordonii
statherin
sialylated mucins
proline-rich protein
salivary agglutinin
bacterial cell fragment
sialylated mucins
salivary agglutinin
alpha-amylase
proline-rich protein
Salivary Pellicle
Tooth Surface


A
B


75 µm


Optimal AI-2 Picomolar Concentration
10,000
1000
100
10
Pathogenic bacterial growth (stage 4)
Transition bacterial growth (stage 3)
Mutualism and commensal bacterial growth (stages 1&2)
Flow
Salivary Pellicle
Tooth Surface
Stage 4
Flow
Salivary Pellicle
Tooth Surface
Stage 3
Flow
Salivary Pellicle
Tooth Surface
Stage 2
Flow
Salivary Pellicle
Tooth Surface
Stage 1
Increasing amount of AI-2 produced
Commensals
Pathogens