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Subgingival biofilm formation

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The human body contains numerous distinctive ecosystems that provide a unique environment for colonizing microorganisms. The periodontal pocket is one such microniche. This environment is partially sheltered from the physical shear forces in the oral cavity and contains the hard, nonshedding surfaces of the tooth root along with the shedding surfaces of gingival mucosa. The junctional epithelium, which is attached to the tooth root, is poorly differentiated, lacks keratinization and has relatively wide intercellular spaces. Consequently, junctional epithelium is permeable and allows the migration of polymorphonuclear leukocytes into the periodontal pocket. Furthermore, the tissues in the periodontal pocket are bathed in gingival crevicular fluid, a serum exudate with antioxidant properties. The initial bacterial colonizers attach to the available surfaces, as discussed elsewhere in this volume of *Periodontology 2000*. Later colonizers attach to the antecedent organisms and assemble into polymicrobial communities. The biofilms on the hard surfaces develop into spatially organized structures that can extend several hundred micrometers from the surface. By contrast, the epithelial surfaces, which are continually being sloughed and replenished, tend to be colonized with monolayers of microorganisms. However, several of the more pathogenic species of bacteria are able to invade the gingival cells and tissues where they can remain viable and thus constitute a nidus of infection.

Interspecies adherence interactions help to shape the temporal and spatial development of the complex bacterial consortia in the gingival crevice. Bacteria within these communities encounter high cell densities and, in consequence, community living involves adaptation to higher (and unevenly distributed) levels of metabolic by-products, secondary metabolites and other secreted molecules, and to the sporadic availability of nutrients and oxygen. Bacterial inhabitants of biofilms are known to both collaborate (e.g. through nutritional cross-feeding) and compete (e.g. through production of bacteriocins) as they strive to optimize their adaptation to these environmental constraints. Bacteria can also communicate with one another through a variety of sensing and response systems based on either cell-to-cell contact or detection of soluble mediators. The signaling molecules are processed through transcriptional and post-transcriptional networks and they allow bacterial inhabitants of biofilms to coordinate activities at a group or community level. An understanding of the mechanisms of subgingival biofilm formation and development needs, therefore, to accommodate the multiple interspecies interactions that occur in polymicrobial communities.

Co-adhesion controls community architecture

The predominant early colonizers of the subgingival plaque biofilms are the Actinomyces species and streptococci (110). A complex microbial community then develops within the space of only a few days (76), and the secondary colonizers tend to be the more pathogenic species such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans*. These later colonizers express numerous adhesions that enable attachment to the earlier bacterial inhabitants of the region, often ‘choosing’ partners that are metabolically compatible. Moreover, a number of the secondary colonizers, in particular *F. nucleatum* and *P. gingivalis*, can bind both to early colonizers and to other, later, colonizers (46, 113), thus

contributing a bridge or node function to the developing polymicrobial consortia. A surface configuration that presents multivalent adhesins, along with multiple adhesins with distinct specificity, as found on *P. gingivalis*, for example, will favor community development.

The importance of co-aggregation or co-adhesion for the development of plaque biofilms has been demonstrated *in vivo*. Slots & Gibbons (95) reported that the introduction of *P. gingivalis* into the mouths of human volunteers resulted in the organism locating almost exclusively on preformed, streptococcal-rich supragingival plaque. A close spatial association between streptococci and *Veillonella*, and between streptococci and *Actinomyces* – pairs of organisms that co-aggregate *in vitro* – has been visualized in developing plaque communities *in vivo* (11, 12, 71, 72). The ability of potential periodontal pathogens to locate and attach to compatible antecedent colonizers may therefore drive the development of pathogenic subgingival plaque.

Mechanisms of interspecies binding

A number of studies addressing co-aggregation among subgingival organisms have started to reveal the mechanistic basis of these interactions. *F. nucleatum* binds to *P. gingivalis* through a galactose-specific lectin-like adhesin that recognizes the sugar moiety in the capsule and lipopolysaccharide of *P. gingivalis* (44, 45, 84). Galactose-containing receptors for attachment to *F. nucleatum* are also provided by the serotype-specific O polysaccharide of *A. actinomycetemcomitans* (85) and by the carbohydrate moieties on the major outer sheath protein of *T. denticola* (83). Moreover, as an illustration of the multiplicity of adhesin expression, an arginine-inhibitable adhesin (RadA) of *F. nucleatum* is responsible for co-adhesion with oral streptococci and accumulation into mixed-species biofilms (39). Hence, binding of *F. nucleatum* to streptococci will not occupy all of the fusobacterial adhesins, and so this configuration of adhesins will allow fusobacteria–streptococci consortia to recruit additional gram-negative pathogens.

T. denticola and *P. gingivalis* have been shown to accumulate into dual-species biofilms. Attachment and accumulation requires functional *T. denticola* flagella, while the long (FimA) fimbriae and Arg-gin-gipain (Rgp) B of *P. gingivalis* also play important roles in biofilm formation (112). Leucine-rich repeat proteins of *T. denticola* and *T. forsythia* participate in interbacterial binding with each other and with *F. nucleatum* (34, 93).

P. gingivalis–*Streptococcus gordonii*

One of the best characterized interspecies co-adhesion systems is the binding of the periodontal pathogen *P. gingivalis* to substrata of *S. gordonii*. This interaction may occur on supragingival surfaces and, indeed, *P. gingivalis* is now known to be a common inhabitant of the supragingival biofilm (28, 58, 100, 110), and can even be detected supragingivally in the absence of subgingival colonization (111). Consequently, *P. gingivalis* will be able to establish a foothold on the supragingival tooth surface, from where colonization of the subgingival area can occur by spreading proliferation or by translocation of dislodged progeny. Alternatively, or concomitantly, the interbacterial binding interaction may occur subgingivally, as *S. gordonii* and related streptococci are common and abundant constituents of subgingival plaque (27, 96, 110, 111). Accumulation of *P. gingivalis* occurs on the streptococcal substrate in the absence of significant growth and division (57), and thus represents a means by which the biomass of *P. gingivalis* in a community can increase through attachment and recruitment of cells from the planktonic phase (Fig. 1).

***P. gingivalis* adhesins**

Co-adhesion between *P. gingivalis* and *S. gordonii* is mediated by two sets of adhesion–receptor pairs: the long (major) and short (minor) fimbrial subunit proteins of *P. gingivalis* that interact with streptococcal glyceraldehyde-3-phosphate dehydrogenase and Ssp surface proteins, respectively (49–51, 56, 75) (Fig. 2). The long fimbriae are composed of the FimA structural subunit protein and extend approximately 3 µm from the cell surface. *fimA* is part of a gene cluster that includes the downstream genes *fimC*, *fimD* and *fimE*, which encode minor components of mature fimbriae (70). FimE is required for the assembly of FimC and FimD onto the fimbrillin (FimA) fiber (70). The two genes upstream of *fimA* are involved in the regulation of *fimA* expression under the control of the FimS–FimR two-component system (32, 69). Expression of *fimA* is also controlled by the levels of FimA protein itself and by the Rgp and Kgp gingipains (106). The expression of *fimA* responds to environmental cues relevant to conditions in the subgingival area, such as temperature and hemin concentration (2, 105). The FimA–glyceraldehyde-3-phosphate dehydrogenase interaction is the initial contact event that allows localization of *P. gingivalis* on the streptococcal surface (50). The binding domains of FimA that mediate attachment to streptococci are localized to a C-terminal region spanning amino acid residues 266–337 (1).

The short fimbriae of *P. gingivalis* are approximately 6.5 nm wide and 103 nm long, and are composed of the Mfa structural subunit protein (75). Similarly to *fimA*, *mfa* is also part of a gene cluster; however, the roles of the downstream gene products in the biogenesis of the short fimbriae remain to be determined. The Mfa protein engages the Ssp proteins on the streptococcal cell surface and increases the avidity of binding to be more resistant to shear forces. Mfa–Ssp interactions also initiate a signal transduction cascade within *P. gingivalis* that prepares the cells for community living (described later). Moreover, as the *P. gingivalis*–*S. gordonii* community develops, the expression of *mfa* is down-regulated, presumably reflecting differing adhesin requirements of the organism as the streptococcal substrate becomes unavailable to *P. gingivalis* arriving later (74).

While FimA and Mfa facilitate the accumulation of *P. gingivalis* on streptococcal substrates, other *P. gingivalis* surface molecules can act to constrain community development. For example, InlJ, a member of the cysteine-rich leucine-rich repeat in-ternalin proteins (86), retards the development of *P. gingivalis*–*S. gordonii* communities (9). The availability of surface effectors that can either promote or reduce community development may allow *P. gingivalis* to fine-tune the extent of accumulation according to environmental conditions (as discussed in greater detail below).

The streptococcal contribution to community development

The Ssp adhesins (SspA and SspB) are major surface proteins of *S. gordonii* and members of the Ag I / II family that is widely distributed in the oral streptococci (37). The SspA and SspB polypeptides are encoded by tandemly arranged, monocistronic chromosomal genes and are independently expressed (19). The Ssp proteins also mediate attachment of *S. gordonii* to the salivary pellicle and their expression is up-regulated by saliva (22), increasing the receptor availability for *P. gingivalis*. The SspA and SspB proteins are structurally conserved and comprise seven discrete regions: a signal peptide; an N-terminal region; alanine-rich repeat blocks; a divergent or variable central region; proline-rich repeat blocks; a C-terminal region; and a cell-wall anchorage domain (37). Structure–function analyses on the mechanism of the Mfa–SspB interaction identified a discrete region of SspB, designated the SspB adherence region, which spans amino acid residues 1167–1193. The SspB adherence region is fully conserved between SspA and SspB and is necessary for attachment to *P. gingivalis* cells or purified Mfa (8). Within the SspB adherence region, residues N1182 and V1185 of an NITVK motif are essential for the recognition of SspB by

Mfa (20), and these residues, along with T1184, are not conserved in SpaP, the *Streptococcus mutans* homolog of Ssp that does not bind to Mfa. The NITVK domain is fully conserved in *Streptococcus oralis* and *Streptococcus sanguinis*, species that also accumulate in dual-species communities with *P. gingivalis*. Substitution of basic amino acids or serine for N1182, and substitution of hydrophobic residues Ile, Trp or Phe for V1185, enhances the degree of *P. gingivalis* attachment to the SspB adherence region, suggesting that both electrostatic and hydrophobic interactions contribute to SspB adherence region–Mfa binding (16). Furthermore, substitution of the α -helix breaking residues Pro or Gly is detrimental for *P. gingivalis* adherence, consistent with the prediction that secondary structure plays a role in *P. gingivalis* adherence (16). The SspB adherence region also possesses a domain immediately upstream of the NITVK motif that resembles the eukaryotic nuclear receptor box (17). Interactions of nuclear receptors with their co-activating proteins is driven by the association of a hydrophobic α -helix of consensus sequence LXXLL, the nuclear receptor box, with a hydrophobic pocket in the nuclear receptor protein. This initial interaction is stabilized by electrostatic interactions that form with charged amino acids that flank LXXLL (90). The SspB adherence region equivalent contains a predicted hydrophobic α -helix of sequence VXXLL that is flanked on each side by positively charged lysine residues. The introduction of amino acids with the potential to disrupt the secondary structure of VXXLL reduces the binding activity of the SspB adherence region, suggesting that the putative α -helical character of VXXLL is important for the interaction of the SspB adherence region with Mfa (17). Furthermore, replacing the lysines that flank VQDLL with acidic amino acids also reduces activity, suggesting that the association of VQDLL with Mfa may be stabilized by a charge clamp.

In addition to adhesins, a number of streptococcal processes contribute to community development with *P. gingivalis* (47). These can be grouped into broad categories, as follows: (i) intercellular or intracellular signaling (chorismate-binding enzyme, pyruvate oxidase, MarR family transcriptional regulator); (ii) cell wall integrity and maintenance of adhesive proteins [methionine sulfoxide reductase, UDP-*N*-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase (MurE)]; (iii) extracellular capsule biosynthesis (cell wall polysaccharide biosynthesis protein); and (iv) physiology (glutamate dehydrogenase, ABC transporter ATP-binding protein, V-type ATP synthase). Deletion of genes encoding these proteins diminishes heterotypic community formation (47). Moreover, several of the genes encoding these proteins are clustered in a 40-kb region on the *S. gordonii* chromosome. This cluster also contains *bfrA / B*, a two-component system, and *bgIB*, a beta glucoside, both of which are involved in monospecies *S. gordonii* biofilm formation (114). As an organism that is adapted to life in oral communities, *S. gordonii* may benefit from homotypic and heterotypic biofilm-related genes being in relatively close proximity.

Monospecies *P. gingivalis* accumulations

While monospecies biofilms are unlikely to be prevalent in the subgingival area, the rapid accumulation of *P. gingivalis* on substrates of other bacteria will result in localized areas of dense *P. gingivalis* cells. Hence, molecules of *P. gingivalis* that are found to be important for monospecies biofilm formation *in vitro* can be predicted to play a role in the developing accretions of *P. gingivalis* *in vivo*. A variety of *in vitro* assays have been utilized to model the formation of *P. gingivalis* monospecies biofilms, ranging from short-term growth in a microtiter well plate, to more complex longer-term chemostat studies. Each of these assays shed light on different aspects of *P. gingivalis* monospecies accumulation, but beyond very simple inferences current understanding does not allow us to contextualize the functional roles of the identified molecules in the temporal development of *P. gingivalis* biofilms.

A number of studies have shown that *P. gingivalis* autoaggregation, and by extension the initiation of a biofilm, is attributable to FimA (48, 102), and that loss of short fimbriae

enhances autoaggregation (102). Other work suggests that the Mfa fimbriae are required for autoaggregation and microcolony formation on solid surfaces (54), and hence the role of the different fimbrial types may be assay- and context-dependent.

In the microtiter plate assay InlJ is required to initiate monospecies biofilms (10). Contrast this to the situation for dual-species biofilms (discussed earlier) where InlJ is detrimental to *P. gingivalis* accumulation and it becomes evident that the process of biofilm maturation is finely tuned and nuanced in order to respond rapidly to changing environmental conditions such as the presence or absence of different species of bacteria. The universal stress protein, UspA, is also required for *P. gingivalis* biofilm development, both in microtiter plate assays and in flow cells (13). Conversely, loss of several gene products results in enhanced biofilm growth of *P. gingivalis*. Inhibitors of homotypic biofilm accumulation include ClpXP, along with ClpC, and GalE (UDP-galactose 4-epimerase) (10, 64). Components of the Clp stress-response system will affect the stability or levels of a number of proteins in *P. gingivalis* that could impact biofilm formation. GalE catalyzes the interconversion of UDP-glucose to UDP-galactose and in its absence the amount of galactose in lipopolysaccharide, exopolysaccharide and on outer membrane proteins, such as OMP85, will be reduced, which may stimulate biofilm development (64, 65). Loss of a glucosyltransferase gene has also been shown to increase monospecies *P. gingivalis* biofilm in microtiter plates (18).

Differential regulation in bacterial communities

Bacteria adapt to community living through orchestrated patterns of gene regulation. Global expression analyses using proteome or transcriptome approaches can provide insights into these complex systems and begin to reveal the distinct characteristics of community-adapted cells.

Proteome and transcriptome of monospecies *P. gingivalis* communities

A proteomic approach has been used to compare envelope proteins of planktonic *P. gingivalis* cells with those of cells cultured as a community in a chemostat (3). Twenty-four proteins increased in abundance and 18 decreased significantly in the biofilm state. Interestingly, the levels of many proteins that were classified into the cell-surface-located C-terminal domain family increased in the biofilm cells. These included RgpA, HagA, InlJ, thioredoxin, CPG70 carboxypeptidase, API extracellular protease and the Pg99 immunoreactive protein. The C-terminal domain region is thought to participate in secretion across the outer membrane and attachment to the surface of the cell, probably via glycosylation (67, 88, 91). As C-terminal domain proteins are surface exposed, they are thus likely to play important roles in *P. gingivalis* virulence. Other proteins that exhibited significant changes in abundance include hemin transport-related proteins (HmuY and IhtB), metabolic enzymes (glyceraldehyde-3-phosphate dehydrogenase and fumarate reductase) and several proteins with unknown function, along with putative proteins.

Transcriptional changes in *P. gingivalis* cells under the same conditions as above have also been investigated (55). Approximately 18% (377 genes) of the *P. gingivalis* genome was differentially expressed in monospecies community cells relative to planktonic cells. Of these genes, 191 were up-regulated and 186 were down-regulated. Genes that were down-regulated in biofilm cells included those involved in cell envelope biogenesis, DNA replication, energy production, biosynthesis of cofactors, prosthetic groups and carriers, fatty acid and phospholipid metabolism, and central intermediary metabolism. These observations suggest a decrease in cell replication and growth rate in biofilm cells. By contrast, a number of genes encoding transport and binding proteins were up-regulated in *P. gingivalis* biofilm cells, as were several genes predicted to encode proteins involved in signal transduction and transcriptional regulation. Correlation between messenger RNA

levels (55) and protein levels (3) was modest, a common observation in other systems (26) and reflective of the multilevel control systems that regulate bacterial physiology.

Gene regulation in mixed *P. gingivalis*–*S. gordonii* communities

As discussed above, *P. gingivalis* develops biofilm microcolonies on the substrata of *S. gordonii* but not on *S. mutans* (50). In a transcriptome analysis, 33 genes showed up-regulation or down-regulation with *S. gordonii*, and the functions of the regulated genes were predominantly related to metabolism and energy production (94). Studies of individual *P. gingivalis* dual-species community-associated genes are still emerging; however, one gene that has been investigated in some detail is *ltp1* (57). The *ltp1* gene encodes a cytoplasmic eukaryotic-type low-molecular-weight tyrosine phosphatase. Interestingly, although expression of Ltp1 was increased in *P. gingivalis*–*S. gordonii* communities, deletion of the *ltp1* gene, or loss of tyrosine phosphatase activity, increases the level of *P. gingivalis* accumulation with *S. gordonii*. Hence, the role of Ltp1 phosphatase activity is to constrain community development, a process that may serve to minimize exposure to oxygen or facilitate influx of nutrients and efflux of waste (77). One mechanism by which Ltp1 functions to control community development is through down-regulating exopolysaccharide production. Ltp1 activity impacts transcription across several exo-polysaccharide production loci, including those involved in K-antigen and anionic polysaccharide production (73). While exopolysaccharide provides a protective matrix for bacterial cells (104), it is energetically costly and some organisms terminate polymer secretion at a high cell density (62). In addition, exopolysaccharide can physically propel individual cells into a more oxygenated environment (104), hence there is a possible benefit to the anaerobic *P. gingivalis* of exopolysaccharide control mechanisms when in a community structure. Ltp1 also contributes to the regulation of LuxS-dependent signaling, a topic discussed elsewhere in this volume of *Periodontology 2000*.

Signaling mechanisms within bacterial communities

Within densely packed subgingival communities there is ample opportunity for communication among bacteria that are in close proximity. Such signaling can be based on direct contact, metabolic co-operation or on diffusible short-range mediators. A major class of short-range mediators, the auto-inducers, will not be discussed here, as they are the topic of another article in this volume of *Periodontology 2000*.

Metabolic communication

Subgingival bacteria often have complex nutritional requirements that can be met, in part, through the release of a metabolite by another organism in the community. In addition, closely associated organisms can compile a communal suite of enzymes for degradation of complex substrates into constituents that can be metabolized by individual members of the community. These interactions can be considered signaling, in the broad sense, in that they represent sensing and responses to environmental conditions by the organisms, although the extent to which cellular responses of participating organisms extend beyond physiological adaptation to nutrient availability remains to be determined in many cases. One well-documented example of such metabolic communication occurs between *P. gingivalis* and *T. denticola*. In culture together these organisms combine synergistically to produce more biomass than the additive amounts in monoculture (25). This nutritional cross-feeding involves the utilization by *P. gingivalis* of succinate produced by *T. denticola*, and, in turn, the growth of *T. denticola* is stimulated by isobutyric acid generated as a metabolic end product by *P. gingivalis* (25). Growth of *T. denticola* can also be enhanced by proteinaceous substrates produced by *P. gingivalis* (68).

Metabolic support for *P. gingivalis* is also provided by *F. nucleatum*, an organism that can tolerate higher levels of oxygen than *P. gingivalis*. When cultured together under aerated conditions, *F. nucleatum* can create a reduced microenvironment that is optimal for *P. gingivalis* growth (7, 21). *F. nucleatum* can also generate ammonia from glutamic and aspartic acids – amino acids found in crevicular fluid – thus elevating the pH to levels preferred by *P. gingivalis* (99).

Metabolic pathways relevant to a ‘periodontal disease-causing’ phenotype—

The *in vivo* relevance of metabolic communication networks is supported by animal virulence testing. A polymicrobial consortium of *P. gingivalis*, *T. denticola*, *T. forsythia* and *F. nucleatum* induces elevated alveolar bone resorption in rats compared with monoinfections (43).

Recently, multivariate machine-learning techniques were utilized for comparing automatically derived metabolic reconstructions of 266 sequenced genomes, including those of *P. gingivalis*, *T. denticola* and *F. nucleatum* (41). A link was found between the potential of microorganisms to cause periodontal disease and their ability to degrade histidine via three biological pathways: histidine2 (degradation of histidine to L-glutamate); fnc1 (glutamate fermentation); and c2 (biosynthesis of 5-formimino-tetrahydrofolate). In addition, this association held through a further comparison with the genomes of *T. forsythia* and *Prevotella intermedia*. These three pathways are interconnected and result in the complete degradation of L-histidine to acetate and three moles of ammonia. Interestingly, two enzymes in the 5-formimino-tetrahydrofolate biosynthesis pathway, Fld (methylenetetrahydrofolate dehydrogenase) and Fhs (formate-tetrahydrofolate ligase), in *P. gingivalis* were significantly up-regulated when in a community with *S. gordonii* (94). Furthermore, we have found (unpublished information; M. Kuboniwa, M. Hackett and R.J. Lamont) that Fhs is significantly up-regulated by *P. gingivalis* in a community with *S. gordonii* and *F. nucleatum*, indicating that the community lifestyle may lead to a more virulent *P. gingivalis* phenotype. The basis of community-derived behavioral changes may lie in metabolic communication related to the formimino-tetrahydrofolate biosynthesis pathway (Fig. 3). *S. gordonii* possesses Cbe, a chorismate-binding enzyme involved in the production of 4-aminobenzoate (pABA), a precursor of tetrahydrofolate (33, 98, 103). *P. gingivalis* is capable of utilizing exogenous pABA (115), and so pABA generated by *S. gordonii* may facilitate degradation of histidine and push *P. gingivalis* towards a more virulent phenotype. Support for this concept is provided by the finding (discussed above) that the loss of Cbe in *S. gordonii* reduces community development with *P. gingivalis* (47), as the streptococcal contribution to the dual-species consortia may no longer be sufficient for the metabolic needs of *P. gingivalis*.

Arginine deiminase

While many species of subgingival bacteria engage in synergistic relationships, a number of examples of antagonism have also been documented. Antagonistic interactions can be based on the production of antimicrobial compounds such as bacteriocins or hydrogen peroxide (see below); however, propagation of a signal by one species, that is designed specifically to inhibit colonization of a second species, also occurs. *Streptococcus cristatus* is distinct from other oral streptococci in that it possesses characteristic tufts of fibrils. Also unlike other oral streptococci, *S. cristatus* cells tend to be later colonizers of plaque and more frequent colonizers of periodontal pockets where they bind to *F. nucleatum* and form distinctive ‘corn-cob’ structures that are readily visible in mature plaque biofilms (30, 52). Contact between *S. cristatus* and *P. gingivalis*, however, initiates a signal transduction cascade in *P. gingivalis* that causes down-regulation of *fimA* expression and consequently fewer long fimbriae are present on the cell surface (107) (Fig. 2). With the reduction in fimbrial adhesin

activity, *P. gingivalis* is unable to bind to or form communities on substrata of *S. cristatus*. Signaling is mediated by arginine deiminase (ArcA) on the surface of *S. cristatus* (109). While ArcA is an enzyme involved in the arginine metabolism pathway that converts arginine to ornithine, ammonia and CO₂, the signaling function of ArcA does not depend on enzymatic activity (109). Although *S. gordonii* also expresses ArcA, the ability of *S. cristatus* to repress FimA production is related to the elevated expression of *arcA* as a result of differences in the *cis* catabolite response elements of *arcA*, and in the expression of trans-acting regulatory proteins (53). The regulatory network within *P. gingivalis* that responds to ArcA signaling involves both transcriptional and post-transcriptional control of FimA expression (108). Regions of the subgingival biofilm that are rich in *S. cristatus* may be resistant to colonization of *P. gingivalis*.

Hydrogen peroxide

Oral streptococci produce hydrogen peroxide, which, as a strong oxidant, is toxic to bacteria; however, streptococci are protected from oxidative self-damage in mixed communities with *Actinomyces naeslundii* (36). Hydrogen peroxide can also act as a signaling molecule for *A. actinomycetemcomitans*. When in coculture with streptococci, *A. actinomycetemcomitans* displays enhanced resistance to killing by human serum. Hydrogen peroxide is sensed by the oxidative stress response regulator, OxyR, which then induces up-regulation of the complement resistance protein, ApiA, in *A. actinomycetemcomitans* (79).

Contact-dependent signaling

Contact-dependent signaling between *P. gingivalis* and *S. gordonii* is discussed above. Gene regulation follows a temporal progression because extended contact between these organisms results in down-regulation of the gene encoding the short fimbrial adhesin Mfa (74). Presumably, once initial adhesion between *P. gingivalis* and *S. gordonii* has been established, Mfa is no longer required for the accumulation of the community. Similarly, in *T. forsythia*, expression of the BspA leucine-rich repeat protein adhesin is down-regulated following contact with *F. nucleatum* or *P. gingivalis* (35).

Genetic exchange within communities

Horizontal gene transfer by transformation, conjugation or transduction is a principal driver of bacterial evolution. The closely packed environment in biofilm communities facilitates genetic exchange among constituent cells (61, 97). The opportunistic pathogen *Pseudomonas aeruginosa*, for example, can undergo extensive genetic diversification during short-term growth in biofilm communities (4). Furthermore, conjugative plasmids themselves express factors that induce their planktonic bacterial hosts to form or enter biofilm communities, which then favors the transfer of the plasmid (24). The diversity and adaptability produced by horizontal gene transfer provide a form of biological insurance (4) that can help biofilm communities to survive in harsh environments. Subgingival biofilms have been less extensively studied; however, there are several mechanisms by which horizontal gene transfer may be operational.

Mobile genetic elements

Mobile genetic elements can be exchanged promiscuously between a broad spectrum of bacteria and contribute to bacterial genome plasticity. Mobile genetic elements include insertion sequences, transposons, integrons, bacteriophages, genomic islands, plasmids and combinations of these elements.

Conjugative transposons are genetic elements capable of excision from the chromosome of the donor genome, transfer to a recipient cell by conjugation and insertion into the resulting

transconjugants' genome (82). Some conjugative transposons are widespread in oral bacteria. Tn916 and its derivatives, for example, have been found in, or have been introduced into, more than 50 different species of bacteria, including the streptococci, *Veillonella parvula*, *A. actinomycetemcomitans* and *F. nucleatum* (14, 59, 80, 81, 89).

The integron-gene cassette system is a mechanism that allows bacteria to accumulate diverse genes at a common locus. Integrons associated with plasmids or transposons have contributed to the increase in antibiotic resistance in many gram-negative pathogens as a result of their ability to acquire, rearrange and spread antibiotic-resistance genes. The basic machinery of an integron is a site-specific recombinase of the *IntI* family, its cognate recombination site and promoters for the expression of *intI* and captured genes. Collectively, these give an integron the potential to both accumulate gene cassettes and express the cassette-encoded genes (29). Interestingly, the *T. denticola* ATCC 35405 genome sequence contains a 65 kb region containing a number of open-reading frames hypothesized to have been acquired by lateral transfer (92), and an unusual integron (InTde35405) covering 58 kb of this region has been identified (15).

Genomic islands are regions of the genome acquired horizontally. Base composition analysis (G+C content, genome signature, codon usage) can be used to identify laterally transferred genes (40); Table 1 shows genomic islands that have been identified in periodontally relevant microbes using base composition analysis and BLAST taxonomy data [Oralgen database (<http://www.oralgen.lanl.gov/>)]. Subsequent BLASTP homology analysis with *Bacteroides* CTn341 and CTnDOT revealed that three periodontal pathogens (*T. forsythia* ATCC43037, *P. intermedia* 17 and *P. gingivalis* W83) have predicted genomic islands that correspond to the *tra* gene cluster, which is the DNA transfer region in CTn341 and CTnDOT (5, 60).

DNA-transfer mechanisms in *P. gingivalis*

Recently, Naito et al. (63) presented the whole genome sequence of *P. gingivalis* ATCC 33277, a strain better adapted for oral colonization and induction of bone loss than strain W83 (42, 78). Comparison between W83 and ATCC 33277 revealed 461 ATCC 33277-specific and 415 W83-specific predicted protein coding sequences. In addition, 175 regions with genomic re-arrangements were observed between the two strains. Both strains contained large numbers of mobile elements, such as conjugative transposons, insertion sequences and miniature inverted-repeat transposable elements. In ATCC 33277, there are four copies of conjugative transposons, designated as CTnPg1-a, CTnPg1-b, CTnPg2 and CTnPg3, all of which are different from conjugative transposon-related gene clusters in W83. CTnPg1-a contains 50 coding sequences, including a set of genes for conjugative transfer and integration, and several of these show moderate sequence homologies to the genes of CTn341 and CTnDOT. The other conjugative transposons (CTnPg1-b, CTnPg2 and CTnPg3) were truncated and disrupted by multiple insertion sequences.

Besides conjugative transposons, a total of 93 insertion sequence elements and 48 miniature inverted-repeat transposable elements were found in ATCC 33277. Insertion sequences are the simplest transposable elements and can be as short as 600–700 bp, simply encoding a transposase. The presence of several closely related insertion sequence elements in the genome allows homologous recombination between unrelated elements, provided that each of the elements carries a copy of the same insertion sequence element. The insertion sequence elements identified in ATCC 33277 were classified into six types, ISPg1–ISPg6, all of which are also present in W83 (66). Miniature inverted-repeat transposable elements comprise a group of small mobile genetic elements. They do not encode transposases by themselves but have terminal inverted repeats that are the same as, or very similar to, those

of some insertion sequence elements, and they are thus transposable by the action of transposase provided *in trans* by the cognate insertion sequence element.

Functional DNA transfer in *P. gingivalis* was studied by Tribble et al. (101). *P. gingivalis* strains ATCC 33277, 381, ATCC 49417, A1A7-28 and a low-passage clinical isolate (MP4-504), were able to transfer the *Bacteroides–Escherichia coli* shuttle vectors, pT-COW and pFD340, to *E. coli* by a mechanism most consistent with conjugation. By contrast, strains W83, W50 and another clinical strain, 5083, did not transfer either plasmid at detectable levels. Horizontal transfer of genomic DNA between *P. gingivalis* W83 and ATCC 33277 was also demonstrated and, moreover, in contrast to plasmid DNA conjugation, both strains were able to transfer chromosomal DNA to each other. Chimeras showed phenotypic changes in the ability to accrete into biofilms, implying that DNA-transfer events are sufficient to generate measurable changes in complex behaviors.

Transformation and transduction

The conserved ability to acquire DNA molecules by natural transformation enables access to DNA as a source of nutrients or to increase genetic variability. Transformation has not been extensively investigated in subgingival biofilms; however, some strains of *A. actinomycetemcomitans* are naturally competent (23).

Horizontal gene transfer through transduction mediated by bacteriophage is responsible for the lysogenic conversion of many different nonpatho-genic bacteria, including *E. coli*, *Vibrio cholerae*, *Listeria* spp. and *Streptococcus* spp., to pathogens (6). Periodontal bacteria, such as *A. actinomycetemcomitans*, fusobacteria and *T. denticola* have been shown to possess bacteriophage (38, 87, 92). In *A. actinomycetemcomitans*, phage Aa phi 23 correlates with population genetic structure, but does not appear to influence virulence (31). The full extent and role of bacteriophage and transduction in the subgingival microbiota remains to be determined.

Conclusions

The subgingival biofilm is more than a random assemblage of organisms seeking shelter from the hostile environment of the oral cavity. Rather, there exists sophisticated social networking, based initially on very specific recognition of surface characteristics, which provides the discrimination necessary for the formation of metabolically compatible, physiologically integrated communities. Community development is controlled by programmed patterns of gene expression and multilevel regulation of protein expression and activity. Organisms within these communities continually monitor the host environment and the nature and intentions of other organisms that may seek to participate in community affairs. Interspecies communication systems may allow rudimentary group decisions to occur. The sub-gingival ecosystem is thus a dynamic environment and it is likely that much community-specific physiology is devoted to adaptation to stimulate an increase in biomass or to limit and stabilize accumulation according to prevailing conditions. Once a degree of stability or maturity is reached, organisms can begin the process of genetic exchange and the production of genetically diverse daughter cells, some of which will exhibit increased fitness. The success of these strategies is evidenced by the fact that in the absence of host intervention, the subgingival area is colonized by biofilm communities from shortly after birth until death.

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Fig. 1. Confocal microscopy of hete-rotypic *Porphyromonas gingivalis*–*Streptococcus gordonii* communities. *S. gordonii* stained with hexidium iodide (red) was cultured on glass plates. *P. gingivalis* stained with fluorescein (green) was reacted with the *S. gordonii* biofilms for 24 h. The colocalized bacteria appear yellow in the merged image. The upper panel shows *x*–*y* projection and the lower panel shows *x*–*z* projection.

Fig. 2.

Schematic (not to scale) representation of differing community-relevant events that occur following the binding of *Porphyromonas gingivalis* to *Streptococcus gordonii* or to *Streptococcus cristatus*. (A) *S. gordonii* cells attach to the saliva-coated tooth surface. *S. gordonii* produces multiple adhesins, many of which have cognate salivary receptors; for simplicity only SspA / B is shown. Initial localization of *P. gingivalis* with *S. gordonii* is mediated by the interaction of FimA with glyceraldehyde-3-phosphate dehydrogenase on the streptococcal surface. Higher-affinity binding occurs after engagement of Mfa with SspA / B. This interaction initiates a signal transduction event that modulates the *P. gingivalis* transcriptome. The resulting phenotypic adaptation of *P. gingivalis*, along with the

production of signaling molecules, allows the recruitment of additional *P. gingivalis* cells from the planktonic phase and the initiation of community development. (B) *S. cristatus* is a later colonizer of tooth surfaces and attaches to other organisms. Contact with arginine deiminase on the surface of *S. cristatus* induces the down-regulation of *fimA* in *P. gingivalis* and the long fimbriae are lost. Consequently, community formation does not occur between *P. gingivalis* and *S. cristatus*.

Fig. 3. Potential contribution of *Streptococcus gordonii* to the conversion of *Porphyromonas gingivalis* to a more virulent phenotype within a community. The chorismate-binding enzyme (Cbe) of *S. gordonii* can produce 4-aminobenzoate (pABA) from chorismate. pABA, which is acquired by *P. gingivalis*, can be converted into 5,6,7,8-tetrahydrofolate (THF). THF can be used to produce 5-formimino-THF, which is used in the degradation of histidine that is associated with increased virulence of *P. gingivalis*. Gene numbers are shown for *S. gordonii* (SGO) and *P. gingivalis* W83 (PG) or 33277 (PGN). Genes transcriptionally upregulated in *P. gingivalis* in the context of a heterotypic community with *S. gordonii* are indicated with red arrows.

Table 1

Genomic islands in periodontal microbes *

Organism	Number of distinct genomic islands	Description
<i>Porphyromonas gingivalis</i> W83	1	<i>Bacteroides</i> conjugative transposon-related island (<i>tra</i> gene cluster)
	1	Hemagglutinin-related cluster
	1	Thiamin biosynthesis cluster
	1	Potassium uptake gene cluster
	1	Transport-related genomic island
	1	Mobilization cluster, ISPg-related
	2	Mobilization cluster
	3	Uncharacterized genomic island
	5	IS-related genomic island
	12	IS-related potential island
<i>Tannerella forsythia</i> ATCC43037	1	<i>Bacteroides</i> conjugative transposon-related island (<i>tra</i> gene cluster)
	1	Conjugative transposon-related genomic island
	1	Transport-related genomic island
	1	Hemolysin-related genomic island
	1	Thermolysin-related genomic island
	1	Glycosyltransferase-related genomic island
	1	Phage-related genomic island
	1	CRISPR-associated genomic island
	1	Type I restriction system genomic island
	1	Electron transport-related genomic island
<i>Prevotella intermedia</i> 17	2	Uncharacterized genomic island
	1	<i>Bacteroides</i> conjugative transposon-related island (<i>tra</i> gene cluster)
	1	Glycosyltransferase gene cluster
	1	<i>N</i> -acetylmuramoyl-L-alanine amidase-containing cluster
	1	ATP synthase and glycosyltransferase gene clusters
	1	Membrane protein gene and ABC transport gene cluster
	1	Mobilization gene cluster with <i>fic</i> -related gene
	1	Uncharacterized genomic island with integrases
	3	Uncharacterized genomic island
<i>Aggregatibacter actinomycetemcomitans</i> HK1651	1	O-antigen biosynthesis and transport gene cluster
	1	Leukotoxin gene cluster
	1	Cytolethal distending toxin gene cluster
	1	Tight adherence gene cluster
	1	LOS biosynthesis enzyme
	3	Uncharacterized genomic island
<i>Treponema denticola</i> ATCC 35405	1	Super integron
	1	ABC transport system
	1	Capsular polysaccharide biosynthesis cluster
	1	<i>sapI</i> -related and hypothetical protein-containing island

Organism	Number of distinct genomic islands	Description
	1	Uncharacterized genomic island
<i>Fusobacterium nucleatum</i> ATCC 25586	0	

* Compiled from the Oralgen database (<http://www.oralgen.lanl.gov/>).

CRISPR, clustered regularly interspaced short palinormic repeats; IS, insertion sequence; LOS, lipooligosaccharide.