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Biofilm formation by *Porphyromonas gingivalis* and *Streptococcus gordonii*

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Confocal scanning laser microscopy (CSLM) was used to visualize and quantify biofilm formation by the oral bacteria *Streptococcus gordonii* and *Porphyromonas gingivalis*. A saliva-coated glass coverslip under continuous bacterial challenge and conditions of low shear force was used to investigate attachment to the salivary pellicle and also the effect of cell–cell interactions on the extent of colonization and biofilm development. *S. gordonii* bound to the salivary pellicle and outcompeted *P. gingivalis* for attachment sites. Both *P. gingivalis* and *S. gordonii* failed to establish substantial biofilm formation independently. However, biofilm formation did occur subsequent to initial adherence of *P. gingivalis* to *S. gordonii* cells deposited on the salivary pellicle. The commensal species *S. gordonii* may, therefore, provide an attachment substrate for colonization and biofilm accretion by the potential pathogen, *P. gingivalis*.

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Dental plaque is a complex and dynamic microbial community that develops on the tooth surface and, in certain circumstances, leads to the initiation and progression of periodontal diseases. Multiple bacterial and host factors are involved in the transformation of a healthy plaque into a pathogenic entity. One aspect of this process that has been well-characterized is the binding interactions that occur among various oral bacteria and contribute to the development of pathogenic plaque (1–3). Thus early, primarily commensal, colonizers, such as oral streptococci, adhere to the enamel pellicle coating the tooth surface and then provide an attachment substrate for the subsequent colonization by potential periodontal pathogens such as *Porphyromonas gingivalis*. Co-adherence between *S. gordonii* and *P. gingivalis* is, therefore, one important factor that facilitates *P. gingivalis* colonization (4, 5). Studies of the molecular basis of the binding interaction between *S. gordonii* and *P. gingivalis* have revealed that the interaction is multimodal; involving several distinct adhesin-receptor pairings. A major streptococcal surface protein, designated Ssp, mediates adherence via a C-terminal functional domain (5, 6). On the *P. gingivalis* surface, fimbriae and a 35 kDa protein participate in binding (7, 8).

On the tooth surface plaque bacteria comprise a biofilm (9, 10). It is well established that sessile bacteria in a biofilm differ phenotypically from their planktonic counterparts (10). Biofilm-induced phenotypic changes include physiological properties, susceptibility to antibiotics and host defense mechanisms and interactions with host tissues (11, 12). In the case of *Pseudomonas aeruginosa*, a biofilm environment induces changes in alginate synthesis and in expression of many outer membrane proteins (10–12). The environmental conditions that prevail within a biofilm, particularly with regard to cell density and surface contact, may participate in the induction of the biofilm phenotype. Cell density can control *P. aeruginosa* alginate expression at the transcriptional level through sigma factor E mediated responses to *N*-acyl homoserine lactone dependent quorum sensing (12, 13). Contact with host cell surfaces can modulate expression of virulence factors such as flagella (in *Salmonella typhimurium*), iron starvation responses (in uropathogenic *Escherichia coli*), and Yop expression (in *Yersinia pseudotuberculosis*) (14).

With regard to oral pathogenesis, studies have revealed that transcription of the *S. mutans gtf* genes is regulated in response to contact with saliva coated hydroxyapatite (15). In addition, the *P. gingivalis fimA* gene (encoding fimbrillin) may be regulated at the transcriptional level by cell density or contact with surfaces (16).

Co-adherence between *P. gingivalis* and *S. gordonii* has been established in a number of model systems (4, 17–19). However, little is known about the interactions between these organisms on a saliva-coated surface under conditions of low shear force, as a model of the conditions prevailing on the tooth surface. Furthermore, the ability of these organisms to form classical biofilm structures has not been investigated. The goal of this study was to determine the ability of *P. gingivalis* to adhere to *S. gordonii* in the context of a simulated oral environment and to assess the subsequent formation of biofilm structures by these organisms.

Materials and Methods

Bacteria and culture conditions

P. gingivalis strain 33277 was grown from frozen stocks in trypticase soy broth supplemented with 1g yeast extract, 5 mg hemin and 1 mg menadione, each per liter. Culture was at 37°C under anaerobic conditions of 85% N_2, 10% H_2 and 5% CO_2. *S. gordonii* strain DL1 was grown from frozen stocks in trypticase peptone broth supplemented with 5 g/l yeast extract and 0.5% glucose. Culture was at 37°C under normal aerobic conditions. Bacterial numbers were determined with a Klett-Summerson photometer.

Saliva

Unstimulated whole saliva was collected from 20 healthy volunteers (laboratory personnel), clarified by centrifugation at 10,000 *g* for 10 min, pooled and stored at −80°C.

Generation of biofilms

Bacteria were suspended in sterile, pre-reduced PBS (pH 7.2) to a concentration of 10^7 cells/ml. No autoaggregation of *P. gingivalis* was observed under these conditions by phase contrast or confocal microscopy, and the streptococci were in discrete chains. Biofilms were generated on a glass coverslip in a flow cell with attached syringe pump. The flow cell comprised a polycarbonate channel with a 0.6 × 1.0 cm cross section and a glass coverslip top, which served as an observation window. The flow cell, connected to the syringe pump by flexible tubing, was mounted on a microscope stage. Prior to insertion in the flow cell, the coverslip was coated with a salivary pellicle by reacting (30 min 37°C) with excess clarified human saliva. Unadsorbed saliva was then removed by washing 3 times with PBS. The flow cell was inoculated sequentially with the 2 strains of bacteria at room temperature. Bacterial challenges were delivered at a constant rate of 4.1 ml/h for a period of up to 4 h each at room temperature. All experiments were repeated at least twice on at least 2 separate occasions. The biofilms that developed on the coverslip were then accessible for *in-situ* microscopic examination.

Confocal scanning laser microscopy (CSLM)

For analysis of the structure of the biofilms, a Bio-Rad MRC600 confocal scanning laser microscope (Kr/Ar) system with an Olympus IMT-2 inverted light microscope and a MS plan 60 × 1.4 NA objective was used. Reflected laser light of combined 488, 546 and 647 nm wavelengths was used to observe biomass. For CSLM, streptococci were stained in situ with propidium iodide (10 mg/l), and *P. gingivalis* was stained in situ with fluorescein (10 mg/l). *S. gordonii* DL1 did not stain with fluorescein, allowing differentiation of the 2 species. CLSM was also used in reflected white light mode of illumination to directly observe biofilm formation within the time frame of the experiment. A series of fluorescent optical sections were collected to create a digitally reconstructed 3-dimensional view of the 2 species with the Slicer (Fortner Research) imaging program.

Results

Biofilm formation by *S. gordonii* and *P. gingivalis* individually

S. gordonii exposure to the saliva-coated glass (SCG) surface resulted in greater than 50% coverage after 30 min of constant challenge (Fig. 1). After 2 h of challenge, a monolayer of *S. gordonii* cells was produced. No microcolonies or structures

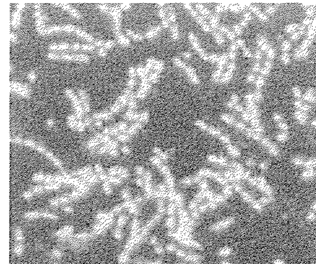

Fig. 1. Confocal image of *S. gordonii* DL1 stained with propidium iodide after 30 min exposure to a SCG surface. Magnification ×2400.

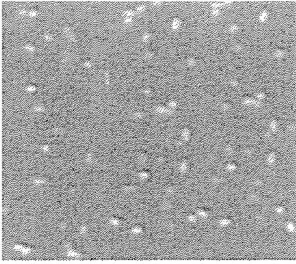

Fig. 2. Confocal image of *P. gingivalis* 33277 stained with fluorescein after 4 h exposure to a SCG surface. Magnification ×2100.

consistent with biofilm development were observed with *S. gordonii* over a 4-h time period. Exposure of the SCG surface to *P. gingivalis* only resulted in about 10% coverage after 4 h of constant challenge. Widely distributed, very small microcolonies (10–20 micron thick) were observed sparsely on the SCG surface (Fig. 2). These experiments demonstrate that under the conditions of low shear force employed, *S. gordonii* has a higher affinity for the SCG surface than *P. gingivalis*. Moreover, individually, *S. gordonii* is incapable of forming biofilm structure and *P. gingivalis* can sporadically form small microcolonies.

Biofilm formation by *S. gordonii* and *P. gingivalis* in combination

P. gingivalis was reacted with a SCG-streptococcal surface produced by a 30 minute exposure of the SCG surface to *S. gordonii*. After 30 min incubation, *P. gingivalis* cells were observed binding to streptococcal cells but rarely directly to the salivary pellicle (Fig. 3). Not all streptococcal cells possessed bound *P. gingivalis* cells, and those that did usually had multiple adhering *P. gingivalis*. After 2 h co-incubation, microcolonies of *P. gingivalis* began to develop over areas of attachment to streptococcal cells. These typical biofilm structures extended 30–60 microns up into the lumen of the flow chamber and were frequently observed (Fig. 4). Figure 5 represents a 3-dimensional reconstruction of representative *P. gingivalis-S. gordonii* biofilm architecture. When the sequence of bacterial challenge was reversed (i.e. *P. gingivalis* followed by *S. gordonii*), *S. gordonii* cells competed with and dislodged the sparsely bound *P. gingivalis* cells and

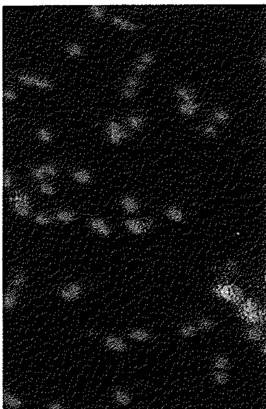

Fig. 3. Confocal image *P. gingivalis* (colored green) and *S. gordonii* (colored red) after 30 min co-incubation on a SCG surface. Co-localized bacteria appear yellow. Magnification ×3000.

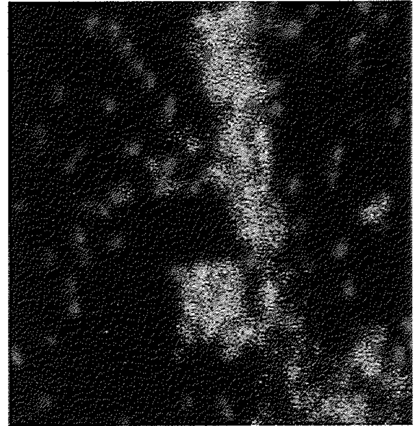

Fig. 4. Confocal image *P. gingivalis* (colored green) and *S. gordonii* (colorized red) after 2 h co-incubation on a SCG surface. Co-localized bacteria appear yellow. Magnification ×3000.

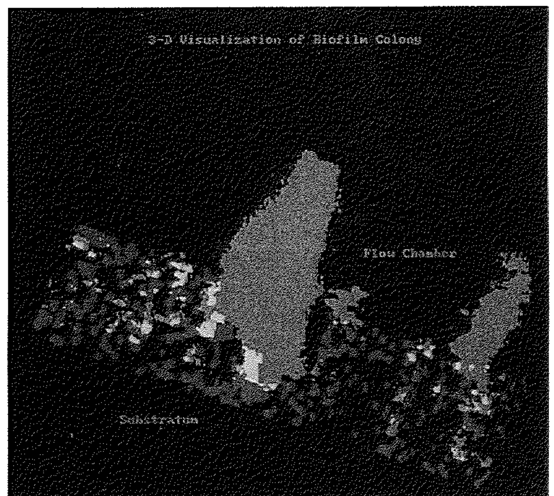

Fig. 5. 3-Dimensional reconstruction (×3000) of *P. gingivalis* (colored green) and *S. gordonii* (colored red) biofilm. Co-localized bacteria appear yellow. Angle orientations are x: 70, y: −70, z: 22.

a biofilm did not develop. These results show that *P. gingivalis* and *S. gordonii* together are capable of forming a biofilm, provided the *P. gingivalis* cells can first adhere to streptococcal cells. This is consistent with the sequence of bacterial colonization observed to occur in dental plaque.

Discussion

Interbacterial binding between *P. gingivalis* and *S. gordonii* has been demonstrated in a number of model systems and is considered an important means by which *P. gingivalis* can colonize developing plaque (4, 17–19). *P. gingivalis* demonstrates a higher affinity for streptococcal cells that are deposited on a solid support, as a facsimile of streptococcal plaque, than to streptococcal cells in suspension (17). This is consistent with the situation that prevails in the oral cavity, where *P. gingivalis* is a secondary colonizer of plaque and will encounter an adherent plaque rich in streptococci and other antecedent colonizers (20). Nonetheless, the ability of *P. gingivalis* to adhere to *S. gordonii* under the stress of low shear forces that will exist in the oral cavity, and the ability of these organisms to form biofilm structures typical of plaque deposits, have not been investigated.

We have developed an *in vitro* model system to study co-adherence and biofilm development by *P. gingivalis* and *S. gordonii*. A saliva coated glass (SCG) surface was utilized as the support matrix in order to mimic the salivary pellicle that is present on the tooth surface. Although tooth enamel (and to a lesser extent root cememtum) is comprised of substituted hydroxyapatite (21), for convenience, glass or plastic can be employed in adherence assays as specific bacterial adhesion occurs to molecules in the deposited salivary coating, not to the support matrix (22–24). Weak shear forces were generated by continuous flow of the bacteria over the solid support.

In accord with its status as a major primary colonizer of enamel salivary pellicle, *S. gordonii* rapidly formed a monolayer on the SCG surface. *P. gingivalis*, by contrast, only achieved intermittent coverage and was readily displaced by streptococcal cells. However, *P. gingivalis* cells did adhere rapidly and in high numbers to a streptococcal surface, with little binding to exposed area of SCG. Thus, under weak shear forces, *P. gingivalis* will preferentially adhere to a streptococcal surface rather than a salivary surface. These findings support the seminal *in vivo* observation of Slots & Gibbons (4), who found that introduction of streptomycin resistant *P. gingivalis* into the mouths of human volunteers resulted in the organism locating almost exclusively on preformed plaque. The model system we have established should, therefore, find application in studies to investigate adherence and colonization mechanisms of *P. gingivalis* in the *in vivo*-relevant context of a developing biofilm.

After initial attachment of *P. gingivalis* to streptococcal cells, the organisms rapidly formed structures typical of fully developed biofilms (10). The streptococcal cells formed a "lawn", over much of the surface, while the *P. gingivalis* cells formed towering microcolonies anchored to the streptococcal monolayer. The time of exposure of *P. gingivalis* to the streptococci was not greater than the optimal dividing time of *P. gingivalis* in complex media. It is unlikely, therefore, that microcolonies were formed by *P. gingivalis* cell division, but rather were the result of accretion of the organism. The biofilm architecture observed is similar to that of biofilms formed by pure cultures of *P. aeruginosa* (25), in that the *Porphyromonas* forms bulbous microcolonies of cells most probably embedded in their exopolysaccharide matrix material. These microcolonies, which widen as they protrude as much as 60 micrometers into the bulk fluid, constituted the predominant sessile mode of growth of the *P. gingivalis* cells. This typical biofilm architecture, in which the sessile population is made up of complex cellular aggregates, is much more extensive when the *P. gingivalis* cells produce microcolonies on a monolayer of streptococcal cells, rather than directly on the saliva coated glass surface. The observed biofilm architecture would lead us to predict that, as in biofilms of other Gram-negative bacteria (26), nutrients would be delivered to all parts of the biofilm by convective flow of the bulk fluid, over the monolayer and between the microcolonies.

Although *P. gingivalis* did form some biofilm structures directly on SCG, biofilm formation in combination with *S. gordonii* was much more efficient. This would suggest that *P. gingivalis* is capable of sensing the presence of *S. gordonii* and responding by initiating biofilm-mode. This signal could be the adherence interaction between the 2 species or a mediator produced by *S. gordonii* to which *P. gingivalis* can respond when in close proximity.

In mature dental plaque over 300 different species comprise the biofilm (2). Our results indicate that in the case of the potential periodontal pathogen, *P. gingivalis*, adherence to the antecedent colonizer *S. gordonii* is an important step for both colonization and biofilm formation. The potentiating or inhibitory effect of other organisms, and of differing environmental conditions, on this process are important areas for further study. None the less, direct synergistic interactions between *P. gingivalis*

and *S. gordonii* can occur and, if operational *in vivo*, may be important in the transition of early commensal plaque into pathogenic plaque that is a direct precursor of periodontal diseases. This interaction may thus provide a suitable target for novel therapeutic agents.

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