

Mutualistic Biofilm Communities Develop with *Porphyromonas gingivalis* and Initial, Early, and Late Colonizers of Enamel[∇]

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Porphyromonas gingivalis is present in dental plaque as early as 4 h after tooth cleaning, but it is also associated with periodontal disease, a late-developing event in the microbial successions that characterize daily plaque development. We report here that *P. gingivalis* ATCC 33277 is remarkable in its ability to interact with a variety of initial, early, middle, and late colonizers growing solely on saliva. Integration of *P. gingivalis* into multispecies communities was investigated by using two in vitro biofilm models. In flow cells, bacterial growth was quantified using fluorescently conjugated antibodies against each species, and static biofilm growth on saliva-submerged polystyrene pegs was analyzed by quantitative real-time PCR using species-specific primers. *P. gingivalis* could not grow as a single species or together with initial colonizer *Streptococcus oralis* but showed mutualistic growth when paired with two other initial colonizers, *Streptococcus gordonii* and *Actinomyces oris*, as well as with *Veillonella* sp. (early colonizer), *Fusobacterium nucleatum* (middle colonizer), and *Aggregatibacter actinomycetemcomitans* (late colonizer). In three-species flow cells, *P. gingivalis* grew with *Veillonella* sp. and *A. actinomycetemcomitans* but not with *S. oralis* and *A. actinomycetemcomitans*. Also, it grew with *Veillonella* sp. and *F. nucleatum* but not with *S. oralis* and *F. nucleatum*, indicating that *P. gingivalis* and *S. oralis* are not compatible. However, *P. gingivalis* grew in combination with *S. gordonii* and *S. oralis*, demonstrating its ability to overcome the incompatibility when cultured with a second initially colonizing species. Collectively, these data help explain the observed presence of *P. gingivalis* at all stages of dental plaque development.

Removal of dental plaque by routine oral hygiene procedures is followed by a repetition of a species succession that starts with initially colonizing streptococci and actinomyces (5, 16). Other species follow as early, middle, and late colonizers, which establishes the following developmental process: successive attachment of saliva-suspended species to already attached bacteria and formation of multispecies communities.

Attachment is a critical event essential to preventing the bacteria from being swallowed by salivary flow. Initial colonizers bind to host-derived receptors in the salivary pellicle coating of the tooth enamel. The remainder of typical plaque development occurs by accretion of saliva-suspended species and growth of attached bacteria, thereby increasing the microbial diversity. Adherence of suspended single cells to attached cells is called coadhesion (1). Some suspended cells are already coaggregated and adhere to attached cells as coaggregates; coaggregation is defined as the specific cell-to-cell recognition and adherence of genetically distinct cell types (8). All human oral bacterial species exhibit coaggregation. For example, *Streptococcus oralis* coaggregates with *Streptococcus gordonii* (intrageneric coaggregation). Both species pair with *Actinomyces oris* (intergeneric coaggregation), and all of them coaggregate with *Fusobacterium nucleatum* (multigeneric coaggregation). Multispecies communities composed of coaggregating species characterize dental plaque biofilms in vivo (3, 17, 18).

To increase our understanding of interactions among spe-

cies, we have employed two in vitro model systems and are testing numerous combinations of seven species for their ability to grow on saliva as their sole nutritional source (20, 21). First, we reported that *F. nucleatum* (middle colonizer) failed to grow when paired with *S. oralis* but grew well when *A. oris* was included in the three-species biofilm (20), indicating specificity by *F. nucleatum* for the presence of a particular initial colonizer. Recently, we showed that *Aggregatibacter actinomycetemcomitans* (late colonizer and periodontopathogen) exhibited mutualistic relationships with *F. nucleatum* and *Veillonella* sp. (early colonizer and commensal organism), illustrating the ability of commensals and pathogens to grow together (21).

Porphyromonas gingivalis, another periodontopathogen, forms three-species communities with *F. nucleatum* and *S. gordonii* (11). Proteomics of *P. gingivalis* in this three-species community revealed a broad increase in proteins involved in protein synthesis, suggesting that a multispecies relationship is advantageous for the porphyromonad (11). This research group had previously reported the presence of differentially regulated porphyromonad genes when *P. gingivalis* and *S. gordonii* were together in biofilms (22). Thus, *P. gingivalis* responds to the presence of other oral species.

P. gingivalis is detected in dental plaque samples within 6 h after professional tooth cleaning (5, 13), and its numbers increase in periodontally diseased sites (15). It forms biofilms with *S. gordonii* but not with *Streptococcus mutans* (12) or *Streptococcus cristatus* (23). *P. gingivalis* required a preformed streptococcal substratum for its incorporation into a biofilm (12). Partner specificity was also noted among four fresh isolates of *P. gingivalis*, which showed no coaggregation with a variety of oral actinomyces, aggregatibacteria, capnocytophagae, and streptococci (9) but coaggregated with *F. nucleatum*

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(7, 10). We show here that *P. gingivalis* exhibits widespread mutualism with initial, early, middle, and late colonizers but also shows specificity with initially colonizing streptococci, which could help explain its early appearance in the development of dental plaque biofilms. The relationship of porphyromonads with initial, early, middle, and later colonizers during biofilm growth on saliva as a sole nutritional source has not been explored previously. We hypothesize that the ability of *P. gingivalis* to coaggregate with *S. gordonii* and *A. oris* (initial colonizers), *Veillonella* sp. (early colonizer), *F. nucleatum* (middle colonizer), and *A. actinomycetemcomitans* (late colonizer) allows these bacteria to form multispecies biofilm communities.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. oralis* 34, *S. gordonii* DL1, and *A. oris* ATCC 43146 were routinely cultured in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, MI) or on THB agar. *Veillonella* sp. PK1910 was grown in THB supplemented with 0.6% lactic acid. *A. actinomycetemcomitans* JP2 (kind gift from D. Demuth, University of Louisville, Louisville, KY) was grown in brain heart infusion medium (BHI; Difco) supplemented with 0.04% sodium bicarbonate and 2% yeast extract. *F. nucleatum* ATCC 10953 was grown in BHI supplemented with 0.25% L-glutamic acid. *P. gingivalis* ATCC 33277 (kind gift from R. J. Lamont, University of Florida, Gainesville, FL) was grown in BHI supplemented with 0.1% yeast extract, 0.5% hemin, and 0.001% menadione. All species were grown in a Bactron anaerobic (N₂:CO₂:H₂, 90:5:5) environmental chamber (Sheldon Manufacturing Inc., Cornelius, OR) at 37°C.

Saliva preparation. Saliva from 6 to 10 healthy individuals was collected on ice, pooled on ice, and treated with 2.5 mM dithiothreitol for 10 min, with stirring to reduce salivary protein aggregation. The saliva was then centrifuged and processed as previously described (19). Briefly, the supernatant was diluted with distilled water to produce 25% saliva and then was filtered through a 0.22- μ m-pore-size SFCA low-protein-binding filter (Nalge Nunc International, Rochester, NY) and stored at -20°C. Prior to use, saliva was thawed and centrifuged to remove any precipitate that resulted from freezing and thawing.

Flow cell preparation. Two tracks (each track was 40 mm long, 3 mm wide, and 2 mm deep) were milled into a high-density polyethylene block, resulting in two chambers, each with a 240- μ l volume. A glass coverslip, which serves as the attachment substratum for the growing biofilm, was secured to the reusable flow cells with a silicone adhesive. The flow cells were cleaned overnight with 0.1 M HCl, rinsed with 5 ml of distilled water, injected with 70% ethanol, and incubated 20 min. The flow cells were then treated with 25% sterile human saliva for 15 min at 37°C in an anaerobic chamber to condition the glass surface with salivary components.

Biofilm growth conditions in flow cells. Overnight bacterial cultures were harvested by centrifugation and washed twice with 25% sterile human saliva, and the optical density at 600 nm was adjusted to 0.1, which was equivalent to about 1×10^7 to 3×10^7 cells/ml. Flow cells were inoculated with one, two, or three species. Two-species and three-species inoculations were first coaggregated (by mixing 0.1-ml portions of appropriate combinations of *P. gingivalis*, *S. oralis*, *S. gordonii*, *A. oris*, *Veillonella* sp., *F. nucleatum*, and *A. actinomycetemcomitans* [equivalent to about 1×10^6 to 3×10^6 cells of each species]), and the flow cells were incubated in the anaerobic chamber to provide an environment favorable to *P. gingivalis*, *Veillonella* sp., and *F. nucleatum*, which are strict anaerobes. The sole nutritional source was sterile 25% saliva supplied at a flow rate of 0.2 ml/min, which approximated unstimulated salivary flow in the mouth (4).

Biofilm staining. Staining of cells varied in the different experiments. When strains were grown as monocultures, they were visualized by primary immunofluorescence with Alexa Fluor 546- (Invitrogen), Alexa Fluor 488- (Invitrogen), Alexa Fluor 546- (Invitrogen), Alexa Fluor 488- (Invitrogen), Alexa Fluor 633- (Invitrogen), Alexa Fluor 488- (Invitrogen), and Alexa Fluor 546- (Invitrogen) conjugated immunoglobulin G of a polyclonal antiserum to *P. gingivalis*, *S. gordonii*, *Veillonella* sp., *A. actinomycetemcomitans*, *S. oralis*, *A. oris*, and *F. nucleatum*, respectively. When multiple species were inoculated, visualization of species was by primary immunofluorescence with Alexa Fluor 546-, Alexa Fluor 633-, Alexa Fluor 488-, Alexa Fluor 488-, Alexa Fluor 633-, Alexa Fluor 488-, and Alexa Fluor 488-conjugated immunoglobulin G of a polyclonal antiserum to *P. gingivalis*, *S. oralis*, *S. gordonii*, *A. oris*, *Veillonella* sp., *F. nucleatum*, and *A. actinomycetemcomitans*, respectively. Immunofluorescence was performed by in-

jecting the antibody (5 μ g/ml in filter-sterilized phosphate-buffered saline containing 1% [wt/vol] bovine serum albumin) into the appropriate flow cell track and incubating for 20 min. A final wash with phosphate-buffered saline preceded confocal laser scanning microscopy. No cross-reactivity with nonhomologous strains was observed, based on staining by antibodies.

Image and statistical analysis. A TCS-SP2 confocal microscope (Leica, Exton, PA) with a 40 \times , 1.25-numerical aperture oil immersion lens was used to record confocal image stacks in five random locations near the center of the flow cells, after which biofilm biovolumes were determined by volumetric analyses (IMARIS version 5.71; Bitplane AG, Zurich, Switzerland). Fluorescence intensity thresholds were set manually for red, green, and blue pixels with cubic voxels used for the biovolume determination. Five confocal datasets were analyzed for each time point, and the mean and standard deviations were calculated. A one-way analysis of variance at the 95% confidence level with a nonparametric Tukey's pairwise comparison test was used to determine if means were statistically different between the 4-h and 18-h time points for each condition (one, two, and three species) in the flow cell studies and between the 12-h, 24-h, and 36-h time points for each condition (one, two, and three species) in the peg studies with static saliva. All images presented in the figures are maximum projections of the entire confocal image stack as produced by the Leica TCS software (Leica).

Biofilm growth conditions on polystyrene pegs (peg biofilms). Biofilms were grown in 25% human saliva on pegs (Nunc-Immuno transferable solid phase; Nunc 445497) (2, 14) mounted in U96 microwell plates (Nunc 163320) (2, 14, 20). Microtiter plate wells were filled with 200 μ l of 25% human saliva, and pegs were then inserted for 30 min at room temperature to build a conditioning film. Overnight cultures (~20 μ l) of *P. gingivalis* ATCC 33277, *S. oralis* 34, *Veillonella* sp. PK1910, *A. actinomycetemcomitans* JP2, and *F. nucleatum* ATCC 10953 were added to the 200 μ l of saliva in the wells; this equals about 1×10^7 to 3×10^7 cells of each species in the microtiter well; plates were placed in a humidity chamber and incubated anaerobically at 37°C for 12, 24, or 36 h. Once the wells were inoculated and the pegs placed in the saliva, the pegs were not transferred and thus remained submerged in the same saliva for 12, 24, or 36 h.

DNA extraction and quantification. DNA was extracted from biofilms by a modified alkaline lysis protocol (3, 6, 20). Biofilm-covered pegs were immersed in 40 μ l of sterile ultrapure water plus 160 μ l of 0.05 M sodium hydroxide incubated at 60°C for 60 min, after which 18.4 μ l of 1 M Tris-HCl (pH 7.0) was added to neutralize the pH. This extract was used as the template DNA for the quantitative real-time PCR (q-PCR) analyses (3, 6, 20). Bacterial genomic DNA for standard curves was extracted from overnight cultures of *P. gingivalis* ATCC 33277, *A. actinomycetemcomitans* JP2, *Veillonella* sp. PK1910, and *F. nucleatum* ATCC 10953 with a DNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Genomic DNA was stored at -20°C.

q-PCR quantification of species in biofilms. Species-specific primers were designed with the Primer 3 program (<http://www.justbio.com>). No cross-hybridization of primers with nonhomologous species was observed. The primers specific for *P. gingivalis* were F, TGGGTTTAAAGGGTGCGTAG, and R, CAATCGGAGTTCCTCGTGAT; the annealing temperature was 60°C. The primers specific for *A. actinomycetemcomitans* JP2 were F, GGACGGTGAGTAATGCTTG, and R, CCCTTACCCACCAACTACC; the annealing temperature was 60°C. The primers specific for *Veillonella* sp. PK1910 were F, CCGTGATGGGATGGAACTGC, and R, CCTTCGCCACTGGTGTCTTC; the annealing temperature was 60°C. The primers specific for *F. nucleatum* ATCC 10953 were F, CTTAGGAATGAGACAGAGATG, and R, TGATGGTAACATACGAAAGG; the annealing temperature was 56°C. Quantification of *P. gingivalis*, *A. actinomycetemcomitans*, and *F. nucleatum* cells in the biofilms was performed with q-PCR using the SYBR green dye to detect the 16S rRNA gene amplicons. Each reaction (final reaction volume, 20 μ l) contained 3 μ l of template, 1.0 μ l of diethyl pyrocarbonate-treated ultra pure water, 10 μ l of fast SYBR green master mix (Applied Biosystems, Foster City, CA), and 3 μ l each of forward and reverse primers at 375 nM. q-PCR was performed in an MX3005P thermocycler (Stratagene, La Jolla, CA) using the following thermocycle recommended for fast SYBR green master mix: 95°C for 20 s, 40 cycles of 3 s at 95°C, and 30 s at 60°C. Dissociation curves were generated by incubating reaction products at 95°C for 1 min and 56°C for 30 s and incrementally increasing the temperature to 95°C. Fluorescence data were collected at the end of the 60°C primer annealing step for 40 amplification cycles and throughout the dissociation curve analysis. The analysis of the melting curves with both primer sets showed a single sharp peak. DNA concentrations (ng/ml) were calculated based on standard curves obtained by using 10-fold serial dilutions of bacterial DNA isolated with a DNA extraction kit (Qiagen) and quantified with a PicoGreen fluorescence assay (Invitrogen). To convert ng DNA to cell numbers, the following weights and genome sizes were used: 2.34 fg/genome and 2.3 Mb for porphyromonads, 2.05 fg/genome and 2.1 Mb for aggregatibacteria, 3.05 fg/genome and 3.0 Mb for veillonellae, and 2.41

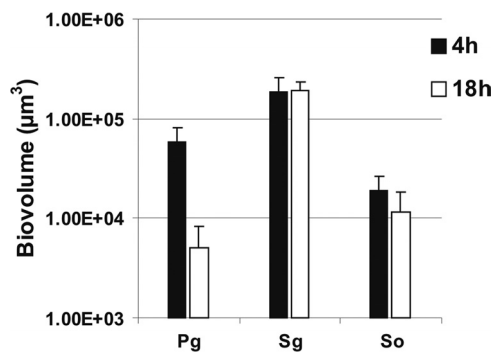


FIG. 1. Time-resolved change in biovolume (μm^3 per field of view) of *P. gingivalis* (Pg), *S. gordonii* (Sg), and *S. oralis* (So) following 4 h and 18 h of incubation as single species in 25% saliva-fed flow cells.

fg/genome and 2.4 Mb for fusobacteria (<http://www.homd.org>). The data presented are from three independent biofilms.

RESULTS

Flow cells inoculated with one species. *P. gingivalis*, *S. gordonii*, and *S. oralis* were inoculated into separate flow cells. Images of the biofilms formed were obtained after 4 h and 18 h of incubation and used to quantify biofilm cells as biovolume (Fig. 1). Each species showed attachment to the saliva-conditioned surface at 4 h. After 18 h of incubation, the number of *P. gingivalis* cells decreased 10-fold, whereas *S. gordonii* and *S. oralis* cells remained nearly constant, indicating their retention but no obvious growth. We reported earlier that *Veillonella* sp. and *A. actinomycetemcomitans* also did not grow as single species on saliva (21) and that *F. nucleatum* monospecies cell numbers decreased (20). Only *A. oris* grew consistently as a single species on saliva (20).

Flow cells inoculated with two species. To investigate the possibility of mutualism between *P. gingivalis* and the other six species, each of the six pairwise mixtures of *P. gingivalis* and a coaggregation partner were inoculated into a saliva-fed flow cell. The biofilms formed were viewed by confocal laser scanning microscopy (Fig. 2 and 3), and the biovolumes of each species were quantified (Fig. 4). Neither species of the *S. oralis*-*P. gingivalis* pair showed growth (Fig. 4A and 2, top panels). In sharp contrast, mutualistic growth was seen with *P. gingivalis* and partners *S. gordonii* (Fig. 4B and 2, middle panels), *Veillonella* sp. (Fig. 4C and 2, bottom panels), *F. nucleatum* (Fig. 4D and 3, top panels), and *A. actinomycetemcomitans* (Fig. 4E and 3, middle panels), and the *P. gingivalis*-*A. oris* pair grew commensally (Fig. 4F and 3, bottom panels). These results document the favorable interaction of *P. gingivalis* with only certain initial colonizers (*S. gordonii* and *A. oris*) and show that *P. gingivalis* exhibits a broad range of favorable interactions with early, middle, and late colonizers as well.

Flow cells inoculated with three species. The inability of *P. gingivalis* to grow with *S. oralis* was explored further by including a third species and examining the formation of multispecies communities in saliva-fed flow cells. Also, substituting *Veillonella* sp. for *S. oralis* in the triple-species biofilms was investigated. The biovolume of *P. gingivalis* increased sixfold between 4 h and 18 h of incubation, when *P. gingivalis* was inoculated as a coaggregate with *Veillonella* sp. and *A. actino-*

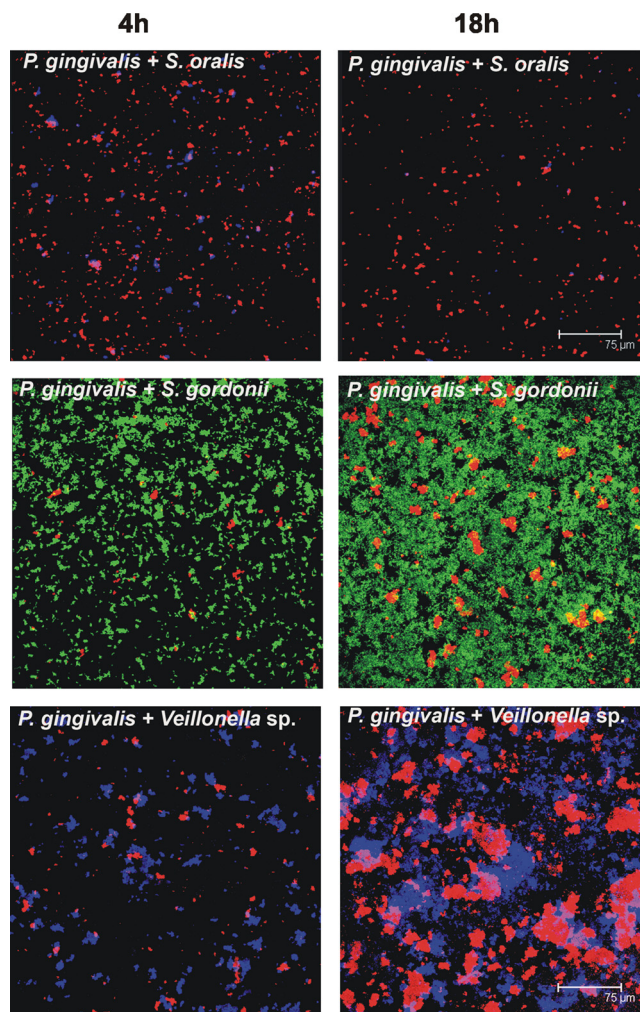


FIG. 2. Representative confocal micrographs of 4-h (left column) and 18-h (right column) biofilms showing no growth (top panels, *P. gingivalis* + *S. oralis*) and mutualistic growth (middle panels, *P. gingivalis* + *S. gordonii*; bottom panels, *P. gingivalis* + *Veillonella* sp.) in flow cells inoculated with two species. Bacterial cells were stained with species-specific fluorophore-conjugated immunoglobulin G (red, *P. gingivalis*; blue, *S. oralis*/*Veillonella* sp., green, *S. gordonii*), and cell-cell contact between species is evident.

mycetemcomitans; *Veillonella* sp. biovolume increased twofold and *A. actinomycetemcomitans* biovolume increased threefold, indicating a productive interaction in this triple-species community (Fig. 5 and 6A). In contrast, substituting *S. oralis* for *Veillonella* sp. inhibited growth of all species, although initial binding to the saliva-coated substratum was excellent (Fig. 6B, 4-h time point); reduced numbers of the three species were seen at 18 h (Fig. 6B). The same trend was observed in triple-species inocula containing *P. gingivalis*, *Veillonella* sp., and *F. nucleatum* (Fig. 6C) versus *P. gingivalis*, *S. oralis*, and *F. nucleatum* (Fig. 6D). The biovolumes of porphyromonads, veillonellae, and fusobacteria increased (Fig. 6C), but the biovolumes of porphyromonads, streptococci, and fusobacteria did not (Fig. 6D), indicating that *Veillonella* sp. is a favorable partner for later colonizers, whereas *S. oralis* is not. Moreover,

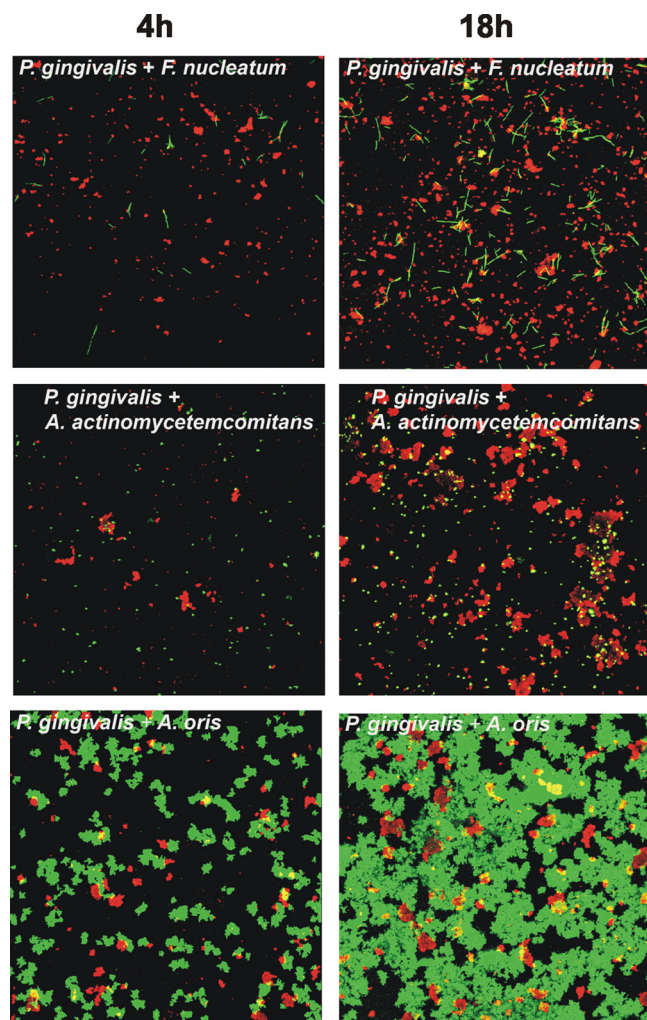


FIG. 3. Representative confocal micrographs of 4-h (left column) and 18-h (right column) biofilms showing mutualistic growth (top panels, *P. gingivalis* + *F. nucleatum*; middle panels, *P. gingivalis* + *A. actinomycetemcomitans*) and commensal growth (*P. gingivalis* + *A. oris*) in flow cells inoculated with two species. Bacterial cells were stained with species-specific fluorophore-conjugated immunoglobulin G (red, *P. gingivalis*; green, *F. nucleatum*/*A. actinomycetemcomitans*/*A. oris*), and cell-cell contact between species is evident.

S. oralis appears to hinder growth of all four gram-negative species.

The specificity of *P. gingivalis* for growth with *S. gordonii* but not *S. oralis* was investigated by inoculating the three species into a flow cell. All species attached well to the saliva-coated substratum; *P. gingivalis* biovolume increased sixfold, and *S. gordonii* biovolume increased twofold, but *S. oralis* failed to grow between 4 h and 18 h (Fig. 7). Thus, *P. gingivalis* is capable of significant growth with a suitable initial colonizer (*S. gordonii*), even in the presence of an unfavorable initial colonizer (*S. oralis*).

q-PCR quantification of species in static biofilms on pegs immersed in saliva. Multispecies biofilm formation in the open, saliva-fed flowing system was compared with biofilms formed in the closed solid-phase polystyrene peg system; pegs remained submerged in the same saliva for 12, 24, or 36 h.

When *P. gingivalis* was inoculated as a single species, its cell numbers did not increase significantly (Fig. 8A), and we reported previously that monospecies *Veillonella* sp. and *F. nucleatum* do not grow in static culture; *A. actinomycetemcomitans* shows significant growth only after 36 h of incubation (21). In combination with *Veillonella* sp., *P. gingivalis* increased fivefold between 12 h and 24 h and decreased slightly between 24 h and 36 h (Fig. 8B). Cell numbers of *Veillonella* sp. increased eightfold between 12 and 24 h. With *F. nucleatum*, *P. gingivalis* increased 5-fold between 12 h and 36 h, and *F. nucleatum* cell numbers increased 22-fold between 12 h and 24 h and another 2-fold by 36 h (Fig. 8C). With *A. actinomycetemcomitans*, *P. gingivalis* increased 10-fold between 12 h and 36 h, and *A. actinomycetemcomitans* increased 5-fold (Fig. 8D). Collectively, these results demonstrate mutualism between *P. gingivalis* and these three gram-negative partners.

In triple-species combination with *Veillonella* sp. and *A. actinomycetemcomitans*, *P. gingivalis* increased 12-fold between 12 h and 24 h and an additional 2-fold between 24 h and 36 h, indicating a dramatic and flourishing interaction with these partners (Fig. 9A). *Veillonella* sp. increased threefold, and *A. actinomycetemcomitans* increased fivefold, indicating a mutualistic three-species relationship. A mutualistic relationship was also discovered by replacing *A. actinomycetemcomitans* with *F. nucleatum*: *P. gingivalis* numbers increased 7-fold between 12 h and 24 h (Fig. 9B); *Veillonella* sp. increased 2-fold, but *F. nucleatum* cell numbers jumped 22-fold between 12 h and 24 h and an additional 2-fold in the next 12 h. Thus, *P. gingivalis* is not simply a late-colonizing periodontopathogen, but rather it forms mutualistic biofilm communities with a variety of oral species spanning initial, early, middle, and late colonizers of enamel surfaces.

DISCUSSION

Coupling the reported observations that *P. gingivalis* is present in dental plaque samples taken in the first 6 h after oral hygiene treatment (5, 13) and our results here, we clearly determined that the periodontopathogen *P. gingivalis* is capable of being an integral component of mutualistic multispecies oral biofilm communities at all stages of dental plaque development. We show that *P. gingivalis* adheres to saliva-coated glass and polystyrene but cannot grow without other species. When combined with a variety of initial (*S. gordonii* and *A. oris*), early (*Veillonella* sp.), middle (*F. nucleatum*), and late (*A. actinomycetemcomitans*) colonizers, *P. gingivalis* is an active member of these mutualistic communities.

Specificity by *P. gingivalis* for coadherence with certain streptococci was reported by Lamont and coworkers (12) and Xie et al. (23). Biofilms were formed when *S. gordonii* was the partner but were not formed with either *S. mutans* or *S. cristatus*. Our results agree with and extend those observations. Although it grows mutualistically with *S. gordonii*, *P. gingivalis* cannot grow when paired with a different initial colonizer, *S. oralis* (Fig. 4), but *P. gingivalis* grows well in *S. gordonii*-*S. oralis* three-species communities (Fig. 7). Thus, *P. gingivalis* shows a high degree of specificity for community partnerships with streptococci, which account for 60 to 90% of initial colonizers of enamel. It has been proposed for many years that anaerobic bacteria, such as *P. gingivalis*, are unsuited for initial colonization and must wait

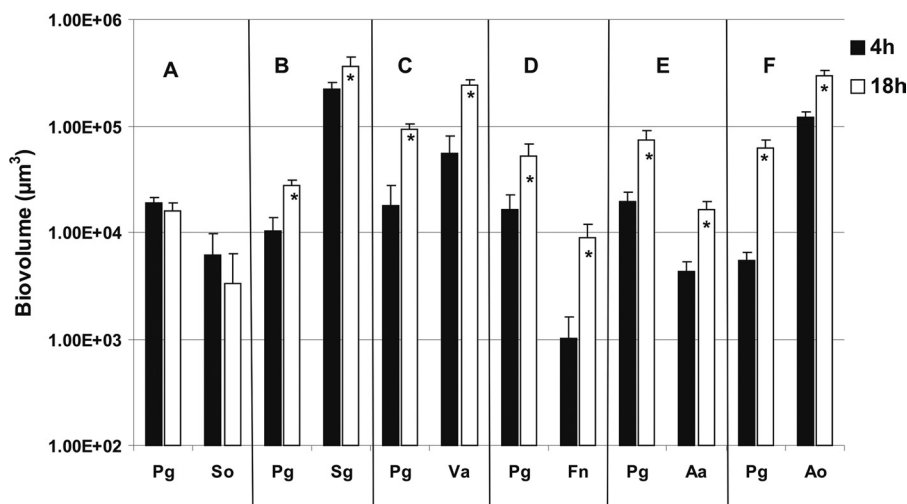


FIG. 4. Time-resolved changes in biovolumes (μm^3 per field of view) of two-species flow cells inoculated with coaggregates of *P. gingivalis* (Pg) and *S. oralis* (So) (A), *S. gordonii* (Sg) (B), *Veillonella* sp. (Va) (C), *F. nucleatum* (Fn) (D), *A. actinomycetemcomitans* (Aa) (E), or *A. oris* (Ao) (F). An asterisk indicates statistically significant increases ($P < 0.05$) in bacterial growth.

for more aerotolerant species, such as streptococci, to reduce the oxygen tension to permissive levels for anaerobic growth. However, our results here and those of Lamont and coworkers (12) and Xie et al. (23) emphasize instead the importance of partner specificity among streptococci and *P. gingivalis* in the establishment of *P. gingivalis* with initial colonizers.

Inability to form productive partnerships with *S. oralis* is not limited to *P. gingivalis*. We reported that *F. nucleatum*, another strict anaerobe, did not grow with *S. oralis* (20). However, *F. nucleatum* paired with initial colonizer *A. oris* with or without *S. oralis* was productive. As with *F. nucleatum*, *P. gingivalis* grows commensally with *A. oris*. Collectively, these results indicate temporal specificity for coaggregation by *P. gingivalis* with certain initial colonizers of enamel. These results suggest that *P. gingivalis* be considered among other early-colonizing species, such as veillonellae, that also coaggregate with initial colonizers. *Veillonella* sp. PK1910 and *P. gingivalis* ATCC 33277 are coaggregation partners and grow mutualistically on saliva. Our results here (Fig. 6) indicate that *Veillonella* sp.

PK1910 but not *S. oralis* 34 promotes growth of *P. gingivalis* in three-species communities with either *A. actinomycetemcomitans* or *F. nucleatum*. In fact, the three-species communities of *S. oralis* 34 and *P. gingivalis* ATCC 33277 with either *A. actinomycetemcomitans* or *F. nucleatum* decreased in cell numbers. Clearly, *P. gingivalis* and veillonellae have a productive pairwise relationship, which supports their placement as early colonizers of enamel.

Our laboratory has been interested in comparing the flow cell system and the static peg-biofilm system as models for in vitro study of multispecies communities of initial and early colonizers of enamel. Generally, the same partner specificities and mutualistic growth on saliva are seen with both systems (3, 20, 21). *P. gingivalis* and the other six species studied here were capable of binding to both saliva-conditioned substrata. As a single-species inoculum in the static system, *P. gingivalis* was retained on the polystyrene surface, although no significant growth occurred (Fig. 8A). However, initial attachment at 4 h to the saliva-coated surface in the flow cell was not maintained during the ensuing 14 h (Fig. 1). It is likely that the loss of porphyromonad biovolume is a result of the flow dynamics, whereby metabolically stressed cells are unable to maintain adherence under conditions of constant flow. Experiments with either model system were conducted under an anaerobic atmosphere, suggesting that redox potential is unlikely to be a factor.

In flow cells, *P. gingivalis* attached to the saliva-coated glass surface at about the same level, whether as a single species or as part of multispecies coaggregates (Fig. 1 to 7). In contrast, *P. gingivalis* binds at higher numbers when it is in two-species biofilms with *Veillonella* sp., *A. actinomycetemcomitans*, or *F. nucleatum* in static biofilm pegs (Fig. 8 and 9). With *Veillonella* sp., *P. gingivalis* cell numbers in the biofilm pegs were fivefold higher than those of *P. gingivalis* as a single species (Fig. 8); with *A. actinomycetemcomitans*, *P. gingivalis* biofilm cells were the same, except that a threefold higher number was noted at 36 h. However, *P. gingivalis* and *F. nucleatum* appear to be

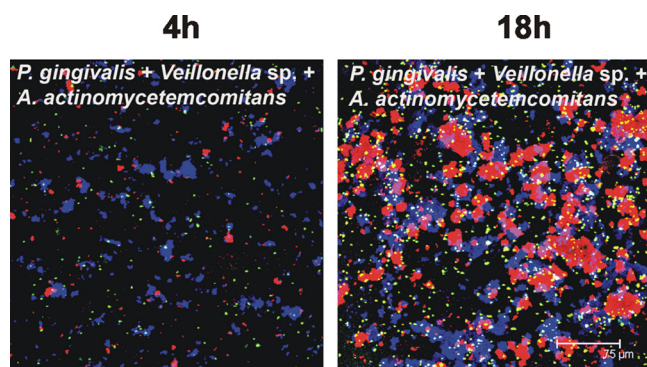


FIG. 5. Representative confocal micrographs of biofilm from three-species-inoculated flow cell. Communities following 4 h and at 18 h of incubation show intimate interaction of *Veillonella* sp. (blue) with *A. actinomycetemcomitans* (green) and *P. gingivalis* (red) to form mutualistic multispecies communities.

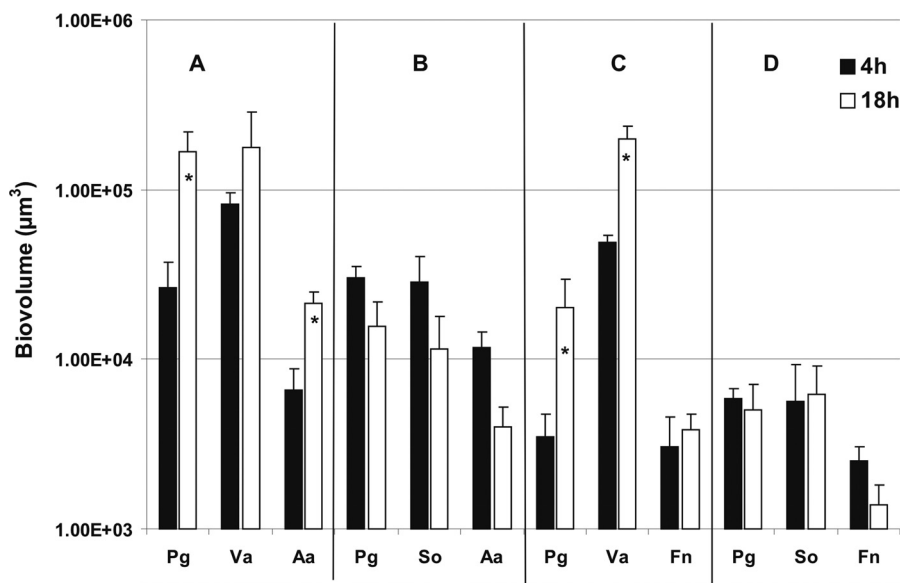


FIG. 6. Time-resolved changes in biovolumes (μm³ per field of view) of three-species-inoculated flow cell following 4-h (black bar) and 18-h (open bar) incubation showing growth enhanced by *Veillonella* sp. and growth inhibited by *S. oralis*. (A) *P. gingivalis* (Pg) + *Veillonella* sp. (Va) + *A. actinomycetemcomitans* (Aa); (B) *P. gingivalis* (Pg) + *S. oralis* (So) + *A. actinomycetemcomitans* (Aa); (C) *P. gingivalis* (Pg) + *Veillonella* sp. (Va) + *F. nucleatum* (Fn); and (D) *P. gingivalis* (Pg) + *S. oralis* (So) + *F. nucleatum* (Fn). An asterisk indicates statistically significant increases ($P < 0.05$) in bacterial growth.

particularly fitted for each other; the numbers of *P. gingivalis* cells are 15- to 20-fold higher at the three time points. These results might be indicative of natural relationships that occur between species during the development of dental plaque. The static peg system is a closed system (compared to flow cells) and allows accumulation of end products of digestion of salivary components by each biofilm member species. Apparently, this accumulation is highly beneficial to community growth and results in enhanced growth of all species in these two-species biofilms.

Three-species communities with *P. gingivalis*, *Veillonella* sp., and either *A. actinomycetemcomitans* or *F. nucleatum* are even better suited for cooperation than the two-species situations. Compared with monospecies peg biofilms, *P. gingivalis* in combination with *Veillonella* sp. and *A. actinomycetemcomitans* ex-

hibited 5-, 15-, and 35-fold greater cell numbers at 12-, 24-, and 36-h time points, respectively (Fig. 9). Likewise, *P. gingivalis* in combination with *Veillonella* sp. and *F. nucleatum* exhibited 12-, 15-, and 25-fold greater cell numbers at 12-, 24-, and 36-h time points, respectively. These data suggest that *Veillonella* sp. plays an integral role in coaggregates with *P. gingivalis* and the two other partners. Indeed, examination of the numbers of *Veillonella* sp. cells in three-species biofilms with *P. gingivalis* and *A. actinomycetemcomitans* revealed 45-, 32-, and 20-fold greater cell numbers at 12-, 24-, and 36-h time points, respectively, compared to those of veillonella single-species biofilms (data not shown). Further, the numbers of *Veillonella* sp. cells in three-species biofilms with *P. gingivalis* and *F. nucleatum* revealed cell numbers at 12-, 24-, and 36-h time points that were 50-, 31-, and 16-fold greater, respectively, than those for veillonella single-species biofilms (data not shown). Likewise, *F. nucleatum* increased 33-fold at 36 h with *P. gingivalis* and *Veillonella* sp., compared to *F. nucleatum* by itself (data not shown). Also, *A. actinomycetemcomitans* in biofilms with *P. gingivalis* and *Veillonella* sp. exhibited numbers that were 51-, 212-, and 14-fold higher than those for *A. actinomycetemcomitans* single-species biofilms (data not shown). Clearly, in static biofilm pegs, these species grow well together, compared to individually, and these data suggest that they are naturally compatible.

In flow cells with *P. gingivalis*, the biovolumes of *F. nucleatum*, *A. actinomycetemcomitans*, and *Veillonella* sp. were 6-, 11-, and 12-fold higher, respectively, than monospecies biovolumes at 18 h (data not shown). Various three-species combinations of *F. nucleatum*, *A. actinomycetemcomitans*, and *Veillonella* sp. with *P. gingivalis* yielded biovolume increases of the first three species of 3- to 15-fold compared to their respective single-species biofilm biovolume values (data not shown). Given that

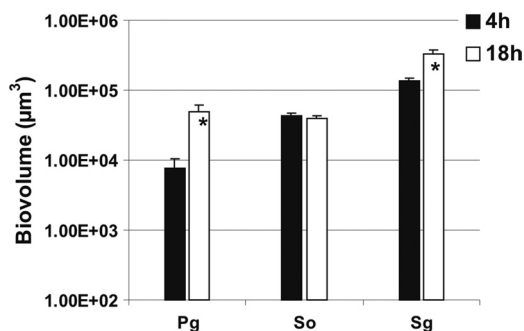


FIG. 7. Time-resolved changes in biovolume (μm³ per field of view) of three-species-inoculated flow cell showing specificity by *P. gingivalis* (Pg) for *S. gordonii* (Sg) in the presence of *S. oralis* (So) following 4-h (black bar) and 18-h (open bar) incubation. An asterisk indicates statistically significant increases ($P < 0.05$) in bacterial growth.

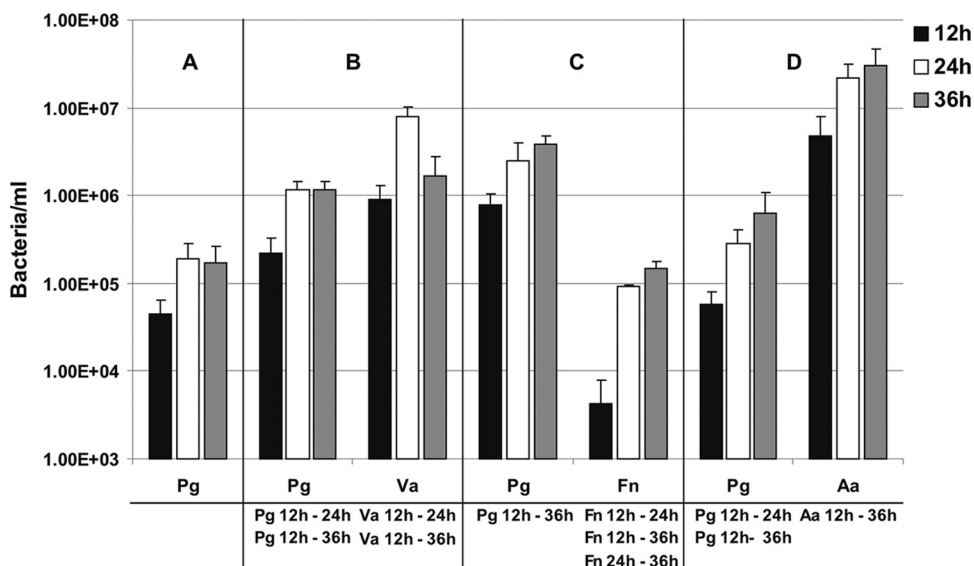


FIG. 8. q-PCR quantification of *P. gingivalis* cells alone in biofilms and with coaggregation partners in two-species-inoculated biofilms grown on polystyrene pegs submerged in 25% saliva and incubated anaerobically for 12, 24, or 36 h. (A) Single species, *P. gingivalis*; (B) two species, *P. gingivalis* (Pg) + *Veillonella* sp. (Va); (C) two species, *P. gingivalis* (Pg) + *F. nucleatum* (Fn); and (D) two species, *P. gingivalis* (Pg) + *A. actinomycetemcomitans* (Aa). Statistically significant increases ($P < 0.05$) in bacterial growth on the pegs is indicated at the bottom of each panel; for example in panel B, “Pg 12 h - 24 h” indicates that significant growth of *P. gingivalis* occurred between 12 h and 24 h in the two-species biofilm with *Veillonella* sp.

the two-model systems are significantly different in design (flow versus static), the high degree of correlation between them is remarkable. Even so, the increases in cell numbers in multispecies communities in flow cells are more modest than the increases in the static peg system. A priori, one might think that the flow cell would support unlimited growth because saliva is replenished continuously. However, our results suggest that a static system provides greater opportunity for the two- and three-species communities to interact metabolically

and flourish. These results may reflect the importance of time-dependent signal accumulation that regulates community metabolism of saliva in the microtiter well. Mutualistic relationships built on this complex nutritional source may require temporal metabolic give and take in which each species contributes its part to enhance the growth of all species. A metabolic contribution will remain in a microtiter well, but in a flow cell, it might be swept away before it has an opportunity to contribute to the community metabolism.

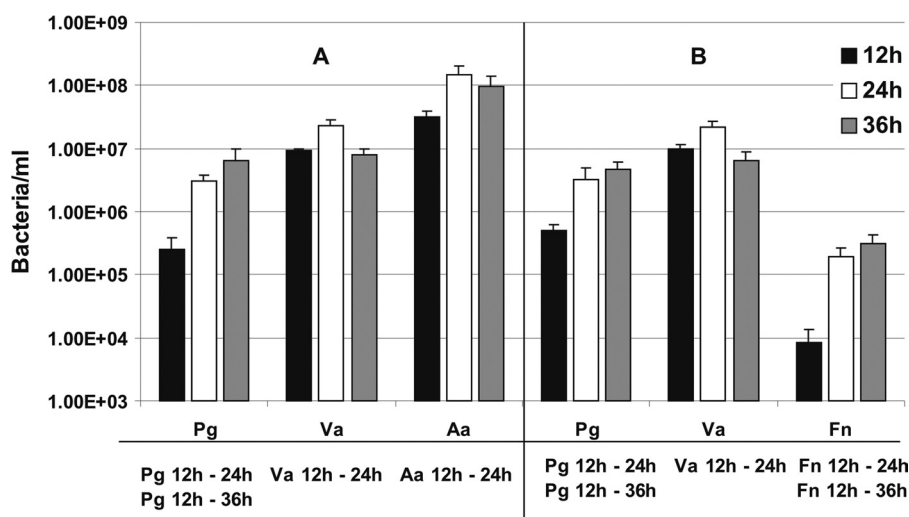


FIG. 9. q-PCR quantification of three-species-inoculated biofilms grown on polystyrene pegs submerged in 25% saliva and incubated anaerobically for 12, 24, or 36 h. Pg, *P. gingivalis*; Va, *Veillonella* sp.; Aa, *A. actinomycetemcomitans*; and Fn, *F. nucleatum*. Statistically significant increases ($P < 0.05$) in bacterial growth on the pegs is indicated at the bottom of each panel; for example in panel A, “Pg 12 h - 24 h” indicates that significant growth of *P. gingivalis* occurred between 12 h and 24 h in the three-species inoculated biofilm with *Veillonella* sp. and *A. actinomycetemcomitans*.

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