Aggregatibacter actinomycetemcomitans Builds Mutualistic Biofilm Communities with Fusobacterium nucleatum and Veillonella Species in Saliva\textsuperscript{\textdagger}

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Human oral bacterial pathogens grow in attached multispecies biofilm communities. Unattached cells are quickly removed by swallowing. Therefore, surface attachment is essential for growth, and we investigated multispecies community interactions resulting in mutualistic growth on saliva as the sole nutritional source. We used two model systems, saliva-coated transferable solid-phase polystyrene pegs (peg biofilms) and flow cells with saliva-coated glass surfaces. Fluorescent antibody staining and image analysis were used to quantify the biomass in flow cells, and quantitative real-time PCR with species-specific primers was used to quantify the biomass in peg biofilms. Veillonella sp. strain PK1910, Aggregatibacter actinomycetemcomitans JP2, and Fusobacterium nucleatum ATCC 10953 were unable to grow as single species in flow cells. Only A. actinomycetemcomitans grew after 36 h when peg biofilms remained submerged in saliva from the time of inoculation. Mixed-species coaggregates were used for two- and three-species inoculation. The biomass in two-species biofilms increased in both systems when Veillonella sp. strain PK1910 was present as one of the partners. Enhanced growth of all strains was observed in three-species biofilms in flow cells. Interestingly, in flow cells F. nucleatum and A. actinomycetemcomitans exhibited mutualism, and, although F. nucleatum was unable to grow with either of the other species in the peg system, F. nucleatum stimulated the growth of Veillonella sp. and together these two organisms increased the total biomass of A. actinomycetemcomitans in three-species peg biofilms. We propose that mutualistic two-species and multispecies oral biofilm communities form in vivo and that mutualism between commensal veillonellae and late colonizing pathogens, such as aggregatibacteria, contributes to the development of periodontal disease.

The human oral cavity is home to a robust microbial community composed of specialized microbes that are well adapted to biofilm growth in this environment with a constant flow. More than 700 bacterial phylotypes are found in dental plaque (1), and one-half of them have not been cultivated yet. By forming multispecies biofilm communities, oral bacteria overcome many environmental challenges, such as salivary flow and low-nutrient conditions. Communities that typify a healthy oral cavity are composed primarily of commensal bacteria, which do no harm to the host. However, oral pathogens, such as Aggregatibacter actinomycetemcomitans, are also present (13), and it is unclear how they compete and grow in established commensal communities.

The factors that play essential roles in biofilm community formation include the initial attachment of bacteria to a surface, coaggregation and coadhesion, communication, and growth of attached cells (2, 6, 11, 12, 27). The initial attachment events involve the adsorption of bacteria to the salivary pellicle, which is composed of many host-derived proteins, including glycoproteins. Attachment requires specific adhesins to be present on the bacterial cell surface; these adhesins are often lectins (carbohydrate-binding proteins), which interact with a complementary carbohydrate-containing receptor in the salivary pellicle. Coaggregation results from cell-cell recognition between genetically distinct bacteria in a planktonic cell suspension. The interaction between a planktonic cell and a surface-attached cell is called coadhesion (2). The physical interactions between cells provide the juxtaposition for communication in a flowing environment (11). Finally, growth of the attached species must occur. The first two factors define mixed-species attachment, but combinations of species often are unable to grow on whole saliva, which is a complex nutritional source; growth might require mutualistic communication among species and a combination of metabolic properties of several species to convert latent nutrition into usable nutrition.

We have been interested in the ability of different combinations of species to grow on saliva as their sole source of nutrients. When inoculated as single species, most bacteria are unable to grow on saliva. However, when accompanied by a second species, some bacteria exhibit mutualism, a beneficial relationship resulting in growth of both species (27). Three gram-negative genera that have received little attention for growth on saliva are Aggregatibacter (formerly Actinobacillus), Fusobacterium, and Veillonella. Metabolic cooperation among bacteria is central to the establishment of stable multispecies biofilm communities (5, 14). Veillonellae are unable to catabolize sugars. For growth, veillonellae depend upon organic acids produced by other species (9, 30). Fusobacterium nucleatum produces acetic, propionic, butyric, and lactic acids from...
peptone-yeast extract-glucose broth (15). *A. actinomycetemcomitans* ferments carbohydrates and produces catalase; one report has stated that *A. actinomycetemcomitans* does not utilize lactate (31), whereas two recent articles indicate that it uses lactic acid preferentially over high-energy carbon sources, such as glucose (3, 29). Thus, these organisms have the potential to cooperate metabolically while they are growing on saliva as the sole source of nutrients.

None of the three genera mentioned above are considered initial colonizers of saliva-coated tooth surfaces. The majority of initial colonization is colonization by streptococci (60 to 90%) and actinomyces (5 to 10%) (26). Veillonellae coaggregate with both streptococci and actinomyces (17) and therefore can participate in a second tier of early colonization, which might occur through coadshesion with adhered streptococci and actinomyces or through coaggregation with these organisms followed by attachment of the coaggregates to the initial biofilm. Indeed, veillonellae comprise 5 to 10% of the early colonizers (10, 26). Likewise, *Aggregatibacter* and *Fusobacterium* coaggregate with *Veillonella*, and all of these taxa coaggregate with streptococci and actinomyces. *A. actinomycetemcomitans* is considered a late colonizing species (18) and a causative agent of localized aggressive periodontitis (23). *F. nucleatum* has been proposed to be a bridge between early and late colonizers (19) on the basis of (i) the finding that other species initiate colonization of enamel (10, 21, 26), (ii) the finding that *F. nucleatum* is the most numerous species at healthy sites and that the numbers of *F. nucleatum* cells increase markedly at periodontally diseased sites (24), and (iii) the finding that *F. nucleatum* coaggregates with all of the initial, early, and late (typically pathogens) colonizers (20). Although most of the late colonizers coaggregate with *F. nucleatum*, they generally do not coaggregate with each other. Thus, the fusobacterial coaggregation bridge between early and late colonizers may partially explain why fusobacteria are so numerous in samples from both healthy and diseased sites.

It is well known that the lactic acid-utilizing veillonellae benefit from lactic acid-producing streptococci and actinomyces and that they might use this metabolic relationship to enhance multispecies community development (5, 11, 17). Streptococcus-veillonella communities have been micromani-pulated from 8-h dental plaque (5). However, the relationship of veillonellae with later colonizers has not been explored. We hypothesize that in the absence of the initial colonizers streptococci and actinomyces, veillonellae are capable of developing multispecies communities with later colonizers. Furthermore, we hypothesize that the ability of *Veillonella* sp. (early colonizer), *F. nucleatum* (secondary colonizer), and *A. actinomycetemcomitans* (late colonizer) to coaggregate with each other allows these organisms to form a multispecies biofilm. We used two in vitro models, a saliva-fed flow cell (open system) and a polystyrene peg immersed in static saliva (closed system). The spatial and temporal organization of the structure of a multispecies community resulting from attachment and growth is preserved and can be examined by confocal laser scanning microscopy and by quantitative PCR (q-PCR) (5, 11, 27, 28). We show here that mutualism occurs in an open flowing environment and in a closed static system. Although initial colonizers (streptococci and actinomyces) were not present in these studies, we show here that *Veillonella* sp., *F. nucleatum* and *A. actinomycetemcomitans* form multispecies communities in vitro. Pathogenic bacteria in vivo might use such a community to gain an advantage and initiate pathogenic sequelae.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *Veillonella* sp. strain PK1910 was grown in Todd-Hewitt broth (Difco Laboratories, Detroit, MI) supplemented with 0.6% lactic acid. *A. actinomycetemcomitans* JP2 (a kind gift from D. Demuth, University of Louisville, Louisville, KY) was grown in brain heart infusion medium (Difco) supplemented with 0.04% sodium bicarbonate and 2% yeast extract. *F. nucleatum* ATCC 10953 was grown in brain heart infusion medium supplemented with 0.25% l-glutamic acid. All species were grown in a Bactron anaerobic (N2,CO2,H2 ratio, 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 (8, 27). Briefly, the supernatant was diluted with distilled water to produce 25% saliva and then was filtered through a 0.2-μm pore-size SFCA (Nalgene) sterile-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 with a volume of 240 μl each. A glass coverslip, which served as the attachment substrate for the growing biofilm, was secured to each reusable flow cell with a silicone adhesive. The flow cells were cleaned overnight with 0.1 M HCl and rinsed with 5 ml of distilled water, which was followed by injection of 7% Tween into the flow cells and incubation for 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. The bacteria used for inoculation of two species and three species were first coaggregated (by mixing 0.1–ml portions of appropriate combinations of *F. nucleatum*, *Veillonella*, and *A. actinomycetemcomitans* [equivalent to about 1 × 10^7 to 3 × 10^7 cells of each species]), which was followed by incubation of the flow cells in the anaerobic chamber to provide an environment favorable for growth of *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 (7).

**Biofilm staining.** The staining of cells was different 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), or Alexa Fluor 546 (Invitrogen)-conjugated immunoglobulin G of a polyclonal antiserum to *Veillonella* sp., *A. actinomycetemcomitans*, or *F. nucleatum*, respectively. When multiple species were inoculated, Alexa Fluor 633 was used in place of Alexa Fluor 546 to visualize *Veillonella* sp. in the presence of *A. actinomycetemcomitans* or *F. nucleatum*. All conjugation procedures with immunoglobulin Gs were performed by using Alexa Fluor labeling kits (Invitrogen) and following the manufacturer’s directions. Immunofluorescence was obtained by injecting the antibody (5 μg/ml in phosphate-buffered saline) into the appropriate flow cell track and incubating the flow cell 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 ×100, 1.25 NA 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, and cubic voxels were used for biovolume determination. Five confocal data sets were analyzed for each time point, and the mean and standard deviation were calculated. A one-way analysis of variance at the 95% confidence level with a nonparametric Tukey pairwise comparison test was used to determine if the means for the 4-h and 18-h time points were statistically different for each species for each condition (one-, two-, and three-species biofilms) in the flow cell studies and if the means for the 12-h, 24-h, and 36-h time points were statistically different for each species for each condition.
condition (one-, two-, and three-species biofilms) in the peg studies with static saliva. All images presented below are maximum projections of the entire confocal image stack produced by the Leica TCS software (Leica).

Coaggregation assay. Microtiter plate wells were filled with 200 μl of 25% human saliva. Overnight cultures (20 μl) of Veillonella sp. strain PK9190, A. actinomycetemcomitans JP2, and F. nucleatum ATCC 10953 were added alone or in different combinations to the 200 μl of saliva, and the microtiter plate was shaken for 1 min on an orbital shaker, after which a sample wet mount of each possible pair combination was examined by phase-contrast microscopy.

Biofilm growth conditions on polystyrene pegs (peg biofilms). Biofilms were grown in 25% human saliva on pegs (Nunc-Immuno TSP; Nunc 445497) (4, 22) mounted in a 96 MicroWell plate (Nunc 163320) (5, 22). Microtiter plate wells were filled with 200 μl of 25% human saliva, and pegs were then inserted and incubated for 30 min at room temperature to obtain a conditioning film. Overnight cultures (~20 μl) of Veillonella sp. strain PK9190, A. actinomycetemcomitans JP2, and F. nucleatum ATCC 10953 were added to the 200 μl of saliva in the wells; this amount was equivalent to about 1 × 10^9 cells of each species in a microtiter well. The 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 were 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 (16). Biofilm-covered pegs were immersed in 40 μl of sterile ultrapure water plus 160 μl of 0.05 M sodium hydroxide and incubated at 60°C for 60 min, after which 18.4 μl of 1 M Tris-HCl (pH 7.0) was added to neutralize the pH. The extract was used as the template DNA for the q-PCR analyses (5, 16). The bacterial genomic DNA used for standard curves were extracted from overnight cultures of Veillonella sp. strain PK9190, A. actinomycetemcomitans JP2, and F. nucleatum ATCC 10953 with a DNA extraction kit (Qiagen, Valencia, CA) used according to the manufacturer’s instructions. Genomic DNA was stored at −20°C.

Real-time 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 Veillonella sp. strain PK9190 were forward primer CCGTGTAGGG ATGGAAACTGC and reverse primer CCTTCGCCACTGGTGTTCTC, and the annealing temperature was 56°C. The primers specific for A. actinomycetemcomitans JP2 were forward primer GGACGGGTGAGTAATGCTTG and reverse primer ATGGAAACTGC and reverse primer CCTTCGCCACTGGTGTTCTC, and the annealing temperature was 56°C. The primers specific for F. nucleatum ATCC 10953 were forward primer CTTTACCCCAACTTACC, and the annealing temperature was 56°C. Quantification of Veillonella sp., A. actinomycetemcomitans, and F. nucleatum in the biofilms was performed by q-PCR analysis using the SYBR green dye to detect the 16S rRNA gene amplicons. Each reaction mixture (final volume, 25 μl) contained 3 μl of template, 3.5 μl of diethyl pyrocarbonate-treated ultrapure water, 12.5 μl of Power SYBR green PCR master mixture (Applied Biosystems, Foster City, CA), and 0.2 μl of each of the forward and reverse primers (375 nM). q-PCR was performed with an MX3005P thermocycler (Stratagene, La Jolla, CA) using the following thermal cycle recommended for the Power SYBR green PCR master mixture: 95°C for 10 min and then 40 cycles of 30 s at 95°C and 1 min at 56°C. Dissociation curves were generated by incubating reaction products at 95°C for 1 min and at 56°C for 30 s and then incrementally increasing the temperature to 95°C. Fluorescence data were collected at the end of the 56°C primer annealing step for 40 amplification cycles and throughout the dissociation curve analysis. Analysis of the melting curves with both primer sets revealed 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 the PicoGreen fluorescence assay (Invitrogen). To convert ng DNA to numbers of cells, the following weights and genome sizes were used: 3.05 fg/ genome and 3.0 Mb for Veillonella, 2.05 fg/ genome and 2.1 Mb for aggregatibacteria, and 2.41 fg/ genome and 2.4 Mb for fusobacteria (http://www.hmod.org). The data presented below are data for three independent biofilms.

RESULTS

Flow cells inoculated with one species. Veillonella sp., A. actinomycetemcomitans, and F. nucleatum monocultures were inoculated into flow cells, and then images of the biofilms formed were obtained (Fig. 1) and their biovolumes were quantified (Fig. 2A) after 4 h and 18 h of incubation. Each species attached to the saliva-conditioned surface within 4 h (Fig. 1, left panels). After 18 h of incubation, the biovolume was less for all species, indicating that the cells detached from the conditioned surface (Fig. 1, right panels, and Fig. 2A).

Flow cells inoculated with two species. In flow cells inoculated with coaggregates of Veillonella sp. and F. nucleatum, both species attached to the saliva-conditioned surface, as shown by labeling with specific antibodies (Fig. 3). The biovolumes of Veillonella sp. and F. nucleatum each increased threefold between 4 h and 18 h (Fig. 2B, left panels), indicating that both species grew on saliva. After inoculation of Veillonella sp. and A. actinomycetemcomitans, the biovolumes of these two species increased three- and twofold, respectively (Fig. 2B, middle panels), indicating that both species grew on saliva. The data for the combination of F. nucleatum and A. actinomycetemcomitans showed that the biovolumes of these two species increased significantly between 4 h and 18 h (Fig. 2B, right panels). Interestingly, these later colonizing species grew in the absence of any initial colonizers. These data obtained using species unable to grow separately but able to grow together indicate that there was mutualistic growth of each pair of organisms.

Flow cells inoculated with three species. Coaggregates of the three species were inoculated into a flow cell, and all species attached to the saliva-conditioned substrate and grew between 4 h and 18 h after inoculation (Fig. 4). The biovolumes of Veillonella sp. (Fig. 2C, left panel) and F. nucleatum (Fig. 2C, right panel) increased threefold, whereas the biovolume of A. actinomycetemcomitans (Fig. 2C, middle panel) increased only twofold, indicating that these three species grow in multispecies communities in saliva.

Coaggregation of members of two- and three-species communities. Coaggregation of Veillonella sp. with F. nucleatum and A. actinomycetemcomitans was examined using pairwise combinations and three species in saliva for samples taken directly from microtiter wells just before a peg was inserted. Coaggregates were visualized by phase-contrast microscopy, and the results revealed the juxtaposition of long, slender F. nucleatum cells with the smaller cells of A. actinomycetemcomitans and Veillonella sp. (Fig. 5). Each species was a coaggregation partner of the other species, and the ability of the organisms to form multispecies biofilm communities in a static system was investigated.

Real-time q-PCR quantification of species in biofilms on pegs immersed in saliva. The peg system uses 1 ml (total volume) of saliva for five replicates instead of the minimum volume of 216 ml for one flow cell track for 18 h, and thus the peg system has great advantages. To investigate potential differences between flow cells and a completely closed system, we kept pegs submerged in the same saliva for 12, 24, or 36 h. When inoculated as single species, neither Veillonella sp. nor F. nucleatum grew (Fig. 6A, left and right panels, respectively). A. actinomycetemcomitans grew, but growth (10-fold increase) was evident only at 36 h (Fig. 6A, middle panel). To quantify the population of each species in the one- two- and three-species biofilms, we used species-specific primers and performed q-PCR for biofilms formed on pegs. In Veillonella sp.-F. nucleatum two-species biofilms, the concentration of Veillonella sp. increased ninefold between 12 h

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and 24 h and decreased slightly between 24 h and 36 h, but *F. nucleatum* did not grow (Fig. 6B, left panel). In two-species biofilms containing *Veillonella* sp. and *A. actinomycetemcomitans*, the concentrations of both species increased between 12 h and 24 h and remained high at 36 h (Fig. 6B, middle panel). Interestingly, the numbers of cells of *A. actinomycetemcomitans* increased between 12 h and 36 h in the presence of *F. nucleatum*, but *F. nucleatum* did not grow (Fig. 6B, right panel).
panel). Veillonella sp. and A. actinomycetemcomitans grew with F. nucleatum in three-species biofilms, but again F. nucleatum failed to grow (Fig. 6C). Although there was no increase in the number of F. nucleatum cells in one-, two-, or three-species cultures, this species was retained in the biofilms at levels equal to or nearly equal to its level at the 12-h time point, suggesting that in a closed system with these community partners F. nucleatum requires different conditions for growth that are provided in a flowing-saliva environment. Collectively, the results for the peg and flow cell model systems show that there are readily attained mutualistic relationships between a known periodontopathogen, A. actinomycetemcomitans, and two other oral species, and they identify a potential mechanism that the pathogen uses to gain an advantage in the host oral environment.

**DISCUSSION**

We investigated multispecies community growth using a periodontopathogen and two other species that are not considered initial colonizers of oral surfaces. We showed that these species were capable of growth as a biofilm community without members of the genera Streptococcus and Actinomyces, the principal initiators of the succession of species that repetitively form dental plaque (10, 26). Initial colonizers naturally cover oral surfaces (26) and thus may facilitate incorporation of other multispecies communities into developing dental plaque.

The buccal (cheek) and lingual (tongue) sides of enamel surfaces are constantly bathed in saliva, suggesting that these surfaces are subjected to freely flowing saliva. In contrast, enamel surfaces between teeth (interproximal) have considerably less exposure to flow and might support communities favored by more static conditions. Indeed, it is well known that dental plaques obtained from buccal and interproximal surfaces contain different proportions of the same species, as well as different species (32).

To explore building multispecies communities without initial colonizers, we chose Veillonella sp. (an early but not initial colonizer), F. nucleatum (a secondary colonizer), and A. actinomycetemcomitans (a late colonizer and periodontopathogen associated directly with localized aggressive periodontitis). Statistical comparisons of the microfloras associated with healthy sites and with localized aggressive periodontitis (formerly called localized juvenile periodontitis) sites (24, 25) revealed that F. nucleatum and A. actinomycetemcomitans were among the species that occurred at at least twofold-greater concentrations in disease sites than in other sites sampled (25). Veillonella spp. were present in disease sites, but the proportions were higher in healthy sites (25). F. nucleatum, Veillonella spp., and A. actinomycetemcomitans accounted for 7, 4, and 1%, respectively, of 1,065 isolates obtained at random from subgingival plaque of subjects with localized aggressive periodontitis (24). Thus, these species are commonly found together. To study the interactions among species, we chose two dramatically different biofilm model systems that mimicked a freely flowing saliva situation and a static saliva situation in the oral cavity. The saliva-conditioned glass surface of a flow cell receiving constant salivary flow was compared with a static saliva-conditioned polystyrene peg immersed in saliva for up to 36 h. The three species were inoculated singly, as two-species aggregates, or as three-species aggregates. Biofilm growth in both model systems was monitored quantitatively.

In flow cells, an open system with fresh saliva continually passing across the biofilm, mutualism occurred and was evident with all species pairs. Instead of a decrease in the biovolume of each species, as observed in one-species biofilms, when two species were inoculated, the biovolumes of the species more
than doubled. The pair that appeared to be best matched is the *Veillonella* sp. strain PK1910-*A. actinomycetemcomitans* JP2 pair. The biovolume of *Veillonella* when this pair was used was higher than its biovolume when it was inoculated singly, and the biovolume of *Aggregatibacter* was 10-fold higher than its biovolume in one-species biofilms. Also, fusobacteria flourished in flow cells, as shown by the >10-fold increase in the biovolume of *Fusobacterium* in biofilms inoculated with three

FIG. 3. Representative confocal micrographs of 4-h (left panels) and 18-h (right panels) biofilms showing mutualistic growth in flow cells inoculated with two species. The organisms used were *Veillonella* sp. strain PK1910, *A. actinomycetemcomitans* JP2, and *F. nucleatum* ATCC 10953. Bacterial cells were stained with species-specific fluorophore-conjugated immunoglobulin G (blue, *Veillonella* sp. strain PK1910; green, *A. actinomycetemcomitans* JP2; red, *F. nucleatum* ATCC 10953), and cell-cell contact is evident.
species at 18 h compared to the biovolume at 18 h when it was inoculated alone. *F. nucleatum* may act as a coaggregation bridge between *Veillonella* sp. and *A. actinomycetemcomitans*, and corncob morphologies may occur (Fig. 3, bottom panels) and by *A. actinomycetemcomitans* and *Veillonella* sp. cells (Fig. 4) are evident and indicate that there is an intimate physical arrangement of cells of these species. *F. nucleatum* ATCC 10953 produces lactic acid (15), and its closeness to *Veillonella* sp. strain PK1910 (blue) with *A. actinomycetemcomitans* JP2 (green) and *F. nucleatum* ATCC 10953 (red) that formed mutualistic multispecies communities.

In summary, the two model systems provide an opportunity to compare and contrast the biofilm-forming characteristics of multiple species chosen based on their abilities to form mutualistic multispecies communities. Some pairs of species (for example, *Streptococcus oralis* and *A. actinomycetemcomitans*) and some groups of three species (for example, *Porphyromonas gingivalis*, *S. oralis*, and *A. actinomycetemcomitans*) do not grow on saliva. We have usually chosen species that, as single species, cannot grow on saliva as the sole nutritional source. When other factors are more important (for example, when *F. nucleatum*, a proposed coaggregation bridge organism, is used because of its ability to integrate with initial colonizing species, such as *S. oralis* and *Actinomyces oris*, that do grow as single species), then our focus changes (28). The flow cell model has the advantage that the spatiotemporal changes in multispecies community structure can be viewed by confocal microscopy and the advantage that the salivary flow constantly refreshes the nutrients and washes out inhibitory molecules (but also, perhaps, important signaling molecules). We have shown that a streptococcus-veillonella pair in a flow cell must be juxtaposed for a streptococcal green fluorescent protein-amylase reporter gene to report (11), indicating the importance of cell-to-cell distance for interspecies communication. The flow cell model has received a lot of attention, but confocal microscopy is limited by its concurrent use of four or possibly five species-specific fluorescently labeled primary antibodies for studies of multispecies community structure and the flow cell model uses a lot of saliva. For these reasons, we have explored the static peg model system. This system has major advantages because it is amenable to high-throughput screening, it uses only a few milliliters of saliva per multispecies biofilm, and, significantly, the number of species that can be tested for mutualistic relationships is not limited to four or five; the number of species-specific primers designed for a multispecies investigation limits this model. Fortunately, workers are beginning to use this model for oral bacterial multispecies biofilms.
One promising research area compatible with the static model is the role of signaling molecules that are produced by the multispecies community and accumulate in the microtiter well. It is possible that the delayed growth of *A. actinomycetemcomitans* seen only after 36 h (Fig. 6A; Fig. 6B, right panel) and the continuous growth over 36 h (Fig. 6B, middle panel; Fig. 6C, middle panel) are results of gene expression regulated by environmentally accumulating signals. The static peg model is conducive to studies of interspecies interactions mimicking environmentally relevant multispecies communities, such as studies of the integration of pathogens into commensal communities.
FIG. 6. q-PCR quantification of biofilms inoculated with one (A), two (B), and three (C) species grown on polystyrene pegs submerged in 25% saliva and incubated anaerobically for 12, 24, or 36 h. (A and C) Open bars, 12 h; black bars, 24 h; gray bars, 36 h. (B) Va, two (B), and three (C) species grown on polystyrene pegs submerged in ATCC 10953. Statistically significant increases in bacterial growth on strain PK1910; Aa, P. nigrescens; Aa, P. niger; Va, P. asaccharolyticus; Fn, S. constellatus; Va, P. gasseri. The y-axis of each graph represents the number of bacteria per ml of biofilm. The x-axis represents the species inoculated and the time of incubation. The bars represent the standard deviation of the mean. 

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REFERENCES


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