Aggregatibacter actinomycetemcomitans Builds Mutualistic Biofilm Communities in Saliva With Fusobacterium nucleatum and Veillonella sp.

Saravanan Periasamy and Paul E. Kolenbrander*

Oral Infection and Immunity Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892.

*Corresponding author:
Paul E. Kolenbrander
National Institutes of Health/NIDCR
Building 30, Room 310
30 Convent Drive, MSC 4350
Bethesda, MD 20892-4350
Phone: 301-496-1497
Fax: 301-402-0396

Short title:
Mutualism among A. actinomycetemcomitans, F. nucleatum and Veillonella sp.
ABSTRACT

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

The human oral cavity is home to a robust microbial community composed of specialized microbes that are well adapted to biofilm growth in this constantly flowing environment. More than 700 bacterial phylotypes are found in dental plaque (1), and half of them have not yet been cultivated. By forming multi-species biofilm communities, oral bacteria overcome many of their 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 A. actinomycetemcomitans are also present (13), and it is unclear how they compete and grow in established commensal communities.

Several factors that play essential roles in biofilm community formation include initial attachment of bacteria to a surface, coaggregation/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 is the cell-cell recognition between genetically distinct bacteria in a planktonic cell suspension. Recognition between a planktonic and a surface-attached cell is called coadhesion (2). These physical interactions between cells provide the juxtaposition for communication in a flowing environment (11). And, lastly, 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 nutrient. When inoculated as mono-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 called *Actinobacillus*), *Fusobacterium*, and *Veillonella*. Metabolic cooperation among bacteria is central to the establishment of stable multi-species biofilm communities (5, 14). Veillonellae are unable to catabolize sugars. For growth, veillonellae depend upon organic acids produced by other species (9, 30). *F. nucleatum* produces acetic, propionic, butyric and lactic acids from peptone-yeast extract-glucose broth (15): *A. actinomycetemcomitans* ferments carbohydrates, produces catalase, and one report states 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 species have the potential to cooperate metabolically while growing on saliva as the sole nutritional source.

None of the three genera are considered initial colonizers of saliva-coated tooth surfaces. The majority of initial colonization is accomplished 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 coadhesion with already 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 coaggregate with streptococci and actinomyces. A. actinomycetemcomitans is considered to be 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) other species initiate colonization of enamel (10, 21, 26), ii) F. nucleatum is the most numerous species in healthy sites, and its numbers increase markedly in periodontally diseased sites (24), and iii) F. nucleatum coaggregates with all of the initial, the early, as well as the 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 multi-species community development (5, 11, 17). Such streptococcal-veillonellae communities have been micromanipulated 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 initial colonizers streptococci and actinomyces, veillonellae are capable of developing multi-species communities with later colonizers. Further, we hypothesize that the ability of Veillonella sp. (early colonizer), F.
nucleatum (secondary colonizer), and A. actinomycetemcomitans (late colonizer) to
coaaggregate with each other provides them juxtaposition to form a multi-species biofilm.
We used two in vitro models, a saliva-fed flowcell (open system) and a polystyrene peg
immersed in static saliva (closed system). The spatial and temporal organization of the
multi-species community structure resulting from attachment and growth is preserved and
can be examined by confocal laser scanning microscopy and by q-PCR (5, 11, 27, 28).
We show that mutualism occurs in an open flowing environment and in a closed static
system. Although initial colonizers (streptococci and actinomyces) were absent in these
studies, we show here that Veillonella sp., F. nucleatum and A. actinomycetemcomitans
form multi-species communities in vitro. Pathogenic bacteria in vivo might use such
community organization to gain an advantage and initiate pathogenic sequelae.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Veillonella sp. PK1910 was grown in
Todd-Hewitt broth (THB, Difco Laboratories, Detroit, MI) supplemented with 0.6%
lactic acid (THBL). A. actinomycetemcomitans JP2 (kind gift from D. Demuth,
University of 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. 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 six to ten 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 0.22-µm-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.

**Flowcell 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-µl volume. A glass coverslip, which serves as the attachment substratum for the growing biofilm, was secured to the reusable flowcells with a silicone adhesive. The flowcells were cleaned overnight with 0.1 M HCl and rinsed with 5 ml of distilled water, followed by injecting 70% ethanol into the flowcells and incubating 20 min. The flowcells 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 flowcells.** Overnight bacterial cultures were harvested by centrifugation, washed twice with 25% sterile human saliva, and the optical density at 600 nm was adjusted to 0.1, which is equivalent to about 1 to 3 x 10⁷ cells/ml. Flowcells were inoculated with one, two or three species. Dual-species and triple-species inoculations were first coaggregated (mixing 0.1 ml of appropriate combinations of each *F. nucleatum, Veillonella* sp., and *A. actinomycetemcomitans*) (equivalent to about 1 to 3 x 10⁶ cells of each species) and followed by incubation of the flowcell in the anaerobic chamber to provide an environment favorable to *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 approximates unstimulated salivary flow in the mouth (7).

**Biofilm staining.** Staining of cells varied in the different experiments. When strains were grown as monocultures, visualization was by primary immunofluorescence with Alexa Fluor 546, Alexa Fluor 488 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 conjugations of immunoglobulins G were done by using AlexaFluor labeling kits (Invitrogen) following the manufacturer’s directions. Immunofluorescence was performed by injecting the antibody (5 µg/ml in PBS) into the appropriate flowcell track and incubating for 20 min. A final wash with PBS preceded confocal laser scanning microscopy. No cross reactivity of staining by antibodies with non-homologous strains was observed.

**Image and statistical analysis.** A TCS-SP2 confocal microscope (Leica, Exton, PA) with a 40x, 1.25 NA oil immersion lens was used to record confocal image stacks in five random locations near the center of the flowcells, after which biofilm biovolumes were determined by volumetric analyses (IMARIS ver. 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 deviation 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 the means were statistically
different between the 4 h- and 18 h-time points for each species for each condition (mono-, dual- and triple-species) in the flowcell studies and if the means were statistically different between the 12 h-, 24 h-, and 36 h-time points for each species for each condition (mono-, dual- and triple-species) in the Peg studies with static saliva. All images presented are maximum projections of the entire confocal image stack as 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. PK1910, *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 one minute on an orbital shaker, after which, a sample wet mount of all possible pair combinations was examined by phase contrast microscopy.

**Biofilm growth conditions on polystyrene pegs (Pegs).** Biofilms were grown in 25% human saliva on Pegs (Nunc-Immuno™ TSP; Nunc 445497) (4, 22) mounted in U96 MicroWell™ Plates (Nunc 163320) (5, 22). Microtiter plate wells were filled with 200 µl of 25% human saliva, and Pegs were then inserted for 30 min at room temperature to build a conditioning film. Overnight cultures (~20 µl) of *Veillonella* sp. PK1910, *A. actinomycetemcomitans* JP2 and *F. nucleatum* ATCC 10953 were added to the 200 µl of saliva in the wells; this equals about 1 to 3 x 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 (16). Biofilm-covered Pegs were immersed in 40 µl of sterile ultrapure water plus 160 µl of 0.05 M sodium hydroxide incubated at 60°C for 60 min, after which 18.4 µl of 1 M Tris-HCl (pH 7.0) were added to neutralize the pH. This extract was used as the template DNA for the q-PCR analyses (5, 16). Bacterial genomic DNA for standard curves was extracted from overnight cultures of *Veillonella* sp. PK1910, *A. actinomycetemcomitans* JP2 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.

**Real-time q-PCR quantification of species in biofilms.** Species-specific primers were designed with Primer 3 program (http://www. justbio.com). No cross-hybridization of primers with non-homologous species was observed. The primers specific for *Veillonella* sp. PK1910 were F: CCGTGATGGGATGGAAACTGC ; R: CCTTCGCCACTGGTGTTCTTC annealing temperature was 56˚C. The primers specific for *A. actinomycetemcomitans* JP2 were F: GGACGGGTGAGTAATGCTTG; R: CCCTTACCCCACCAACTACC; annealing temperature was 56˚C. The primers specific for *F. nucleatum* ATCC 10953 were F: CTTAGGAATGAGACAGAGATG, R: TGATGGTAACATACGAAAGG; annealing temperature was 56˚C. Quantification of *Veillonella* sp., *A. actinomycetemcomitans* and *F. nucleatum* in the biofilms was performed with q-PCR reaction using the SYBR ® Green dye to detect the 16S-rRNA gene amplicons. Each reaction (final reaction volume 25 µl) contained 3 µl template, 3.5 µl of DEPC-treated ultra pure water, 12.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 3 µl each of forward and reverse primers at
375 nM. Q-PCR was performed in an MX3005P thermocycler (Stratagene, La Jolla, CA) using the thermocycle recommended for Power SYBR Green PCR Master Mix: 95°C for 10 min, and 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, 56°C for 30 s and 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. 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 ten-fold serial dilutions of bacterial DNA isolated with a DNA extraction kit (Qiagen) and quantified with PicoGreen® fluorescence Assay (Invitrogen). To convert ng DNA to cell numbers, the following weights and genome sizes were used: 3.05 fg/genome and 3.0 Mb for veillonellae; 2.05 fg/genome and 2.1 Mb for aggregatibacteria; 2.41 fg/genome and 2.4 Mb for fusobacteria (http://www.homd.org). The data presented are from three independent biofilms.
RESULTS

Mono-species inoculated flowcells. Veillonella sp., A. actinomycetemcomitans, and F. nucleatum monocultures were inoculated into flowcells, and the biofilms formed were imaged (Fig. 1) and their biovolumes quantified (Fig. 2A) after 4 h and 18 h incubation. Each species attached to the saliva-conditioned surface within 4 h (Fig. 1, left column). After 18 h incubation, less biovolume was evident for all species, indicating that the cells detached from the conditioned surface (Fig. 1, right column and Fig. 2A).

Dual-species inoculated flowcells. In flowcells inoculated with coaggregates of Veillonella sp. and F. nucleatum, both species attached to the saliva-conditioned surface, as seen by their labeling with specific antibody (Fig. 3). The biovolumes of Veillonella sp. and F. nucleatum increased 3-fold for each species between 4 h and 18 h (Fig. 2B, left panel), indicating that both species grew on saliva. Dual species inoculations with Veillonella sp. and A. actinomycetemcomitans exhibited biovolume increases of 3- and 2-fold, respectively, (Fig. 2B, middle panel) indicating that both species grew on saliva. The pair combination of F. nucleatum and A. actinomycetemcomitans showed that biovolumes of both species significantly increased between 4 h and 18 h (Fig. 2B, right panel). Interestingly, these later-colonizing species grew in the absence of any initial colonizers. These data using species unable to grow separately but able to grow together represent mutualistic growth in each pairwise combination.

Triple-species inoculated flowcells. Coaggregates of the three species were inoculated into a flowcell, and all species attached to the saliva-conditioned substratum and grew between 4 h and 18 h incubation (Fig. 4). The biovolumes of Veillonella sp. (Fig. 2C, left panel) and F. nucleatum (Fig. 2C, right panel) increased 3-fold, whereas A.
actinomycetemcomitans (Fig. 2C, middle panel) biovolume increased only 2-fold, indicating a coordination by these three species to grow in multi-species communities in saliva.

**Coaggregation of dual- and triple-species community members.**

Coaggregation of Veillonella sp. with F. nucleatum or with A. actinomycetemcomitans was examined in pairwise combinations and as triple species in saliva in samples taken directly from microtiter wells just before inserting the Peg. Coaggregates were visualized by phase-contrast microscopy and revealed the juxtapositioning 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 others, and their ability to form multi-species biofilm communities in a static system was investigated.

**Real-time q-PCR quantification of species in biofilms on saliva-immersed Pegs.** Compared to flowcells, the Peg system uses 1 ml saliva total for five replicates versus a minimum of 216 ml for one flowcell track for 18 h, and thus the Peg system is highly advantageous. To investigate potential differences between flowcells versus 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. or F. nucleatum grew (Fig. 6A, left, 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 mono- dual- and triple-species biofilms, we used species-specific primers and conducted q-PCR on biofilms formed on Pegs.

In Veillonella sp. and F. nucleatum dual-species biofilm, Veillonella sp. increased (9-fold) between 12 h and 24 h and decreased slightly between 24 h and 36 h, but F.
nucleatum did not grow (Fig. 6B, left panel). In dual-species biofilms of Veillonella sp. and A. actinomycetemcomitans, both increased between 12 h and 24 h and remained at high cell numbers at 36 h (Fig. 6B, middle panel). Interestingly, the cell numbers of A. actinomycetemcomitans increased between 12 h and 36 h with F. nucleatum, but F. nucleatum did not grow (Fig. 6B, right panel). Veillonella sp. and A. actinomycetemcomitans grew with F. nucleatum in triple-species biofilms, but again F. nucleatum failed to grow (Fig. 6C). Although F. nucleatum exhibited no increase in cell numbers in monoculture, dual- or triple-culture, it was retained in the biofilms at levels equal or nearly equal to its attachment level at the 12 h time point, suggesting that in a closed system with these community partners it requires different conditions for growth that are provided in a flowing saliva environment. Collectively, the results from the Peg and flowcell model systems show readily attained mutualistic relationships between a known periodontopathogen, A. actinomycetemcomitans, and two other oral species and identify a potential mechanism that the pathogen uses to gain advantage in the host oral environment.
DISCUSSION

We investigated multi-species 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 multi-species communities into developing dental plaque.

The buccal (cheek side) and lingual (tongue side) of enamel surfaces are constantly bathed in saliva, suggesting that those surfaces are subject 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 plaque obtained from buccal and interproximal surfaces contain different proportions of the same species as well as different species (32).

To explore building multi-species 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 in at least 2-fold greater concentrations in disease sites as compared with other sites sampled (25).
Veillonella spp. were present in disease sites but found in higher proportions in healthy sites (25). Of 1065 isolates taken at random from subgingival plaque of localized aggressive periodontitis subjects, *F. nucleatum*, *Veillonella* spp., and *A. actinomycetemcomitans* constituted 7, 4, and 1 percent, respectively (24). Thus, these species are commonly found together. To study the interactions among species, we chose two dramatically different biofilm model systems that could mimic a freely flowing saliva and a static saliva situation in the oral cavity. The saliva-conditioned glass surface of a flowcell 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 pairwise coaggregates or as three-species coaggregates. Biofilm growth in both model systems was monitored quantitatively.

In flowcells, an open system with fresh saliva continually passing across the biofilm, mutualism occurred and was evident with all possible species pairs. Instead of each species showing a decrease in biovolume as observed in mono-species biofilms, when inoculated pairwise, species biovolumes more than doubled. The pair that appeared best matched is the *Veillonella* sp. PK1910-*A. actinomycetemcomitans* JP2 pair. Veillonella biovolume in this pair was higher than its biovolume when inoculated singly; aggregatibacter biovolume was 10-fold higher than its biovolume in mono-species biofilms. Also, in flowcells, fusobacteria flourished as shown by a more than 10-fold increase in biovolume in triple-species inoculated biofilms at 18 h compared to the biovolume at 18 h when inoculated singly. *F. nucleatum* may be acting as a coaggregation bridge between *Veillonella* sp. and *A. actinomycetemcomitans* and appear as corncob morphologies (Fig. 3, bottom panels with *A. actinomycetemcomitans*; and Fig. 4, bottom panels with *F. nucleatum*).
4). In both figures, corncob arrangements composed of the slender fusobacterium surrounded by *A. actinomycetemcomitans* (Fig. 3 bottom panels) and *A. actinomycetemcomitans* and *Veillonella* sp. (Fig. 4) are evident and indicate an intimate physical cellular arrangement of these species. *F. nucleatum* ATCC 10953 produces lactic acid (15); the close physical presence to *Veillonella* sp. PK1910 in a flowing system enhances the potential for metabolic exchange, as we have reported for a streptococcus-veillonella pair in a flowing system (11). Overall, these data demonstrate metabolic cooperation among these three species that is manifested as mutualistic growth on a complex substrate, human saliva.

However, in the closed, static system, a major difference from the open, flowing system was noted: fusobacteria were retained in the biofilm, but cell numbers did not increase. *Veillonella* sp. grew well (9-fold increase) with *F. nucleatum*, and generally, veillonellae and aggregatibacteria dominated. Importantly, cell numbers of *A. actinomycetemcomitans* and *Veillonella* sp. reached the highest we have observed in the Peg system. Compared with the values at 12, 24, and 36 h in mono-species biofilms, *Veillonella* sp. PK1910 exhibited increases in cell numbers of 29-, 82-, and 11-fold, respectively, in the triple-species inoculated biofilm. Likewise, compared with cell numbers in mono-species biofilms, *A. actinomycetemcomitans* JP2 exhibited 14-, 31-, and 11-fold increases in cell numbers in triple-species inoculated biofilms for these time points. When compared with other species examined in Peg biofilms (5, 28), significantly, the cell numbers of *A. actinomycetemcomitans* were nearly 10-fold higher. The results presented here indicate that a known oral pathogen can establish itself in a
multi-species biofilm community, which has implications on the nature of potential reservoirs containing pathogens in the oral cavity.

In summary, the two model systems provide an opportunity to compare and contrast the biofilm-forming characteristics of multiple species chosen for their ability to form mutualistic multi-species communities. Some pairs (for example, *Streptococcus oralis* with *A. actinomycetemcomitans*) and triple species (for example, *Porphyromonas gingivalis* with *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, such as when choosing *F. nucleatum*, a proposed coaggregation-bridge organism, for its ability to integrate with initial colonizing species, *S. oralis* and *Actinomyces oris*, that do grow as single species, then our focus changes (28). The flowcell model has the advantages that the spatiotemporal changes in multi-species community structure can be viewed by confocal microscopy, and that the salivary flow constantly refreshes the nutrient and washes out inhibitory molecules but also, perhaps, important signaling molecules. We have shown that a streptococcal-veillonellae pair in the flowcell must be in juxtaposition for a streptococcal GFP-amylase reporter gene to report (11), indicating the importance of cell-to-cell distance for inter-species communication. The flowcell model has received a lot of attention, but confocal microscopy is limited by concurrent use of four or possibly five species-specific fluorescently labeled primary antibodies for studies of multi-species community structure, and it uses a lot of saliva. For these reasons, we have explored the static Peg model system. This model has major advantages in that it is amenable to high throughput screening, it uses only a few milliliters of saliva per multi-species 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 multi-species investigation limits this model. Fortunately, use of this model for oral bacterial multi-species biofilms is beginning (5, 28). One promising research arena compatible with the static model is the role of signaling molecules produced by the multi-species community and accumulated in the microtiter well. It is possible that the delayed growth of \textit{A. actinomycetemcomitans} seen only after 36 h (Fig. 6A; Fig. 6B, right panel) and the continuous growth over the 36 h (Fig. 6B, middle panel; Fig. 6C, middle panel) is a result of gene expression regulated by environmentally accumulating signals. The static Peg model is conducive to studies of inter-species interactions mimicking environmentally relevant multi-species communities such as the integration of pathogens into commensal communities.

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FIGURE LEGENDS

FIG. 1. Confocal micrographs of biofilms formed in mono-species inoculated flowcells after 4 h (left column) and 18 h (right column) of growth on 25% saliva. Top panels, *Veillonella* sp. PK1910; middle panels, *A. actinomycetemcomitans* JP2; and bottom panels *F. nucleatum* ATCC 10953. Colonization (4 h) and growth (18 h) of each species was monitored. Bacterial cells are stained with species-specific fluorophore-conjugated immunoglobulin G. Bar marker, 75 μm.

FIG. 2. Time-resolved change in biovolume (μm³ per field-of-view) of *Veillonella* sp. PK1910 (Va), *A. actinomycetemcomitans* JP2 (Aa), and *F. nucleatum* ATCC 10953 (Fn) following 4 h and 18 h incubation in 25% saliva-fed flowcells. For clarity and to correspond with the text, the figure is divided into three sections, top, middle, and bottom, divided by thick black horizontal lines. The top section (A) contains biovolume values for mono-species inoculated flowcells (Fig. 1); the middle section (B) contains biovolume values for the three pairs of dual-species inoculated flowcells (Fig. 3); and the bottom section (C) contains biovolume values for the triple-species inoculated flowcell (Fig. 4). An asterisk indicates statistically significant growth.

FIG. 3. Representative confocal micrographs of 4 h (left column) and 18 h (right column) biofilms showing mutualistic growth in dual-species inoculated flowcells. Top panels, *Veillonella* sp. PK1910 + *F. nucleatum* ATCC 10953; middle panels, *Veillonella* sp. PK1910 + *A. actinomycetemcomitans* JP2; bottom panels, *A. actinomycetemcomitans* JP2 + *F. nucleatum* ATCC 10953. Bacterial cells are stained with species-specific
fluorophore-conjugated immunoglobulin G (blue, *Veillonella* sp. PK1910; green, *A. actinomycetemcomitans* JP2; and red, *F. nucleatum* ATCC 10953) and show cell-cell contact with each other. Bar marker, 75 µm.

FIG. 4. Representative confocal micrographs of biofilm from triple-species inoculated flowcell. Communities at 4 h and at 18 h show intimate interaction of *Veillonella* sp. PK1910 (blue) with *A. actinomycetemcomitans* JP2 (green) and *F. nucleatum* ATCC 10953 (red) to form the mutualistic multi-species communities. Bar marker, 75 µm.

FIG. 5. Phase contrast micrographs of mono-species suspensions (left panels), dual-species coaggregates (right panels), and triple-species coaggregates (bottom panel) in microtiter wells immediately before immersion of polystyrene Peg. *Veillonella* sp. PK1910 (Va), *A. actinomycetemcomitans* JP2 (Aa), *F. nucleatum* ATCC 10953 (Fn). Bar marker, 5 µm.

FIG. 6. Q-PCR quantification of mono- (A), dual- (B), and triple- (C) species inoculated biofilms grown on polystyrene Pegs submerged in 25% saliva and incubated anaerobically for 12, 24, or 36 h. (A and C); 12 h (white bars), 24 h (black bars), 36 h (gray bars). (B); *Veillonella* sp. PK1910 (Va, black), *A. actinomycetemcomitans* JP2 (Aa, gray), and *F. nucleatum* ATCC 10953 (Fn, white). Statistically significant increases in bacterial growth on the Pegs is indicated in the lower part of each panel; for example at the middle left, ‘Va 12h - 24h’ indicates significant
growth of *Veillonella* sp. PK1910 between 12 h and 24 h in dual-species inoculated Peg biofilm. As in Fig. 2, thick black horizontal lines separate the three sections.
REFERENCES


