

Fusobacterium nucleatum ATCC 10953 Requires *Actinomyces naeslundii* ATCC 43146 for Growth on Saliva in a Three-Species Community That Includes *Streptococcus oralis* 34[∇]

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Formation of dental plaque is a developmental process involving initial and late colonizing species that form polymicrobial communities. Fusobacteria are the most numerous gram-negative bacteria in dental plaque, but they become prevalent after the initial commensal colonizers, such as streptococci and actinomyces, have established communities. The unusual ability of these bacteria to coaggregate with commensals, as well as pathogenic late colonizers, has been proposed to facilitate colonization by the latter organisms. We investigated the integration of *Fusobacterium nucleatum* into multispecies communities by employing two in vitro models with saliva as the sole nutritional source. In flow cell biofilms, numbers of cells were quantified using fluorescently conjugated antibodies against each species, and static biofilms were analyzed by quantitative real-time PCR (q-PCR) using species-specific primers. Unable to grow as single-species biofilms, *F. nucleatum* grew in two-species biofilms with *Actinomyces naeslundii* but not with *Streptococcus oralis*. However, enhanced growth of fusobacteria was observed in three-species biofilms, indicating that there was multispecies cooperation. Importantly, these community dynamics yielded an 18-fold increase in the *F. nucleatum* biomass between 4 h and 18 h in the flow cell inoculated with three species. q-PCR analysis of static biofilms revealed that maximum growth of the three species occurred at 24 h to 36 h. Lower numbers of cells were observed at 48 h, suggesting that saliva could not support higher cell densities as the sole nutrient. Integration of *F. nucleatum* into multispecies commensal communities was evident from the interdigitation of fusobacteria in coaggregates with *A. naeslundii* and *S. oralis* and from the improved growth of fusobacteria, which was dependent on the presence of *A. naeslundii*.

The human mouth contains microbiologically diverse communities. While collectively humans harbor more than 700 bacterial phylotypes, each individual is estimated to have fewer than 100 such phylotypes (1), and approximately 50% of human oral bacteria have yet to be cultivated. Although biofilm communities on tooth enamel are polymicrobial (3, 20), more than 60 to 90% of the bacteria found in initial plaque on saliva-coated tooth enamel are streptococci (6, 19). Other bacterial genera that are among the initial commensal colonizers include *Actinomyces*, *Veillonella*, and *Neisseria* (6, 16, 19), and these organisms contribute to the polymicrobial nature of initial plaque.

The structure of a community is dependent upon the nature of the foundation. An integral feature of an oral bacterial biofilm foundation is the ability to coaggregate, which is defined as cell-cell recognition and binding between genetically distinct bacteria. After routine oral hygiene treatment, freshly cleaned tooth enamel is quickly coated with a salivary pellicle, which provides a set of receptor molecules recognized by primary colonizing bacteria, such as streptococci and actinomyces. Besides recognizing salivary receptors, these bacteria co-

aggregate and provide a foundation for the subsequent attachment and growth of other bacteria, such as veillonellae, that form close metabolic relationships with streptococci (12, 15). As initial colonizers develop into biofilm communities with anaerobic microenvironments, incorporation of the obligate anaerobic fusobacteria into these communities becomes possible. Fusobacteria as a group coaggregate with all other oral bacteria and have been suggested, therefore, to be a crucial link between primary colonizing species and later colonizing pathogens (13, 14). Thus, a foundation consisting of coaggregating streptococci, actinomyces, and veillonellae populates the tooth surface, and these organisms are recognized by fusobacteria, which colonize and become the dominant gram-negative bacterial species. The new foundation is a substratum containing fusobacterial surface receptors available for recognition by late colonizing pathogens. Supporting the crucial link is clinical evidence that fusobacteria appear in dental plaque after commensal species and before the pathogenic “red” complex consisting of *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* (22, 23).

Coaggregation partnerships are highly specific. A significant role for coaggregation in the formation of dental plaque biofilms and particularly in accretion of secondary colonizers to the pioneer species in plaque has been proposed (14) and has been demonstrated for the development of a spatially organized community (20). However, coaggregation may also provide some metabolic advantages (e.g., cross feeding and enzyme complementation) to neighboring cells by facilitating

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physical juxtaposition of partner cells, as has been shown for glucose metabolism of coaggregates of actinomyces and streptococci (7, 8). One aim of the present study was to examine the structures of two- and three-species communities composed of *Actinomyces naeslundii*, *Streptococcus oralis*, and *Fusobacterium nucleatum* in model biofilm systems. The first two species are initial colonizers and are considered commensals, whereas fusobacteria are secondary colonizers and are postulated to be a coaggregation bridge between initial and late colonizers (14). Our second aim was to investigate the integration and growth of fusobacteria in polymicrobial communities.

A variety of experimental methods have been developed to study the formation of biofilms. Model systems often rely on the flow of nutrients over a surface on which bacteria are able to attach and grow. In the present study we used two distinct *in vitro* models, a saliva-fed flow cell and a polystyrene peg immersed in static saliva. Biofilm communities form naturally and are undisturbed (3, 20, 21). The spatial organization of a multispecies community resulting from colonization and growth is preserved and can be examined noninvasively by confocal laser scanning microscopy (CLSM). In the static system, the amount of each species in multispecies biofilms formed on polystyrene pegs can be measured by real-time quantitative PCR (q-PCR). We show here with both models that fusobacteria are unable to grow as single species, but they integrate into commensal streptococcus-actinomyces communities and grow. Integration and growth are required for fusobacteria to become crucial links between commensal communities and later colonizing pathogenic communities. In the three-species community studied here, *A. naeslundii* is required for *F. nucleatum* to integrate and grow.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. oralis* 34 and *A. naeslundii* ATCC 43146 were routinely cultured in Todd-Hewitt broth (Difco Laboratories, Detroit, MI) or on Todd-Hewitt agar. *F. nucleatum* ATCC 10953 was grown in brain heart infusion (Difco) broth 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.

Coaggregation assay. Coaggregation reactions were investigated by combining *F. nucleatum*, *S. oralis*, and *A. naeslundii* pairwise and by mixing all three species in saliva. Equal volumes (0.1 ml) of microbial suspensions (about 1×10^9 cells/ml) were combined in glass tubes (12 by 75 mm) and mixed for 10 s with a vortex mixer. After mixing, the suspensions were scored immediately for coaggregation. The scoring system of Cisar et al. (4) was used to evaluate the degree of coaggregation in the suspensions by viewing the tubes with the naked eye. The scores ranged from 0 to +4, as follows: 0, no change in turbidity and no evidence of coaggregates in the mixed suspensions; +1, turbid supernatant with finely dispersed coaggregates which did not precipitate immediately; +2, definite coaggregates easily seen, but the suspension remained turbid and there was no immediate settling of coaggregates; +3, slightly turbid supernatant and formation of large precipitating coaggregates; and +4, clear supernatant and large coaggregates that precipitated immediately. The coaggregating partners *S. oralis* 34, *A. naeslundii* ATCC 43146, and *F. nucleatum* ATCC 10953 were used as inocula for formation of multispecies biofilms.

Saliva preparation. Saliva from at least six 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 (5, 21). Briefly, the supernatant was diluted with distilled water to obtain 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 having a volume of 240 μ l. A glass coverslip, which served as the attachment substratum for the growing biofilm, was secured to each of the reusable flow cells with a silicone adhesive. The flow cells were cleaned overnight with 0.1 M HCl and rinsed with 5 ml of distilled water. To disinfect the flow cells, 70% ethanol was injected into the flow cells and the flow cells were incubated 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. Flow cells were inoculated with one, two, or three species. Two-species and three-species inoculants were first coaggregated (by mixing 0.1-ml portions of appropriate combinations of *S. oralis* 34, *A. naeslundii* ATCC 43146, and *F. nucleatum* ATCC 10953), which was followed by incubation of the flow cell in the anaerobic chamber to provide an environment favorable for *F. nucleatum*, a strict anaerobe. Sterile 25% saliva was supplied at a flow rate of 0.2 ml/min as the sole source of nutrients.

Biofilm staining. Cells were stained with BacLight Live/Dead (Invitrogen, Carlsbad, CA) when strains were grown as monocultures. When multiple species were inoculated, organisms were visualized by using primary immunofluorescence with Alexa Fluor 633 (Invitrogen)-, Alexa Fluor 488 (Invitrogen)-, and Alexa Fluor 546 (Invitrogen)-conjugated immunoglobulin G of a polyclonal antiserum to *S. oralis*, *A. naeslundii*, and *F. nucleatum*, respectively. The immunofluorescence analysis was performed by injecting the antibody (5 μ g/ml in phosphate-buffered saline) into the appropriate flow cell track and incubating the preparation for 20 min. A final wash with 1% bovine serum albumin in phosphate-buffered saline (filter sterilized with a 0.22- μ m-pore-size SFCA low-protein-binding filter [Nalge Nunc International]) preceded CLSM. No cross-reactivity of staining by antibodies with nonhomologous strains was observed.

Image and statistical analysis. A TCS-SP2 confocal microscope (Leica, Exton, PA) with a 40 \times , 1.25 NA oil immersion lens was used to record confocal image stacks in five random locations near the center of each flow cell, 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 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'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 (one, two, or three species) in the flow cell studies and if the means were statistically different between the 12-h, 24-h, 36-h, and 48-h time points for each species for each condition (one, two, or three species) 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).

Biofilm growth conditions on polystyrene pegs. Static biofilms were grown in 25% human saliva on polystyrene pegs (Nunc-Immuno TSP; Nunc catalog no. 445497) (2, 17) mounted in U96 MicroWell plates (Nunc catalog no. 163320) (3, 17). Microtiter plate wells were filled with 200 μ l of 25% human saliva, and the pegs were then inserted for 30 min at room temperature to obtain a conditioning film. Overnight cultures (~20 μ l) of *S. oralis*, *A. naeslundii*, and *F. nucleatum* were added to the 200 μ l of saliva in the wells to obtain an optical density at 600 nm of 0.1, which was equivalent to about 1×10^7 to 3×10^7 cells of each species in the microtiter wells; the plates were placed in a humidity chamber and incubated anaerobically at 37°C for 48 h. After 12-h intervals, the pegs were transferred to fresh, prerduced saliva in microtiter plate wells.

DNA extraction and quantification. DNA was extracted from biofilms by using a modified alkaline lysis protocol (10). Biofilm-covered pegs were immersed in 40 μ l of sterile ultrapure water plus 160 μ l of 0.05 M sodium hydroxide and incubated at 60°C for 45 to 60 min, after which 18.4 μ l of 1 M Tris-HCl (pH 7.0) was added to neutralize the pH. The resulting extract was used as the template DNA for the q-PCR analyses (3, 10). Bacterial genomic DNA for construction of standard curves was extracted from overnight cultures of *S. oralis*, *A. naeslundii*, and *F. nucleatum* with a DNA extraction kit (Qiagen, Valencia, CA) used according to the manufacturer's instructions. Genomic DNA was stored at -20°C.

Quantification of species in static biofilms by real-time q-PCR. Species-specific primers were designed with AlleleID6 (PREMIER Biosoft International, Palo Alto, CA); no cross-reactivity of primers with nonhomologous species was observed. The primers specific for *S. oralis* were forward primer GATACATA GCCGACTGAG and reverse primer TCCATTGCCGAAGATTCC, and the annealing temperature was 56°C. The primers specific for *A. naeslundii* were forward primer GGCTGCGATACCGTGAGG and reverse primer TCTGCGA TTACTAGCGACTCC, and the annealing temperature was 56°C. The primers

specific for *F. nucleatum* were forward primer CTTAGGAATGAGACAGA GATG and reverse primer TGATGGTAAACATACGAAAGG, and the annealing temperature was 56°C. Quantification of *S. oralis*, *A. naeslundii*, and *F. nucleatum* in the biofilms was performed by q-PCR using the SYBR green dye to detect the 16S rRNA gene amplicons. Each reaction mixture (final volume, 25 μ l) contained 3 μ l of template, 3.5 μ l of diethyl pyrocarbonate-treated ultrapure water, 12.5 μ l of Power SYBR green PCR master mixture (Applied Biosystems, Foster City, CA), and 3 μ l each of the forward and reverse primers at a concentration of 375 nM. q-PCR was performed with an MX3005P thermocycler (Stratagene, La Jolla, CA) using the thermocycle recommended for Power SYBR green PCR master mixture (95°C for 10 min, followed by 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 each of the three primer sets revealed a single sharp peak. DNA concentrations (in 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 nanograms of DNA to numbers of cells, the following weights and genome sizes were used: 2.41 fg/genome and 2.4 Mb for fusobacteria, 2.05 fg/genome and 2 Mb for streptococci, and 3.08 fg/genome and 3.0 Mb for actinomyces (<http://www.homd.org>). The data presented below are data for three independent biofilms.

RESULTS

Coaggregation of three-species community members. Pairwise coaggregation of *F. nucleatum* with *A. naeslundii* in saliva yielded a coaggregation score of +2. A weak coaggregation score, +1, was obtained for *F. nucleatum* and *S. oralis*, and examination of the coaggregates by phase-contrast microscopy revealed numerous corn-cob cellular arrangements consisting of long, slender fusobacterial cells surrounded by coccoid streptococci. In three-species interactions *F. nucleatum* participated in very strong coaggregations with a score of +4. Pairwise coaggregations of *S. oralis* and *A. naeslundii* gave a score of +3. Thus, each species is a coaggregation partner of the other two species, and the ability of the species to form multispecies biofilm communities in two model systems was investigated.

Flow cells inoculated with one species. *S. oralis*, *A. naeslundii*, and *F. nucleatum* monocultures were inoculated into flow cells, and images of the biofilms formed were obtained after 4 h and 18 h of incubation (Fig. 1). The level of cell attachment to the substratum was quantified by determining the biovolume (Fig. 2A). Each species attached to the saliva-conditioned surface within 4 h (Fig. 1, left panels). After 18 h of incubation, the number of *F. nucleatum* cells decreased, indicating that there was no growth (Fig. 1, top right panel). In most experiments in this anaerobic environment, *S. oralis* grew well (Fig. 1, middle right panel, and Fig. 2A). Occasionally, *S. oralis* grew poorly, as we observed previously for aerobic environments (21). Only *A. naeslundii* grew consistently (Fig. 1, lower right panel, and Fig. 2A). To determine whether the cells in the biofilms were viable after 4 h and 18 h, cells were stained with the BacLight Live/Dead viability stain (Invitrogen), a fluorescent marker of membrane integrity (Fig. 1). Most cells (90%) in biofilms containing *S. oralis* or *A. naeslundii* appeared to have no membrane damage (green cells) at 4 h or 18 h. Also, at 4 h *F. nucleatum* appeared to have no membrane damage, which is consistent with the presence of a proper environment for the anaerobic physiology of this organism. However, after 18 h in saliva, fewer *F. nucleatum* cells were present, and most cells exhibited membrane damage (red cells), suggesting that

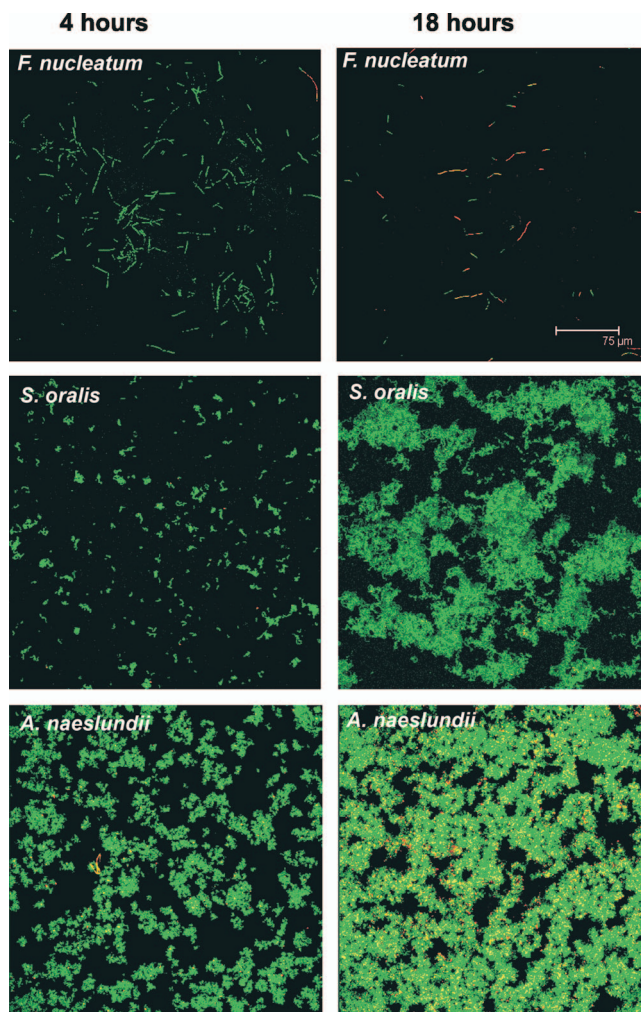


FIG. 1. Confocal micrographs of biofilms formed in flow cells inoculated with one species (*F. nucleatum*, *S. oralis*, or *A. naeslundii*) after 4 h and 18 h of growth on 25% saliva. BacLight Live/Dead stain was used to assess the vitality of cells. Red cells have impaired membrane activity, whereas green cells have fully functional membranes.

saliva did not provide nutrients for growth. Thus, while *F. nucleatum* was unable to grow by itself, the commensals streptococcus and actinomyces formed one-species biofilms.

Flow cells inoculated with two species. Saliva-conditioned flow cells were inoculated with pairwise combinations of coaggregating species, and the species were distinguished by labeling them with specific antibodies (Fig. 3). In the presence of *A. naeslundii*, *F. nucleatum* flourished (Fig. 3 and Fig. 2B). Although *F. nucleatum* attached to the substratum equally well in one-species biofilms (Fig. 1 and Fig. 2A) and in coaggregates with *A. naeslundii* (Fig. 2B and Fig. 3), its biovolume increased ninefold when it was in coaggregates with *A. naeslundii* (Fig. 2B), indicating that *F. nucleatum* required the actinomyces for cooperative growth. The *A. naeslundii* biovolume increased fivefold (Fig. 2B). In the presence of *S. oralis*, *F. nucleatum* did not grow, although the initial attachment to the substratum at 4 h was excellent (Fig. 3), whereas the *S. oralis* biovolume increased ninefold (Fig. 3 and Fig. 2B). As determined by Live/Dead staining, fusobacteria were green at 4 h with either

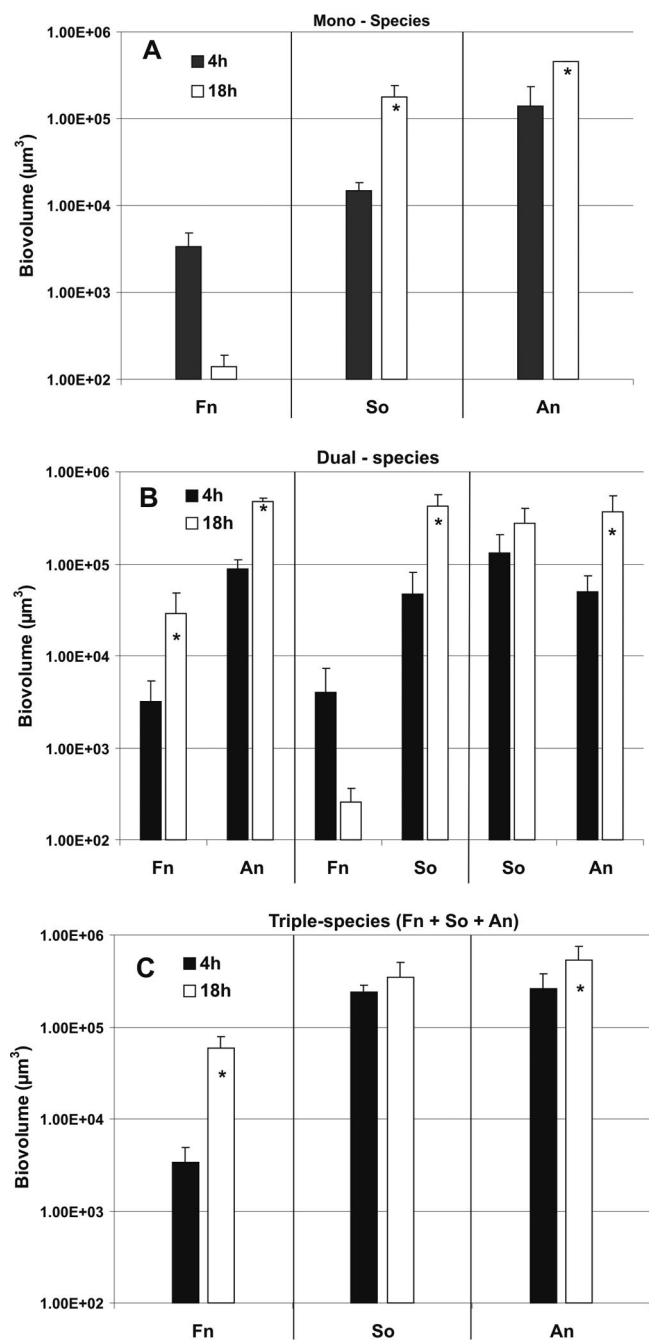


FIG. 2. Time-resolved changes in the biovolume (µm³ per field of view) of *A. naeslundii* (An), *S. oralis* (So), and *F. nucleatum* (Fn) following 4 h and 18 h of incubation in flow cells fed 25% saliva. (A) Biovolumes of single species (see Fig. 1). (B) Biovolumes of each species in two-species biofilms (see Fig. 3). (C) Biovolumes of each species in three-species biofilms (see Fig. 4). An asterisk indicates statistically significant growth.

coaggregation partner and at 18 h with *A. naeslundii*, but most of the cells were red with *S. oralis* after 18 h (data not shown), suggesting that the membrane integrity of *F. nucleatum* cells was compromised when they were paired with *S. oralis*. The biovolumes of *S. oralis* and *A. naeslundii* increased between 4 h and 18 h (Fig. 2B), and the increase in the *A. naeslundii* bio-

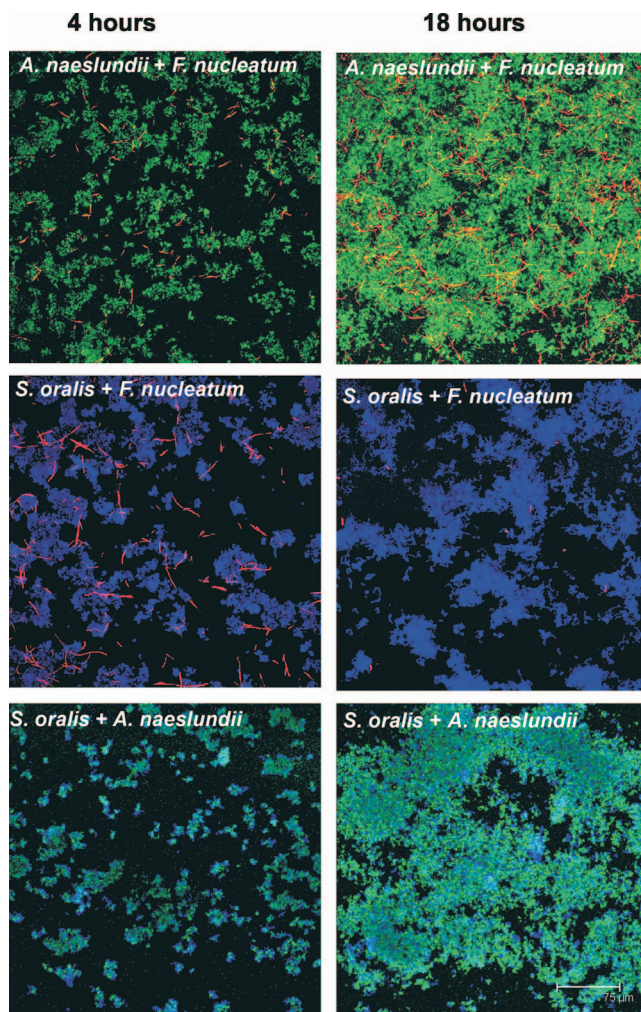


FIG. 3. Representative confocal micrographs of 4-h and 18-h two-species biofilms grown in flow cells. The primary immunofluorescence indicates *S. oralis* (blue), *A. naeslundii* (green), and *F. nucleatum* (red) and shows that there is intimate contact among species.

volume was statistically significant. Thus, the three species grew in saliva in some but not all pairwise combinations, leaving open the possibility of unchanged pairwise relationships or unanticipated cooperative growth of all members in three-species communities.

Flow cell inoculated with three species. Coaggregates of the three species were inoculated into a flow cell, and all of the species attached to the saliva-conditioned substratum and grew between 4 h and 18 h during incubation (Fig. 4A and 4B). A dramatic increase in the biovolume of *F. nucleatum* was evident (Fig. 4C and 4D). The biovolume of *F. nucleatum* increased 18-fold (Fig. 2C), indicating that the cooperation between fusobacteria and actinomyces is stronger when the three species are present than when fusobacteria and actinomyces form two-species communities in the flowing saliva model system. Further, these data suggest that the outcome of interactions among three species cannot be predicted on the basis of two-species interactions.

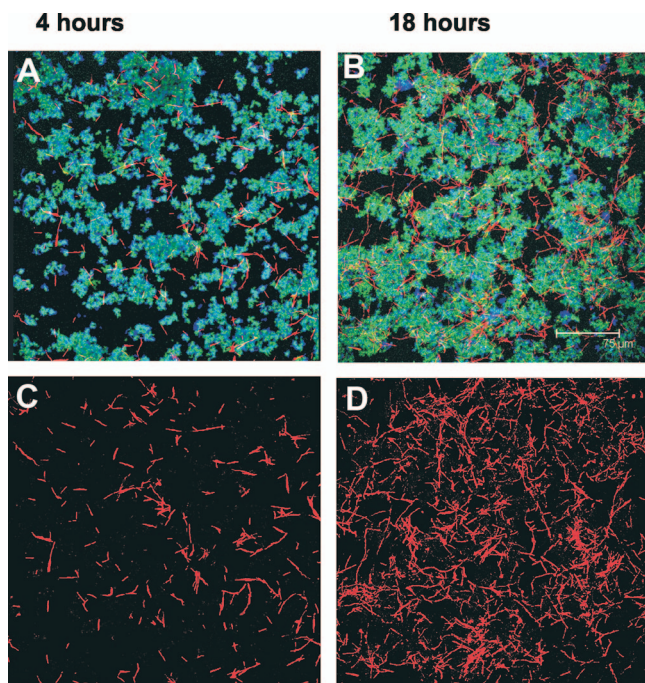


FIG. 4. Representative confocal micrographs of three-species biofilms grown in a flow cell. (A and B) Communities at 4 h and at 18 h, showing that there is an intimate interaction of *F. nucleatum* (red) with *S. oralis* (blue) and *A. naeslundii* (green) that forms the integrated multispecies communities. (C and D) Micrographs showing only the fusobacteria at 4 h and at 18 h to emphasize the dramatic increase in fusobacterial growth in the three-species communities.

Real-time q-PCR quantification of species in static biofilms on pegs immersed in saliva. The second model system, the system with saliva-fed transferable solid-phase polystyrene pegs, was used to compare static systems and flowing systems.

This system uses much less saliva and can be easily manipulated anaerobically. Coaggregates were formed easily by inoculating the species sequentially into the saliva in a well (Fig. 5). Single cells, two-species coaggregates, and large multispecies coaggregates are evident in Fig. 5. A corn-cob morphology consisting of a fusobacterial cell with several streptococci alongside it is also shown Fig. 5. Preliminary measurements of biomass accumulation in biofilms were obtained by crystal violet staining (data not shown) using a previously described method (3). An increased amount of accumulated stain on a peg can indicate an increase in biomass, which is acceptable for one-species biofilms and was very helpful as a screening tool for identifying potential target multispecies communities, but this technique cannot identify which bacteria are growing in two-species and three-species systems. To quantify each species in the multispecies biofilms, we used species-specific primers and conducted q-PCR with biofilms formed on pegs. When inoculated as single species, *F. nucleatum* did not grow, *S. oralis* grew rapidly between 12 h and 24 h, and the level of *A. naeslundii* increased gradually between 12 h and 36 h but the numbers of cells leveled off between 36 h and 48 h (Fig. 6A), suggesting that nutrient limitation had occurred. In *F. nucleatum*-*A. naeslundii* two-species biofilms, the numbers of *F. nucleatum* and *A. naeslundii* cells doubled (Fig. 6B). In contrast, in two-species biofilms with *S. oralis* no increase in the number of *F. nucleatum* cells was observed; the number of *S. oralis* cells increased sixfold (Fig. 6B). For the *S. oralis*-*A. naeslundii* two-species biofilm there was a 10-fold increase in the numbers of cells of both species (Fig. 6B).

Significantly, the number of *F. nucleatum* cells increased fivefold between 12 h and 24 h when this organism was inoculated with *S. oralis* and *A. naeslundii* (Fig. 6C). This rapid increase in the number of fusobacterial cells within 12 h was accompanied by sharp increases in the numbers of streptococcus cells (14-fold) and actinomyces cells (4-fold), suggesting

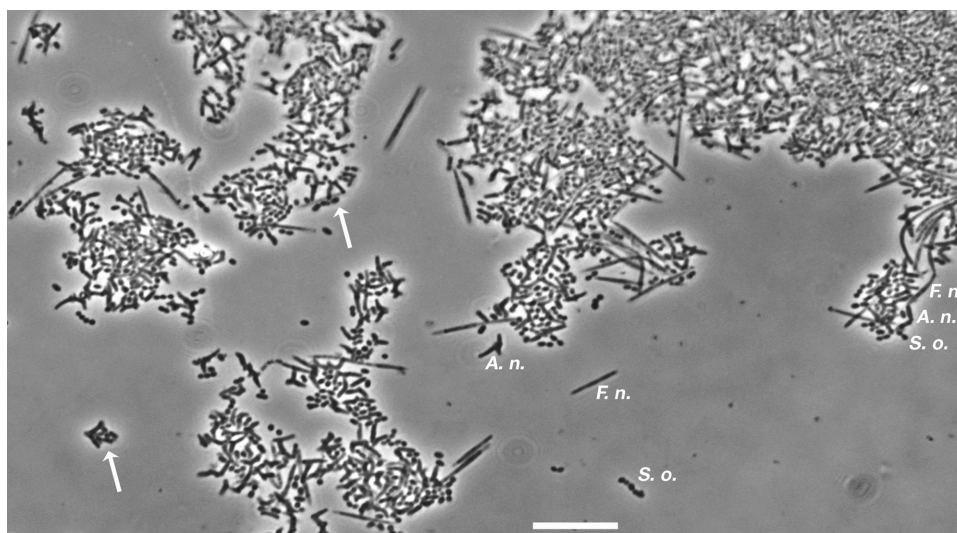


FIG. 5. Phase-contrast micrograph of a suspension of the three-species inoculum for a peg biofilm, showing mostly two-species and three-species coaggregates. The morphologies of the three species, *A. naeslundii* (A. n.), *F. nucleatum* (F. n.), and *S. oralis* (S. o.), are shown. The arrows indicate coaggregates consisting of *A. naeslundii* and *S. oralis* (left arrow) and consisting of *F. nucleatum* and *S. oralis* (right arrow). A chain consisting of *S. oralis*, *A. naeslundii*, and *F. nucleatum* attached end to end is on the right. Numerous other multispecies coaggregates are present. Bar, 10 μ m.

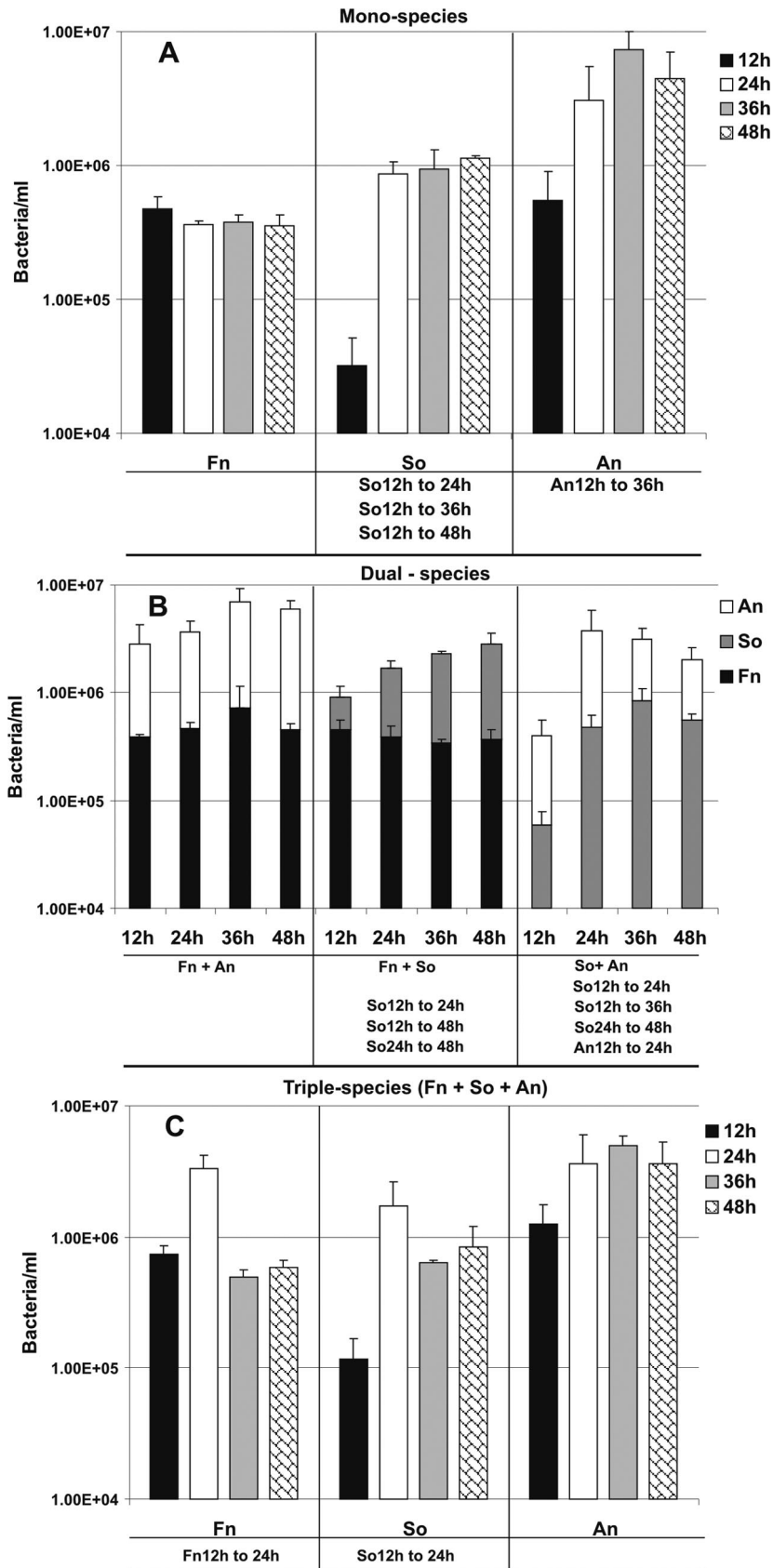


FIG. 6. q-PCR quantification for one-species (A), two-species (B), and three-species (C) biofilms grown for 12 h, 24 h, 36 h, or 48 h on polystyrene pegs submerged in 25% saliva. An, *A. naeslundii*; So, *S. oralis*; Fn, *F. nucleatum*. (A) Numbers of cells in one-species peg biofilms; (B) numbers of cells of each species in two-species peg biofilms; (C) numbers of cells of each species in three-species peg biofilms. Statistically significant increases ($P < 0.05$) in bacterial growth on the pegs are indicated at the bottom of each panel; for example, in the middle section of panel A, “So12h to 24h” indicates that there was significant growth of *S. oralis* 34 between 12 h and 24 h in a peg biofilm inoculated with one species.

that the multispecies community was particularly advantageous for all of the species. However, the rapid increases could not be sustained through 36 h and 48 h, suggesting that the maximum cell density that could be supported was approximately 1.0×10^7 cells per ml of saliva.

Collectively, the results for both model systems emphasize that it is not possible to predict multispecies community growth based solely on one-species and two-species growth patterns. Clearly, the two model systems, although distinctly different (flow versus static), yielded similar results, which may reflect a natural relationship among the three species. Of particular interest was the integration of *F. nucleatum* into the growing community; instead of no growth, the number of *F. nucleatum* cells increased 5-fold (static conditions) and 18-fold (flow cell) when *A. naeslundii* was part of the community.

DISCUSSION

We used two saliva-fed biofilm model systems and two quantitative methods to investigate multispecies community growth under an anaerobic atmosphere. In both model systems, the bacterial cells first had to attach to the saliva-conditioned substratum or they would be washed away by salivary flow (flow cell model) or when the substratum was lifted out of the saliva pool and inserted into a fresh saliva pool (transferable solid-phase polystyrene peg model). Although distinctly different in terms of design and operation, the two models yielded similar growth patterns for single species and multiple species, indicating that attachment to a substratum is a key element. Further, the data show that community composition and participant relationships can be consistent under very different environmental conditions. For example, flowing saliva can remove inhibitory as well as beneficial molecules; static incubation with saliva allows accumulation of these substances. Also, flowing saliva provides constant nourishment; static incubation with saliva provides a finite amount of nourishment. Given these significant differences, our data suggest that the three-species community possesses properties relevant to the development of dental plaque, such as integration of *F. nucleatum* into biofilms containing the initially colonizing organisms *A. naeslundii* and *S. oralis*.

In an earlier study, we failed to obtain growth of *F. nucleatum* in a multispecies flow cell community (9). In that study, flow cells were placed in an aerobic incubator, and it was assumed that the streptococci, actinomyces, and veillonellae used converted the biofilm environment to an environment favorable for anaerobiosis. Fusobacteria are known anaerobes, and it is likely that there was not a favorable anaerobic environment those flow cells. In the current study we incubated the saliva and the flow cells or the transferable pegs in an anaerobic chamber. Although *F. nucleatum* could not grow by itself on saliva as the sole source of nutrients, it grew well in two-species biofilms with *A. naeslundii* and exhibited significantly enhanced growth (compare Fig. 2B with Fig. 2C and Fig. 6B with Fig. 6C) in three-species biofilms with *A. naeslundii* and *S. oralis*. This dramatic shift in growth of fusobacteria in vitro is consistent with the secondary appearance of fusobacteria in dental plaque biofilms; first, streptococci, actinomyces, and veillonellae colonize enamel, and this is followed by a dramatic increase in the number of fusobacterial cells (18). Accordingly,

we propose that either of the models used in this study is suitable for investigating the integration of additional species, such as the late colonizing pathogens *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, into these developing communities.

It is significant that *F. nucleatum* was unable to grow on saliva without *A. naeslundii*. Curiously, the numbers of *S. oralis* cells increased when *S. oralis* was paired with *F. nucleatum* (Fig. 2B and 6B), although *F. nucleatum* did not appear to benefit from this relationship. Such results emphasize the need to include multiple species in investigations of oral bacterial community development. Our use of q-PCR to quantify the numbers of cells of each species was essential for clarifying which species was growing in the peg biofilms. For example, only *S. oralis* grew after two-species inoculation with *F. nucleatum*, whereas both *F. nucleatum* and *A. naeslundii* grew after inoculation of these two species and all three species grew when the three species were inoculated. The transferable peg system and q-PCR are not limited in terms of the number of species, and therefore additional species, such as in vivo late colonizing pathogens, can be included.

In this study, we established that there was fusobacterial interdigitation with initial colonizing commensal species by performing CLSM with undisturbed flow cell biofilm communities. We showed that fusobacteria required *A. naeslundii* for biofilm growth and improved the initial colonization of streptococci in two-species biofilms. Initial colonizing streptococci produce hydrogen peroxide, which inhibits the growth of anaerobes. *A. naeslundii* produces catalase, which inactivates the hydrogen peroxide and could promote the growth of anaerobic fusobacteria. We reported recently that *A. naeslundii* protected *Streptococcus gordonii* from self-produced hydrogen peroxide (11); a similar protective effect of actinomyces on fusobacteria could have contributed to the enhanced growth of fusobacteria in the presence of streptococci in three-species biofilms in the current study. We can now extend these observations and design multispecies communities that mimic developing dental plaque at initial stages, secondary stages, and late stages of colonization and quantify each species in the communities.

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ADDENDUM IN PROOF

After this paper was in press, we became aware of the name change for *Actinomyces naeslundii* genospecies 2, which is now called *Actinomyces oris* (U. Henssge et al., Int. J. Syst. Evol. Microbiol. **59**:509–516, 2009). *A. naeslundii* ATCC 43146 is a genospecies 2 strain and is thus *A. oris* ATCC 43146.

REFERENCES

- Aas, J. A., B. J. Paster, L. N. Stokes, I. Olsen, and F. E. Dewhirst. 2005. Defining the normal bacterial flora of the oral cavity. *J. Clin. Microbiol.* **43**:5721–5732.
- Ceri, H., M. E. Olson, C. Stremick, R. R. Read, D. Morck, and A. Buret. 1999. The Calgary biofilm device: new technology for rapid determination of

- antibiotic susceptibilities of bacterial biofilms. *J. Clin. Microbiol.* **37**:1771–1776.
3. Chalmers, N. I., R. J. Palmer, Jr., J. O. Cisar, and P. E. Kolenbrander. 2008. Characterization of a *Streptococcus* sp.-*Veillonella* sp. community micromanipulated from dental plaque. *J. Bacteriol.* **190**:8145–8154.
 4. Cisar, J. O., P. E. Kolenbrander, and F. C. McIntire. 1979. Specificity of coaggregation reactions between human oral streptococci and strains of *Actinomyces viscosus* or *Actinomyces naeslundii*. *Infect. Immun.* **24**:742–752.
 5. De Jong, M. H., and J. S. Van der Hoeven. 1987. The growth of oral bacteria on saliva. *J. Dent. Res.* **66**:498–505.
 6. Diaz, P. I., N. I. Chalmers, A. H. Rickard, C. Kong, C. L. Milburn, R. J. Palmer, Jr., and P. E. Kolenbrander. 2006. Molecular characterization of subject-specific oral microflora during initial colonization of enamel. *Appl. Environ. Microbiol.* **72**:2837–2848.
 7. Distler, W., A. Kagermeier-Callaway, and A. Petschelt. 1992. Prevention of coaggregation by a dialysis membrane—influences on the glucose metabolism of mixtures of *Actinomyces* and streptococci. *J. Dent. Res.* **71**:735.
 8. Distler, W., A. Kagermeier, and A. Kroencke. 1991. Influence of coaggregation on the glucose metabolism of mixtures of *Actinomyces* and streptococci. *J. Dent. Res.* **70**:764.
 9. Foster, J. S., and P. E. Kolenbrander. 2004. Development of a multispecies oral bacterial community in a saliva-conditioned flow cell. *Appl. Environ. Microbiol.* **70**:4340–4348.
 10. Hoshino, T., T. Fujiwara, and M. Kilian. 2005. Use of phylogenetic and phenotypic analyses to identify nonhemolytic streptococci isolated from bacteremic patients. *J. Clin. Microbiol.* **43**:6073–6085.
 11. Jakobovics, N. S., S. R. Gill, M. M. Vickerman, and P. E. Kolenbrander. 2008. Role of hydrogen peroxide in competition and cooperation between *Streptococcus gordonii* and *Actinomyces naeslundii*. *FEMS Microbiol. Ecol.* **66**:637–644.
 12. Jenkinson, H. F., and R. J. Lamont. 2005. Oral microbial communities in sickness and in health. *Trends Microbiol.* **13**:589–595.
 13. Kolenbrander, P. E., R. N. Andersen, D. S. Blehert, P. G. Eglund, J. S. Foster, and R. J. Palmer, Jr. 2002. Communication among oral bacteria. *Microbiol. Mol. Biol. Rev.* **66**:486–505.
 14. Kolenbrander, P. E., and J. London. 1993. Adhere today, here tomorrow: oral bacterial adherence. *J. Bacteriol.* **175**:3247–3252.
 15. Kolenbrander, P. E., R. J. Palmer, Jr., A. H. Rickard, N. S. Jakobovics, N. I. Chalmers, and P. I. Diaz. 2006. Bacterial interactions and successions during plaque development. *Periodontol.* **2000** **42**:47–79.
 16. Li, J., E. J. Helmerhorst, C. W. Leone, R. F. Troxler, T. Yaskell, A. D. Haffajee, S. S. Socransky, and F. G. Oppenheim. 2004. Identification of early microbial colonizers in human dental biofilm. *J. Appl. Microbiol.* **97**:1311–1318.
 17. Mampel, J., T. Spirig, S. S. Weber, J. A. Haagensen, S. Molin, and H. Hilbi. 2006. Planktonic replication is essential for biofilm formation by *Legionella pneumophila* in a complex medium under static and dynamic flow conditions. *Appl. Environ. Microbiol.* **72**:2885–2895.
 18. Moore, W. E., and L. V. Moore. 1994. The bacteria of periodontal diseases. *Periodontol.* **2000** **5**:66–77.
 19. Nyvad, B., and M. Kilian. 1987. Microbiology of the early colonization of human enamel and root surfaces in vivo. *Scand. J. Dent. Res.* **95**:369–380.
 20. Palmer, R. J., Jr., S. M. Gordon, J. O. Cisar, and P. E. Kolenbrander. 2003. Coaggregation-mediated interactions of streptococci and actinomyces detected in initial human dental plaque. *J. Bacteriol.* **185**:3400–3409.
 21. Palmer, R. J., Jr., K. Kazmerzak, M. C. Hansen, and P. E. Kolenbrander. 2001. Mutualism versus independence: strategies of mixed-species oral biofilms in vitro using saliva as the sole nutrient source. *Infect. Immun.* **69**:5794–5804.
 22. Socransky, S. S., and A. D. Haffajee. 2005. Periodontal microbial ecology. *Periodontol.* **2000** **38**:135–187.
 23. Socransky, S. S., A. D. Haffajee, M. A. Cugini, C. Smith, and R. L. Kent, Jr. 1998. Microbial complexes in subgingival plaque. *J. Clin. Periodontol.* **25**:134–144.