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Short communication

Synergy between *Tannerella forsythia* and *Fusobacterium nucleatum* in biofilm formation

A. Sharma[1], S. Inagaki[1], W. Sigurdson[2], H. K. Kuramitsu[1]
[1]Department of Oral Biology, School of Dental Medicine and [2]Confocal Faculty, State University of New York, Buffalo, New York, USA

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During dental plaque formation, the interaction of different organisms is important in the development of complex communities. *Fusobacterium nucleatum* is considered a 'bridge-organism' that facilitates colonization of other bacteria by coaggregation-mediated mechanisms and possibly by making the environment conducive for oxygen intolerant anaerobes. These studies were carried out to determine whether coaggregation between *F. nucleatum* and *Tannerella forsythia* is important in the formation of mixed species biofilms. Further, the role of BspA protein, a surface adhesin of *T. forsythia,* in coaggregation and biofilm formation was investigated. The results showed the development of synergistic mixed biofilms of *F. nucleatum* and *T. forsythia* when these bacteria were cocultured. The BspA protein was not involved in biofilm formation. Though BspA plays a role in coaggregation with *F. nucleatum*, presumably other adhesins are also involved. The synergistic biofilm formation between the two species was dependent on cell–cell contact and soluble components of the bacteria were not required. This study demonstrates that there is a positive synergy between *F. nucleatum* and *T. forsythia* in the development of mixed biofilms and that the cell–cell interaction is essential for this phenomenon.

Key words: *Tannerella forsythia*; *Fusobacterium nucleatum*; biofilm synergy

Ashu Sharma, Department of Oral Biology, 211 Foster Hall, State University of New York, Buffalo, NY 14214, USA
Tel. +1 716 829 2759;
Fax: +1 716 829 3942;
e-mail: sharmaa@buffalo.edu
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Dental plaque comprises biofilm communities of mixed species of oral bacteria. More than 300 bacterial species have been assumed to reside in the subgingival periodontal pocket of humans (10, 12). In the development of dental plaque, coaggregation processes are believed to be an important first step in the colonization of the secondary colonizers (15). *Fusobacterium nucleatum* is considered an important bacterium in the development of complex dental plaque biofilms; a 'bridge bacterium', it mediates multiple coaggregation interactions with other oral bacteria (1). In the complex ecology of dental plaque, *F. nucleatum* is also believed to support the growth of other anaerobes of the dental plaque (2).

Tannerella forsythia is one of the periodontal pathogens recently implicated in the development of periodontal diseases (5, 6, 17, 18). The mechanism by which *T. forsythia* colonizes the oral cavity and becomes part of the dental plaque is poorly understood. This is partly due to the lack of information on the putative adhesins of *T. forsythia* that may be involved in the colonization process. Recently, we identified a cell surface-associated putative adhesin of *T. forsythia*, the BspA protein (16). BspA belongs to the leucine-rich-repeat (LRR) protein family characterized by the presence of leucine-rich-repeat motifs. Most proteins belonging to the LRR family are involved in receptor–ligand recognition via protein–protein interactions (8).

Bacterial interactions between *F. nucleatum* and bacteria of the oral cavity have been demonstrated in a number of studies using different model systems. The bacterium is considered important in the colonization of late colonizers in dental plaque formation (1, 9, 15, 19). We are specifically interested in studying bacterial interactions between *F. nucleatum* and *T. forsythia* and the benefits they derive in terms of dental plaque development and/or acquiring novel virulence properties. Since coaggregation is one of the mechanisms by which bacterial interactions and cell–cell communications are facilitated, we tested whether *F. nucleatum* and *T. forsythia* coaggregate. The bacteria used in the present study were *F. nucleatum* ATCC 10953. *T. forsythia* ATCC 43037 and a mutant strain BFM-571 defective in the expression of the BspA protein (7). Bacteria were grown anaerobically in Brain Heart Infusion

(Difco Laboratories, Detroit, MI) broth containing 5 μg/ml hemin, 0.5 μg/ml menadione, 0.001% N-acetyl muramic acid and 5% fetal bovine serum (Life Technologies, Grand Island, NY). Coaggregation between *F. nucleatum* and *T. forsythia* was evaluated by the turbidimetric method as described previously (13). The coaggregation was monitored at 37°C by measuring the decrease in absorbance at 600 nm in 10 mM phosphate-buffered saline containing 0.15 M NaCl (pH 7.0). The results of coaggregation assays (Fig. 1) demonstrated coaggregation between *F. nucleatum* and *T. forsythia* wild-type strains. In contrast, a reduced coaggregation activity was observed between *F. nucleatum* and the BspA-defective mutant BFM571 of *T. forsythia*. We also studied the biofilm formation by each of these bacterial species and investigated whether coaggregation between *F. nucleatum* and *T. forsythia* would be beneficial in biofilm formation. Biofilms were studied under static culture conditions on polystyrene surfaces. Briefly, bacterial cultures of *T. forsythia* and *F. nucleatum* were grown in half-strength growth medium to an absorbance of 0.05 at 600 nm. Cells were then dispensed (0.5 ml per well) in triplicate wells of 24-well culture plates and incubated anaerobically. For mixed biofilms, each bacterial culture was adjusted to an absorbance of 0.05, dispensed into the wells and incubated as above. After incubation for 3 days, planktonic cells were aspirated and the wells were washed three times with phosphate-buffered saline (PBS) followed by staining of biofilms on plastic surfaces with crystal violet (0.1% crystal violet for 15 min). Bound dye was solubilized in an acetone : alcohol (1 : 1) mixture and absorbance read at 595 nm. Total biofilm was calculated by normalizing dye binding (absorbance at 595 nm) to total bacterial growth (biofilm cells + planktonic cells) determined from parallel identical wells by measuring the absorbance at 600 nm. The following methods were used to calculate specific *T. forsythia* or *F. nucleatum* biofilm bacteria in mixed biofilms. *T. forsythia* was estimated by its sialidase activity using 2′-(4-methylumbelliferyl)-alpha-D-N-acetylneuraminic acid as a fluorogenic enzyme substrate as per the manufacturer's recommendations (Sigma Chemical Co., St. Louis, MO). *T. forsythia* cells were lysed in 1% Triton X-100 prior to enzyme assay. *T. forsythia* biofilms were expressed as sialidase units associated with the biofilm divided by sialidase activity associated with total bacteria (planktonic + biofilm). Since the sialidase activity per cell did not differ significantly in the planktonic and biofilm cells (data not shown), sialidase assays were utilized as a measure of *T. forsythia* in mixed biofilm and planktonic cultures or in cultures with *T. forsythia* alone. The sialidase activity was not detected in *F. nucleatum* (data not shown). For enumerating *F. nucleatum*, a flow cytometric-based Bacteria Counting kit was used according to the manufacturer's recommendations (Molecular Probes, Eugene, OR). *F. nucleatum* biofilms were expressed as the number of bacteria in the biofilm divided by the number of total bacteria (planktonic + biofilm).

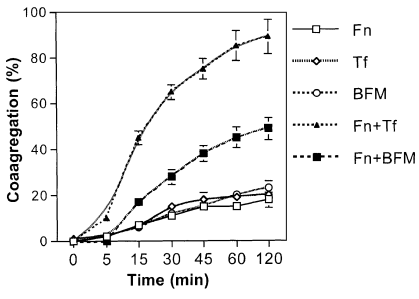

Fig. 1. Coaggregation of *F. nucleatum* with *T. forsythia* or BspA-mutant BFM571. *F. nucleatum* and *T. forsythia* (Fn + Tf) showed increased coaggregation activity as compared to *F. nucleatum* and the BspA-mutant BFM571 (Fn + BFM571). Autoaggregation by *F. nucleatum* and *T. forsythia* was minimal. Data points represent mean±standard error of triplicate samples. Data are representative of three independent experiments.

The results showed that although both *F. nucleatum* and *T. forsythia* formed a weak biofilm alone, a robust biofilm was formed when the two species were present as mixed cultures (Fig. 2). Comparable levels of synergistic biofilms were observed when *F. nucleatum* was cocultured with either the wild-type or the BspA-mutant of *T. forsythia*. These results suggest that although coaggregation between the two species is dependent upon the BspA protein, the formation of synergistic biofilms is independent of the BspA protein. In addition, the levels of *F. nucleatum* in mixed biofilms were significantly higher than the levels in biofilms of *F. nucleatum* alone. There was at least a twofold increase in the *F. nucleatum* levels in mixed biofilms as compared to biofilms of *F. nucleatum* alone, suggesting that *T. forsythia* plays a beneficial role. In comparison with *F. nucleatum*, the effect on *T. forsythia* was much more pronounced. The levels of *T. forsythia* in biofilms were up to sixfold higher in mixed cultures as compared to biofilms of *T. forsythia* alone (biofilms units of ~3 in mixed biofilms vs. 0.5 for *T. forsythia* alone). These results therefore demonstrated a positive cooperativity between *F. nucleatum* and *T. forsythia* in relation to biofilm formation. In order to

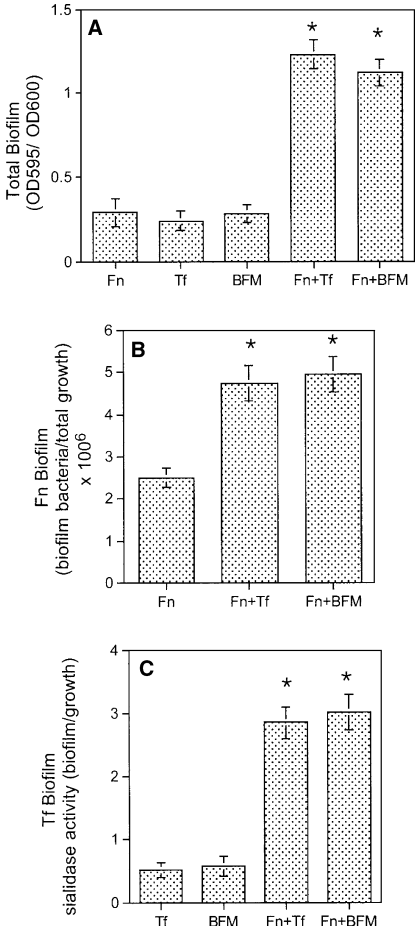

Fig. 2. In vitro biofilm assay. A) Total biofilms formed in polystyrene wells were quantified by crystal violet dye staining (OD595) normalized to total bacterial growth (OD600). Fn, *F. nucleatum* alone biofilm; Tf, *T. forsythia* alone biofilm; Fn + Tf, mixed biofilm of *T. forsythia* and *F. nucleatum*. * $P<0.01$ when compared to *F. nucleatum* or *T. forsythia* alone biofilms; Fn + BFM, mixed biofilm of *F. nucleatum* and BFM571 (BspA mutant). * $P<0.01$ when compared to biofilms of *F. nucleatum* or *T. forsythia* alone. B) *F. nucleatum* biofilm quantified as number of cells in biofilms normalized to growth (planktonic + biofilm). A twofold increase in *F. nucleatum* cells in mixed biofilms as compared to biofilms of *F. nucleatum* alone was observed. * $P<0.05$. C) *T. forsythia* in biofilms quantified based on sialidase activity showed up to a sixfold increase in mixed biofilms compared to *T. forsythia* alone. Wild-type *T. forsythia* and the BspA-mutant (BFM) formed comparable amounts of biofilms when present alone or as mixed cultures with *F. nucleatum*. Bars represent mean±standard error of triplicate samples. Data are representative of four independent experiments. * $P<0.01$ when compared to biofilms of *T. forsythia* or BFM alone. There was no significant difference between *F. nucleatum* + *T. forsythia* and *F. nucleatum* + BFM mixed biofilms.

determine whether soluble factors of one bacterial species, such as autoinducer molecules, could induce biofilm formation of the other species, experiments were carried out in Transwells (Costar Scientific, Cambridge, MA) where the two species were physically separated by a 0.4 μm filter allowing exchange only of soluble factors. Briefly, one of the members of the pair (*F. nucleatum* or *T. forsythia*) was placed in the upper compartment and the other in the lower compartment (and *vice versa*) of a Transwell (0.4 μm). Components secreted by bacterial species in the upper compartment can diffuse and influence the biofilm formation of the bacteria placed in the polystyrene well. Each bacteria was seeded in half strength broth at an OD_{600nm} of 0.05. Biofilms formed in the polystyrene wells were quantified as described above. The results showed that the levels of biofilms formed by each partner in the presence of the other in a Transwell were not significantly different from the levels formed in the absence of the partner (data not shown). Synergistic biofilms were formed only when the two species were present as mixed cultures. The results therefore ruled out a requirement for soluble components secreted from *F. nucleatum* in biofilm formation by *T. forsythia,* and *vice versa*. Experiments were also carried out to test the role of bacterial outer membrane vesicles in biofilm formation as these represent the majority of the outer membrane components in gram-negative bacteria. Vesicles possess multiple properties that include bacterial attachment and coaggregation (11). Bacterial vesicles from each organism were prepared by ultracentrifugation of the spent culture medium as previously described (4). Vesicles were washed twice with cold PBS and the concentration was estimated with the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA). The results showed that *F. nucleatum* vesicles (50 μg/ml) did not enhance biofilm formation by *T. forsythia*. Likewise, *T. forsythia* vesicles failed to enhance *F. nucleatum* biofilms (data not shown). Interestingly, *F. nucleatum* vesicles enhanced the aggregation of *T. forsythia* (data not shown). The levels of biofilms formed by each of the bacterial species (*F. nucleatum* or *T. forsythia*) were similar regardless of the presence or absence of vesicles of the other species. These results demonstrate that soluble components or bacterial vesicles are not required for synergistic biofilm formation. A physical cell–cell communication between the two species is likely responsible for the development of synergistic biofilms. The direct interactions between cell surface-associated ligand/receptor molecules on each species may result in the expression of biofilm-specific genes in each bacterium.

For analysis of the structure of the biofilms, a Bio-Rad MRC1024 confocal canning laser (Kr/Ar) microscope attached to a Nikon Diaphot microscope and an APO Plan 60 × 1.4 NA objective available in the Confocal Microscopy and 3-D Imaging Facility (School of Medicine and Biomedical Sciences, UB, Buffalo, NY) was used. For CSLM, biofilms were formed in 6-well polystyrene plates as per the protocol described above and stained with a Bacterial Live-Dead viability kit according to the manufacturer's recommendations (Molecular Probes). The staining allowed differentiation of live (green) and dead (red) bacteria and identification of *F. nucleatum* and *T. forsythia* due to their distinct bacterial morphology (*F. nucleatum* as long rods vs. small fusiform *T. forsythia* cells). A three-dimensional view of the biofilm was then digitally constructed from fluorescent optical sections with the IMAGEJ program (http://rsb.info.nih.gov/ij/). Biofilms were observed at 1-day intervals. On day 1, *F. nucleatum* cells were found attached to bottom of the plates (data not

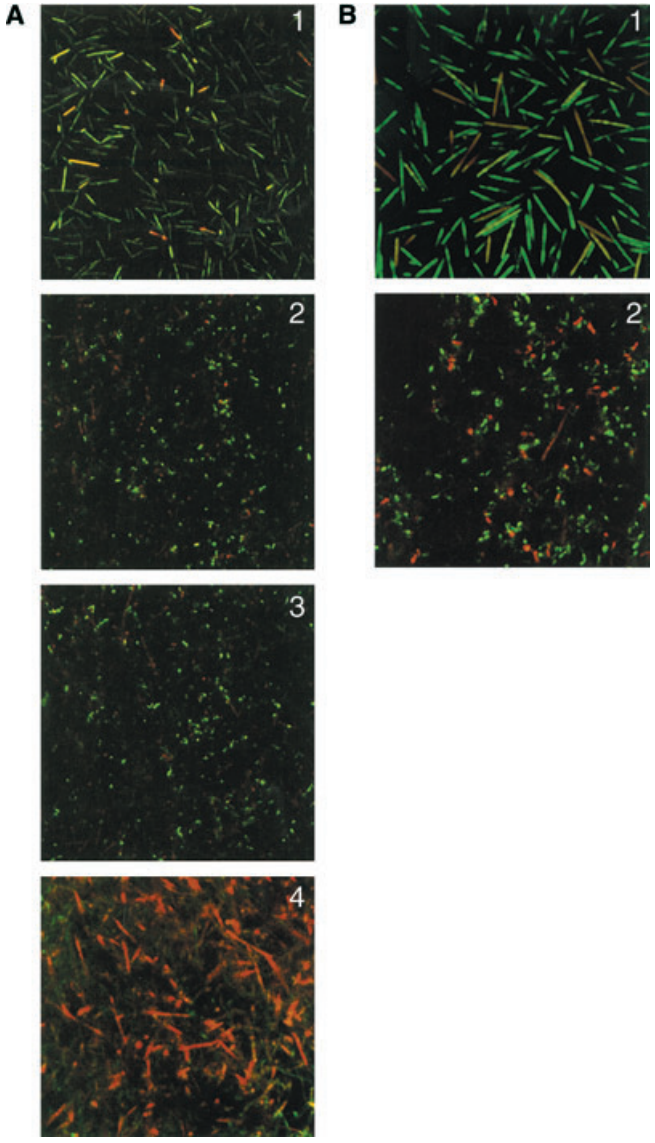

Fig. 3. Panel A: Optical slices through mixed biofilm of *F. nucleatum* and *T. forsythia*. Three-day-old mixed biofilm was stained with SYTO9 and propidium iodide dyes to differentiate live (green) and dead or damaged bacteria (red) in the biofilm. Total thickness 70 μm. Inset 1, optical slice 2.5 μm deep from top of the biofilm showing majority of live *F. nucleatum* cells. Insets 2 (17.5 μm) and 3 (35 μm) show *T. forsythia* as the major bacteria. Inset 4, shows dead/damaged *F. nucleatum* cells attached to the substratum. Panel B: Insets 1, biofilm cells of *F. nucleatum*; 2, biofilm cells of *T. forsythia*.

shown). By day 2, most of the surface-attached *F. nucleatum* were found damaged or dead. Also at day 2, *T. forsythia* cells were observed attaching to *F. nucleatum* and beginning to form more structured biofilm layers (data not shown). The mixed biofilm matured by day 3. The three-dimensional reconstruction of the confocal images of 3-day-old biofilms yielded a total thickness of approximately 70 μm with extensive labeling of biofilms with SYTO 9 (green) mostly in the upper two thirds of the biofilm. Cells in the bottom layers adjacent to the substratum had more damaged cells (red). Representative optical slices (0.5 μm thick) of the biofilm are shown in Fig. 3. Live *F. nucleatum* cells were present within the upper slices of the biofilm (5 μm from top; panel A, inset 1), but *T. forsythia* was the major species within most of the biofilm (Panel A, insets 2 & 3). Mostly dead *F. nucleatum* cells were present at the bottom of the biofilm and attached to the substratum (panel A, inset 4). The structure of mixed *F. nucleatum* and *T. forsythia* biofilms demonstrated an interesting biofilm topology in that live *F. nucleatum* were mainly localized in the upper layers of the biofilm, whereas dead *F. nucleatum* cells formed the substratum. Live and damaged or dead *F. nucleatum* cells were frequently interspersed within the biofilm layers. A general view concerning the formation of dental plaque suggests that *F. nucleatum* initially colonizes the tooth and gingival surfaces to form the substrate onto which later colonizers such as *T. forsythia* adhere via coaggregation-based mechanisms and then migrate to deeper periodontal pockets for colonization. Based on the biofilm architecture observed in this study, we propose the following model of mixed biofilm formation by *F. nucleatum* and *T. forsythia*. Since the uppermost biofilm layers which comprise immature and developing young biofilm contained mainly live *F. nucleatum* cells and *T. forsythia* was found abundantly in the deeper layers along with *F. nucleatum* cells, *F. nucleatum* present in the developing top biofilm layers likely serve as the substratum to which *T. forsythia* cells from the planktonic state adhere and initiate the development of biofilm. Once the biofilm between *T. forsythia* and *F. nucleatum* has formed, new planktonic *F. nucleatum* cells adhere to the already formed biofilm and the above process begins again. *F. nucleatum* in this process is important not only as the substratum for *T. forsythia* attachment but possibly also because it creates a favorable environment for *T. forsythia*. It has been demonstrated that *F. nucleatum* is able to generate reducing conditions which may be beneficial for strict anaerobes less tolerant of oxygen (3). To determine whether the association of *F. nucleatum* and *T. forsythia* in biofilm formation would lead to increased virulence, studies will be carried out in the mouse model of periodontal disease. Since *T. forsythia* alone is able to induce alveolar bone loss in mice following oral infection (unpublished results), it would be of interest to determine whether coinfection with *F. nucleatum* would have a synergistic effect on alveolar bone loss.

In conclusion, our studies demonstrate synergistic biofilm formation between *F. nucleatum* and *T. forsythia*. Further, we show that direct cell–cell contact mediated communication is necessary for such synergistic effects. To our knowledge, there is only one other report that describes a mutualistic relationship in coaggregated colonies of oral bacteria to form biofilms (14). In that report, *Actinomyces naeslundii* and *Streptococcus oralis* formed luxuriant biofilms when paired together in coaggregated microcolonies. It will be of interest to determine whether this property is shared by other bacteria in natural biofilms. It has recently been demonstrated that *Treponema denticola* and *Porphyromonas gingivalis* also form synergistic biofilms (Ikegami and Kuramitsu, unpublished results). Studies have been initiated in our laboratory to understand the mechanisms underlying this interesting phenomenon.

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