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Stimulation of *Fusobacterium nucleatum* biofilm formation by *Porphyromonas gingivalis*

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Saito Y, Fujii R, Nakagawa K-I, Kuramitsu HK, Okuda K, Ishihara K. Stimulation of Fusobacterium nucleatum *biofilm formation by* Porphyromonas gingivalis*.*
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Background/aims: Bacterial infection is a major cause of periapical periodontitis. Eradication of these microorganisms from apical lesions is essential to the success of endodontic treatment. The aim of this study was to clarify the molecular interaction between *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and other microorganisms associated with periapical periodontitis.
Methods: Microorganisms isolated from periapical lesions were inoculated into type-I collagen-coated polystyrene microtiter plates and maintained at 37°C under anaerobic conditions for 2 days, after which, the quantity of organized biofilm on the plates was evaluated by crystal violet staining. Growth enhancement via soluble factor was evaluated by separated coculture using a 0.4-μm membrane filter.
Results: *F. nucleatum* exhibited strong adherence to type-I collagen-coated polystyrene microplates. Biofilm formation by *F. nucleatum* was significantly enhanced by *P. gingivalis*. It was complemented by compartmentalized coculture with *P. gingivalis*. Enhancement of biofilm formation by *P. gingivalis* was only slightly reduced by inactivation of its autoinducer-2-producing gene *luxS*.
Conclusion: The results suggest that *P. gingivalis* enhances biofilm formation by *F. nucleatum* by releasing diffusible signaling molecules other than autoinducer-2.

Key words: biofilm; *Fusobacterium nucleatum*; periapical periodontitis; *Porphyromonas gingivalis*; synergistic effect

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Microbial biofilms are the etiological agents of persistent infectious disease. The microorganisms in biofilms possess a different phenotype to planktonic cells. Enhancement and inhibition have both been observed among bacterial species during the process of biofilm formation (7, 12). After biofilm formation, microorganisms are resistant not only to host defense mechanisms, such as phagocytosis, but also to antimicrobial agents. Quorum sensing plays a role in phenotypic changes in certain bacteria through its control of bacterial gene expression via autoinducers (AI) in biofilms (30).

Periapical periodontitis occurs as a sequel to pulpal infection or infection of periapical tissue arising from marginal periodontitis (17). The immune response results in local inflammation and destruction of the surrounding tissue of the infected apical foramen (18). The infected apical 5 mm of a root canal is dominated by a mixed bacterial flora in which obligate anaerobes are predominant (1). Bacteria in an infected periapical region can survive by utilizing serum-like fluid transudate from periapical tissue and necrotic pulp (4) forming biofilms on the surface of the tooth in areas where periapical lesions have occurred. Biofilms have been reported to form in periapical lesions such as those on the external surface of the root apex (13, 20), and apical biofilms are clinically important in periapical periodontitis. Such microbial biofilms are inherently resistant to antimicrobial agents and are difficult to remove by mechanical means alone, resulting in persistent infection. Recently, pathogens of marginal periodontitis were isolated from necrotic pulp and apical periodontitis (22). Multiple species, including periodontopathic bacteria such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, have been detected in biofilms associated with periapical periodontitis lesions (19). Therefore, it is important to clarify the nature of interbacterial communication among these biofilm-forming bacteria.

The role of intercellular signaling molecules such as AI-2 in biofilm formation resulting in periapical periodontitis lesions remains to be clarified. In this study, we

investigated synergistic effects in the process of biofilm formation and communication among bacteria, focusing particularly on *F. nucleatum* and *P. gingivalis*.

Materials and methods

Culture conditions and bacterial strains

All procedures used in this study were approved by the Review Board of the Tokyo Dental College Ethical Committee. Written informed consent was obtained from each subject before sample collection. Twenty clinical strains isolated aseptically from apical periodontitis lesions in 23 patients were used in this study. The apices of the teeth were aseptically isolated during apicoectomy. Root apices were placed in reduced transport medium (25) containing glass beads, and microorganisms on the surface of the teeth were dispersed with a vortex mixer for 30 min. The microorganisms were then serially diluted from 10^{-1} to 10^{-5} and inoculated onto tryptic soy agar (TSB; Becton Dickinson Microbiology System, Cockeysville, MD) containing 5 μg/ml hemin, 0.5 μg/ml menadione and 10% horse blood (blood agar plate). The isolated strains were identified by 16S ribosomal DNA (rDNA) sequencing using the Full Gene 16S rDNA Bacterial Identification Kit (Applied Biosystems, Foster City, CA). These strains were maintained on blood agar plates.

Evaluation of biofilm-forming ability

Biofilm formation on the root canal apex or on periodontal tissues is affected by the ability of the bacteria to adhere to surface-located type-I collagen. The biofilm-forming activities of a total of 20 isolated strains were quantified according to the method of Takahashi et al. (26) with minor modifications. Briefly, the microorganisms were inoculated into TSB (Becton Dickinson) supplemented with 5 μg/ml hemin and 0.5 μg/ml menadione, and precultured anaerobically at 37°C for 2 days. Fifty-microliter cultures were then inoculated into collagen type-I-coated 96-well flat-bottom microplates (Asahi Techno glass, Funabashi, Japan) containing 150 μl of the same medium, and cultured anaerobically at 37°C for 2 days. The culture medium was then removed from each well and 50 μl of 0.1% (weight/volume) crystal violet solution was added. After 15 min, the wells were rinsed twice with 350 μl distilled water and air-dried. The crystal violet remaining in the biofilm was solubilized and extracted with 200 μl 99% ethanol. Biofilm mass was evaluated at an optical density of 595 nm using a microplate reader (BIO-RAD, Hercules, CA).

Evaluation of synergistic effects in multi-species biofilms

Biofilm formation by coculture of *F. nucleatum* TDC100 with partner strains was also evaluated. *P. gingivalis* ATCC33277, FDC381 and two gram-positive strains (*Streptococcus sanguinis* TDC15 and *Staphylococcus epidermidis* TDC78) were used as partner strains. To evaluate the effect of AI-2 on biofilm formation, a *P. gingivalis luxS*-deficient mutant, CW221 (32), constructed from strain *P. gingivalis* FDC381, was used. Each microorganism was inoculated into TSB and precultured overnight under anaerobic conditions. Bacterial cells from 300 μl precultured *F. nucleatum* TDC100 and 300 μl precultured partner strain were inoculated into a collagen type-I-coated 12-well flat-bottom microplate (IWAKI, Funabasi, Japan) containing 1200 μl of the same medium and cultured anaerobically at 37°C for 2 days. In the case of *F. nucleatum* alone, 600 μl precultured *F. nucleatum* was inoculated. Biofilm mass was measured as described above.

To investigate the induction of signaling between species by diffusible bacterial mediators, a two-compartment separated culture system was used according to the method of Yoshida et al. (32). Five hundred microliters TSB was placed in each well of a type-I collagen-coated polystyrene 12-well plate (IWAKI), which was then designated the lower well. Two hundred and fifty microliters of each strain, cultured overnight, was then inoculated into each lower well. An insert (Transwell, Corning, Corning, NY) was then placed in each well, and designated the upper well. Next, 500 μl TSB was placed in each upper well. Finally, 250 μl partner strain, cultured overnight, was inoculated into each upper well. The organisms were cocultured physically separated by a porous membrane (pore size, 0.4 μm; Falcon cell culture insert; BD Labware, Lincoln Park, NJ). After incubation at 37°C for 2 days, the inner-well insert was removed and the biofilm mass in the lower well was measured as described above. Biofilm formation by the species in the two-compartment system was quantified according to the following formula: (biofilm mass of the cocultured species evaluated by crystal violet)/(biofilm mass of the species alone evaluated by crystal violet).

Effects of AI-2 on biofilm formation

F. nucleatum TDC100 and *P. gingivalis* FDC381 or CW221, which lacks *luxS* (32), were cocultured, and biofilm formation was evaluated as described above. *F. nucleatum* TDC100 was inoculated into the lower compartment and *P. gingivalis* FDC381 or CW221 were inoculated into the upper compartments and incubated as described above. After incubation, the mass of the biofilm formed by *F. nucleatum* was assayed as described above.

Statistical analysis

Two-group comparisons were performed using Student's *t*-test. In comparing data from more than three groups, evaluation was carried out using an analysis of variance and the Newman–Keuls multiple-comparison test.

Results

Biofilm-forming activity assay

The biofilm-forming activities of 20 strains from among 74 isolates are shown in Table 1. Among these species, *F. nucleatum* and *Propionibacterium acnes* were frequently isolated together with other species. These microorganisms showed strong adherence to type-I collagen. *F. nucleatum* TDC100 showed the strongest adherence activity, so further investigation of the effects of other members of apical periodontal lesions on *F. nucleatum* biofilm formation was performed using mainly this strain.

Synergistic effect on biofilm formation by coculture

The effects of coculture on *F. nucleatum* biofilm formation are summarized in Fig. 1. Biofilm formation by coculture with *S. epidermidis* TDC78, and *P. gingivalis* FDC381 and ATCC33277 was 1.8, 3.1 and 2.8 times greater ($P < 0.001$), respectively, than that by *F. nucleatum* TDC100 alone. Similar enhancement was also observed in *F. nucleatum* TDC845. However, coculture with *S. sanguinis* TDC15 resulted in almost the same level of biofilm formation as that by *F. nucleatum* TDC100 alone (data not shown).

Evaluation of involvement of intercellular signaling molecules on biofilm formation

Effects of complementation by *P. gingivalis* strains on the enhancement of biofilm formation of microorganisms isolated from

Table 1. Biofilm-forming activity on type-I collagen-coated polystyrene plates of 20 strains isolated from refractory apical periodontitis lesions

Species	Biofilm formation (OD_{595})[1]
P. acnes TDC 18	0.642 ± 0.048
P. acnes TDC58	0.307 ± 0.025
P. acnes TDC95	0.567 ± 0.130
P. acnes TDC103	0.549 ± 0.059
P. acnes TDC121	0.457 ± 0.082
Pseudomonas aeruginosa TDC612	0.285 ± 0.148
P. aeruginosa TDC66	0.491 ± 0.070
P. aeruginosa TDC72	0.350 ± 0.016
F. nucleatum TDC845	0.424 ± 0.060
F. nucleatum TDC100	0.667 ± 0.083
K. pneumoniae TDC116	0.557 ± 0.163
K. pneumoniae TDC120	0.602 ± 0.156
Staphylococcus epidermidis TDC78	0.474 ± 0.045
S. epidermidis TDC86	0.551 ± 0.023
Staphylococcus hominis TDC54	0.402 ± 0.075
Staphylococcus pasteuri pasteuri TDC563	0.391 ± 0.025
C. rectus TDC67	0.364 ± 0.070
V. atypica TDC96	0.237 ± 0.067
S. sanguinis TDC15	0.357 ± 0.028
A. naeslundii genotype 2 TDC107	0.658 ± 0.171

[1]Biofilm formation was quantified according to the method of Takahashi et al. (26).

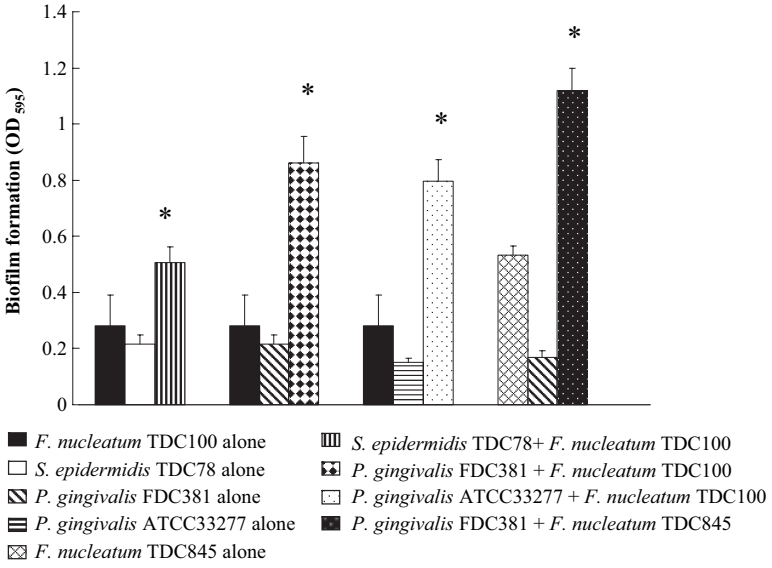

Fig. 1. Biofilm formation by coculture of *Fusobacterium nucleatum* TDC100 and TDC845 with partner strains. Pairs of microorganisms were cultured either alone or cocultured together on type-I collagen-coated microtiter plates. After 48 h of cultivation, the mass of organized biofilms was evaluated by staining with crystal violet. Error bars indicate standard deviations. Data are representative of three independent runs of each experiment ($n = 18$); *$P < 0.001$ compared with culture of each strain alone by analysis of variance and Newman–Keuls multiple-comparison test.

periapical periodontitis lesions were evaluated by a two-compartment system. *P. gingivalis* ATCC33277 enhanced biofilm formation by *Veillonella atypica* TDC96, *Campylobacter rectus* TDC67 and *F. nucleatum* TDC100 (Table 2). The enhancement was especially significantly elevated for the combination of *P. gingivalis* ATCC33277 and *F. nucleatum* TDC100. For combination of the gram-positive bacteria, only *S. epidermidis* TDC78 enhanced at 1.75 times the growth of *S. sanguinis* biofilms (data not shown, $P < 0.05$).

The effects of coculture with other species on the enhancement of biofilm formation by *F. nucleatum* TDC100 using the two-compartment system are shown in Table 3. When each strain was inoculated into the upper well and *F. nucleatum* TDC100 was inoculated into the lower well, all the strains shown in Table 3, except *S. sanguinis* TDC15, significantly enhanced the formation of *F. nucleatum* TDC100 biofilms ($P < 0.001$), and the activity of *P. gingivalis* was statistically higher than that of *S. epidermidis*. This enhancement of biofilm formation was also detected with *F. nucleatum* TDC845 (Table 3).

When *F. nucleatum* TDC100 was inoculated into the upper wells with each partner strain in the lower wells, biofilm formation by *P. gingivalis* ATCC33277 and FDC381 was 1.5 and 1.6 times higher, respectively, than that of each strain alone. On the other hand, coculture with *S. sanguinis* TDC15 yielded lower biofilm formation than that obtained with *S. sanguinis* TDC15 alone (data not shown).

Evaluation of effects of AI-2 on biofilm formation

To determine the effects of AI-2 from *P. gingivalis* on biofilm formation by *F. nucleatum* TDC100, biofilm formation of *F. nucleatum* TDC100 at 24 and 48 h was evaluated using *P. gingivalis* FDC381 or its *luxS*-deficient mutant CW221. As shown in Fig. 2, enhancement of biofilm formation by coculture of *F. nucleatum* TDC100 with *P. gingivalis* wild-type or *luxS* mutant CW221 was higher than that by *F. nucleatum* TDC100, *P. gingivalis* FDC381 or CW221 alone ($P < 0.001$). The results from the two-compartment system are shown in Fig. 3. Enhancement of biofilm formation by *F. nucleatum* TDC100 with *P. gingivalis* FDC381 was only slightly higher than that with *P. gingivalis* CW221 when the partner strains were separated by membrane filters. However, this difference was statistically significant at 24 h ($P < 0.001$).

Discussion

We have found that many bacterial species isolated from surgical materials obtained from patients with periapical periodontitis lesions form biofilms on collagen-coated polystyrene plates. Most of these strains are frequently isolated from apical periodontitis (19, 24). Among them, biofilm-forming ability is strongest in *F. nucleatum* TDC100. *F. nucleatum* is also frequently isolated from the lesions of periapical periodontitis (24, 27). In lesions of apical periodontitis, the surfaces of the dentin and periodontal tissue contain type-I collagen. The ability of *F. nucleatum* to bind to type-I collagen noted in the present study agrees

Table 2. Effects of *Porphyromonas gingivalis* inoculated into upper wells on biofilm formation by microorganisms isolated from apical periodontitis lesions

Lower compartment	Biofilm formation[1]
P. acnes TDC18	1.14 + 0.13
S. sanguinis TDC15	1.01 + 0.12
S. pasteuri TDC563	0.91 + 0.07
A. naeslundii TDC107	1.061 + 0.09
P. aeruginosa TDC 66	0.98 + 0.13
S. hominis TDC54	1.10 + 0.29
V. atypica TDC96	1.67 + 0.18*
F. nucleatum TDC100	4.19 + 0.43*
C. rectus TDC67	1.19 + 0.25**

[1]Relative quantity of biofilms formed by *Fusobacterium nucleatum* TDC100 in the two-compartment system was calculated according to the following formula: (biofilm mass of the cocultured species evaluated by crystal violet)/(biofilm mass of the species alone evaluated by crystal violet). NT, not tested.
Data are representative of three independent runs of each experiment ($n = 18$); *$P < 0.001$, **$P < 0.05$ compared with the species alone by Student's *t*-test ($P < 0.001$).

Table 3. Effects of partner strains inoculated into upper wells on biofilm formation by *Fusobacterium nucleatum* TDC100 and TDC845

Upper compartment	Biofilm formation[1] by *F. nucleatum* TDC100 (OD_{595})	Biofilm formation[1] by *F. nucleatum* TDC845 (OD_{595})
None	1.00 + 0.11 (0.116 + 0.013)	1.00 + 0.054 (0.312 + 0.017)
S. epidermidis TDC78	1.85 + 0.57*	NT
P. gingivalis FDC381	4.15 + 0.46*	2.47 + 0.06*
P. gingivalis ATCC33277	4.19 + 0.43*	2.56 + 0.12*

[1]Relative quantity of biofilms formed by *F. nucleatum* TDC100 was calculated according to the following formula: (biofilm mass of cocultured *F. nucleatum* TDC100 evaluated by crystal violet)/(biofilm mass of *F. nucleatum* TDC100 alone evaluated by crystal violet).NT, not tested.
Data are representative of three independent runs of each experiment ($n = 18$); *statistically higher than *F. nucleatum* alone by Student's *t*-test ($P < 0.001$).

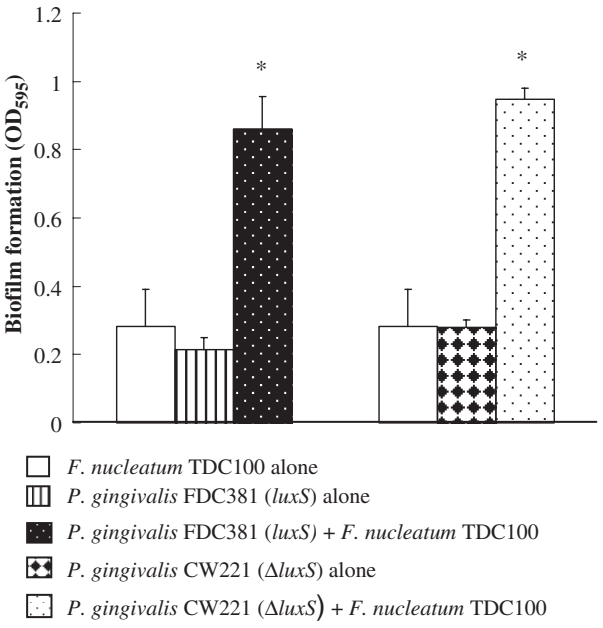

Fig. 2. Effects of *Porphyromonas gingivalis* wild-type and *luxS*-deficient mutant on biofilm formation by *Fusobacterium nucleatum* TDC100. The *P. gingivalis* strain and *F. nucleatum* were either cultured alone or were cocultured together on type-I collagen-coated microtiter plates. After a 48-h cultivation, the mass of organized biofilms was evaluated by staining with crystal violet. Error bars indicate standard deviations. Data are representative of three independent runs of each experiment ($n = 18$); *$P < 0.001$ compared with culture of each strain alone by analysis of variance and Newman–Keuls multiple-comparison test.

with the prevalence of *F. nucleatum* in periapical lesions.

In the present study, we demonstrated the enhancement of biofilm formation by *F. nucleatum*, *V. atypica* and *S. epidermidis* by *P. gingivalis,* and of *F. nucleatum* by *S. epidermidis.* These synergistic effects suggest that *P. gingivalis* enhances subsequent colonization and biofilm formation by *F. nucleatum*, *C. rectus*, *S. epidermidis* and *V. atypica.* Yamada et al. (31) reported that *P. gingivalis* strongly enhanced biofilm formation by *Treponema denticola in vitro.* These species have often been isolated from abscesses in human dento-alveolar lesions (19, 23, 29). *In vitro* studies have shown that *F. nucleatum* exhibited the ability to coaggregate with gram-positive cocci such as *S. sanguinis*, *Peptostreptococcus micros,* as well as with *P. gingivalis* (8, 10, 11). *F. nucleatum* initially adheres to early colonizers, including gram-positive cocci, and enhances the adherence of periodontopathic bacteria such as *P. gingivalis* and *T. denticola* in periodontal lesions (9). These reports suggest that *F. nucleatum* plays an important role in biofilm formation via its strong adherence activity. Noguchi et al. (19) detected *F. nucleatum* with *Tannerella forsythia* and *P. gingivalis* in extraradicular biofilms from clinical specimens. Taken together with the results of the present study, this suggests that such synergistic effects may play an important role in biofilm formation.

The synergistic effect between *P. gingivalis* and *F. nucleatum* was strongest among the species isolated from the periapical periodontitis lesions used in this study. A synergistic effect on pathogenicity between *P. gingivalis* and *F. nucleatum* was also reported using a murine model (3). However, the synergistic effect between these two microorganisms was reported only for growth support of *P. gingivalis* by *F. nucleatum* in oxygenated and carbon dioxide-depleted environments (2). The results of the present study showed that *P. gingivalis* enhanced the growth of *F. nucleatum*, providing direct evidence of periodontal bacteria exerting a synergistic effect on biofilm formation by *F. nucleatum*.

The slight reduction in biofilm formation by *F. nucleatum* TDC100 with *P. gingivalis* CW221 compared with the wild-type strain suggests that AI-2 is likely to be only marginally involved in the enhancement of biofilm formation. Autoinducers were reported to mediate changes in gene expression in microorganisms within biofilms (16). Several oral microorganisms have been reported to produce

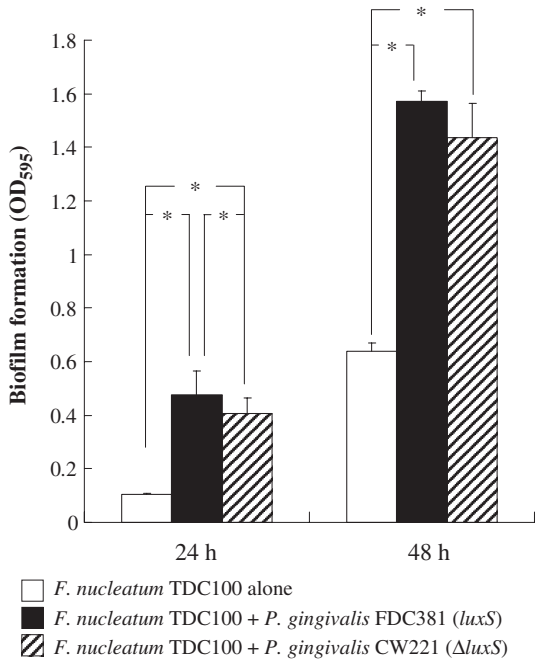

Fig. 3. Effects of *Porphyromonas gingivalis* wild-type and *luxS*-deficient mutant inoculated into upper wells on biofilm formation by compartmentalized *Fusobacterium nucleatum* TDC100. Error bars indicate standard deviations. Data are representative of three independent runs of each experiment ($n = 18$); *$P < 0.001$ compared with *F. nucleatum* TDC100 monoculture by Student's *t*-test.

AI-2 (5, 6, 32). The involvement of AI-2 in biofilm formation in oral microorganisms *in vitro* has also been reported (21, 32). Yoshida et al. (32) reported that biofilm formation by the *luxS* mutant of *Streptococcus mutans* was complemented by *Streptococcus gordonii*, *Streptococcus sobrinus* or *P. gingivalis* 381, but not by the *P. gingivalis luxS*-deficient mutant CW221. McNab et al. (15) also suggested that *S. gordonii* produced an AI-2-like signaling molecule that regulated various aspects of carbohydrate metabolism in some microorganisms. Furthermore, LuxS-dependent intercellular communication is essential for biofilm formation between *P. gingivalis* and *S. gordonii*. In the present study, enhanced biofilm formation was observed with both *P. gingivalis* FDC381 and its *luxS* mutant CW221, with little difference between the two. This suggests that molecules other than AI-2 are involved in the enhancement of biofilm formation by *F. nucleatum.* Loo et al. (14) showed that several genes of *S. gordonii*, including those coding for signaling molecules, were involved in biofilm formation. It has also been suggested that single-species biofilm formation is not affected by inactivation of *luxS* (15, 28). Rickard et al. (21) reported that the optimal concentration of 4,5-dihydroxy-2,3-pentanedione (DPD), a product of the LuxS enzyme in biofilm formation by *Actinomyces naeslundii* and *Streptococcus oralis*, was 100-fold lower than the detection limit of the commonly utilized AI-2 assay. *F. nucleatum* also produces AI-2 (5). Therefore, production of sufficient amounts of AI-2 by *F. nucleatum* to form multispecies biofilms may have masked the potentially stimulatory effects of AI-2 secreted by *P. gingivalis*. In addition, sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of *F. nucleatum* TDC100 revealed that some proteins, including an approximately 35 kDa protein, were predominantly expressed in the separated cocultures with *P. gingivalis* FDC381 and CW221 strains, but not in *F. nucleatum* TDC100 alone (data not shown). Further analysis will be required to clarify how induction is initiated and the role of the 35 kDa protein in biofilm formation by *F. nucleatum.*

Taken together, these results suggest that *P. gingivalis* secretes a molecule other than AI-2 to enhance biofilm formation by *F. nucleatum* TDC100, and that synergistic effects on biofilm formation are an important factor in polymicrobial tooth apical infections.

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