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

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Running title: *P. gingivalis* activate biofilm formation by *F. nucleatum*

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Abstract

Introduction: 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 0.45 μm membrane filter.

Results: *Fusobacterium nucleatum* exhibited strong adherence to type-I collagen-coated polystyrene microplates. Biofilm formation by *F. nucleatum* was significantly enhanced by *Porphyromonas gingivalis*. Enhancement of *F. nucleatum* biofilm formation was complemented by compartmentalized coculture with *P. gingivalis*. Enhancement of biofilm formation by *P. gingivalis* was only slightly reduced by inactivation of its AI-2 producing gene *luxS*.

Conclusion: The results suggest that *P. gingivalis* enhances biofilm formation by *F. nucleatum* by releasing diffusible signaling molecules other than AI-2.
**Introduction**

Microbial biofilms are etiologic agents of persistent infectious disease. Microorganisms in biofilms possess a different phenotype to that of 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 change in certain bacteria through its control of bacterial gene expression via auto-inducers (AI) in biofilm (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 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 media (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 (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 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. 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 consisting of Tryptic soy broth (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-µl 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% (wt/vol) crystal violet (CV) solution was added. After 15 min, the wells were rinsed twice with 350 µl distilled water and air-dried. The CV remaining in the biofilm was solubilized and extracted with 200 µl of 99% ethanol. Biofilm mass was evaluated at an optical density of 595 nm using a microplate reader (BIO-RAD, Herucules, 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, 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 *P. gingivalis* FDC381 strain was used.

Each microorganism was inoculated into TSB and precultured over-night anaerobically. 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 µl 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 fifty µl overnight culture of each strain 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 overnight culture partner strain 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, N.J.). After incubation at 37 °C for 2 days, the inner-well insert was removed and biofilm mass in the lower well was measured as described above. Biofilm formation by the species in the two-compartment system was quantitated 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 biofilm formed by *F. nucleatum* was assayed as described above.

Statistical analysis

Two-group comparisons were performed using the student 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 *P. acnes* were frequently isolated together with other species. These microorganisms showed strong adherence to type-I collagen. As *F. nucleatum* TDC100 showed the strongest adherence activity, 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, *P. gingivalis* FDC 381 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 periapical periodontitis lesions were evaluated by a two-compartment system. *P. gingivalis* ATCC33277 enhanced biofilm formation by *Veillonella atypica* TDC 96, *C. recuts* and *F. nucleatum* TDC100 (Table 2). The enhancement is 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 of the growth of *S. sanguinis* biofilms (data not shown, p<0.05).

The effects of coculture with other species on 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 strains in Table 3, except *S. sanguinis* TDC15, significantly enhanced 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 FDC 381 was 1.5 and 1.6 times higher, respectively, than that of each strain alone, as shown in Table 3. 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 h 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 formed 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 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 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 *T. 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 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* under oxygenated and carbon-dioxide-depleted environments (2). The results of the present study showed that *P. gingivalis* enhanced 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* TDC 100 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 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 CW 221, 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, SDS-PAGE 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* TDC 100, and that synergistic effects on biofilm formation are an important factor in polymicrobial tooth apical infections.

**Acknowledgements**

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REFERENCES


22. Rocas IN, Siqueira JF, Jr., Santos KR, Coelho AM, De Janeiro R. "Red complex" (*Bacteroides* *forsythus*, *Porphyromonas gingivalis*, and *Treponema denticola*) in


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

<table>
<thead>
<tr>
<th>Species</th>
<th>Biofilm formation (OD595)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Propionibacterium acnes</em> TDC 18</td>
<td>0.642 ± 0.048</td>
</tr>
<tr>
<td><em>Propionibacterium acnes</em> TDC 58</td>
<td>0.307 ± 0.025</td>
</tr>
<tr>
<td><em>Propionibacterium acnes</em> TDC 95</td>
<td>0.567 ± 0.130</td>
</tr>
<tr>
<td><em>Propionibacterium acnes</em> TDC 103</td>
<td>0.549 ± 0.059</td>
</tr>
<tr>
<td><em>Propionibacterium acnes</em> TDC 121</td>
<td>0.457 ± 0.082</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> TDC 612</td>
<td>0.285 ± 0.148</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> TDC 66</td>
<td>0.491 ± 0.070</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> TDC 72</td>
<td>0.350 ± 0.016</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em> TDC 845</td>
<td>0.424 ± 0.060</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em> TDC 100</td>
<td>0.667 ± 0.083</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> TDC 116</td>
<td>0.557 ± 0.163</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> TDC 120</td>
<td>0.602 ± 0.156</td>
</tr>
<tr>
<td>Microorganism</td>
<td>Biofilm Formation</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em> TDC 78</td>
<td>0.474 ± 0.045</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em> TDC 86</td>
<td>0.551 ± 0.023</td>
</tr>
<tr>
<td><em>Staphylococcus hominis</em> TDC 54</td>
<td>0.402 ± 0.075</td>
</tr>
<tr>
<td><em>pasteuri pasteuri</em> TDC 563</td>
<td>0.391 ± 0.025</td>
</tr>
<tr>
<td><em>Campylobacter rectus</em> TDC 67</td>
<td>0.364 ± 0.070</td>
</tr>
<tr>
<td><em>Veillonella atypica</em> TDC 96</td>
<td>0.237 ± 0.067</td>
</tr>
<tr>
<td><em>Streptococcus sanguinis</em> TDC15</td>
<td>0.357 ± 0.028</td>
</tr>
<tr>
<td><em>Actinomyces naeslundii</em> genotype 2 TDC 107</td>
<td>0.658 ± 0.171</td>
</tr>
</tbody>
</table>

*Biofilm formation was quantified according to method of Takahashi et al. (26).*
Table 2. Effects of *P. gingivalis* inoculated into upper wells on biofilm formation by microorganisms isolated from apical periodontitis lesions.

<table>
<thead>
<tr>
<th>Lower compartment</th>
<th>Biofilm formation$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. acnes</em> TDC18</td>
<td>1.14 ± 0.13</td>
</tr>
<tr>
<td><em>S. sanguinis</em> TDC15</td>
<td>1.01 ± 0.12</td>
</tr>
<tr>
<td><em>S. pasteuri</em> TDC563</td>
<td>0.91 ± 0.07</td>
</tr>
<tr>
<td><em>A. naeslundii</em> TDC107</td>
<td>1.061 ± 0.09</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> TDC 66</td>
<td>0.98 ± 0.13</td>
</tr>
<tr>
<td><em>S. hominis</em> TDC54</td>
<td>1.10 ± 0.29</td>
</tr>
<tr>
<td><em>V. atypica</em> TDC96</td>
<td>1.67 ± 0.18*</td>
</tr>
<tr>
<td><em>F. nucleatum</em> TDC100</td>
<td>4.19 ± 0.43*</td>
</tr>
<tr>
<td><em>C. rectus</em> TDC67</td>
<td>1.19 ± 0.25**</td>
</tr>
</tbody>
</table>

$^a$Relative quantity of biofilms formed by *F. nucleatum* TDC100 in the tow-compartment system was calculated according to following formula:

\[
\frac{\text{[Biofilm mass of the cocultured species evaluated by crystal violet]}}{\text{[Biofilm mass of the species alone evaluated by crystal violet]}}
\]

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 t-test (p<0.001)
Table 3. Effects of partner strains inoculated into upper wells on biofilm formation by *F. nucleatum* TDC100 and TDC845

<table>
<thead>
<tr>
<th>Upper compartment</th>
<th>Biofilm formation(^a) by <em>F. nucleatum</em> TDC100 (OD(_{595}))</th>
<th>Biofilm formation(^a) by <em>F. nucleatum</em> TDC845 (OD(_{595}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.00 + 0.11 (0.116 + 0.013)</td>
<td>1.00 + 0.054 (0.312 + 0.017)</td>
</tr>
<tr>
<td><em>S. epidermidis</em> TDC78</td>
<td>1.85 + 0.57*</td>
<td>NT</td>
</tr>
<tr>
<td><em>P. gingivalis</em> FDC381</td>
<td>4.15 + 0.46*</td>
<td>2.47 + 0.06*</td>
</tr>
<tr>
<td><em>P. gingivalis</em> ATCC33277</td>
<td>4.19 + 0.43*</td>
<td>2.56 + 0.12*</td>
</tr>
</tbody>
</table>

\(^a\)Relative quantity of biofilms formed by *F. nucleatum* TDC100 was calculated according to the following formula:

\[
\frac{\text{Biofilm mass of cocultured } F. \text{ nucleatum TDC100 evaluated by crystal violet}}{\text{Biofilm mass of } F. \text{ nucleatum TDC100 alone evaluated by crystal violet}}
\]

NT: Not tested

Data are representative of three independent runs of each experiment (n=18) *Statistically higher than *F. nucleatum* alone by student t-test (p<0.001)
Figure legends

Fig. 1. Biofilm formation by coculture of *F. 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 cultivation, 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.

Fig. 2. Effects of *P. gingivalis* wild type and *luxS*-deficient mutant on biofilm formation by *F. nucleatum* TDC100. *P. gingivalis* strain and *F. nucleatum* were cultured either alone alone or cocultured together on type-I collagen-coated microtiter plates. After 48 h cultivation, 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.

Fig. 3 Effects of *P. gingivalis* wild type and *luxS*-deficient mutant inoculated into upper wells on biofilm formation by compartmentalized *F. 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 t-test.
Fig. 1.

- **F. nucleatum** TDC100 alone
- **S. epidermidis** TDC78 alone
- **P. gingivalis** FDC381 alone
- **P. gingivalis** ATCC33277 alone
- **F. nucleatum** TDC845 alone

- **S. epidermidis** TDC78 + **F. nucleatum** TDC100
- **P. gingivalis** FDC381 + **F. nucleatum** TDC100
- **P. gingivalis** ATCC33277 + **F. nucleatum** TDC100
- **P. gingivalis** FDC381 + **F. nucleatum** TDC845

Biofilm formation (OD595)
Fig. 2

- F. nucleatum TDC100 alone
- P. gingivalis FDC381 (luxS) alone
- P. gingivalis FDC381 (luxS) + F. nucleatum TDC100
- P. gingivalis CW221 (ΔluxS) alone
- P. gingivalis CW221 (ΔluxS) + F. nucleatum TDC100

Biofilm formation (OD$_{595}$)
Fig. 3

*Biofilm formation (OD$_{595}$)*

- **F. nucleatum** TDC100 alone
- **F. nucleatum** TDC100 + **P. gingivalis** FDC381 (*luxS*)
- **F. nucleatum** TDC100 + **P. gingivalis** CW221 (*ΔluxS*)