Coaggregation of *Fusobacterium nucleatum* with *Prevotella intermedia* or *Prevotella nigrescens* in biofilm formation

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Running title: *F. nucleatum-Prevotella species Coaggregation*

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Background and Objective: The formation of biofilm by anaerobic, Gram-negative bacteria plays an important role in the development of chronic periodontitis. The aim of this study was to characterize the role of coaggregation between *Fusobacterium nucleatum* and Prevotella species in biofilm formation.

Materials and Methods: ATCC and clinically isolated strains of *F. nucleatum, P. intermedia* and *P. nigrescens* were subjected. Coaggregation between these species was determined by visual assay. Biofilm formation was assessed by crystal violet staining. Effect of co-culture of *F. nucleatum* with Prevotella species was evaluated. Enhancement of biofilm formation via soluble factor was also examined by addition of culture supernatant and a two-compartment separated co-culture system. Production of autoinducer-2 (AI-2) by the tested organisms was evaluated using *Vibrio harveyi* BB170.

Results: Cells of all *F. nucleatum* strains coaggregated with *P. intermedia* or *P. nigrescens*. Addition of EDTA or L-lysine inhibited coaggregation, the other sugars and amino acids tested did not. Coaggregation disappeared after heating of *P. intermedia* and *P. nigrescens* cells, but not after heating of *F. nucleatum* cells. Proteinase K treatment of *P. nigrescens* cells affected coaggregation with *F. nucleatum,* but no significant effect was observed with the treatment of cells of *P. intermedia* or *F. nucleatum*. The quantities of biofilm formation by strains of *F. nucleatum, P. intermedia* and *P. nigrescens* varied. Co-culture of *F. nucleatum* ATCC 25586 with *P. intermedia or P. nigrescens* strains increased biofilm formation than by the single culture ($p<0.05$). Production levels of AI-2 in strains of *F. nucleatum*, *P. intermedia* and *P.*

nigrescens varied. Addition of culture supernatant of *P. intermedia* or *P. nigrescens* did not enhance biofilm formation by *F. nucleatum,* and no biofilm formation enhancement by *F. nucleatum* was observed by the two-compartment co-culture system.

Conclusion: These findings indicate that physical contact by coaggregation of *F. nucleatum* strains with *P. intermedia* or *P. nigrescens* is the key factor in the formation of biofilm by these strains, rather than the AI-2 signaling molecule.

Introduction

Dental plaque, a multispecies biofilm, is organized on the tooth surfaces and periodontal tissues of the human oral cavity (1). More than 500 taxa of bacteria have been identified in the human oral cavity (2), and an increase in specific anaerobic, Gram-negative bacteria in the biofilm is involved in the progression of periodontal disease (3). Bacterial gene expression in the biofilm is regulated by quorum sensing according to increase in cell density (1). After incorporation into the biofilm, microorganisms become resistant not only to host defense mechanisms, but also antimicrobial agents. Moreover, this change has been suggested to be involved in the persistence of infection by such microorganisms in the subgingival crevice (4).

In dental plaque biofilm formation, early colonizers, including streptococci, adhere to pellicles, while late colonizers, including periodontopathic bacteria such as *Porphyromonas gingivalis* and *Prevotella intermedia*, adhere to antecedent microorganisms on the tooth surface (5). Fusobacterium species play a central role in biofilm formation, mediating coaggregation between early and late colonizers (5, 6, 7). Fusobacterium species including *F. nucleatum*, are detected in high frequency in lesions of gingivitis and chronic periodontitis (8, 9). *P. intermedia* and *Prevotella nigrescens* are frequently isolated from the periodontal lesions of patients with chronic periodontitis (8, 9, 10), aggressive periodontitis (11, 12), puberty-associated gingivitis (13, 14), and acute necrotizing ulcerative gingivitis (15). Recently, our research group reported that the average number of periodontopathic bacterial species, including *P. intermedia* and *P. nigrescens*, was significantly higher in plaque samples

harboring *F. nucleatum* and/or *F. periodontium* than in those without in Japanese children and mothers (16). Previous studies showed that *P. intermedia* and *P. nigrescens* possessed specific adherence activity (17, 18), and that they were late colonizers (6). However, the colonization mechanism of *P. intermedia* and *P. nigrescens* strains remains to be clarified. The aim of this study was to characterize the role of coaggregation between *F. nucleatum* and Prevotella species in the formation of biofilm by these strains.

Materials and Methods

Bacterial strains and culture conditions

F. nucleatum ATCC 25586, *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 33563 (kindly provided by Dr. Yoshida, A, Kyusyu Dental College), *P. gingivalis* ATCC 33277 and 5 strains of *F. nucleatum* isolated from dental plaque samples of 5 Japanese adults were used. These bacterial strains were grown in Tryptic soy (TS) broth (Becton Dickinson and Co., Sparks, MD) supplemented with hemin (5 μg/ml) and menadione (0.5 μg/ml) at 37°C under anaerobic conditions (N_2: 80%, H_2: 10%, CO_2: 10%). The biochemical characteristics of clinically isolated strains of YA-F60, MA-F1, SHI-F1, HI-F2 and ABC-F4 were identical to those of *F. nucleatum* ATCC 25586 and all hemagglutinated human O-type erythrocytes. *P. intermedia* M-Q, MA-P1 and MA-P2 and *P. nigrescens* TA-1, B-1, and Q-1 isolated from Japanese adults were also used. The biochemical characteristics of the isolated *P. intermedia* and *P. nigrescens* strains were identical to those of *P. intermedia* ATCC 25611, as well as *P. nigrescens* ATCC 33563 that did not agglutinate human O-type erythrocytes. All subjected clinical

strains of *F. nucleatum*, *P. intermedia* and *P. nigrescens* were identified by the 16S ribosomal RNA sequencing as described previously (19). Briefly, genomic DNA was isolated from each strain and the 16S ribosomal RNA (rRNA) coding sequence amplified and sequenced with the MicroSeq Full Gene 16S rDNA Bacterial Identification Kit (Applied Biosystems, Foster City, CA). The obtained sequence was compared with the 16S rRNA coding locus in the public sequence database (GenBank) and species containing identical sequences identified.

Coaggregation assay

Visual assay of coaggregation of *F. nucleatum* with *P. intermedia* or *P. nigrescens* was carried out by the method of Cisar *et al*. (20) and Kolenbrander *et al*. (21). Two-day cultures of tested strains were harvested by centrifugation at 10,000 g for 20 min. Cells were washed twice with phosphate-buffered saline (PBS, pH 7.2) and re-suspended in coaggregation buffer, which consisted of 1 mM phosphate buffer (pH 8.0) containing 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 150 mM NaCl. Each cell suspension was adjusted to an optical density of 0.5 at 660 nm using the UV-2550 spectrophotometer (Shimazu, Kyoto, Japan). Equal volumes (1.0 ml) of each cell suspension were added to a glass test tube (10 mm by 75 mm) and vortex-mixed for 5 sec. Two hr after mixing, a coaggregation score ranging from 0 to 4 (0: no change in turbidity and no visual coaggregates; 1: finely dispersed coaggregates in a turbid background; 2: definite coaggregates were visible, but did not settle immediately; 3: the formation of large, settling coaggregates, but slightly turbid supernatant; 4: maximum coaggregation, that is, large coaggregates settling immediately,

leaving a water-clear supernatant) was given to each pair. Coaggregation reactions were also confirmed by phase-contrast microscopy as described previously (22).

Effect of chelating agent, sugars or amino acid on coaggregation

The effect of 100 mM ethylenediamine tetraacetic acid (EDTA), sugar or amino acid on coaggregation was examined. The tested sugars were lactose, D-galactose, D-mannose, *N*-acetyl-D-galacosamine and *N*-acetylneuraminic acid. Amino acids tested were L-arginine, L-histidine, L-lysine, L-proline, and L-serine.

Effect of heating and proteinase K treatment

To investigate the effect of heat treatment on coaggregation, coaggregation activity was measured after bacterial cells were heated at 100°C for 10 min. Proteinase K (Sigma Chemical Co., St. Louis, Mo, USA) treatment of cells was carried out by incubation of cell suspension in coaggregation buffer containing 100 µg/ml proteinase K at 37°C for 2 hr. The cells were washed twice with coaggregation buffer and suspended to the original volume with the coaggregation buffer and coaggregation activity determined.

Quantification of biofilm

Quantity of biofilm was achieved by staining with crystal violet as described previously (23). An aliquot of 200 µl cell suspension in TS broth was inoculated into 96-well (flat-bottomed) cell culture plates (Sumitomo Bakelite Co., LTD,

Tokyo, Japan). Collagen type I-coated microplates (Iwaki Brand Asahi Glass Co., Ltd., Tokyo, Japan) and human saliva-coated plates were also used as described by Oliveire *et al.* (24).

After 48 hr incubation in an anaerobic chamber, the culture medium was removed and the wells were washed twice with 200 µl distilled water. The adhered bacteria were stained with 50 µl of 0.1% crystal violet for 15 min at room temperature. After rinsing twice with 200 µl distilled water, the dye bound to the biofilm was extracted with 200 µl of 99% ethanol over 20 min. The extracted dye was then quantified by measuring absorbance at 595 nm with a microplate reader (Spectra MAX M5; Molecular Device, Sunnyvale, CA).

Effect of co-culture with Prevotella strains and addition of culture supernatant on biofilm formation by *F. nucleatum*

To investigate intercellular communication in the biofilm formation, we examined the effect of co-culture of *F. nucleatum* with *P. intermedia* or *P. nigrescens.* Each cell suspension of 100 µl *F. nucleatum* and Prevotella species was inoculated into 96-well cell culture plates. A two-compartment separated co-culture system was also used according to the method of Yoshida *et al*. (25) and as described previously (26). Briefly, *F. nucleatum* ATCC 25586 cell suspension of 750 µl TS broth was placed in each well of a type-I collagen coated polystyrene 12-well plate (Iwaki Brand Asahi Glass), which was then designated the lower well. An insert (Tanswell, Corning, Corning, NY) was then placed in each well, and designated the upper well. Next, partner cell

suspension of 750 μl TS broth was then placed into each upper well. The plates were co-cultured physically separated by a porous membrane (pore size, 0.4 μm: Falcon cell culture insert; BD Labware, Lincon cell culture insert; BD Laware, Lincoln Park, NJ). After incubation at 37°C for 3 days, the inner well insert was removed and the biofilm mass in the lower well was measured as described above.

The effect of addition of cell-free culture supernatant of *P. intermedia* or *P. nigrescens* on biofilm formation by *F. nucleatum* was also examined. Twenty-four-hr culture supernatant of *P. intermedia* or *P. nigrescens* was adjusted to a pH of 7.2 with NaOH and the resulting supernatant filtered through 0.2-μm pore size filters. *F. nucleatum* was inoculated into 96-well cell culture plates containing 20-μl aliquots of cell-free supernatant and 180 μl TS broth. After 48 hr culture at 37°C under anaerobic conditions, biofilm formation level was measured as described above.

Detection of AI-2

AI-2 levels in the culture supernatants of *F. nucleatum*, *P. intermedia* and *P. nigrescens* were evaluated using the *Vibrio harveyi* reporter strain BB170 (kindly provided by Dr. Bassler, B.L., Princeton University) as described by Surette *et al*. (27, 28). Briefly, after incubation in an anaerobic chamber for 24 hr, each culture broth was centrifuged at 10,000 g for 20 min. After adjusting the pH to 7.2 with NaOH, the resulting supernatant was filtered through 0.2-μm pore size filters. To determine *V. harveyi* bioluminescence, 30 μl cell-free supernatant of each tested strain was added to 270 μl of the 1:5,000 diluted overnight culture of

V. harveyi BB170 with fresh AB medium. The mixtures were shaken in a rotary shaker at 200 rpm at 30°C. After 2.5 hr incubation, light production from the reporter strain was measured using the Auto-LUMI-Counter 1422EX (Microtech-Nichion, Funabashi, Japan). Data, reported as fold activation, were obtained by dividing the light produced by the reporter after addition of TS broth alone.

Statistical analysis

Each experiment was performed at least in triplicate. The following analyses were performed. The Kruskal-Wallis test was used to compare quantity of biofilm formation. Dunn tests were used to analyze the difference between groups. $P<0.05$ was considered statistically significant.

RESULTS

Coaggregation of *F. nucleatum* with *P. intermedia* or *P. nigrescens*

The coaggregation reactions of *F. nucleatum* with *P. intermedia* or *P. nigrescens* strains are summarized in Table 1. All tested strains of *F. nucleatum* coaggregated with both *P. intermedia* ATCC 25611 and *P. nigrescens* ATCC 33563, with a score of more than 3. Coaggregation between *F. nucleatum* ATCC 25586 and *P. intermedia* ATCC 25611 observed by phase-contrast microscopy is shown in Fig. 1. All tested strains of *F. nucleatum* coaggregated with clinical isolates of *P. intermedia* or *P. nigrescens* with various scores.

Effect of EDTA, sugars or amino acid on coaggregation

The effects by addition of 100 mM/ml EDTA, sugar or amino acid on coaggregation between *F. nucleatum* ATCC 25586 and *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 33563 or *P. gingivalis* ATCC 33277 are summarized in Table 2. Coaggregation between *F. nucleatum* and *P. intermedia, P. nigrescens* or *P. gingivalis* was completely inhibited by addition of 100 mM/ml EDTA or L-lysine (Table 2 and Fig. 2). *N*-acetyl-D-galactosamine or L-arginine inhibited coaggregation of *F. nucleatum* with *P. gingivalis*, but not with *P. intermedia* or *P. nigrescens*. Other tested sugars and amino acids showed no significant inhibitory effect on coaggregation of *F. nucleatum* with *P. intermedia* or *P. nigrescens*.

Effect of heating and proteinase K treatment on coaggregation

The effects of heating of bacterial cells on coaggregation of ATCC strains of *F. nucleatum* with *P. intermedia* or *P. nigrescens* are summarized in Table 3. When *P. intermedia* or *P. nigrescens* cells were heated at 100°C for 10 min, coaggregation with both untreated and heated cells of *F. nucleatum* was completely abolished. Heating of *F. nucleatum* cells, however, did not significantly affect coaggregation with untreated cells of *P. intermedia* or *P. nigrescens*.

Proteinase K treatment of *P. nigrescens* cells affected coaggregation with *F. nucleatum*, but did not significantly the treatment of *P. intermedia* (Table 4). Proteinase K digestion of *F. nucleatum* cells partially reduced coaggregation with untreated cells of *P. nigrescens*.

Biofilm-forming activity

Quantity of biofilm formation by strains of *F. nucleatum*, *P. intermedia* and *P. nigrescens* is shown in Fig. 3. No significant difference was observed in the biofilm forming activities of the examined strains of *F. nucleatum, P. intermedia* or *P. nigrescens* among untreated plates, collagen type I-coated plates or human saliva-coated plates (data not shown). In untreated plates, *F. nucleatum* ATCC 25586 showed moderate biofilm forming activity of approximately twice that of clinically isolated strains of *F. nucleatum*. The quantity of biofilm formation by *P. intermedia* strains was greater than that of the *F. nucleatum* strains. The quantity of biofilm formation by *P. nigrescens* strains was almost the same as that of *F. nucleatum* ATCC 25586.

Effect of co-culture of *F. nucleatum* with *P. intermedia* or *P. nigrescens*

The results of biofilm formation by co-culture of *F. nucleatum* ATCC 25586 with *P. intermedia* or *P. nigrescens* strains are shown in Fig. 4. The quantity of biofilm formation by co-culture of *F. nucleatum* with *P. intermedia* ATCC 25611 or MA-P1 and with *P. nigrescense* ATCC 33563 was significantly greater than that by *F. nucleatum* single culture ($p<0.01$). The quantity of biofilm formation by co-culture of *F. nucleatum* with *P. intermedia* ATCC 25611 and MA-P1 strains was greater than that of by each *P. intermedia* strain ($p<0.01$). No significant effect of co-culture of *F. nucleatum* ATCC 25586 with *P. nigrescens* TA-1 strain on the biofilm formation.

The effect of co-culture using the two-compartment system on enhancement of biofilm formation of *F. nucleatum* ATCC 25586 was examined.

P. intermedia ATCC 25611 and *P. nigrescens* ATCC 33563 placed in upper well did not enhance the biofilm formation by *F. nucleatum* ATCC 25586 (data not shown).

We examined the effect of addition of culture supernatant of *P. intermedia* ATCC 26611 or MA-P1 or *P. nigrescens* ATCC 33563 or TA-1 on biofilm formation of *F. nucleatum* ATCC 25586. Addition of a final concentration of 20% culture supernatant exerted no effect on the biofilm formation by *F. nucleatum* (data not shown).

Detection of AI-2

To investigate signaling molecule involving to biofilm formation between *F. nucleatum* and *P. intermedia* or *P. nigrescens*, we evaluated AI-2 activity. Levels of luminescence in *V. harveyi* BB170 with addition of culture supernatant of *F. nucleatum*, *P. intermedia* or *P. nigrescens* are summarized in Table 5. A1-2 activity in *F. nucleatum* ATCC 25586 was the highest in *F. nucleatum* strains examined, the levels in *F. nucleatum* YA-F60, MA-F1 and SHI-F1 at 41%-74% of that in *F. nucleatum* ATCC 25586. *P. nigrescens* ATCC 33563 showed the highest level of AI-2 activity among the examined strains, and *P. intermedia* MA-P1 the lowest. Levels of AI-2 production in clinical isolates of *P. intermedia* MA-P1 and *P. nigrescens* TA-1 were 8.9% and 14.5% of their respective ATCC strains.

DISCUSSION

Periodontal disease-associated biofilm-formation is important in the process of colonization by various anaerobic bacteria in a hostile environment such as the gingival crevice, where saliva flows continuously and anti-microbial agents are present within the gingival crevicular fluid (29, 30). An important role has been suggested for *F. nucleatum* in this complex microbial milieu (31, 32). Coaggregation has been shown to be a highly specific mechanism in the formation of biofilm, in which periodontopathic bacteria physically interact (1, 7, 21). In the present study, we investigated the characteristics of the coaggregation reaction between *F. nucleatum* and *P. intermedia* or *P. nigrescens.* All *F. nucleatum* strains coaggregated with *P. intermedia* or *P. nigrescens* with a varied coaggregation score. Kolenbrander *et al.* (31) demonstrated that coaggregation of *F. nucleatum* with *P. gingivalis* was inhibited by N-acetyl-D-galacosamine, lactose, or D-galactose. We confirmed that coaggregation of *F. nucleatum* with *P. gingivalis* was inhibited by these sugars or L-arginine. The present study showed coaggregation of *F. nucleatum* with *P. intermedia* or *P. nigrescens* was not inhibited by these sugars or L-arginine. Coaggregation of *F. nucleatum* with *P. intermedia* or *P. nigrescens* was completely inhibited by addition of 100 mM of EDTA or L-lysine, but not affected significantly by other amino acids or sugars. The results of the inhibition study suggest that the adhesin and/or receptor involved in coaggregation of *F. nucleatum* with *P. intermedia* or *P. nigrescens* is different from that involved in its coaggregation with *P. gingivalis*.

When cells of *P. intermedia* or *P. nigrescens* were heated at 100°C for 10

min, coaggregation with untreated or heated cells *F. nucleatum* was completely abolished. Heating of *F. nucleatum* cells, however, did not affect coaggregation with untreated cells of *P. intermedia* or *P. nigrescens*. Kolenblander *et al*. (6) reported that heating Fusobacterium species abolished coaggregation in every partnership of oral Treponema species. The present results, however, indicate that the adhesin involved in the coaggregation of *F. nucleatum* with *P. intermedia* or *P. nigrescens* is heat stable, suggesting that it differs from that involved in coaggregation between Fusobacterium species and oral Treponema species. The present study showed proteinase K treatment of *P. nigrescens* cells affected coaggregation with *F. nucleatum*, but the treatment of *P. intermedia* did not. Proteinase K digestion of *F. nucleatum* did not abolish reactivity with untreated cells of *P. intermedia*, and decreased weakly coaggregation with untreated *P. nigrescens*. It is possible that a heat-treatment stable and proteinase K-resistant surface component on *F. nucleatum* cells plays a role in its coaggregation with *P. intermedia* or *P. nigrescens.* Several types of adhesion mechanism have been described in various *F. nucleatum* strains: (i) galactose-sensitive lectin-like adhesion (33, 34, 35, 36); (ii) adhesion via protein-protein interaction, including hemagglutinating activity (37, 38); and (iii) a capsular polysaccharide or lipopolysaccharide of periodontal pathogenic bacteria that possesses adherence activity (39, 40). The present results suggest that a heat-stable and protenase-K resistant adhesin such as a capsular polysaccharide or lipopolysaccharide on *F. nucleatum* is involved in its coaggregation with *P. intermedia* or *P. nigrescens.*

In this study, we observed that cell-free culture supernatants of *P.*

intermedia or *P. nigrescens* strains partially inhibited growth of the *F. nucleatum* strains tested. Kaewscrichen *et al*. (41) showed that *P. nigrescens* produced a bacteriocin, nigrescin, which exhibited a bactericidal mode of action against *P. gingivalis*, *P. intermedia*, *Tannerella forsythensis* and Actinomyces species. We investigated the bacteriocin-like activity of *P. intermedia* or *P. nigrescens* strains against *F. nucleatum* strains by the stub culture method as described previously (42). No bacteriocin-like activity was observed in *P. intermedia* or *P. nigrescens* strains against *F. nucleatum*. After adjusting the supernatant pH to 7.2, inhibition of growth vanished. These results revealed that neither *P. intermedia* nor *P. nigrescens* inhibited growth of *F. nucleatum* strains.

Communication based on AI-2 is widespread among Gram-negative and Gram-positive bacteria, and the AI-2 pathway can control the expression of genes involved in a variety of metabolic pathways and pathogenic mechanisms (25, 43, 44, 45). Fries *et al.* (46) showed that strains of *F. nucleatum, P. intermedia* and *P. gingivalis* produced AI-2, but that they were unable to produce homologues of acylhomoserine lactones. In our previous study, *P. gingivalis* enhanced biofilm formation by *F. nucleatum* by releasing diffusible signaling molecules other than AI-2 (26). The present study showed an increase in biofilm formation by co-culture of *F. nucleatum* strains with *P. intermedia* or *P. nigrescens*, while no stimulation of biofilm formation was induced by addition of cell-free culture supernatant of these same species.

In summary, these findings indicate that physical contact by coaggregation of *F. nucleatum* strains with *P. intermedia* or *P. nigrescens* is the key factor in the formation of biofilm by these strains, rather than the AI-2 signaling molecule.

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Table 1. Coaggregation reactions of strains of *F. nucleatum* with *P. intermedia* or *P. nigrescens*

	Coaggregation score[a]					
F. nucleatum	ATCC 25586	YA-F60	MA-F1	SHI-F1	HI-F2	ABC-F4
P. intermedia strain						
ATCC 25611	4	3	3	4	4	4
M-Q	3	1	1	2	1	2
MA-P1	3	1	1	2	1	3
MA-P2	3	1	1	4	1	4
P. nigrescens strain						
ATCC 33563	3	1	2	2	1	2
TA-1	2	2	2	1	1	2
B-1	3	1	2	1	1	1
Q-1	2	1	2	2	2	1

a: 1: finely dispersed coaggregates in turbid background; 2: definite coaggregates were visible, but did not settle immediately; 3: formation of large settling coaggregates, but slightly turbid supernatant; 4: maximum coaggregation, that is, large coaggregates settling immediately, leaving water-clear supernatant.

Table 2. Effect of 100 mM EDTA, sugar or amino acid on coaggregation of *F. nucleatum* ATCC 25586 with *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 33563 or *P. gingivalis* ATCC 33277

	Coaggregation score[a] with *F. nucleatum* ATCC 25586		
	P. intermedia ATCC 25611	*P. nigrescens* ATCC 33563	*P. gingivalis* ATCC 33277
Coaggregation buffer	4	3	3
Added 100 mM			
EDTA	0	0	0
lactose	3	3	1
D-galactose	3	2	1
D-mannose	4	3	3
N-acetyl-D-galactosamine	3	3	0
N-acetylneuraminic acid	4	3	2
L-arginine	4	3	0
L-histidine	4	3	3
L-lysine	0	0	0
L-proline	4	3	3
L-serine	4	3	3

a: 0: no change in turbidity and no visual coaggregates; 1: finely dispersed coaggregates in turbid background; 2: definite coaggregates were visible, but did not settle immediately; 3: formation of large settling coaggregates, but slightly turbid supernatant; 4: maximum coaggregation, that is, large coaggregates settling immediately, leaving water-clear supernatant.

Table 3. Effect of heat treatment on coaggregation of *F. nucleatum* ATCC 25586 with *P. intermedia* ATCC 25611 or *P. nigrescens* ATCC 33563

	Coaggregation score[a]	
	F. nucleatum ATCC 25586	
	untreated cells	heated cells
P. intermedia ATCC 25611		
untreated cells	4	4
heated cells	0	0
P. nigrescens ATCC 33563		
untreated cells	3	3
heated cells	0	0

a: 0: no change in turbidity and no visual coaggregates; 3: formation of large settling coaggregates, but slightly turbid supernatant; 4: maximum coaggregation, that is, large coaggregates settling immediately, leaving water-clear supernatant.

Table 4. Effect of proteinase K treatment on coaggregation reactions of *F. nucleatum* ATCC 25586 with *P. intermedia* ATCC 25611 or *P. nigrescens* ATCC 33563

	Coaggregation score[a]	
	F. nucleatum ATCC 25586	
	untreated cells	proteinase K treated cells
P. intermedia ATCC 25611		
untreated cells	4	4
proteinase K treated cells	4	3
P. nigrescens ATCC 33563		
untreated cells	3	2
proteinase K treated cells	0	0

a: 0: no change in turbidity and no visual coaggregates; 2: definite coaggregates were visible, but did not settle immediately; 3: formation of large settling coaggregates, but slightly turbid supernatant; 4: maximum coaggregation, that is, large coaggregates settling immediately, leaving water-clear supernatant.

Table 5. Induction of luminescence in *V. harveyi* BB170 strain by culture fluids from strains of *F. nucleatum*, *P. intermedia* or *P. nigrescens*

	Relative luminescence
Control	1.00
F. nucleatum strain	
ATCC 25586	53.60
YA-F60	30.74
MA-F1	39.89
SHI-F1	22.09
P. intermedia strain	
ATCC 25611	64.30
MA-P1	5.73
P. nigrescens strain	
ATCC 33653	88.48
TA-1	12.87

Figure legends

Fig. 1. Phase-contrast photomicrographs of coaggregation between *F. nucleatum* ATCC 25586 and *P. intermedia* ATCC 25611. Coaggregated bacterial mass can be seen in mixture.

Fig. 2. Results of coaggregation inhibition test. 1: cells of *F. nucleatum* ATCC 25586; 2: cells of *P. intermedia* ATCC 25611; 3: mixture of cells of *F. nucleatum* and *P. intermedia*; 4: containing 100 mM EDTA; 5: containing 100 mM lactose; 6: containing 100 mM D-galactose; 7: containing 100 mM N-acetylneuraminic acid; 8: containing 100 mM L-arginine; 9: containing 100 mM L-lysine.

Fig. 3. Quantification of biofilm formation by ATCC strains and isolated strains of *F. nucleatum*, *P. intermedia* or *P. nigrescens.* Values represent means ± standard deviations of triplicate independent determinations from typical experiment.

Fig. 4. Quantification of biofilm formation by co-culture of *F. nucleatum* ATCC 25586 with *P. intermedia* ATCC 25611 or MA-P1 or *P. nigrescens* ATCC 33563 or TA-1. Values represent means ± standard deviations of 8 wells independent determinations from typical experiment. Quantity of biofilm by co-culture of *F. nucleatum* ATCC 25586 with *P. intermedia* ATCC 25611 or *P. intermedia* MA-P1 was significantly higher than those of their culture alone ($p<0.01$). Quantity of biofilm by co-culture with *P. nigrescens* ATCC 33563 was higher than *F. nucleatum* ATCC 25586 alone ($p<0.01$), and co-culture with *P. nigrescens* TA-1 was higher than that of *P. nigrescens* TA-1 alone ($p<0.01$).

Figure 1

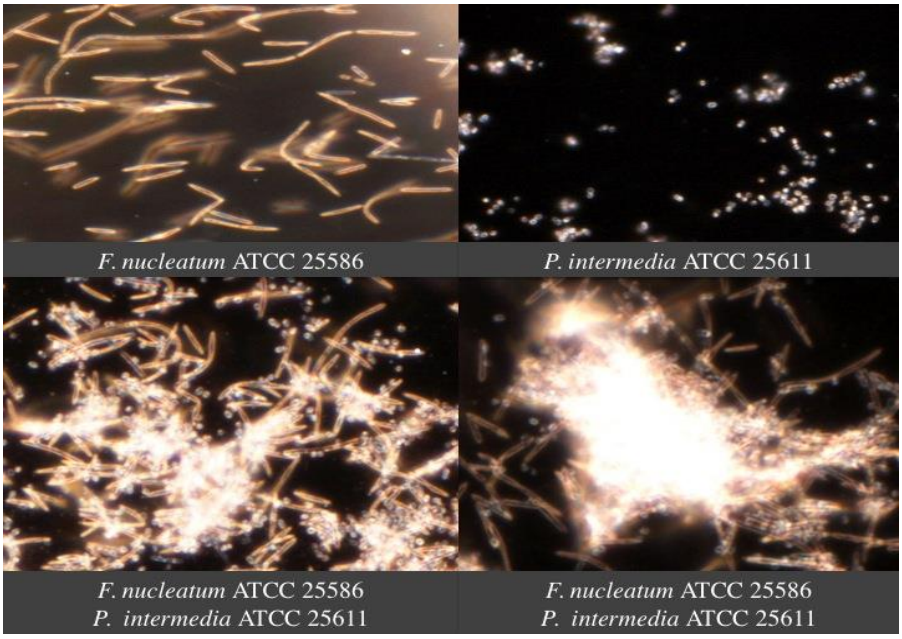

F. nucleatum ATCC 25586
P. intermedia ATCC 25611
F. nucleatum ATCC 25586
P. intermedia ATCC 25611
F. nucleatum ATCC 25586
P. intermedia ATCC 25611

Figure 2

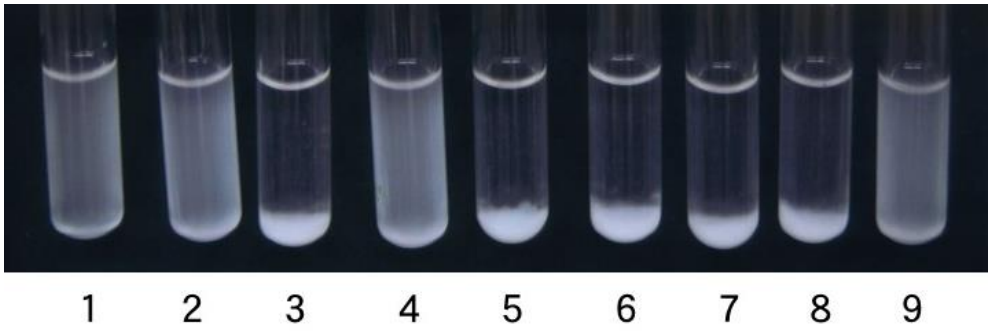


Figure 3

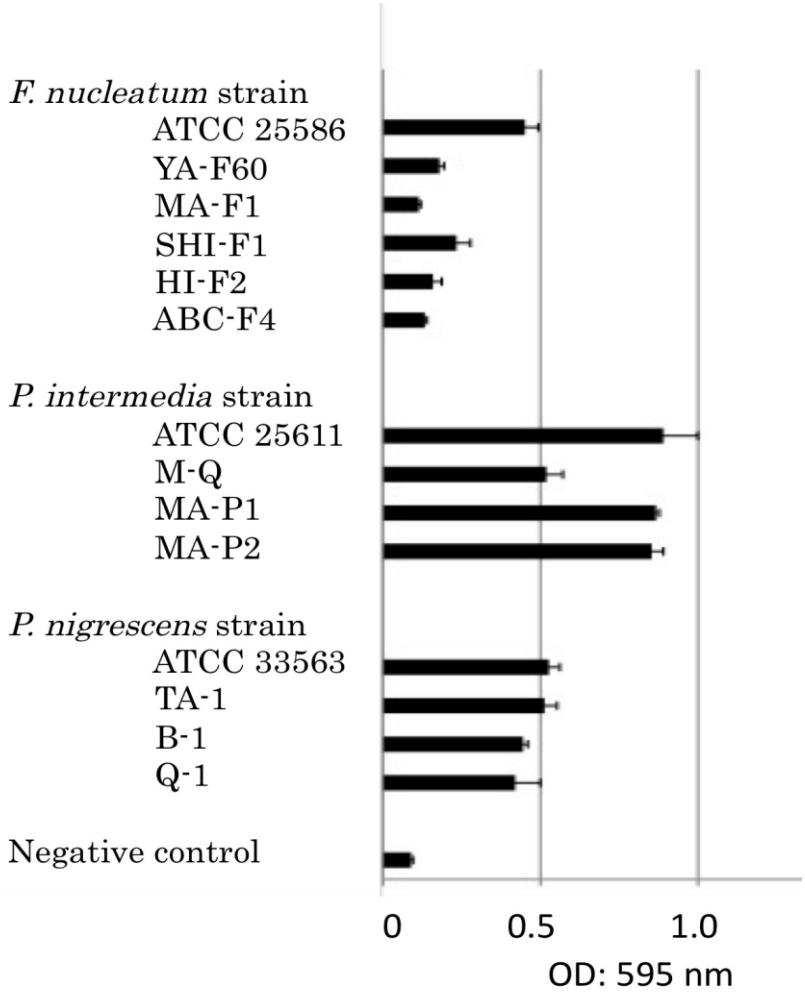
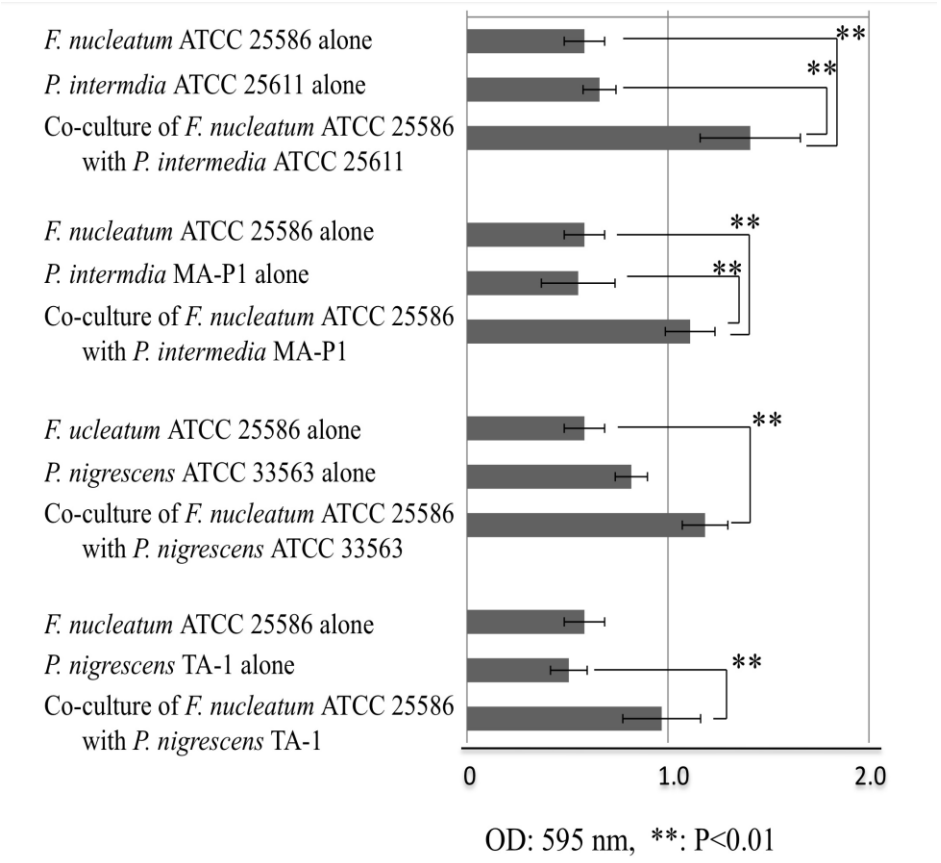

F. nucleatum strain
ATCC 25586
YA-F60
MA-F1
SHI-F1
HI-F2
ABC-F4
P. intermedia strain
ATCC 25611
M-Q
MA-P1
MA-P2
P. nigrescens strain
ATCC 33563
TA-1
B-1
Q-1
Negative control
0
0.5
1.0
OD: 595 nm

Figure 4


F. nucleatum ATCC 25586 alone
P. intermdia ATCC 25611 alone
Co-culture of F. nucleatum ATCC 25586 with P. intermedia ATCC 25611
F. nucleatum ATCC 25586 alone
P. intermdia MA-P1 alone
Co-culture of F. nucleatum ATCC 25586 with P. intermedia MA-P1
F. ucleatum ATCC 25586 alone
P. nigrescens ATCC 33563 alone
Co-culture of F. nucleatum ATCC 25586 with P. nigrescens ATCC 33563
F. nucleatum ATCC 25586 alone
P. nigrescens TA-1 alone
Co-culture of F. nucleatum ATCC 25586 with P. nigrescens TA-1
**
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**
**
0
1.0
2.0
OD: 595 nm, **: P<0.01