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Porphyromonas gingivalis displays a competitive advantage over *Aggregatibacter actinomycetemcomitans* in co-cultured biofilm

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Background and Objective: Biofilm formation occurs through the events of cooperative growth and competitive survival among multiple species. *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are important periodontal pathogens. The aim of this study was to demonstrate competitive or cooperative interactions between these two species in co-cultured biofilm.

Material and Methods: P. gingivalis strains and gingipain mutants were cultured with or without *A. actinomycetemcomitans*. Biofilms formed on glass surfaces were analyzed by crystal violet staining and colony counting. Preformed *A. actinomycetemcomitans* biofilms were treated with *P. gingivalis* culture supernatants. Growth and proteolytic activities of gingipains were also determined.

Results: Monocultured *P. gingivalis* strains exhibited a range of biofilm-formation abilities and proteolytic activities. The ATCC33277 strain, noted for its high biofilm-formation ability and proteolytic activity, was found to be dominant in biofilm co-cultured with *A. actinomycetemcomitans*. In a time-resolved assay, *A. actinomycetemcomitans* was primarily the dominant colonizer on a glass surface and subsequently detached in the presence of increasing numbers of ATCC33277. Detachment of preformed *A. actinomycetemcomitans* biofilm was observed by incubation with culture supernatants from highly proteolytic strains.

Conclusion: These results suggest that *P. gingivalis* possesses a competitive advantage over *A. actinomycetemcomitans*. As the required biofilm-formation abilities and proteolytic activities vary among *P. gingivalis* strains, the diversity of the competitive advantage is likely to affect disease recurrence during periodontal maintenance.

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Periodontitis is the result of inflammatory responses to bacterial challenge in the gingival crevicular area. As a large number of bacterial species in subgingival plaque exist within biofilm communities (1), biofilm formation occurs through the events of cooperative growth and competitive survival among multiple species (2–4). The development of dental biofilms starts with the adherence of initial colonizers, which is followed by the cooperative binding of late colonizers (5–8). Some bacteria may compete with their neighbors for space and resources. A competitive relationship between oral bacteria was observed in an *in-vitro* experiment, in which *Streptococcus mitis* was able to inhibit the adherence of *Porphyromonas gingivalis* to the surface of a flow chamber (9). However, there are only a few reports demonstrating a competitive relationship among subgingival bacteria in either *in-vitro* or *in-vivo* experiments.

P. gingivalis and *Aggregatibacter actinomycetemcomitans* are important periodontal pathogens (1, 10–12). In a study performed by Johansson *et al.* (13), *P. gingivalis* strongly inhibited *A. actinomycetemcomitans* leukotoxicity. In addition, this study demonstrated an antagonistic effect of *P. gingivalis* on *A. actinomycetemcomitans*. However, little is known regarding whether these two species are cooperative or competitive in the process of biofilm formation. In the oral niche, *P. gingivalis* can colonize abiotic surfaces after the initial stages of biofilm formation by coaggregation with antecedent bacteria, such as *Streptococcus gordonii* or *Actinomyces naeslundii* (4, 8). While *A. actinomycetemcomitans* is also generally recognized as a late colonizer (4), some reports indicate that this species could colonize both primate and human enamel within a 6-h period after tooth prophylaxis (14,15). Fine *et al.* (16) showed colonization of *A. actinomycetemcomitans* on the surface of hydroxyapatite 6 h after it had been inserted in human pockets suggesting that *A. actinomycetemcomitans* did not require any other microbes for colonization on a tooth-like surface. Therefore, *A. actinomycetemcomitans,* as well as *Streptococcus* and *Actinomyces* species, could be initial colonizers of tooth surfaces. Antecedent *Streptococcus* and *Actinomyces* species can remain on the surface during the colonization process of *P. gingivalis* (4, 8). However, it is unclear how antecedent *A. actinomycetemcomitans* is affected by *P. gingivalis*.

There are at least four possible theoretical relationships between *P. gingivalis* and *A. actinomycetemcomitans* in the process of biofilm formation: (i) the two species have cooperative interactions; (ii) *P. gingivalis* has a competitive advantage over *A. actinomycetemcomitans*; (iii) *P. gingivalis* is competitively excluded by *A. actinomycetemcomitans*; and (iv) there is no biological interaction between the two species. The aim of this study was to clarify which type of theoretical relationship exists in dual-species biofilm formation of *P. gingivalis* and *A. actinomycetemcomitans*. Of the various cell-surface components of *P. gingivalis*, gingipains are well characterized and are involved in biofilm formation (17,18). Arginine-specific cysteine proteinase (Arg-gingipain; encoded by *rgpA* and *rgpB*) and lysine-specific proteinase (Lys-gingipain; encoded by *kgp*) are the predominant extracellular proteolytic enzymes produced by *P. gingivalis* (19,20). Therefore, in this study, *P. gingivalis* strains and gingipain mutants with varying biofilm formation and proteolytic activities were prepared and co-cultured with *A. actinomycetemcomitans* to demonstrate biofilm formation.

Material and methods

Preparation of bacterial strains

The *P. gingivalis* strains used in this study are listed in Table 1. *P. gingivalis* was cultured at 37°C anaerobically (10% CO_2, 10% H_2 and 80% N_2) on blood agar or in brain–heart infusion (BHI) broth supplemented with menadione (1 μg/mL) and hemin (5 μg/mL). For blood agar plates, defibrinated laked sheep blood was added to tryptic soy agar (19) at 5%. When appropriate, blood agar was supplemented with 200 μg/mL of kanamycin. *A. actinomycetemcomitans* strain ATCC29523 was cultured on modified sTSB agar (3% trypticase soy broth, 0.3% yeast extract, 5% heat-inactivated horse serum and 1.5% agar) (22) or in BHI broth at 37°C in 5% CO_2. As in previous studies (23,24), monospecies biofilms of *P. gingivalis* and *A. actinomycetemcomitans* were generated by static incubation of broth cultures for 24 h and 18 h, respectively. The dual-species biofilm of *P. gingivalis* and *S. gordonii* was generated through static incubation of broth culture for 16 h (24). Therefore, in this study, monocultures and co-cultures of *P. gingivalis* and *A. actinomycetemcomitans* were used for monospecies and dual-species biofilm formation when at the middle or late logarithmic phases of bacterial growth.

Table 1. The *Porphyromonas gingivalis* strains used in this study

Strain	Characteristics	Reference
ATCC33277	Wild type, *fimA* type I	ATCC
ATCC53977	Wild type, *fimA* type II	ATCC
KDP133	ATCC33277 derivative, *rgpA*::Tc^r *rgpB*::Em^r	21
KDP129	ATCC33277 derivative, *kgp*::Cm^r	21
KDP136	ATCC33277 derivative, *rgpA*::Tc^r *rgpB*::Em^r *kgp*::Cm^r	21

Monospecies biofilm formation followed by spectrophotometry assay

As some *P. gingivalis* strains showed slower growth rates than others, *P. gingivalis* strains were subcultured repeatedly in broth and the size of the inoculum in the final biofilm formation assay was adjusted to account for the variation in growth rates. In brief, *P. gingivalis* colonies from blood agar plates were inoculated into 3 mL of BHI broth and cultured overnight. An aliquot (100 μL) of this overnight

culture was transferred into 3 mL of fresh BHI broth and cultured for 18 h. Then, an appropriate volume of inoculum (30–50 μL, depending on the growth rate of the *P. gingivalis* strain) of this culture was transferred into 3 mL of fresh BHI broth in a glass tube (borosilicate glass JR-2; Iwaki, Tokyo, Japan) prepared for the biofilm-formation assay. After 24 h of incubation under anaerobic and static conditions, formation of monospecies *P. gingivalis* biofilms on the glass surface was evaluated. Following agitation for 5 s using a vortex, nonbiofilm cells became separated and the glass tube was rinsed with phosphate-buffered saline (PBS, pH 7.4) to remove loosely attached cells. The separated nonbiofilm cells became quantified by measuring the optical density (OD) at 590 nm using a spectrophotometer (Colorwave CO7500 Colorimeter; Biochrom Ltd., Cambridge, UK). Biofilm cells on the bottom surface of the glass tube were then stained with 0.1% crystal violet. After destaining with 95% ethanol, the absorbance at 590 nm (A_{590}) was determined. All assays were performed in triplicate.

Dual-species biofilm formation followed by colony counting

P. gingivalis and *A. actinomycetemcomitans* were cultured in BHI broth separately for 24 h and then transferred separately to fresh BHI for a further 18 h of incubation. Both species were added to 9 mL of BHI broth in a Petri dish containing coverslips (18 mm × 18 mm). *A. actinomycetemcomitans* [3×10^7 colony-forming units (CFUs)/mL] was co-incubated with the appropriate concentration of *P. gingivalis* ($3–5 \times 10^7$ CFUs/mL) for 18 h at 37°C under anaerobic and static conditions. As controls, a monoculture of each species was performed as for co-culture. After incubation, nonbiofilm cells were removed from the bottom of the vessel by pipetting and the whole supernatant was separated. Coverslips were removed from the dish and washed with PBS to remove loosely attached cells. The remaining adherent cells were collected with a sterile cell scraper (Sarstedt, Newton, NC, USA), dispersed in 1 mL of PBS by vigorous vortexing for 30 s, serially diluted in 10-fold steps and seeded onto culture plates for colony counting. The nonbiofilm cells in the separated supernatants were also diluted and seeded onto culture plates for colony counting. For *P. gingivalis* strains ATCC33277 and ATCC53977, inoculated blood agar plates were incubated anaerobically at 37°C for 5 d and the numbers of CFUs were determined by counting black colonies. The colonies of strain KDP136 were counted on anaerobic blood agar plates supplemented with kanamycin. For *A. actinomycetemcomitans*, sTSB agar plates were inoculated and incubated in 5% CO_2 for 5 d, and opaque colonies were then counted. After removal of the biofilms, the coverslips were gram stained and visualized using an optical light microscope (BX50-DP70; Olympus, Tokyo, Japan), and the number of residual cells on each coverslip was counted ($1.23 \pm 0.33 \times 10^5$ cells/cm^2). All assays were performed in triplicate.

Biofilm detachment assay

A preformed *A. actinomycetemcomitans* biofilm was prepared as follows. *A. actinomycetemcomitans* was cultured overnight in BHI broth and then 100 μL of this overnight culture was transferred to a glass tube filled with 3 mL of fresh BHI broth and incubated for a further 18 h. After vigorous mixing using a vortex, the liquid was discarded. Residual cells on the bottom of the tube were rinsed twice with 3 mL of PBS, generating a preformed *A. actinomycetemcomitans* biofilm.

The supernatant of an 18-h culture of *P. gingivalis* was harvested by centrifugation at 6000 *g* for 15 min. Aliquots of this culture were boiled for 10 min or were treated with 500 μg/mL of proteinase K (Wako Pure Chemical Industries Ltd., Osaka, Japan) at 37°C for 60 min. Proteinase K was then inactivated by boiling the culture for 10 min. In the subsequent detachment procedure, preformed *A. actinomycetemcomitans* biofilm was incubated with 3 mL of *P. gingivalis* culture supernatant, boiled supernatant, proteinase K-treated supernatant or fresh BHI broth as a control, at 37°C for 1 h. The supernatant or broth was then discarded and the glass tube was rinsed twice with 3 mL of PBS. The biofilms remaining on the surface were quantified by crystal violet staining, as described above. All assays were performed in triplicate.

Proteolytic activities of gingipains

P. gingivalis cells were grown for 24 h in 3 mL of BHI broth, and then the cell-free culture supernatants and cells of *P. gingivalis* were obtained by centrifugation at 6000 *g* for 15 min at 4°C. The cells were washed and resuspended in PBS (pH 7.4) to the same cell density (OD at 590 nm = 1.0). The resuspended cells were analyzed immediately for Arg-gingipain and Lys-gingipain proteolytic activity using *N*-α-benzoyl-DL-Arg-*p*-nitroanilide (BApNA; Sigma-Aldrich, St Louis, MO, USA) and *N*-*p*-Tosyl-Gly-Pro-Lys-*p*-nitroanilide (TGPLpNA; Sigma-Aldrich), respectively (21). In brief, 200 μL of cell suspension was added to 800 μL of a reaction mixture containing 0.25 mM BApNA and 0.3 M Tris–HCl (pH 8.5) for Arg-gingipain or 800 μL of a reaction mixture containing 0.25 mM TGPLpNA and 0.3 M Tris-HCl (pH 8.5) for Lys-gingipain. The reaction mixtures were incubated at 37°C for 10 min. During the incubation, absorbance at 405 nm was continuously measured on a spectrophotometer (Jasco Ubest-30 UV/VIS Spectrophotometer; Jasco, Tokyo, Japan). One unit of enzyme activity is defined as the amount of enzyme that hydrolyzes 1 mmol of substrate per min under the conditions of the assay.

Statistical analysis

The results are expressed as mean values with standard deviations. Student's *t*-tests were used to determine whether the observed differences were statistically significant. A *p*-value of < 0.05 was considered statistically significant. Calculations were performed using Microsoft Excel software (Windows 2007; Microsoft Co., Seattle, WA, USA).

Results

Preparation of *P. gingivalis* strains

Our laboratory strains were examined for their monospecies biofilm-forming capacity. As some strains showed relatively slow growth rates, the size of the inoculum was varied among strains to generate similar growth in the final culture tubes. Biofilms that formed on the bottom of the glass tubes were quantified by crystal violet staining. *P. gingivalis* strains exhibited a diversity of biofilm volume. Moreover, the adherence of various *P. gingivalis* strains to a glass surface varied. Of our stock strains, ATCC33277 was noted to form the most developed biofilm (Fig. 1B); in contrast, the volume of biofilm formed by strain ATCC53977 was approximately four times lower. Given that strain ATCC33277 demonstrated strong biofilm-formation ability, mutants generated from this strain were also examined. A substantial decrease in biofilm formation was noted in the KDP136 gingipain-null mutant (Fig. 1B). Growth of nonbiofilm cells of the three strains was confirmed to be almost identical (Fig. 1A).

With regard to the proteolytic activities of cell-associated gingipains and supernatant-secreted gingipains, ATCC53977 and ATCC33277 had relatively high Arg-gingipain and Lys-gingipain activities (Fig. 1C–F). As expected, KDP136 was completely deficient in proteolytic activity. Based on the results of monospecies biofilm and gingipain assays, ATCC33277 was classified as the greatest biofilm former as a result of its high biofilm-formation ability and high proteolytic activity. Strain ATCC53977 had low biofilm-formation ability and high proteolytic activity. The gingipain-null mutant, KDP136, had low biofilm-formation ability and low proteolytic activity.

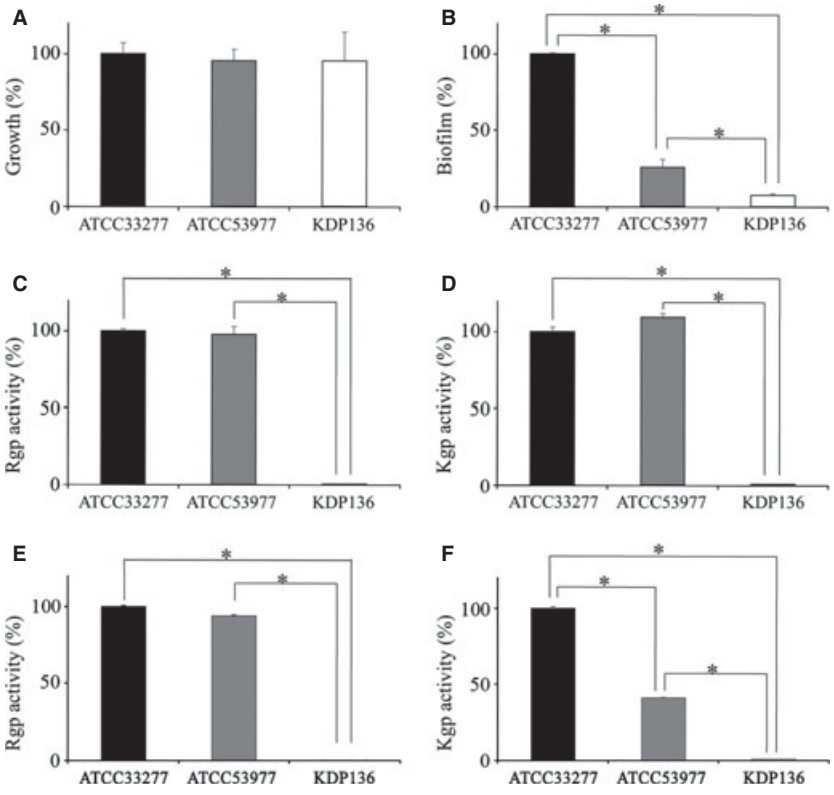

Fig. 1. Characteristics of *Porphyromonas gingivalis* strains in monoculture. (A) The optical density (OD) at 590 nm of monoculture supernatants was measured directly. The mean OD of the supernatant of ATCC33277 is shown as 100%. (B) Monoculture biofilms were stained with crystal violet and quantified by dissolving the dye in 95% ethanol and measuring the A_{590}. The mean absorbance of ATCC33277 is shown as 100%. (C, D) Arg-gingipain and Lys-gingipain activities of the cell lysates were assayed by measuring the absorbance at 405nm (A_{405}). (E, F) Arg-gingipain and Lys-gingipain activities in the culture supernatants were assayed by measuring the A_{405}. The activity of ATCC33277 was set as 100%. Kgp, Lys-gingipain; Rgp, Arg-gingipain. Error bars show standard deviation. *Statistically significant difference ($p < 0.05$).

Competitive advantage of *P. gingivalis* against *A. actinomycetemcomitans* in biofilm

Each of the selected *P. gingivalis* strains was co-cultured with *A. actinomycetemcomitans*. Table 2 indicates the numbers of *P. gingivalis* and *A. actinomycetemcomitans* cells in the supernatant or biofilm derived from monocultures and co-cultures. As shown by the supernatant data (i.e. nonadherent cells), each *P. gingivalis* and *A. actinomycetemcomitans* strain grew to the same extent in monocultures and co-cultures. Co-cultured *P. gingivalis* and *A. actinomycetemcomitans* were unlikely to affect each other's growth rates. Biofilm assays revealed that *P. gingivalis* biofilm formation was independent of the presence of *A. actinomycetemcomitans*. Hence, ATCC33277, ATCC53977 and KDP136 showed similar levels of biofilm cells in both monocultured and co-cultured biofilms (i.e. the high biofilm formation of strain ATCC33277 was maintained in co-culture). Strains ATCC53977 and KDP136 showed low levels of biofilm formation, irrespective of monoculture or co-culture.

To be competitively dominant, *P. gingivalis* may need to reduce the threat represented by opposing species. As shown in Table 2, the biofilm formation of *A. actinomycetemcomitans* was altered upon co-culture with highly proteolytic *P. gingivalis* strains. Compared with monoculture, when co-cultured with the highly proteolytic strains ATCC33277 and ATCC53977, *A. actinomycetemcomitans* showed decreased biofilm formation. The gingipain mutant KDP136 did not decrease the biofilm-formation ability of *A. actinomycetemcomitans*. It therefore seems that high gingipain

Table 2. Influence of co-cultures on growth and biofilm formation of the bacterial strains investigated in this study

Strain	Nonadherent cells (Log_{10} CFUs/mL ± SD)	Biofilm cells (Log_{10} CFUs/cm^2 ± SD)
Monocultured strains		
P. gingivalis ATCC33277	9.46 ± 0.20	7.59 ± 0.02
P. gingivalis ATCC53977	9.35 ± 0.05	6.22 ± 0.16
P. gingivalis KDP136	9.42 ± 0.07	5.21 ± 0.15
A. actinomycetemcomitans	9.38 ± 0.08	7.72 ± 0.04*
Co-cultured strains; #1		
P. gingivalis ATCC33277	9.68 ± 0.21	7.90 ± 0.05
A. actinomycetemcomitans	9.39 ± 0.16	6.19 ± 0.23
Co-cultured strains; #2		
P. gingivalis ATCC53977	9.32 ± 0.12	6.42 ± 0.21
A. actinomycetemcomitans	9.45 ± 0.11	6.58 ± 0.26
Co-cultured strains; #3		
P. gingivalis KDP136	9.14 ± 0.16	5.58 ± 0.51
A. actinomycetemcomitans	9.34 ± 0.14	7.72 ± 0.36

A. actinomycetemcomitans, *Aggregatibacter actinomycetemcomitans*; CFUs, colony-forming units; *P. gingivalis*, *Porphyromonas gingivalis*; SD, standard deviation.
*Significant differences were detected ($p < 0.05$).

proteolytic activity was involved in the decreased biofilm-formation capacity of *A. actinomycetemcomitans*. Therefore, the high biofilm-formation ability and proteolytic activity of strain ATCC33277 may provide a competitive advantage over *A. actinomycetemcomitans*.

The changes, over time, in the population densities of each species in co-cultured biofilm were assessed (Fig. 2). At the time of inoculation, just before incubation, low numbers of bacteria (4.2×10^4 CFUs/cm^2 of *P. gingivalis* and 2.4×10^4 CFUs/cm^2 of *A. actinomycetemcomitans*) were detected on the glass surface. Given this background, the vertical axis in Fig. 2 shows the number of biofilm cells as greater than 1×10^5 CFUs/cm^2. At 6 h, *A. actinomycetemcomitans* predominated over *P. gingivalis* strain ATCC33277 in the biofilm. However, this initial predominance was reversed during the late log phase at 18 h. Biofilm of *A. actinomycetemcomitans* decreased from 6 h to 18 h, whereas that of *P. gingivalis* increased, eventually covering the glass surface.

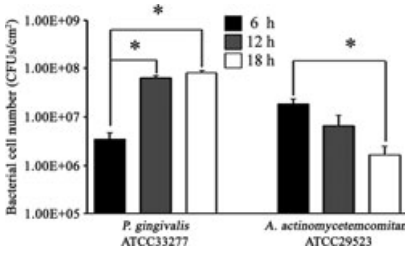

Fig. 2. Dual-species biofilm formation by *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* strain ATCC33277 on coverslips. Both species were anaerobically co-cultured for up to 18 h in brain–heart infusion (BHI) broth containing a coverslip, from which bacterial cells were harvested and colonies were counted. CFUs, colony-forming units. *Statistically significant difference ($p < 0.05$).

Detachment of preformed *A. actinomycetemcomitans* biofilm by *P. gingivalis*

Preformed *A. actinomycetemcomitans* biofilms were treated with culture supernatants of ATCC33277, ATCC53977 and gingipain mutants. Supernatants of the highly proteolytic strains ATCC33277 and ATCC53977 were able to detach *A. actinomycetemcomitans* from the surface (Fig. 3). Compared with the wild-type strain ATCC33277, the Arg-gingipain mutant KDP133 and the Lys-gingipain mutant KDP129 showed reduced detachment ability. Furthermore, the gingipain-null mutant KDP136 showed only a negligible effect on the detachment of *A. actinomycetemcomitans* biofilms. Boiled or proteinase K-treated supernatants of strain ATCC33277 failed to detach *A. actinomycetemcomitans* biofilms.

Discussion

To prepare *P. gingivalis* strains with varying levels of biofilm formation, we grew our stock strains as monospecies biofilms. Data indicated that the level of biofilm formation on a glass surface varied among *P. gingivalis* strains and was independent of *fimA* genotype (data not shown). The subunit of the major fimbriae is a 41-kDa protein (FimA) encoded by the *fimA* gene (25). FimA are classified into six genotypes (types I–V and Ib) based on the diversity of the *fimA* genes (26). Mutations in the *fimA* gene did not influence the ability to form biofilm on the abiotic surface (data not shown). A previous study by Kuboniwa *et al*. (27) also reported varying degrees of monospecies biofilm formation by *P. gingivalis* strains. The authors used one strain from each *fimA* genotype, but did not ascertain whether the variation in biofilm volume was *fimA* genotype- or strain-specific. Recently, pathogenic heterogeneity has been noted among *fimA* type II strains (28). Therefore, bacterial phenotypes, including the ability to form a biofilm, are likely to vary in each *fimA* genotype of *P. gingivalis*.

Gingipains are major processing enzymes for the maturation of various biofilm-related proteins, including long and short fimbriae (29). The gingipain-null mutant KDP136 showed markedly decreased monospecies biofilm formation (Fig. 1). Our results are consistent with those reported by Grenier *et al*., in which the Arg-gingipain mutant KDP112 and the gingipain-null mutant KDP128 generated less biofilm than did the wild-type strain ATCC33277 (18). However, in the study carried out by Kuboniwa *et al*., the gingipain-null mutant KDP136 showed increased levels of biofilm formation (30). Hence, whether gingipains are positively involved in biofilm formation remains controversial. These studies determined the role of

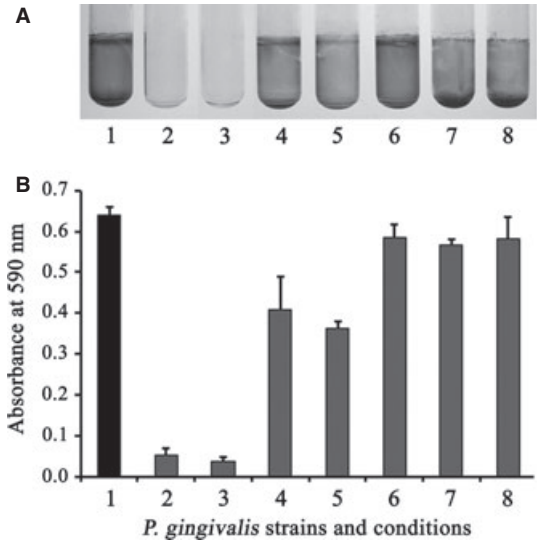

Fig. 3. Detachment of *Aggregatibacter actinomycetemcomitans* biofilm by *Porphyromonas gingivalis* culture supernatants. Preformed *A. actinomycetemcomitans* biofilms were incubated with native, boiled or proteinase K-treated *P. gingivalis* supernatants of stock and mutant strains for 60 min at 37°C. *A. actinomycetemcomitans* biofilms were (A) visualized and (B) quantified using 0.1% crystal violet staining. Lanes: 1, fresh broth (control); 2; ATCC33277 (wild type); 3, ATCC53977; 4, KDP133 (Δ*rgpA*, Δ*rgpB*); 5, KDP129 (Δ*kgp*); 6, KDP136 (Δ*rgpA*, Δ*rgpB*, Δ*kgp*); 7, proteinase K-treated; 8, boiled.

gingipain by comparing gingipain mutants and wild-type strains. To settle this controversy, gingipains should be examined using different approaches, such as inhibition of proteolytic activity and blocking of the regions critical for biofilm formation.

Our results regarding monospecies biofilm formation agree with those of Wakabayashi *et al*., in that *P. gingivalis* strain ATCC33277 exhibited greater biofilm formation than did the other strains (31). Based on their monospecies biofilm-forming ability and gingipain proteolytic activity, three strains were selected for co-culture experiments (Table 2): ATCC33277 (high biofilm-formation ability and high proteolytic activity); ATCC53977 (low biofilm-formation ability but high proteolytic activity); and the mutant strain KDP136 (low biofilm-formation ability and no proteolytic activity). These three strains demonstrated the factors that provide *P. gingivalis* with a competitive advantage over *A. actinomycetemcomitans* during biofilm formation on an abiotic surface. In a study by Suzuki *et al*. (32), *P. gingivalis* ATCC33277 was not able to co-aggregate with *A. actinomycetemcomitans*, except for serotype c strains. As *A. actinomycetemcomitans* ATCC29523 (used in this study) is a serotype a strain, these species are unlikely to co-exist in a biofilm through coaggregation. Moreover, *P. gingivalis* appears to compete with *A. actinomycetemcomitans* during initial colonization to a glass surface (Fig. 2). *A. actinomycetemcomitans* was the dominant primary colonizer of the surface and was subsequently detached by the increasing numbers of *P. gingivalis*. Gingipains may be effective in inducing detachment of preformed *A. actinomycetemcomitans* biofilm from a glass surface (Fig. 3). These data strongly suggest that high gingipain proteolytic activity as well as high biofilm-formation ability is required for *P. gingivalis* to compete with *A. actinomycetemcomitans* in a co-cultured biofilm. Considering the possibility that *A. actinomycetemcomitans* cells were digested by the proteolytic activity of gingipains shown in Fig. 3, we examined the bactericidal effect of *P. gingivalis* supernatant on *A. actinomycetemcomitans* cells. The results demonstrated that the number of viable *A. actinomycetemcomitans* cells did not differ significantly between those treated with the *P. gingivalis* supernatant and the BHI control treatment (data not shown). Therefore, the reduced volume of preformed *A. actinomycetemcomitans* biofilm was unlikely to be caused by the digestion of *A. actinomycetemcomitans* cells.

The biological relationship between *P. gingivalis* and *A. actinomycetemcomitans* is unlikely to be cooperative, as the nature of these species in the oral cavity is different. According to Asikainen *et al*., transmission of *A. actinomycetemcomitans* can occur from carrier parents to periodontally healthy children, but the major route of infection with *P. gingivalis* occurs between cohabiting adults (33). Socransky *et al*. (34) proposed that *P. gingivalis*, along with *Tannerella forsythia* and *Treponema denticola*, form a tightly related complex in human subgingival plaque; however, *A. actinomycetemcomitans* does not belong to this complex. In the natural environment, *P. gingivalis* and *A. actinomycetemcomitans* might inhabit a biofilm community without establishing a cooperative relationship. Moreover, Johansson *et al*. (13) demonstrated an antagonistic effect of *P. gingivalis* on *A. actinomycetemcomitans* (i.e. *P. gingivalis* strongly inhibited *A. actinomycetemcomitans* leukotoxicity). The present study found another competitive relationship between these two species in the initial colonization on an abiotic surface. Our data suggest that *P. gingivalis* gingipains secreted in the subgingival environment are critical components in promoting the detachment of antecedent *A. actinomycetemcomitans* cells from the surface. There may be other competitive relationships between *P. gingivalis* and *A. actinomycetemcomitans*, and therefore it is important to investigate these two species in greater detail.

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