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NOTES

Intergeneric Communication in Dental Plaque Biofilms

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Dental plaque is a complex biofilm that accretes in a series of discrete steps proceeding from a gram-positive streptococcus-rich biofilm to a structure rich in gram-negative anaerobes. This study investigated information flow between two unrelated plaque bacteria, *Streptococcus cristatus* and *Porphyromonas gingivalis*. A surface protein of *S. cristatus* caused repression of the *P. gingivalis* fimbrial gene (*fimA*), as determined by a chromosomal *fimA* promoter-*lacZ* reporter construct and by reverse transcription-PCR. Signaling activity was associated with a 59-kDa surface protein of *S. cristatus* and showed specificity for the *fimA* gene. Furthermore, *P. gingivalis* was unable to form biofilm microcolonies with *S. cristatus*. Thus, *S. cristatus* is capable of modulating virulence gene expression in *P. gingivalis*, consequently influencing the development of pathogenic plaque.

The study of the ability of bacterial cells to communicate with one another and coordinate behavior is a burgeoning field with relevance to a number of microbial ecosystems (5, 6, 11, 12, 17). The plaque biofilm that accumulates on tooth surfaces comprises over 30 genera representing more than 500 species (9, 16). Despite this complexity, plaque formation is highly choreographed. Initial colonization by a group of gram-positive organisms, mainly streptococci, is followed by a succession of species that culminates in the arrival of gram-negative anaerobic bacteria such as *Porphyromonas gingivalis*, a predominant pathogen in severe adult periodontitis (13). Colonization of the dental biofilm by *P. gingivalis* is thus a pivotal event in the transition from a commensal plaque to a pathogenic entity. *P. gingivalis* colonization is contingent upon fimbria-mediated adhesion to oral surfaces (1, 7). The *fimA* gene that encodes the major subunit protein of fimbriae (FimA) can be regulated by environmental cues (2, 19). However, the extent to which plaque bacteria can modulate fimbrial gene expression in *P. gingivalis* through intercellular signaling mechanisms is largely unknown.

Expression of *fimA* is regulated by *S. cristatus*. To identify signaling mechanisms of oral biofilm organisms that could affect expression of the *fimA* gene, we utilized *P. gingivalis* strain UPF, which contains a chromosomal fusion between the *fimA* promoter and a *lacZ* reporter gene (18). *P. gingivalis* UPF was grown in Trypticase soy broth (TSB) or on 1.5% TSB blood agar plates supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml), and menadione (1 μg/ml) at 37°C in an anaerobic (85% N_2, 10% H_2, 5% CO_2) chamber. When appropriate, the culture medium contained the antibiotics erythromycin (20 μg/ml) and gentamicin (100 μg/ml). The organisms tested for signaling activity were *Streptococcus gordonii* G9B and M5, *Streptococcus sanguis* 10556, *Streptococcus mutans* KPSK2, *Streptococcus cristatus* CC5A, and *Actinomyces naeslundii* NC-3, all of which were grown in Trypticase Peptone broth supplemented with yeast extract (5 mg/ml) and 0.5% glucose at 37°C aerobically; *Treponema denticola* GM-1, which was cultured for 5 days anaerobically in GM broth (15); and *Fusobacterium nucleatum* 10953, which was cultured anaerobically in the same way as *P. gingivalis*. A surface extract of test organisms was prepared by sonication (30 s) of late-log-phase cultures. Whole cells were removed by centrifugation (13,000 × *g* for 30 min) followed by filtration (0.2-μm pore size). The protein concentration of the surface extract in the supernatant was determined by the Bio-Rad protein assay.

Bacterial extracts were reacted with 10^5 cells of *P. gingivalis* UPF, and the mixture (20 μl) was spotted onto a TSB blood agar plate. After anaerobic culture for 24 to 36 h, *P. gingivalis* cells were harvested, washed, and resuspended in phosphate-buffered saline (PBS) to an optical density at 600 nm of 0.4 to 0.6. Expression of the *lacZ* gene under the control of the *fimA* promoter was measured by the standard Miller spectrophotometric β-galactosidase assay with *o*-nitrophenyl-β-D-galactopyranoside as the substrate as previously described (10, 18). *P. gingivalis fimA* expression was dramatically downregulated by *S. cristatus* CC5A but not by other common plaque constituents (Table 1). As shown in Fig. 1, the effect of the CC5A extract on *fimA* expression was dose dependent. An increase in extract concentration reduced *fimA* promoter activity a maximum of 12-fold compared to controls. The results show that many common constituents of both early commensal and late pathogenic plaque organisms do not influence *fimA* transcriptional activity. These organisms and *P. gingivalis* therefore exist "incommunicado," at least with regard to fimbria production. However, the plaque commensal *S. cristatus* is capable of inducing downregulation of *fimA* expression and thus, *S. cristatus* has the potential to impede *P. gingivalis* colonization of plaque. A reduction in the expression of fimbrial adhesin may delay attachment of *P. gingivalis* and render the organism more susceptible to elimination by salivary flow.

The *S. cristatus* signal shows specificity for the *fimA* gene. The *S. cristatus* CC5A extract was tested to determine whether the signaling activity could modulate the expression of another

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TABLE 1. Effects of oral bacteria on *fimA* expression in *P. gingivalis*

Extract source[a]	Mean β-galactosidase level[b] ± SE
None (PBS control)	138 ± 2
S. gordonii G9B	154 ± 7
S. gordonii M5	127 ± 5
S. mutans KPSK2	117 ± 13
P. cristatus CC5A	43 ± 4
S. sanguis 10556	126 ± 11
A. naeslundii NC-3	135 ± 2
T. denticola GM-1	133 ± 5
F. nucleatum 10953	134 ± 6

[a] Extract (10 μg of protein) was reacted with 10^5 *P. gingivalis* cells.
[b] Miller units are shown. The assay was performed at least three times.

P. gingivalis virulence gene. A *P. gingivalis* strain containing a transcriptional chromosomal fusion between the *lacZ* reporter and protease gene *prtT* or *rgpA* (14; kindly provided by H. Kuramitsu) was utilized. Transcriptional activity of *rgpA* and *prtT* was not affected by the CC5A extract (Table 2). Further evidence of specificity and confirmation of the reporter gene assay were provided by reverse transcription-PCR. Steady-state levels of *fimA* mRNA decreased approximately 60%

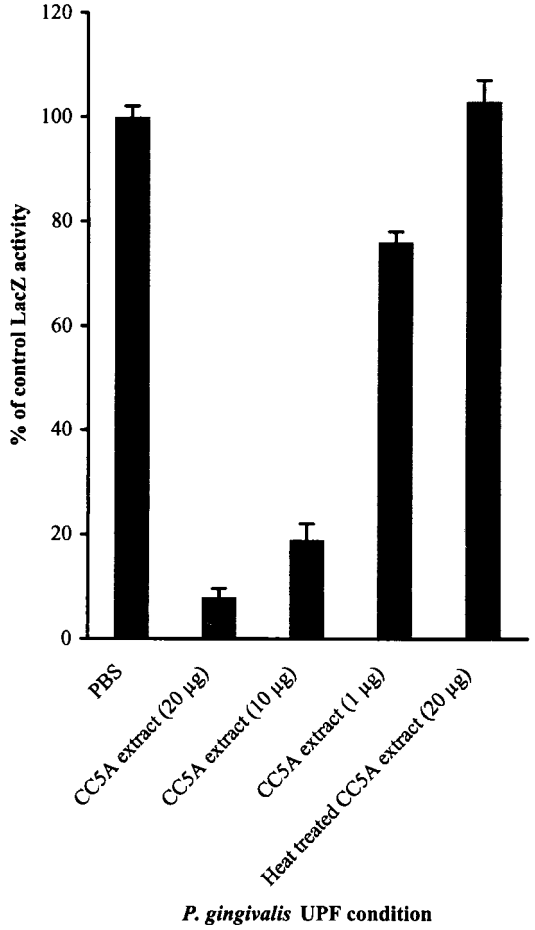

FIG. 1. Effect of CC5A extract on *fimA* transcriptional activity. *P. gingivalis* UPF containing a *fimA-lacZ* promoter-reporter fusion was cultured with the CC5A extract noted. *lacZ* activity was determined as previously described (10), and the values obtained with PBS were set to 100%. Error bars represent standard deviations ($n = 3$).

TABLE 2. Effects of *P. cristatus* CC5A extract on virulence gene expression in *P. gingivalis*

P. gingivalis gene with transcriptional *lacZ* fusion[a]	Supplement added to bacteria[b]	Mean % of LacZ activity[c] ± SE
fimA	PBS	100 ± 2
fimA	CC5A extract	8 ± 16
rgpA	PBS	100 ± 5
rgpA	CC5A extract	99 ± 7
prtT	PBS	100 ± 8
prtT	CC5A extract	98 ± 2

[a] Gene products: *fimA*, fimbrillin; *rgpA*, arginine protease; *prtT*, minor protease.
[b] CC5A (extract (20 μg of protein) or PBS was mixed with 10^5 *P. gingivalis* cells.
[c] Values (Miller units) relative to that of the control bacteria with PBS (100%) are shown. The assay was performed at least three times.

when *P. gingivalis* 33277 (the parent of UPF) was grown with CC5A extract, while mRNA levels of *rgpA* were unaffected (data not shown).

Formation of *P. gingivalis* biofilms is inhibited by *S. cristatus*. As the results suggested that *S. cristatus* CC5A could prevent colonization of plaque by the pathogen *P. gingivalis*, biofilm formation by *P. gingivalis* with CC5A was investigated as described previously (4). Streptococci (10^7 cells/ml) were labeled with hexidium iodide and passed over a saliva-coated glass slide in a flow chamber (0.6 by 1.0 cm) for 4 h at a flow rate of 2 ml/h. Following the deposition of streptococci, *P. gingivalis* 33277 (10^7 cells/ml) was labeled with fluorescein and passed through the flow cell (containing the streptococci) in PBS at 2 ml/h for 4 h. The *P. gingivalis*-streptococcal biofilm was examined by confocal microscopy (Bio-Rad MRC600), and images were generated using Slicer imaging software (4).

P. gingivalis cells did not bind to or accrete on CC5A cells

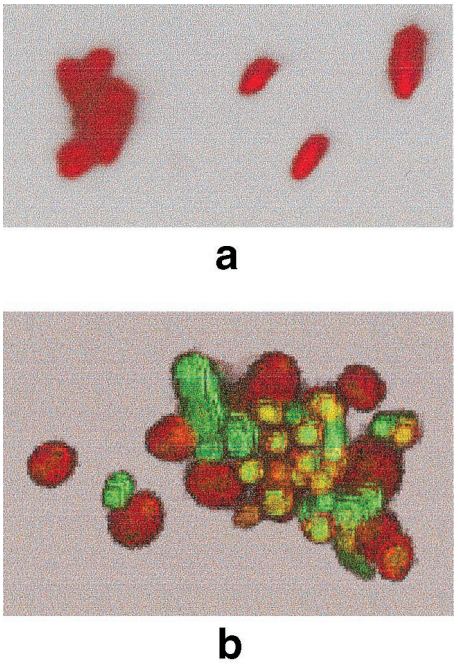

FIG. 2. Confocal image of *S. cristatus* CC5A (a) or *S. gordonii* G9B (b) reacted with *P. gingivalis* 33277 in a flow chamber. Streptococcal cells are red, *P. gingivalis* are green, and colocalized bacteria are yellow.

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(Fig. 2a). In contrast, *P. gingivalis* binds to and accumulates (in the absence of cell division and growth) in biofilm microcolonies with sessile *S. gordonii* G9B, a major component of early plaque (Fig. 2b). Thus, downregulation of *fimA* expression by *S. cristatus* following initial contact between the organisms appears to inhibit subsequent longer-term adhesion and accumulation of *P. gingivalis*. These results corroborate the antagonism of *S. cristatus* toward *P. gingivalis* colonization and show that signaling can occur between whole cells of the two species. It is noteworthy that *S. cristatus* strains are capable of binding to other oral organisms such as *F. nucleatum*, resulting in the formation of corncob structures that are readily visible in plaque (8, 9). Thus, the *S. cristatus* signal has specificity for *P. gingivalis* and may not affect the maturation of commensal plaque.

***S. cristatus* signaling activity is associated with a 59-kDa surface protein.** To characterize the *S. cristatus* signaling molecule, the extract was subjected to heat treatment by incubation for 1 h at 100°C. As shown in Fig. 1, exposure to heat abolished activity, suggesting the involvement of a proteinaceous molecule. Treatment of the extract with proteinase K also abrogated activity (data not shown). Fast protein liquid chromatographic separation of the CC5A extract showed a peak of activity in fractions eluting at 59 kDa. The active fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis but still contained more than one polypeptide; identification of the specific protein is in progress. However, the size of the molecule suggests that it is likely to be distinct from the short signaling peptides secreted by other gram-positive bacteria (6).

Conclusions. The physical nature of the plaque biofilm provides opportunities for close-range cell-cell signaling interactions. However, the role of intercellular communication in the formation and development of plaque is poorly understood. Based on our observations, we can postulate that the shift from commensal plaque to periodontitis-related plaque may depend on the relative proportions of the early colonizers. *S. gordonii* and related species provide an attachment substrate for *P. gingivalis* through the interaction of specific adhesin-receptor pairs, including recognition of the FimA protein by *S. gordonii* surface molecules (3, 7). In contrast, the molecular dialogue between *S. cristatus* and *P. gingivalis* restrains *fimA* expression and as a result, *P. gingivalis* will be unable to adhere and will be more readily shorn from the biofilm and eventually eliminated from the oral cavity. It is likely that complete elucidation of the components of this signaling mechanism will provide insight into the nature of dental plaque formation.

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