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Periodontal pathogens interfere with quorum-sensing-dependent virulence properties in *Streptococcus mutans*

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Abstract

Background and objective—The mechanism by which periodontal pathogens dominate at disease sites is not yet understood. One possibility is that these late colonizers antagonize quorum-sensing systems of early colonizers and render those early colonizers less resistant to environmental factors. In this study, we utilized *Streptococcus mutans*, a well-documented oral Streptococcus with many quorum-sensing dependent properties, as an example of targeted earlier colonizers that are antagonized by periodontal pathogens.

Material and Methods—*S. mutans* NG8, LT11, and BM71 were used in this study for assessment of transformation and bacteriocin production, respectively. The effects of *Porphyromonas gingivalis* and *Treponema denticola* on these competence-stimulating peptide (CSP)-dependent properties were evaluated in mixed broth assays.

Results—Both *P. gingivalis* (either live bacteria or membrane vesicles) and *T. denticola* antagonized transformation in *S. mutans* NG8 and LT11. *S. mutans* BM71 bacteriocin production was also inhibited by *P. gingivalis* and *T. denticola*. Boiling of these late colonizers before mixing the broth cultures abolished their ability to inhibit *S. mutans* transformation and bacteriocin production. *P. gingivalis* and *T. denticola* inactivated *S. mutans* exogenous CSP, whereas the boiled bacteria did not.

Conclusions—This study demonstrated that periodontal pathogens antagonized *S. mutans* quorum-sensing properties. This may render *S. mutans* less virulent and less resistant to environmental antibacterial factors.

Keywords

periodontal pathogen; *Porphyromonas gingivalis*; *Treponema denticola*; *Streptococcus mutans*; quorum sensing

Periodontal diseases are among the most common chronic infectious diseases occurring in humans. According to the National Institute of Dental and Craniofacial Research, periodontal diseases affect the majority of the American population (1). Oral chronic

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infectious diseases not only lead to tooth loss but may also act as a risk factor for systemic diseases such as cardiovascular diseases, respiratory diseases, and pre-term birth (2-4).

Quorum sensing involves the control of gene expression in response to cell density when a minimum population unit, or quorum, is reached (5). In streptococci such as *Streptococcus mutans*, one of the quorum sensing signaling systems depends on a competence-stimulating peptide (CSP, encoded by the *comC* gene) and a two-component signal transduction system which is encoded by the *comD* and *comE* genes, corresponding to a histidine protein kinase (receptor for CSP) and a response regulator, respectively (6-8). Quorum sensing systems modulate a variety of virulence activities in *S. mutans*. Disruption of the Com quorum sensing system in *S. mutans* results in attenuated virulence activities such as biofilm formation (9,10), acid tolerance (11), bacteriocin production (12) and genetic competence (13,14). In addition, Matsumoto-Nakano and Kuramitsu (15) have recently demonstrated that the *comC* gene of strain GS5 also modulates the sensitivity of *S. mutans* to a variety of antimicrobial agents such as triclosan, fluoride, antibiotics and histatin-5. Therefore, CSP represents a potential target for control of *S. mutans* infection.

Some bacteria such as *S. mutans* and *Streptococcus gordonii* are naturally transformable, being able to take up naked DNA from the extracellular environment (16). Homologous recombination of foreign DNA into the host chromosome following transformation is believed to play a major role in the evolution of bacteria. This was demonstrated by both the rapid emergence of penicillin resistance following the acquisition of low-affinity penicillin binding proteins (17) and evidence for the occurrence of frequent recombination events in the evolution of virulence factors in *Streptococcus pneumoniae* (18,19). Experimental evidence also has shown that oral bacteria such as *S. gordonii* can take up free extracellular DNA from saliva *in vitro* (20). Furthermore, transformation of bacteria has been demonstrated after infection in animal hosts (21). Such events suggest that the expanded capacities conferred by acquired genes could result in more virulent bacterial species. Since bacteria can acquire resistance to compromising environments by taking up extracellular DNA, the ability to transfer genetic material among bacteria should be considered as a possible virulence attribute of bacteria involved in caries and periodontal disease.

The production of bacteriocins by microorganisms is also one of the important mechanisms used by bacteria for antagonistic interference (22). Although these peptide molecules are not required for growth, they may help the microorganisms that produce them to compete for the limited nutrients in their environment (23).

Some late colonizers of dental plaque such as *Porphyromonas gingivalis* and *Treponema denticola* have been implicated as pathogens causing chronic periodontitis (24,25). Colonization by these pathogens can result in a proportional decrease in the population of early colonizers such as streptococci leading to the domination of sites of periodontal diseases by the former (24). How these periodontal pathogens dominate in dental plaque is not yet understood. One possibility is that these late colonizers antagonize the quorum sensing of the early colonizers and therefore render the early colonizers less virulent and less resistant to endogenous antimicrobial agents such as histatins, peroxide, and lysozyme. The objective of the present study was to determine if these late colonizers interfere with quorum sensing in *S. mutans*.

The Com quorum-sensing system has been identified in several early colonizers of dental plaque (8,26,27) and may be generalized targets for bacterial antagonism by late colonizers. In this study, we used *S. mutans*, a well-documented oral streptococcus with many CSP-dependent quorum-sensing properties, as an example of an earlier colonizer which is antagonized by periodontal pathogens such as *P. gingivalis* and *T. denticola*.

Materials and methods

Bacterial strains and media

S. mutans BM71, *S. mutans* NG8, and their *com* mutants were maintained on Tryptic Soy Agar (TSA) plates supplemented with erythromycin (10 μg/mL) where indicated. Bacteria were routinely cultured in Todd Hewitt broth (THB). A group C streptococcal strain RP66 was used as an indicator for assays of *S. mutans* bacteriocin activity (28). *P. gingivalis* 381 was grown anaerobically in enriched tryptic soy broth (TSB) medium (containing, per liter, 40 g of TSB, 5 g of yeast extract, 0.5 g of cysteine, 10 mg of hemin, and 1 mg of vitamin K_1) and maintained on TSA blood agar plates (containing, per liter, TSB plus 15 g of agar and 50 mL of sheep blood). *T. denticola* 35405 was routinely maintained in tryptone yeast extract-gelatin-volatile fatty acids-serum (TYGVS) medium under anaerobic conditions. Bacteria used in this study are listed in Table 1.

Natural genetic transformation

Recipient *S. mutans* NG8 or LT11 in the stationary phase were diluted twenty-fold in THB with 10% horse serum (THB-HS) and cultured at 37°C for 30 minutes to induce competence. For the transformation of mixtures of *S. mutans* with other periodontal pathogens, bacteria at the stationary phase were diluted twenty-fold in THB-HS and mixed in 1:1 ratio for competence induction. For some experiments, the periodontal pathogens were boiled for 10 minutes before mixed culturing. Competent *S. mutans* recipient cells (100 μL) were exposed to exogenous donor plasmid or chromosomal DNA [1 μg of pTet (29) or 10 μg of DNA*gtfD* (30)] for 2 hours. Transformants were then sonified and selected on TSA agar plates supplemented with tetracycline (10 μg/mL). Transformation frequencies were determined after 48-72 hours of anaerobic incubation at 37°C.

Isolation of membrane vesicles from *P. gingivalis* 381

Membrane vesicles (MVs) from a culture of *P. gingivalis* 381 in early stationary growth phase in TSB were isolated from the supernatants of the cultures by filtrating through 0.22-μm-pore-size filters. Vesicles were recovered from the resulting filtrates by ultra-centrifugation at 150,000 X g for 2 hours at 4°C with a Ti 75 rotor (Beckman Instruments, Inc., Fullerton, CA). Isolated MVs were resuspended in PBS (100-fold concentrated from the original cultures) and stored at -70°C for further transformation experiments. For transformation experiments, MVs (40 μL) were added to 100 μL twenty-fold diluted *S. mutans* NG8 for competence induction.

Bacteriocin production and assays

S. mutans BM71 cells (10^7 cfu) or mixtures with *P. gingivalis* 381 or *T. denticola* 35405 (10^7 or 5×10^7 cfu) were cultured in 1.0 mL of THB supplemented with 3% yeast extract (THBY) at 37°C for 24 hours. For some experiments, the periodontal pathogens were boiled for 10 minutes before mixed culturing. Supernatants containing bacteriocin from the cultures were neutralized to pH 7.0 with NaOH, filtered through 0.22 μm pore size filters, and either assayed immediately or frozen at -20°C for subsequent assays. RP66 cells (2×10^5 cfu) in 0.7 mL THB were grown in the presence of the above-mentioned supernatant fluids (300 μL) and incubated at 37°C for 5-6 hours. OD_{600nm} measured thereafter indicated the presence or the absence of bacteriocin.

Effects of periodontal pathogens on the CSP of *S. mutans*

Supernatants from *P. gingivalis* 381 or *T. denticola* 35405 grown to the stationary phase in TSB or TYGVS respectively were filtered through 0.22 μm filters to eliminate cellular components. The supernatants were then neutralized with NaOH to pH 7.0-7.5. Exogenous

synthetic *S. mutans* CSP [amino acid sequence: SGSLSTFFRLFNRSFTQALGK (13), synthesized by Sigma-Genosys, The Woodlands, TX, 2.5 μg/mL final concentration] was incubated with the supernatants at 37°C for 2 hours. CSP thus treated was added to *S. mutans* BM71 *comC* mutant cells (10^7 cfu) in 0.9 mL of THBY to a final concentration of 0.25 μg/mL. After 24-hour incubation at 37°C, the supernatants from the cultures were passed through 0.22 μm filters and the bacteriocin level produced by the *S. mutans* BM71 *comC* mutant was determined by the agar well assay.

Agar well assays

The supernatant fluids containing bacteriocin from the *S. mutans* BM71 *comC* mutant cultures were obtained as described above. The supernatants were added into pre-cut wells in THB agar plates and incubated at 37°C for 24 hours to facilitate the absorption of the supernatants into the agar surrounding the wells. The wells were then filled using THB with 1% low melting temperature agarose and the plates were overlaid with the indicator strain RP66 (10^6 cfu) in 3 mL THB with 1% low melting temperature agarose. After 24 hours of further incubation at 37°C under anaerobic conditions, the diameters of the inhibition zones surrounding the wells were measured.

Statistical analysis

Student's t-Test was performed to determine significance. A difference was considered significant when a p value < 0.05 was obtained.

Results

Attenuation of *S. mutans* natural transformation by *P. gingivalis*

Experiments were performed to determine the efficiencies of *S. mutans* natural transformation. *S. mutans* natural transformation is mediated by CSP and the *comC* knockout mutant exhibited minimum transformation efficiency, relative to its parent strain (Table 2). A late colonizer, *P. gingivalis*, either live bacteria or MVs, significantly antagonized natural transformation in *S. mutans* NG8.

Boiling of periodontal pathogens abolished their antagonism for *S. mutans* natural transformation

We then compared the ability to antagonize *S. mutans* natural transformation in different periodontal pathogens. As shown in Table 3, the transformation efficiency of *S. mutans* LT11, like *S. mutans* NG8, was significantly antagonized by *P. gingivalis*. Another periodontal pathogen, *T. denticola* 35405, also significantly antagonized the natural transformation of *S. mutans* strain LT11. Boiling of periodontal pathogens before mixed incubation with *S. mutans* abolished their antagonism for *S. mutans* natural transformation.

Periodontal pathogens abolished *S. mutans* bacteriocin

To investigate interactions among oral bacteria relative to another CSP-dependent property in *S. mutans*, we performed broth assays to determine bacteriocin production by *S. mutans* BM71. Strain BM71 was chosen for the study, since it produces bacteriocin that kills an indicator strain, RP66, whereas NG8 and LT11 do not. Both periodontal pathogens, *P. gingivalis* 381 and *T. denticola* 35405, completely abolished bacteriocin production by *S. mutans* BM71 in the mixed cultures, which was demonstrated by no inhibition of RP66 growth (Table 4).

We did titration to determine the amount of bacteriocin from *S. mutans* BM71 monocultures for the complete inhibition of RP66 growth. <50 μL bacteriocin supernatants did not

consistently inhibit RP66 growth, while >100 μL volumes did. We therefore utilized 300 μL bacteriocin supernatants in the bacteriocin assays, which ensured that any growth of RP66 is due to the decrease in bacteriocin, not from experimental error. These volumes of bacteriocin supernatants (300 μL) completely inhibited the growth of RP66 and did not change over time such as after overnight incubation. However, the growth of RP66 without bacteriocin (either with or without the supernatants from the mixed cultures of periodontal pathogens) did increase over time.

Periodontal pathogens inactivated exogenous *S. mutans* CSP

We have demonstrated previously that *S. gordonii* inactivated *S. mutans* CSP, which in turn interfered with bacteriocin production, a Com quorum-sensing-dependent phenomenon. Since *P. gingivalis* 381 and *T. denticola* 35405 interfered with two of the CSP-dependent properties in *S. mutans* and boiling of these two periodontal pathogens abolished the interference (Tables 2, 3, and 4), we carried out experiments to determine the ability of *P. gingivalis* 381 and *T. denticola* 35405 to inactivate *S. mutans* CSP. As shown in Fig. 1, the supernatant fluids from both *P. gingivalis* 381 and *T. denticola* 35405 inactivated *S. mutans* CSP. Boiling of the supernatants before *S. mutans* CSP inactivation abolished such inhibition.

We carried out additional experiments to exclude the possibility that the supernatant fluids from *P. gingivalis* 381 and *T. denticola* 35405 inactivated the bacteriocin produced by the *S. mutans*, instead of inactivating *S. mutans* CSP per se. The supernatant fluids from *P. gingivalis* 381 or *T. denticola* 35405 were mixed with existing *S. mutans* bacteriocin (produced by the *S. mutans comC* mutant in the presence of CSP) at the same ratio (1:10 in volume) as in the above-mentioned well assays for the CSP activity assessment. After 2 h incubation at 37°C, *S. mutans* bacteriocin in the mixtures was still functional and inhibited RP66 growth (data not shown).

Discussion

Periodontal pathogens such as *P. gingivalis* and *T. denticola* can dominate at the sites of periodontal diseases. It is not yet understood how these late colonizers antagonize the earlier dental plaque colonizers. The *comCDE* operon has been demonstrated in multiple oral streptococci such as *S. mutans*, *S. gordonii*, *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus sanguinis* (26,27) and may be generalized targets for bacterial antagonism. We chose *S. mutans* as an example of oral streptococci in the present study, since it has several CSP-dependent virulence properties. However, other oral streptococci do not have demonstrable CSP-dependent properties *in vitro*, despite the presence of CSP-dependent quorum sensing pathways. We realize that *S. mutans* is not commonly found in subgingival sites and therefore our findings may or may not be applicable to other oral streptococcal species mentioned above. However, our preliminary data has demonstrated that the Com-dependent natural transformation in *S. gordonii* was also abolished by *P. gingivalis* in mixed broth assays (data not shown). This observation suggests that our findings may be relevant for other oral streptococci as well.

We have previously reported that proteases produced by other species of oral streptococci such as *S. gordonii* interfere with the quorum sensing properties of *S. mutans* (12). It has been well documented that some of the periodontal pathogens produce proteases. Using BANA assays, we confirmed the presence of proteases in *P. gingivalis* 381 and *T. denticola* 35405 (data not shown). Our studies were designed to determine if periodontal pathogens such as *P. gingivalis* or *T. denticola* interrupt quorum sensing in *S. mutans* and if multiple CSP-dependent quorum-sensing properties in *S. mutans* are antagonized by these late colonizers as did at least one early colonizer.

Our results indicated that both *P. gingivalis* and *T. denticola* can attenuate some of the virulence properties of *S. mutans* by altering the quorum-sensing-dependent properties of the organism. Tables 2, 3, and 4 demonstrated that both *P. gingivalis* and *T. denticola* antagonized two quorum-sensing dependent properties in *S. mutans*. In transformation assays, strains NG8 and LT11 were used for transformation efficiency assessment since *S. mutans* NG8 and LT11 exhibit high CSP-dependent transformation efficiencies. However, *S. mutans* NG8 or LT11 do not produce bacteriocin that kills the indicator strain, RP66, while *S. mutans* BM71 does.

The THB medium and static aerobic culturing conditions utilized in our experiments do not support the growth of periodontal pathogens. In addition, the levels of periodontal pathogens in the mixed cultures are relatively low (10^7 or 5×10^7 cfu). We have also carried out experiments to determine *S. mutans* viability in monocultures and its mixtures with *P. gingivalis* under the conditions used for both natural transformation and bacteriocin production. There was no decrease in viability of *S. mutans* in the mixed cultures relative to its monocultures (data not shown).

Our results indicated that antagonism of *S. mutans* quorum sensing is not restricted to a particular species of oral bacteria and periodontal pathogens affected multiple quorum-sensing properties in several *S. mutans* strains. Since it is well-documented that both *P. gingivalis* and *T. denticola* produce multiple proteases, our results indicated that heat-sensitive proteins, speculated to be proteases, from periodontal pathogens are responsible for inactivation of *S. mutans* CSP.

It has been reported that a large number of Gram-negative bacteria form and release MVs during growth (31). Because of their small dimensions (approximately 50-150 nm), MVs might more easily reach inaccessible areas such as the interior of biofilms, relative to their whole bacteria counterparts. Furthermore, MVs could have bacteriolytic effects on both Gram-positive and Gram-negative bacteria (32). Since *P. gingivalis* generates MVs, we compared live bacteria and MVs of *P. gingivalis* for their ability to antagonize *S. mutans* natural transformation. As shown in Table 2, *P. gingivalis* MVs exhibited a similar capacity to antagonize *S. mutans* transformation, relative to live bacteria.

Taken together, our results suggest that the presence of periodontal pathogens in dental plaque could modulate the virulence properties of *S. mutans* by interfering with its Com quorum sensing system. Since the Com quorum sensing system exists in many species of earlier dental plaque colonizers, this interference of quorum sensing by periodontal pathogens such as *P. gingivalis* and *T. denticola* could, at least in part, be a mechanism of bacterial antagonism in periodontal diseases.

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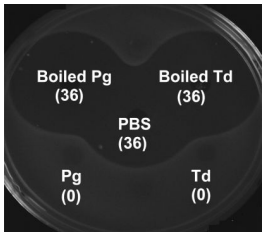

Figure 1.

The supernatants from broth cultures of *P. gingivalis* 381 or *T. denticola* 35405 (with or without boiling) were incubated with exogenous *S. mutans* CSP for 2 h and added to the *comC* mutant cells of *S. mutans* BM71. The bacteriocin activity was assayed by inoculating the supernatants into pre-cut wells in THB plates. After absorption of the supernatants, the plates were overlaid with RP66 indicator cells and incubated at 37°C overnight. The presence of bacteriocin in the culture supernatants of the *comC* mutant would inhibit RP66 growth, which indicated the presence of functional exogenous CSP. The diameters of the inhibition zones surrounding the wells (mm) were shown in brackets.

Table 1

Bacteria used in this study

S. mutans NG8	WT, for genetic transformation assays
S. mutans LT11	WT, for genetic transformation assays
S. mutans BM71	WT, for bacteriocin assays
S. mutans comC mutants	No CSP production due to inactivation of the *comC* gene
RP66	Group C *Streptococcus*, an indicator strain that is sensitive to *S. mutans* bacteriocin
P. gingivalis 381	WT, late colonizer
T. denticola 35405	WT, late colonizer

Table 2

Competent *S. mutans* NG8 and mixtures with *P. gingivalis* or MVs from *P. gingivalis* were transformed with exogenous chromosomal DNA *gtfD* and the transformation frequencies are indicated as numbers of tetracycline-resistant colonies/100 μL. Assessments were performed in triplicate in two independent experiments. Data presented are the means ± standard deviation (n=6).

Species	Transformation Efficiency (colonies/100 μL)
S.m. NG8	437.5 ± 53.9
S.m. NG8 + *P.g.* 381	11.6 ± 4.7
S.m. NG8 + *P.g.* 381 MVs	14.6 ± 3.8
S.m. NG8 *comC*$^{-}$	0.3 ± 0.5

Table 3

Competent *S. mutans* LT11 and mixtures with *P. gingivalis* 381 or *T. denticola* 35405 were transformed with pTet, which transforms only *S. mutans. P. gingivalis* and *T. denticola* cells were boiled for 10 minutes before mixed culturing in the experimental groups 3 and 5. The transformation frequencies are indicated as numbers of tetracycline resistant colonies/100 μL. Assessments were performed in triplicate in two independent experiments. Data presented are the means ± standard deviation (n=6).

Species	Transformation Efficiency (colonies/100 μL)
S.m. LT11	691.8 ± 67.0
S.m. LT11 + *P.g.* 381	0.5 ± 0.8
S.m. LT11 + Boiled *P.g.* 381	682.0 ± 90.9
S.m. LT11 + *T.d.* 35405	13.5 ± 3.5
S.m. LT11 + Boiled *T.d.* 35405	703.8 ± 62.9

Table 4

Bacteria were cultured in THB broth at 37°C for 24 hours. Supernatants containing bacteriocin from the cultures were neutralized to pH 7.0, filtered through 0.22 μm pore size filters. RP66 cells were grown in the presence of the supernatants and incubated at 37°C for 5-6 hours. Assessments were performed in triplicate in two independent experiments. Data presented are the means ± standard deviation (n=6).

Supernatant Sources	**RP66 Growth (OD_{600})**
S.m. BM71	0.020 ± 0.009
S.m. BM71 + *P.g.* 381	0.601 ± 0.071
S.m. BM71 + Boiled *P.g.* 381	0.019 ± 0.011
S.m. BM71 + *T.d.* 35405	0.598 ± 0.062
S.m. BM71 + Boiled *T.d.* 35405	0.020 ± 0.009
None	0.586 ± 0.046