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

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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 the 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 an earlier colonizer antagonized by periodontal pathogens.

Material and Methods: In this study, *S. mutans* NG8 and *S. mutans* LT11 were used in experiments assessing transformation, and *S. mutans* BM71 was used in experiments investigating bacteriocin production. The effects of the periodontal pathogens *Porphyromonas gingivalis* and *Treponema denticola* on these competence-stimulating peptide-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. The production of bacteriocin by *S. mutans* BM71 was also inhibited by *P. gingivalis* and *T. denticola*. Boiling of these late colonizers before addition to the broth cultures abolished their ability to inhibit *S. mutans* transformation and bacteriocin production. *P. gingivalis* and *T. denticola* inactivated *S. mutans* exogenous competence-stimulating peptide, whereas the boiled bacteria did not.

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

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Periodontal diseases are among the most common chronic infectious diseases to occur in humans. According to the National Institute of Dental and Craniofacial Research, periodontal diseases affect the majority of the American population (1). Oral chronic 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 preterm 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 that is encoded by the *comD* and *comE* genes, corresponding to a histidine protein kinase (a 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 *S. mutans* GS5 also modulates the sensitivity of *S. mutans* to a variety of antimicrobial agents, such as triclosan, fluoride, antibiotics and histatin-5. Therefore, the CSP represents a potential target for controlling infection with *S. mutans*.

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 has also 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 bacterial species with increased virulence. As 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 an important antagonistic mechanism used by bacteria (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 these 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 early colonizer that is antagonized by periodontal pathogens such as *P. gingivalis* and *T. denticola*.

Material 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 in 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, 40 g of 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. The 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 20-fold in THB containing 10% horse serum and cultured at 37°C for 30 min to induce competence. For the transformation of mixtures of *S. mutans* with other periodontal pathogens, bacteria at the stationary phase were diluted 20-fold in THB containing 10% horse serum and mixed at a 1 : 1 ratio to induce competence. In some experiments, the periodontal pathogens were boiled for 10 min before mixing. Competent *S. mutans* recipient

Table 1. Bacteria used in this study

Streptococcus 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 because of inactivation of the *comC* gene
RP66	Group C *Streptococcus*, an indicator strain that is sensitive to *S. mutans* bacteriocin
Porphyromonas gingivalis 381	WT, late colonizer
Treponema denticola 35405	WT, late colonizer

CSP, competence-stimulating peptide; WT, wild type.

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 h. Transformants were then sonicated for 3 times using a Branson sonifier (output 3, cycle 30) and selected on TSA agar plates supplemented with tetracycline (10 μg/mL). Transformation frequencies were determined after 48–72 h of anaerobic incubation in an anaerobic jar 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 filtration through 0.22-μm-pore-size filters. Vesicles were recovered from the resulting filtrates by ultracentrifugation (150,000 *g* for 2 h at 4°C) in a Ti 75 rotor (Beckman Instruments, Inc., Fullerton, CA, USA). Isolated MVs were resuspended in phosphate-buffered saline (in a volume that was 100-fold smaller than the original culture supernatant volume) and stored at −70°C for use in transformation experiments. For transformation experiments, MVs (40 μL) were added to 100 μL of 20-fold-diluted *S. mutans* NG8 for induction of competence.

Bacteriocin production and assays

S. mutans BM71 cells [10^7 colony-forming units (CFU)], or mixtures of *S. mutans* BM71 (10^7 CFU) with *P. gingivalis* 381 or *T. denticola* 35405 (10^7 or 5×10^7 CFU, respectively), were cultured in 1 mL of THB supplemented with 3% yeast extract, at 37°C for 24 h. For some experiments, the periodontal pathogens were boiled for 10 min before use in mixed cultures. Supernatants containing culture-derived bacteriocin 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 use in subsequent assays. RP66 cells (2×10^5 CFU) in 0.7 mL of THB were grown in the presence of the above-mentioned supernatant fluids (300 μL) and incubated at 37°C for 5–6 h. The optical density at 600 nm (OD_{600nm}) was measured and used to indicate 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 stationary phase in TSB or TYGVS, respectively, were filtered through 0.22-μm-pore-size 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: SGSLSTFFRL FNRSFTQALGK (13); synthesized by Sigma-Genosys, The Woodlands, TX, USA; 2.5 μg/mL final concentration] was incubated with the supernatants at 37°C for 2 h. The CSP thus treated was added, to a final concentration of 0.25 μg/mL, to *S. mutans* BM71 *comC* mutant cells (10^7 CFU) in 0.9 mL of THB supplemented with 3% yeast extract. After 24 h of incubation at 37°C, the supernatants from the cultures were passed through 0.22-μm-pore-size filters and the bacteriocin production by the *S. mutans* BM71 *comC* mutant was determined using the agar-well assay.

Agar-well assays

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

Statistical analysis

The Student's *t*-test was performed to determine significance. The difference was considered significant when a p value of < 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 therefore the *comC* knockout mutant exhibited minimum transformation efficiency, relative to its parent strain (Table 2). Both live *P. gingivalis* and MVs of *P. gingivalis* significantly antagonized natural transformation of *S. mutans* NG8.

Boiling of periodontal pathogens abolished their ability to antagonize the natural transformation of *S. mutans*

We then compared the ability of the periodontal pathogens to antagonize

Table 2. Effect of *Porphyromonas gingivalis* 381, or membrane vesicles (MVs) from *P. gingivalis* 381, on the transformation efficiency of competent *Streptococcus mutans* NG8

Species	Transformation efficiency (colonies/100 μL)
S. mutans NG8	437.5 ± 53.9
S. mutans NG8 + *P. gingivalis* 381	11.6 ± 4.7
S. mutans NG8 + *P. gingivalis* 381 MVs	14.6 ± 3.8
S. mutans NG8 *comC*$^-$	0.3 ± 0.5

Competent *Streptococcus mutans* NG8, alone, or mixed with *Porphyromonas gingivalis* or with MVs from *P. gingivalis* were transformed with exogenous chromosomal DNA *gtfD* and the transformation frequencies (indicated as the numbers of tetracycline-resistant colonies per 100 μL) were measured. The *S. mutans* NG8 *comC*$^-$ mutant was included as a control. Assessments were performed in triplicate in two independent experiments. Data presented are the means ± standard deviation ($n = 6$).

Table 3. Effect of boiled and native *Treponema denticola* 35405 and *Porphyromonas gingivalis* 381 on the transformation efficiencies of competent *Streptococcus mutans* LT11

Species	Transformation efficiency (colonies/100 μL)
S. mutans LT11	691.8 ± 67.0
S. mutans LT11 + *P. gingivalis* 381	0.5 ± 0.8
S. mutans LT11 + boiled *P. gingivalis* 381	682.0 ± 90.9
S. mutans LT11 + *T. denticola* 35405	13.5 ± 3.5
S. mutans LT11 + boiled *T. denticola* 35405	703.8 ± 62.9

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

the natural transformation of *S. mutans*. 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* LT11. Boiling of the periodontal pathogens before mixed incubation with *S. mutans* abolished their ability to antagonize the natural transformation of *S. mutans*.

Periodontal pathogens abolished *S. mutans* bacteriocin

To investigate the 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 because 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 performed titration to determine the amount of bacteriocin from *S. mutans* BM71 monocultures required to completely inhibit the growth of RP66. Bacteriocin supernatants of < 50 μL did not consistently inhibit RP66 growth, while those of > 100 μL did. We therefore utilized 300 μL of bacteriocin supernatants in the bacteriocin assays, which ensured that any growth of RP66 was caused by the decrease in bacteriocin, not from experimental error. These volumes of bacteriocin supernatants (300 μL) completely inhibited the growth of RP66 and this 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.

Table 4. Effect of bacteriocin on the growth of RP66 cells

Supernatant sources	RP66 growth (OD_{600})
Streptococcus mutans BM71	0.020 ± 0.009
S. mutans BM71 + *Porphyromonas gingivalis* 381	0.601 ± 0.071
S. mutans BM71 + Boiled *P. gingivalis* 381	0.019 ± 0.011
S. mutans BM71 + *Treponema denticola* 35405	0.598 ± 0.062
S. mutans BM71 + Boiled *T. denticola* 35405	0.020 ± 0.009
None	0.586 ± 0.046

Bacteria were cultured in Todd Hewitt broth at 37°C for 24 h. Culture supernatants containing bacteriocin were neutralized to pH 7.0 and then 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 h. Assessments were performed in triplicate in two independent experiments. Data presented are the means ± standard deviation ($n = 6$).

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. As *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 2–4), 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 culture supernatants

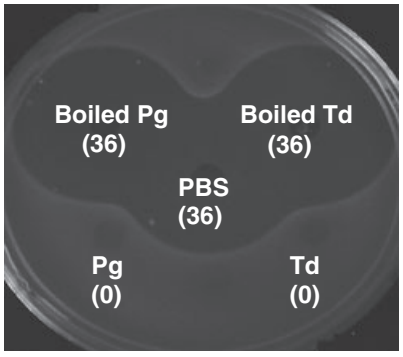

Fig. 1. The supernatants from broth cultures of *Porphyromonas gingivalis* 381 or *Treponema denticola* 35405 (with or without boiling) were incubated with exogenous *Streptococcus mutans* competence-stimulating peptide (CSP) for 2 h and added to the *comC* mutant cells of *S. mutans* BM71. The bacteriocin activity was assayed by inoculating the supernatants into pre-cut wells in Todd Hewitt broth (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 inhibited RP66 growth, which indicated the presence of functional exogenous CSP. The diameters of the inhibition zones surrounding the wells (mm) are shown in brackets. Pg, *Porphyromonas gingivalis* 381; Td, *Treponema denticola* 35405.

from both *P. gingivalis* 381 and *T. denticola* 35405 inactivated *S. mutans* CSP. Boiling of the supernatants before inactivation of *S. mutans* CSP abolished such inhibition.

We carried out additional experiments to exclude the possibility that the culture supernatants 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 culture supernatants 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, v/v) as in the above-mentioned well assays used to assess the CSP activity. After 2 h of 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 found in multiple oral streptococci such as *S. mutans*, *S. gordonii*, *Streptococcus mitis*, *Streptococcus oralis* and *Streptococcus sanguinis* (26,27) and may be a general target for bacterial antagonism. We chose *S. mutans* as an example of oral streptococci in the present study because 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 the other oral streptococcal species mentioned above. However, our preliminary data have 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 also be applicable to other oral streptococci.

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 periodontal pathogens produce proteases. Using N-benzoyl-DL-arginine-naphthylamide 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 they were by 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. The results shown in Tables 2–4 demonstrated that both *P. gingivalis* and *T. denticola* antagonized two quorum-sensing-dependent properties in *S. mutans*. In transformation assays, *S. mutans* strains NG8 and LT11 were used to assess transformation efficiency because they 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 culture conditions utilized in our experiments do not support the growth of periodontal pathogens. In addition, the levels of periodontal pathogens in the mixed cultures were relatively low (10^7 or 5×10^7 CFU). We carried out experiments to determine the viability of *S. mutans* in monocultures and when mixed with *P. gingivalis* under the conditions used for both natural transformation and bacteriocin production. There was no decrease in the viability of *S. mutans* in mixed cultures relative to 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. As 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 the 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 reach inaccessible areas, such as the interior of biofilms, more easily than their whole-bacteria counterparts. Furthermore, MVs could have bacteriolytic effects on both gram-positive and gram-negative bacteria (32). Because *P. gingivalis* generates MVs, we compared the ability of live bacteria and isolated MVs of *P. gingivalis* to antagonize the natural transformation of *S. mutans*. 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. Because 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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