

Interactions between Oral Bacteria: Inhibition of *Streptococcus mutans* Bacteriocin Production by *Streptococcus gordonii*

Bing-Yan Wang and Howard K. Kuramitsu*

Department of Oral Biology, State University of New York, Buffalo, New York

Received 28 May 2004/Accepted 25 August 2004

***Streptococcus mutans* has been recognized as an important etiological agent in human dental caries. Some strains of *S. mutans* also produce bacteriocins. In this study, we sought to demonstrate that bacteriocin production by *S. mutans* strains GS5 and BM71 was mediated by quorum sensing, which is dependent on a competence-stimulating peptide (CSP) signaling system encoded by the *com* genes. We also demonstrated that interactions with some other oral streptococci interfered with *S. mutans* bacteriocin production both in broth and in biofilms. The inhibition of *S. mutans* bacteriocin production by oral bacteria was stronger in biofilms than in broth. Using transposon Tn916 mutagenesis, we identified a gene (*sgc*; named for *Streptococcus gordonii* challisin) responsible for the inhibition of *S. mutans* bacteriocin production by *S. gordonii* Challis. Interruption of the *sgc* gene in *S. gordonii* Challis resulted in attenuated inhibition of *S. mutans* bacteriocin production. The supernatant fluids from the *sgc* mutant did not inactivate the exogenous *S. mutans* CSP as did those from the parent strain Challis. *S. gordonii* Challis did not inactivate bacteriocin produced by *S. mutans* GS5. Because *S. mutans* uses quorum sensing to regulate virulence, strategies designed to interfere with these signaling systems may have broad applicability for biological control of this caries-causing organism.**

Cell-cell communication is a fundamental activity performed by most types of cells. Previously, bacteria were considered to exist as individual cells that scavenge for nutrients and multiply independently. However, recent studies have shown that bacteria are capable of coordinated activities, such as quorum sensing (13). Quorum sensing is the control of gene expression in response to cell density. The mediators for this intercellular communication among bacterial cells are self-secreted signaling molecules called autoinducers. These signaling molecules include *N*-acylhomoserine lactones, used by gram-negative bacteria (8), and small peptides, effectors of gram-positive bacteria (6). Gram-positive bacteria use two-component signal transduction systems for detection of autoinducers (13). Quorum sensing also regulates virulence in many human pathogens (2). Examples of quorum-sensing-dependent systems in gram-positive bacteria include the formation of biofilms (18, 33), the development of genetic competence (10, 16), the acid tolerance response (18), and the production of antimicrobial peptides (12), which may all provide a competitive advantage over other organisms. Recent studies have shown that quorum sensing modulates both intra- and interspecies cell-cell communications. For example, there is cross-inhibition by quorum-sensing pheromones between *Staphylococcus aureus* and *Staphylococcus epidermidis* (21). *S. epidermidis* pheromones inhibited the *S. aureus agr* response, which may explain the predominance of *S. epidermidis* on the skin. Interaction between *Bacillus subtilis* and *Erwinia carotovora* provides another example (5), since the former produces an enzyme (AiiA) that inactivates the *E. carotovora* autoinducers. Inactivation of the

autoinducers by AiiA attenuated the virulence of *E. carotovora*.

In the oral cavity, bacteria interact with each other as they attempt to establish themselves in this environment. Oral streptococci are gram-positive, facultative anaerobes that initiate the formation of biofilms (known as dental plaque) on tooth surfaces. *Streptococcus mutans*, one of the colonizers of dental plaque, exhibits an acid tolerance response (16). Some *S. mutans* strains are naturally transformable (14, 15) and produce bacteriocins (3, 22–24). Recent studies have demonstrated that biofilm formation, acid tolerance, and the transformation of *S. mutans* are mediated by quorum sensing (4). This signaling system depends on a competence-stimulating peptide (CSP) and a two-component signal transduction system, which are encoded by the *comCDE* genes, corresponding to the CSP precursor, a histidine kinase (receptor for CSP), and a response regulator, respectively (4, 13, 20). By interfering with this cell-cell signaling mechanism, caries-causing *S. mutans* bacteria that use quorum sensing to control virulence could potentially be rendered avirulent.

Many gram-positive bacteria produce antimicrobial peptides called bacteriocins (11, 28). 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 (31). For example, a mixed culture of bacteriocin-producing *S. mutans* SW31 with sensitive *Streptococcus sanguis* Ny101 resulted in a plaque composed primarily of *S. mutans* SW31. A nonbacteriocinogenic mutant of *S. mutans* SW31 allowed substantial growth of *S. sanguis* Ny101 (32). It has been shown that production of these antimicrobial peptides is frequently regulated by a two-component regulatory system through a quorum-sensing system in some gram-positive bacteria (12). However, this has not yet been demonstrated for *S. mutans* bacteriocin production.

Fabio et al. (7) studied a sample of human streptococci

* Corresponding author. Mailing address: Department of Oral Biology, State University of New York at Buffalo, 3435 Main St., Buffalo, NY 14214. Phone: (716) 829-2068. Fax: (716) 829-3942. E-mail: kuramits@buffalo.edu.

(mainly *S. mutans* species) from dental plaques to evaluate the production frequency and activity spectrum of bacteriocins. Most of the 55 *S. mutans* strains (89%) produced substances with a wide spectrum of antibacterial activity. The bacteriocins produced showed a marked inhibitory activity against gram-positive bacteria. How interspecies interactions with other oral bacteria affects *S. mutans* bacteriocin production is not yet understood. The objectives of the present study were to demonstrate that *S. mutans* bacteriocin production is a quorum-sensing-controlled phenomenon and to establish the role of interactions with other oral bacteria on bacteriocin production by *S. mutans*.

MATERIALS AND METHODS

Bacterial strains and media. *S. mutans* GS5 and its *comC*, *comD*, and *comE* mutants (33), *S. mutans* BM71 and its *com* mutants (obtained from D. G. Cvitkovich, University of Toronto, Toronto, Ontario, Canada), *S. mutans* NG8, *Streptococcus gordonii* Challis, *S. gordonii* 10558, *S. sanguis* 10556, *Streptococcus mitis* 10712, and *Streptococcus oralis* KS32AR were maintained on tryptic soy agar (TSA) (Difco, Detroit, Mich.) plates supplemented with erythromycin (10 µg/ml) where indicated. Bacteria were routinely cultured in Todd-Hewitt broth (THB) (Difco). A group C streptococcal strain RP66 was used as an indicator for assays of *S. mutans* bacteriocin activity (22). *Escherichia coli* DH5α competent cells (Life Technologies, Gaithersburg, Md.) were used in construction of an *S. gordonii* Challis inactivation vector.

Bacteriocin production and assays. (i) **Agar plate assays.** *S. mutans* GS5 or BM71 cells in the presence or absence of other oral streptococcal cells were inoculated into THB agar plates with or without the addition of exogenous synthetic CSP (3 µg in 3 µl) of *S. mutans* (the amino acid sequence of CSP was SGSLSTFFRLFNRSFTQALGK [17]) (synthesized by Sigma-Genosys, The Woodlands, Tex.) for stab cultures. After 24 h of incubation at 37°C under anaerobic conditions, the plates were overlaid with indicator RP66 cells (10⁶ CFU) in 3 ml of THB with 1% low-melting-point agarose (BioProducts, Rockland, Maine). After 24 more hours of incubation at 37°C under anaerobic conditions, the diameters of the clearance zones surrounding the inoculated bacteria (which indicated the presence of bacteriocin produced by *S. mutans*) were measured.

(ii) **THB broth assays.** *S. mutans* GS5 or BM71 cells (10⁷ CFU) or mixtures with other oral streptococcal cells at equal densities were cultured in 1.0-ml portions of THB supplemented with 3% yeast extract (THBY) with or without the addition of exogenous synthetic CSP (final concentration, 1.0 µg/ml) for 24 h at 37°C. Supernatant fluids containing bacteriocin from the cultures were filtered through 0.22-µm-pore-size filters and either assayed immediately or frozen at -20°C for subsequent assays. RP66 cells (2 × 10⁵ CFU) in 0.7 ml of THB were grown in the presence of the supernatant fluids mentioned above and incubated at 37°C for 5 to 6 h. The optical density at 600 nm (OD₆₀₀) was measured and indicated the presence or absence of bacteriocin. The presence or absence of bacteriocin using arbitrary units (microliters of supernatant used to obtain complete inhibition of RP66 growth) was scored as follows: 10 µl, +++; 100 µl, ++; 300 µl, +; and no inhibition, -.

(iii) **Agar well assays.** The supernatant fluids containing bacteriocin from *S. mutans* GS5 cultures were added into precut wells in THB agar plates and incubated at 37°C for 24 h. The wells were then filled using THB with 1% low-melting-point agarose, and the plates were overlaid with the indicator strain RP66 (10⁶ CFU) in 3 ml of THB with 1% low-melting-point agarose. After 24 more hours of incubation at 37°C under anaerobic conditions, the diameters of the inhibition zones surrounding the wells were measured.

(iv) **Killing assays.** A 100-µl aliquot from *S. mutans* GS5 or BM71 or mixtures with *S. gordonii* Challis from broth or biofilm cultures was mixed with 5 × 10⁴ CFU of RP66 indicator cells (in 100 µl of THB) and incubated at 37°C for 1 h (modified version of the method of Paul and Slade [22]). Two-microliter aliquots of the cultures were plated on the surfaces of TSA plates and incubated for another 24 h at 37°C under anaerobic conditions. The percent killing of RP66 cells was calculated as follows: [100 - (number of colonies in the presence of supernatants/number of colonies in THB)] × 100.

Effects of oral streptococci on the CSP of *S. mutans*. Supernatant fluids from various oral streptococci grown to the stationary phase in THB were filtered through 0.22-µm-pore-size filters to eliminate cellular components. The supernatant fluids were then neutralized with NaOH to pH 7.0 to 7.5. Exogenous

synthetic *S. mutans* CSP (final concentration, 2.5 µg/ml) was incubated with supernatant fluid at 37°C for 0.5 to 3 h. For some experiments, part of the supernatant fluid was boiled for 10 min before the incubation with CSP. CSP thus treated was added to *S. mutans* GS5 *comC* mutant cells (10⁷ CFU) in 1.0 ml of THBY to a final concentration of 0.25 µg/ml. After a 24-h incubation at 37°C, the supernatant fluids from the cultures were passed through 0.22-µm-pore-size filters, and the bacteriocin level produced by the *S. mutans* GS5 *comC* mutant was determined by the agar well assays.

Transposon mutagenesis of *S. gordonii* Challis. *S. gordonii* Challis cells were randomly mutagenized by transformation with pAM620, a plasmid containing transposon Tn916 (9). The individual clones of the transformants on TSA plates supplemented with tetracycline (10 µg/ml) were cultured in THB. Mixtures with *S. mutans* GS5 in THB (0.7:1 by volume at the stationary phase) were inoculated into THB agar plates for stab cultures, and the clones with decreased ability to inhibit *S. mutans* GS5 bacteriocin production were selected for further investigation.

Sequencing of chromosomal DNA flanking Tn916 from insertional mutants of *S. gordonii* Challis. Chromosomal DNA was extracted from a Tn916 insertion mutant of *S. gordonii* Challis (designated 41A9) using a Puregene DNA isolation kit (Gentra System, Inc., Minneapolis, Minn.). The DNA was digested with BclI (with a cutting site 2.2 kb from the N terminus of Tn916) and a combination of BclI with SalI, HindIII, PstI, KpnI, SacI, or EcoRI. Southern blot analysis was performed according to the protocol for the ECL detection system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom) using PCR products of the N-terminal end of Tn916 as the probe. The DNA fragments digested with PstI and BclI were ligated with pUC19 (PstI and BamHI digested) using T4 ligase. The ligation mixtures were then used as PCR templates. PCR was performed using one primer of the Tn916 sequence (5'-CGCTCGTATCAAAGCTC-3') and another primer from the pUC19 vector sequence (5'-AGCTC ACTCATTAGGCAC-3'). Both primers were designed in the 3' direction toward the chromosomal DNA flanking Tn916. The resulting PCR fragments were sequenced by the sequencing facility at the Roswell Park Cancer Institute (Buffalo, N.Y.).

Construction of the *S. gordonii* Challis protease knockout mutant BYW1. pPE (Challis protease knockout inactivation vector) was constructed as described in Results (see Fig. 3). A plasmid, pACYC184, that replicates only in *E. coli*, was digested with BstYI. The resulting 2.67-kb fragment was blunt ended with T4 DNA polymerase and self-ligated with T4 ligase to obtain pACYC-b. A 1.9-kb PCR product, obtained using primers at the N-terminal end of the *S. gordonii* Challis *sgc* gene (forward primer, 5'-ACGAAGCTTTACGAGGTGTCAG-3'; reverse primer, 5'-CAAGTCGCAAGCTTCTGAAG-3'), was digested with HindIII (HindIII sites were designed into the primers) and ligated with pACYC-b (digested with HindIII), using T4 ligase. The resulting plasmid, pACYC-1.9, was then digested with BstYI to delete a 1.0-kb fragment of the *sgc* gene fragment. The remaining 3.57-kb pACYC-1.9 fragment was blunt ended using T4 DNA polymerase and ligated to the SmaI-digested Erm cassette (released from pEMnP [B. Wang, personal communication]), using T4 ligase. The final plasmid, pPE, contained two 400-bp fragments of the *sgc* gene flanking the Erm cassette. *S. gordonii* Challis strains with knockout mutations of the *sgc* gene were constructed via a double crossover event into the Challis chromosome at the predicted *sgc* site by transforming Challis using XbaI-linearized pPE. The mutants were selected on TSA plates supplemented with erythromycin (10 µg/ml).

Zymography of supernatant fluids from *S. gordonii* Challis and its protease mutants. The supernatant fluids from broth cultures of *S. gordonii* Challis or *sgc* mutants grown overnight were filtered through 0.22-µm-pore-size filters and precipitated with 40% (wt/vol) ammonium sulfate at room temperature for 1 h, with constant shaking. After 30 min of centrifugation at 15,000 × g, the pellets were resuspended in phosphate-buffered saline (PBS) and 30-K Centricon ultrafilters (Millipore Co., Bedford, Mass.) were utilized to further concentrate the supernatant fluids. The supernatant fluids (concentrated 500-fold) were applied to ready-made casein gels (Bio-Rad, Hercules, Calif.), and zymography was performed following the supplier's protocol.

Azocasein assays for the protease activity of *S. gordonii* Challis. Azocasein assays were performed by a modified version of the method of Sojar et al. (27). Briefly, 50-ml cultures of *S. gordonii* Challis or its *sgc* mutants grown overnight were centrifuged at 4,000 × g for 10 min. The supernatant fluids from these 50-ml cultures were then concentrated as described above for the zymography experiments. The cell pellets and the concentrated supernatant fluids from 50-ml cultures were utilized in the azocasein assays. The OD₄₂₀ measured in these assays indicated the amount of protease present.

Biofilm formation. *S. mutans* BM71 cells (10⁷ CFU) or mixtures with other oral streptococcal cells were inoculated into 2-ml portions of one-quarter strength THB supplemented with 0.01% mucin (Sigma Chemical Co., St. Louis,

TABLE 1. Production of bacteriocin by *S. mutans* GS5 or BM71 in agar plate assays^a

Bacterium	Diameter of inhibition zone (mm) ^b			
	Without CSP		With CSP	
	GS5	BM71	GS5	BM71
Wild-type	38.3 ± 1.7	39.0 ± 0.8	39.0 ± 2.4	39.3 ± 1.2
<i>comC</i> mutant	3.7 ± 2.9	1.7 ± 1.2	38.7 ± 2.1	39.0 ± 0.8
<i>comD</i> mutant	2.7 ± 2.1	1.3 ± 0.5	2.3 ± 1.7	1.7 ± 0.5
<i>comE</i> mutant	2.3 ± 1.7	1.7 ± 0.5	3.0 ± 2.2	2.3 ± 0.5

^a *S. mutans* GS5 or BM71 or their *com* mutant cells were inoculated into THB agar plates with or without the addition of exogenous synthetic CSP (3 µg in 3 µl) for stab cultures. After 24 h of incubation at 37°C under anaerobic conditions, the plates were overlaid with indicator RP66 cells (10⁶ CFU) in 3 ml of THB with 1% low-melting-point agarose. After 24 more hours of incubation at 37°C under anaerobic conditions, the diameters of the clearance zones surrounding the inoculated bacteria were measured.

^b Values are means ± standard deviations from three experiments.

Mo.) and 0.1% glucose (biofilm medium [BM]) (17) in six-well polystyrene microtiter plates to form biofilms. After 24 h of incubation at 37°C under aerobic conditions, the planktonic cells were discarded, and 1.5 ml of THBY was added to each well containing biofilm cells and incubated at 37°C for another 24 h for bacteriocin production by *S. mutans* BM71 cells.

Reverse transcription-PCR. Total RNA from *S. mutans* BM71 or GS5 alone and in mixtures with other streptococci was isolated from 15-ml samples of cultures (OD₆₀₀ readings of 1.0 to 1.2). The cells were centrifuged at 4,000 × *g* for 10 min at 4°C. The cell pellets were resuspended in diethyl pyrocarbonate-treated water, and the suspensions were mixed vigorously with Lysing Matrix B (Q. Biogene, Carlsbad, Calif.) and Trizol (Invitrogen, Carlsbad, Calif.). After two washes with chloroform, RNA was precipitated with isopropanol. The RNA pellets were washed with 70% ethanol and resuspended in diethyl pyrocarbonate-treated water. After DNase (Promega, Madison, Wis.) treatment, 1 µg of RNA from each sample was utilized for reverse transcription, using SuperScript III RNase H⁻ reverse transcriptase (Invitrogen) and a forward primer for the *S. mutans* GS5 bacteriocin gene (5'-CTGCAGTACTCCGGCATGTGCAATT-3') (H. Yonezawa and H. K. Kuramitsu, submitted for publication), a forward primer for the *S. mutans* GS5 regulatory region of the bacteriocin operon (5'-TCCGAGCTCTGTGTGGAAACAAGGACTGATAA-3'), or a primer for *S. mutans* fructosyltransferase (*fff*) gene (5'-GATCAAGCAGCTACCGTAAC-3') (26). PCR was then performed using the cDNAs indicated as templates and primers for the *S. mutans* GS5 bacteriocin gene (forward, 5'-CTGCAGTACTCCGGCATGTGCAATT-3'; reverse, 5'-CCCTTTATTTCCCAATACAATG-3'), the *S. mutans* GS5 bacteriocin operon regulatory region (forward, 5'-TCCGAGCTCTGTGTGGAAACAAGGACTGATAA-3'; reverse, 5'-GGGGTACCTCACTCTGCTATCCAGA-3'), or the *S. mutans* *fff* gene (forward, 5'-TTGGGATCTTGGCCAGTTC-3'; reverse, 5'-CAATGACATGTGGATCCGC-3').

RESULTS

Bacteriocin production by *S. mutans* GS5 and BM71 is mediated by the CSP. *S. mutans* GS5, BM71, or NG8 and their *comC*, *comD*, and *comE* knockout mutants were assayed for the production of bacteriocin using the agar plate assays. Of three strains surveyed, the wild-type *S. mutans* GS5 and BM71 produced significant amounts of bacteriocin as indicated by the diameter of the inhibition zone of the indicator strain RP66. However, their *com* mutants produced minimum amounts of bacteriocin (Table 1). The addition of exogenous CSP of *S. mutans* in the bacterial stab cultures restored the bacteriocinogenic ability of the *comC* mutant, but not that of the *comD* or *comE* mutant. These results indicated that bacteriocin production by *S. mutans* GS5 or BM71 is a CSP-dependent phenomenon. Recent results have demonstrated that BM71 produces the same bacteriocin as GS5 (Yonezawa and Kuramitsu, submitted). However, *S. mutans* NG8 did not produce detect-

TABLE 2. Inhibition of *S. mutans* GS5 bacteriocin production by other oral streptococci in agar plate assays^a

Bacterial mixtures ^b	Diameter of inhibition zone (mm) ^c	
	Without CSP	With CSP
GS5 + NG8	38.5 ± 2.5	39.0 ± 3.0
GS5 + Challis	5.5 ± 2.5	38.5 ± 2.5
GS5 + 10558	20.5 ± 3.5	38.5 ± 2.5
GS5 + 10556	22.5 ± 1.0	38.5 ± 2.5
GS5 + 10712	14.0 ± 5.0	39.0 ± 3.0
GS5 + KS32AR	22.0 ± 6.0	38.5 ± 2.5

^a *S. mutans* GS5 cells in the presence or absence of other oral streptococcal cells were inoculated into THB agar plates with or without the addition of exogenous synthetic CSP (3 µg in 3 µl) for stab cultures. After 24 h of incubation at 37°C under anaerobic conditions, the plates were overlaid with indicator RP66 cells (10⁶ CFU) in 3 ml of THB with 1% low-melting-point agarose. After 24 more hours of incubation at 37°C under anaerobic conditions, the diameters of the clearance zones surrounding the inoculated bacteria were measured.

^b Mixtures of *S. mutans* GS5 and NG8, *S. gordonii* Challis and 10558, *S. sanguis* 10556, *S. mitis* 10712, and *S. oralis* KS32AR were used.

^c Values are means ± standard deviations from two experiments.

able bacteriocin against RP66, even with the addition of exogenous CSP (data not shown). Therefore, the dependence (on CSP) of the production of the antimicrobial substances active against RP66 is strain specific.

To confirm the results of the agar plate assays, another assay system, using THB, was used to investigate the production of bacteriocin in broth by *S. mutans* GS5 or BM71. Similar to the results in agar plate assays, *S. mutans* GS5 or BM71 produced bacteriocin in broth, but not its *com* mutants (data not shown). The addition of exogenous synthetic CSP also restored bacteriocin production by *S. mutans* GS5 *comC* mutant or BM71 *comC* mutant but did not restore bacteriocin production by the *comD* or *comE* mutant.

Inhibition of *S. mutans* bacteriocin production by other oral streptococci. To investigate interactions among oral streptococci relative to bacteriocin production by *S. mutans*, other oral streptococcal cells were mixed with *S. mutans* GS5 cells (1:1 by volume at the stationary phase) and inoculated into THB agar plates with or without the addition of exogenous synthetic CSP. The inhibition zones of overlaid indicator strain RP66 were measured and are shown in Table 2. All of the other oral streptococci, except *S. mutans* NG8, inhibited bacteriocin production by *S. mutans* GS5 to different degrees. The addition of exogenous CSP fully restored bacteriocin production by *S. mutans* GS5 in the indicated mixed stab cultures. All of these other species of oral streptococci did not produce detectable bacteriocin against RP66 or GS5 in agar plate assays (data not shown).

THB broth assays were also performed to confirm the results from the agar plate assays. All of the oral streptococci, except *S. mutans* NG8, completely inhibited bacteriocin production by *S. mutans* GS5 or BM71 in broth (data not shown). *S. mutans* NG8 did not inhibit bacteriocin production by *S. mutans* GS5 or BM71 at all. The addition of exogenous CSP fully restored bacteriocin production in the mixed cultures to the level of wild-type *S. mutans* GS5 or BM71. We also examined the potential effects of the streptococci on the viability of *S. mutans* in mixed broth cultures. The cell numbers of *S. mutans* GS5 or BM71 in the mixtures with other species were comparable to those in the mixtures with NG8 (data not shown). These results

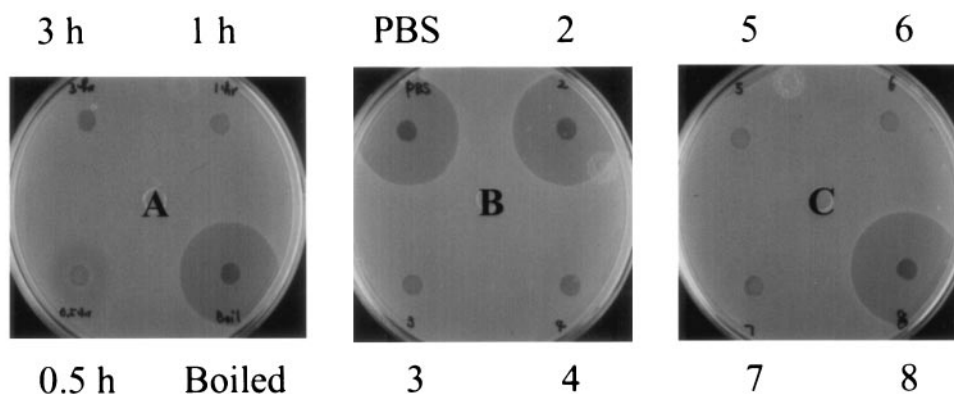


FIG. 1. Determination of the effect of supernatant fluids from oral streptococci on *S. mutans* CSP in agar well assays. Exogenous synthetic *S. mutans* CSP (final concentration, 2.5 $\mu\text{g/ml}$) was incubated with the supernatant fluids from oral streptococci at 37°C for 0.5 to 3 h. CSP thus treated was added to *S. mutans* GS5 *comC* mutant cells (10^7 CFU) in 1.0 ml of THBY to a final concentration of 0.25 $\mu\text{g/ml}$. After a 24-h incubation at 37°C, the supernatant fluids from the cultures were passed through 0.22- μm -pore-size filters, and the bacteriocin level produced by the *S. mutans* GS5 *comC* mutant was determined by the agar well assays. (A) Supernatant fluid from *S. gordonii* Challis. Part of the supernatant fluid was boiled for 10 min before the incubation with CSP. (B and C) PBS, a CSP positive control treated with PBS instead of supernatant fluid; zone 2, supernatant fluid from *S. gordonii* Challis mutant 41A9; zones 3 to 7, supernatant fluid from *S. gordonii* Challis, *S. gordonii* 10558, *S. sanguis* 10556, *S. mitis* 10712, and *S. oralis* KS32AR, respectively; and zone 8, supernatant fluid from *S. mutans* GS5. CSP was incubated with the supernatant fluid for 3 h in panels B and C.

indicated that inhibition of bacteriocin production by other species in the mixed cultures was not due to a reduction in cell numbers of *S. mutans* GS5 or BM71, since the amounts of bacteriocin produced by the mixtures of *S. mutans* GS5 or BM71 with NG8 were similar to those produced by *S. mutans* GS5 or BM71 single strain cultures.

Effects of supernatant fluids from oral streptococci on the CSP activity of *S. mutans*. Next, experiments were performed to determine whether supernatant fluids from other species of oral streptococci could inactivate exogenous synthetic *S. mutans* CSP. *S. mutans* GS5 *comC* mutant cells were used in this experiment as an indicator of the levels of active CSP, since the mutant requires exogenous CSP to restore bacteriocin production (Table 1). As shown in Fig. 1A, incubating CSP with the supernatant fluid from *S. gordonii* Challis for more than 1 h completely inactivated CSP, while a 0.5-h incubation only partially inactivated CSP. Heating the supernatant fluid from *S. gordonii* Challis at 100°C for 10 min destroyed the inhibitory activity on *S. mutans* CSP, indicating that the substance(s) responsible for *S. mutans* CSP inactivation is heat sensitive. Figure 1B demonstrated that the supernatant fluid from *S. gordonii* Challis mutant 41A9 (described below) lost its ability to inactivate *S. mutans* CSP, since *S. mutans* GS5 *comC* cells produced a similar amount of bacteriocin in the presence of CSP treated with either 41A9 supernatant fluid or PBS. Supernatant fluids from other oral streptococci, namely, *S. gordonii* 10558, *S. sanguis* 10556, *S. mitis* 10712, and *S. oralis* KS32AR, also inactivated *S. mutans* CSP as did the supernatant fluid from *S. gordonii* Challis (Fig. 1B and C). However, the supernatant fluid from *S. mutans* GS5 did not inactivate its own CSP (Fig. 1C).

***S. gordonii* Challis does not inactivate the *S. mutans* GS5 bacteriocin.** To determine whether *S. gordonii* Challis inactivated the bacteriocin activity of *S. mutans* GS5 as it inactivated the CSP of *S. mutans*, supernatant fluids (containing bacteriocin) from GS5 grown in broth cultures in THBY supplemented with 1% glucose, 1% sucrose, or 0.1 μg of CSP per ml

were mixed with *S. gordonii* Challis (10^7 CFU) and incubated in precut wells in THB agar plates for 24 h at 37°C. If *S. gordonii* Challis inactivates *S. mutans* GS5 bacteriocin present in the supernatant fluids, there would be a reduction in the inhibition zone diameters in the presence of *S. gordonii* Challis cells compared to the zones in the absence of *S. gordonii* Challis cells. The presence of *S. gordonii* Challis cells grown in the *S. mutans* supernatant fluids did not reduce the diameter of the inhibition zones (Table 3), indicating that *S. gordonii* Challis has limited ability to inactivate the bacteriocin produced by *S. mutans* GS5.

Isolation of an *S. gordonii* Challis mutant defective in attenuating bacteriocin production by *S. mutans* GS5. To determine the mechanism of the interspecies inhibition of *S. mutans* GS5 bacteriocin production, *S. gordonii* Challis cells were randomly mutagenized using Tn916. The resulting insertion mutants were screened for mutants that displayed decreased ability to inhibit *S. mutans* GS5 bacteriocin production. Of 1,500 clones screened, one mutant, named 41A9, exhibited this property in

TABLE 3. Effect of *S. gordonii* Challis on bacteriocin produced by *S. mutans* GS5^a

Supplement in THBY	Diameter of inhibition (mm) ^b	
	Without Challis	With Challis
None	0 \pm 0	0 \pm 0
Glucose	0 \pm 0	0 \pm 0
Sucrose	24.5 \pm 0.5	24.0 \pm 1.0
CSP	39.5 \pm 0.5	39.5 \pm 0.5

^a Supernatant fluids from *S. mutans* GS5 grown in broth cultures in THBY supplemented with 1% glucose, 1% sucrose, or 0.1 μg of CSP per ml were mixed with *S. gordonii* Challis (10^7 CFU) and incubated in precut wells in THB agar plates for 24 h at 37°C. The wells were then filled using THB with 1% low-melting-point agarose, and the plates were overlaid with the indicator strain RP66 (10^6 CFU) in 3 ml of THB with 1% low-melting-point agarose. After 24 more hours of incubation at 37°C under anaerobic conditions, the diameters of the inhibition zones surrounding the wells were measured.

^b Values are means \pm standard deviations from two experiments.

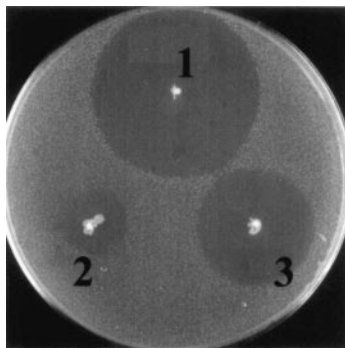


FIG. 2. Screening Tn916 insertional mutants of *S. gordonii* Challis in agar plate bacteriocin assays. *S. mutans* GS5 alone or in mixtures with *S. gordonii* Challis or with *S. gordonii* Challis mutant 41A9 were inoculated into THB agar plates. After 24 h of incubation at 37°C under anaerobic conditions, the plates were overlaid with indicator RP66 cells (10^6 CFU) in 3 ml of THB with 1% low-melting-point agarose. After 24 more hours of incubation at 37°C under anaerobic conditions, the diameters of the clearance zones surrounding the inoculated bacteria were measured. Zones: 1, *S. mutans* GS5 alone; 2, mixture of wild-type *S. gordonii* Challis and *S. mutans* GS5; and 3, mixture of *S. gordonii* Challis mutant 41A9 and *S. mutans* GS5.

agar plate bacteriocin assays (Fig. 2, zone 3). The transformants of wild-type *S. gordonii* Challis with chromosomal DNA extracted from 41A9 all showed the same phenotype as the original 41A9 transformant in the plate bacteriocin assays, with 19- to 24-mm inhibition zone diameters. These results confirmed that the phenotype of 41A9 was determined by the insertional mutation of Tn916 into the host chromosome.

Sequencing the chromosomal DNA flanking Tn916 from mutant 41A9. As described in Materials and Methods, restriction enzyme digestions and Southern blot analysis revealed a 1.4-kb fragment of 41A9 chromosomal DNA that flanked the transposase proximal end of Tn916. Using appropriate primers, PCR of the ligation mixture of digested 41A9 DNA fragments with pUC19 resulted in the predicted 1.4-kb PCR product. Subsequent sequencing of the PCR product and analysis in The Institute for Genomic Research (TIGR) *S. gordonii* Challis unfinished sequence database (<http://www.ncbi.nlm.nih.gov/genomes/framik.cgi?db=genome&gi=5078>) revealed that Tn916 interrupted a putative subtilisin-like serine protease gene in *S. gordonii* Challis. The complete sequence of this protease gene (4,541 bp) was identified in the unpublished TIGR database (M. M. Vickerman, personal communication). This protease has 53% identity and 66% similarity to PstS, a cell envelope protease in *Streptococcus thermophilus* and 49% identity and 63% similarity to CspA, a cell-surface-associated protease in a group B streptococcus. We have named this gene *sgc* (for *S. gordonii* challisin) gene, as it codes for the challisin protease.

Construction of the *S. gordonii* Challis *sgc* mutant. As depicted in Fig. 3, pPE, a vector containing the disrupted *sgc* gene was constructed so that it could be used to inactivate the *sgc* gene in *S. gordonii* Challis after selection with erythromycin. To confirm *sgc* inactivation in Challis transformants, chromosomal DNA was extracted from wild-type Challis and its *sgc* mutant (BYW1). The chromosomal DNA was digested with three different restriction enzymes with known digestion sites

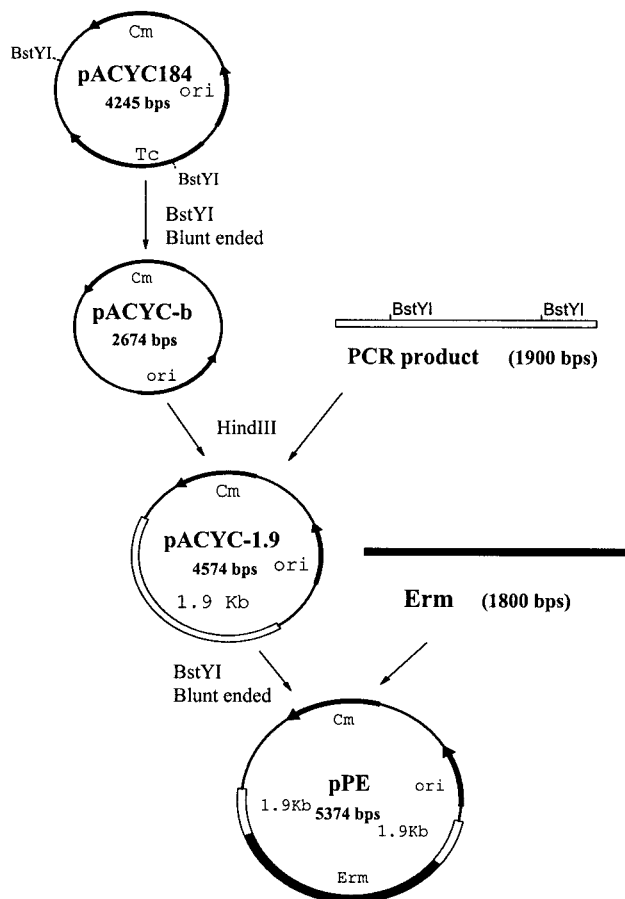


FIG. 3. Construction of the *S. gordonii* Challis vector pPE. As described in the text, pPE was constructed containing an interrupted *sgc* gene of *S. gordonii* Challis.

that flank the integration site of the Erm cassette but with no digestion sites in the Erm cassette. AflIII, EcoRI, and PvuII digestions of the chromosomal DNA produced the predicted fragments after Southern blot analysis (2.3-, 2.1-, and 1.3-kb fragments for Challis and 4.1-, 3.9-, and 3.1-kb fragments for BYW1, respectively).

The *S. gordonii* Challis *sgc* mutant BYW1 exhibited the same phenotype as the insertional mutant, 41A9. Agar plate and agar well assays were performed to determine whether the BYW1 mutant had the same phenotype as 41A9, the Tn916-insertion-inactivated mutant of the *S. gordonii* Challis *sgc* gene. Strain BYW1, compared to its parent, Challis, indeed exhibited attenuation in its ability to inhibit *S. mutans* GS5 bacteriocin production in the agar plate assay to the same extent as 41A9. Both the BYW1 and 41A9 mutants yielded 19- to 24-mm inhibition zone diameters compared to the parent strain Challis (3- to 8-mm inhibition zone diameters) in the mixed stab culture with *S. mutans* GS5 in agar plate assays.

The effects of *S. gordonii* Challis and its *sgc* mutants on *S. mutans* CSP were compared using the agar well assays. The synthetic *S. mutans* CSP was incubated with supernatant fluids from *S. gordonii* Challis, 41A9, or BYW1 or with PBS (as a positive control) for 2 h, since more than 1-h incubation was sufficient to inactivate CSP by Challis supernatant fluid (Fig.

TABLE 4. Protease activity of *S. gordonii* Challis by azocasein assays^a

<i>S. gordonii</i> strain	OD ₄₂₀ ^b	
	Cell pellet	Supernatant
Challis	0.174 ± 0.055	0.452 ± 0.044
41A9	0.011 ± 0.034	0.255 ± 0.016
BYW1	0.103 ± 0.031	0.233 ± 0.013

^a The cell pellets and supernatant fluids were from cultures of *S. gordonii* Challis grown overnight in THB. The supernatant fluids were concentrated as described in Materials and Methods. All of the cell pellets and the concentrated supernatant fluids from 50-ml cultures were utilized in the assays.

^b Values are means ± standard deviations from three experiments.

1). The treated CSP was then used to restore bacteriocin production by the *S. mutans comC* mutant cells as described earlier in this study. The Challis supernatant fluid completely inactivated *S. mutans* CSP, while those from 41A9 or BYW1 did not inactivate *S. mutans* CSP, since the *S. mutans comC* cells responded to the CSP treated with the mutant supernatant fluids to a degree similar to that seen with the CSP positive control (data not shown).

Protease activity of *S. gordonii* Challis resides in its supernatant fluids. To determine whether *S. gordonii* Challis primarily secretes the challisin protease or expresses it on the cell surface, azocasein assays were performed as described above. The supernatant fluids exhibited higher protease activity than the cell pellets from equivalent numbers of cells (Table 4), indicating that there was a higher level of protease activity in the supernatant fluid than in the cell surface. These experiments also demonstrated that the levels of protease activity of protease mutants, 41A9 and BYW1, were lower than that of the parental strain Challis.

A 168-kDa protease was missing in *sgc* mutants of *S. gordonii* Challis. To further characterize the proteases secreted by *S. gordonii* Challis, concentrated supernatant fluids from Challis and its *sgc* mutants were assayed by zymography. As shown in Fig. 4, a protease present in the supernatant fluid of parental strain Challis at approximately 160 kDa could not be detected

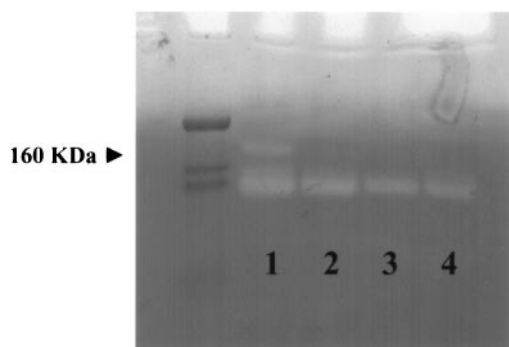


FIG. 4. Casein zymography of *S. gordonii* protease activity. The supernatant fluids from broth cultures of *S. gordonii* Challis or *sgc* mutants grown overnight were concentrated 500-fold and applied to ready-made casein gels. Concentrated supernatant fluids from parental strain Challis (lane 1), insertional mutant 41A9 (lane 2), and two clones of knockout mutant BYW1 (lanes 3 and 4) were used. The approximate position of the 160-kDa band is indicated by the arrowhead.

TABLE 5. Comparison of bacteriocin production by mixed cultures of *S. mutans* BM71 *S. gordonii* Challis in broth or biofilms^a

Supernatant from strain(s)	% Killing of indicator RP66 ^b	
	Broth	Biofilms
BM71 alone	98.3 ± 0.3	99.6 ± 0.4
BM71 + Challis	0 ± 0 (537/406)	0 ± 0 (517/353)
BM71 + BYW1	97.1 ± 1.0 (447/356)	52.9 ± 7.0 (479/146)

^a A 100 µl aliquot from *S. mutans* BM71 alone or in a mixture with *S. gordonii* Challis from a broth or biofilm culture was mixed with 5×10^4 CFU of RP666 indicator cells (in 100 µl of THB) and incubated at 37°C for 1 h. Two micro liters of the culture was plated on the surface of a TSA plate and incubated for another 24 h at 37°C under anaerobic conditions.

^b Values are means ± standard deviations from two experiments. The values in parentheses are the total number of *S. mutans* cells (10^6)/total number of *S. gordonii* cells (10^6) counted on mitis salivarius plates.

in the supernatant fluids of the protease mutants. Since the size of the *S. gordonii* Challis challisin gene is 4,541 bp (M. M. Vickerman, personal communication) and therefore a predicted protein size for challisin protease is 168 kDa, the missing 160-kDa activity in the *sgc* mutants detected by zymography is consistent with the suggestion that *sgc* gene expression had been interrupted.

Comparison of bacteriocin production in broth or biofilms by *S. mutans* in mixed cultures with either wild-type *S. gordonii* Challis or *sgc* mutant BYW1. To compare bacteriocin production in broth or biofilms, *S. mutans* BM71 was used instead of *S. mutans* GS5, since BM71, but not GS5, consistently formed stable biofilms in six-well polystyrene plates in BM. *S. mutans* BM71 or *S. gordonii* Challis or BYW1 were cultured in THB to a density of 0.5 at OD₆₀₀. For broth bacteriocin production, strain BM71 alone (40 µl) or in mixtures with either Challis or BYW1 (40 µl of BM71 and 20 µl of Challis or BYW1) were cultured in 1.0 ml of THBY at 37°C for 24 h. For biofilm bacteriocin production, the same amounts of cells were inoculated in 2 ml of BM in six-well plates to form biofilm. THBY was used subsequently to obtain biofilm bacteriocin supernatant fluids as described earlier in this study. We used different media for biofilm bacteriocin production, since *S. mutans* does not form stable biofilms in rich medium (THBY) and does not produce as much bacteriocin in BM medium. The numbers of cells counted on mitis salivarius agar plates for broth cultures and biofilm cultures were comparable (Table 5). Killing assays were performed to compare the amounts of bacteriocin produced by *S. mutans* BM71. There was no difference in bacteriocin production by *S. mutans* BM71 in biofilms or broth cultures when it was the single strain (Table 5). Supernatant fluids from mixtures of *S. mutans* BM71 with wild-type *S. gordonii* Challis in biofilms or broth cultures exhibited no killing of RP66 indicator cells. However, the killing percentage of RP66 cells by supernatant fluid from a mixture of *S. mutans* BM71 and mutant BYW1 cells in broth culture was similar to that from *S. mutans* BM71 single cultures, while the supernatant fluids from mixtures of *S. mutans* BM71 and mutant BYW1 in biofilms exhibited 52.9% killing (Table 5).

***S. gordonii* Challis down-regulated bacteriocin gene expression in *S. mutans*.** *S. mutans* BM71 alone and in mixtures with *S. gordonii* Challis, the *sgc* mutant BYW1, and the *S. mutans* BM71 *comC* mutant were cultured in THBY. Total RNA was extracted, and RT-PCR was performed to examine the expres-

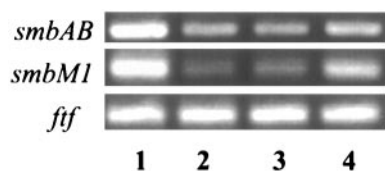


FIG. 5. *S. mutans* bacteriocin gene expression in mixed cultures. Total RNA from *S. mutans* BM71 alone or in a mixture with *S. gordonii* or from *S. mutans* BM71 *comC* mutant cells was isolated from broth cultures in THBY (OD₆₀₀ readings of 1.0 to 1.2). Reverse transcription-PCR products were electrophoresed in 2% agarose gels and demonstrated bands of 274, 357, and 482 bp for the *smbAB*, *smbMI*, and *ftf* genes, respectively. Lanes: 1, *S. mutans* BM71; 2, *S. mutans* BM71 *comC* mutant, 3, *S. mutans* BM71 mixed with *S. gordonii* Challis; 4, *S. mutans* BM71 mixed with *S. gordonii* mutant BYW1.

sion of the *S. mutans* bacteriocin gene *smbAB*, its modification gene *smbMI* (Yonezawa and Kuramitsu, submitted), or the *ftf* gene (the internal control). These three genes are expressed only in *S. mutans* and not in *S. gordonii* (data not shown). The expression of *S. mutans* *smbAB* and *smbMI* genes was significantly down-regulated in the *S. mutans* BM71 *comC* mutant compared to its wild type (Fig. 5), indicating that CSP also regulates the expression of *S. mutans* bacteriocin genes. *S. gordonii* Challis down-regulated the expression of *smbAB* and *smbMI* genes of *S. mutans* BM71 in the mixed cultures to an extent similar to that of the *S. mutans* BM71 *comC* mutant, while the *sgc* mutant BYW1 only slightly down-regulated the expression of these genes (Fig. 5). This is another indication that the expression of *S. mutans* bacteriocin genes is regulated by CSP, since Challis completely inactivated *S. mutans* CSP, while the *sgc* mutants did not (Fig. 1). DNase-treated RNA without reverse transcription (as negative controls) did not produce the 274- or 357-bp PCR products for *S. mutans* *smbAB* and *smbMI* genes, respectively (data not shown). The *ftf* gene of *S. mutans* was used as an internal control for RT-PCR, and the expression of the *ftf* gene did not exhibit regulation in the mixed cultures as the *S. mutans* bacteriocin gene and its modification gene did.

DISCUSSION

Previous studies have shown that many clinical isolates and laboratory strains of *S. mutans* produce antimicrobial substances termed bacteriocin (mutacins) that exhibit various degrees of inhibition of other bacteria. The operons coding for four mutacins (mutacins I, II, III, and IV) have been identified (3, 23, 24). On the other hand, bacteriocins produced by many *S. mutans* strains have been identified only by the bacterial strains that they inhibited and have not yet been further characterized. *S. mutans* GS5 produces a mutacin that inhibits a group C streptococcal strain RP66, among other sensitive indicator bacteria (22). Among the three *S. mutans* strains tested in the present study, GS5 and BM71 inhibited the growth of RP66, while their corresponding *com* mutants did not. These results indicated that bacteriocin production by *S. mutans*, at least in the two strains tested, is a quorum-sensing-dependent phenomenon (4). The addition of exogenous CSP to the *com* mutants restored the ability of bacteriocin production only in the *comC* mutant, not in the *comD* or *comE* mutant. These

results further confirmed that *S. mutans* bacteriocin production is a CSP-dependent phenomenon, since the *comC* mutant is defective in CSP itself, while the *comD* and *comE* mutants are defective in the CSP receptor and response regulator, respectively.

The inhibition zone sizes measured in plate assays have been previously used by many investigators to evaluate production of bacteriocin by *S. mutans* (3, 24). Since the bacteriocin from strain GS5 has not been purified, it is not clear to what extent zone size is directly related to bacteriocin concentration. Nevertheless, it is a reliable method to indicate relative levels of bacteriocin produced by *S. mutans*. In our study, the stab cultures of the *com* mutants of *S. mutans* resulted in much smaller zones than those of the wild type (0 to 6 mm compared to 36 to 40 mm), indicating that the *com* mutants of *S. mutans* produce minimal amounts of bacteriocin. This was further confirmed by another semiquantitative method (broth assays) using supernatant fluids from broth cultures. However, the plate assay does have limitations, since bacteriocin produced by *S. mutans* has to diffuse through agar. In broth assays, the addition of exogenous CSP resulted in enhanced bacteriocin production by wild-type *S. mutans*, which was not reflected in the plate assays.

Mutacin production is affected by the composition of the culture medium. Paul and Slade (22) reported that *S. mutans* GS5 consistently produced bacteriocin in Trypticase soy broth (TSB) supplemented with 5% horse serum, but not in THB plus horse serum. However, Rogers (25) reported that the addition of yeast extract enhanced bacteriocin production of *S. mutans*. We utilized THB supplemented with 3% yeast extract in our experiments of broth bacteriocin production to compare the results with those of agar stab culture assays. THBY, compared with TSB plus serum, produced an even larger amount of bacteriocin when assayed in broth assays (data not shown). THBY that had been filtered through 0.22- μ m-pore-size filters instead of being autoclaved produced a slightly increased amount of bacteriocin (data not shown).

The addition of 1% sucrose in THBY significantly increased bacteriocin production by *S. mutans* GS5 in broth (Table 3). This seems to be correlated with aggregations of *S. mutans* GS5, since *gtfB* (glucosyltransferase) (1) and *gtfC* (30) mutants of GS5 did not produce as much bacteriocin as their parent strain did in the presence of sucrose, while SP2 (a spontaneous mutant of GS5 with *gtfB* and *gtfC* genes that had undergone recombination [29]) did not exhibit enhanced bacteriocin production in the THBY supplemented with sucrose (data not shown). Agar well assays used in Table 3 allow bacteriocins in the supernatant fluids to diffuse into agar surrounding the wells. The assays produced reproducible results without much variation. However, the agar well assays were not as sensitive as other assays. The supernatant fluids of *S. mutans* GS5 from either THBY or THBY plus 1% glucose did not produce detectable inhibition zones in agar well assays, while they inhibited the growth of RP66 in broth or viable count assays. Agar well assays were valuable for the detection of bacteriocin activities beyond a certain threshold (e.g., the supernatant fluids from *S. mutans* GS5 cultures in THBY plus 1% sucrose or in the presence of exogenous CSP), since the agar well assay is quantitative and exhibits little variation.

Mixtures of *S. mutans* GS5 with other oral streptococci re-

sulted in attenuated bacteriocin production by *S. mutans* GS5. Bacteriocin production by *S. mutans* GS5 was completely abolished in mixed broth cultures for all species but was inhibited by different degrees in mixed agar stab culture assays. This may be explained by the difference in the differential inhibition of the growth of other species by *S. mutans* GS5. *S. mutans* GS5 did not inhibit *S. gordonii* Challis growth in agar stab culture assays, while it weakly inhibited the growth of other oral bacteria (data not shown). This difference might be detected only in stab culture assays, since the inoculated bacteria were in much smaller numbers in stab culture assays than in broth assays.

Supernatant fluids from other oral streptococci inactivated CSP, but not the bacteriocin of *S. mutans* (Fig. 1 and Table 3). The concentration of CSP (2.5 µg/ml) that was inactivated by the supernatants from other oral streptococci was 25 times higher than that sufficient to restore the ability of bacteriocin production in the *S. mutans* GS5 or BM71 *comC* mutant (0.1 µg/ml). This indicated that a substance(s) in the supernatant fluids from other oral streptococci was responsible for this interspecies interaction. The subsequent identification of the *sgc* gene and the construction of *sgc* mutants confirmed that this substance was produced by *S. gordonii* Challis.

A relatively high concentration (3 µl of 1 mg/ml) of CSP was added to the mixed stab cultures in plate assays. We have titrated the CSP and discovered that as little as 3 µl of 10-µg/ml CSP (100-fold diluted) could induce bacteriocin production by the *comC* mutant of *S. mutans* GS5 or BM71 in plate assays (data not shown). Therefore, even though some CSP was degraded by protease produced by other species of oral streptococci, the remaining CSP would still be sufficient to induce maximal amounts of bacteriocin production by *S. mutans* in the mixed stab cultures.

There are proteases present in *S. gordonii* Challis other than the challisin protease (Fig. 4 and Table 4). Therefore, knocking out the *sgc* gene in *S. gordonii* Challis would not completely abolish its interference with the CSP-dependent phenomenon of *S. mutans*. This was demonstrated in Fig. 2 where the mixed stab culture of *S. mutans* GS5 and the insertional mutant 41A9 produced a smaller zone of inhibition than did *S. mutans* GS5 itself. This was also true for the knockout mutant BYW1 and 41A9.

Inhibition of *S. mutans* bacteriocin production by other oral streptococci was more significant in biofilms than in broth. This was demonstrated in Table 5, where BYW1 exhibited no inhibition of *S. mutans* bacteriocin production in broth but around 50% inhibition of that in mixed biofilms.

Recently, the bacteriocin genes of *S. mutans* GS5 have been identified and sequenced and appear to code for a dipeptide mutacin (Yonezawa and Kuramitsu, submitted). Using primers for the newly identified *S. mutans* GS5 bacteriocin genes, we demonstrated that *S. gordonii* Challis, but not its *sgc* mutant, significantly down-regulated bacteriocin gene expression in strain GS5 (data not shown). Strain BM71 appears to contain the same bacteriocin genes as GS5, as was demonstrated in the present study.

Quorum-sensing systems in *S. mutans* govern the competence of genetic transformation (17), biofilm formation (19, 33), and acid tolerance (18). Therefore, the CSP-dependent quorum-sensing system and the virulence response it mediates

offer new targets for strategies to reduce the cariogenicity of organisms, such as *S. mutans*. We demonstrated here that *S. mutans* bacteriocin production is another quorum-sensing-dependent phenomenon. We also demonstrated that the interactions with other oral streptococci interfered with *S. mutans* bacteriocin production. At this time, we are also examining the inhibitory effects of other oral streptococci on *S. mutans* genetic transformation. An understanding of interactions among oral streptococci may provide valuable insight into the prevention of oral infections, such as dental caries.

ACKNOWLEDGMENTS

We thank F. Scannapieco for helpful discussions. We also thank M. M. Vickerman for assistance in identifying the complete *sgc* gene sequence of *S. gordonii* Challis in the unpublished TIGR database. B.-Y.W. is grateful to the members of the Kuramitsu laboratory, especially B. Wang, W. Chen, and M. S. Qi, for their support and advice.

These studies were supported in part by grants DE03258 and T32-DE07034 from the National Institute of Dental and Craniofacial Research.

REFERENCES

- Aoki, H., T. Shiroza, M. Hayakawa, S. Sato, and H. K. Kuramitsu. 1986. Cloning of a *Streptococcus mutans* glucosyltransferase gene coding for insoluble glucan synthesis. *Infect. Immun.* **53**:587–594.
- Bassler, B. L. 1999. How bacteria talk to each other: regulation of gene expression by quorum sensing. *Curr. Opin. Microbiol.* **2**:582–587.
- Chen, P., F. Qi, J. Novak, and P. W. Caufield. 1999. The specific genes for antibiotic mutacin II biosynthesis in *Streptococcus mutans* T8 are clustered and can be transferred en bloc. *Appl. Environ. Microbiol.* **65**:1356–1360.
- Cvitkovitch, D. G., Y. H. Li, and R. P. Ellen. 2003. Quorum sensing and biofilm formation in streptococcal infections. *J. Clin. Investig.* **112**:1626–1632.
- Dong, Y. H., J. L. Xu, X. Z. Li, and L. H. Zhang. 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc. Natl. Acad. Sci. USA* **97**:3526–3531.
- Dunny, G. M., and B. A. Leonard. 1997. Cell-cell communication in gram-positive bacteria. *Annu. Rev. Microbiol.* **51**:527–564.
- Fabio, U., M. Bondi, G. Manicardi, P. Messi, and R. Neglia. 1987. Production of bacteriocin-like substances by human oral streptococci. *Microbiologica* **10**:363–370.
- Fuqua, C., and E. P. Greenberg. 1998. Self perception in bacteria: quorum sensing with acylated homoserine lactones. *Curr. Opin. Microbiol.* **1**:183–189.
- Harris, G. S., S. M. Michalek, and R. Curtiss III. 1992. Cloning of a locus involved in *Streptococcus mutans* intracellular polysaccharide accumulation and virulence testing of an intracellular polysaccharide-deficient mutant. *Infect. Immun.* **60**:3175–3185.
- Havarstein, L. S., G. Coomaraswamy, and D. A. Morrison. 1995. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. *Proc. Natl. Acad. Sci. USA* **92**:11140–11144.
- Jack, R. W., J. R. Tagg, and B. Ray. 1995. Bacteriocins of gram-positive bacteria. *Microbiol. Rev.* **59**:171–200.
- Kleerebezem, M., and L. E. Quadri. 2001. Peptide pheromone-dependent regulation of antimicrobial peptide production in Gram-positive bacteria: a case of multicellular behavior. *Peptides* **22**:1579–1596.
- Kleerebezem, M., L. E. Quadri, O. P. Kuipers, and W. M. de Vos. 1997. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol. Microbiol.* **24**:895–904.
- Kuramitsu, H. K., and C. M. Long. 1982. Plasmid-mediated transformation of *Streptococcus mutans*. *Infect. Immun.* **36**:435–436.
- Kuramitsu, H. K., and V. Trapa. 1984. Genetic exchange between oral streptococci during mixed growth. *J. Gen. Microbiol.* **130**:2497–2500.
- Li, Y.-H., M. N. Hanna, G. Svensäter, R. P. Ellen, and D. G. Cvitkovitch. 2001. Cell density modulates acid adaptation in *Streptococcus mutans*: implications for survival in biofilms. *J. Bacteriol.* **183**:6875–6884.
- Li, Y.-H., P. C. Y. Lau, J. H. Lee, R. P. Ellen, and D. G. Cvitkovitch. 2001. Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *J. Bacteriol.* **183**:897–908.
- Li, Y.-H., P. C. Y. Lau, N. Tang, G. Svensäter, R. P. Ellen, and D. G. Cvitkovitch. 2002. Novel two-component regulatory system involved in biofilm formation and acid resistance in *Streptococcus mutans*. *J. Bacteriol.* **184**:6333–6342.
- Li, Y.-H., N. Tang, M. B. Aspiras, P. C. Y. Lau, J. H. Lee, R. P. Ellen, and

- D. G. Cvitkovitch. 2002. A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. *J. Bacteriol.* **184**:2699–2708.
20. Morrison, D. A. 1997. Streptococcal competence for genetic transformation: regulation by peptide pheromones. *Microb. Drug Resist.* **3**:27–37.
21. Otto, M., H. Echner, W. Voelter, and F. Götz. 2001. Pheromone cross-inhibition between *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Infect. Immun.* **69**:1957–1960.
22. Paul, D., and H. D. Slade. 1975. Production and properties of an extracellular bacteriocin from *Streptococcus mutans* bacteriocidal for group A and other streptococci. *Infect. Immun.* **12**:1375–1385.
23. Qi, F., P. Chen, and P. W. Caufield. 2001. The group I strain of *Streptococcus mutans*, UA140, produces both the lantibiotic mutacin I and a nonlantibiotic bacteriocin, mutacin IV. *Appl. Environ. Microbiol.* **67**:15–21.
24. Qi, F., P. Chen, and P. W. Caufield. 1999. Purification of mutacin III from group III *Streptococcus mutans* UA787 and genetic analyses of mutacin III biosynthesis genes. *Appl. Environ. Microbiol.* **65**:3880–3887.
25. Rogers, A. H. 1972. Effect of the medium on bacteriocin production among strains of *Streptococcus mutans*. *Appl. Microbiol.* **24**:294–295.
26. Shiroza, T., and H. K. Kuramitsu. 1988. Sequence analysis of the *Streptococcus mutans* fructosyltransferase gene and flanking regions. *J. Bacteriol.* **170**:810–816.
27. Sojar, H. T., J.-Y. Lee, G. S. Bedi, and R. J. Genco. 1993. Purification and characterization of a protease from *Porphyromonas gingivalis* capable of degrading salt-solubilized collagen. *Infect. Immun.* **61**:2369–2376.
28. Tagg, J. R., A. S. Dajani, and L. W. Wannamaker. 1976. Bacteriocins of gram-positive bacteria. *Bacteriol. Rev.* **40**:722–756.
29. Ueda, S., and H. K. Kuramitsu. 1988. Molecular basis for the spontaneous generation of colonization-defective mutants of *Streptococcus mutans*. *Mol. Microbiol.* **2**:135–140.
30. Ueda, S., T. Shiroza, and H. K. Kuramitsu. 1988. Sequence analysis of the *gtfC* gene from *Streptococcus mutans* GS-5. *Gene* **69**:101–109.
31. Vining, L. C. 1990. Functions of secondary metabolites. *Annu. Rev. Microbiol.* **44**:395–427.
32. Weerkamp, A., L. Bongaerts-Larik, and G. D. Vogels. 1977. Bacteriocins as factors in the in vitro interaction between oral streptococci in plaque. *Infect. Immun.* **16**:773–780.
33. Yoshida, A., and H. K. Kuramitsu. 2002. Multiple *Streptococcus mutans* genes are involved in biofilm formation. *Appl. Environ. Microbiol.* **68**:6283–6291.