

Role of Bacteriocin Immunity Proteins in the Antimicrobial Sensitivity of *Streptococcus mutans*[▽]

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Bacteria utilize quorum-sensing systems to modulate environmental stress responses. The quorum-sensing system of *Streptococcus mutans* is mediated by the competence-stimulating peptide (CSP), whose precursor is encoded by the *comC* gene. A *comC* mutant of strain GS5 exhibited enhanced antimicrobial sensitivity to a wide variety of different agents. Since the addition of exogenous CSP did not complement this phenotype, it was determined that the increased tetracycline, penicillin, and triclosan sensitivities resulted from repression of the putative bacteriocin immunity protein gene, *bip*, which is located immediately upstream from *comC*. We further demonstrated that the inactivation of *bip* or *smbG*, another bacteriocin immunity protein gene present within the *smb* operon in *S. mutans* GS5, affected sensitivity to a variety of antimicrobial agents. Furthermore, both the *bip* and *smbG* genes were upregulated in the presence of low concentrations of antibiotics and were induced during biofilm formation relative to in planktonic cells. These results suggest, for the first time, that the antimicrobial sensitivity of a bacterium can be modulated by some of the putative bacteriocin immunity proteins expressed by the organism. The implications of these observations for the evolution of bacteriocin immunity protein genes as well as for potential new chemotherapeutic strategies are discussed.

Streptococcus mutans has been implicated as a primary causative agent of dental caries in humans (6). One of the important virulence properties of these organisms is their ability to form biofilms known as dental plaque on tooth surfaces.

Biofilm formation is initiated by interaction between planktonic bacteria and a surface in response to appropriate environmental signals (2, 5, 11, 12, 16–18). In addition to responses to physical and chemical signals, bacteria regulate diverse physiological processes in a cell density-dependent manner; this is commonly called quorum sensing (1, 10). Bacteria utilize quorum-sensing systems to modulate environmental stress responses. Recently, some studies demonstrated that biofilm formation, acid tolerance, and the transformation of *S. mutans* are mediated by quorum sensing (3). The quorum-sensing system in *S. mutans* consists of at least five gene products, including a 21-amino-acid competence-stimulating peptide (CSP) whose precursor is encoded by *comC*, a histidine kinase sensor protein encoded by *comD*, and the cognate response regulator expressed from the *comE* gene (13). The *comCDE* genes are located in the same locus and together constitute a quorum-sensing system for generating and responding to CSP (13). By interfering with this cell-cell signaling mechanism, caries-causing *S. mutans* bacteria which use quorum sensing to control virulence could potentially be attenuated.

Many gram-positive bacteria produce antimicrobial peptides called bacteriocins (4, 8, 20, 24). 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 (22). It has been shown that production of some of these antimicrobial peptides is regulated

by the *comC* quorum-sensing system in *S. mutans* (9, 21). Bacteria in biofilms frequently express much greater resistance to antimicrobial agents than they do in the corresponding planktonic cultures (5). Since biofilm formation in some organisms is modulated by quorum sensing, we investigated the possibility that the *comC* quorum-sensing system in *S. mutans* modulates its sensitivity to antimicrobial compounds. Although some of the CSP-induced genes might be important for efficient genetic exchange under natural conditions, many of these gene products likely aid in other cell density-adaptive functions; i.e., several CSP-induced genes have stress-related roles or participate in the synthesis and transport of bacteriocin-like peptides (21, 24). The present results suggest for the first time that the antimicrobial sensitivity of *S. mutans* GS5 is modulated by some of the putative bacteriocin immunity proteins expressed by the organism. Since the bacteriocin operons are regulated, in turn, by quorum sensing, this suggests how quorum sensing can regulate the antimicrobial sensitivity of an organism.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. Strains of *S. mutans* were cultured and maintained in Todd-Hewitt broth (THB) (Difco Laboratories, Grand Island, N.Y.). Transformants of *S. mutans* were selected following growth on mitis salivarius agar (Difco Laboratories) plates supplemented with erythromycin (10 µg/ml).

DNA manipulations. DNA isolation, endonuclease restriction, ligation, and transformation of competent *Escherichia coli* were carried out as previously described (19). Transformation of *S. mutans* was accomplished by procedures routinely carried out in this laboratory (19).

Construction of the *bip* and *smbG* mutants. The *bip* and *smbG* mutants (Fig. 1) were constructed following double-crossover homologous recombination via insertion of an erythromycin resistance determinant into each gene. The plasmids used for disruption of the *bip* and *smbG* genes were prepared as follows. A 1.6-kb DNA fragment containing the entire *bip* or *smbG* gene together with the respective flanking regions were PCR amplified from GS5 genomic DNA with the following primers: for *bip*, forward primer 1736A (5'-TGCGGTCTATTGACCTCCTC-3') and reverse primer ComCsmmC (5'-CGGGGTACCTTGATTA

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
<i>S. mutans</i> strains		
GS5	wt ^a ; Em ^s Km ^s	SUNYaB ^b
UA159	wt; Em ^s Km ^s	J. Ferretti, OU-ACGT ^c
Δ <i>bip</i>	GS5::pMM503; Bip ⁻ Em ^r	This study
Δ <i>smbG</i>	GS5::pMM504; SmbG ⁻ Em ^r	This study
H1	GS5 with transposon Tn916 inserted at the upstream of Smb operon; Tet ^r	24
GS5 Δ <i>comC</i>	GS5::pAYCA1301; ComC ⁻ Em ^r	25
UA159 Δ <i>comC</i>	UA159::pAYCA1301; ComC ⁻ Em ^r	This study
<i>E. coli</i> DH5a	Cloning host	GIBCO BRL
Plasmids		
pCR2.1	Cloning vector; Amp ^r	Invitrogen
pUC19	Cloning vector	Promega
pMM501	pUC19 harboring a <i>bip</i> gene; Amp ^r	This study
pMM502	pUC19 harboring an <i>smbG</i> gene; Amp ^r	This study
pMM503	pUC19 harboring an inactivated <i>bip</i> gene with an Em ^r cassette; Em ^r Amp ^r	This study
pMM504	pUC19 harboring an inactivated <i>smbG</i> gene with an Em ^r cassette; Em ^r Amp ^r	This study

^a wt, wild type.

^b SUNYaB, culture collection at the Department of Oral Biology, State University of New York, Buffalo, N.Y.

^c OU-ACGT, University of Oklahoma Advanced Center for Genome Technology.

TTTAACCC-3'); for *smbG*, forward primer SMBT2/PCRFO (5'-CGTATGGA GCTGTGGGAATAC-3') and reverse primer SMBT2/PCRRE (5'-GGTACT GTGAGCCAATCCAC-3'). Initially, the DNA fragments including the *bip* and *smbG* genes were cloned into the pCR2.1-TOPO vector (Invitrogen), and the resulting plasmids were digested with EcoRI. The fragments were then cloned into pUC19 to generate resulting plasmids pMM501 and pMM502, respectively. Both plasmids were digested with SpeI and BamHI at their respective unique sites, which were located within the target genes, followed by blunt ending and ligation with an *erm* resistance cassette from pUC119Em (19). The resulting plasmids, pMM503 and pMM504, were linearized with KpnI and HindIII, respectively, downstream from the inserted fragments, and the linearized plasmids were used to transform *S. mutans* GS5 (19). Confirmation of plasmid insertions causing gene disruption was performed by PCR (data not shown).

The *bip* H1 double mutant was constructed as follows. The plasmid pMM503, which included the *bip* gene and an *erm* resistance cassette, was linearized with KpnI and transformed into strain H1 (24). The *bip* H1 double mutant construction was confirmed by PCR.

Growth inhibition by antibiotics. Each *S. mutans* strain grown overnight at 37°C was inoculated into THB containing several concentrations of each antimicrobial in duplicate. After the strains were grown overnight at 37°C, the cell density was measured by determining the optical density at 600 nm.

Agar plate bacteriocin assays. Loopfuls of stationary-phase cultures of the *S. mutans* strains were stabbed into a THB agar plate. After 24 h of incubation at 37°C under anaerobic conditions, the plates were overlaid with the *bip* H1 double mutant in 3 ml of THB with 1% low-melting-point agarose (Bioproducts, Rockland, Maine). After 24 h of additional incubation at 37°C under anaerobic conditions, the diameters of the clearance zones surrounding the inoculated bacteria were measured.

Biofilm formation. *S. mutans* GS5 was inoculated into 5-ml portions of half-strength THB in six-well polystyrene microtiter plates to form biofilms. After

48 h of incubation, the planktonic cells and biofilm cells were separated for quantitation as previously described (25). Briefly, biofilm formation was quantitated following crystal violet staining of biofilms, while growth was determined by measuring the turbidities (optical density at 570 nm) of parallel wells following resuspension of the sessile organisms with planktonic cells.

Real-time quantitative RT-PCR. Total RNA was isolated from 15 ml of log-phase cell cultures. After centrifugation, the cells were suspended in 0.3 ml of diethylpyrocarbonate-treated water. The samples were transferred to FastRNA tubes with blue caps (Qbiogene, Inc., Carlsbad, Calif.), and 0.9 ml of TRIzol reagent (Invitrogen) was then added. Cells were broken with a FastPREP FP120 homogenizer (Qbiogene) at a speed setting of 0.6 for 30 s. After samples were placed on ice for 2 min, 0.2 ml of chloroform was added and the tubes were vortexed and centrifuged again as described above. The RNA was finally precipitated from the aqueous phase with isopropanol, and the resulting pellets were dried and resuspended in 20 μ l of diethylpyrocarbonate-treated water. For reverse transcription-PCR (RT-PCR) analysis, RNA samples were treated for 15 min at 37°C with 1.0 U of RNase-free DNase (Amersham Biosciences Corp., Piscataway, N.J.) per ml to remove contaminating DNA. Reverse transcription was carried out with SuperScript III (Invitrogen) according to the directions of the supplier. The real-time RT-PCR was performed on cDNA samples with either the 16S rRNA primers (as internal controls) or *bip*-specific primers (1737/F, 5'-CAGCGCAGCTGATAGCTGTTTGTCT-3'; 1737/R, 5'-CTGCTG GCAAATTCGCTTACTTG-3'), using IQ-Supermix PCR reagent (Bio-Rad) in the iCycler thermal cycler according to the manufacturer's recommendations (Bio-Rad Laboratories). Relative expression levels of *bip* transcripts were then calculated by normalizing the levels of *bip*-specific RNA to the levels of 16S rRNA. By normalizing the cycle threshold (C_T) values for *bip* to the total amount of 16S rRNA, all samples were compared, and the relative fold changes in the samples were calculated using the $-\Delta\Delta C_T$ method described for the MyIQ

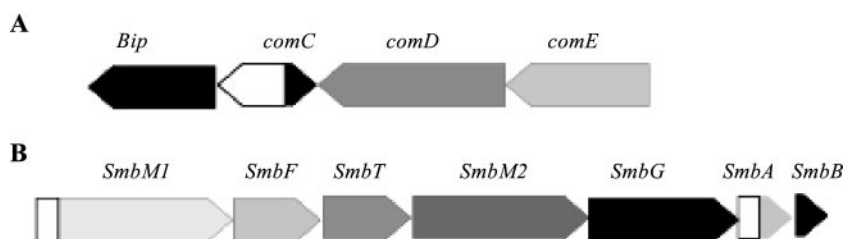


FIG. 1. Relative orientations of the *bip* and *smbG* genes in *S. mutans* GS5. (A) The *bip* gene is located upstream of the *comCDE* locus. (B) The *smbG* gene is located upstream of the bacteriocin structural genes, *smbA* and *smbB*, within the *smb* operon. Open boxes, putative promoter regions of the operons. The transcription of the genes is depicted by the orientation of the boxed arrows.

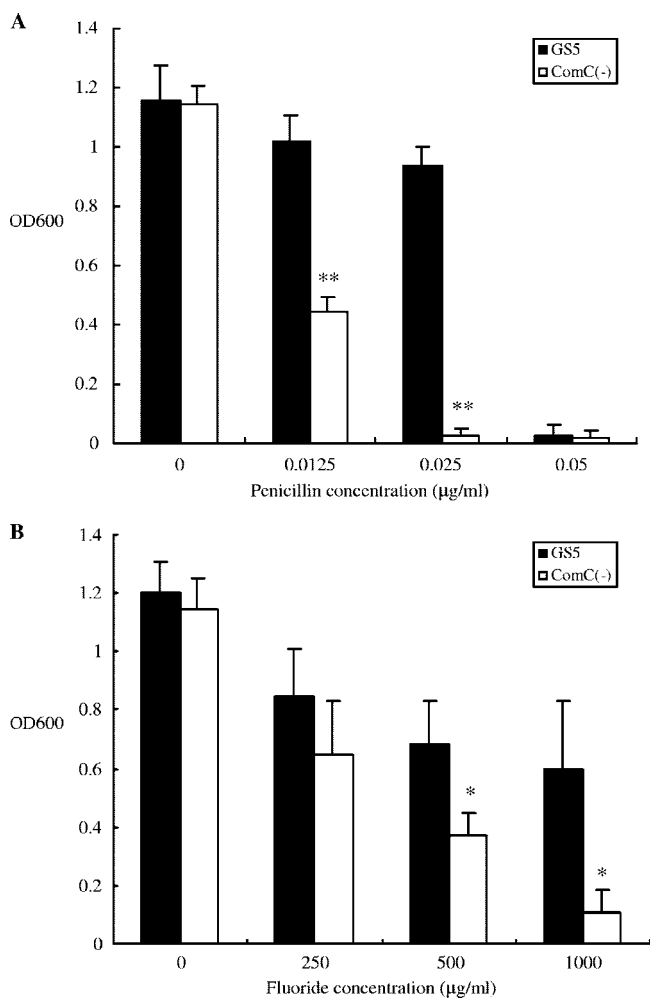


FIG. 2. Antimicrobial sensitivities of GS5 and the *comC* mutant. Penicillin (A) and fluoride (B) at the indicated concentrations were added to cultures grown as described in the text. There are statistically significant differences between the growth of GS5 and that of the *comC* mutant (*, $P < 0.05$; **, $P < 0.001$ [Fisher's PLSD]). Error bars indicate standard deviations.

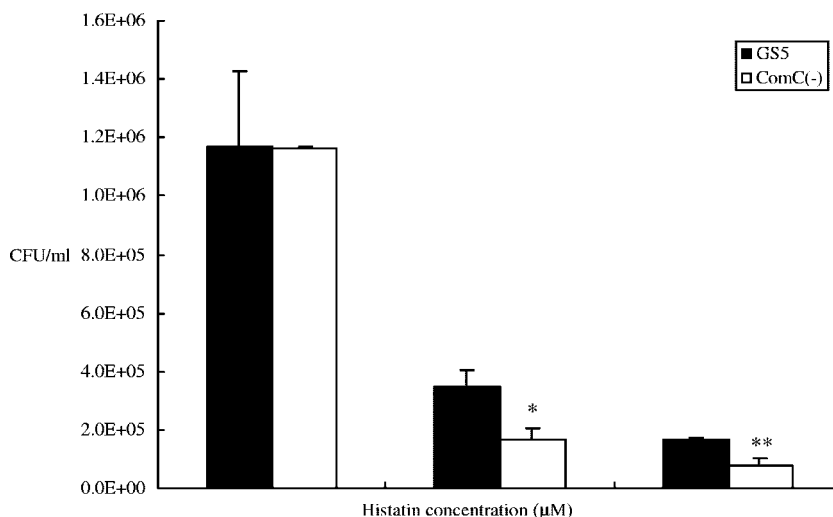


FIG. 3. Histatin sensitivities of GS5 and the *comC* mutant. The GS5 and *comC* mutant strains were grown overnight, and cells were washed twice with sodium phosphate buffer. Cells were resuspended at 2.5×10^5 CFU/ml and incubated with the indicated concentration of histatin. There are statistically significant differences between the growth of GS5 and that of the *comC* mutant (*, $P < 0.05$; **, $P < 0.01$ [Fisher's PLSD]). Error bars indicate standard deviations.

real-time PCR detection system (Bio-Rad Laboratories). A similar approach was used to quantitate *smbG* mRNA

Fluorescence efflux measurement. The tested strains were grown to mid-log phase, and then cells were then harvested at $7,000 \times g$ for 10 min, washed once with 100 mM NaCl–50 mM sodium phosphate buffer (pH 7.0), and suspended again in the same buffer at an optical density at 600 nm of 0.1 in the presence of glycerol. Experimental measurements were generally performed within 2 h after cell preparation. Fluorescence measurements were performed by a modification of methods described previously (15). The fluorescent probe, *N*-phenyl-1-naphthylamine (NPN; Sigma), was initially dissolved in absolute methanol. Fluorescence emission intensity was measured with a Twinkle LB970 fluorometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Excitation and emission wavelengths for NPN were 355 nm and 460 nm, respectively.

Statistical analysis. Intergroup differences between various factors were estimated using a statistical analysis of variance for factorial models. Fisher's protected least-significant differences (PLSD) test was used to compare individual groups.

RESULTS

Antimicrobial sensitivity and quorum sensing. Since relatively dense biofilms have been shown to be more resistant to antimicrobial agents than planktonic cells (5), it was of interest to determine the role of quorum sensing in this property. Therefore, the effects of a *comC* mutation in *S. mutans* GS5 on antimicrobial sensitivity were examined. These results clearly demonstrated that the mutation increased the sensitivity of the mutant to two distinct antimicrobials, fluoride and penicillin (Fig. 2). In addition, the *comC* mutant was much more sensitive to a variety of other distinct antimicrobial agents, including triclosan (an antimicrobial agent used in commercial toothpastes), tetracycline, and the endogenous antimicrobial compound histatin-5 (Fig. 2 and 3). These effects were not strain specific, as the *comC* mutants of strains UA159 (Fig. 4) and BM71 and NG8 (data not shown) also displayed increased sensitivity to the antimicrobial agents. However, surprisingly, the addition of synthetic CSP did not complement these effects in the *comC* mutant. Therefore, this prompted a closer examination of the method utilized to construct the *comC* mutant. Such an analysis revealed that the replacement of the *comC*

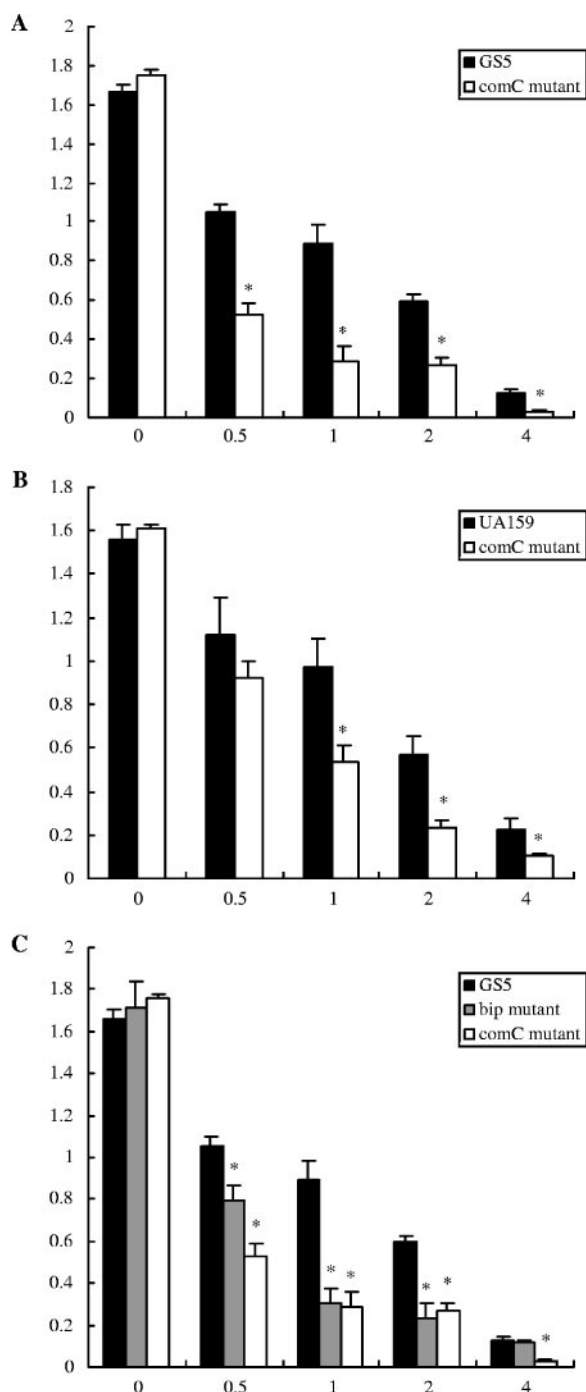


FIG. 4. Tetracycline sensitivities of the *bip* and *comC* mutants. The mutants were incubated in THB containing tetracycline and grown as described in the text. (A) GS5 and GS5 *comC* mutant. (B) UA159 and UA159 *comC* mutant. (C) GS5, GS5 *comC* mutant, and GS5 *bip* mutant. There are statistically significant differences between the growth of the wild-type strains and that of the mutants (*, $P < 0.05$ [Fisher's PLSD]). Each experiment was repeated three times with triplicate samples. Error bars indicate standard deviations.

gene with an *Erm^r* gene also removed a portion of the nucleotide sequence immediately upstream of the gene. The *erm* cassette replaced part of the 3' end of the *comD* gene, all of *comC* and some bases upstream of *comC*. This later region

corresponded to a potential promoter region for a bacteriocin-like cassette containing open reading frames Smu1737 and Smu1738 (Los Alamos National Laboratory database [www.oragen.lanl.gov]) (Fig. 1). The initial gene in this region, Smu1738, displayed weak similarity to a bacteriocin-like protein, BIpO. The adjacent open reading frame, Smu1737, corresponded to a putative immunity-like protein with a molecular size of approximately 15 kDa. This gene, recently referred to as *immA* (21), was named *bip*, for bacteriocin-like immunity protein, in the present study based upon its properties described below and to differentiate it from the other potential immunity protein genes present in the *S. mutans* genome. In order to examine the possibility that construction of the *comC* mutant had also affected expression of the bacteriocin-like operon, expression of this operon was examined using RT-PCR, which showed no detectable expression of this operon in the mutant relative to that in wild-type GS5 (data not shown). This result suggested that the effects of the *comC* mutation on antimicrobial sensitivity could actually be due, in part, to attenuation of the expression of the bacteriocin immunity protein-like gene.

In order to directly test the hypothesis that alterations in the expression of the bacteriocin immunity protein upstream of the *comC* gene affected sensitivity of strain GS5 to a variety of antimicrobial agents, a monospecific *bip* mutant was constructed and was shown to be also altered in antimicrobial sensitivity (Fig. 4).

Therefore, these results suggested that the increased sensitivity of strain GS5 to a wide variety of antimicrobial agents was primarily due to the inactivation of the *bip* gene, which codes for a putative immunity protein.

The SmbG immunity protein also affects antimicrobial sensitivity in *S. mutans* GS5. Since an operon coding for the bacteriocin Smb in strain GS5 has recently been described (24) and was shown to contain a putative immunity protein gene, *smbG*, it was of interest to determine if this gene also influences the sensitivity of strain GS5 to antimicrobial agents. Therefore, an *smbG* mutant was constructed and its antimicrobial sensitivities determined. Like the *bip* mutation, the *smbG* mutation also resulted in increased sensitivity to a variety of antimicrobial agents, including tetracycline and triclosan, relative to wild-type GS5 (Fig. 5). This suggested that the alteration in antimicrobial sensitivity resulting from the *bip* mutation was not limited to this putative immunity protein gene alone.

It was also of interest to examine the antimicrobial sensitivity of a double *bip* *smbG* mutant. However, repeated attempts to construct such a mutant were unsuccessful. Nevertheless, it was possible to isolate a mutant containing both the *bip* mutation and an *smb* promoter mutation (H1) (24) which prevented expression of all of the genes of this operon, including the Smb structural genes *smbA* and *smbB* (24). These results suggested that the *bip* gene could also play a role in the sensitivity of strain GS5 to the Smb bacteriocin. This further suggested that either the *bip* or *smbG* gene is required to protect strain GS5 against the inhibitory effect of the Smb bacteriocin. To test this hypothesis, a comparison of the sensitivities of the wild-type GS5 and the *bip* H1 double mutant to Smb was carried out (Fig. 6). These results indicated that the double mutant, but not the wild-type strain, was very sensitive

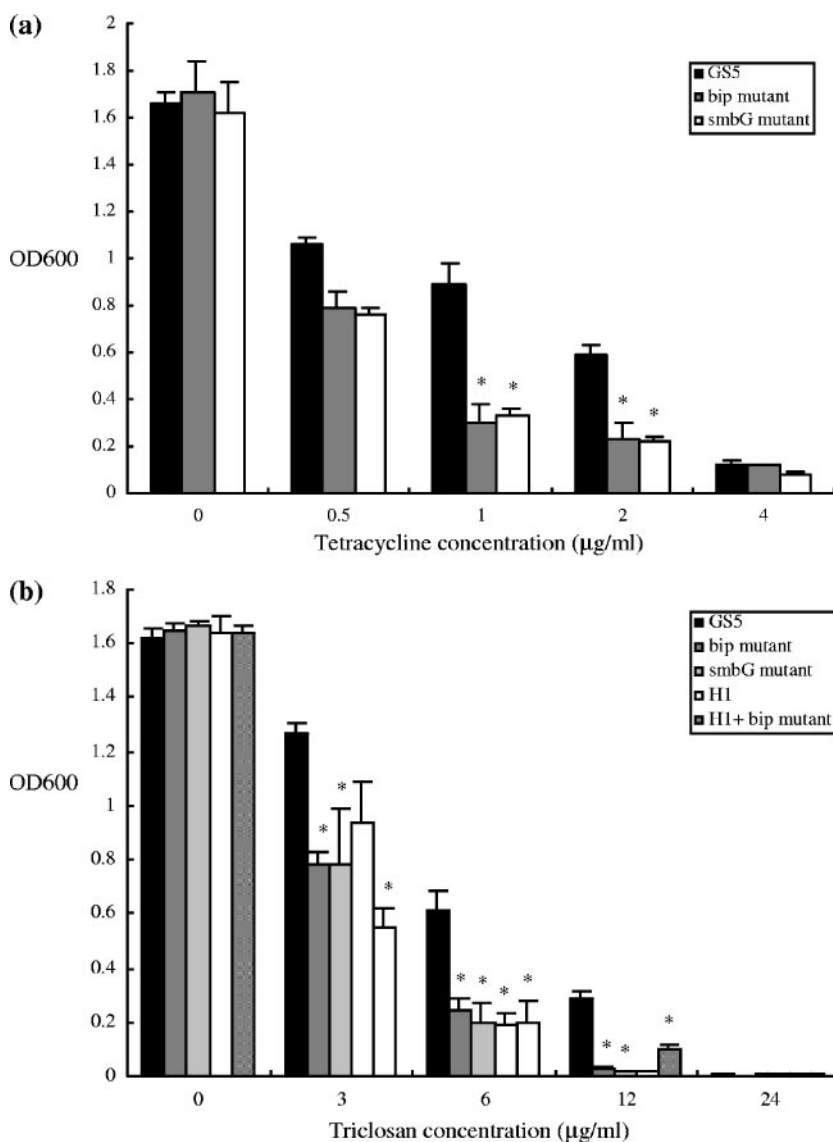


FIG. 5. Antimicrobial sensitivities of GS5 and the *smbG* mutant. Tetracycline (a) and triclosan (b) at the indicated concentrations were added to cultures grown as described in the text. There are statistically significant differences between the growth of GS5 and that of the *smbG* mutant (*, $P < 0.001$ [Fisher's PLSD]). Each experiment was repeated three times with triplicate samples. Error bars indicate standard deviations.

to the bacteriocin. Moreover, the *bip* and *smbG* mutants each grew well in the presence of Smb. These results are consistent with a redundant role for both proteins in immunity to Smb. Therefore, attenuation of either gene significantly increased the sensitivity of strain GS5 to a variety of antimicrobial agents but not that to the endogenous bacteriocin Smb.

***bip* gene expression in biofilm and planktonic cells.** Since the present results suggested a role for bacteriocin immunity proteins in the relatively sensitivity of strain GS5 to a variety of antimicrobial agents and since biofilm cells are more resistant to such agents than planktonic cells, it was of interest to examine the regulation of immunity protein expression during biofilm formation. Therefore, a comparison of *bip* expression in planktonic versus biofilm cells was carried out (Fig. 7). *bip* gene expression appeared to be induced more than twofold during the development of biofilms. Similarly, expression of the

smbG gene was also upregulated under these biofilm-forming conditions (Fig. 7). Therefore, these results are compatible with a role for the putative bacteriocin immunity proteins in the increased resistance of biofilm cells to antimicrobial agents, although the magnitude of the changes detected suggests that *bip* gene expression is not the sole determinant of this phenotype.

***bip* and *smbG* gene expression in the presence of antimicrobial agents.** A role for bacteriocin immunity proteins in resistance to some noncognate bacteriocins was suggested by the demonstration that the H1 mutant defective in Smb production expressed lower levels of *bip* transcripts than wild-type GS5 (Fig. 8). This suggested that the expression of the immunity proteins could be regulated by heterologous antimicrobial agents. To determine whether the presence of antimicrobial agents could affect *bip* and *smbG* expression at the transcriptional level, we used real-time RT-PCR to compare the tran-

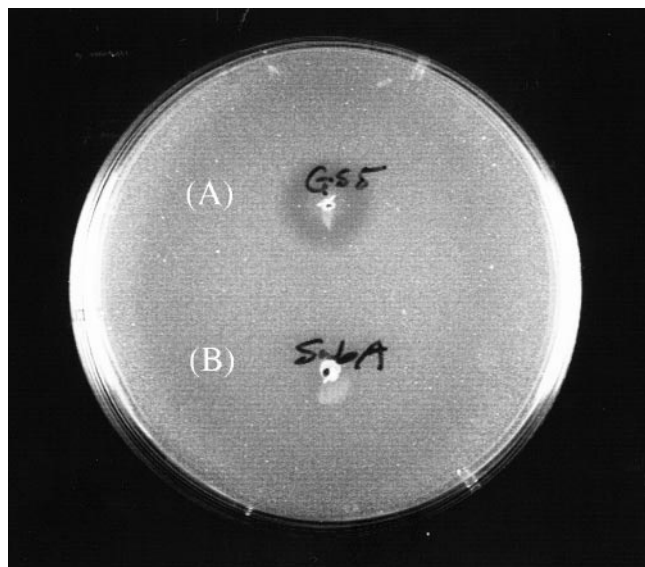


FIG. 6. Sensitivity of the *bip* H1 double mutant to the Smb bacteriocin. Wild-type strain GS5 (A) and the *smbA* mutant (defective in Smb production) (B) were spotted onto a THB agar plate from overnight cultures. After incubation for 1 day, the plate was overlaid with the indicator strain, the *bip* H1 double mutant, and incubated for an additional 24 h.

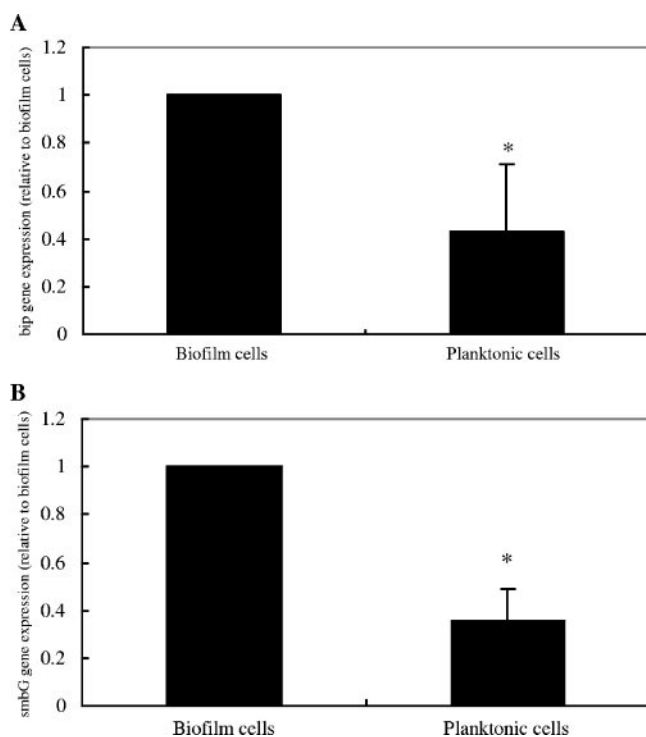


FIG. 7. Expression of the *bip* (A) and *smbG* (B) genes in biofilms and planktonic cells. The quantity of *bip* gene cDNA measured by real-time RT-PCR was normalized to the 16S rRNA cDNA abundance within each unique reaction. Each experiment was repeated three times with triplicate samples. Error bars indicate the variance between triplicate samples within the real-time RT-PCR. There are statistically significant differences between biofilm cells and planktonic cells (*, $P < 0.05$ [Fisher's PLSD]).

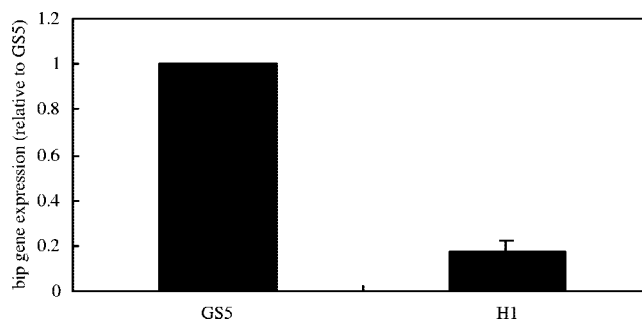


FIG. 8. Effects of *smb* mutation on *bip* gene transcription. The RNA was extracted and quantified by real-time RT-PCR for *bip* gene expression. The quantity of *bip* cDNA measured by real-time RT-PCR was normalized to the 16S cDNA abundance within each unique reaction.

scriptional levels of *bip* and *smbG* in the presence of low concentrations of tetracycline (Fig. 9). The expression of both the *bip* and *smbG* genes was significantly increased in the presence of low concentrations of tetracycline, but the expression of the *bip* gene was induced much more than that of the *smbG* gene.

Extrusion of intracellular compounds in the *bip* and *smbG* mutants. Immunity proteins commonly are export proteins which secrete intracellularly accumulated antimicrobial agents. Therefore, it was of interest to determine if both Bip and SmbG might be involved in the export of antimicrobial compounds. For this purpose, the fluorescent model export agent NPN was utilized to compare the export properties of the mutants with those of the parental GS5 strain. This agent has been utilized previously to quantitate the export of drugs from bacteria (15). Both the *bip* and *smbG* mutants were impeded in exporting NPN relative to the wild-type strain (Fig. 10). Another *Erm*^r mutant, altered in the *gtfB* gene (23), was demonstrated to export NPN similarly to the wild-type strain, indicating that the *Erm*^r marker was not responsible for these differences. Therefore, these results are consistent with a role for both the Bip and SmbG proteins in the export of a variety of distinct antimicrobial agents.

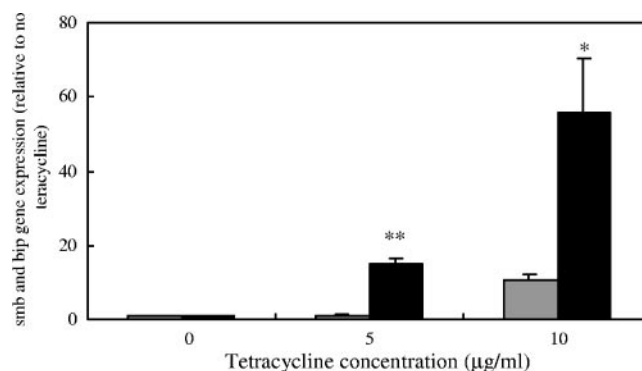


FIG. 9. Immunity protein gene expression in the presence of tetracycline. Shaded bars, *smbG* gene; solid bars, *bip* gene. There are statistically significant differences between strain GS5 in the presence and absence of different concentrations of tetracycline (*, $P < 0.01$; **, $P < 0.001$ [Fisher's PLSD]). Each experiment was repeated three times with triplicate samples. Error bars indicate standard deviations.

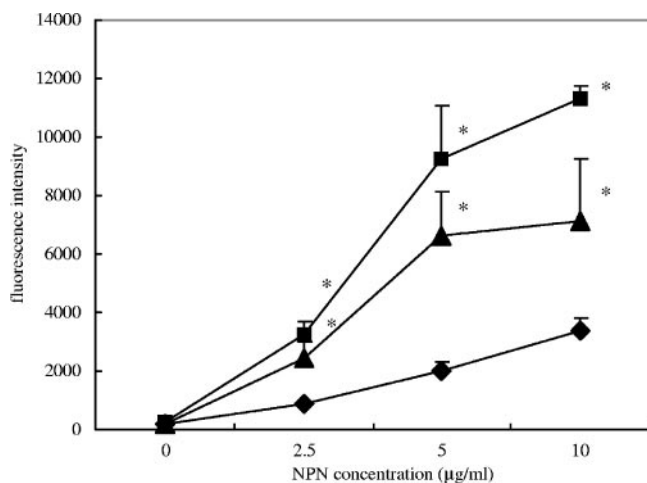


FIG. 10. Roles of Bip and SmbG in export as determined using a model export molecule, fluorescent NPN. Fluorescence of the cells was determined in the presence of different concentrations of NPN as described in the text. ♦, GS5; ■, *bip* mutant; ▲, *smbG* mutant. There are statistically significant differences between GS5 and the two mutants (*, $P < 0.05$ [Fisher's PLSD]). Each experiment was repeated three times with triplicate samples. Error bars indicate standard deviations.

DISCUSSION

Although some of the CSP-induced genes are obviously important for transformation, it is becoming increasingly clear that CSP mediates a variety of different physiological responses (3, 19, 25). Since relatively dense biofilms express increased resistance to antimicrobial agents, we hypothesized that the antimicrobial sensitivity of *S. mutans* might be affected by CSP-dependent quorum-sensing systems. Indeed, the *comC* mutant of strain GS5 exhibited enhanced antimicrobial sensitivity to a variety of different agents. However, CSP did not complement the phenotype of increased antimicrobial sensitivity. These results suggested that attenuation of *comC* expression alone was not responsible for this alteration. The present results suggested that the attenuation of *bip* gene expression was primarily responsible for the increased antimicrobial sensitivity displayed by the *comC* mutant used in this study. Therefore, the gene replacement strategy for generating the *comC* mutant used in this investigation apparently altered the promoter activity corresponding to the *bip*-containing operon. Despite the fact that it has been recently demonstrated that CSP is involved in the regulation of expression of this operon (21), the inability to complement these effects in the *comC* mutant by addition of exogenous CSP suggests that it is the promoter alteration which is primarily responsible for the phenotype displayed by the *comC* mutant. Nevertheless, it is predicted that a true *comC* mutant of *S. mutans* without an alteration in the *bip* operon should also be more sensitive to a variety of antimicrobial agents due to repression of *bip* and *smbG* expression. Thus, in this manner, quorum sensing mediated by CSP can indirectly affect the relative sensitivity of *S. mutans* to antimicrobial agents. In addition, to our knowledge, this is the first demonstration that putative bacteriocin immunity proteins display broad activities with a variety of heterologous antimicrobial agents.

The present results further suggest that other bacteriocin immunity proteins in addition to Bip in strain GS5 could also affect antimicrobial sensitivity. For example, the *smbG* gene, coding for another putative immunity protein, also appears to play a role in this sensitivity. Recent results have also suggested that CSP is also involved in the regulation of expression of the *smb* operon (24). However, the inability to complement the *comC* mutation and alter antimicrobial sensitivity with exogenous CSP under conditions where bacteriocin production is complemented (24) suggests that *smbG* is not primarily responsible for the increased *comC* mutant antimicrobial sensitivities. Nevertheless, the *smbG* mutation also increased the sensitivity of strain GS5 to antimicrobial agents, indicating that this gene product does have an effect on this property. Thus, it is suggested that the multiple bacteriocin-like immunity proteins expressed by organisms such as *S. mutans* could play roles in the resistance of these organisms to a variety of antimicrobial agents.

It has been widely reported that bacteria growing as a surface biofilm are more resistant to various stress challenges and antimicrobial agents than planktonic cells (2, 7, 14). Of medical importance is the observation that biofilm cells can be 1,000-fold more resistant to antibiotics than planktonic cells. The present results suggesting increased expression of the putative immunity protein genes during in vitro biofilm formation are compatible with a general role for the bacteriocin immunity proteins in such resistance. However, the magnitude of the induction demonstrated suggests that this may not be the major factor associated with biofilm resistance.

The present study also demonstrated that the expression of the *bip* and *smbG* genes was markedly increased in the presence of tetracycline. The induced expression of the *bip* gene in the presence of low concentrations of the antibiotic was more pronounced, approximately 1,000-fold higher than that of the *smbG* gene. Such a large increase might reflect the weak expression of the *bip* gene in the absence of exogenous antimicrobial agents. In addition, the present results suggesting a redundant role for SmbG and Bip relative to Smb sensitivity further suggest that these proteins are not absolutely specific for resistance to their cognate bacteriocins. However, the putative bacteriocin associated with Bip has not yet been characterized.

The present results are also consistent with a role for both the Bip and SmbG proteins in the export of antimicrobial agents, as suggested for many bacteriocin immunity proteins (12). Both the *bip* and *smbG* mutants were attenuated in exporting NPN relative to the wild-type strain. An interesting question raised by these results involves the molecular basis for the broad specificity displayed by the *S. mutans* putative immunity proteins. Although it would appear that these proteins evolved to ensure the relative resistance of the producer strain to their respective bacteriocins, it appears that these proteins have much broader specificity. Further investigation into the structures and mechanisms of action of these proteins will be required to determine the molecular basis for these properties.

Several general implications of the present results are suggested both for the evolution of immunity proteins and for antimicrobial therapy. It is reasonable to propose that bacteriocin-producing bacteria may have evolved as a result of the environmental advantage of producing bacteriocins. These an-

timicrobial products generally affect organisms closely related to the producers. The present results suggest that the selection for bacteriocin immunity proteins may also have been an important factor in the evolution and transmission of these operons. The broad specificity of these proteins as demonstrated in the present study may provide protection against a variety of inhibitory substances (i.e., metals, antibiotics, bacteriocins, etc.) in different environments. The demonstration of the presence of genes such as those in the *bip*-containing operon of *S. mutans* without demonstrated antimicrobial activity is also compatible with such a hypothesis. Thus, the activity of the immunity proteins, rather than their cognate bacteriocins, may have become important for the survival of some bacteria in specific environments.

The present results further suggest a novel approach for chemotherapy against certain bacteria. It may be possible to increase the effectiveness of commonly used antimicrobial agents by specifically inhibiting the bacteriocin immunity protein activities of these organisms. For example, the effectiveness of the commonly utilized antiplaque agent triclosan against cariogenic *S. mutans* could be enhanced by developing specific inhibitors of Bip activity. Moreover, since CSP is involved in regulating the expression of multiple bacteriocin operons in these organisms, specific inhibitors of CSP activity may also produce comparable effects. Such approaches would also have the advantage of targeting specific pathogenic organisms without comparable effects on endogenous bacteria. Further investigation will be required to determine if such approaches can prove to be useful in chemotherapy against specific diseases.

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