Genetic Analysis of a Unique Bacteriocin, Smb, Produced by Streptococcus mutans GS5

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A dipeptide lantibiotic, named Smb, in Streptococcus mutans GS5 was characterized by molecular genetic approaches. The Smb biosynthesis gene locus is encoded by a 9.5-kb region of chromosomal DNA and consists of seven genes in the order smbM1, -T, -F, -M2, -G, -A, -B. This operon is not present in some other strains of S. mutans, including strain UA159. The genes encoding Smb were identified as smbA and smbB. Inactivation of smbM1, smbA, or smbB attenuated the inhibition of the growth of the indicator strain RP66, confirming an essential role for these genes in Smb expression. Mature Smb likely consists of the 30-amino-acid SmbA together with the 32-amino-acid SmbB. SmbA exhibited similarity with the mature lantibiotic lacticin A2 from Lactococcus lactis, while SmbB was similar to the mersacidin-like peptides from Bacillus halodurans and L. lactis. We also demonstrated that Smb expression is induced by the competence-stimulating peptide (CSP) and that a com box-like sequence is located in the smb promoter region. These results suggest that Smb belongs to the class I bacteriocin family, and its expression is dependent on CSP-induced quorum sensing.

Dental caries has plagued humans since the dawn of civilization and still constitutes one of the most common human infectious diseases. Multiple species of bacteria inhabit the human oral cavity, and the species most commonly associated with human caries is Streptococcus mutans (22). Among the attributes thought to contribute to the virulence of S. mutans is its ability to elaborate antmicrobial or bacteriocin-like substances, which may provide a selective advantage for initial or sustained colonization in a milieu of densely packed competing organisms found in dental plaque (38, 48).

Bacteriocins are a family of ribosomally synthesized peptide antibiotics that are produced by bacteria (11, 14, 18, 37). They are subdivided into four different classes based on biochemical and genetic characteristics (14, 16, 17). Class I and class II bacteriocins are by far the most extensively studied because they are the most abundant and most prominent in industrial applications (26). Class I bacteriocins, named lantibiotics, contain two modified amino acid residues, lanthionine and/or methyllanthionines, which are formed posttranslationally (7).

The primary product of the lantibiotic structural gene is a precursor with an N-terminal leader sequence followed by a C-terminal propeptide which undergoes modification. Once modified within the cell, the bacteriocin is secreted by a dedicated transporter and the N-terminal leader sequence is cleaved by a protease (12, 49).

Some strains of S. mutans produce antimicrobial substances called mutacins (3, 4, 30, 34, 35, 36). Mutacins have been classified into two families: the lantibiotics and the nonlantibiotics. Classification of mutacin-producing strains based on their bactericidal activities, their sensitivities to other or self-produced mutacins, and the presence of plasmids divides the mutacins into four types, I, II, III, and IV (28, 34, 35, 36). Mutacins I, II, and III belong to the lantibiotic family, while mutacin IV is a dipeptide nonlantibiotic bacteriocin. The structural genes for the prepropeptides of mutacins I, II, III, and IV have been sequenced, and their biosynthetic loci are composed of multiple genes, including those involved in regulation, cleavage, transport, and immunity to the produced mutacins (28, 34, 35, 36). However, a recent report suggests that the bacteriocin previously demonstrated to be synthesized by S. mutans GS5 (31) is not a member of the mutacin I, II, or III family (23).

Quorum sensing in gram-positive bacteria has been found to regulate a number of physiological activities, including competence development in Streptococcus pneumoniae (19) and S. mutans (21). A quorum-sensing system essential for genetic competence in S. mutans was recently identified (21). This cell-cell signaling system involves at least five gene products encoded by comAB (33) and comCDE (21). The comC genes encode a competence-stimulating peptide (CSP) precursor. Recently, several competence-specific genes which are likely involved in the DNA uptake process and in recombination, such as cilA (ssb2, a gene for single-stranded breaks), cilB (similar to dprA in Haemophilus influenzae) (15), cilC (ccl, similar to comC in Bacillus subtilis), cilD (cglABCDE), cilE (celAB), and coi (2, 32), were identified. These operons contain a conserved consensus sequence, TACGAATA (com box), at position −10 from the transcription start site and a T-rich region at −25 (2).

In the present study, we have used Tn916 mutagenesis and the single-specific-primer PCR (SSP-PCR) (29, 43, 44) technique to characterize a novel smb (S. mutans bacteriocin) operon in S. mutans GS5. We demonstrate that the smb genes are present in an operon structure using transcriptional analysis. Targeted gene integration mutagenesis was also used to probe the essentiality of the operon genes for Smb production. In addition, the results of sequence analysis and homology searches demonstrated that Smb is a class I two-component bacteriocin and is regulated by CSP.
**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** *S. mutans* GS5 was used in this study for the production and characterization of the Smb bacteriocin. RP66 (group C streptococcus) was used as an indicator strain for Smb activity assays (31). *S. mutans* GS5 and RP66 were grown in Todd-Hewitt (TH) medium (Becton Dickinson) in an anaerobic atmosphere of 85% N<sub>2</sub>, 10% CO<sub>2</sub>, and 5% H<sub>2</sub>. Transformants of *S. mutans* were selected following their growth on TH broth agar plates supplemented with 10 μg of erythromycin per ml or 3 μg of tetracycline per ml.

**Agar plate assays.** Loopfuls of stationary-phase cultures of *S. mutans* strains were stabbed into a TH broth agar plate. The plate was incubated at 37°C for 24 h. RP66, to be assayed for sensitivity, was grown to an optical density of 0.2 at 550 nm. Each culture was then diluted 1:100, and 0.2 ml of this dilution was pipetted into a tube containing 4 ml of molten Trypticase soy broth containing 1% agar (Becton Dickinson and Co.). This solution was mixed and poured evenly onto the surfaces of the plates and incubated at 37°C for an additional 24 to 48 h, and the diameters of the zones of inhibition were measured.

**Characterization of the Tn<sub>916</sub> insertion region.** The broad-host-range conjugative transposon Tn<sub>916</sub>, originally identified on the chromosome of Enterococcus faecalis (9), has been used as a mutagen in streptococci. SSP-PCR (29, 43, 44) was performed for the characterization of the Tn<sub>916</sub> insertion sites. Briefly, chromosomal DNA from a transposon-containing GS5 mutant was isolated and digested with the restriction endonuclease EcoRI and then ligated into EcoRI-digested pUC19. The ligation mixture served as the template for amplification with transposon-specific primers (6) and an M13 primer. Subsequently, the PCR product was used as a template for sequencing. Amino acid homology searches and comparisons were carried out with the FASTA and BLAST network services of GenBank.

**Construction of the smb mutants.** The mutants of the smbM1, smbC, smbD, and smbA-smbB genes were created by double-crossover homologous recombination via insertion of an erythromycin resistance determinant into each gene. The plasmids used for disruption of the smbM1, smbA, and smbB genes were prepared as follows. The PCR fragments of the upstream and downstream regions of each gene were amplified with pairs of primers and chromosomal DNA from GS5 as a template. Initially, PCR products of the downstream region were ligated into the pResEmMCS10 plasmid (42) containing the Erm cassette. A 2.5-kb fragment was generated from the THL0-2 and M13-Fw primer set using ligation mixtures of EcoRI-digested H1 mutant chromosomal DNA and plasmid pUC19. The other flanking region of the Tn<sub>916</sub> insertion was not shown). Transformation of strain GS5 with the DNA isolated from the H1 mutant chromosomal DNA (data not shown). Transformation of strain GS5 with DNA isolated from mutant H1 resulted in mutants with reduced Smb production (data not shown).

**Identification of the bacteriocin gene locus following transposon mutagenesis.** The broad-host-range conjugative transposon Tn<sub>916</sub>, originally identified on the chromosome of *E. faecalis* (9), was used to mutagenize *S. mutans* GS5. One of 8,000 Tn<sub>916</sub> transformants of strain GS5, mutant H1, exhibited a pronounced bacteriocin-negative phenotype when it was screened on agar plates with indicator strain RP66 (Fig. 1). Southern blotting indicated the presence of a single copy of the Tn<sub>916</sub> transposon in the H1 mutant chromosomal DNA (data not shown). Transformation of strain GS5 with the DNA isolated from mutant H1 resulted in mutants with reduced Smb production (data not shown).

In order to characterize the Tn<sub>916</sub> insertion site, SSP-PCR was performed. A 2.5-kb fragment was generated from the THL0-2 and M13-Fw primer set using ligation mixtures of EcoRI-digested H1 mutant chromosomal DNA and plasmid pUC19. The other flanking region of the Tn<sub>916</sub> insertion was also amplified by using the same technique with the TnLO and F13-Rev primer set. Sequence analysis of these regions indicated that the GS5 bacteriocin genes, designated smb, were located downstream of the Tn<sub>916</sub> insertion site (Fig. 2). Additional primers were then designed according to the newly
derived DNA sequences. The original \textit{smb} locus was amplified by PCR from the wild-type strain GS5, and the PCR products were used as templates for further sequence analysis. Comparison of the sequence adjacent to the Tn916 insertion region of H1 with the wild-type GS5 sequence showed that Tn916 was inserted into the promoter region of the \textit{smb} locus. A total of about 11 kb of DNA was sequenced in this region. The \textit{smb} locus is not present in the \textit{S. mutans} UA159 database (http://www.genome.ou.edu.smutans.html). Sequence analysis revealed that there are 12 putative open reading frames in the region of \textit{cyl}\textit{-smbM1-smbF-smbT-smbM2-smbG-smbA-smbB} (Fig. 2), which were followed by three predicted transposases and an ABC transporter gene. The \textit{cyl} and ABC transporter genes are found adjacent to one another in the UA159 database. The \textit{cyl} gene encodes a leucyl-tRNA synthetase and therefore was presumed to define the upstream border of the \textit{smb} gene locus in strain GS5.

\textbf{Sequence analysis of the \textit{smb} locus and homology of the \textit{Smb} gene products with other proteins}. Inspection of the upstream region of \textit{smbM1} revealed a potential ribosomal binding site with the sequence AAGGG, which was 6 bp upstream of the predicted initiating codon, GTG. A GenBank search for similar peptides revealed that the first gene, \textit{smbM1}, encoded a protein of 958 aa, which showed significant similarity to the lantibiotic-mersacidin-modifying enzyme from \textit{Bacillus halodurans} (46) and \textit{scnM} from \textit{Streptococcus pyogenes} (13). The second gene, \textit{smbF}, began 11 bp after the stop codon for \textit{smbM1}. This gene would encode a protein of 274 aa which shows homology with the SpaF protein from \textit{Bacillus subtilis} (45) and MutF from another bacteriocin-producing strain of \textit{S. mutans}, UA877 (36). The SpaF and MutF proteins are immunity proteins for lantibiotic bacteriocins. The third gene, \textit{smbT}, encoded 243 aa and bore similarity to an ABC transporter (27). The reading frames of \textit{smbF} and \textit{smbT} overlapped in 5 bp. The fourth gene in the operon, \textit{smbM2}, encoded a protein of 876 aa, which resembled the salivaricin A modification protein from \textit{S. pyogenes} (1) and the lantibiotic-modifying enzyme from \textit{Staphylococcus aureus} (51). The reading frame of \textit{smbM2} overlapped with that of \textit{smbT} by 16 bp and is followed closely by \textit{smbG}. The latter gene encoded a putative protein of 968 aa and exhibits similarity with the \textit{phnG} immunity gene from \textit{Lactobacillus plantarum} (8). A GenBank search for similar peptides revealed that SmbA was homologous to lactacinA2 (39) and displayed 46.9% identity and 75% similarity with the lactacin at the amino acid level. Furthermore, SmbB was similar to a mersacidin-like peptide (24, 46), with the putative mature peptide sharing 52.9% identity and 70.6% similarity with the mersacidin-like peptide (Fig. 3). LacticinA2 produced by \textit{Lactococcus lactis}, the mersacidin-like peptide produced by \textit{B. halodurans}, and lacticinA1 secreted by \textit{L. lactis} are components of lantibiotic bacteriocins (24, 46). These results suggested that the two peptides, SmbA and SmbB, belong to the dipeptide lantibiotic bacteriocin family (10, 24, 25, 39, 40).

\textbf{Inactivation of the \textit{smb} genes and their effects on \textit{Smb} production}. To determine whether the two putative structural genes \textit{smbA} and \textit{smbB} are required for \textit{Smb} activity, we disrupted the two genes individually or together by inserting an erythromycin resistance gene cassette within the genes (Fig. 4a). The three resulting mutants, GS5SmbAEm (H2A), GS5SmbBEm (H2B), and GS5SmbABEm (H2AB), were assayed for \textit{Smb} production by plate assays against RP66 (Fig. 4b). Although RP66 displays some sensitivity to most \textit{S. mutans} strains, including strain UA159 (data not shown), which lacks the \textit{smb} genes, each of the mutants was markedly attenuated in its ability to inhibit the growth of indicator strain RP66 compared with that of parental strain GS5. This result suggests that both the \textit{smbA} and \textit{smbB} genes are necessary for \textit{Smb} activity. We also disrupted the \textit{smbA} gene by insertional inactivation and also constructed a mutant with the Erm cassette inserted into the same region disrupted by Tn916 in mutant H1. The fact that we detected transcription of the genes downstream from the Erm cassette in mutants H2A and H2M1 by RT-PCR (data not shown) demonstrated that the H2A and H2M1 mutations did not produce polar effects. The resulting mutants, GS5SmbM1Em (H2M1) and GS5SmbPEm (H2P), were also markedly attenuated in bacteriocin production (Fig. 4b).

\textbf{Transcriptional analysis of the \textit{smb} operon}. To determine how many transcription units comprise the Smb biosynthetic locus, Northern blotting and RT-PCR were performed with wild-type GS5. We performed Northern blot analysis with DIG-labeled RNA probes (200 to 300 bp) specific to each of the seven \textit{smb} genes. Hybridization with the \textit{smbA} or \textit{smbB} probe detected the same transcript of about 500 bp in size (Fig. 5). This transcript size was approximately equal to that predicted for cotranscription of the two genes. This result suggested that transcripts for both \textit{smbA} and \textit{smbB} corresponded to an initiation site upstream of \textit{smbA}. Multiple attempts at detection of \textit{smb} operon transcripts by Northern blot analysis of GS5 using the other probes were unsuccessful, even when 10 times the amount of RNA was analyzed (data not shown).

FIG. 2. Smb biosynthesis genes. The orientation of the genes and their relative distances are shown. The \textit{cyl} gene encodes leucyl tRNA synthetase and is therefore presumed not to be part of the \textit{smb} biosynthesis gene operon. The arrows represent promoters (P), and a potential terminator (black oval) is also depicted.
Therefore, we hypothesized that the mRNA encoding \textit{smbM1} to \textit{smbG} or \textit{smbB} is transcribed together and is too large to be detected following Northern blot analysis. Furthermore, it is unlikely that these negative results were due to weak transcription from the promoter upstream of \textit{smbM1}. We further tested this hypothesis using the RT-PCR approach. The results of RT-PCR analysis indicated that mRNA encoding SmbM1 was carried on the same transcript as that encoding SmbF, since a product of the expected size was amplified with specific \textit{smbM1} forward and \textit{smbF} reverse primers from wild-type GS5 (data not shown). A similar RT-PCR analysis revealed that the \textit{smbF} transcript was cotranscribed with \textit{smbT}, \textit{smbT} was cotranscribed with \textit{smbM2}, and \textit{smbT} was cotranscribed with \textit{smbA}, indicating that the \textit{smb} locus represents a seven-gene operon. Taken together, these data suggest that there is a single \textit{smb} operon with two promoters, one upstream of \textit{smbM1} and the other flanking \textit{smbA} with a terminator sequence downstream of \textit{smbB}.

The CSP is required for the transcription of \textit{smbA} and \textit{smbB}. Recent results have indicated that a GS5 \textit{comC} null mutant (CC1301) (52) was attenuated in bacteriocin production (49a). However, addition of synthetic CSP to the culture of this mutant restored the production of Smb. With cocultured samples, it was demonstrated that mutants H2AB, H2M1, and H2P also complemented the \textit{comC} mutant for bacteriocin production (data not shown). These data indicated
that these smb mutants produce CSP and that their inability to secrete bacteriocin was not due to a defect in CSP secretion. In order to determine which smb genes are affected by the competence of strain GS5, Northern blot analysis and RT-PCR were used to examine expression of these genes in the presence and absence of CSP. Transcription of the smbA and smbB genes appeared to be weaker in the comC mutant than in the parental strain GS5 following Northern blot analysis (Fig. 6). Furthermore, even weaker expression of smbA and smbB was apparent in the mutant whose promoter region upstream of smbM1 was altered. This result suggests that this promoter may be the major promoter for regulating the expression of these two genes. RT-PCR analysis also confirmed these results (data not shown). These results are consistent with the agar plate assay results demonstrating that the comC mutant inhibition zone against RP66 is much smaller than that of GS5 but larger than that of H2P (data not shown). Using RT-PCR, the other transcripts of the smb operon (M1 to G) were detected at the same levels in all of strains (data not shown). These results show that the reduction of Smb production in the comC and H2P mutants resulted from decreased transcription of the smb operon.

Most of the CSP-induced genes in S. pneumoniae and S. mutans have a com box (TACGAATA) sequence located in their promoter regions (9, 20, 50). We also identified candidate sites which shared sequence elements with the com box and which were located in the apparent extragenic regions. We identified a com box-like sequence upstream of smbA (Fig. 7a), suggesting that the promoter upstream of smbA may be regulated by the competence state of the cells. We also observed that the smb operon promoter region (upstream of smbM1), containing the transposon insertion site in mutant H1, was very highly homologous to the promoter region of the comC gene. The two sequences are very closely related, with over 93% identity (Fig. 7b). Based on the results of H2P Northern blot analysis and the reduction of the levels of transcription of smbA and smbB, these com box sequences likely are involved in the regulation of the two Smb structural genes.

**DISCUSSION**

In the present study, we identified an S. mutans GS5 unique bacteriocin locus, smb, following Tn916 mutagenesis. One of the resulting transformants, H1, exhibited a defective phenotype for the production of Smb and was isolated following screening of approximately 8,000 Tn916 transformants. Sequence analysis of wild-type S. mutans (lane 1), the comC mutant (lane 2), and the H2P mutant (lane 3) with the DIG-labeled smbAB probe. The arrow indicates the smbA-smbB transcript. The RNA molecular size marker is present in the leftmost lane (lane M).
that is known to immediately precede the cleavage site in several bacteriocins (47). In SmbB, the presence of Gly-Ala may play a similar role (7). A GenBank search for similar peptides revealed that these two peptides have homologies with lantibiotic peptides; SmbA was similar to lacticin A2 encoded by \textit{L. lactis}, and SmbB was homologous to mersacidin-like peptides expressed by \textit{B. halodurans} and \textit{L. lactis}. Inactivation of the \textit{smbA} or \textit{smbB} gene resulted in marked attenuation of the inhibitory effects on indicator strain RP66. That the products of both genes are required for bacteriocin activity was further suggested by the observation that inactivation of the \textit{smbA} gene did not interfere with transcription of the \textit{smbB} gene, ruling out possible polar effects in the \textit{smbA} mutant (data not shown). However, we did not obtain direct evidence that Smb is a dipeptide. Nevertheless, these data show that both SmbA and SmbB are required for bacteriocin activity and that either the active bacteriocin is a dipeptide or each peptide acts in a synergistic manner to produce inhibition against the indicator strain RP66. Purification and chemical characterization of the active bacteriocin will be required to resolve this issue.

Recently, several dipeptide lantibiotics, including cytolysin produced by \textit{E. faecalis} (10), staphylococcin C55 produced by \textit{S. aureus} C55 (25), and lacticin 3147 produced by \textit{L. lactis} subsp. \textit{lactis} DPC3147 (39, 40), have been identified. It has been observed for these bacteriocins that equivalent amounts of both peptides are required for an interaction with target cells. This observation suggests that both SmbA and SmbB may be required at equivalent levels for bacteriocin activity. The Smb operon also appears to contain two modification genes. The biosynthesis of lantibiotics involves several posttranslational modification steps (5, 7, 41). Following translation of the \textit{smb} transcript into prepropeptides, these products must be modified. The observation of the presence of two putative modification genes (\textit{smbM1} and \textit{smbM2}) and two structural genes (\textit{smbA} and \textit{smbB}) within the \textit{smb} gene cluster suggests several possibilities: (i) both propeptides may be modified sequentially by both modification enzymes, (ii) either one of the modification enzymes may alter both propeptides (with the second modification protein being redundant), and (iii) each propeptide may be modified by one specific modification enzyme. In the \textit{smb} operon, a gene coding for a potential transporter (\textit{smbT}) is also present in the \textit{smb} operon. Typically, the bacteriocin-encoding genes are processed and secreted out of the cell via a dedicated transporter, which is a typical feature of most class I and class II bacteriocins (6). In most of the bacteriocin operons described to date, a gene encoding an immunity protein which protects the producing bacteria against autotoxicity is usually located downstream of the bacteriocin structural genes (26). The bacteriocin- and immunity protein-encoding genes are generally cotranscribed to ensure that the producer strain is not killed by its own bacteriocin (26). For the strain GS5 bacteriocin, \textit{smbF} and \textit{smbG} are good candidates to encode Smb immunity proteins. Significant homology among immunity proteins in the bacterial databases has been observed. For example, SmF showed homology with the immunity proteins of the bacterial databases has been observed. For example, SmF showed homology with the immunity proteins of the bacterial databases has been observed. For example, SmF showed homology with the immunity proteins of the bacterial databases has been observed.
nity proteins SpaF and MutF (36, 45), while SmbG exhibited similarity with the PlnG immunity protein (8).

Recently, it was observed that a GSS comC mutant, CC1301 (52), was attenuated in Smb expression (49a). Addition of synthetic CSP was able to restore Smb production to the comC mutant. S. mutans uses a typical gram-positive CSP secretion and detection system for quorum sensing which affects several physiological properties (21). Interestingly, the promoter regions of both comC and the smb operon share similar sequences. The H1 and H2P mutants are disrupted by insertion of transposon Tn916 or the Erm cassette, respectively, resulting in a reduction in Smb expression. This decrease in Smb production results from a reduction in the transcription of the smb operon, including the smbA and smbB genes. However the mechanism by which these identical promoter sequences are involved in the transcription of smbA and smbB is still unknown. A recent report suggests that ComE regulated transcription results from a reduction in the transcription of the smb operon, including the smbA and smbB genes. However the mechanism by which these identical promoter sequences are involved in the transcription of smbA and smbB is still unknown. A recent report suggests that ComE regulated transcription directly by interacting with the major RNA polymerase and the direct repeats in the comC promoter region. Based on these reports, Smb production may be directly regulated by ComE. The smb promoter region, upstream of smbM1, has a com box-like structure present at the end of the common sequence of both the comC and smb promoter regions. The smb locus may have originally been inserted into the strain GSS5 chromosome on an insertion element (direct repeat of a bacteriocin in S. mutans). This is the first demonstration that CSP regulates the expression of a bacteriocin in S. mutans.

In summary, we have identified the genes for a putative dipeptide lantibiotic-type bacteriocin produced by S. mutans strain GSS5. Other strains of this organism, including strain BM71, also appear to produce the same bacteriocin (unpublished results). Smb is unique and distinct from the other previously characterized bacteriocins, mutacin I to IV, produced by other S. mutans strains and characterized by Caufield’s group (3, 34, 35, 36). In addition, we have determined that strain GSS5 also produces a nonlantibiotic mutacin IV bacteriocin (data not shown). Thus, bacteriocin production may be used by S. mutans as a means to compete with other oral bacteria present in dental plaque. However, the in vivo role of the S. mutans bacteriocins in dental caries formation still remains to be determined.

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