

SmbFT, a Putative ABC Transporter Complex, Confers Protection against the Lantibiotic Smb in Streptococci

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Streptococcus mutans, a dental pathogen, secretes different kinds of lantibiotic and nonlantibiotic bacteriocins. For self-protection, a bacteriocin producer strain must possess one or more cognate immunity mechanisms. We report here the identification of one such immunity complex in *S. mutans* strain GS-5 that confers protection against Smb, a two-component lantibiotic. The immunity complex that we identified is an ABC transporter composed of two proteins: SmbF (the ATPase component) and SmbT (the permease component). Both of the protein-encoding genes are located within the *smb* locus. We show that GS-5 becomes sensitized to Smb upon deletion of *smbT*, which makes the ABC transporter nonfunctional. To establish the role SmbFT in providing immunity, we heterologously expressed this ABC transporter complex in four different sensitive streptococcal species and demonstrated that it can confer resistance against Smb. To explore the specificity of SmbFT in conferring resistance, we tested mutacin IV (a nonlantibiotic), nisin (a single peptide lantibiotics), and three peptide antibiotics (bacitracin, polymyxin B, and vancomycin). We found that SmbFT does not recognize these structurally different peptides. We then tested whether SmbFT can confer protection against haloduracin, another two-component lantibiotic that is structurally similar to Smb; SmbFT indeed conferred protection against haloduracin. SmbFT can also confer protection against an uncharacterized but structurally similar lantibiotic produced by *Streptococcus gallolyticus*. Our data suggest that SmbFT truly displays immunity function and confer protection against Smb and structurally similar lantibiotics.

Oral biofilm is complex and diverse in nature. As many as 700 different bacterial species colonizes in our mouth; unfortunately, the identity of many of them is still unknown (1, 2). The complexity of biofilm greatly depends on the interspecies interaction during both early and maturing phases of biofilm formation. The participation of these organisms is not restricted to dental disease only, since they can cause bacteremia and in some cases infective endocarditis (3–5). Endocarditis is generally caused by viridans group of streptococci. Some of these viridians streptococci are commensal in oral cavity, and the others are involved in disease formation (5, 6). Some of the commensal streptococci have a protective role against oral disease development (7). The exact *in vivo* mechanisms for this interspecies interference are currently not well understood; however, several studies strongly suggest an important role for hydrogen peroxide and bacteriocin in this process (8–10).

In general, bacteriocins are ribosomally synthesized small peptides with bactericidal or bacteriostatic activity on other species. Bacteriocins are broadly categorized into two groups: nonlantibiotic and lantibiotic. The nonlantibiotic group contains peptides that do not require any modifications for their biological activity (11). In contrast, the lantibiotic groups contain peptides that require posttranslational modification for their antimicrobial activity (12, 13). In lantibiotics, most of the serine and threonine residues are dehydrated to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively, by modifying enzymes encoded within the lantibiotic synthesis operon. With neighboring cysteine residues, Dha and Dhb can form thioether-linked lanthionine and 3-methyl lanthionine bridges, respectively. Sometimes Dha, Dhb, and other modified residues can be present as unlinked residues (for reviews, see references 13, 14, 15, 16, and 17). Based on the nature of the enzymes responsible for the modifications, lantibiotics are divided into three classes (13). Class I lantibiotics (such as nisin and subtilin) are modified by two enzymes generally

referred to as LanBC system. Class II lantibiotics (such as lactacin 481 and mersacidin) are modified by a single enzyme often referred to as LanM-type enzyme. Class II lantibiotics also include two-component lantibiotics (such as lactacin 3147 and haloduracin), and the antimicrobial activity requires synergistic interaction of both peptides (18, 19). Lantibiotics that belong to class III are lanthionine peptides that have no antimicrobial activity (20, 21). The advantage of bearing modified residues, as well as cyclization, is to make lantibiotics more stable against heat, pH, and protease degradation compared to nonlantibiotics (22). This stable nature of lantibiotics makes them a major candidate for commercial application; for example, nisin is extensively used as a preservative in the food industry (23).

Lantibiotics are generally produced by Gram-positive bacteria and usually have a variable spectrum of inhibition (15, 22–25). The producer organisms usually encodes specific immunity proteins that protect themselves from the deleterious effect of their own lantibiotics, and the immunity protein encoding genes are often present within the same lantibiotic biosynthesis operon. Currently, two types of immunity protein have been identified. The first type includes dedicated ABC transporters that presumably pumps lantibiotics out of the membrane and thus prevent accumulation to inhibitory levels (for reviews, see references 14 and 26). The second type includes small proteins that are weakly

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TABLE 1 Oligonucleotides used in this study

Oligonucleotide	Sequence (5'-3')	Purpose ^a
fsnsmbAupF	GTTGGCGAAAGTGTTCTGGTAAG	Inactivation of <i>smbAB</i> (up)
fsnsmbAupR	GCCGCCATGGCGGCCGGAGTAATAAATTACTTTTCATTTTA	Inactivation of <i>smbAB</i> (up)
fsnsmbBdnF	CGCGGCCGCTGCAGGTGACCGAATGCATGAGAAAATTGTAG	Inactivation of <i>smbAB</i> (dn)
fsnsmbAdnR	GACCGCTTTCATATTGTTTCAGCAC	Inactivation of <i>smbAB</i> (dn)
locussmbAF	GGAAGGAATATAGGGTGAAGAAG	<i>smbAB</i> locus amplification
locussmbdnR	CGTATTACTTACTACAATTCTCATGC	<i>smbAB</i> locus amplification
NcoI-Kan-D7-F	CTCCCGGCCCATGGCGGCCGC	Antibiotic cassette (middle)
PstI-Kan-D7-R	GGTCGACCTGCAGGCGGCCGCG	Antibiotic cassette (middle)
fsnSmbTupF	CCAGCCACATCTTACAAAATTTGGAGC	Deletion of <i>smbT</i> (up)
fsnrtSmbTupR	GCGGCCGCATGGCGGCCGGAGGTCCTTTTAAATTCTCAATAG	Deletion of <i>smbT</i> (up)
fsnSmbTdnF	CGCGGCCGCTGCAGGTGACGCTGATTTGACTAAATATATTCC	Deletion of <i>smbT</i> (dn)
fsnSmbTdnR	CCTTTGAATATAATTACAAAACAAC	Deletion of <i>smbT</i> (dn)
SMBFT-XHO-R	GCCCTCGAGCCACTATGCATAACCCCATTTACGATAAATC	Complementation of <i>smbFT</i>
SMBFT-BGLII-RBSGTF-F	CGCAGATCTTGGAGGTTCTTAATGAGAATTTTAGACATTCAAAATTTAAA	Complementation of <i>smbFT</i>

^a up, upstream; dn, downstream.

associated with the membrane and often sequester specific lantibiotics at the cell surface before they could cause cell damage (27–29).

Streptococcus mutans, an oral streptococci and a major etiological agent of human dental caries, often produces several kinds of bacteriocins, collectively known as mutacins. A majority of the mutacins characterized to date belong to lantibiotics, such as the mutacins I, II, III, K8, B-Ny266, Smb, and 1140 (30–37). Based on the primary amino acid sequences, mutacins belonging to lantibiotics are further subdivided into two subclasses: AI and AII (38–40). Subclass AI contains the most well-characterized peptides such as mutacins I and III (mutacin 1140), which are similar to nisin and subtilin. Detailed structural information of these mutacins is currently lacking. Although the lantibiotic mutacins are widely present in *S. mutans* (37, 41), the first sequenced reference strain UA159, surprisingly, does not encode any lantibiotic but only encodes nonlantibiotics (42). In contrast, another recently sequenced *S. mutans* strain GS-5 encodes both Smb and nonlantibiotics (35, 43). The majority of the mutacins encoding genes are acquired by the *S. mutans* by horizontal gene transfer mechanism. For example, the strains that produce mutacin K8 contains the K8-encoding *muk* locus that is inserted in between SMU.1811 and SMU.1812 genes of the corresponding UA159 genome (37). Similarly, the *smb* locus, which contains all of the genes necessary for Smb biosynthesis, is integrated in between SMU.1942 and *syl* locus. Smb is the only two-component lantibiotic identified thus far in *S. mutans* and appears to be encoded only by a few strains (37).

The *smb* locus contains seven open reading frames in the following order: *smbM1*, *smbF*, *smbT*, *smbM2*, *smbG*, *smbA*, and *smbB*. Putative transposase encoding genes are also present near the *smb* locus (35). Two genes, *smbA* and *smbB*, are the structural genes for the Smb prepeptides and two putative modification enzymes encoded by *smbM1* and *smbM2* are also present in the *smb* locus. A recent report indicated that SmbG appears to play a role in Smb immunity and sensitivity to antimicrobial agents (44). Two other genes, *smbF* and *smbT*, show homology with the ATP binding cassette transporter and were previously predicted to be involved in processing and secretion of Smb prepeptides (35). We noticed that this SmbFT ABC transporter complex does not have a signal peptidase domain and hence might not be involved in the processing and secretion of the Smb prepeptide. We predicted that

SmbFT might provide immunity to Smb-producing *S. mutans* strains. In the present study, we provide evidence that SmbFT indeed confers resistance to Smb in the producer strain and show that deletion of *smbT* gene in GS-5 leads to sensitivity toward Smb. We also demonstrate that SmbFT can confer resistance to sensitive streptococci belonging to nonmutans group. Furthermore, we show that SmbFT has a narrow specificity, since it only recognizes structurally similar two-peptide lantibiotics.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* was routinely grown in Luria-Bertani (LB) medium and, when necessary, supplemented with 100 µg of kanamycin/ml. *S. mutans* and other Gram-positive bacteria were normally grown at 37°C in Todd-Hewitt medium (BBL, BD) supplemented with 0.2% yeast extract (THY) under microaerophilic conditions. When necessary, 5 µg of erythromycin/ml or 500 µg of kanamycin/ml was included in the THY medium. All of the streptococcal strains, except for *S. pyogenes*, were transformed by means of natural transformation according to a standard protocol with the addition of competence stimulating peptides (45). For *S. pyogenes*, electrotransformation was carried out as previously described (46).

Inactivation of *smbAB* and *smbT* genes in GS-5. A previously described fusion PCR method was used for the construction of Δ *smbAB* and Δ *smbT* mutant strains (47). In short, 500-bp upstream (up) and downstream (dn) flanking regions of the respective genes were separately PCR amplified using GS-5 genomic DNA as a template (see Table 1 for the primers). A third fragment (middle) containing an erythromycin resistance cassette was amplified using pIBM01 (I. Biswas, unpublished data), a plasmid containing an *ermB* gene, as a template. This plasmid was created by amplifying the *ermB* gene from pGhost9 plasmid (48) and inserted into the pGEM-T Easy vector by TA cloning. The 5' end of this middle PCR fragment has a 20-bp complementary sequence to the 3' end of the up fragments, whereas the 3' end of this middle fragment has a 20-bp complementary region to the dn fragment. To generate fusion PCR products, equal molar ratios of the three fragments were mixed and used as a template for next round of PCR amplification with the flanking primer pairs of the respective genes. The final PCR products were verified by gel electrophoresis for correct sizes and transformed into *S. mutans* GS-5 using standard protocol. Transformants were selected on THY agar plate containing erythromycin.

Construction of plasmids for complementation. Plasmid pIB184-Km was used as vector for cloning (47). This plasmid contains P23 promoter from lactococcal phage (pOri23). To clone the *smbFT* genes, a fragment containing the coding regions was amplified from GS-5 genomic DNA.

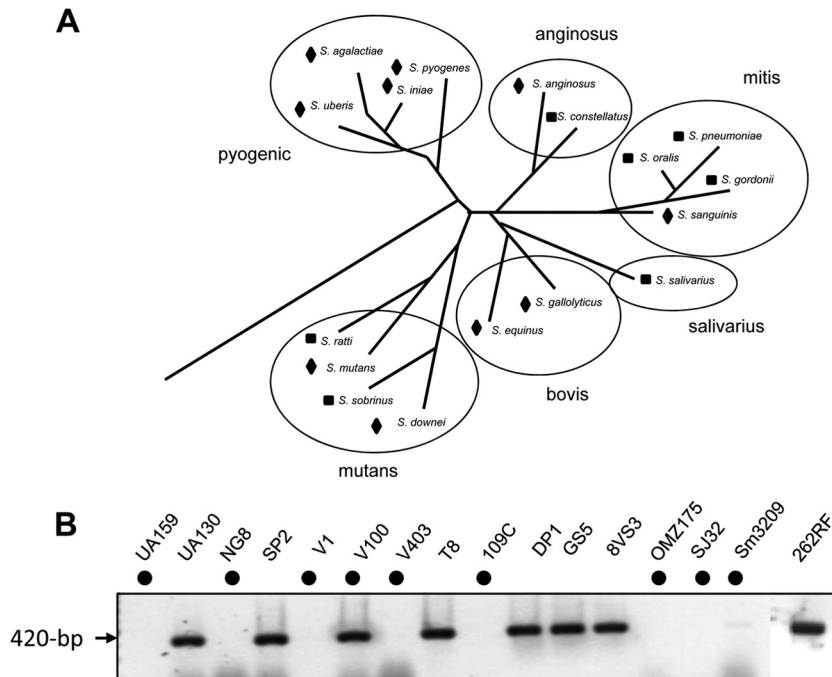


FIG 1 Smb activity spectrum and distribution of *smb* locus in various *S. mutans* strains. (A) Member species of different phylogenetic group of streptococci were tested against mutacin Smb produced by GS-5. GS-5 and a Δ *smbAB* mutant strain were stabbed onto THY-agar plate and incubated overnight in microaerophilic condition at 37°C. The next day, an overnight-grown culture of indicator strain was mixed with soft agar and overlaid onto the plate. The overlaid plates were incubated again overnight under the same condition. The following day, the diameter of the zone of inhibition (ZOI) was measured. The observation is based on two separate experiments with four replicates. Diamonds and squares indicate ZOIs of >10 mm or <10 mm in diameter, respectively. (B) Presence of the *smb* locus in 16 different *S. mutans* strains. Analysis of PCR products of *smbAB* genes by agarose gel electrophoresis. Solid circles indicate the strains that are susceptible to Smb inhibition (see Table S1 in the supplemental material). The observation is based on at least three independent experiments.

The upstream primer was designed in such a way that it also includes the ribosome-binding site (see Fig. 2). The amplified fragment was digested with BglII and XhoI and cloned into BamHI-XhoI-digested pIB184-Km to generate pIBM09. This plasmid and the vector were introduced into various streptococci.

Bacteriocin assay (zone of inhibition). GS-5 and its mutant derivatives were stabbed on THY-agar plates and incubated overnight under microaerophilic condition at 37°C (49). The following day, the plates were overlaid with freshly grown indicator strain cultures by mixing with soft agar. When the indicator strains contain plasmids, kanamycin was also included in the soft agar. The overlaid plates were incubated again overnight under the same condition as described above. The diameter of the clearing zone was measured afterward. Assays were repeated at least twice with four replicates.

Antibiotic sensitivity assay. Disk diffusion assays were performed for vancomycin, bacitracin, and nisin against different strains, including wild-type (WT) and Δ *smbT* strains. Briefly, disks (BRL) were placed on THY agar plates and overlaid with soft agar containing overnight grown *S. mutans* or other strains of choice. The plates were incubated at 37°C under microaerophilic condition. Zones of inhibition were measured after 24 h.

RESULTS

Smb-sensitive strains do not encode *smb* locus. Smb is the only lantibiotic that is secreted by *S. mutans* GS-5 (43), and a previous study suggested that Smb is active against many oral streptococci (50). Since most of the lantibiotics, including nisin, have wider spectra than the nonlantibiotics (11, 22, 39), we wanted to determine whether Smb could inhibit various streptococci belonging to different phylogenetic groups (51). We first constructed an *smbAB* deletion mutant strain (Δ *smbAB*) to include in our inhibition as-

says to make sure that the activity that we observed was due to lantibiotic Smb and not to other bacteriocins. We then used GS-5 and Δ *smbAB* strains as testers against 18 streptococci and 16 *S. mutans* isolates (Fig. 1A). We observed that all of the streptococci strains, except some *S. mutans* isolates (see below), were inhibited by Smb. We divided the activity spectra in to two classes, a zone of inhibition (ZOI) smaller than 10 mm and larger than 10 mm (Fig. 1A). Two groups of streptococci, the pyogenic and the bovis groups, consistently produced larger ZOI than the other groups, suggesting that the streptococci belonging to these groups are more susceptible to Smb than the other groups. *S. mutans* strains generally do not inhibit the growth of other *S. mutans* strains. However, we found that 9 of 16 *S. mutans* also produced large ZOI (Table 2). Furthermore, two *S. ratti* strains also produced some ZOI, while the FA strain produced small (~8 mm) but clear ZOI, and the BHT strain produced diffused ZOI (~10 mm). Among the mitis group, most of the isolates produced smaller ZOI except *S. sanguinis* SK36, which produced a large ZOI (~20 mm).

It was surprising to observe that many of the *S. mutans* isolates were insensitive to Smb. Since the lantibiotics have the potential to attack the producer strains, the producer strains must contain some self-protection mechanisms to protect against their own lantibiotics. Therefore, it is possible that the *S. mutans* strains that were insensitive may actually encode the *smb* locus. To examine the presence of *smb* locus, we PCR amplified the *smbAB* structural genes from all of the 16 *S. mutans* isolates. Eleven of these isolates belong to serotype c, including common laboratory strains (UA159, NG8, and GS5), and one each from serotype e (V100)

TABLE 2 Susceptibility of various *S. mutans* strains to Smb

<i>S. mutans</i> strain ^a	Mean zone of inhibition diam (mm) ± SD	
	GS-5 strain	Δ <i>smbAB</i> mutant
UA159*	11.0 ± 1.0	2.0 ± 2.0
NG-8*	11.0 ± 1.0	0.0 ± 0.0
GS-5	0.0 ± 0.0	0.0 ± 0.0
SP-2	0.0 ± 0.0	0.0 ± 0.0
Sm3209	9.5 ± 1.5	2.5 ± 1.5
T8	0.0 ± 0.0	0.0 ± 0.0
SJ32	13.5 ± 1.5	0.0 ± 0.0
8VS3	4.1 ± 1.0	0.0 ± 0.0
109C*	16.5 ± 0.5	0.0 ± 0.0
OMZ175*	15.5 ± 0.5	0.0 ± 0.0
V100	11.5 ± 1.5	0.0 ± 0.0
262RF	0.0 ± 0.0	0.0 ± 0.0
V403*	18.0 ± 2.0	0.0 ± 0.0
UA130	0.0 ± 2.0	0.0 ± 0.0
DP1	0.0 ± 1.0	0.0 ± 0.0
V1	10.0 ± 1.0	0.0 ± 0.0

^a *, strains used for heterologous expression of SmbFT.

and serotype f (OMZ175); the rest were taken from unknown serotypes. We found the presence of an expected 420-bp size band in eight of the isolates. As expected, these strains were also immune to Smb, indicating the presence of the same immune mech-

anism(s) in these strains as in the producer strain. The only exception was V100 strain, which encodes the *smb* locus but was sensitive to Smb-mediated inhibition. The eight strains that did not produce a PCR product all produced clear ZOIs against Smb. These data support the notion that strains that encode the *smb* locus are all immune to Smb-mediated killing, except for strain V100. V100 is unique among the *S. mutans* strains since does not develop natural competence (data not shown). We speculate that it does not express the *smb* locus.

SmbFT, a putative ABC transporter complex, provides immunity against Smb. Since the Smb-encoding operon also encodes *smbFT* that shares high homology to a putative ABC transporter complex, and since ABC transporters often function as immunity proteins, we wanted to verify whether SmbFT can protect *S. mutans* from Smb-mediated killing. SmbFT transporter is composed of two polypeptides. SmbT encodes permease component and is 238 residues long with six transmembrane domains. SmbF, on the other hand, encodes a 274 residues long protein with ATPase signature sequences (52). The promoter for *smb* locus lies ~3.0 kb upstream of the *smbFT* genes. Sequence analysis suggests the presence of a weak ribosome-binding site located five nucleotides upstream of *smbF* start codon and an overlap of nine codons between the *smbF* and *smbT* coding sequences (Fig. 2A). Because the promoter lies far from the *smbF* and since *smbFT* are linked

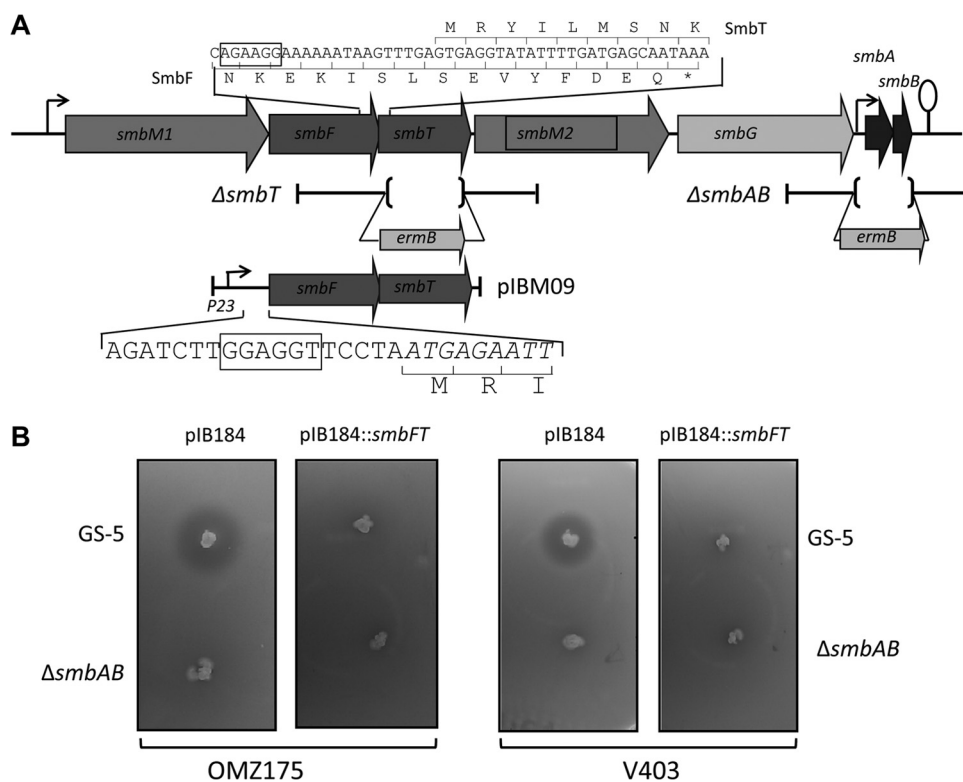


FIG 2 Smb and its immunity protein. (A) Genomic organization of *smb* locus in GS-5. The regions used for fusion PCR for deletion constructions are shown at the bottom. Brackets indicate the regions that were deleted from the genome, and an erythromycin resistance gene (*ermB*) was inserted. A plasmid used for the heterologous expression of *smbFT* (pIBM09) is shown. This plasmid contains a ribosome-binding site from the *gftB* gene and a P23 promoter used for *smbFT* expression. Relevant sequences near the *smbF* start codon and the overlapping region between *smbF* and *smbT* are shown. Bent arrows and lollipops indicate promoters and transcription termination sites. Block arrows indicate gene orientation. (B). Immunity activity of SmbFT in *S. mutans* strains. GS-5 and an Δ *smbAB* strain were stabbed into THY agar, followed by incubation overnight at 37°C under microaerophilic conditions. The plates were overlaid with soft agar containing the indicator strains. The ZOIs of the indicator strains were evaluated after overnight incubation. The indicator strains are OMZ175 (left panel) and V403 (right panel) containing the vector (pIB184) or the vector with SmbFT. These plates are representative of three independent assays.

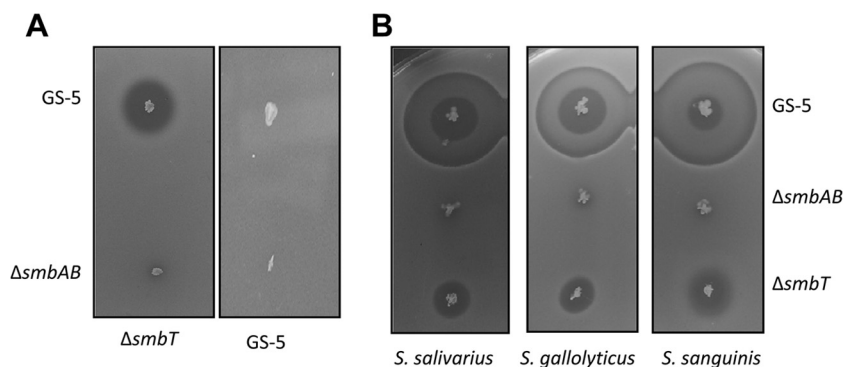


FIG 3 Sensitivity of the Δ *smbT* strain to Smb and the secretion of Smb by the Δ *smbT* strain. (A) Agar diffusion assays were carried out with GS-5 and Δ *smbAB* strains as tester strains with GS-5 and Δ *smbT* strains as indicator strains. (B) The production of Smb by the Δ *smbT* strain was verified by agar diffusion assay using *S. salivarius* BAA491, *S. gallolyticus* BAA2069, and *S. sanguinis* SK36 as indicator strains. The GS-5 and Δ *smbAB* strains were used as positive and negative controls, respectively. The plates are representative of three independent replicates.

together, we decided to express both *smbFT* from a low-copy-number plasmid under a heterologous promoter (P23) as a single transcript. We selected a stable theta-replicating plasmid, pIB184Km (46), to clone and express *smbFT*. We also replaced the native weak ribosome-binding sequence with a strong sequence from the *gftB* gene. To examine the role in immunity, we heterologously expressed SmbFT in five *S. mutans* strains that are susceptible to Smb. These strains are UA159, NG-8, V403, 109c, and OMZ175; all of these strains produced a clear ZOI in our susceptibility assay (Fig. 1). As a control, we also transformed the vector plasmid into the *S. mutans* strains. These strains were then used as indicators in an agar plate assay. Heterologous expression of SmbFT in all of the five *S. mutans* strains provided complete protection against Smb compared to the empty-vector carrying strains (Fig. 2A, data not shown). Thus, our findings support the prediction that SmbFT indeed functions as an immunity protein against Smb.

Inactivation of *smbT* makes the strain susceptible to Smb.

Since SmbFT can confer protection against Smb lantibiotic, we wanted to determine whether SmbFT has a redundant function in providing immunity against Smb in GS-5. This is because a previous study suggests that two other factors, Bip (SMU.1914) and SmbG (encoded by *smb* operon), also provide protection against Smb (44). We first constructed an *smbT* deletion mutant (Δ *smbT*) in GS-5 by replacing the *smbT* coding sequence with a nonpolar erythromycin resistance cassette. We used the Δ *smbT* mutant as an indicator strain in previously described plate assays against Smb producer. As shown in Fig. 3A, we observed that Δ *smbT* is more susceptible to Smb compared to the GS-5 strain. The ZOI produced by the Δ *smbT* strain was very clear and as large as the ZOI produced by the *S. salivarius* strain. This result supports that SmbFT functions primarily as an immunity protein in GS-5, and its absence makes the strain more susceptible to the lantibiotic.

To investigate the influence of SmbFT in Smb production, we performed a plate assay to measure ZOI formation by Δ *smbT* strain against *S. salivarius* indicator strain with GS-5 and Δ *smbAB* strains as controls. Although GS-5 produced a larger and clear ZOI, including a secondary halo, the ZOI produced by the Δ *smbT* mutant was clear and small. On the other hand, the Δ *smbAB* mutant did not produce a visible ZOI. The larger secondary ZOI that was seen with GS-5 is not always visible, and it depends on the age of the indicator culture (Fig. 3B, data not shown). To confirm that

the Δ *smbT* mutant could produce a clear ZOI, we used two other indicator strains, *S. gallolyticus* and *S. sanguinis*. As shown in Fig. 3B (right panels), the Δ *smbT* mutant produced a clear ZOI in both strains compared to the Δ *smbAB* control strain, which did not produce a halo. As expected, the GS-5 strain produced a larger ZOI with a secondary halo against both indicator strains. Taken together, our data suggest that Smb is secreted at low levels even in the absence of *smbT*.

SmbFT can confer immunity in heterologous streptococcal hosts. Oral cavity harbors over 700 different bacterial species and streptococci constitute the majority (53). Many streptococci contain ABC transporters that are similar to SmbFT, and yet they are sensitive to Smb. To verify whether SmbFT can confer resistance in other oral streptococci, we selected a mitis group of streptococci, *S. sanguinis*, which plays a beneficial role in the oral cavity and is known to compete with *S. mutans* for subsequent colonization (8, 10). Another reason for selecting *S. sanguinis* was that the organism is naturally competent and therefore easy to manipulate (54). When we overexpressed SmbFT from the plasmid in *S. sanguinis* SK36, we observed a complete protection against Smb-mediated killing (Fig. 4A). In fact, there was very little or no ZOI in strain overexpressing SmbFT, whereas ZOI of ~20 mm in diameter was observed in strain containing the vector only (Fig. 4A).

Since SmbFT conferred protection against Smb in streptococcus belonging to mitis group, we wanted to test whether it can confer protection in other group. For this we selected the pyogenic group, since all of the four tested species produced larger and clearer ZOI (Fig. 1A). We introduced the plasmid overexpressing SmbFT and the vector control in *S. pyogenes* JRS4 strain, an M6 serotype strain. As shown in Fig. 4B, the overexpression of SmbFT in *S. pyogenes* also provided protection against Smb in the *S. pyogenes* strain. However, the level of protection was less drastic, and the Δ *smbAB* strain, which was included as a control, also produced a clear ZOI in both vector- and SmbFT-overexpressing strains. Nevertheless, SmbFT conferred protection against this lantibiotic in *S. pyogenes*. Taken together, our results suggest that SmbFT can function as a universal immunity protein for Smb in streptococci.

The immunity provided by SmbFT is specific toward Smb and related lantibiotics. Cross-immunity is a rare occurrence among lantibiotic producer strains and has only been found in strains that produces very closely related lantibiotics, such as nu-

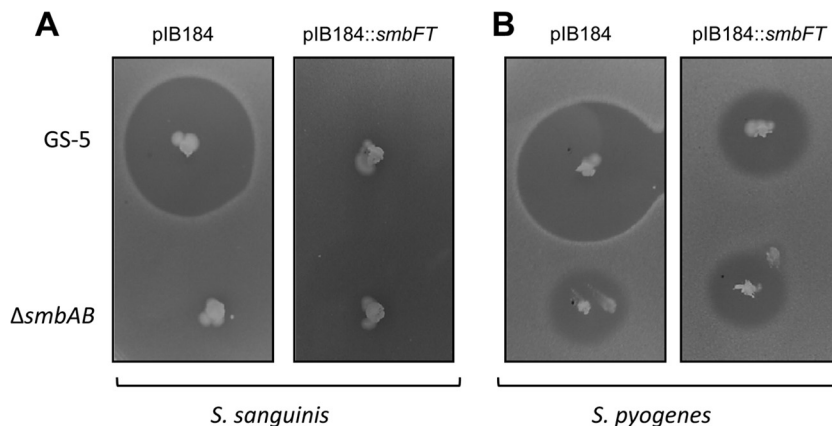


FIG 4 SmbFT confers protection in heterologous hosts agar diffusion assays were carried out with the GS-5 and Δ smbAB strains as tester strains. The indicator strains are *S. sanguinis* SK36 (left panel) and *S. pyogenes* JRS4 (right panel) containing the vector (pIB184) or the vector with SmbFT. These plates are representative of three independent assays.

kacin ISK-1 and lactacin 481 (55). Since SmbFT was able to confer protection in various streptococci, we wondered whether SmbFT could provide cross-protection against other bacteriocins. Toward this end, we first tested against haloduracin, a two-component lantibiotic produced by *Bacillus halodurans* and shares structural similarity with Smb (18). *S. sanguinis* and *S. mutans* V403 strains containing either smbFT expression plasmid or the vector alone were used as indicator strains in an agar diffusion assay with 10 μ l of 100 μ M purified haloduracin (kindly provided by W. A. van der Donk) spotted onto a plate, and the results are shown in Fig. 5A. Haloduracin produced a clearer and larger ZOI in *S. sanguinis* containing the vector, whereas *S. sanguinis* expressing SmbFT produced a much smaller and cloudier ZOI. The ZOI in *S. mutans* V403 with the vector was much smaller and cloudier, suggesting that haloduracin is not very effective against *S. mutans*. However, no ZOI was observed in V403 expressing SmbFT, indi-

cating that, as in *S. sanguinis*, SmbFT provided protection against haloduracin in *S. mutans*.

S. gallolyticus BAA-2069 possess a unique 23-kb genetic island in the genome, coding for bacteriocin-associated genes (SGBAA2069_c00810-c00960). These genes encode for a lantibiotic that share high degree of similarity with Smb and haloduracin. Hinse et al. (56) have shown that the BAA-2069 strain indeed produces a lantibiotic that is active. Therefore, we tested *S. mutans* V403, *S. sanguinis* SK36, and *S. pyogenes* JRS4 strains overexpressing SmbFT or containing the vector. All of these streptococci produced much smaller ZOIs when SmbFT was present compared to vector-only strains (data not shown). This finding indicates that SmbFT is also active against the lantibiotic produced by BAA-2069.

We then tested whether SmbFT could provide protection against other bacteriocins. For this, we chose *S. mutans* UA159 that primarily produces mutacin IV (NlmAB, a two-component

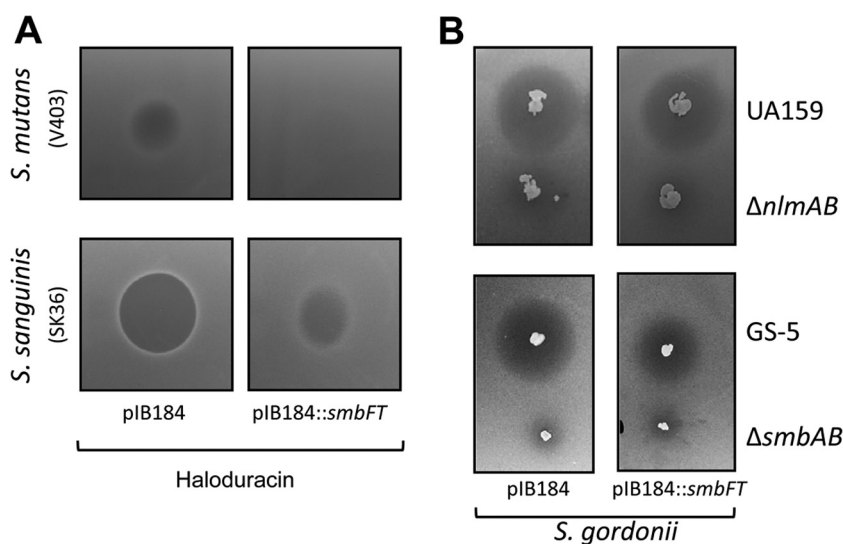


FIG 5 Substrate specificity of SmbFT. (A) Purified haloduracin (1:1 mixture of Hal α and Hal β) was spotted on THY plate and then overlaid with indicator strains containing the vector or the vector with SmbFT. (B) UA159 and Δ nlmAB strains were stabbed into THY agar, and a deferred antagonism assay was carried out with *S. gordonii* DL-1 strain containing the vector or the vector with SmbFT. GS-5 and Δ smbAB strains were used as control. Assays were repeated at least three times, and a representative plate is shown.

nonlantibiotic). *S. gordonii* is generally used as an indicator strain for mutacin IV. As shown in Fig. 5B, *S. gordonii* with or without SmbFT produced similar size ZOI, indicating that SmbFT could not confer protection (the control $\Delta nlmAB$ strain did not produce any ZOI in either cases). Next, we checked the effect of SmbFT on protection against purified nisin, a well-studied mono-peptide lantibiotic that is structurally different from Smb. As expected, SmbFT did not provide protection against nisin (data not shown). ABC transporters are often associated with tolerance to various peptide antibiotics, such as vancomycin and bacitracin (57). Therefore, we also checked sensitivity to different cell wall-specific peptide antibiotics, such as bacitracin, polymyxin B, and vancomycin, by disc diffusion assay. We did not observe any noticeable differences between the strain containing SmbFT or the vector control (data not shown). Taken together, our results suggest that SmbFT is specific in conferring protection against Smb or closely related lantibiotics.

DISCUSSION

Lantibiotic producer strains possess multiple mechanisms to protect themselves against their own inhibitory activity. One of the most effective mechanisms that provide the maximum protection is through various dedicated ABC transporters. ABC transporters that are involved in self-immunity or protection against other lantibiotics are generally classified into multiple categories (58). ABC transporters such as NisT and SunT family members are encoded by a single polypeptide, where the ATPase domain is fused with the permease domain. Some members of SunT also encode an N-terminal peptidase C39 domain. SmbG, which was shown to confer protection against Smb (44), belongs to the SunT family. The other ABC transporter families all contain two separate polypeptides, one with permease activity and the other with ATPase function. SmbFT, the ABC transporter that we studied here, belong to the BcrAB family that contains two separate polypeptides. ABC transporters belonging to BcrAB family are not extensively characterized, except for two transporters from bacilli (59, 60). A recent evolutionary relationship study indicates that BcrAB family proteins are closely related to LanFEG family transporters. The latter family proteins, which contain two separate permeases (LanE and LanG) and one ATPase (LanF), are all involved in self-immunity of lantibiotic-producing bacteria. On the other hand, BcrAB family transporters are involved in resistance against bacitracin (59, 61). Since the genes are encoded within the biosynthesis locus for bacitracin, BcrAB family proteins appear to confer self-immunity (61). The SmbFT transporter that we studied here is the first example of a BcrAB family protein that is involved in lantibiotic resistance.

As already mentioned above, the SmbFT transporter is composed of two polypeptides: SmbT, which encodes the permease component, and SmbF, which encodes the ATPase where two-thirds of the N-terminal region comprises of P-loop containing nucleoside triphosphate hydrolase signature sequences (62). Although *S. mutans* encodes numerous permease proteins (42), a sequence similarity search with SmbT against the genome returned no results, suggesting that SmbT encodes a unique sequence. In contrast, a search using SmbF as the query against the genome returned several ABC transporter-related ATPases. Four ATPases—SMU.238, SMU.654, SMU.1035, and SMU.1811—showed maximum similarity (E-value ≥ 30) with SmbF (data not shown). At present, we do not know whether any of these ATPases

can form a productive complex with SmbT permease to counteract Smb.

We expressed SmbFT in at least four different streptococcal strains other than *S. mutans*. In each of these streptococcal strains, the heterologous expression of SmbFT conferred full protection against the Smb lantibiotic. This means that SmbFT does not need any other accessory proteins for its activity. Alternatively, the accessory protein could be a highly conserved protein present in all of the streptococcal strains tested here. As previously mentioned, lantibiotic producer strains often encode two types of transporters for full protection. For example, a *Lactococcus lactis* strain that produces lacticin 3147, a two-component lantibiotic similar to Smb, possesses both LtnI, a lipid-anchored membrane protein, and LtnFE systems (63). Draper et al. (63) have demonstrated that, in a heterologous system, LtnI and LtnFE act synergistically to confer complete protection. The *smb* locus does not encode any LtnI homolog, but it does encode SmbG, a SunT homolog, containing the ABC transporter motif and a C39 peptidase motif. It has been proposed that SmbG functions as an immunity protein against Smb (44); however, an *smbG* mutant strain is not susceptible to Smb. An immunity-like protein encoded by SMU.1913 (referred to as Bip in reference 44) has also been proposed to function as an Smb immunity protein. Surprisingly, a *bip* single-mutant strain is also not susceptible to Smb, indicating other proteins can confer resistance. In contrast, our data suggest that SmbFT alone can confer complete protection against Smb in a variety of streptococcal strains; none of these strains contain either SmbG or Bip. Therefore, unlike other lantibiotic immunity systems, we speculate that SmbG and SmbFT provide different functions in *S. mutans*. We believe that the primary role of SmbG is to secrete Smb peptides from the cytoplasm to the milieu and, when SmbG is absent, the strain does not secrete active Smb. Indeed, we found that an *smbG* mutant is unable to produce Smb and functionally behave like the $\Delta smbAB$ mutant. We also believe that the primary function of SmbFT is to confer self-protection and not secretion. This is because we found that the $\Delta smbT$ mutant produced a clear ZOI against multiple strains (Fig. 3), indicating Smb production; however, the ZOI was much smaller than that observed with the GS-5 strain. There are several reasons that can explain the smaller ZOI produced by the $\Delta smbT$ strain. Since the strain was constructed by insertion of an *ermB* gene (erythromycin resistance), it might have interfered with the transcription of the downstream genes in the *smb* operon and so, as a result, less *smbAB* was produced. Often lantibiotic production is subject to feedback inhibition and is positively influenced by the immunity proteins (64, 65). Therefore, it is also possible that SmbFT positively influences *smb* production. Whether SmbFT has a positive role in Smb production remains to be tested.

It appears that SmbFT could confer protection not only against Smb but also against haloduracin and an uncharacterized lantibiotic produced by BAA-2069 (we named this lantibiotic gallolactacin). All three lantibiotics share high degree of sequence similarity (see Fig. S1 in the supplemental material) and probably structurally similar as well. All three lantibiotics are composed of two modified peptides, α and β . Although no information is available for Smb or gallolactacin, a detailed structure has been proposed for haloduracin that is based on mass spectrometry data and modeled on nuclear magnetic resonance structure of lacticin 3147 (66). The structural information, together with the sequence similarity, suggests that all α -peptides have the same topology with three C-ter-

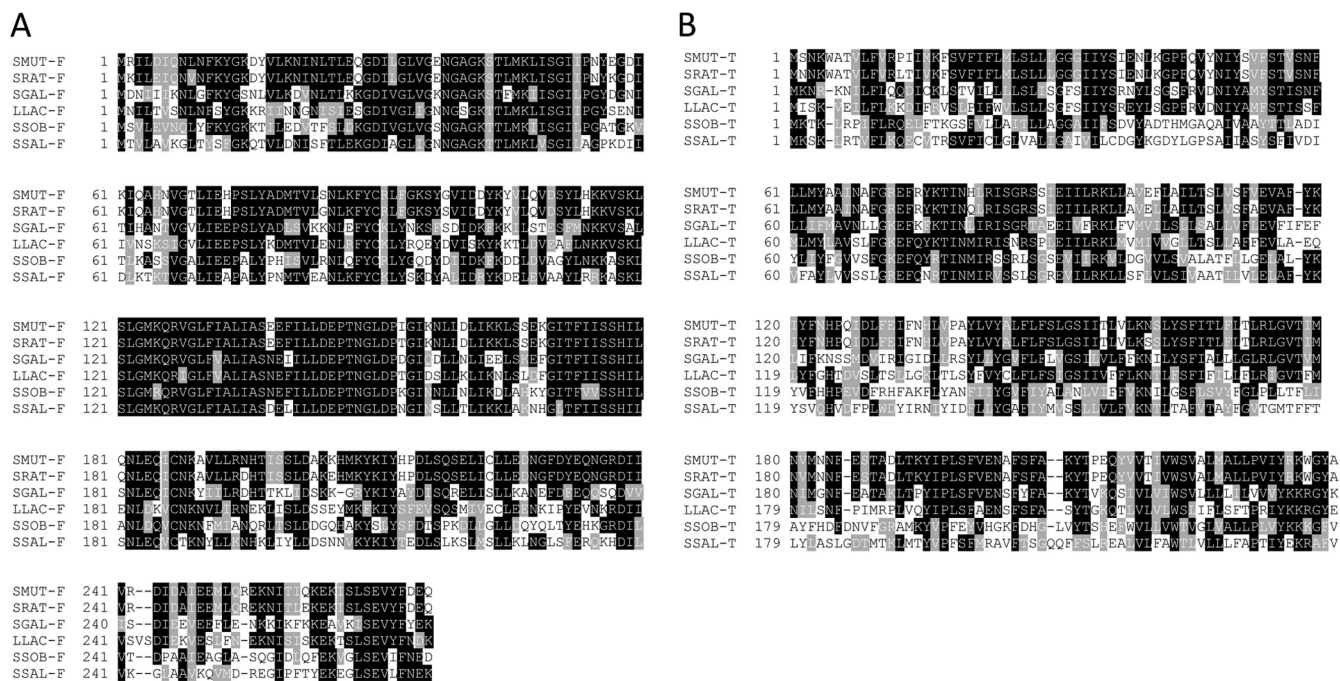


FIG 6 Multiple sequence alignment of SmbF (A) and SmbT (B) with their closest homologs. The alignment was performed using CLUSTAL W. Degree of shading was done using BoxShade where black and gray blocks indicate identical and similar amino acids, respectively. Sequences were obtained from GenBank (accession numbers are in parentheses). The following strains were tested: *S. ratti* (SRAT-F, WP_003086779.1; SRAT-T, WP_003086777.1), *S. gallolyticus* (SGAL-F, YP_004287007.1; SGAL-T, YP_004287008.1), *L. lactis* (LLAC-F, YP_004761484.1; LLAC-T, YP_004761485.1), *S. sobrinus* (SSOB-F, WP_019770819.1; SSOB-T, WP_019776383.1), and *S. salivarius* (SSAL-F, YP_004728647.1; SSAL-T, YP_004728646.1).

minal rings (13) (see Fig. S1 in the supplemental material). The β -peptides appear to contain three or four rings; however, the B ring is present only in haloduracin and is absent in all of the others. Since SmbFT recognized all three lantibiotics, we speculate that the transporter complex predominantly recognizes the conserved topology and structurally similar lantibiotics. Based on this observation, we also speculate that SmbFT will confer protection against two-component lantibiotics that share this overall topology. Interestingly, the β -peptides of two lantibiotics, lactacin 3147 and staphylococcus strain C55 lack the first ring (ring A) but contain three other rings (rings B to D). It would be of interest to test whether SmbFT could recognize lactacin 3147 and staphylococcus C55.

Thus far, all of the ABC transporters that belong to BcrAB family have been shown to act specifically against cyclic peptide bacitracin (59, 61). Although sequence similarity indicates that SmbFT belongs BcrAB family, we found that SmbFT does not recognize bacitracin; it also recognizes neither vancomycin nor polymyxin B, two other cyclic peptide antibiotics. SmbFT also did not recognize nisin, a heavily modified single-peptide lantibiotic. A BLASP search using SmbF or SmbT peptides as a query sequences against the GenBank database returned sequences mostly from streptococci. The top hit sequences include *S. ratti* (strains BHT and FA-1), *S. gallolyticus* BAA-2069, *S. sobrinus* DSM20742, *S. salivarius* CCHS3, and *Lactococcus lactis* (Fig. 6). We propose that SmbFT and its homolog constitute an ABC transporter family distinct from BcrAB and specifically recognize two-component lantibiotics. It should be noted that although *S. gallolyticus* BAA2069 produces gallolactacin and encodes an SmbFT homolog, which we named GalFT; the strain was sensitive to Smb (data not

shown). Therefore, we speculate that whereas SmbFT confers protection against gallolactacin, GalFT from BAA2069 cannot confer protection against Smb. A sequence alignment between SmbF and SmbT with the corresponding GalF and GalT sequences identified presence of several unique amino acid residues in both GalF and GalT. A hybrid analytical approach is under way wherein we are combining SmbF with GalT and vice versa to identify the subunit necessary for substrate recognition and/or discrimination.

Although the mechanisms related to protection against lantibiotics have been extensively studied, the molecular mechanism of lantibiotic transport is still poorly understood. Several recent studies suggest that, unlike general ABC transporters that transport substrates across the membranes, the ABC transporters related to immunity function transport specific lantibiotics from the membrane to the extracellular space (28, 29, 55). A recent study identified the presence of a conserved motif, termed the E-loop, in the ABC transporters belonging to LanFEG and BcrAB families (52). This E-loop structure plays an important role in the function of these transporters and perhaps induces structural changes in the transmembrane domains. Whether the E-loop is involved in substrate recognition and/or binding remains to be evaluated. Additional structure-function studies are necessary in order to understand the molecular mechanism of lantibiotic transport by immunity-related ABC transporter systems.

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