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Salivaricin A2 and the Novel Lantibiotic Salivaricin B Are Encoded at Adjacent Loci on a 190-Kilobase Transmissible Megaplasmid in the Oral Probiotic Strain *Streptococcus salivarius* K12[▽]

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The commercial probiotic *Streptococcus salivarius* strain K12 is the prototype of those *S. salivarius* strains that are the most strongly inhibitory in a standardized test of streptococcal bacteriocin production and has been shown to produce the 2,368-Da salivaricin A2 (SalA2) and the 2,740-Da salivaricin B (SboB) lantibiotics. The previously uncharacterized SboB belongs to the type AII class of lantibiotic bacteriocins and is encoded by an eight-gene cluster. The genetic loci encoding SalA2 and SboB in strain K12 have been fully characterized and are localized to nearly adjacent sites on pSsal-K12, a 190-kb megaplasmid. Of 61 strongly inhibitory strains of *S. salivarius*, 19 (31%) were positive for the *sboB* structural gene. All but one (strain NR) of these 19 strains were also positive for *salA2*, and in each of these cases of double positivity, the two loci were separated by fewer than 10 kb. This is the first report of a single streptococcus strain producing two distinct lantibiotics.

Streptococcus salivarius is a primary and predominant colonizer of oral mucosal surfaces in humans and is not known to initiate infections in healthy individuals (4). A feature of *S. salivarius* is its production of a variety of bacteriocin-like inhibitory substances (BLIS), the activity of which appears to be particularly strong against *Streptococcus pyogenes*, a potentially pathogenic species that, like *S. salivarius*, has adapted to a principally oral mucosal existence in humans. This laboratory utilizes a deferred antagonism method with blood agar to test streptococci for their patterns of BLIS activity against nine standard indicators, and these patterns are referred to in code form as production (P) types (21). Our investigations of *S. salivarius* strains have shown that they can produce a large number of different P type patterns and that children harboring strains with P types 677 or 777 appear less likely to acquire *S. pyogenes* (7). Approximately 10% of *S. salivarius* strains exhibit P type 677 activity, a pattern corresponding to the inhibition of all nine standard indicators other than indicator 3 (I3) (7). Only around 1% of *S. salivarius* strains display P type 777 activity (all nine indicators inhibited) (our unpublished results).

Our wide-ranging application of the P-typing procedure has shown the production of BLIS activity to be both frequent and varied within the genus *Streptococcus* (2, 6, 21, 23, 24). Much of the streptococcal BLIS activity detected to date has subsequently been ascribed to peptides of the bacteriocin class, and several of these, including SA-FF22 (9), streptin (12, 28), and salivaricin A (SalA) (16), are of the lantibiotic subclass. These "lanthionine-containing antibiotics" (18), the prototype of which is nisin (8), have now been reported to be produced by strains of a wide variety of gram-positive species (5, 10). Strain 20P3, the prototype P type 677 *S. salivarius* strain, produces SalA, an atypical lantibiotic in that it (i) contains no dehydrated residues in its biologically active propeptide form (16) and (ii) appears to exhibit bacteriostatic rather than bactericidal activity toward its probable target bacterium (in this case, *S. pyogenes*) (11). Another intriguing finding has been that almost all tested *S. pyogenes* strains harbor large remnants of the SalA locus (19), with to date only serotype M-4 *S. pyogenes* having an intact locus and expressing functional (inhibitory) SalA activity in vitro (29). Interestingly, it is a homolog of SalA, named SalA1, that is encoded by *S. pyogenes* strains. The SalA locus in *S. pyogenes*, like those in many other lantibiotic-producing species, encodes an autoinducing two-component regulatory system (25). We have now detected five variants of SalA (SalA1 through SalA5), produced by strains of four different streptococcal species, all of which are capable of cross-inducing the production of the entire family of SalA peptides (29). Specific inducing activity can be detected in the saliva of subjects following their colonization with SalA-producing *S. salivarius* (29).

Our studies of the more broadly inhibitory (P type 777) *S. salivarius* strains have focused on strain K12, a bacterium now widely used as a probiotic for the treatment of halitosis and the maintenance of throat health (3, 4, 22). In the present study, we demonstrate that strain K12 produces two lantibiotics, SalA2 (29) and salivaricin B (SboB), the genetic loci for which are closely linked in strain K12 and localized to a large, transmissible plasmid. This, to our knowledge, is the first example of a streptococcus strain being shown to produce two different types of lantibiotics.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. All *S. salivarius* strains (K12, NR, 20P3, and those listed in Table 2) were isolated from human saliva. The nine standard indicator strains (I1, *Micrococcus luteus*; I2, *S. pyogenes* M type 52; I3, *Streptococcus constellatus*; I4, *Streptococcus uberis*; I5, *S. pyogenes* M type 4; I6, *Lactococcus lactis*; I7, *S. pyogenes* M type 28; I8, *S. pyogenes* M type 87; and I9, *Streptococcus dysgalactiae*) used for P typing have been described previously (21). Deferred antagonism studies were performed with Columbia agar base (Difco) supplemented with 5% human blood (vol/vol) and 0.1% calcium carbonate (wt/vol).

Deferred antagonism method. The method of deferred antagonism originally described by Tagg and Bannister (21) was used, either to determine the patterns (P types) of BLIS activities of the test strains or to compare the relative susceptibilities of different bacterial strains to the BLIS activities produced in agar media. The test strain was inoculated diametrically across the surface of the supplemented agar base as a 1-cm-wide streak. After incubation (18 h at 37°C in 5% CO_2 in air), the visible growth of the test strain was removed using a glass slide and the surface of the agar was sterilized by exposure to chloroform vapors for 30 min. The plate was then aired for 15 min prior to inoculation with 18-h Todd-Hewitt broth (THB) cultures of the indicator strains across the line of the original producer growth. The plates were then incubated as before for 24 h and examined for zones of interference with the indicator growth. Definite inhibition of indicator growth was recorded as +. For the purposes of P typing, the inhibitory activity toward the nine standard indicators was recorded in code form (as the P type) by considering the indicators as three triplets (i.e., I1, I2, I3; I4, I5, I6; and I7, I8, I9). Inhibition of the first member of an indicator triplet was given a score of 4; that of the second, 2; and that of the third, 1. Lack of inhibition of an indicator was scored as 0. The complete P type code was recorded as a sequence of three numbers representing the sum for each triplet. All tests were performed in duplicate, and further testing was undertaken if significant discrepancies were detected in the inhibition patterns obtained.

In a variation of the deferred antagonism test designed to assess the relative heat stability of the BLIS activities produced by the test strain, the agar plates were heated at 80°C for 30 min and then cooled to room temperature prior to the application of the indicator bacteria. Decreased inhibition of an indicator compared to that on the unheated (control) plate indicated heat lability of the inhibitory agent.

Purification and characterization of SalA2 and SboB. The purification procedure was based on that developed previously for SalA (16). Cotton swabs charged by immersion in an 18-h THB culture of *S. salivarius* K12 were used to seed lawn cultures on plates containing a 1-liter total volume of M17 agar medium supplemented with 0.5% sucrose, 0.1% calcium carbonate, and 0.8% bacteriological agar. The cultures were grown for 18 h in an atmosphere of 5% CO_2 in air, followed by freezing at −80°C and thawing at 4°C. After clarification of the exudate by centrifugation (15,000 × *g* for 25 min), the exudate was passed through an XAD-2 column with a 500-ml bed volume and was followed by 1 liter of 80% (vol/vol) methanol and then 1 liter of acidified (pH 2) 95% methanol to elute the inhibitory activity. Spot testing was carried out as described previously to identify fractions harboring inhibitory activity (28). Briefly, 10 μl of each fraction was spotted onto the surface of a blood agar plate and allowed to dry. After sterilization of the agar surface by exposure to chloroform vapors, a swab charged by immersion in an 18-h THB culture of either I1 or I3 was used to inoculate the agar surface, followed by overnight incubation. A zone of no growth demonstrates which fractions harbor inhibitory molecules. Fractions having inhibitory activity were concentrated 10-fold using a rotary evaporator and then fractionated on a C_8 (Brownlee) column by fast protein liquid chromatography using an isocratic (26%) acetonitrile gradient followed by high-pressure liquid chromatography using a C_{18} column with an acetonitrile gradient of 33 to 44% over 35 min. This enabled the separation of fractions having inhibitory activity toward both I1 and I3 (i.e., containing SboB) from those inhibitory only to I1 (i.e., due to SalA2). Mass spectrometry analysis and N-terminal sequencing was carried out at the Protein Microchemistry Facility, Department of Biochemistry, University of Otago.

DNA isolation, sequencing, and analysis. Chromosomal DNA was extracted from 18-h THB cultures. Briefly, the pelleted cells from 1.5 ml of culture were resuspended in 300 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). After the addition of 100 μl of lysozyme (20 mg/ml) and 9 units of mutanolysin and incubation at 37°C for 1 h, 60 μl of 10% sodium dodecyl sulfate (wt/vol) was added, followed by further incubation at 65°C for 15 min. The addition of 10 μl of proteinase K (13.6 mg/ml) and a 30-min incubation at 37°C were followed by the addition of 170 μl of 8 M LiCl and a 30-min incubation on ice. After clarification by centrifugation (16,100 × *g* for 10 min), 500 μl of the supernatant was mixed with 1 ml of absolute ethanol, precipitated, washed with 0.5 ml of 70% ethanol, and then resuspended in 50 μl of TE buffer containing 0.2 μg of RNase A. PCR and inverse PCR using appropriate specific primers were used to amplify individual regions and ultimately the entire salivaricin B locus, which was sequenced using an ABI 377 sequencer (Centre for Gene Research, University of Otago). Sequences were analyzed using the DNAStar suite of DNA analysis programs (Lasergene). Putative open reading frames (ORFs) were compared to known sequences and searched for known motifs by using the Web-based software programs BLAST and PredictProtein (1, 17).

Dot blot hybridization screening. The distribution of *sboA*, the structural gene encoding SboB, among *S. salivarius* and various other BLIS-producing bacteria was determined by dot blotting. A 5-μl portion of each DNA sample (prepared as outlined above) was applied to a nylon membrane (Hybond-N+ [Amersham Pharmacia Biotech, Inc.]) by using a vacuum manifold, followed by 100 μl of 2× SSC (0.3 M NaCl plus 0.03 M sodium citrate). Denaturation of the DNA was effected by exposure to two 2-min washes with 0.4 M NaOH, followed by two 2-min washes with 1 M Tris-HCl. The membrane was then exposed to UV light for 5 min and probed with a digoxigenin-dUTP (Roche Diagnostics, Ltd., Lewes, England)-labeled probe corresponding to the *sboA* gene. The probe was generated by PCR using HotMaster *Taq* DNA polymerase (Eppendorf) and *S. salivarius* K12 genomic DNA as the template with the following amplification parameters: 30 cycles, with denaturation at 95°C for 30 s, annealing at 40°C for 30 s, and extension at 65°C for 30 s.

Nucleotide sequence accession number. The GenBank accession number for the sequence determined in this project is DQ889451.

RESULTS

Production, purification, and partial characterization of salivaricin A2 and salivaricin B from *S. salivarius* K12. Two different lantibiotic bacteriocins that together appear to account for most of the relatively wide-ranging BLIS activity of strain K12 have been detected and characterized in this study. SalA2 and SboB were recovered from lawn cultures of *S. salivarius* K12 grown on M17 sucrose agar. Low levels of SalA2-like inhibitory activity and no SboB-like activity were detected in THB cultures, and when M17 agar was supplemented with 1% (wt/vol) glucose or lactose instead of sucrose, SboB-like activity was reduced (data not shown).

Preparations of both of these inhibitory peptides in a liquid form were obtained by freeze-thaw extraction of M17 sucrose agar cultures of *S. salivarius* K12. Two high-pressure liquid chromatography fractions inhibitory (i) only to I1 (putative SalA2 activity) or (ii) to both I1 and I3 (putative SboB activity) were identified. The peak corresponding to SalA2 eluted from the C_{18} column in 36% acetonitrile and had a mass of 2,368 Da (Fig. 1A). A second peak, eluting in 38% acetonitrile, contained a single inhibitory peptide (SboB) with a mass of 2,740 Da (Fig. 1B). Amino acid analysis of the SboB peptide detected lanthionine, a defining characteristic of lantibiotic peptides. Edman degradation established a partial SboB N-terminal amino acid sequence of G-G-G-V-I-Q-X-I-X-H-E-X-R-M-N-X-Q-F-L-F. The 21 residues sequenced included four that were unidentifiable (X) and are presumed to be involved in lanthionine ring formation.

Sequence analysis of the salivaricin B structural gene. The salivaricin B structural gene (*sboA*) in *S. salivarius* K12 was localized to a 1.8-kb EcoRI-HindIII restriction fragment by Southern hybridization using a degenerate DNA oligonucleotide probe (5′-TCNTGGCAATTTTTRTTTACT-3′) designed using the partial N-terminal amino acid sequence established for SboB (see above). The 1.8-kb fragment was cloned into pUC-19 and sequenced using universal and specifically designed primers. Inverse PCR was then used to sequence the

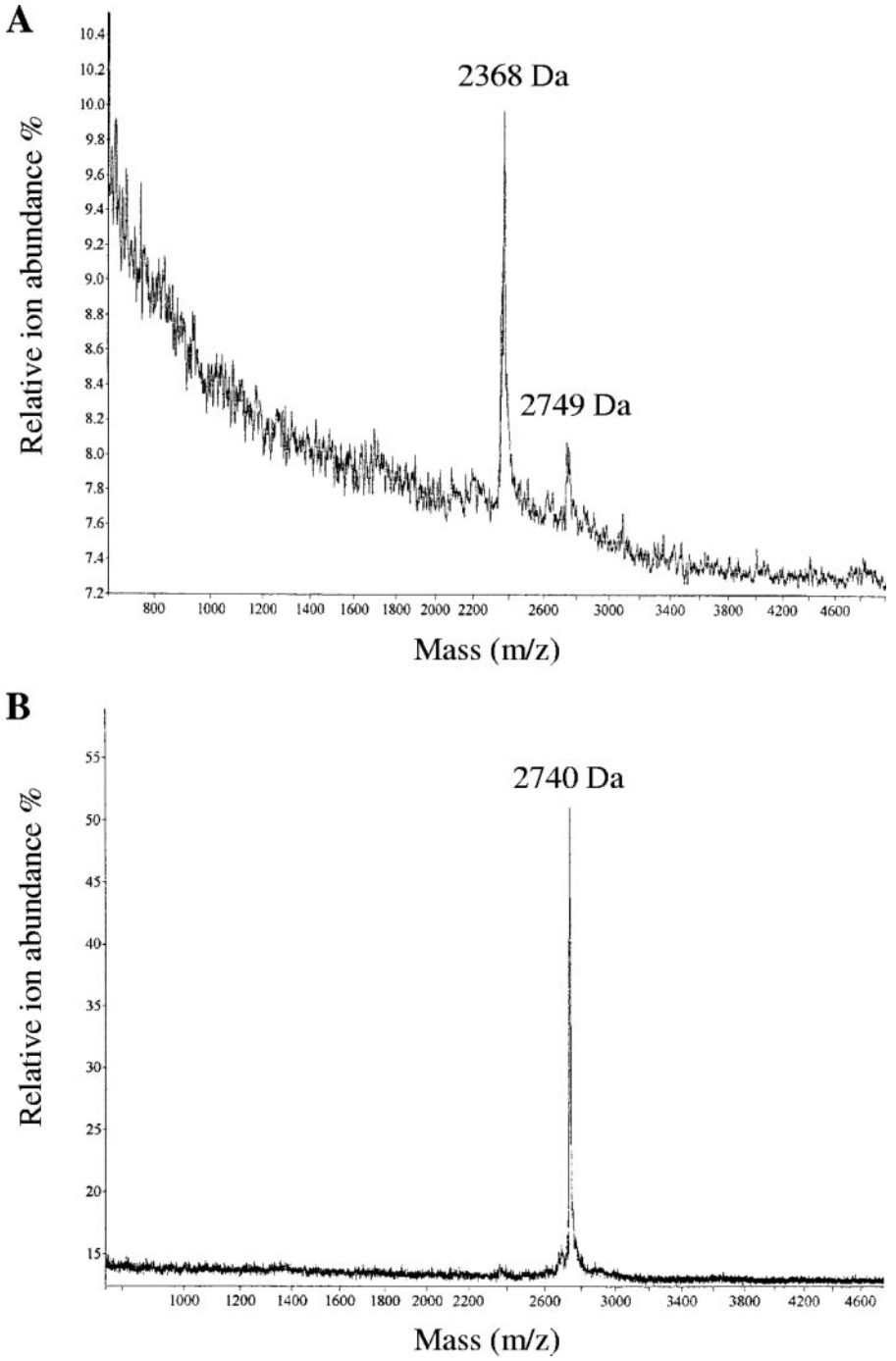

FIG. 1. Matrix-assisted laser desorption ionzation–time of flight analysis plots for purified salivaricin A2 (A) and salivaricin B (B). The sample of salivaricin B was also tested and found to be positive for lanthionine, indicating that salivaricin B is a lantibiotic.

region encompassing the EcoRI site, which was internal to the *sboA* gene. The SboA amino acid sequence aligned strongly with those of other members of the group AII lantibiotic bacteriocins (Fig. 2A). Conservation in the leader sequences of lantibiotics is often low. This holds true for the group AII lantibiotics, being limited to the eight amino acids directly preceding the cleavage site. The 25-amino-acid propeptide differs by five and six amino acids from the variacin and lacticin 481 propeptides, respectively. The residues involved in lanthionine ring formation for these peptides are fully conserved in the SboA sequence. A possible secondary structure of the SboA propeptide, based on the resolved structures of the lacticin 481 (15, 26) and mutacin II (13) peptides, is shown in Fig. 2B.

Identification and analysis of the locus defining SboB production in *S. salivarius* strain K12. Inverse PCR and standard

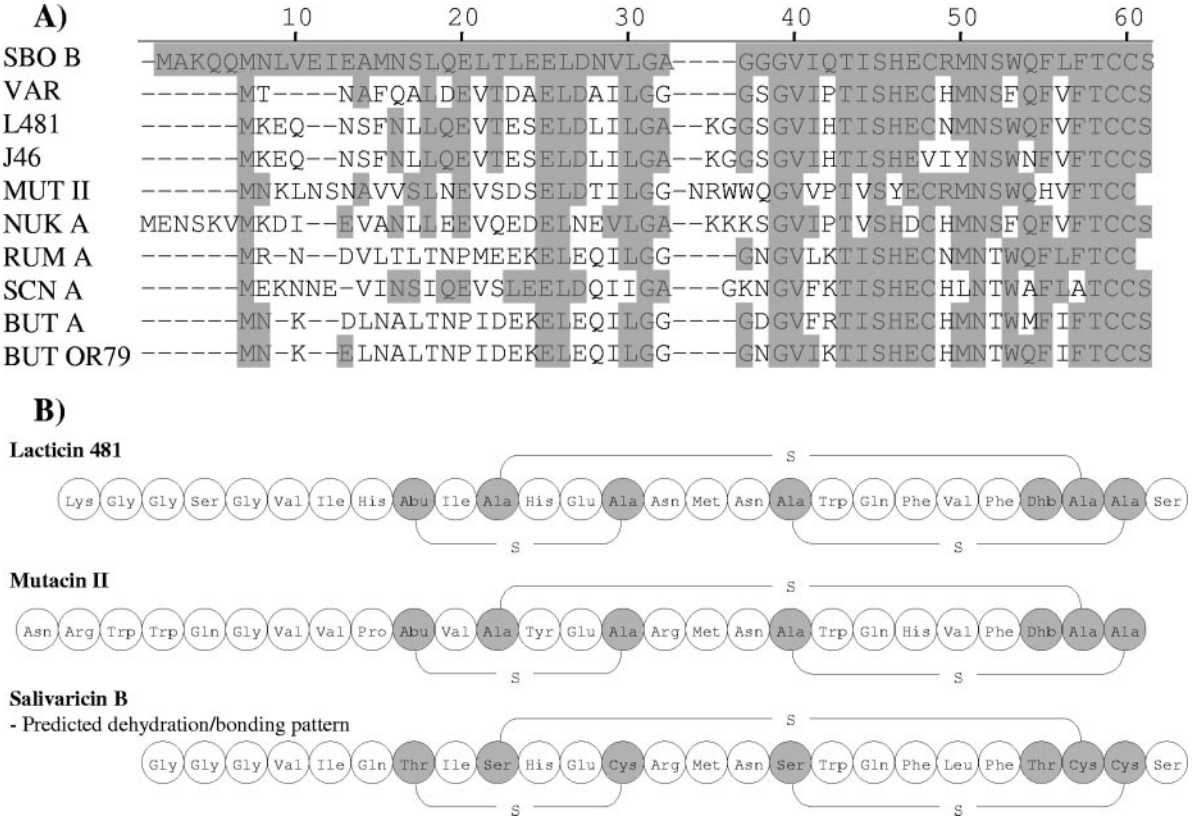

FIG. 2. Comparison of salivaricin B to similar lantibiotics. (A) Alignment of the amino acid sequences of lantibiotic precursors similar to salivaricin B, with residues matching those in the salivaricin B sequence highlighted. Sequences are labeled as follows (GenBank accession numbers are in parentheses): SBO B, salivaricin B (DQ889451); VAR, variacin (CAA63706); L481, lacticin 481 (P36499); J46, bacteriocin J46 (CAA61674); MUT II, mutacin II (O54329); NUK A, nukacin A (NP_940772); RUM A, ruminococcin A (P83675); SCN A, streptococcin A-FF22 (P36501); BUT A, butyrivibriocin A (AAK32692); and BUT OR79, butyrivibriocin OR79 (AAC19355). Numbers along the top indicate amino acid positions. (B) Predicted structure of salivaricin B based on the resolved structures of lacticin 481 and mutacin II. Dehydrated residues and residues linked by lanthionine rings are highlighted. S, thioether sulfur.

PCR were subsequently used to amplify and sequence the remainder of the SboB operon. Eight ORFs with similarity to genes of known functions were identified (Table 1). Putative proteins involved in SboB regulation, modification, transport, and immunity are encoded at this locus, with the genes probably arranged in a two-operon structure. The first predicted operon encodes the putative proteins SboK and SboR, thought to be involved in the autoregulation of SboB production. The second predicted operon comprises the *sboAMTFEG* gene cluster, the products of which have homologies to type AII lantibiotic structural (SboA), modification (SboM), transporter (SboT), and immunity (SboFEG) proteins (Table 1). Putative transcription terminators were identified downstream of *sboK* and *sboG* as well as a weaker terminator downstream of *sboA* that may allow readthrough into *sboMTFEG* (Fig. 3).

TABLE 1. Genes of the *sboB* locus

Gene	Size of product (aa)[a]	Predicted function[b]	Protein most similar to gene product (% ID)[c]
sboK	456	Regulation	*S. pyogenes* ScnK (47)
sboR	232	Regulation	*S. pyogenes* ScnR (60)
sboA	56	SalB precursor	*Kocuria varians* variacin (65)
sboM	933	Modification	*S. pyogenes* ScnM (39)
sboT	693	Transport	*S. mutans* MukT (55)
sboF	303	Immunity	*S. pyogenes* ScnF (60)
sboE	249	Immunity	*S. pyogenes* ScnE (41)
sboG	242	Immunity	*S. pyogenes* ScnG (40)

[a] aa, amino acids.
[b] Data are based on functions of homologous genes in other lantibiotic-encoding loci.
[c] % ID refers to the percentage of identity at the amino acid level.

Three repeat regions were identified at each end of the SboB locus (Fig. 3). The inverted repeats 1 and 3 share 89.5% identity over a 1,470-bp region, while repeat 2, which is in the same orientation as repeat 3, is shorter, sharing 97% identity with repeat 3 over a 537-bp stretch. Similarity searching reveals that the two longer repeats are similar to transposase genes, which have undergone multiple frameshift mutations and therefore can no longer encode functional proteins.

Distribution of the SboB locus and its proximity to the SalA locus in *S. salivarius*. The SalA gene cluster and variants thereof have been found in at least four different species of streptococci (29). Moreover, 31 of 61 BLIS-active *S. salivarius* strains were positive for *salA*. The distribution of the SboB gene cluster was investigated by dot blot hybridization using *sboA* as a probe. Nineteen of 61 strongly BLIS-active *S. sali-*

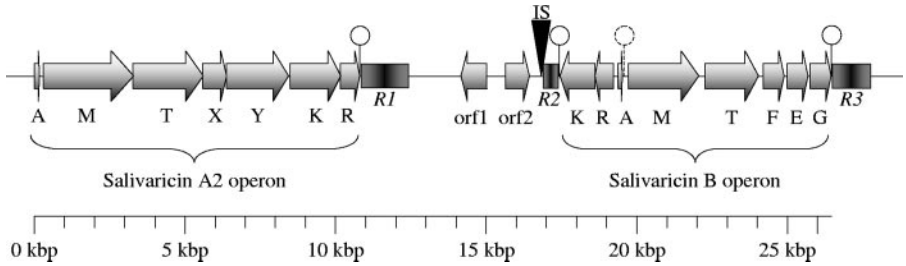

FIG. 3. Arrangement of the two gene clusters encoding salivaricins A and B in *S. salivarius* K12. Predicted transcriptional terminators for both loci are indicated by stem-loop symbols, with the dashed stem-loop showing a possible weak terminator. Two putative genes are located between the two lantibiotic loci. The three repeat regions (R1, R2, and R3) identified are also indicated, as is the position of a small insertion sequence (IS; black triangle) identified upstream of the salivaricin B locus in some strains (Table 2).

varius strains but none of 25 *S. pyogenes* strains (of different M types) or 12 *S. uberis* strains were positive. A single strain of *Streptococcus mitis*, identified for its ability to produce SboB-like activity, was positive for the *sboA* gene. Sequencing of the *sboA* gene from this *S. mitis* strain showed that the gene differed from the K12 *sboA* gene by a single point mutation from guanine to adenine at position 131, resulting in an arginine-to-histidine change at amino acid 44. Of 19 *sboA*-positive *S. salivarius* strains, 18 were also positive for *salA*: only strain NR was *sboA* positive and *salA* negative (Table 2).

The K12 SalA2 and SboB loci have recently been localized to a megaplasmid of approximately 190 kb (pSsal-K12) (27). Sequencing upstream of the SboB locus in strain K12 showed that the two lantibiotic loci are separated by only 7.5 kb. Only two predicted protein-encoding ORFs (named *orf1* and *orf2*) were identified in this 7.5-kb region. Orf1 showed some similarity to repressor proteins expressed by some staphylococcal phages, while Orf2 showed no similarity to any database-listed proteins. PCR analysis of the 18 *salA*-positive, *sboA*-positive *S. salivarius* strains showed that whereas the two loci are separated by 7.5 kb in 13 strains, the remaining 5 carry an additional 1.5 kb encoding a putative insertion sequence very similar to ISSmu2 found in *Streptococcus mutans* (GenBank accession number AE014133).

Comparative analysis of the inhibitory activities of SboB and related lantibiotics. Purified salivaricin B was used to assess the spectrum of activity against the nine standard indicators by using surface spot assays. All nine standard indicators were inhibited by the purified salivaricin B preparation, although I4 (*S. uberis*) was less sensitive than the other eight indicator strains (data not shown). The spectrum of activity of *S. salivarius* strain NR in deferred antagonism studies gives the same result, with activity against all nine standard indicators and weaker activity against I4. Deferred antagonism results for strain NR were therefore used as a close approximation of the spectrum of activity of salivaricin B.

Previous studies have shown that *S. salivarius* 20P3 is active against all *S. pyogenes* strains tested (6). The demonstration of the extended activity spectra of *S. salivarius* strains 20P3 (producer of SalA), NR (producer of SboB), and K12 (producer of SalA2 and SboB) was undertaken using a variety of indicator strains (summarized in Table 3). This enabled putative categorization of the indicators according to whether they appeared to be inhibited by both, either, or neither of the two lantibiotics. *S. salivarius* K12 had a much broader overall spectrum than the single-lantibiotic-producing strains NR and 20P3. Experiments using purified SalA and SboB showed no evidence of these two lantibiotics displaying synergistic inhibitory activity (data not shown). Of particular interest was the identification of strains inhibited by strain K12 but not by either strain NR or strain 20P3. Two separate activities, apparently unrelated to either SalA2 or SboB, were identified:

TABLE 2. Distribution of salivaricins A and B in 61 strongly BLIS-active *S. salivarius* strains

S. salivarius strain(s)[a]	Gene PCR result for:		Length (kb) of linkage PCR product[b]
	salA	*sboA*	
1-1, C78, H21-finger, K19, and SF-1 (5 strains)	+	+	9.0
152T, 161T, 3B, G69, H28, H31, K12, K28, K30, min5, N45, Strong-SA, T18A (13 strains)	+	+	7.5
NR	−	+	
13 strains	+	−	
30 strains	−	−	

[a] Only salivaricin B-positive strains have been named.
[b] The linkage PCR results indicate the distance between the salivaricin A and salivaricin B loci in the named strains.

TABLE 3. Indicator strains that differentiate the inhibitory spectra of *S. salivarius* strains K12, NR, and 20P3

Indicator(s)	Indicator(s) inhibited by[a]:		
	K12	NR	20P3
I1 through I9	Y (777)	Y (777)	Y (677)
S. salivarius K12	N	Y	Y
S. salivarius NR	N	N	Y
S. salivarius 20P3	N	Y	N
S. mutans 10449	Y	N	N
S. mutans OMZ175	Y	N	Y
S. sobrinus OMZ176	Y	N	Y
S. mitis 15914	Y	Y	N
Staphylococcus hominis 2203	Y (HL)	N	N
Enterococcus faecalis ATCC 19433	Y (HR)	N	N
Actinomyces naeslundii NCTC 10301	Y (HR)	N	N

[a] Y, yes; N, no; HL, heat-labile inhibitory activity; HR, heat-stable inhibitory activity. Numbers in parentheses are P types.

TABLE 4. Comparative inhibitory spectra of *S. salivarius* K12, *Micrococcus varians* 1482, and *Lactococcus lactis* C2102

Producer strain	P type	Inhibitory activity against strain[a]:			No. of tested *S. salivarius* strains inhibited ($n = 25$)
		NR	1482	C2102	
S. salivarius NR	777	−	+++	+++	14
Micrococcus varians 1482	777	++++	−	+++	25
Lactococcus lactis C2102	636	−	−	−	0

[a] Levels of inhibition were scored from −, for no inhibition, to ++++, for complete inhibition.

one that was heat stable and directed against *Enterococcus faecalis* strain ATCC 19433 and *Actinomyces naeslundii* strain NCTC 10301 and another that appeared relatively heat labile (inactivated by heating at 80°C for 30 min) and was directed against *Staphylococcus hominis* strain 2203. Both of these activities were absent from the plasmid-cured derivative of K12. The molecules responsible for these inhibitory activities are yet to be identified.

The inhibitory spectrum of *S. salivarius* NR (SboB producer) was compared with that of *Micrococcus varians* 1482, which produces variacin, and that of *Lactococcus lactis* C2102, which produces lacticin 481 (Table 4). Deferred antagonism cross-testing of the three producer strains showed that each was immune to their own (i.e., the homologous) inhibitor. Strains NR and 1482 were inhibitory to the other two strains and appeared to have broader inhibitory activities than strain C2102. Strains NR and 1482 were both P type 777 (inhibitory to all nine standard indicators), whereas strain C2102 was P type 636. Further deferred antagonism testing with a set of 25 *S. salivarius* strains differentiated strain NR (inhibitory to 14 of 25 *S. salivarius* strains) and 1482 (inhibitory to all 25 strains) from strain C2102 (not inhibitory to any *S. salivarius* strains tested).

DISCUSSION

In the present study, the probiotic *S. salivarius* strain K12 was shown to produce a novel type AII lantibiotic, SboB, in addition to the previously defined lantibiotic salivaricin A2 (29). Both were recovered from M17 sucrose agar cultures of *S. salivarius* K12. Although some low levels of inhibitory activity attributable to SalA2 appeared to be produced in THB cultures of strain K12, no SboB activity could be detected in these cultures. Moreover, the production of SboB was also found to be reduced in agar cultures supplemented with either glucose or lactose at 1% (wt/vol) rather than sucrose, indicating that the SboB locus is susceptible to catabolite repression.

The molecule responsible for the inhibition of *S. constellatus* (I3 in the set of nine standard indicators) was characterized as a 25-amino-acid, 2,740-Da peptide, the partial N-terminal sequence of which matched the predicted posttranslationally modified product of *sboA*. The secondary structure of SboB is proposed to resemble those of lacticin 481 and mutacin II, each of which contains two lanthionine residues and one methyl-lanthionine residue (Fig. 2). The amino acid sequence of the active SboA peptide differs from the sequences of the variacin and lacticin 481 peptides by only five and six amino acids, respectively. Analysis of the activities of the strains *S. salivarius* NR (SboA producer), *Micrococcus varians* 1482 (variacin producer), and *Lactococcus lactis* C2102 (lacticin 481 producer) was carried out to identify differences in the inhibitory activity spectra of these three lantibiotics. Lacticin 481 had no activity against the SboA and variacin producer strains and generally showed less inhibitory activity toward other indicators tested. There was a lack of cross-immunity among the three producer strains, and the variacin and SboA producers showed different inhibitory profiles when tested against a set of 25 *S. salivarius* indicator strains. These results clearly demonstrated that these three inhibitors differ significantly in their inhibitory activity.

The present study has shown that SboB production appears to be defined by an 11-kb locus consisting of eight ORFs organized into two putative operons (Fig. 3), the first of which consists of genes encoding a putative two-component sensor kinase-response regulator system (*sboK* and *sboR*) and the second comprising genes encoding the SboB prepeptide (*sboA*) and those responsible for the modification (*sboM*) and export and activation (*sboT*) of SboB and for producer self-immunity (*sboFEG*). The presence of the two-component regulatory system indicates that SboB production could be regulated in a manner similar to that of the SalA and nisin loci (i.e., autoinduced by the active peptide) (14, 25). SboB production on agar was shown to be inducible by the addition of a crude, SboB-containing preparation (M17-sucrose agar freeze-thaw exudate from the SboB producer strain *S. salivarius* NR), although the exact nature of the inducing signal molecule has not yet been defined (data not shown).

The SboB locus is flanked by large inverted repeat sequences which show similarity to transposase gene sequences that have undergone multiple frameshift mutations. These repeat regions are probably small insertion sequences that are no longer active. Small insertion sequences that encode solely a transposase gene are quite common in streptococci (20), as evidenced by the appearance of the ISSmu2-like element between the SalA and SboB loci in a subset of SalA/SboB producer strains.

Nineteen of 61 BLIS-producing *S. salivarius* strains were *sboA* positive, and 31 were *salA* positive. This shows that there are still many antimicrobial activities produced by *S. salivarius* that have not yet been characterized. Eighteen of the 19 strains positive for *sboA* were also positive for *salA*, indicating that these two lantibiotic loci are commonly linked in *S. salivarius*. Further sequence analysis of *S. salivarius* strain K12 showed that the SalA and SboB loci are localized to the 190-kb plasmid pSsal-K12 (27), separated by just 7.5 kb. Only two open reading frames likely to encode proteins were identified in this interlocus region (*orf1* and *orf2*) (Fig. 3), the functions of which are unknown. Screening the sensitivity of a large number of indicator strains to *S. salivarius* strains K12 (SalA/SboB producer), NR (SboB producer), and 20P3 (SalA producer) revealed that K12 produces at least two further molecules with BLIS activity and that both of these are also carried by pSsal-K12.

Variants of *salA* have been shown to be widely distributed within the streptococci, being present in more than 90% of *S. pyogenes* strains tested (19). Six *salA* variants have been iden-

tified, five of which were identified in *S. salivarius* (29). By contrast, *sboB* genes are identical in all *S. salivarius* strains in which they have been identified and one variant in a single strain of *S. mitis* has been identified.

The spectrum of inhibitory activity of purified SboB was assayed against the set of nine standard indicators. The growth of all nine indicator strains was strongly inhibited with the exception of that of I4, which was only weakly inhibited. These results were equivalent to the results obtained with *S. salivarius* strain NR, which appears to produce only the SboB inhibitor. *S. salivarius* strain K12 was compared with *S. salivarius* strains NR (SboB producer) and 20P3 (SalA producer) in an attempt to dissect the inhibitory activities related to the production of SalA and SboB and the additional activity produced by strain K12. Indicators that appear to be specific for SalA are the two strains *S. mutans* OMZ175 and *Streptococcus sobrinus* OMZ176 since these are the only indicators inhibited by only K12 and 20P3. SboB-specific indicators (i.e., those inhibited by only strains K12 and NR) are more common and include *S. constellatus* (I3) and numerous non-SboB-producing *S. salivarius* strains. Some indicators that were inhibited by only strain K12 were also identified (Table 3). Of the two putative extra inhibitors that have been identified, one was heat stable and active against *Enterococcus faecalis* ATCC 19433 and *Actinomyces naeslundii* NCTC 10301 and the second was heat labile and active against *Staphylococcus hominis* 2203. Neither of these extra inhibitory activities was produced by the plasmid-cured K12 derivative, indicating they are also located on the 190-kb pSsal-K12 and showing that this plasmid may act as a general repository for BLIS-encoding loci.

We have found that megaplasmids expressing a plethora of different BLIS activities are commonly and characteristically present in *S. salivarius* (27). Since the harboring and expression of complex bacteriocin loci imposes a substantial genetic investment and metabolic burden upon the host bacterium, it appears to us that the striking propensity of many *S. salivarius* strains to produce multiple anticompetitor molecules indicates that these bacteria may have a central population surveillance and controlling role within the complex bacterial zoo that is human oral microflora. Future work will aim to characterize the other inhibitory molecules expressed by pSsal-K12 and by numerous other plasmid-carrying strains of *S. salivarius*.

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