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Isolation and Characterization of the Lantibiotic Salivaricin A and Its Structural Gene *salA* from *Streptococcus salivarius* 20P3

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A bacteriocin-like inhibitory substance, salivaricin A, was purified from cultures of *Streptococcus salivarius* 20P3 and was shown by ion spray mass spectrometry to have a molecular mass of 2,315 ± 1.1 Da. Amino acid composition analysis demonstrated the presence of lanthionine, indicating that salivaricin A may be a member of the lantibiotic class of antibiotic substances. The sequence of eight amino acids at the N terminus of the molecule was determined by Edman degradation, and mixed oligonucleotide probes based on part of this sequence (GSGWIA) were used to detect the salivaricin A structural gene. A 6.2-kb *Eco*RI fragment of chromosomal DNA from strain 20P3 that hybridized with the probes was cloned, and the hybridizing region was further localized to a 379-bp *Dra*I-*Alu*I fragment. Analysis of the nucleotide sequence of this fragment indicated that salivaricin A is synthesized as a 51-amino-acid prepeptide that is posttranslationally modified and cleaved to give a biologically active 22-residue peptide containing one lanthionine and two β-methyllanthionine residues. The secondary structure of presalivaricin A was predicted to be similar to that of type A lantibiotics, with a hydrophilic α-helical leader sequence and a propeptide region with potential for β-turn formation and a lack of α-helicity. The sequence around the cleavage site of presalivaricin A differed from that of other type A lantibiotics but was similar to that of several bacteriocin-like inhibitory substances produced by lactic acid bacteria.

Numerous isolates of a wide variety of streptococcal species have been shown to produce antibiotic substances that appear to be similar to the bacteriocins produced by some gram-negative bacteria (37, 41). The term bacteriocin-like inhibitory substance (BLIS) has recently been recommended for use when these substances are described (38). BLIS may be defined as extracellularly released bacterial peptide or protein molecules that in low concentrations are able to kill some closely related bacteria by a mechanism against which the producer bacterium itself exhibits some specific immunity (38). One species of streptococci that has a particularly high incidence of BLIS-producing strains is *Streptococcus salivarius* (7, 42). This species is a numerically prominent colonist of oral epithelial surfaces in humans and is one of the first organisms to become established in the mouths of neonates (4). Interestingly, several of the first reports of the inhibitory activity of *S. salivarius* isolates showed that this activity was directed against potential oral and respiratory pathogens, such as *Mycobacterium tuberculosis* (6), *Corynebacterium diphtheriae* (2), *Streptococcus pneumoniae* (15), and *Streptococcus pyogenes* (7, 32).

BLIS-producing strains of streptococci may be categorized according to the patterns of inhibitory activity that they produce against a set of nine standard indicator bacteria (39). In practice, these patterns are converted to numerical code designations called BLIS production (P) types. When the standard BLIS typing procedure was applied to 5,750 *S. salivarius* isolates from 180 subjects, 13 different P types were detected (42); 19 of the subjects carried either P type 676 or P type 677 *S. salivarius* isolates, and strains 20P3 and 5 were adopted as the prototype producers of these two similar patterns of BLIS activity, respectively. Both of these BLIS-producing strains strongly inhibited the growth of all 81 *S. pyogenes* strains tested by using an in vitro deferred antagonism procedure (7). In a follow-up study, it was shown that there was a significantly higher proportion of BLIS-resistant organisms among the gram-positive alpha-hemolytic cocci isolated from the dorsa of the tongues of four individuals who had large populations of P type 677 *S. salivarius* than among gram-positive alpha-hemolytic isolates obtained from 13 control individuals who did not appear to have any BLIS-positive *S. salivarius* (44). It was suggested that this apparent selection of BLIS-resistant bacteria may indicate that P type 677 *S. salivarius* BLIS is produced and biologically active within the oral ecosystem.

The first *S. salivarius* BLIS to be isolated was streptococcin sal P (43). Production of this BLIS was significantly greater in cultures growing on a solid nutrient substrate than in cultures growing in liquid media. Partial characterization of the inhibitory agent indicated that it was a low-molecular-weight proteinaceous substance which was stable when boiled for 10 min at either pH 2 or pH 10. The inhibitory spectrum of streptococcin sal P included a wide variety of gram-positive bacteria, and the bactericidal activity of this BLIS was particularly evident against susceptible cells that were metabolically active. Similarly, in an artificial-mouth test system, killing of a preestablished population of BLIS-susceptible *Streptococcus sanguis* by a challenge inoculum of the streptococcin sal P producer strain occurred only when a carbohydrate source utilizable by the *S. sanguis* cells was provided (29).

Several types of BLIS have recently been shown to be ribosomally synthesized polycyclic peptides containing thioether amino acids, such as lanthionine and β-methyllan-

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TABLE 1. Bacterial strains, plasmids, and phages used in this study

Bacterial strain, plasmid, or phage	Description	Source (reference)
Bacteria		
S. salivarius 20P3	BLIS positive	Dempster and Tagg (7)
Micrococcus luteus T-18	BLIS sensitive	Tompkins and Tagg (44)
E. coli JM101	F′ *traD36 lacI*^{q}Δ*(lacZ)M15 proAB/supE thi*Δ*(lac-proAB)*	Messing (23)
Plasmids		
pUC18	Amp^r	Yanisch-Perron et al. (46)
pKRE8	pUC18 containing a 6.2-kb *Eco*RI fragment from strain 20P3	This study
pKRD19	pUC18 containing an 800-bp *Dra*I fragment from pKRE8	This study
pKRA21	pUC18 containing a 184-bp *Alu*I fragment from pKRE8	This study
pKRDA1	pUC18 containing a 294-bp *Dra*I-*Acc*I fragment from pKRD19	This study
Phages		
M13mp18		Norrander et al. (25)
M13mp19		Norrander et al. (25)

thionine (16). These lanthionine-containing antibiotics are now referred to collectively as lantibiotics (35). The first streptococcal BLIS established to be a lantibiotic is streptococcin A-FF22, a 2,795-Da product of *S. pyogenes* FF22 (13). In this paper we describe the purification of salivaricin A, a BLIS produced by the prototype P type 676 *S. salivarius* strain, its characterization as a probable lantibiotic, and the cloning and sequencing of the salivaricin A structural gene, *salA*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *S. salivarius* 20P3 and salivaricin A-sensitive indicator strain *Micrococcus luteus* T-18 were regularly subcultured on blood agar (Columbia agar base [GIBCO, Ltd., Paisley, United Kingdom] supplemented with 5% [vol/vol] human blood). *Escherichia coli* JM101 was used for plasmid propagation.

Detection and purification of salivaricin A. Salivaricin A activity was titrated by using an agar surface assay (14). Drops (20 μl) of twofold saline dilutions of a sample were assayed against *Micrococcus luteus* T-18 on Columbia agar base; the highest dilution that produced a definite zone of inhibition of growth of the indicator lawn was defined as containing 1 arbitrary unit of salivaricin A activity per ml. Salivaricin A was purified from M17 glucose agar (MGA) cultures of *S. salivarius* 20P3. MGA contained M17 broth (Difco Laboratories, East Molesey, United Kingdom), modified by addition of 0.5% (wt/vol) glucose instead of lactose, and 1.5% (wt/vol) agar (Davis Gelatine, Ltd., Christchurch, New Zealand). The surfaces of the MGA plates in each production batch were uniformly seeded by using cotton swabs charged with cells of strain 20P3 that had just been grown on blood agar for 18 h at 37°C in an atmosphere containing 5% CO_2 in air. These growth conditions were also used to obtain salivaricin A production on MGA. Fluid was extracted from the cultures by freezing and thawing, followed by centrifugation to clarify the extracted liquor (40).

A 2-liter volume of the clarified liquor was applied to an XAD-2 column (diameter, 5.0 cm; bed volume, 150 ml; Serva) and washed with 7 bed volumes of 50% (vol/vol) methanol. BLIS activity was eluted with 5 bed volumes of 90% (vol/vol) methanol (adjusted to pH 2 with 11.6 M HCl) and was concentrated by evaporation at 50°C under reduced pressure. Following fourfold dilution in 100 mM Tris-HCl (pH 6.5), this material was applied to a DEAE-Sephadex column (diameter, 3 cm; bed volume, 21 ml; Sigma) equilibrated with 100 mM Tris-HCl (pH 6.5). The effluent was concentrated and desalted on an XAD-2 column (diameter, 1.5 cm; bed volume, 10 ml), which was then washed with 10 bed volumes of Milli Q-purified water before the salivaricin A activity was eluted with 90% (vol/vol) acidified methanol and concentrated by evaporation to a volume of approximately 80 ml. This material was diluted 1:4 with 30 mM acetate buffer (pH 4.5) and applied to a CM-Sephadex column (diameter, 3 cm; bed volume, 20 ml; Pharmacia) equilibrated with the same buffer. The column was washed with 20 bed volumes of buffer, and the salivaricin A activity was eluted with a 500-ml linear salt gradient (0.0 to 0.5 M NaCl in 30 mM acetate buffer [pH 4.5]) at a flow rate of 1.25 ml/min. The active fractions were pooled and lyophilized. The lyophilized material was redissolved in 10 ml of 50 mM sodium phosphate (pH 6) containing 30% (vol/vol) acetonitrile, and 0.5-ml volumes were fractionated on a Superose 12HR 10/30 column (Pharmacia) equilibrated with 50 mM sodium phosphate (pH 6) by using a Pharmacia fast protein liquid chromatography system.

The active fractions from each of five fractionation runs were pooled, and 1-ml volumes were loaded onto a C_8 reversed-phase high-performance liquid chromatography (HPLC) column (Aquapore RP 300; pore size, 7 μm; 30 by 4.6 mm; Applied Biosystems, Inc.) equilibrated in Milli Q-purified water containing 0.1% trifluoroacetic acid (TFA), using a Waters/Millipore HPLC system. Salivaricin A activity was eluted by using a linear gradient (0 to 40% acetonitrile containing 0.085% TFA) over a period of 80 min at a constant flow rate of 1 ml/min. A_{214} was monitored, and fractions corresponding to the various peaks were collected manually. The active fractions from each C_8 run were pooled, lyophilized, and redissolved in 1 ml of Milli Q-purified water containing 0.1% TFA. Aliquots (200 μl) were loaded onto a C_{18} reversed-phase HPLC column (Pep RPC HR 5/5; Pharmacia) equilibrated as described above. The BLIS activity was eluted by using a two-step linear gradient (0 to 25% acetonitrile containing 0.085% TFA for 5 min, followed by 25 to 40% acetonitrile containing 0.085% TFA for 30 min at a constant flow rate of 0.7 ml/min). The fractions containing inhibitory activity (purified salivaricin A) were pooled and stored at −20°C.

SDS-PAGE. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed in a 20% discontinuous gel by using the method of Laemmli (21), a Mini-Protean II electrophoresis system (Bio-Rad, Richmond, Calif.), and the chemicals and protocols recom-

mended by the manufacturer. The gel was stained with Coomassie brilliant blue R-250, and molecular weights were estimated by comparing migration distances with migration distances of peptide standards (Pharmacia).

Ion spray mass spectrometry. Mass spectrometry was performed by Stefan Freund (Institut für Organische Chemie, University of Tübingen, Tübingen, Germany), who used a Sciex API III triple-quadrupole mass spectrometer equipped with an IonSpray source (Sciex, Thornhill, Ontario, Canada). Purified salivaricin A was lyophilized and redissolved in 50 μl of 0.1% formic acid before it was introduced into the ion spray interface with a syringe infusion pump (model 22; Harvard Apparatus) at a flow rate of 5 μl/min.

Amino acid analysis. Purified salivaricin A was lyophilized and hydrolyzed in 6 M HCl at 110°C for 24, 48, and 72 h. Each hydrolysate was analyzed by using a Waters/Millipore HPLC amino acid analyzer and postcolumn σ-phthaldialdehyde (OPA) detection (45a). Proline was detected after reaction with hypochlorite (included in the detection system), and cystine was detected as cysteic acid following performic acid treatment of the hydrolysate (11). For detection of lanthionine, 18-h hydrolysates of purified salivaricin A were reacted with OPA and separated by C_{18} reversed-phase HPLC as described by Sahl et al. (30). Amino acids and lanthionine were identified, and their yields were determined by comparing their integrated peak areas with those of amino acid and lanthionine (Sigma) standards. There is no commercial standard for β-methyllanthionine available, and it is not clear from the literature whether lanthionine and β-methyllanthionine can be differentiated by using reversed-phase HPLC.

N-terminal sequence analysis. An N-terminal sequence analysis of purified salivaricin A was performed by Edman degradation, using an Applied Biosystems model 470A protein sequencer equipped with an on-line model 120A phenylthiohydantoin analyzer and the chemicals and procedures recommended by the manufacturer.

DNA isolation procedures and manipulations. Chromosomal DNA from *S. salivarius* 20P3 was isolated by the method of Spanier and Cleary (36), except that after lysozyme treatment mutanolysin (Sigma) was added to a final concentration of 5 U/ml and the preparation was incubated for an additional 60 min. After pronase treatment, the DNA was extracted once with phenol and three times with phenol-chloroform-isoamyl alcohol (25:24:1) before precipitation with 0.1 volume of 3 M sodium acetate (pH 6.0) and 2 volumes of ethanol. Plasmid DNA for restriction enzyme analysis was isolated by the alkali lysis method (31). Small-scale preparation of *E. coli* plasmid DNA for screening transformants by hybridization was accomplished as follows. Transformant colonies were patched onto Luria-Bertani agar plates containing 100 μg of ampicillin per ml and incubated at 37°C overnight. Growth from each patch was suspended in 40 μl of TE buffer (pH 7.5) by using a toothpick, and 40 μl of phenol-chloroform-isoamyl alcohol (25:24:1) was added. The mixture was homogenized by vortex mixing and then centrifuged at 15,000 × *g* for 5 min. A 5-μl aliquot of 5× loading buffer containing 1 mg of DNase-free RNase per ml (20:1) was added gently to the top phase, and the mixture was left to stand for 5 min before 15 μl of the top phase was loaded onto a gel.

Standard procedures were used for restriction enzyme digestion, ligation, and agarose gel electrophoresis (31). Restriction enzyme-cut DNA for cloning was extracted from agarose by using GeneClean (Bio 101, Inc., La Jolla, Calif.). Competent cells were prepared and the procedure for transformation was performed as described by Hanahan (9).

Nucleic acid hybridizations. Four 17-mer oligonucleotides (oligonucleotides ON1 through ON4) were constructed on the basis of the sequence of amino acids (Gly-Ser-Gly-Trp-Ile-Ala) at positions 3 to 8 in the salivaricin A peptide sequence, as follows: ON1, 5′-GG(A/T)TC(A/T)GG(A/T)TGGATTGC-3′; ON2, 5′-GG(A/T)AG(C/T)GG(A/T)TGGATTGC-3′; ON3, 5′-GG(A/G/C/T)TC(A/G/C/T)GG(A/G/C/T)TGGAT(T/C/A)GC-3′; and ON4, 5′-GG(A/G/C/T)AG(C/T)GG(A/G/C/T)TGGAT(T/C/A)GC-3′.

These oligonucleotides were prepared with an Applied Biosystems model 380B DNA synthesizer and were used for hybridization without further purification. The oligonucleotides were end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham, Buckinghamshire, United Kingdom) by using T4 polynucleotide kinase (31). The DNA to be tested for hybridization was transferred to a Hybond N^+ nylon membrane (Amersham) by using the Southern capillary blotting procedure followed by alkali fixation according to the manufacturer's instructions. Hybridization was performed overnight in 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) containing 0.1% sodium pyrophosphate, 0.5% SDS, and 0.5 mg of heparin per ml at 45°C; this was followed by washing at 50°C in 5× SSC containing 0.1% SDS.

Nucleotide sequencing and computer analysis. Single-stranded recombinant phage template DNA for sequencing was prepared as described by Sambrook et al. (31). Restriction fragments were cloned into M13mp18 and M13mp19, and nucleotide sequencing was accomplished by the dideoxy chain termination method (33), using a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio) and a synthetic 17-base universal primer. α-^{35}S-labeled dATP (1,000 Ci/mmol; Amersham) was used for labeling.

DNA sequences were compiled and translated by using GeneJockey (BIOSOFT, Cambridge, England) and an Apple Macintosh computer. The GenBank, translated GenBank, and National Biomedical Research Foundation sequence data banks were searched for similar DNA or amino acid sequences by using the FASTA program of Pearson (26) and a mini-Vax computer. By using RDF2, an option of FASTA, all previously published lantibiotic sequences were analyzed for similarities to salivaricin A. Secondary structure was predicted by using the parameters of Chou and Fasman (5) and a program written by Peter A. Stockwell (Biochemistry Department, University of Otago, Dunedin, New Zealand) and based on the work of Rawlings et al. (28). Hydropathicity was plotted by using the method of Kyte and Doolittle (20) and GeneJockey. The isoelectric point of the propeptide was predicted by using the Chargpro program from PC-GENE.

Nucleotide sequence accession number. The nucleotide sequence reported below has been assigned GenBank accession number L07740.

RESULTS

Purification of salivaricin A. Salivaricin A yield was found to be highest from cultures grown on solid MGA, and so this medium was used for growth of lawns of strain 20P3 for salivaricin A purification by the protocol described in Materials and Methods. The titers of salivaricin A in freeze-thaw extracts of the MGA cultures were generally 2 to 4 arbitrary units per ml. The final purification step in which C_{18} reversed-phase HPLC was used resulted in a single homoge-

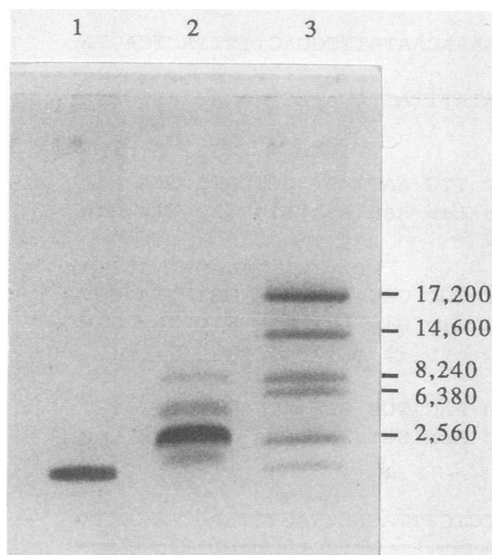

FIG. 1. Coomassie brilliant blue R-250-stained SDS-PAGE gel of purified salivaricin A. Lane 1, purified salivaricin A; lane 2, purified Pep5 monomer (3,489 Da) and dimer (6,978 Da) (note that some degradation of Pep5 occurred during 4°C storage); lane 3, molecular size standards. Molecular sizes (in daltons) are shown on the right.

TABLE 2. Amino acid composition of salivaricin A[a]

Amino acid	No. of residues per molecule from:	
	Amino acid analysis	Nucleotide sequence
Ala	1 (1.1)	1
Arg	1 (1.1)	1
Asn/Asp	3 (3.4)	1/2
Cys	0 (0.0)	3
Gln/Glu	0 (0.2)	0
Gly	2 (2.2)	2
His	0 (0.0)	0
Ile	2 (1.8)	2
Leu	0 (0.2)	0
Lys	1 (1.2)	1
Met	0 (0.0)	0
Phe	1 (1.0)	1
Pro	1 (1.5)	1
Ser	1 (1.1)	2
Thr	0 (0.0)	2
Trp	ND[b]	1
Tyr	0 (0.0)	0
Val	2 (2.1)	2
Lanthionine or β-methyllanthionine	3 (3.1)	

[a] Data were derived either by direct amino acid analysis or by translation of the nucleotide sequence. The number of amino acid residues was determined from the molar ratio relative to alanine, and the predicted mole ratios are given in parentheses.
[b] ND, not determined.

neous peak eluting at 34 to 35% acetonitrile. Only 3 to 5% of the original salivaricin A activity was recovered in the purified preparation. The purity of the salivaricin A preparation was further established by the appearance on SDS-PAGE gels of a single band at a molecular mass of about 2,000 Da (Fig. 1), and ion spray mass spectrometry indicated that the molecular mass of salivaricin A was 2,315 ± 1.1 Da.

Amino acid analysis. The amino acid composition of purified salivaricin A after acid hydrolysis is shown in Table 2. The amount of tryptophan, which is acid labile, could not be determined by this method, but a spectral scan (190 to 320 nm) of purified salivaricin A gave a profile in the 230- to 300-nm region that was characteristic of tryptophan (data not shown).

Edman analysis of purified salivaricin A revealed the following N-terminal sequence: Lys^1-Arg^2-Gly^3-Ser^4-Gly^5-Trp^6-Ile^7-Ala^8-Xaa^9-Ile^{10}-Xaa^{11}-Asp^{12}-Asp^{13}-Xaa^{14}-Pro^{15}-Asn^{16}. Xaa at positions 9, 11, and 14 indicates blank cycles in which no amino acid derivative was detected. The amino acid yields decreased substantially after residue 8, and unambiguous sequence information could not be obtained beyond cycle 16, although an additional six cycles were analyzed. There was no indication that any sequence-blocking residues had interfered with the degradation.

Lanthionine detection. The absence of cysteine and the occurrence of blank cycles during Edman sequencing are characteristics of lanthionine-containing peptides (16). In order to test for the presence of lanthionine, an 18-h hydrolysate of purified salivaricin A was reacted with OPA, and the OPA amino acid derivatives were separated by C_{18} reversed-phase HPLC. A comparison of the peak areas with the peak areas of amino acid and lanthionine standards suggested that salivaricin A contained a total of three residues of lanthionine and/or β-methyllanthionine (Table 2).

Identification and cloning of the salivaricin A structural gene. Four oligonucleotide probes (ON1 through ON4) designed on the basis of residues 3 through 8 of the salivaricin A sequence were initially used in Southern hybridization analyses to localize the salivaricin A structural gene. When an *Eco*RI digest of *S. salivarius* 20P3 chromosomal DNA was probed, a single band was observed at 6.2 kb when ON1 and ON3 were used. No bands were detected under these conditions with probes ON2 and ON4. All additional hybridizations were carried out with ON1 since it had a lower level of redundancy than ON3. The DNA from the 6.2-kb area of a gel containing *Eco*RI-digested strain 20P3 DNA was extracted from the agarose by using GeneClean and was cloned in *E. coli* by using pUC18 as the vector. Of the 150 clones containing inserts, 5 hybridized to ON1, and all of these contained a single 6.2-kb *Eco*RI fragment. Recombinant plasmid pKRE8 from one of these clones was further restricted with *Dra*I and with *Alu*I, and hybridizing bands at approximately 800 and 200 bp, respectively, were detected following Southern transfer and probing with ON1. These fragments were subcloned into the *Hin*cII site of pUC18 to form recombinant plasmids pKRD19 and pKRA21. To confirm that the DNA encoded a sequence that was homologous to the salivaricin A sequence, the 200 bp fragment from pKRA21 was subcloned as an *Eco*RI-*Pst*I fragment into M13mp18 and M13mp19 for sequencing. The sequence was translated, and a region which exhibited identity to the N-terminal sequence derived from purified salivaricin A was detected. Further analysis indicated that the *Alu*I fragment contained the 3′ portion of the salivaricin A structural gene but not its 5′ end. However, there was an *Acc*I site 20 bp downstream of the termination codon of the gene, and this site, together with a *Sma*I site in the polylinker region of the vector, was used to obtain a fragment suitable for sequencing the entire gene. Plasmid pKRD19, which contained the 800-bp *Dra*I fragment, was cut with *Acc*I and *Sma*I, end filled, and religated to form pKRDA1, which contained the

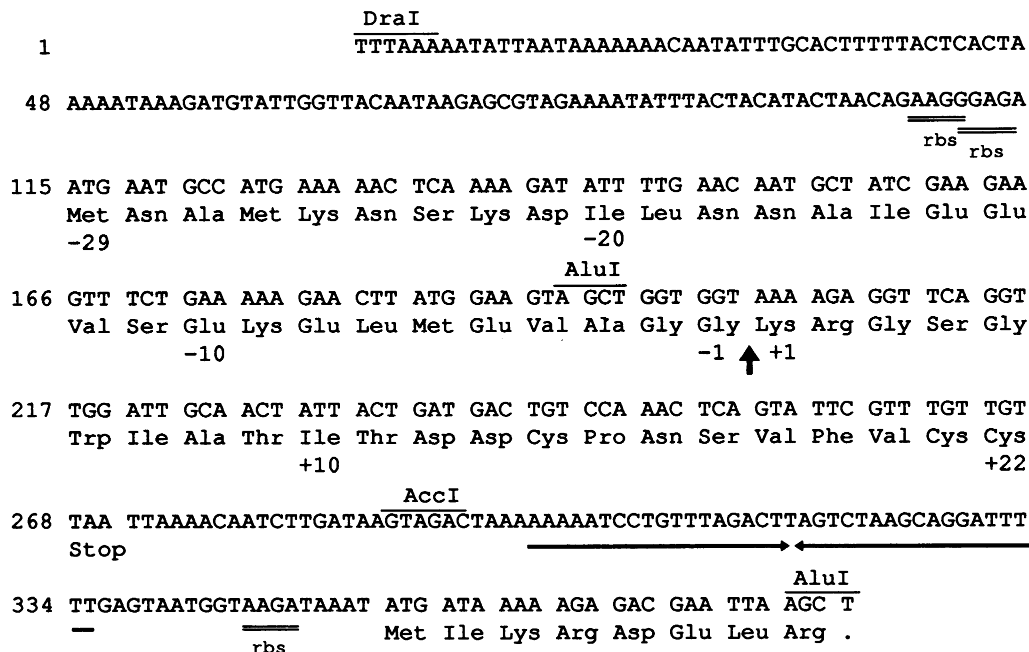

FIG. 2. Nucleotide sequence of salivaricin A structural gene *salA* and flanking DNA and deduced amino acid sequences of the open reading frames. Potential ribosome binding sites are double underlined, and the site at which presalivaricin A is cleaved to form the propeptide is indicated by an arrow. The amino acids of presalivaricin A are numbered from the cleavage site. The inverted repeat downstream of *salA* is underlined. The restriction endonuclease sites used to generate clones for sequencing are also shown.

hybridizing fragment as a 294-bp insert. This fragment was subcloned as an *Eco*RI-*Pst*I fragment into M13mp18 and M13mp19 to allow sequencing in both orientations. The sequence of the 379-bp *Dra*I-*Alu*I fragment revealed one 153-bp open reading frame containing the structural gene that we designated *salA* (Fig. 2). The translation initiation site was arbitrarily assigned to the first of two ATG (methionine) codons, each of which was preceded by a potential ribosome binding site. A 37-bp inverted repeat sequence was present 28 bp downstream of the *salA* stop codon and was followed by the start of a second open reading frame and its associated potential ribosome binding site (Fig. 2).

An amino acid sequence corresponding to the N-terminal sequence of purified salivaricin A was located starting at residue 30 of the predicted product of the *salA* gene. Hence, it appears that salivaricin A is synthesized as a 51-amino-acid prepeptide that is processed between Gly^{-1} and Lys^{+1} to produce a 22-residue propeptide. The amino acid composition of the predicted propeptide sequence was consistent with the amino acid composition obtained from the purified peptide, except that three cysteine residues, two threonine residues, and one serine residue were not detected in the amino acid analysis (Table 2). This can be explained by the formation of one lanthionine and two β-methyllanthionine residues during the maturation of the propeptide to its biologically active form. The calculated molecular mass of salivaricin A, based on the predicted amino acid sequence and taking into account the presence of one lanthionine and two β-methyllanthionine residues, was 2,316.9 Da. This value is in close agreement with the molecular mass of 2,315 ± 1.1 Da obtained by ion spray mass spectrometry of purified salivaricin A.

A search of protein and DNA data bases by using FASTA and a comparison of salivaricin A with other previously sequenced lantibiotics by RDF2 analysis revealed no significant homologies. Analyses of presalivaricin A by using the parameters of Chou and Fasman (5) suggested that the leader sequence had an α-helical structure. The sequence around the cleavage site showed a high probability for β-turn formation. The prolantibiotic part was predicted to be predominantly β-sheet in conformation, with a β-turn. An analysis of hydropathicity by using the parameters of Kyte and Doolittle (20) and a window length of 6 suggested that the leader sequence and propeptide regions were hydrophilic except for the C-terminal five amino acids of the propeptide region, which were hydrophobic (data not shown). The predicted isoelectric point of the propeptide was 5.9.

DISCUSSION

Like the production of many another streptococcal BLIS (41), the production of salivaricin A was poor in liquid media. However, enough of this inhibitory agent for purification purposes was obtained by freeze-thaw extraction of cultures of *S. salivarius* 20P3 grown on MGA plates. The recovery of salivaricin A activity from cultures grown on a glucose-supplemented medium indicates that, unlike the production of some other types of streptococcal BLIS (41), the production of salivaricin A is not subject to strong glucose catabolite repression. The salivaricin A purification protocol was not optimized, as our principal objective was to obtain enough pure peptide for an amino acid sequence determination. The adsorption of salivaricin A to the hydrophobic resin XAD-2, followed by methanol elution, was a important component of the purification protocol and has also been successfully applied in previous studies to the purification of a variety of lantibiotic molecules (13, 16).

Several observations made in this study support the contention that salivaricin A is a lantibiotic. Edman analysis of salivaricin A revealed the sequence of the eight N-terminal

amino acids before a blank cycle (a blank cycle generally signifies in lantibiotics the presence of either a lanthionine residue or a β-methyllanthionine residue [19]). The presence of three lanthionine or β-methyllanthionine residues was directly confirmed by reversed-phase HPLC of OPA-derived amino acids. This procedure has been used previously to demonstrate the presence of lanthionine (30), but our data indicate that it does not distinguish between lanthionine and β-methyllanthionine. A comparison of the amino acid composition of purified salivaricin A with the composition predicted from the translated nucleotide sequence also confirmed that salivaricin A was a lantibiotic. The predicted sequence contained one serine, two threonine, and three cysteine residues that were not present in the purified product, indicating that salivaricin A contained one lanthionine residue (Ser-Cys derived) and two β-methyllanthionine residues (Thr-Cys derived). No other disparities were found between the predicted and actual amino acid compositions, showing that salivaricin A does not contain any other modified amino acids, such as the didehydroalanine and didehydroaminobutyric acid residues commonly detected in previously characterized lantibiotics (16). The lack of premature termination of the Edman degradation reaction also indicates that these amino acids were not present in salivaricin A as their presence results in the formation of an N-terminal 2-oxobutyryl residue that is resistant to degradation (19).

A variety of lanthionine-containing peptides having diverse biological activities have now been isolated, and Jung (16) suggested that two basic types could be defined on the basis of charge, conformation, and biological activity differences. The characteristic type A lantibiotics nisin, subtilin, epidermin, gallidermin, and Pep5 are strongly cationic (with two to seven net positive charges), have molecular masses of more than 2,100 Da, and are long, screw-shaped molecules exhibiting clear structural similarities in their sequences and ring structures. Their bactericidal activity is mediated through the formation of voltage-dependent cytoplasmic membrane channels. By contrast, type B lantibiotics, such as the duramycins, cinnamycin, and ancovenin, carry no more than one net positive charge, have molecular masses of less than 2,100 Da, and exhibit a more globular structure. These peptides, which are predominantly produced by streptomycetes, have been studied principally because of their immunological or enzyme inhibitory activities, and there is little information available about the extent of their bactericidal activities. Two other lantibiotics, mersacidin and actagardine, resemble the type A lantibiotics in their patterns of ring formation, but differ because of their smaller sizes (1,825 and 1,890 Da, respectively) and noncationic nature. Actagardine, which exhibits bactericidal activity against streptococci, carries a net charge of −1 (16), indicating that a net cationic nature is not a prerequisite for the bactericidal activity of these peptides. This finding is supported by our observation that salivaricin A has a net positive charge of only +1 and a predicted isoelectric point of 5.9. It appears that salivaricin A represents another variation on the basic type A lantibiotic theme in that, although the size of the molecule (2,315 Da) is within the typical range, the molecule is not strongly cationic.

In the previously characterized type A lantibiotics nisin, subtilin, epidermin, and gallidermin the first thioether-linked residue is at position 3 from the N terminus (16). By contrast, the first such residue in salivaricin A was at position 9. Similarly, the first lanthionine-like residue in the *S. pyogenes* lantibiotic streptococcin A-FF22 does not occur until position 8 (13). The amino acids Lys-Arg at the N terminus of salivaricin A should confer a strong positive charge on this region of the peptide. These residues also represent potential cleavage sites for trypsin and are not found elsewhere in the propeptide. Since salivaricin A is inactivated by trypsin (data not shown), it is likely that the N terminus has an important role in the killing action of the molecule. A similar conclusion has been drawn regarding the role of the N-terminal amino acids of streptococcin A-FF22 since a variant form of the peptide that was missing only the N-terminal four amino acids was found to have no inhibitory activity (13).

All previous studies have indicated that lantibiotics are formed as prepeptides; then posttranslational enzymatic modification of the prolantibiotic region and cleavage of a leader sequence occur (16). The structural genes of the following five type A lantibiotic prepeptides have been sequenced: preepidermin (35), pregallidermin (34), prenisin (3, 8, 17), presubtilin (1), and pre-Pep5 (18). A comparison of the predicted prepeptide sequences shows that all contain a characteristic leader region that is hydrophilic and strongly charged and has an α-helical conformation (16). The 29-amino-acid N-terminal leader sequence of presalivaricin A conforms to this pattern. However, presalivaricin A differs from the other type A prelantibiotics in the nature of its cleavage site. The other type A lantibiotics have a turn-inducing proline at position −2, while the residue at position +1 is usually hydrophobic, the residue at position −1 is positively charged or polar, the residue at position −3 is negatively charged or polar, and the residue at position −4 is hydrophobic (16). The cleavage site of presalivaricin A is also predicted to have a β-turn conformation but contains a Gly at position −2 and positively charged Lys and Arg residues at positions +1 and +2, respectively (Table 3). Interestingly, the Gly residues at positions −1 and −2 of presalivaricin A are also found in these positions in several other non-lanthionine-containing peptide antibiotics produced by some lactic acid bacteria (Table 3). Like salivaricin A, these BLIS types also differ from the type A lantibiotics in having a positively charged amino acid residue at position +1 or +2, and it seems likely that these molecules are cleaved by a leader peptidase different from the one involved in the processing of typical type A prelantibiotics.

Despite the similarities in predicted secondary structures, the salivaricin A amino acid sequence exhibits no significant similarity to the amino acid sequences of previously described lantibiotics or the non-lanthionine-containing BLIS types produced by the lactic acid bacteria. The recently described N-terminal sequence (KGGSGVI) of the lanthionine-containing bacteriocin lacticin 481 (27) differs from the corresponding region of salivaricin A only at positions +2 and +6. However, the reported amino acid composition of lacticin 481 is quite different from that of salivaricin A, suggesting that the remainders of the molecules may not be similar. Of the previously sequenced type A lantibiotics, nisin, subtilin, gallidermin, and epidermin exhibit significant similarity to each other and presumably arose from a common ancestor, whereas Pep5 exhibits no sequence conservation with the other molecules. The lack of sequence similarity between salivaricin A and other lantibiotics suggests that salivaricin A does not share common ancestry with these other molecules. It is not yet known whether the organization of the genes required for salivaricin A production resembles the organization of genes found for typical type A lantibiotics. However, the structural genes for nisin and subtilin are followed by inverted repeats that can act as

TABLE 3. Comparison of the peptidase cleavage site of salivaricin A with the peptidase cleavage sites of peptide antibiotics from lactic acid bacteria and type A lantibiotics

Compound	Residues before the peptidase cleavage site	Residues after the peptidase cleavage site	Reference
Salivaricin A	Glu-Val-Ala-Gly-Gly	Lys-Arg-Gly-Ser	This study
Peptide antibiotics			
Lactococcin M	Gly-Ile-Asn-**Gly-Gly**[a]	Ile-**Arg-Gly**-Thr	45
Lactococcin A	**Glu**-Ala-Asn-**Gly-Gly**	**Lys**-Leu-Thr-Phe	12
Pediocin PA-1	Asn-Ile-Ile-**Gly-Gly**	**Lys**-Tyr-Tyr-Gly	22
Lactacin F	Val-**Val**-Val-**Gly-Gly**	Arg-Asn-Asn-Trp	24
Leucocin A-UAL 187	Gln-**Val**-Val-**Gly-Gly**	**Lys**-Tyr-Tyr-Gly	10
Lantibiotics			
Nisin	Gly-Ala-Ser-Pro-Arg	Ile-Thr-Ser-Ile	3
Subtilin	Lys-Ile-Thr-Pro-Gln	Trp-Lys-Ser-Glu	1
Epidermin	Gly-Ala-Glu-Pro-Arg	Ile-Ala-Ser-Lys	35
Gallidermin	Gly-Ala-Glu-Pro-Arg	Ile-Ala-Ser-Lys	34
Pep5	**Glu**-Leu-Glu-Pro-Gln	Thr-Ala-**Gly**-Pro	18

[a] Amino acid residues that are found in the same position in salivaricin A and other peptides are in boldface type.

transcriptional terminators, and *salA* is also followed by a large inverted repeat. Genes required for the processing of nisin, epidermin, and subtilin are genetically linked to the structural genes for these lantibiotics, and it would be interesting to determine whether the genes required for the processing of salivaricin A are genetically linked to *salA* and whether they are similar to other lantibiotic processing genes.

BLIS activity is widespread among streptococcal strains and species, and the two fully characterized BLIS types from streptococcal strains have both proven to be lantibiotics, suggesting that lantibiotic production is a common occurrence in streptococci. Since BLIS-positive *S. salivarius* strains have particularly strong in vitro inhibitory activity against *S. pyogenes* strains, but may differ in the patterns of inhibitory activity that they produce against a set of nine standard BLIS detection strains, it will be interesting to determine how many of these patterns reflect the production of peptides that are very similar to salivaricin A. It is also important to determine whether these lantibiotics are produced in the oral cavity and, if so, whether their production leads to the concomitant suppression of potential oral pathogens, such as *S. pyogenes*.

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