Identification of Bacteriocin-Like Inhibitors from Rumen *Streptococcus* spp. and Isolation and Characterization of Bovicin 255†

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Streptococci obtained from rumen sources were tested for the production of antibacterial compounds using a deferred-antagonism plating assay. Of 35 isolates tested, 7 were identified that inhibited the growth of other streptococci. None of the inhibitory activity was due to bacteriophage. Three isolates, LRC0253, LRC0255, and LRC0476, were selected for further characterization. Analysis of 16S ribosomal DNA indicated that LRC0476 was a strain of *Streptococcus bovis*, while isolates LRC0253 and LRC0255 are likely strains of *Streptococcus gallolyticus*. The antibacterial compounds produced by these bacteria were protease sensitive, remained active in a pH range from 1 to 12, and did not lose activity after heating at 100°C for 15 min. The inhibitory peptide from strain LRC0255 was purified using pH-dependent adsorption and desorption to bacterial cells, followed by ammonium sulfate precipitation and reversed-phase chromatography and gel filtration. The peptide was 6 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. An oligonucleotide probe based on the N-terminal sequence of the purified peptide was used to identify the gene encoding the inhibitory peptide. The antibacterial peptide has characteristics that are very similar to those described for class II bacteriocins of gram-positive bacteria.

Bacteriocins are a heterogeneous group of antibacterial peptides and proteins characterized by their ability to inhibit closely related, and sometimes more distantly related, strains of bacteria (10). It has been proposed that bacteriocins may play a key role in bacterial population dynamics (25). In particular, the bacteriocin may give the producing strains a competitive advantage by killing bacteria in the same environment competing for the same resources (23). The diversity and density of the microbial population of the rumen suggest that this environment might favor the evolution of bacteriocins as competitive factors (11, 22). If this can be confirmed, bacteriocins would have potential applications as agents for the modification of rumen microbial populations (13, 30). However, the frequency and significance of bacteriocins in the rumen ecosystem is only starting to be examined.

Bacteriocin-like inhibitory substances (BLIS) have been detected in several genera of rumen bacteria, including *Streptococcus bovis* (9), staphylococci isolated from preruminant calves and lambs (16), *Enterococcus faecium* (17, 18), *Ruminococcus albus* (4, 22), and *Butyrivibrio fibrisolvens* (13). BLIS from two strains of *B. fibrisolvens* have been purified and characterized. The inhibitors were small, hydrophobic peptides similar to lantibiotic (12) and nonlantibiotic (13) bacteriocins of gram-positive bacteria. To date, the two bacteriocins from *B. fibrisolvens* strains are the only BLIS from rumen bacteria to be confirmed as bacteriocins.

The predominant streptococcus isolated from the rumen of

animals is *S. bovis*. *S. bovis* is a highly amylolytic, fast-growing, acid-tolerant, gram-positive bacterium, fermenting starch primarily to lactic acid. *S. bovis* is of particular interest because of its role in the development of lactic acidosis in cattle and sheep fed an excess of starch (28). *S. bovis* strains are currently defined as a genomically heterogeneous group (2), with at least two distinct genetic groups identified in the rumen of cattle, based on hybridization with a 16S rRNA probe and from genomic DNA hybridizations (19). Results from one study suggest that bacteriocin production by rumen streptococci is uncommon; only one of 23 strains examined produced BLIS activity (9).

This study is part of an ongoing project, examining diverse bacteria of rumen origin for BLIS production. Here, we reexamine BLIS production by streptococci obtained from a number of different ruminants. We also purify, characterize, and determine the DNA sequence of one of the inhibitors.

MATERIALS AND METHODS

Bacterial cultures and media. Bacterial isolates used in this study were obtained from the Lethbridge Research Centre Culture Collection. Bacteria were grown in L10 broth (3), containing 0.2% (wt/vol) each of glucose, maltose, cellobiose, and starch or on plates containing L10 with 1.5% agar. Cultures were grown at 39°C in an atmosphere consisting of H₂ and CO₂ (10:90 [vol/vol]).

Phylogenetic analysis. Genomic DNA was prepared as described by Pospiech and Neumann (24). 16S rRNA genes (rDNA) were amplified from genomic DNA using primers FP1 (5'-AGA GTT YGA TYC TGG CT-3') and R1492 (5'-TAC GGY TAC CTT GTT ACG ACT-3') based on primers described by Lane (15). Reactions (100 µl) were set up in thin-walled tubes (Gordon Technologies, Mississauga, Ontario, Canada) containing 100 ng of template DNA, 1× *Pfu* buffer, 50 pmol of each primer, 0.1 mM concentrations of each deoxynucleoside triphosphate, and 2.5 U of *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.). Samples were amplified using a PTC-100-60 thermocycler (MJ Research, Inc., Watertown, Mass.). The program was 20 s at 94°C, 30 s at 50°C, and 3 min at 72°C for 30 cycles. PCR products were cloned and sequenced as previously described (33). Four clones were sequenced for each isolate using IRD800-

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labeled M13 forward and reverse primers (LI-COR Inc., Lincoln, Nebr.), plus the IRD800-labeled 16S rDNA specific primers EUB338f (5'-ACT CCT ACG GGA GGC AG-3'), 519r (5'-GWA TTA CCG CGG CKG CTG-3'), 926f (5'-AAA CTY AAA KGA ATT GAC GG-3'), and 1100r (5'-AGG GTT GCG CTC GTT G-3') (15).

Sequences were aligned with related 16S rDNA sequences retrieved from the Ribosomal Database Project II (www.cme.msu.edu/RDP/html/) using tkDCSE (5). Phylogenetic analysis was performed using a neighbor-joining method with pairwise gap removal, the Kimura-2 correction, and evaluation of 1,000 bootstrap trees as implemented in the PHYLO_WIN package (7).

Detection of bacteriocin activity. Screening of isolates for BLIS was performed using a deferred-antagonism assay (29). Fresh overnight cultures were spotted onto L10 plates and incubated overnight at 39°C in an anaerobic chamber. Resulting colonies were removed using a bent glass rod under a stream of water, and the plates were sterilized under UV light (254 nm) for 20 min. Plates were returned to the anaerobic hood for several hours, and then 5 ml of an L10 overlay containing 0.6% agar and 5 µl of an overnight culture of the indicator strain was poured onto the plates. Plates were again incubated overnight at 39°C and then examined for zones of growth inhibition. During characterization and purification steps antibacterial activity was monitored by a diffusion well assay (29).

Characterization of bacteriocin activity. Zones of growth inhibition were tested for the presence of phage essentially as previously described (13). Similarly, the protease sensitivity of the BLIS was determined as previously described (13). Proteases used in this assay included pronase (protease type XIV; Sigma, St. Louis, Mo.), proteinase K (Sigma), pepsin A (Sigma), and peptidase (porcine intestinal mucosa; Sigma), each at a final concentration of 50 µg/ml. To determine the pH stability of the BLIS, the pH of culture supernatants was adjusted using 1 M HCl or NaOH. The supernatants were incubated at room temperature for 2 h and then tested for activity. For the determination of temperature stability, the pH of culture supernatants was adjusted to pH 7.0 and the supernatants were incubated at 60 or 100°C for 15 min, cooled to room temperature, and then assayed for activity. The indicator strain used throughout the characterization process was LRC5522. This isolate is very similar to *S. bovis* based on phenotypic and phylogenetic analysis (current study and unpublished results).

Purification of bacteriocin from LRC0255. The BLIS produced by LRC0255 was primarily cell associated during the stationary phase of growth. However, the majority of the BLIS could be released from the cells by incubation of the cells at a pH of 2.0 (35). For purification of the inhibitor, L10 broth (400 ml) was inoculated with 0.1% of a fresh overnight culture of LRC0255. Following overnight growth at 39°C, the cells were pelleted by centrifugation at 10,000 × g for 15 min at 4°C, resuspended in 100 ml of 50 mM sodium phosphate (pH 2.0), and stirred for 2 h at room temperature. Cells were again pelleted, and the supernatant was decanted. The inhibitor was precipitated from the supernatant by the addition of solid (NH₄)₂SO₄. The (NH₄)₂SO₄ was added in a series of steps (20, 40, 60, and 80% saturation) to fractionate the supernatant. Once the desired percent saturation with (NH₄)₂SO₄ was reached, the solution was stirred for 1 h at room temperature, and precipitated protein was recovered by centrifugation at 10,000 × g for 20 min. Precipitates were dissolved in 1 ml of distilled water (dH₂O) and tested for antibacterial activity. All of the detectable antibacterial activity was recovered in the 40% saturation fraction as a pellicle floating on the surface of the (NH₄)₂SO₄ solution. The pellicle was lifted from the surface of the (NH₄)₂SO₄ using a sterile glass rod and dissolved in dH₂O.

The inhibitory peptide was purified using a combination of reversed-phase chromatography (RPC) and gel filtration analysis on a fast protein liquid chromatography system (Amersham Pharmacia Biotech, Inc., Baie d'Urfe, Quebec, Canada). RPC was performed on the ammonium sulfate fraction using a Pro-RPC HR5/10 column (Amersham Pharmacia). Samples were eluted from the column using a linear gradient from dH₂O–0.1% trifluoroacetic acid (TFA) to 100% acetonitrile–0.1% TFA. Active fractions from RPC were run on a Superdex Peptide HR 10/30 gel filtration column (Amersham Pharmacia), using 30% acetonitrile–0.1% TFA as the eluent. Column eluates were monitored at 206 nm, and the activity of collected fractions was determined using a well diffusion assay with LRC5522 as the indicator. Acetonitrile and TFA were removed from fractions using a rotary evaporator before testing for antibacterial activity.

SDS-PAGE and assay for antibacterial peptide. Fractions from gel filtration chromatography were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a Tris–tricine system (26). After electrophoresis, one-half of the gel was silver stained using Silver Stain Plus (Bio-Rad Laboratories, Ltd., Mississauga, Ontario, Canada), while the second half of the gel was washed three times for 10 min each time in dH₂O. The washed gel was placed on an L10 agar plate and embedded in L10 (0.6% agar) seeded with an indicator bacterium as described above. Plates were examined for zones of inhibition following overnight incubation at 39°C.

TABLE 1. Production of BLIS by ruminal streptococci

Producer	No. of indicators tested ^a	No. sensitive to BLIS
LRC0077	35	1
LRC0253	35	16
LRC0255	35	15
LRC0452	35	2
LRC0476	35	24
LRC5183	35	3
LRC5204	35	2

^a The indicator strains were other ruminal isolates tentatively identified as streptococci.

N-terminal peptide sequence determination. After SDS-PAGE, peptides were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.) and stained with GelCode Blue (Pierce, Rockford, Ill.). N-terminal amino acid sequencing was performed on a Perkin-Elmer ABI 476A automated sequencer using Edman chemistry by the Nucleic Acids/Protein Service Unit, University of British Columbia, Vancouver, British Columbia, Canada.

Cloning of the gene encoding the bacteriocin. Southern blots containing restriction-digested genomic or plasmid DNA (21) from LRC0255 were probed with an oligonucleotide based on the N-terminal sequence of the antibacterial peptide purified from LRC0255 (5'-AAA CCI GTI TAY GCI GCI AAY GGI TA-3', where I represents inosine). Plasmid DNA or restriction enzyme-digested genomic DNA was separated on an agarose gel and transferred to a Hybond N (Amersham Pharmacia) membrane by capillary transfer in 20× SSC (3 M NaCl, 0.3 M trisodium citrate; pH 7.0). DNA was fixed to the membrane with UV light according to the manufacturer's recommendations. Blots were hybridized overnight at 40°C in hybridization solution (5× SSC, 0.1% SDS, 10 mg of sonicated denatured fish sperm DNA per ml) containing 5 pmol of a ³²P-end-labeled oligonucleotide probe. Blots were washed in 1× SSC–0.1% SDS for 20 min at 40°C and exposed to BioMax film (Eastman Kodak Co., Rochester, N.Y.) for 24 h. Under these conditions the probe hybridized to a 2.3-kbp *Hind*III fragment in genomic digests. The 2.3-kbp region was purified from an agarose gel and cloned into pGEM7zf(–) (Promega Corp., Madison, Wis.) and transformed into electrocompetent *Escherichia coli* XL1-Blue cells (Stratagene) by electroporation according to the manufacturer's specifications.

Transformants were lifted onto Hybond N disks and processed according to the manufacturer's directions (Amersham Pharmacia). Colony lifts were hybridized with the oligonucleotide probe and washed as described for the Southern blot. Plasmid DNA was prepared and cycle sequenced from positive colonies using IRD800-labeled M13 forward and reverse primers (LI-COR, Inc.) using Thermosequenase (Amersham Pharmacia). Sequencing products were resolved on a LI-COR model 4000L DNA sequencer. Sequences were analyzed using Sequencher version 3.1 (Gene Codes Corp., Ann Arbor, Mich.) and Gene Inspector version 1.5 (Textco, Inc., West Lebanon, N.H.). Similarity searches of the sequences were carried out using the BLAST program (1) at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Nucleotide sequence accession numbers. The nucleotide sequence encoding bovicin 255 has been deposited in GenBank under accession number AF298196. The 16S rRNA gene sequences of LRC0253, LRC0255, and LRC0476 have been deposited in GenBank under accession numbers AF298197, AF298198, and AF298199, respectively.

RESULTS

Screening of isolates for BLIS production. Thirty-five rumen isolates which had been identified as streptococci based on phenotypic characteristics were tested against each other in a deferred antagonism assay for the production of BLIS. Seven isolates (20%) produced inhibitory activity against other rumen streptococci (Table 1). None of the inhibitory activity was due to lytic phage. Four isolates produced inhibitory activity against no more than three other isolates, while three isolates (LRC0253, LRC0255, and LRC476) produced BLIS that inhibited 16, 15, and 24 other strains of rumen streptococci, respectively (Table 1). LRC0253 and LRC0255 were originally isolated from the rumen of a moose in Alberta, Canada, and

TABLE 2. Sensitivities of ruminal bacteria to BLIS produced by ruminal streptococci

Indicator	Sensitivity of producing strains ^a :		
	LRC0253	LRC0255	LRC0476
<i>Streptococcus bovis</i> B-b-3	+	—	+
<i>Streptococcus bovis</i> ATCC 33317	+	—	—
<i>Clostridium perfringens</i> ATCC 13124	—	—	+
<i>Clostridium clostridioforme</i> ATCC 25537	—	—	—
<i>Eubacterium ruminantium</i> ATCC 17233	—	—	—
<i>Enterococcus</i> sp. strain OR21	+	+	+
<i>Succinivibrio dextrinosolvens</i> ATCC 19716	—	—	—
<i>Megasphaera elsdenii</i> ATCC 25940	—	—	—
<i>Butyrivibrio fibrisolvens</i> ATCC 19171	—	—	—
<i>Butyrivibrio fibrisolvens</i> OB156	—	—	+
<i>Butyrivibrio fibrisolvens</i> OB250	+	+	+
<i>Selenomonas ruminantium</i> ATCC 12561	—	—	—
<i>Lachnospira multipara</i> ATCC 19207	—	—	—
<i>Lactobacillus ruminis</i> ATCC 27780	+	+	+
<i>Bifidobacterium thermophilum</i> ATCC 25866	—	—	—
<i>Ruminobacter amylophilus</i> ATCC 29744	—	—	—

^a +, Sensitivity of the indicator strain to the tested BLIS.

LRC0476 was from a mouse deer in Malaysia. These three isolates were selected for further characterization.

Phylogenetic analysis of selected producing strains. To determine their phylogenetic positions, the 16S rDNA sequences of LRC0253, LRC0255, and LRC0476 were determined and analyzed using comparative sequence analysis against known 16S rDNA sequences. The comparative sequence analysis confirmed that all three isolates were members of the genus *Streptococcus*. The 16S rDNA sequence from LRC0476 had a similarity value of 99.8% with the type strain, *S. bovis* ATCC 33317, and contained sequences identical to the oligonucleotides used by Whitehead and Cotta (32) to identify ruminal *S. bovis* isolates. The 16S rDNA sequences of LRC0253 and LRC0255 were also very similar to *S. bovis* ATCC 33317 (98.7 and 98.4%, respectively); however, they were most similar to the 16S sequences of *S. gallolyticus* ACM3611 and ACM3969 (99.2 and 99.0%, respectively). Also, they did not contain sequences identical to the oligonucleotides used to identify ruminal *S. bovis* (32).

Spectrum of BLIS activity against ruminal bacteria. LRC0253, LRC0255, and LRC0476 were tested for inhibitory activity in a deferred antagonism assay against a variety of genera of gram-positive and gram-negative bacteria isolated from ruminants. None of the BLIS were inhibitory to the gram-negative bacteria tested. However, all three strains produced BLIS that were inhibitory to strains of *E. faecium*, *B. fibrisolvens*, and *Lactobacillus ruminis* (Table 2). LRC0476 also inhibited a strain of *Clostridium perfringens* and *B. fibrisolvens* OB156. (Table 2). None of the other bacteria tested were inhibited by the BLIS from the three rumen streptococci.

Characterization of the BLIS. BLIS from LRC0253, LRC0255, and LRC0476 all retained activity after treatment at pH ranging from 1.0 to 12.0. Similarly, they retained full activity after being heated at 100°C for 15 min at a neutral pH. All three BLIS were completely inactivated by pronase and proteinase K but were unaffected by treatment with pepsin A or peptidase isolated from porcine intestinal mucosa.

Purification of the BLIS from LRC0255. The BLIS produced by LRC0255 appears to be primarily associated with the

producing cells during the stationary phase of growth in liquid culture (not shown). As a result, the BLIS was initially recovered by extraction of the cells at pH 2.0 (35). The inhibitory activity was then precipitated using ammonium sulfate. All of the BLIS activity was found in the 40% saturation ammonium sulfate fraction. This fraction was run on an RPC column; the inhibitory activity eluted from the column in 30% acetonitrile. The BLIS was further purified by gel filtration chromatography.

Tricine-SDS-PAGE of the active fraction recovered from gel filtration showed of a single band of approximately 6 kDa following silver staining of the gel (Fig. 1a). The inhibitory

FIG. 1. Tricine-SDS-PAGE of purified bovicin 255. (a) Silver-stained 16.5% gel. (b) Gel overlaid with a bacteriocin-sensitive indicator bacterium, LRC5522, in L10 agar demonstrating the zone of growth inhibition. An arrowhead at the right of the figure indicates the position of the zone of growth inhibition. The position of the protein standards with molecular masses in kilodaltons is shown at the left of the figure.


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1  AAAATATGAA AAAATTTACC ATTTACGACC TAAAAACAAC AAATTACGAC TTGGACATGC ATTAACCTCA CAATCTGTTA
81  GACTAAATC TGTCAGTTGC AACGACAAAA TATTATTGGAGGATTTCAA ATGAATACAA AAACCTTTGA ACAATTTGAT
      1► M N T K T F E Q F D
161 GTATGACAG ATGAAGCACT TTCTACTGTG GAAGGCGGAG GAAAAGGATA CTGTAAGCCT GTTTATTATG CAGCAAACGG
      11► V M T D E A L S T V E G G G K G Y C K P V Y Y A A N G
      1► G K G Y X K P V Y Y A A N G
241 ATATCATGT CGATATAGCA ATGGTGAGTG GGGTTATGTT GTTACTAAAG GTGCTTTTCA AGCAACAACA GATGTAATCG
      37► Y S C R Y S N G E W G Y V V T K G A F Q A T T D V I
      14► Y S X R Y S N
321 CAAATGGTTG GGTATCGTCT CTTGGTGGAG GATATTTTGG AAAACCATAG
      64► A N G W V S S L G G G Y F G K P

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FIG. 2. Nucleotide and deduced amino acid sequences for the bovicin 255 gene. A putative ribosome-binding site is boxed. The N-terminal sequence determined from the purified peptide is shown directly below the deduced amino acid sequence. Residues designated by an "X" represent blank cycles.

activity of this peptide was confirmed by overlaying a gel containing the peptide with an indicator bacterium (Fig. 1b).

N-terminal peptide sequence. The first 21 amino acids from the 6-kDa antibacterial peptide produced by LRC0255 were determined (Fig. 2). No amino acid product was identified in cycles 5 and 16 of the Edman sequencing.

Location of the gene encoding the antibacterial peptide. Three plasmid bands were identified in agarose gels of plasmid DNA prepared from LRC0255 (not shown). To determine whether the gene encoding the BLIS purified from LRC0255 was located on a plasmid or in the chromosome, Southern blots containing plasmid DNA or restriction enzyme-digested genomic DNA were probed with a ³²P-labeled oligonucleotide based on the N-terminal amino acid sequence of the 6-kDa peptide. No hybridization occurred between the oligonucleotide and the plasmid DNA. A single *Hind*III band of approximately 2,300 bp was detected in the genomic digest using the oligonucleotide probe (not shown).

Identification of the gene encoding the antibacterial peptide. The *Hind*III fragment which hybridized to the oligonucleotide probe was cloned and sequenced. The 2,280-bp fragment was found to contain an open reading frame (ORF) with a deduced amino acid sequence identical to that determined from N-terminal sequence analysis of the 6-kDa antibacterial peptide (Fig. 2). The two blank cycles from the N-terminal analysis corresponded to cysteine residues in the deduced amino acid sequence. The ORF was preceded by a ribosome-binding site

(GGAG) located at position -11 to the first methionine residue (Fig. 2).

Analysis of bacteriocin sequence. The ORF encodes a 79-amino-acid peptide with a predicted molecular weight of 8,500.8. This peptide contains a protease processing site, with glycine residues at -1 and -2 to the processing site, which is found in many bacteriocins from gram-positive bacteria (10). The processed peptide is a glycine-rich peptide of 56 amino acids with a predicted molecular weight of 5,967.9. The isoelectric point for this peptide was calculated to be 9.18, and the net charge at pH 7 would be +2.90.

The deduced 79-amino-acid prepeptide shares homology with the nonantibiotic (class II) bacteriocins lactococcin A (8) of *Lactobacillus lactis* (identities, 29%; positives, 48%) and thermophilin A (31) of *Streptococcus thermophilus* (identities, 35%; positives, 42%). However, the homology with thermophilin A seems to reside primarily in the leader sequence (identities, 65%; positives, 73%) with no significant similarities found in the processed peptide, whereas homology with lactococcin A is distributed along the length of the prepeptide (Fig. 3).

DISCUSSION

Only one other paper has been published examining the frequency of occurrence of BLIS in streptococci of ruminal origin (9). In that work only 1 of 23 strains of *S. bovis* examined

bovicin 255	M N T K T F E Q F D V M T D E A L S T V E G G G K G Y C K P V Y Y A A N G Y S C - - R Y S
lactococcin A	M K N Q L N F N I V S D E E L S E A N G G K L T F I Q S T - - A A G D L Y Y - - N T N
thermophilin A	M N T I T I C K F D V L D A E L L S T V E G G Y S G - - K D C L K D M G G Y A L A G A G S
bovicin 255	N G E W G Y V V T K G A F Q A T T D V I A N G W V S S L G G G Y F G K P
lactococcin A	T H K Y V Y Q Q T Q N A F G A A A N T I V N G W M G G A A G G - F G L H H
thermophilin A	G A L W G - - A P A G G V G A L P G A F V G A H V G A I A G G F A C M G G M I G N K F N

FIG. 3. Alignment of the deduced amino acid sequence of bovicin 255 with homologous bacteriocin sequences lactococcin A (8) and thermophilin A (31). The alignments were performed using the BLAST program (1). Identical residues are boxed; conservative substitutions are indicated by a gray background. Dashes represent gaps introduced during the alignment.

were found to produce BLIS in a deferred antagonism assay. In the current study we found BLIS production by 7 of 35 strains examined, suggesting that bacteriocin production by rumen streptococci may be more common than was previously thought. One problem encountered in assays used to detect the production of BLIS is the need to simulate environmental conditions that will result in the production of the antibacterial compound and facilitate effective interaction with a susceptible organism (10, 29). In this study the use of a growth medium specifically designed for nonselective growth of rumen bacteria in an anaerobic atmosphere likely provided conditions more similar to those encountered in the rumen than the conditions used by Iverson and Mills (9). This may in part account for the higher number of BLIS producing bacteria detected in this work.

The BLIS from LRC0255 was purified and its gene cloned and sequenced. The peptide, bovicin 255, appears to have a number of features in common with class II bacteriocins isolated from gram-positive bacteria. First, the prepeptide is processed at a double-glycine site (20). The mature peptide is heat stable and hydrophobic (14), has a high content of small amino acids such as glycine, is cationic, and has a pI calculated to be between 8 and 11 (20). Finally, the peptide sequence showed significant similarity to two other class II bacteriocins: lactococcin A (8) and thermophilin A (31).

Bovicin 255 is the third bacteriocin to be isolated and characterized from rumen bacteria. The first two peptides isolated were bacteriocins related to class I and class II bacteriocins of gram-positive bacteria (12, 13). Both bacteriocins were isolated from strains of the rumen anaerobe *B. fibrisolvens*. Although these three peptides are not closely related they do share the feature of being resistant to gastric proteases, which would likely be a positive attribute in the highly proteolytic rumen environment.

Comparative analysis of 16S rDNA sequences was used to establish the phylogenetic positions of three of the BLIS-producing *Streptococcus* strains. The results confirmed that the three isolates were related to the type strain, *S. bovis* ATCC 33317. The rDNA sequence of LRC0476 was very similar to the rDNA sequence of *S. bovis* ATCC 33317. The LRC0476 rDNA sequence also contained sequences identical to those used in oligonucleotides to distinguish ruminal strains of *S. bovis*. Based on these data it appears that LRC0476 is a ruminal strain of *S. bovis*. The rDNA sequences from LRC0253 and LRC0255 were very similar to the rDNA sequences of *S. gallolyticus*, suggesting that these two bacteria are strains of *S. gallolyticus*. However, further phenotypic analysis would be required before this placement could be confirmed. Specifically, the presence of tannase and gallate decarboxylase activities, which are characteristic of *S. gallolyticus* (27), would need to be established.

Comparative analysis of the rDNA from other rumen bacteria has shown that species diversity in the rumen is much higher than was previously thought. For example, the genus *Butyrivibrio* has been shown to encompass a very diverse taxonomic group (6, 34). About 50% of *B. fibrisolvens* isolates examined have been shown to produce BLIS (13), and two of these have been shown to be bacteriocins (12, 13). In this study about 20% of the *Streptococcus* isolates examined were shown to produce BLIS. Because of the high population density in the

rumen and the large number of strains that appear to be competing for the same ecological niche, it seems likely that bacteriocins would play a role in competition in the rumen (30). As other groups of rumen bacteria are examined it is likely that the occurrence of bacteriocin production will be found to be widespread.

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