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Distribution and Genetic Diversity of Bacteriocin Gene Clusters in Rumen Microbial Genomes

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Some species of ruminal bacteria are known to produce antimicrobial peptides, but the screening procedures have mostly been based on *in vitro* assays using standardized methods. Recent sequencing efforts have made available the genome sequences of hundreds of ruminal microorganisms. In this work, we performed genome mining of the complete and partial genome sequences of 224 ruminal bacteria and 5 ruminal archaea to determine the distribution and diversity of bacteriocin gene clusters. A total of 46 bacteriocin gene clusters were identified in 33 strains of ruminal bacteria. Twenty gene clusters were related to lanthipeptide biosynthesis, while 11 gene clusters were associated with sactipeptide production, 7 gene clusters were associated with class II bacteriocin production, and 8 gene clusters were associated with class III bacteriocin production. The frequency of strains whose genomes encode putative antimicrobial peptide precursors was 14.4%. Clusters related to the production of sactipeptides were identified for the first time among ruminal bacteria. BLAST analysis indicated that the majority of the gene clusters (88%) encoding putative lanthipeptides contained all the essential genes required for lanthipeptide biosynthesis. Most strains of *Streptococcus* (66.6%) harbored complete lanthipeptide gene clusters, in addition to an open reading frame encoding a putative class II bacteriocin. Albusin B-like proteins were found in 100% of the *Ruminococcus albus* strains screened in this study. The *in silico* analysis provided evidence of novel biosynthetic gene clusters in bacterial species not previously related to bacteriocin production, suggesting that the rumen microbiota represents an underexplored source of antimicrobial peptides.

The production of low-molecular-weight antimicrobial peptides is a trait widely distributed among species of bacteria and archaea (1). Although a variety of functions has been assigned to these compounds (e.g., toxins, virulence factors, bacterial hormones), the bacteriocins have mainly been investigated due to their potential as alternatives to antibiotics and food preservatives (2–4). With the rise in antibiotic resistance among commensal and pathogenic strains of bacteria, there is an urgent need to identify and develop novel therapeutic strategies for clinical applications in human and animal health (5).

Bacteriocins are often defined as ribosomally synthesized antimicrobial peptides produced by bacteria or archaea (6). Bacteriocins show great diversity in their chemical structures and mechanisms of action, and at least four main groups have been proposed to classify these antimicrobial agents (7). Class I contains posttranslationally modified antimicrobial peptides, such as lanthipeptides, sactipeptides, and lasso peptides, which differ in their molecular structures, their mechanisms of action, and the enzymatic apparatuses involved in modifying the precursor peptides (7, 8). Class II bacteriocins consist of antimicrobial peptides without posttranslationally modified residues and can be subdivided into four subtypes: subtype IIa, the pediocin-like antimicrobial peptides; subtype IIb, two-component bacteriocins; subtype IIc, circular antimicrobial peptides; and subtype IId, linear non-pediocin-like one-peptide bacteriocins (9). The class III bacteriocins consist of heat-labile proteins of more than 10 kDa, also referred to as bacteriolysins (6). These proteins show homology to endopeptidases, and the C-terminal domain appears to be involved in the specificity of the protein with the target cells (10). Class IV was assigned to include the cyclic posttranslationally modified bacteriocins that were previously grouped in the IIc subtype (7).

Most antimicrobial peptides and bacteriolysins have been identified using laborious screening methods and extensive biochemical characterization, but bioinformatics-based evidence increased the notion that *in silico* approaches can be applied to identify novel antimicrobial peptides in microbial genome sequences (11, 12). In a previous study, Begley et al. (13) identified more than 60 genes homologous to *lanM* after performing genome mining of different databases. Most of these genes were found in bacterial species in which the ability to produce lanthipeptides had not yet been reported (13). Another study investigating 58 genomes of cyanobacteria found a total of 145 gene clusters harboring 290 genes encoding putative bacteriocin precursors, demonstrating that the cyanobacteria can be a potential source of bacteriocins (12). Additionally, when Sing and Sareen (14) aligned the peptidase C39 domain of the protein involved in the transport of haloduracin using the PSI-BLAST tool of the GenBank database, eight new clusters encoding putative two-component lanthipeptides were identified in several species of bacteria, including *Bacillus*, *Ruminococcus*, *Staphylococcus*, and *Clostridium* (14).

However, these previous studies did not account for the distribution or genetic diversity of antimicrobial peptides produced

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within a phylogenetic group of bacteria or archaea or consider the bacteriocinogenic potential of organisms found in complex microbial communities, such as the rumen ecosystem. Additionally, there is a lack of studies investigating the occurrence of different classes of bacteriocins within ruminal bacterial strains. Recent global collaboration initiatives have provided a growing number of rumen microbial genome sequences from cultivated ruminal bacteria and methanogenic archaea (http://www.hungate1000.org.nz/), allowing the use of *in silico* strategies to identify and characterize gene clusters related to bacteriocin biosynthesis in rumen microbes. Therefore, we hypothesized that genome mining could guide the identification of antimicrobial peptides in ruminal bacteria and also reveal the organization and diversity of the biosynthetic machinery required for bacteriocin production. We predicted that these computational analyses could provide a basis for the *in vitro* optimization of isolation procedures and biochemical characterization of antimicrobial peptides in the rumen ecosystem.

Previous work demonstrated that strains of *Butyrivibrio* (15), *Enterococcus* (10), *Ruminococcus* (16), and *Streptococcus* (17, 18) had antagonistic activity against other aerobic and anaerobic bacteria, and this inhibitory effect has been related to the production of antimicrobial peptides. However, until now only a few bacteriocins produced by ruminal bacteria have been biochemically characterized, and even fewer studies have been carried out to identify the genes involved in bacteriocin biosynthesis (19–22). Some ruminal bacteriocins have shown great antimicrobial activity and low cytotoxicity, demonstrating potential applications in food preservation and livestock production (4, 23). In this study, we performed an extensive molecular screening of bacteriocin gene clusters in 229 genomes of ruminal bacteria and archaea. Our results revealed novel groups of bacteriocins in species of ruminal bacteria and demonstrate that bacteriocin gene clusters are prevalent among *Butyrivibrio*, *Ruminococcus*, and *Streptococcus* strains.

MATERIALS AND METHODS

Data sets. Complete and partial genome sequences of 224 ruminal bacteria and 5 ruminal archaea were downloaded in the fasta (.fa) format from the Joint Genome Institute (JGI) (http://genome.jgi.doe.gov/) or the Genome Database of NCBI (http://www.ncbi.nlm.nih.gov/genome/). The search words used to identify the genomes of ruminal bacteria and archaea were "Hungate 1000" for the JGI database and "rumen" for the NCBI database. Genome sequence files were accessed from November 2011 to January 2015, and the accession numbers are shown in Table S1 in the supplemental material.

Gene cluster identification and classification. Genomes in the fasta format were individually uploaded to BAGEL3 (24) and antiSMASH (25) software to identify putative proteins related to bacteriocin production. Result files containing the amino acid sequences of the identified proteins were copied as text (.txt) files for sequence comparisons. All gene clusters were initially grouped according to the classification schemes defined by each program using the default parameters. For comparative analyses of the gene clusters within each class of bacteriocins, at least two proteins involved in biosynthesis according to cutoff criteria of an E value of <0.00001 and an identity of $\geq 20\%$ were considered a cluster (12). The domains of each candidate protein in the NCBI Conserved Domain database were identified using the full amino acid sequence of the protein. Multiple-sequence alignments and comparisons of the amino acid sequences were then performed using MEGA (version 6.0) and DNAman (version 5.2.2; Lynnon Biosoft) software (26). To identify precursor genes, a 20-kb region upstream and downstream of the each candidate protein was investigated by scanning the scaffold using the Chromosome Viewer function available on the JGI website.

Phylogenetic analysis. Strains with bacteriocin gene clusters and in which 16S rRNA sequences with a size of ≥ 900 bp were available were used for the phylogenetic reconstruction of inference by the neighbor-joining (NJ) method with 5,000 bootstrap replicates. The ClustalW program (27) was initially used to align the sequences, and some corrections were made manually using MEGA (version 6.0) software (26).

RESULTS

In this study, at least one bacteriocin gene cluster was identified in 45.4% of the 229 genomes of ruminal bacteria and archaea downloaded from the JGI and GenBank databases (103 clusters in *Bacteria* and 1 cluster in *Archaea*) (Table 1). The number of bacteriocin gene clusters in rumen microbial genomes varied from 1 ($n = 55$) to 6 ($n = 1$), and the genomes of some bacterial strains had clusters encoding different classes of bacteriocins.

When the relative abundance of bacteriocin gene clusters in the most representative strains was calculated on the basis of the clusters identified by BAGEL3 and antiSMASH software, it was observed that class I bacteriocins (lanthipeptides, lasso peptides, and sactipeptides) were prevalent among strains of the genus *Butyrivibrio* (58.5%), the family *Lachnospiraceae* (69.2%), and the genus *Ruminococcus* (59.1%) (Fig. 1A). The production of class III bacteriocins was also abundant among these bacteria, while class II bacteriocins were found only in *Streptococcus* strains (Fig. 1A).

When the individual gene clusters were manually curated by considering an E value greater than $1e^{-6}$ and a minimum amino acid sequence identity with the amino acid sequences of other known products of bacteriocin gene clusters of 20%, a total of 46 gene clusters distributed among 33 bacterial strains were considered to be related to bacteriocin biosynthesis, indicating that 14.4% of ruminal strains could potentially produce antimicrobial peptides. These results considerably expand knowledge regarding the abundance of bacteriocin gene clusters among species of ruminal bacteria. Phylogenetic analysis of strains harboring bacteriocin gene clusters indicated that bacteriocin production is a trait widely distributed among ruminal bacteria (Fig. 1B). Lanthipeptide gene clusters were distributed in different species of ruminal bacteria and occurred frequently in *Streptococcus* (66.6%). The presence of sactipeptide gene clusters was higher in the *Clostridiales*, and all *Ruminococcus albus* strains examined in this study contained open reading frames (ORFs) encoding putative class III bacteriocins (Fig. 1B).

Novel sactipeptide gene clusters found in ruminal bacteria. Sactipeptide gene clusters that showed ABC transporters and recombinant *S*-adenosylmethionine (rSAM) proteins in a 20-kb region adjacent to the precursor peptides were selected. This analysis indicated 11 gene clusters varying in size from 5.1 kb (*Butyrivibrio hungatei* XBD2006) to 10.1 kb (*Peptostreptococcus anaerobius* C) in different bacterial genomes (Fig. 2B). The organization pattern of the gene clusters and the number of genes with a putative function in sactipeptide biosynthesis differed considerably among the bacterial strains. Our *in silico* analysis also indicated sactipeptide gene clusters containing a histidine kinase (HK) and a response regulator (RR), suggesting that some of these sactipeptides were subjected to a two-component regulatory system which had not been previously described for other known sactipeptides.

In *Blautia schinkii* DSM 10518, *Blautia* sp. strain SF-50, and *Clostridium aerotolerans* DSM 5434, the structural peptides

TABLE 1 Bacteriocin gene clusters identified in the genomes of ruminal microorganisms

Strain[a]	No. of bacteriocin gene clusters					
	Class I					
	Lanthipeptide	Sactipeptide	Class II	Class III	Unclassified	Total[b]
Anaerovibrio lipolyticus LB2005	0	2	0	0	0	2
Anaerovibrio sp. strain RM50	0	1	0	0	0	1
Blautia schinkii DSM 10518	0	2	0	0	0	2
Blautia sp. strain SF-50	1	2	0	0	0	3
Butyrivibrio fibrisolvens MD2001	0	0	0	0	1	1
Butyrivibrio fibrisolvens WCD2001	0	0	0	0	2	2
Butyrivibrio hungatei NK4A153	0	0	0	0	1	1
Butyrivibrio hungatei XBD2006	1	1	0	1	0	3
Butyrivibrio proteoclasticus B316	0	0	0	1	1	2
Butyrivibrio proteoclasticus FD2007	0	0	0	0	1	1
Butyrivibrio proteoclasticus P6B7	0	0	0	1	0	1
Butyrivibrio sp. strain AD3002	2	0	0	0	0	2
Butyrivibrio sp. strain AE2015	0	0	0	1	4	5
Butyrivibrio sp. strain AE3003	0	0	0	1	0	1
Butyrivibrio sp. strain AE3009	0	2	0	0	0	2
Butyrivibrio sp. strain FCS006	0	0	0	1	2	3
Butyrivibrio sp. strain IN11A18	0	1	0	1	0	2
Butyrivibrio sp. strain LB2008	0	0	0	1	0	1
Butyrivibrio sp. strain LC3010	1	0	0	0	1	2
Butyrivibrio sp. strain NC2007	0	0	0	1	3	4
Butyrivibrio sp. strain NC3005	0	3	0	1	0	4
Butyrivibrio sp. strain MC2013	0	1	0	0	0	1
Butyrivibrio sp. strain Su6	0	0	0	1	0	1
Butyrivibrio sp. strain TB	0	0	0	1	0	1
Butyrivibrio sp. strain VCB2006	0	0	0	1	2	3
Butyrivibrio sp. strain VCD2006	2	0	0	0	0	2
Butyrivibrio sp. strain XBB1001	0	0	0	1	0	1
Butyrivibrio sp. strain XPD2006	0	0	0	1	2	3
Butyrivibrio sp. strain YAB3001	0	0	0	1	0	1
Clostridiales bacterium NK3B98	0	0	0	0	1	1
Clostridium aerotolerans DSM 5434	0	2	0	0	0	2
Clostridium algidicarnis B3	0	0	1	0	0	1
Clostridium beijerinckii HUN142	1	1	3	1	0	6
Clostridium clostridiforme ATCC25537	0	0	1	0	0	1
Clostridium glycolicum KPPR9	0	1	0	0	0	1
Clostridium lundense DSM 17049	0	2	0	0	0	2
Desulfotomaculum ruminis DSM 2154	0	1	0	0	0	1
Dorea longicatena AGR2136	0	0	1	0	0	1
Enterococcus faecalis 68A	0	0	0	1	0	1
Kandleria vitulina WCE2011	0	1	0	0	0	1
Lachnobacterium bovis C6A12	1	1	0	0	0	2
Lachnobacterium bovis DSM 14045	0	1	0	0	0	1
Lachnobacterium bovis NK4b19	0	1	0	0	0	1
Lachnospira multipara MC2003	0	1	0	0	0	1
Lachnospiraceae bacterium AB2028	0	1	0	0	0	1
Lachnospiraceae bacterium AC2014	0	0	0	1	0	1
Lachnospiraceae bacterium AC2028	0	0	0	1	0	1
Lachnospiraceae bacterium AC3007	0	1	0	0	0	1
Lachnospiraceae bacterium C6A11	1	0	0	0	0	1
Lachnospiraceae bacterium ND2006	0	1	0	0	0	1
Lachnospiraceae bacterium NK4A136	0	0	0	1	0	1
Lachnospiraceae bacterium NK4A144	0	0	0	1	0	1
Lachnospiraceae bacterium NK4A179	0	1	0	0	1	2
Lachnospiraceae bacterium XPB1003	0	1	0	0	0	1
Lactobacillus mucosae AGR63	0	0	0	1	0	1
Lactobacillus plantarum AG30	0	0	1	0	0	1
***Lactobacillus ruminis* ATCC 27782**	0	0	1	0	0	1
Lactococcus lactis subsp. *lactis* 511	1	1	1	1	0	4

(Continued on following page)

TABLE 1 (Continued)

Strain[a]	No. of bacteriocin gene clusters					
	Class I					
	Lanthipeptide	Sactipeptide	Class II	Class III	Unclassified	Total[b]
Methanobrevibacter wolinii SH	0	1	0	0	0	1
Mitsuokella jalaludinii DSM 13811	0	2	0	0	0	2
Oribacterium sp. strain FC2011	0	2	0	0	0	2
Oribacterium sp. strain P6A1	0	2	0	0	0	2
Peptostreptococcaceae bacterium VA2	0	3	1	0	0	4
Peptostreptococcus anaerobius C	0	1	0	0	0	1
Prevotella ruminicola 23	0	1	0	0	0	1
Propionibacterium sp. strain MB3007	0	0	1	0	0	1
Pseudobutyrivibrio ruminis AD2017	0	0	0	0	1	1
Pseudobutyrivibrio ruminis CF1b	0	0	0	0	1	1
Pseudobutyrivibrio sp. strain YE44	1	0	0	0	0	1
Ruminococcaceae bacterium AB4001	0	1	0	0	0	1
Ruminococcaceae bacterium AE2021	0	1	0	0	2	3
***Ruminococcus albus* 7**	0	0	0	1	0	1
***Ruminococcus albus* 8**	0	2	0	1	0	3
Ruminococcus albus AD2013	0	0	0	3	0	3
Ruminococcus albus AR67	0	2	0	1	0	3
Ruminococcus albus DSM 20455	0	1	0	1	0	2
Ruminococcus albus SY3	0	1	0	2	0	3
Ruminococcus flavefaciens AE3010	0	1	0	0	0	1
***Ruminococcus flavefaciens* FD1**	1	0	0	0	0	1
Ruminococcus flavefaciens MA2007	0	1	0	0	1	2
Ruminococcus flavefaciens ND2009	0	1	0	0	1	2
Ruminococcus flavefaciens YL228	0	1	0	0	0	1
Ruminococcus sp. strain FC2018	0	1	0	0	2	3
Selenomonas ruminantium TAM6421	0	0	0	0	1	1
Selenomonas ruminantium ATCC 12561	1	1	0	0	3	5
Selenomonas ruminantium C3	0	1	0	0	0	1
Selenomonas ruminantium GACV9	0	1	0	0	0	1
Selenomonas ruminantium subsp. *lactilytica* DSM 2872	0	2	0	0	0	2
Selenomonas sp. strain AE3005	0	1	0	0	0	1
Selenomonas sp. strain FC4001	0	1	0	0	0	1
Selenomonas sp. strain ND2010	0	1	0	0	0	1
Sharpea azabuensis DSM 18934	0	2	0	0	0	2
Slackia heliotrinireducens DSM 20476	0	0	0	0	2	2
Staphylococcus epidermidis AG42	1	0	1	0	0	2
Streptococcus equinus 2B	0	0	1	0	0	1
Streptococcus equinus AG46	0	0	1	0	0	1
Streptococcus equinus B315	2	0	0	0	1	3
***Streptococcus equinus* HC5**	1	0	1	0	0	2
Streptococcus equinus JB1	0	0	1	0	0	1
Streptococcus equinus SN033	2	0	0	0	1	3
Streptococcus equinus GA1	2	0	1	0	0	3
Streptococcus gallolyticus VTM1R27	1	0	1	0	0	2
Streptococcus henryi A4	1	0	0	0	0	1
***Wolinella succinogenes* DSM 1740**	0	0	0	2	0	2
Total	24	68	18	35	38	183

[a] The genomes were obtained from the Genome portal of the JGI (The Hungate1000 Project) and the GenBank databases over the period of this study, from October 2013 to January 2015. Strains in bold have previously been shown to produce bacteriocins *in vitro* or to harbor bacteriocin-encoding genes.
[b] Total number of gene clusters found for each strain.

showed motifs belonging to the protein family TIGR04065 (Table 2). When the amino acid sequences of the four structural peptides of *B. schinkii* DSM 10518 were aligned, the identity of the amino acid residues was 52.3%. A greater amino acid sequence identity (74.7%) was obtained for the sequences of the six structural peptides found in the genome of *R. albus* DSM 20455, and 63.1% identity was obtained for the sequences of the seven structural peptides found in the genome of *R. albus* AR67. The rSAM proteins varied in size from 333 to 510 amino acid (aa) residues and shared an amino acid sequence identity of 21.5%. In 45.4% of the sactipeptide gene clusters, the rSAM proteins showed conserved motifs (YGGEPLXXFXLIK) of the Cys-rich peptide rad-

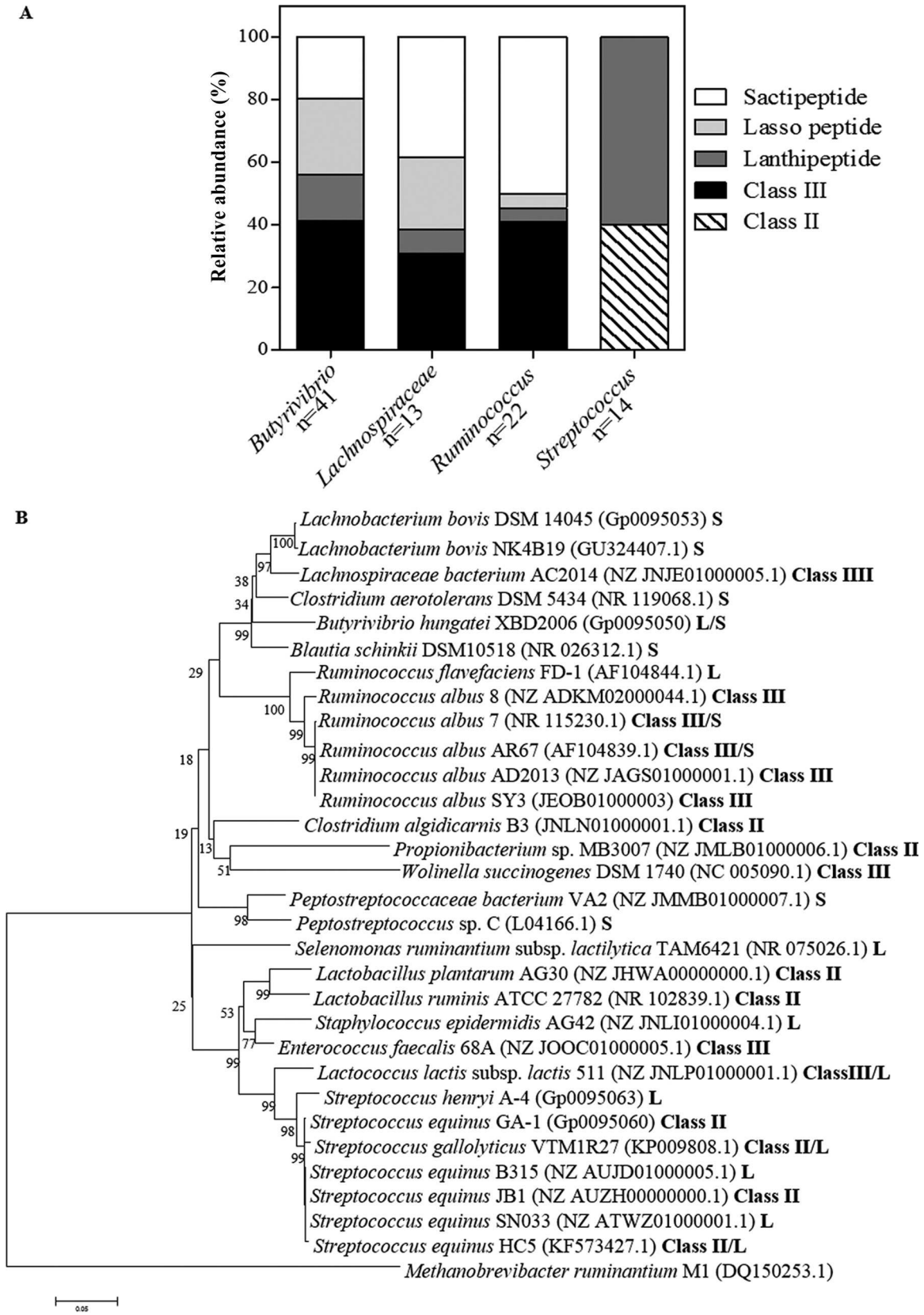

FIG 1 Distribution of bacteriocin gene clusters in the genomes of ruminal bacteria. (A) Relative abundance of bacteriocin gene clusters among different genera of ruminal bacteria. The number of bacteriocin gene clusters (*n*) used in this analysis is indicated on the *x* axis. (B) Phylogenetic tree showing the distribution of bacteriocin gene clusters in species of ruminal bacteria. The consensus tree, generated on the basis of the neighbor-joining (NJ) method, was constructed with 5,000 repetitions using the MEGA (version 6.0) program. Tree construction was based on the 16S rRNA nucleotide sequences of more 900 bp from the ruminal bacteria (30 strains) used in this study. The GenBank accession numbers of the bacterial strains are indicated in parentheses. The scale bar represents 5 nucleotide changes per 100 nucleotides analyzed. The bacteriocin gene clusters are indicated in bold: S, sactipeptide; L, lanthipeptide; class II, class II bacteriocin; class III, class III bacteriocin.

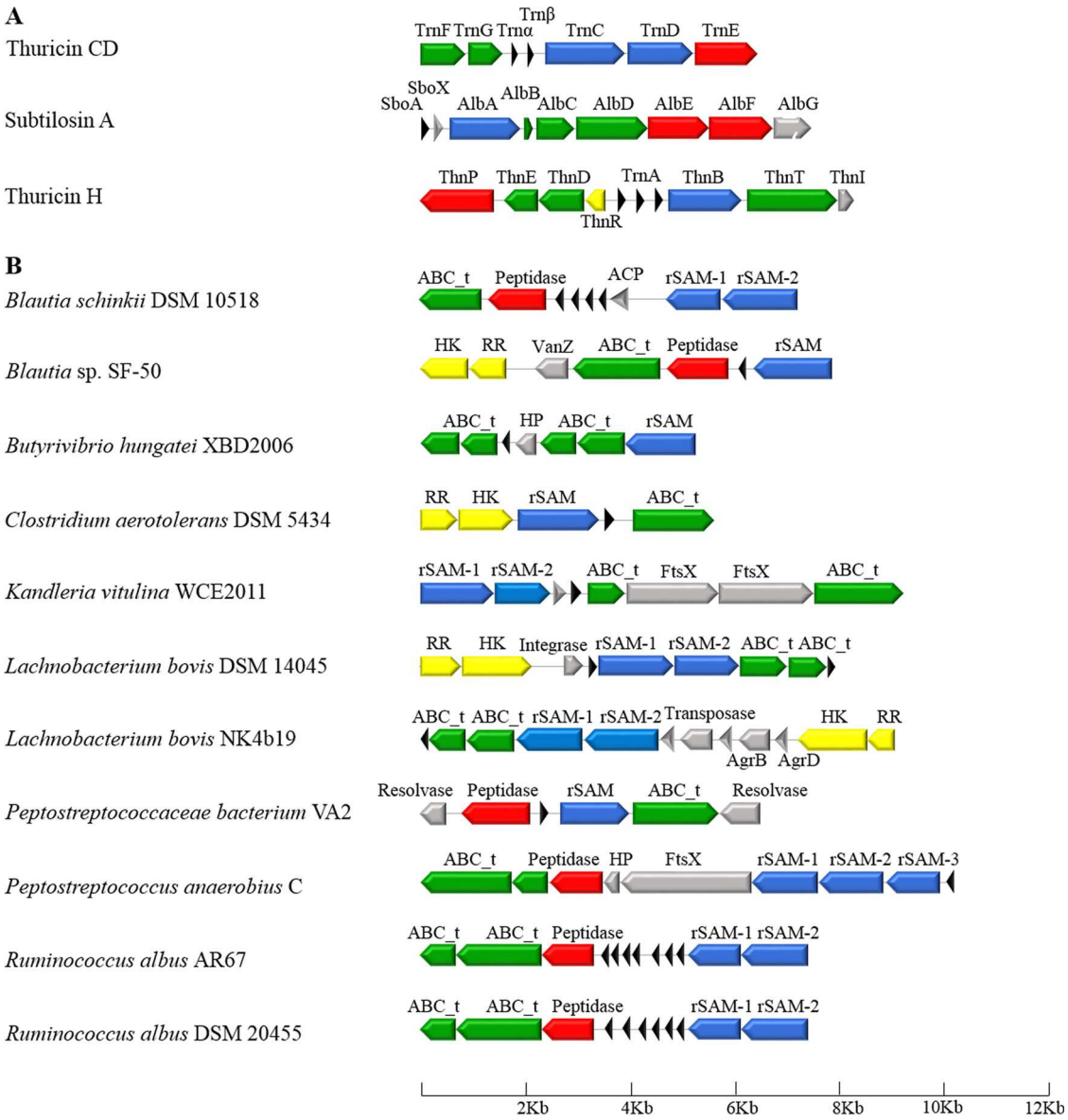

FIG 2 The biosynthetic gene clusters of the sactipeptides of ruminal bacteria. (A) Diagrammatic representation of the previously characterized sactipeptides thuricin CD, produced by *B. thuringiensis* DPC 6431 (GenBank accession number HQ446454.1); subtilosin A, produced by *B. subtilis* 168 (GenBank accession number NC_000964.3); and thuricin H, produced by *B. thuringiensis* SF361 (GenBank accession number FJ977580.1). (B) Sactipeptide gene clusters found in the genomes of ruminal bacteria. Green, genes involved in transport; black, structural genes; blue, rSAM; red, protease proteins; gray, undefined proteins.

ical *S*-adenosylmethionine (SAM) maturase of the protein family TIGR04068 (Table 2).

The putative ABC transporters for sactipeptides varied in size from 219 to 579 amino acid residues, and the average amino acid sequence identity was only 20.89%. The majority of these proteins belonged to the ABC-type multidrug system, but 26.3% of the ABC transporters contained the conserved motifs SGGXXQ and DEPT of the ABC-type antimicrobial peptide transport system (Table 2). *B. hungatei* XBD2006 has four proteins of the ABC-type multidrug transporter adjacent to the rSAM protein, while the genome of *Kandleria vitulina* WCE2011 harbors a protein with 230 amino acid residues related to the transport of antimicrobial peptides.

The genomes of *B. schinkii* DSM 10518, *Peptostreptococcaceae* bacterium VA2, *P. anaerobius* C, *R. albus* AR67, and *R. albus* DSM 20455 showed genes encoding putative peptidases involved in the cleavage of the leader peptide, and proteins of a two-component regulatory system were found in the genomes of *Blautia* sp. SF-50, *C. aerotolerans* DSM 5434, *Lachnobacterium bovis* DSM 14045, and *L. bovis* NK4b19.

Several ruminal bacteria contain a complete biosynthetic gene cluster associated with lanthipeptide production. When genome mining was performed to identify genes related to lanthipeptide biosynthesis in ruminal bacteria, 20 gene clusters contained the putative structural genes and the enzymes required for posttranslational modification and transport of the peptide across the cell membrane (Fig. 3). These bacteriocin gene clusters were classified either as subtype I lanthipeptides, when the modification proteins LanB and LanC were identified in the gene clusters

TABLE 2 Conserved domains of the putative sactipeptide biosynthetic proteins found in ruminal bacteria

Protein	Size (no. of aa)	Conserved domain	Motif[a]	Alignment start position[b]	Frequency[c] (%)
Precursor peptide	64–78	TIGR04065 (putative bacteriocin precursor)			27.3
	32–66	ND[d]			72.7
rSAM1	461–510	TIGR04068 (Cys-rich peptide radical SAM maturase)	YGGEPLXXFXLIK	141–162	45.4
	333	TIGR03974 (SCIFF rSAM maturase)			18.0
rSAM2	398–444	TIGR04085 (radical SAM domain)			72.7
	349–452	TIGR04066 (peptide maturation)			18.1
ABC transporter	219–230	COG1136 (ABC-type antimicrobial peptide transport system)	SGGXX(Q/D)EPT	142–180	26.3
	536–579	COG1132 (ABC-type multidrug transporter)			72.7
Peptidase	285–669	COG1404 (subtilisin-like serine proteases)			54.5

[a] X, any amino acid.
[b] Position in the protein where the conserved motif starts.
[c] Frequency refers to the number of times that clusters containing the specified protein with a particular domain were identified in this study.
[d] ND, not determined.

(Fig. 3A), or as subtype II lanthipeptides, when the bifunctional maturation enzyme LanM was present (Fig. 3B).

The lanthipeptide gene clusters varied in size from 7.4 kb (*Blautia* sp. SF-50) to 18.7 kb (*Lachnospiraceae* bacterium C6A11) in different bacterial genomes (Fig. 3), and the LanFEG immunity system was found in 50% of the lanthipeptide gene clusters. A putative histidine kinase and a response regulator (LanKR) were present in 40% of the clusters, while genes encoding putative peptidases were identified only in lanthipeptide gene clusters belonging to subtype I (Fig. 3A).

Analysis of the LanB proteins (subtype I lanthipeptides) revealed three different consensus sequences in the N-terminal region (RXTPFG) and C-terminal region (RYGG and NRLXGI) (Table 3). The putative LanC proteins varied in size from 398 aa to 444 aa and contained the consensus regions RDAWCYGGP and CHGYXG. The LanT proteins were present in all the lanthipeptide gene clusters belonging to subtype I and showed domains of the protein family COG1132 (ABC-type multidrug system), and two motifs were located in the central region (GXXGSGKST) and the C-terminal region (LSGGQWQT). In 62.5% (5/8) of the clusters grouped in subtype I and 41.6% (5/12) of the clusters grouped in subtype II, the ORFs identified to be LanF showed three conserved regions (NAGKST, FSLGMKQRLG, and LLILDEPTNGLDPI) (Table 3). However, the amino acid sequence of the LanM proteins varied considerably among the strains, and truly conserved regions could not be accurately identified.

In this work, the majority of the *Streptococcus equinus* strains tested (5/7) harbored genes encoding putative lanthipeptides in their genomes. *S. equinus* B315 and *S. equinus* SN033 had virtually identical putative gene clusters encoding subtype I and II lanthipeptides. These gene clusters were also very similar to the nisin U biosynthetic cluster, and the sequences of the precursor peptides showed 84% identity with the sequence of nisin U. The sequences of other ORFs showed identities of greater than 49% with those of corresponding genes involved in the biosynthesis of nisin U. On the other hand, the clusters encoding subtype II lanthipeptides were highly related to the operon of streptococcin A-FF22. In *S. equinus* HC5, the precursor peptide encoding bovicin HC5 showed an identity of 72% with the streptin produced by *Streptococcus pyogenes* BL-T.

In *Clostridium beijerinckii* HUN142, two clusters of subtype I lanthipeptides were adjacent in the chromosome (Fig. 3A). The first cluster contained all the expected genes required for lanthipeptide biosynthesis, including a protease with 63% identity to NisP, four peptide precursors (containing 49, 46, 43, and 47 amino acid residues, respectively) with 42% identity to other lanthipeptides, a modification enzyme homologous to LanB, an ABC transporter, a modification enzyme homologous to LanC, and five putative ORFs related to regulatory functions and immunity (LanFEGRK). The second cluster showed a precursor peptide of 51 amino acid residues with 45% identity to subtilin from *Bacillus amyloliquefaciens* VB2, in addition to a LanB-like gene, an ABC exporter of the CcmA protein family, a LanC-like protein, and two ORFs (LanRK) related to the regulation of lanthipeptide biosynthesis.

Staphylococcus epidermidis AG42 harbored a gene cluster with an organization that was highly similar to that of epicidin 280, and the sequences of the putative gene products always had identities greater than 54% to those of the lanthibiotic biosynthetic machinery produced by *S. epidermidis* BN 280 (28).

The gene cluster found in the genome of *Ruminococcus flavefaciens* FD1 (Fig. 3B) has been previously reported in another *in silico* study (14) and is not discussed here.

Distribution of class II bacteriocins in the genomes of ruminal bacteria. In this study, 18 gene clusters that could be related to the production of class II bacteriocins were identified (Table 1). From these, seven gene clusters contained an ORF encoding an ABC-type transport protein related to bacteriocin secretion (Fig. 4). These transport proteins had sizes of between 689 and 742 amino acid residues and domains characteristic of those of the TIGR01193 family (ABC-type bacteriocin transporter). A C39 domain (LPXXFFXTRXTGE) that has been recognized to be a peptidase involved in the cleavage of a double glycin motif during the secretion of class II bacteriocins was found in the N-terminal region of the transport proteins. The alignment of the amino acid sequences of all the transport proteins indicated that their sequences were 54.83% identical (Table 4).

Additionally, the precursor peptides contained a conserved domain (YGNGVXCXXXXCXVXWXXA) belonging to the class IIa bacteriocins. These peptides are often referred to as pediocin-

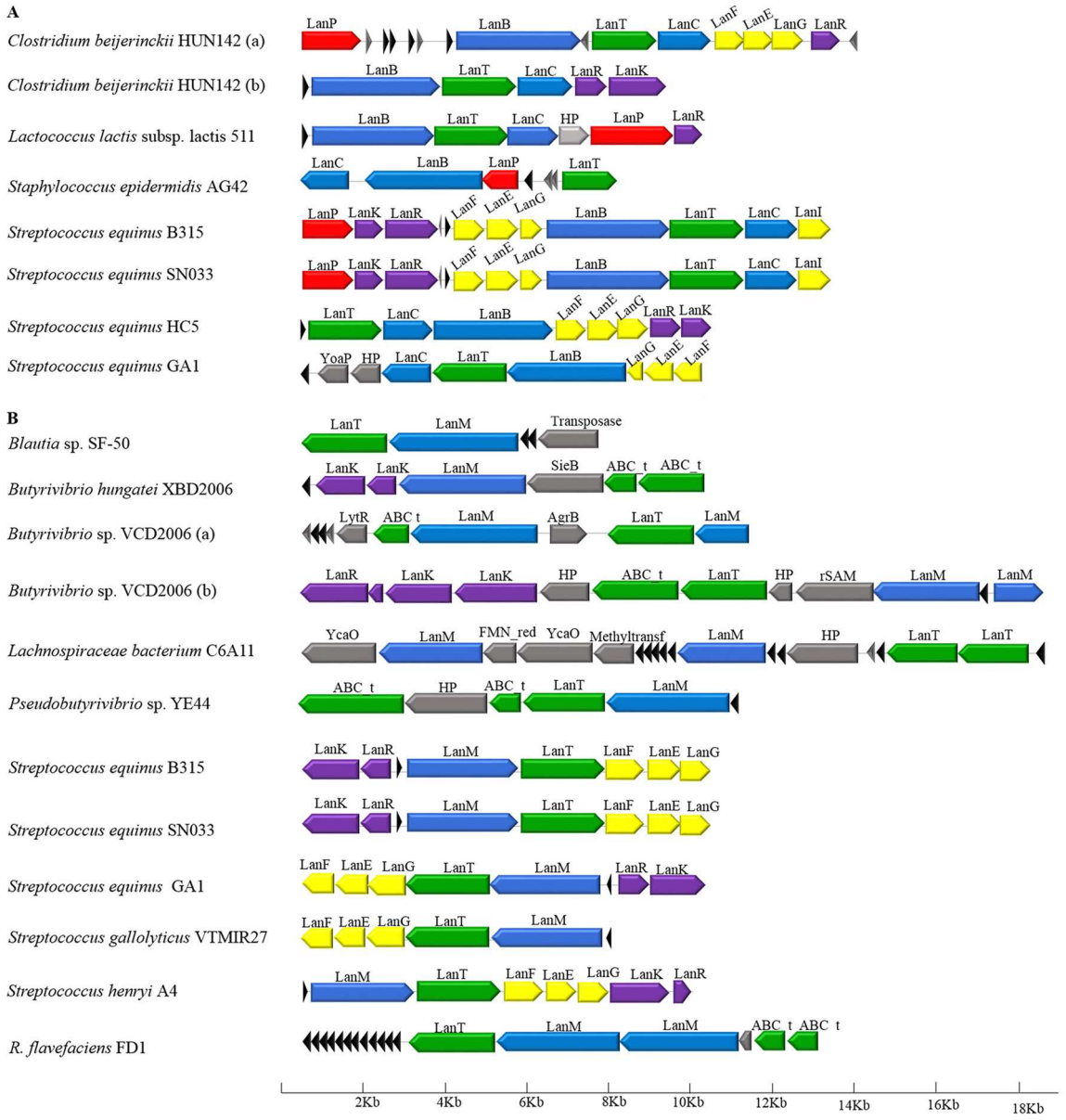

FIG 3 Biosynthetic gene clusters of lanthipeptides found in ruminal bacteria. The gene clusters of the lanthipeptides were classified into class I (A) or class II (B) on the basis of the presence of biosynthetic enzymes involved in thioether cross-links. Black, structural peptides; green with LanT, LanT (processing and transport); blue, modification enzymes (LanB, LanC and LanM); red, LanP (protease); purple, regulatory genes; yellow, immunity genes; green with ABC t, ABC transporters; gray, undefined proteins; Methyltransf, methyltransferase.

like bacteriocins after pediocin PA-1, the first member of this group to be characterized. All precursor peptides identified in the *Streptococcus* cluster had the conserved region EQFXXMTXXXLS XVXGG (Table 4).

More than one putative structural gene was found in *Dorea longicatena* AGR2136, *Lactobacillus plantarum* AG30, *Streptococcus gallolyticus* VTM1R27, and *S. equinus* HC5. The leader peptide in the *S. equinus* HC5 strain showed a high degree of conservation with bovicin 255, a class II bacteriocin produced by *S. gallolyticus* LRC0255.

Our results also suggest that the class II bacteriocins produced by ruminal bacteria are regulated by two-component systems, as histidine kinases and response regulators were found to be associated with some of the putative gene clusters involved in bacteriocin production (Fig. 4).

Class III bacteriocins are highly conserved in the genomes of ruminal bacteria. A total of 35 structural genes were identified as being related to the production of class III bacteriocins in ruminal bacteria (Table 1). However, most of the putative proteins encoded by these genes ($n = 25$) showed amino acid sequence identities with other known class III bacteriocins of less than 20% and were analyzed separately from albusin B-like proteins in this study. All these bacteriocins had functional domains that were homologous to the metalloproteases of the M23 family. These proteins also showed an N-terminal catalytic domain possessing endopeptidase activity and a C-terminal cell wall recognition do-

TABLE 3 Conserved domains of the putative lanthipeptide biosynthetic proteins found in ruminal bacteria

Subtype and Lan protein	Size (no. of aa)	Conserved domain	Motif(s)[a]	Alignment start position[b]	Frequency[c] (%)
Subtype I					
LanA	56–57	TIGR03731 (gallidermin/nisin family)	FNLD	6–8	50
	60	pfam08130 (type A lantibiotic family)			12.5
	43–51	ND[d]			37.5
LanB	967–1042	pfam04738 (lantibiotic dehydratase)	RXTPFG	76–95	100
		—[e]	RYGG	816–866	100
		—	NRLXGI	936–1011	100
LanC	398–444	pfam05147 (LanC-like)	RDAWCYGGP	261–296	100
			CHGYXG	323–346	100
LanT	512–602	COG1132 (ABC-type multidrug)	GXXGSGKST	365–392	100
		—	LSGGQWQT	470–496	
LanP	285–669	cd07482 (peptidase S8 family lantibiotic)			50
	416–669	pfam00082 (subtilase family)			12.5
		—			37.5
LanE	224–250	TIGR0373 (MutE/EpiE family)			62.5
		—			37.5
LanF	225–233	TIGR03740 (gallidermin-lantibiotic protection)	NGAGKST	22–47	62.5
		—	FSLGMKQRLG	112–137	
		—	LLILDEPTNGLDPI	132–157	
		—			37.5
LanG	236–245	TIGR03733 (MutG family)			25
	165–216	ND			37.5
		—			37.5
LanK	300–468	COG0642 (histidine kinase)			75
		—			25
LanR	227–253	COG0745 (response regulators)			75
		—			25
Subtype II					
LanA	53–67	pfam04604 (type A lantibiotic)			441.6
	57–69	Pfam (lantibiotic alpha)			88.3
	56–66	ND			550.1
LanM	411–1050	TIGR03897 (type 2 lantibiotic biosynthesis)			1100
LanT	647–744	COG2274 (ABC-type lantibiotic exporters)	GXSGSGKST	365–392	991.6
	265	COG1136 (ABC-type antimicrobial peptide)			88.4
LanE	207–249	pfam12730 (ABC-2 family transporter)			4.,6
		—			58.4
LanF	225–233	TIGR03740 (gallidermin-lantibiotic protection)	NGAGKST	22–47	41.6
		—	FSLGMKQRLG	112–137	
		—	LLILDEPTNGLDPI	132–157	
		—			58.4
LanG	180–253	pfam12730 (ABC-2 family transporter)			41.6
		—			58.4

[a] X, any amino acid.
[b] Position in the protein where the conserved motif starts.
[c] Frequency refers to the proportion of the time that clusters containing the specified protein with a particular domain were identified in this study.
[d] ND, not determined.
[e] —, undefined protein.

main that confers target specificity (10). Functional annotation by clusters of orthologous groups (COG) further indicated that these proteins were produced by different strains ($n = 16$) of *Butyrivibrio*. The alignment of the amino acid sequences from all 10 of these proteins found in *Butyrivibrio* showed an identity as high as 72.3% (data not shown). However, when these proteins were compared with other known bacteriolysins belonging to the subfamily M23B (e.g., lysostaphin, zoocin A, enterolysin A, and helveticin J), sequence identity was always less than 13.0%.

Manual analysis of the structural genes encoding class III bacteriocins using the BLASTP and DNAman programs revealed the presence of putative genes in five strains of *R. albus*, one strain of *Wolinella succinogenes*, one strain of *Enterococcus faecalis*, one strain of a *Lachnospiraceae* bacterium, and one strain of *Lactococcus lactis* (Fig. 5). The amino acid sequence of albusin B from *R. albus* 7 has been described (29), and linocin was previously reported to be a putative class III bacteriocin produced by *W. succinogenes* DSM 1740, as determined using *in silico* analysis (30). Nonetheless, the analysis of the *R. albus* 7 genome revealed the genetic organization of the albusin B gene cluster, demonstrating the presence of an ABC transporter adjacent to albusin B and two ORFs encoding putative transposases related to the IS*1595* and

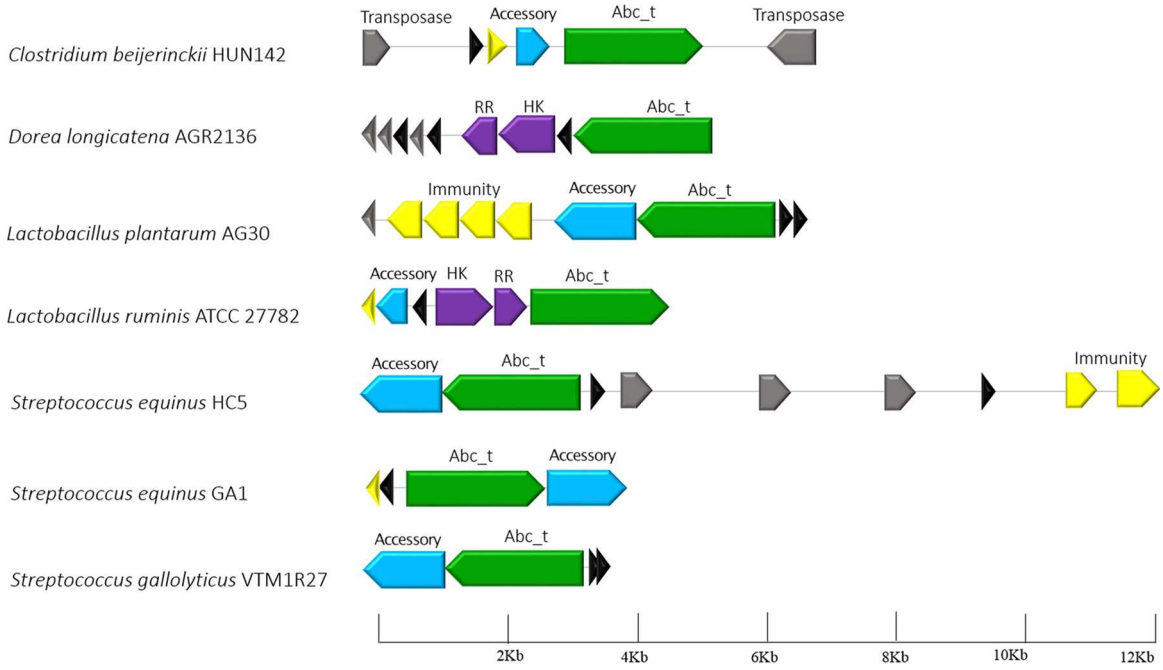

FIG 4 Diversity of class II biosynthetic gene clusters among different species of ruminal bacteria. Black, precursor peptides; green, ABC transporter; purple, regulatory genes; yellow, immunity genes; gray, adjacent ORFs.

IS*66* families. When the amino acid sequences of the putative class III bacteriocins produced by *R. albus* were aligned, an identity as high as 80.4% was obtained (Fig. 6A), indicating that the structural gene is highly conserved among strains of this bacterial species. In fact, the putative class III bacteriocins in *R. albus* AD2013 and *R. albus* SY3 had identical amino acid sequences.

The genome sequence of *E. faecalis* 68A contained an ORF with 100% identity to the enterolysin produced by *E. faecalis* LMG 2333, while *L. lactis* subsp. *lactis* 511 had an ORF with only 30.9% identity to enterolysin. The alignment of these sequences indicated a consensus region in the C-terminal portion of these proteins (Fig. 6B).

DISCUSSION

Most studies demonstrating the inhibitory effects between a producer and a target organism are based on agar plate diffusion assays or direct assays, such as the spot-on-lawn procedure (11). These *in vitro* methods are very useful for assessment of the diversity of inhibitors produced by culturable microorganisms and will certainly continue to be applied for the identification of novel antimicrobial agents of microbial origin (31). However, these methods usually do not discriminate the inhibition caused by bacteriocins, low-molecular-weight antibiotics, bacteriophages, lytic enzymes, and metabolic by-products, such as organic acids and hydrogen peroxide, among others. Additionally, the ability to produce antimicrobial agents *in vitro* can be affected, for example, by the medium composition (32), stress conditions (33), and the presence of target cells (34). Therefore, false-negative results can be obtained if the conditions of incubation are not adequate for the production of these antimicrobial compounds by producer strains.

Besides all the efforts to characterize bacteriocins produced by ruminal bacteria, until now only three lantibiotics (butyrivibriocin OR79A, butyrivibriocin AR10, bovicin HC5), four class II bacteriocins (lichenin, bovicin 255, enterocin BC25, enterocin CCM4231), and two class III bacteriocins (enterolysin, albusin B) have been identified or at least partially characterized at the biochemical and genetic levels among strains of ruminal bacteria (10, 15, 16, 18, 19, 21, 35–37). Moreover, although the occurrence of

TABLE 4 Comparison of the class II biosynthetic gene clusters with the domains of each candidate protein

Protein	Size (no. of aa)	Conserved domain	Motif[a]	Alignment start position[b]	Frequency[c] (%)
Precursor peptide	67–85	pfam10439 (bacteriocin class II)	EQFXXMTXXXLSXVXGG	5–21	42.8
	59–62	pfam01721 (class II bacteriocin)	YGNGVXCXXXXCXVXWXXA	18–21	28.5
ABC transporter	689–742	COG2274 (ABC-type bacteriocin/lantibiotic exporters)	LPXXFFXTRXTGE	250–252	100
Accessory protein	458–549	TIGR01000 (bacteriocin secretion accessory protein)			42.8
	159–172	TIGR01295 (bacteriocin transport accessory protein)			28.5
Immunity	95–110	pfam08951 (enterocin A immunity)			42.8

[a] X, any amino acid.
[b] Position in the protein where the conserved motif starts.
[c] Frequency refers to the proportion of the time that clusters containing the specified protein with a particular domain were identified in this study.

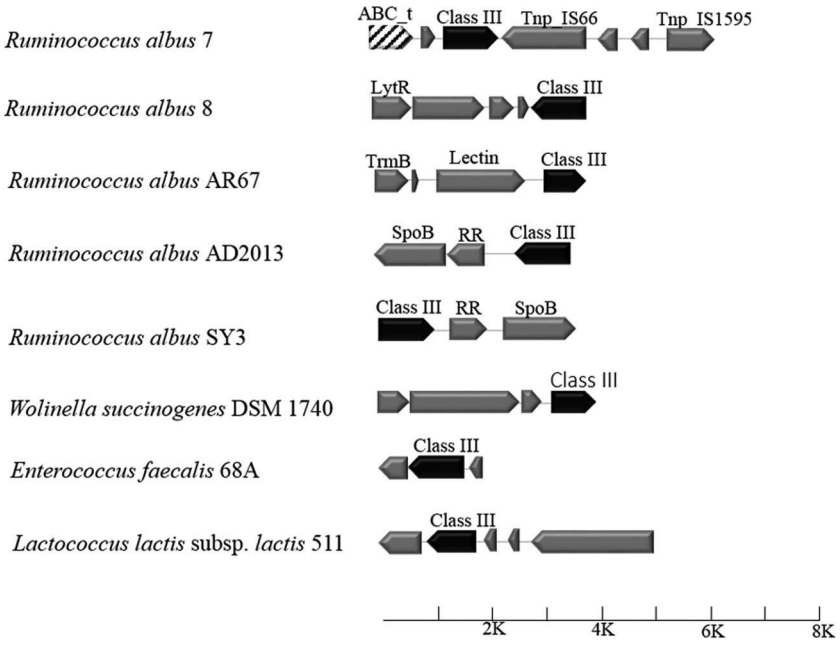

FIG 5 Diagrammatic representation of the class III bacteriocin gene clusters found in ruminal bacteria. Arrow with hatched lines, genes involved in transport; black arrows, structural genes; gray arrows, genes unrelated to the biosynthesis of bacteriocins.

antimicrobial activity among different strains of ruminal bacteria has been demonstrated by *in vitro* screening methods (4), the rumen microbiota is still underexplored as a potential source of antimicrobial peptides.

In this study, 46 new putative bacteriocin structural genes or gene clusters were identified in the genome sequences of 229 ruminal bacteria and archaea. In previous *in vitro* and *in silico* studies, only 6 strains out of the 229 strains (2.6%) investigated in this work had their antimicrobial activity demonstrated *in vitro* (*Lactobacillus ruminis* ATCC 27782, *R. albus* 7, *R. albus* 8, and *S. equinus* HC5) or were reported to contain putative genes related to bacteriocin production (*R. flavefaciens* FD1 and *W. succinogenes* DSM 1740) (16, 20, 38, 39).

Eleven novel sactipeptide gene clusters which had not previously been associated with sactipeptide production were identified in different bacterial genera, including *Blautia*, *Clostridium*, *Kandleria*, *Lachnobacterium*, *Peptostreptococcus*, and *Ruminococcus*. To our knowledge, *Bacillus subtilis* and *Bacillus thuringiensis* strains isolated from soil (40) and human feces (41), respectively, were the only bacterial species in which the production of sactibiotics have been demonstrated *in vitro*. The sactibiotics (sulfur-to-alpha-carbon antibiotics) are posttranslationally modified antimicrobial peptides containing intramolecular linkages between cysteine residues and an alpha carbon of another amino acid residue located on the opposite side of the peptide. These cross-links are catalyzed by rSAM enzymes that cleave the radical *S*-adenosylmethionine (8, 40). In order to distinguish the rSAM proteins that are involved in the maturation of bacteriocins from rSAM proteins participating in other biosynthetic pathways (e.g., cofactors, methyltransferases, modifications of ribosomal proteins), the criteria proposed by Haft and Basu (42) were applied to identify potential sactipeptide-encoding genes in the genome sequences of ruminal bacteria. Therefore, only gene clusters showing one or more precursor peptides containing cysteine residues as well as an ABC-type bacteriocin transport system, in addition to the rSAM proteins, were considered potential biosynthetic gene clusters encoding sactipeptides. Most gene clusters harbored two rSAM proteins, a feature that has been associated with the processing of two or more structural genes encoding sactipeptides (8). Moreover, our *in silico* analysis revealed the presence of multiple precursor peptides in the sactipeptide gene clusters found in *B. schinkii* DSM 10518, *R. albus* AR67, and *R. albus* DSM 20455, a feature that has been used as a genotypic trait to define sactipeptide-producing systems in complete and draft genomes of bacteria (42). The alignment of the rSAM enzymes of the sactipeptide gene clusters identified in this work showed the same conserved motif (YGGEPL) identified by Murphy and colleagues (43) in 16 clusters homologous to TrnC during genome mining of the GenBank database for sactibiotic-like gene clusters.

Although our initial screening of genomic databases indicated 68 gene clusters potentially associated with sactipeptide production in the genomes of ruminal bacteria, 13 of these clusters contained structural genes encoding putative peptides with six cysteine residues and sizes ranging from 45 to 49 amino acid residues. The alignment of these precursor peptides revealed a consensus sequence (SCQSACKTSC) toward its C-terminal region (see Fig. S1 in the supplemental material) that had not been previously reported. Although this conserved region is identical to the SCIFF (six Cys in forty-five) system frequently found in *Clostridium* strains (42), in this study SCIFF peptides were not considered sactipeptides, as dedicated ABC transporters were never found within these gene clusters. Considering that rSAM proteins can account for up to 0.5% of the total protein content of anaerobic bacteria and that diverse functions have been assigned to these enzymes (42), the prevalence of sactipeptides among ruminal bacteria could be much greater than that predicted by computational analysis.

Lantibiotics have been identified in strains of *Butyrivibrio fi-*

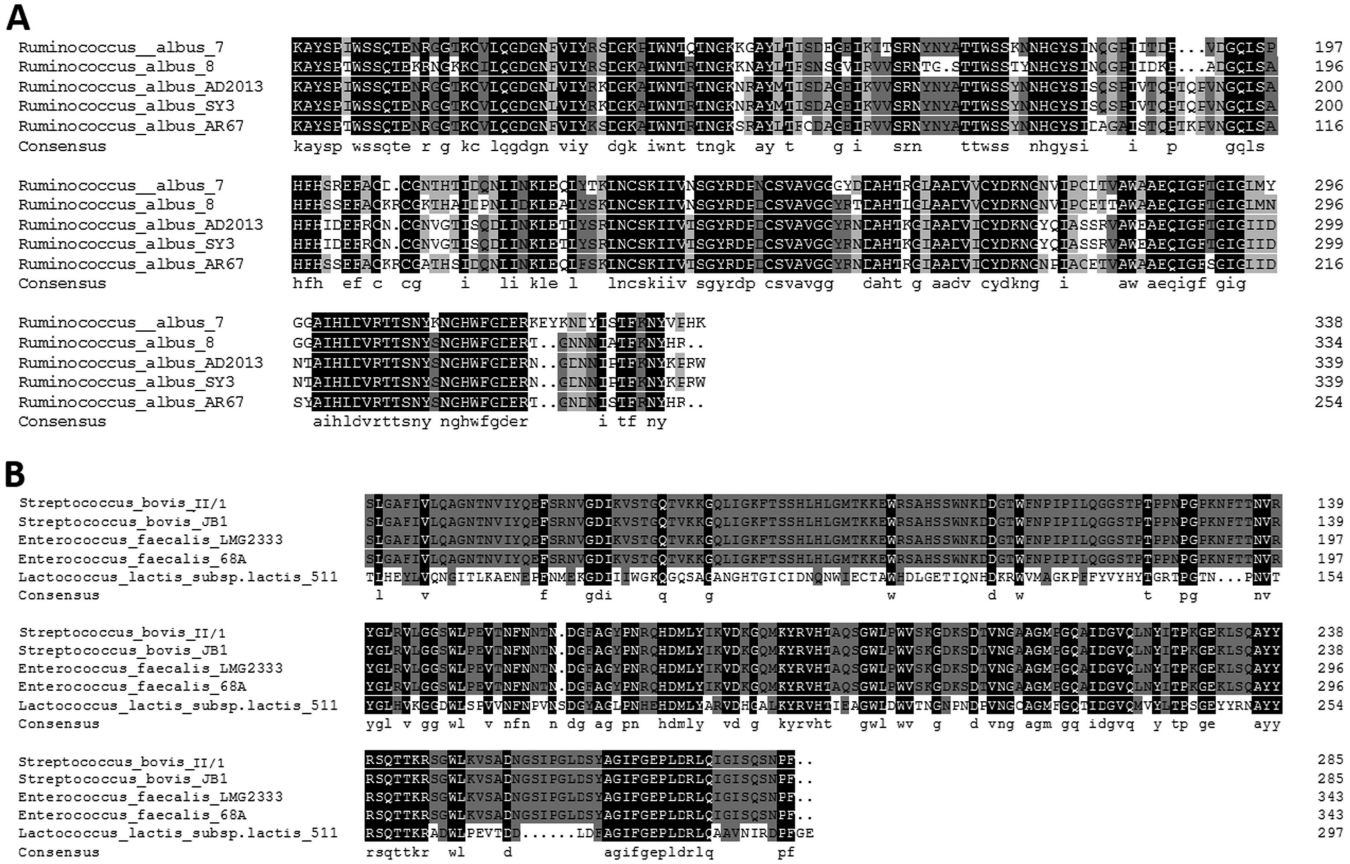

FIG 6 Multiple-sequence alignments of the class III bacteriocins identified in ruminal bacteria. (A) Amino acid sequence alignments of albusin-like proteins of *R. albus* strains showing the consensus regions. (B) Amino acid sequence alignments of enterolysin A-like proteins identified in ruminal bacteria with enterolysin A of *E. faecalis* LMG2333 (GenBank accession number AAG29099) and streptococcin II/1 of *Streptococcus bovis* II/1 (GenBank accession number DQ090994.1). Black background, consensus regions; light gray background, amino acid sequence similarity greater than 50%; dark gray background, amino acid sequence similarity greater than 75%; dots, gaps introduced to improve sequence alignment.

brisolvens (butyrivibriocin OR79A) and *S. equinus* (bovicin HC5 and bovicin Sb15) isolated from the rumen (17, 20, 22). The potential application of bovicin HC5 to manipulate ruminal fermentation was demonstrated *in vitro*. When mixed cultures of ruminal bacteria were incubated with H_2 and CO_2 in the presence of increasing concentrations of bovicin HC5, methane production was inhibited up to 50% (57). Additionally, Lima et al. (44) verified that bovicin HC5 extracts (50 activity units ml^{-1}) reduced amino acid degradation by rumen microorganisms to an extent similar to that of the ionophore antibiotic monensin. These results suggest that antimicrobial peptides could also be useful *in vivo* to protect dietary protein passing through the rumen.

The identification of biosynthetic gene clusters encoding lanthipeptides was facilitated by the fact that several genes involved in lantibiotic biosynthesis, such as LanC, LanM, and LanT, have a relatively conserved nature (45–47). These characteristics have been incorporated into antiSMASH (version 3) software, which combines analysis for signature motifs for biosynthetic enzymes and analysis for lanthipeptide-specific cleavage site motifs to identify gene clusters encoding lanthipeptides in genomic sequences (48). The majority of the gene clusters encoding lanthipeptides that were initially identified using BAGEL3 or antiSMASH software were also confirmed when individual ORFs were subjected to BLAST analysis.

Both class I and class II lanthipeptides were identified in the genomes of ruminal bacteria. Biosynthetic gene clusters encoding class I lanthipeptides typically have two enzymes (LanB and LanC) involved in posttranslational modification, while the gene clusters encoding class II lanthipeptides depend on a multifunctional enzyme (LanM) for the dehydration and cyclization reactions of the precursor peptides (45). The precursor peptides modified by the LanBC enzymes often have a conserved proline at position −2 relative to the cleavage site and an FNLD motif at about positions −20 and −15 in the leader peptide, and these play crucial roles in the posttranslational modification of the antimicrobial peptide (49). In our study, only the putative precursor peptides produced by *S. equinus* strains demonstrated the presence of the FNLD motif, in addition to a proline in the −2 position of the leader peptide. On the other hand, putative precursor peptides encoded by other species of ruminal bacteria showed an FDLD motif and a proline in the −2 position of the leader peptide. In this work, LanP was identified only in lanthipeptide gene clusters belonging to class I. LanP is a membrane-bound extracellular protease which cleaves the N terminus of the precursor peptide on the P(QRS) motif (49). This motif was found in precursor peptides of the class I lanthipeptides of all ruminal bacteria harboring the protein LanP, except for *S. equinus* GA1. On the other hand, biosynthetic gene clusters encoding class II lanthipeptides had a bifunctional protein (LanT)

involved in the processing and transport of lanthipeptides containing a double glycin motif (49).

Some gene clusters associated with the production of class II lanthipeptides in ruminal bacteria contained more than one precursor peptide and up to two putative LanM enzymes. In previous studies, it was demonstrated that the low sequence identity between the precursor peptides of lichenicidin and haloduracin was related to the presence of two separate LanM enzymes in the lantibiotic gene cluster (50, 51). In this work, *Butyrivibrio* sp. strain VCD2006 showed three precursor peptides with 12.1% sequence identity, while *Lachnospiraceae* bacterium C6A11 contained nine precursor peptides sharing 10.25% sequence identity. In both organisms the gene clusters associated with the production of class II lanthipeptides contained two distinct LanM enzymes. In contrast, *Blautia* sp. SF-50 harbors two putative precursor peptides with 66% sequence identity, and only one LanM enzyme was found in its class II lanthipeptide gene cluster.

Until now, the majority of the class II bacteriocins reported for ruminal bacteria have been shown to be produced by Firmicutes, especially bacteria of the orders *Lactobacillales* and *Clostridiales* (9). In this study, we have identified two new peptides belonging to class IIa bacteriocins. These pediocin-like peptides are known to contain an N-terminal consensus sequence (YGNGVXCXXXX CXVXWXXA) that was also found in our *in silico* screening of the genome sequence of *C. beijerinckii* HUN142 and *L. ruminis* ATCC 27782. This N-terminal consensus region is important for the physical interaction of the peptide with the extracellular loop of the Man-phosphotransferase IIC protein, involved in the transport and phosphorylation of mannose and other sugars through the cytoplasmic membrane (9). Additionally, two genes potentially associated with the production of class II bacteriocins were identified in the genomes of *D. longicatena* AGR2136 and *S. equinus* HC5. *D. longicatena* AGR2136 and *S. equinus* HC5 contained more than one putative class II precursor peptide, sharing between them 30.52% and 33.83% amino acid sequence identities, respectively. Even though it is conceivable that these peptides might belong to class IIb, in which the complementary activity of two peptides is required for full inhibition of target organisms, additional structural and biochemical data will be required to accurately assign these peptides to a particular subgroup of the class II bacteriocins.

Several approaches have been used to classify bacteriocins on the basis of their biochemical properties, molecular sizes, and modes of action. Although these classification schemes have been well established and accepted for low-molecular weight antimicrobial peptides, the same is not true regarding the classification of large proteins (>10 kDa) showing antimicrobial activity, such as bacteriolysins (6, 52).

Bacteriolysins are modular proteins with an N-terminal catalytic domain that shows homology to endopeptidases and a C-terminal domain that contains the target recognition site. These cell wall-active enzymes are often produced when the culture enters the stationary phase, which is possibly advantageous for the producer strain to compete for available resources (10). Class III bacteriocins also show great structural diversity, and comparison of the amino acid sequences of enterolysin A, helveticin J, lysostaphin, and zoocin A demonstrated that identity was never greater than 16%. Nonetheless, within a single bacterial species or genus, the degree of sequence conservation can be much higher. For example, our *in silico* analysis indicated that the genes encoding putative class III bacteriocins in strains of *Butyrivibrio* and *Ruminococcus* had identities as high as 77.75% and 71.24%, respectively. Although experimental work and biochemical characterization are needed to confirm the production of class III bacteriocins in *Butyrivibrio*, to our knowledge, this is the first report of gene clusters associated with class III bacteriocin production in this bacterial genus. Because these proteins identified in *Butyrivibrio* appear to be a distinct group of class III bacteriocins, a low level of identity of the amino acid sequence with those of other previously reported bacteriolysins was found.

The genomes of five strains of *R. albus* encoded putative class III bacteriocins that showed high degrees of homology to albusin B. Previous work reported the production of albusin B by *R. albus* 7 and *R. albus* 8, and two new *R. albus* strains whose genomes encode albusin-like bacteriocins were identified in this study. Albusin B was initially described to be a relatively hydrophilic, heat-labile, high-molecular-mass (>30 kDa) protein showing a narrow range of antibacterial activity (29). The putative class III bacteriocins in *R. albus* AD2013 and *R. albus* SY3 had identical amino acid sequences. *R. albus* strain SY3 was isolated from anaerobic cellulose roll tubes (53), and *R. albus* AD2013 was obtained from the rumen of a New Zealand cow as part of a study to isolate previously uncultured ruminal bacteria (54).

When genes encoding albusin homologues were subjected to BLASTp analysis, a relatively high degree of identity with sequences of peptidases belonging to the M15 family was found. Family M15 includes predominantly D-Ala–D-Ala carboxypeptidases, which are important for processing the precursor of the cross-linking peptide of the bacterial cell wall. Although the factors affecting the production of albusin B by *R. albus* have been studied, much less regarding the mechanisms of action of these bacteriolysins is understood. Nonetheless, when recombinant *Saccharomyces cerevisiae* DBY 747 expressing albusin B of *R. albus* 7 was fed (at 2.5 g kg of body weight^{-1}) to broiler chickens, an improvement of growth performance was observed, suggesting that ruminal bacteriocins could have a role as potential alternatives to the growth promoters used in livestock production (55).

Our study also demonstrated that *E. faecalis* 68A, isolated from the ovine rumen, has a putative class III bacteriocin with 100% amino acid sequence identity with the amino acid sequence of enterolysin A of *E. faecalis* LMG2333. Nigutova et al. (56) first detected the presence of enterolysin homologues in 5 of the 33 isolates of *E. faecalis* isolated from the bovine rumen using primers designed to amplify the structural gene encoding enterolysin A.

In conclusion, novel classes of antimicrobial peptides were described for the first time among strains of ruminal bacteria. Bacteriocin production appears to be a widespread ability among *Butyrivibrio*, *Ruminococcus*, and *Streptococcus* strains. Further studies focused on the structural and biochemical characterization of these antimicrobial peptides might be useful to understand their spectrum of activity and mode of action and to develop new strategies to manipulate ruminal fermentation and improve ruminant productivity. These results demonstrate that the *in silico* exploration of potential producers of antimicrobial peptides among bacteria from complex ecosystems, such as the rumen, may be a promising and complementary strategy alongside traditional screening methods to discover novel antimicrobial agents with potential applications in agriculture, industry, and human or animal therapy. The information provided in this study will also be useful in gaining further information on the mechanism involved

in bacterial competition and bacteriocin production in the rumen ecosystem.

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