The use of PCR for the identification and characterisation of bacteriocin genes from bacterial strains isolated from rumen or caecal contents of cattle and sheep

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Abstract

PCR primers were designed to amplify the gene that encodes bovicin 255 from Streptococcus galloyticus LRC0255 and the bacteriocin genes from Butyrivibrio fibrisolvens strains AR10 and OR79A (bviD and bvi79A) in order to screen for their incidence in rumen and caecal B. fibrisolvens and Streptococcus bovis-like isolates from New Zealand and North American ruminants. None of the B. fibrisolvens-like strains (n = 34) isolated from New Zealand or North America had the genes encoding for butyrivibriocins AR10 (bviD) or OR79 (bvi79A). However, seven S. bovis isolates from New Zealand ruminants and three from North American animals had the bovicin 255 gene. Sequence comparison of cloned bovicin 255 PCR products indicated a 92.9–95.7% similarity to that of the corresponding bovicin 255 gene sequence of S. galloyticus. Four of the New Zealand bovicin 255 positive S. bovis isolates were from the caecal contents of the same sheep and had identical PFGE profiles. Two other S. bovis isolates sharing the same PFGE profile were isolated from a separate animal from the same flock. PFGE analysis of the North American strains indicated that all three were closely related as two of three had identical PFGE profiles with the remaining isolate differing only by a single band position. The 16S rRNA gene sequences of the 10 isolates were at least 99.8% identical to S. bovis. All 10 S. bovis isolates having the gene for bovicin 255 produced bacteriocin activity that inhibited the growth of Peptostreptococcus anaerobius D1 in a deferred antagonism plating (DAP) assay. Certain S. bovis isolates obtained from ruminants have bacteriocin activity associated with a distinct bovicin 255 gene sequence but it appears that bacteriocin production by the rumen anaerobe B. fibrisolvens may be uncommon in strains isolated from cattle and sheep in New Zealand.

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Keywords: Bacteriocin; Streptococcus bovis; Butyrivibrio fibrisolvens; Bovicin 255; Rumen; Caecum

1. Introduction

The ruminant ecosystem is a complex and diverse environment where symbiotic bacteria, fungi and protozoa interact with the host in a dynamic relationship to degrade plant material by anaerobic fermentation [1]. The animal provides the microorganisms with a habitat for their growth whilst the microorganisms provide the animal with fermentation acids, microbial protein and vitamins.

The microbial environment within the rumen may be regulated, in part, by the expression of proteaceous antimicrobial agents such as bacteriocins. These molecules are a heterogeneous group of peptides and proteins that are characterised by their ability to inhibit closely and sometimes more distantly related strains of bacteria [2–5], thereby potentially playing a key role in the bacterial population dynamics within the rumen.
HC5, a bacteriocin isolated from S. bovis Lactococcus lactis non-lantibiotic bacteriocins such as lactococcin A of processing site [9]. It shares homology with class II from a 8.5 kDa prepeptide that contains a protease LRC0255, has an active peptide of 5.97 kDa processed vicin 255, isolated from Streptococcus gallolyticus Streptococcus gallolyticus ria, such as produce bacteriocins [8].

Several commonly isolated species of rumen bacteria, such as Streptococcus gallolyticus [9], Streptococcus bovis [10], Butyrivibrio fibrisolvens [6,7,11], Rumino-ococcus albus [12] and Enterococcus faecium [13–16], produce bacteriocins, but their effect on rumen ecology has not been clearly defined. S. bovis is a rapidly growing, acid-tolerant, gram-positive bacterium that ferments starch to lactic acid. Excessive growth of this species may lead to lactic acidosis in the rumen of cattle and sheep fed excess starch [17]. Two inhibitory molecules, bovicin 255 [9] and bovicin HC5 [10], have been identified from rumen Streptococcus species. Bovicin 255, isolated from Streptococcus gallolyticus LRC0255, has an active peptide of 5.97 kDa processed from a 8.5 kDa prepeptide that contains a protease processing site [9]. It shares homology with class II non-lantibiotic bacteriocins such as lacticoccin A of Lactococcus lactis [18]. More recently, the bovicin HC5, a bacteriocin isolated from S. bovis HC5, has been shown to have a broader antibacterial spectrum than bovicin 255 [10,19]. Initial analysis indicated that the active peptide was 2.44 kDa [10] and that the only other bacteriocin that had significant similarity was the type A1 lantibiotic streptin (srtA) of Streptococcus pyogenes [20].

Butyrivibrio fibrisolvens is a frequently isolated member of the obligately anaerobic bacterial population of the rumen and is important for fibre digestion with most strains degrading hemicellulose, xylans, pectin and starch [17]. Several studies indicate that bacteriocin-like activity is a common characteristic of B. fibrisolvens [6,7] and inhibitory molecules from B. fibrisolvens AR10 [7,21] and OR79 [11,22] have been purified and characterised. The inhibitors identified were small hydrophobic peptides similar to a lantibiotic (butyrivibriocin OR79A) and non-lantibiotic peptide (butyrivibriocin AR10) [7,11,21]. The structural gene encoding butyrivibriocin OR79 (bvi79A) produced a prepeptide of 47 amino acids and a mature peptide, butyrivibriocin OR79A, of 25 amino acids [11]. The prepeptide had significant homology to other lantibiotics which contain a double glycine leader peptidase cleavage site, such as variacin from Micrococcus vari- ans [23] and lacticin 481 from L. lactis [24]. The active form of butyrivibriocin AR10 was determined to be approximately 3.9 kDa [7]. This inhibitor molecule demonstrated significant homology to the bacteriocin acidocin B from Lactobacillus acidophilus [25]. Both these butyrivibriocins were able to inhibit a variety of gram-positive ruminal bacteria including Ruminococcus spp., S. bovis, other B. fibrisolvens and listerial food isolates [6,7,11,21].

In this study we used the DNA sequences of the genes encoding butyrivibriocins OR79A and AR10 and bov- icin 255 to design primers for PCR screening of rumen S. bovis, B. fibrisolvens, or other similar bacterial isolates from cattle and sheep in New Zealand [26] for the presence of butyrivibriocins OR79A and AR10 and bovicin 255.

2. Materials and methods

2.1. Bacterial strains and media

Streptococcus gallolyticus LRC0255 was originally isolated from the rumen of a moose in Alberta, Canada [9] B. fibrisolvens OR79 was originally isolated from the rumen of a dairy cow [11] and B. fibrisolvens AR10 was originally isolated from the rumen of a sheep in Australia [7]. All three strains were kindly supplied by R.M. Teather (Lethbridge Research Centre, Alberta, Canada). Peptostreptococcus anaerobius D1 [27] was obtained from the Rumen Microbiology Culture Collection at the AgResearch Grasslands Research Centre, Palmerston North, New Zealand. CC, L10 and RX media were prepared as described previ- ously [28–30]. HAP medium was the same as the basal medium of Chen and Russell [31] with minor modifi- cations [27]. Purified agar (1.5% or 0.75% [w/v]; Difco) was used to make agar plates or top agar overlays respectively.

Rumen samples were collected from fistulated ani- mals as described previously [27], or from the caecal contents of animals post mortem. One millilitre of sample was diluted anaerobically in RX medium (without xylose), serially diluted in both CC and RX broths and incubated at 39 °C in an anaerobic glove box (90% CO₂, 10% H₂). Individual colonies were purified by repeated streaking onto fresh agar plates and colony purity was confirmed by Gram stain and microscopic examination. Presumptive identification of isolates as B. fibrisolvens or S. bovis was as described previously.
2.3. Phylogenetic analysis

Library under Accession Nos. AY338260 to AY338269. PCR products have been deposited in the GenBank data Inc., MD, USA). The DNA sequences of the bovicin 255 using the AlignX programme (Vector NTI, InforMax Biosystems), using M13 Forward (BigDye Terminator v.3.1 Cycle Sequencing kit (Applied manufacturer’s instructions and sequenced using the (Invitrogen, Auckland, New Zealand) according to Rockland, ME, USA).

Gel-Star (Bio-Whittaker Molecular Applications, gels (1.5%) after staining with ethidium bromide or /C176 at 4°

S. gallolyticus from late and aligned with the bovicin 255 gene sequence verse primers. Two clones were sequenced for each iso-

2.2. PCR and sequencing of bacteriocin genes

A single well-spaced colony of the strain to be tested was resuspended in 50 μl sterile water. Template DNA was prepared from this sample by placing in a vigorously boiling water bath for 5 min and placing on ice until required. PCR primers (Table 1) were diluted to a final concentration of 5 pM μl−1. The PCR conditions were an initial denaturation step (94 °C, 5 min) followed by 30 cycles of denaturation (94 °C, 30 s), annealing (52 °C, 30 s) and extension (72 °C, 30 s) in a FTS-320 Thermal Sequencer (Corbett Research) using 23.5 μl Platinum PCR Supermix (Invitrogen), 0.5 μl each of forward and reverse primers and 0.5 μl of template DNA. After thermal extension (72 °C, 5 min), samples were kept at 4 °C. The PCR products were analysed on agarose gels (1.5%) after staining with ethidium bromide or Gel-Star (Bio-Whittaker Molecular Applications, Rockland, ME, USA).

PCR products were cloned into the pCR2.1 vector (Invitrogen, Auckland, New Zealand) according to manufacturer’s instructions and sequenced using the BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems), using M13 Forward (−20) and M13 Reverse primers. Two clones were sequenced for each isolate and aligned with the bovicin 255 gene sequence from S. gallolyticus LRC0255 retrieved from GenBank, using the AlignX programme (Vector NTI, InforMax Inc., MD, USA). The DNA sequences of the bovicin 255 PCR products have been deposited in the GenBank data library under Accession Nos. AY338260 to AY338269.

2.3. Phylogenetic analysis

Eubacterial primers fD1 and rD1 (5 pM μl−1) [33] were used to amplify the 16S rRNA genes from bovicin 255 PCR positive S. bovis-like isolates (Table 1). The PCR conditions were 30 s at 94 °C, 30 s at 57 °C and 1 min at 72 °C for 30 cycles with template preparation, initial denaturation and final extension steps as described above. PCR products were cloned and sequenced as described above using fD1, rD1, F1 and R5 primers and aligned with other related 16S rRNA gene sequences retrieved from GenBank.

2.4. Pulsed-field gel electrophoresis

The procedure used to prepare genomic DNA in agarose blocks for pulsed-field gel electrophoresis has been described previously [34]. DNA embedded in agarose was digested with Smal (New England Biolabs, Beverly, MA), loaded into the wells of 1% agarose gels (pulsed field certified agarose, Bio-Rad Laboratories, Hercules, CA), and run at 5.0 V cm−1 for 20 h at 14 °C in 0.5× Tris–borate buffer using a CHEF DR III PFGE apparatus cooled using a model 1000 mini chiller (Bio-Rad).

2.5. Deferred antagonism plating assay

Screening of bovicin 255 positive S. bovis isolates for the production of inhibitory molecules was performed using the deferred antagonism plating (DAP) assay [7,35]. Strains to be tested for inhibitor production were spotted onto L10 or CC plates and incubated anaerobically at 39 °C for 24 h. The plates were removed from the anaerobic chamber and all bacterial growth was scraped off the agar plate using a sterile spreader. Excess bacterial debris was washed off using 2 ml sterile water. The plate was then inverted (5 min) over a filter paper disc that had been bathed in chloroform to sterilise the surface of the agar. Excess chloroform vapour was then allowed to dissipate (5 min) before the agar plate was re-introduced to the anaerobic chamber to remove oxygen (5 h). Molten HAP top agar was inoculated with approximately 10⁷

Table 1

Characteristics of the oligonucleotide sequences used for PCR and sequence analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplicon</th>
<th>Length (bp)</th>
<th>Accession Nos. and/or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALC1</td>
<td>5′-AAC AAA TTA GTA GGA ACT GTA-3′</td>
<td>boiD</td>
<td>212</td>
<td>AF076529 [21]</td>
</tr>
<tr>
<td>ALC2</td>
<td>5′-AAG CTC CAG CCG CTT CTC GTA-3′</td>
<td>Butyrivibrio AR10</td>
<td>209</td>
<td>AF298196 [9]</td>
</tr>
<tr>
<td>ALC3</td>
<td>5′-TGG AAC AAT TTG ATG TAA TGA-3′</td>
<td>Bovicin 255</td>
<td>146</td>
<td>AF062647 [22]</td>
</tr>
<tr>
<td>ALC4</td>
<td>5′-ATG TTC CTC CAC CAA GAG ACG-3′</td>
<td>but79A</td>
<td>1540</td>
<td>[33]</td>
</tr>
<tr>
<td>ALC5</td>
<td>5′-AGT AAT CCG GAG AAT TCA GAC-3′</td>
<td>Butyrivibrio OR79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALC6</td>
<td>5′-GAT TAT GAT CAT GGC TCA G-3′</td>
<td>Eubacterial 16S rRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fD1</td>
<td>5′-GAT TAT GAT CAT GGC TCA G-3′</td>
<td>Sequencing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rD1</td>
<td>5′-GAT TAT GAT CAT GGC TCA G-3′</td>
<td>Sequencing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>5′-CTTG CAA CCG GAG GCA GTA-3′</td>
<td>Sequencing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R5</td>
<td>5′-GGG TTG CGC TCG TG-3′</td>
<td>Sequencing</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[26,32]. Bacterial strains were stored at −85 °C on CC or L10 agar slopes.
indicator organisms (*P. anaerobius* D1), mixed gently and poured over the DAP assay plates. The plates were re-incubated at 39 °C for 18 h and each isolate was scored for its ability to create a distinct zone of inhibition in the agar overlay.

**3. Results**

**3.1. Isolation of strains**

Caecal and ruminal fluid dilutions in RX or CC broth were spread onto CC agar plates and incubated anaerobically overnight at 39 °C. Resulting colonies that were large, and white to orange in colour were Gram stained and identified as Gram-positive cocci. Slower growing pale colonies were identified as Gram-negative curved rods. Based on these characteristics the isolates were initially identified as *S. bovis*- and *B. fibrisolvens*-like isolates, respectively.

**3.2. PCR primer design**

PCR primers for the amplification of the genes encoding bovicin 255 and butyrivibriocin AR10 (*bvi*D) were designed using the gene sequences from *S. gallo-lyticus* LRC0255 (AF298196) and *B. fibrisolvens* AR10 (AF076529), respectively. Degenerate bases were included in the ALC5 and ALC6 primers to account for the sequence variation found within the *bvi*79A genes encoding the *B. fibrisolvens* butyrivibriocin OR79A-like bacteriocins (AF062647 and AF349664 to AF349674) [22]. Both forward and reverse primers were designed internally to the gene encoding the bacteriocins to ensure that any variations in flanking DNA sequence did not affect identification of strains possessing the target gene sequence.

**3.3. PCR amplification from bacterial isolates**

A PCR product of the expected size was amplified from both *B. fibrisolvens* OR79 (146 bp) and AR10 (213 bp) (Fig. 1). However, an additional non-specific amplicon slightly larger than the anticipated PCR product was often seen (Fig. 1), but only from the positive control strains. Attempts were made to eliminate the non-specific amplification by changing the PCR reaction parameters, however this resulted in reduced levels of amplification of the desired amplicon. Thirty four *B. fibrisolvens*-like isolates were screened by PCR for the presence of bovicin 255. Ten strains gave a PCR amplicon of an indistinguishable size from the product obtained from *S. gallo-lyticus* LRC0255 (Fig. 2). Seven were New Zealand isolates: four from the caecum of one sheep, two from the caecum of another sheep and the final strain was isolated from the rumen contents of a sheep from a separate flock. All sheep were pasture (ryegrass-clover) fed. The other three strains were isolated from grain-fed cattle in North America (D. Krause, personal communication).

**3.4. Sequence analysis of bovicin 255 PCR products**

All PCR products amplified using the ALC3 and ALC4 primers, specific for bovicin 255, were cloned and sequenced. The sequence of the PCR product derived from *S. gallo-lyticus* was identical to the GenBank sequence, however all other cloned sequences differed

![Fig. 1. PCR amplification of the genes encoding butyrivibriocins OR79A and AR10 (lanes 2 and 4) from *B. fibrisolvens* OR79 and AR10 respectively. Negative controls (lanes 1 and 3).](https://academic.oup.com/femsec/article-abstract/48/2/199/468421)

![Fig. 2. PCR amplification of the gene encoding bovicin 255 from *Streptococcus* spp. Lane 1, Negative control; 2, *S. gallo-lyticus* LRC0255; 3, *S. bovis* 7–2; 4, *S. bovis* 7–25; 5, *S. bovis* 7–26; 6, *S. bovis* GKF1; 7, *S. bovis* 22/01 F; 8, *S. bovis* 24/01 B; 9, *S. bovis* 24/01 H; 10, *S. bovis* 4b; 11, *S. bovis* 13a; and 12, *S. bovis* 13b.](https://academic.oup.com/femsec/article-abstract/48/2/199/468421)
from the LRC0255 sequence (209 bp) by between 11 and 15 bp substitutions. Isolates may be grouped on the basis of their amino acid sequences into Group 1, Group 2 and LRC0255 (Fig. 3). Group 1 strains have an identical processed peptide amino acid sequence to LRC0255 whilst the Group 2 strains have four base pair substitutions that result in three amino acid changes (asparagine to serine, alanine to threonine and asparagine to threonine) in the processed peptide (Fig. 3). Four additional base pair changes in Groups 1 and 2 result in three amino acid changes (aspartic acid to glutamic acid, glutamic acid to alanine and alanine to glutamic acid) in the pre-peptide (Fig. 3). The remaining base pair substitutions are silent.

3.5. Phylogenetic analysis of bovicin 255 producing S. bovis-like isolates

To determine the identity of the bovicin 255-producing isolates, their 16S rRNA genes were sequenced and aligned against other known streptococcal sequences. The comparative sequence analysis confirmed that all 10 isolates producing bovicin 255 shared almost identical 16S rRNA genes (99.7–100%) to those of S. bovis B315 and C14b#1, strains previously isolated from ryegrass-clover fed cattle in New Zealand [36], S. bovis JB1 and H2-1 [37] and also contained the same PCR primer sites used by Whitehead and Cotta [37] to identify ruminal S. bovis isolates.

3.6. Pulsed-field gel electrophoresis of bovicin 255 producing S. bovis-like isolates

DNA from each S. bovis-like isolate giving a positive PCR reaction for the amplification of bovicin 255 was screened by pulsed-field gel electrophoresis (PFGE) analysis to determine strain similarity (Fig. 4). Four of the New Zealand S. bovis isolates from the same sheep had identical PFGE profiles. Another two isolates from a different animal within the same flock also had identical PFGE profiles. The other New Zealand strain, GKF1, isolated in a separate study from the rumen contents of a ryegrass-fed sheep, had a distinctly different profile. Of the three North American isolates, two had identical PFGE profiles, the other differing by the position of one DNA band. The PFGE profiles of all the bovicin 255 positive S. bovis isolates were dissimilar to...
the PFGE profile of the bovicin 255 control strain *S. galloyticus* LRC0255 (Fig. 4).

### 3.7. Measuring inhibitory activity

To determine whether the possession of the bovicin 255 amplicon was associated with inhibitory activity, the PCR positive isolates were tested in the DAP assay. When each of the PCR positive streptococci isolates were grown on L10 agar plates, overlaid with agar containing *P. anaerobius* D1 as an indicator strain, zones of inhibition of the indicator organism were observed (Fig. 5). Strain 3–36 which did not possess the bovicin 255 gene using the PCR assay was used in the DAP assay as a negative control and did not give any zones of inhibition in the indicator overlay (Fig. 5). All *S. bovis* isolates possessing the bovicin 255 gene produced inhibition zones of varying sizes with *P. anaerobius* D1 overlays.

### 4. Discussion

Bacteriocins are a large group of microbial compounds that have yet to be fully evaluated for use within the rumen environment with a view to limiting the loss of energy and nitrogen through the microbial production of methane and ammonia, respectively. Inhibitory molecules such as monensin are often included as feed supplements to improve the efficiency of ruminal fermentation through reductions of Gram-positive ruminal strains. The use of novel bacteriocin molecules as feed additives to increase the overall efficiency of ruminal fermentation has attracted considerable commercial interest and research focus. In this study we used PCR and DNA sequence analysis to identify and characterise genes encoding novel bacteriocin variants from rumen and caecal bacterial isolates and examined the relationship between bacteriocin DNA sequence PFGE types.

Previously, phenotypic tests were used to screen for bacteriocin activity from *B. fibrisolvens* and *S. bovis* strains isolated from the rumen [7,9,11,19]. This is the first study where PCR amplification, used as a diagnostic tool, and bacteriocin sequence diversity from multiple rumen or caecal *S. bovis*-like isolates having inhibitory activity, have been linked to source animal, and where the bacterial gene profile of the bacteriocin producer strains has been assessed using PFGE to examine clonality. In a study by Whitford et al. [22] PCR amplification and DNA sequence analyses indicated that 24 of 39 (61%) *B. fibrisolvens*-like isolates whose bacteriocin production was identified on the basis of the DAP assay, had homologues to the *but79A* gene that encodes the butyrivibriocin OR79. The *but79A* positive bacteriocin-producing strains could be organised into 3 groups and one variant was found in *Butyrivibrio crosstotus* [22]. However, no data are available that have identified further *B. fibrisolvens*-like isolates other than strain AR10 that possesses the gene encoding butyrivibriocin AR10. In this study none of the *B. fibrisolvens*-like isolates screened in the PCR assay were found to be positive for the butyrivibriocins AR10 or OR79 and as far as we are aware, butyrivibriocins AR10 or OR79 have not been identified from *B. fibrisolvens*-like isolates from the Southern Hemisphere.

*Butyrivibrio fibrisolvens*-like strains are often assigned on the basis of their butyrate-production and other common phenotypic and metabolic characteristics [38–44], and recent classification has further clarified the complex *Butyrivibrio* group with the identification of *Butyrivibrio hungatei* and *Pseudobutyrivibrio xylanivorans* [45]. However, as none of the field strains in this study gave amplicons with primers specific for butyrivibriocins AR10 or OR79, further characterisation of the *B. fibrisolvens*-like isolates to the 16S rRNA gene level or classification using recently developed methods [45] were not performed.

The identification, cloning and sequencing of bovicin 255 from *S. galloyticus* LRC0255 was the first full characterisation of a bacteriocin from rumen streptococcal spp. [9]. Whilst this isolate was one of seven strains that inhibited the growth of other streptococci, the other six strains having inhibitory activity were not examined for the presence of the bovicin 255 gene. Thus far no other studies have identified any additional streptococcal spp. containing the bovicin 255 gene. Using PCR, however, this study has identified a further 10 streptococcal strains having a bacteriocin with considerable sequence homology (92.9–95.7%) to that of the *S. galloyticus* LRC0255 from which the bovicin 255 gene was first sequenced. Whether the sequence variation is a characteristic of *S. bovis* isolates is not known as additional *S. galloyticus* isolates having the bovicin 255 gene sequence, apart from LRC0255, remain to be identified. The presence of such similar bacteriocins having undergone sequence divergence in two separate rumen streptococcal spp. is noteworthy. Similarly, sequence divergence in the nisin biosynthetic genes found on the conjugative transposon of *L. lactis* N8 results in the substitution of asparagine for histidine at amino acid position 27 in nisin Z compared to nisin A without any apparent loss of antimicrobial activity [46,47]. Amino acid substitutions within the processed bovicin 255 peptide of the *S. bovis* isolates are relatively conserved and do not appear to result in any significant change of inhibitory activity in the DAP assay compared to that of the homologous peptide from *S. galloyticus* LRC0255. There may, however, be some significance of bovicin 255 amino acid sequence variation within the rumen environment that is impossible to quantify in vitro.

The extent and diversity of bacteriocin production by *S. bovis*-like isolates has not been fully investigated.
However, in a recent study [19] approximately 50% and 60% of *S. bovis* isolates from hay and grain fed cattle, respectively, had activities that inhibited the growth of *S. bovis* JB1. PCR detection using primers different to those used in this study, were successfully used to amplify the bovicin 255 gene from the *S. galloyticus* positive control but indicated that none of the inhibitor producer isolates had the gene for bovicin 255 [19]. Sequence data analysis indicates that at least a single base pair change is present between the BOV302R primer sequence [19] and the corresponding PCR annealing site in 50% (5 of 10) of the *S. bovis* isolates having bovicin 255 gene homologues sequenced in this study. Therefore primer design in conserved areas of the gene sequence may be important for accurate identification of streptococcal spp. containing bovicin 255-like gene sequences. Several other rumen *S. bovis* isolates, including HC5, were identified as having bacteriocin activity [19] but whether sequence divergence or the presence of other as yet uncharacterised bacteriocin molecules contributes to this activity is unknown.

Although the bovicin 255 gene may be found from diverse geographical locations, including New Zealand and North America, its distribution is uncertain. On the basis of bovicin 255 sequence analysis, both Groups 1 and 2 organisms were isolated from New Zealand. The three isolates from North America may be placed into Group 1. Only one group type was isolated from any one animal although strains from both Groups 1 and 2 were isolated from separate sheep within the same flock. The seven New Zealand strains were isolated from only three ryegrass-clover fed animals and strains within each animal had a different PFGE profile. It is unclear whether bovicin 255 positive *S. bovis* strains preferentially inhabit ryegrass-fed animals or whether one *S. bovis*-like clonal type may predominate in each animal based on only the few isolates examined from a small number of animals. It is interesting to note that 6 of the 7 New Zealand strains were caecal isolates from two animals from the same sheep flock. The extent to which *S. bovis* colonises the length of the ruminant gastrointestinal tract and whether the caecum may be an additional site in which strains producing bovicin 255 may persist has not been fully established. However, *S. bovis* isolates are uncommon in the rectal contents of pre-weaned calves, but an increase in the levels of *S. bovis* and an associated reduction in the presence of enterococcal spp. in calves and dairy cattle [48] may be due, in part, to the colonisation of the rumen through faecally contaminated forage as calves are weaned. This may indicate that colonisation is throughout the gastrointestinal tract and that excretion may be one mechanism by which initial colonisation with *S. bovis* occurs.

The phylogenetic position of the *S. bovis*-like isolates having the bovicin 255 gene was determined using comparative analysis of the 16S rRNA gene sequences. Unlike the positive control organism, *S. galloyticus* LRC0255, isolated from the rumen of a moose in Alberta, Canada, all isolates described in this study were almost the same or identical to *S. bovis* 16S rRNA gene sequences available in DNA databases. Based on these data it appears that the bovicin 255 gene may be more commonly associated with *S. bovis* ruminal isolates than *S. galloyticus*.

In summary, our investigations have shown that the butyryvibriocin genes may not be widely distributed in *B. fibrisolvens*-like strains isolated in New Zealand, despite attempts to overcome sequence variation with the design of degenerate primers. Any significance in vivo of the *S. bovis* bovicin 255 gene sequence variants are unknown but may represent a convenient method of sub-typing those that have gene sequences homologous to bovicin 255. Careful choice of PCR primers is required to ensure accurate identification of bacteriocins from those isolates having inhibitory activity. It has become apparent that the bacterial diversity of the rumen is much greater than once thought and as examples of bacteriocin-like activity among obligate and facultative anaerobes isolated from the rumen of both sheep and cows have been described, it is conceivable that many other ruminal bacteriocins remain to be discovered. Future investigations are required to examine this rumen bacterial strain diversity and the production of bacteriocin molecules to assess their impact on the overall rumen ecology and to begin to understand the interbacterial mechanisms that influence the flux and diversity of microbial species in the rumen.

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References


