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ORIGINAL ARTICLE

Ruminococcus bromii, identification and isolation as a dominant community member in the rumen of cattle fed a barley diet

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Abstract

Aims: To identify dominant bacteria in grain (barley)-fed cattle for isolation and future use to increase the efficiency of starch utilization in these cattle.
Methods and Results: Total DNA was extracted from samples of the rumen contents from eight steers fed a barley diet for 9 and 14 days. Bacterial profiles were obtained using denaturing gradient gel electrophoresis (DGGE) of the PCR-amplified V2/V3 region of the 16S rRNA genes from total bacterial DNA. Apparently dominant bands were excised and cloned, and the clone insert sequence was determined. One of the most common and dominant bacteria present was identified as *Ruminococcus bromii*. This species was subsequently isolated using traditional culture-based techniques and its dominance in the grain-fed cattle was confirmed using a real-time *Taq* nuclease assay (TNA) designed for this purpose. In some animals, the population of *R. bromii* reached densities above 10^{10} *R. bromii* cell equivalents per ml or approximately 10% of the total bacterial population.
Conclusions: *Ruminococcus bromii* is a dominant bacterial population in the rumen of cattle fed a barley-based diet.
Significance and Impact of the Study: *Ruminococcus bromii* YE282 may be useful as a probiotic inoculant to increase the efficiency of starch utilization in barley-fed cattle. The combination of DGGE and real-time TNA has been an effective process for identifying and targeting for isolation, dominant bacteria in a complex ecosystem.

Introduction

When cattle are fed diets containing a high proportion of cereal grain, the pH of the rumen contents often falls to very low levels (pH < 5) (Dunlop 1972; Nocek 1997; Owens *et al*. 1998). This decreases the efficiency by which a substrate is converted to volatile fatty acids and microbial protein for animal production (Strobel and Russell 1986). The drop in pH is often associated with the accumulation of lactic acid, which can lead to acute lactic acidosis (Dawson and Allison 1988).

A range of preventative measures, including the use of antibiotics (Godfrey *et al*. 1995; Owens *et al*. 1998), probiotic bacteria (Kung and Hession 1995; Wiryawan and Brooker 1995; Klieve *et al*. 2003) and immunization against *Streptococcus bovis* (Shu *et al*. 1999), have been considered. We have pursued the probiotic approach (Klieve *et al*. 2003), whereby lactic-acid-utilizing and competitive starch-utilizing bacteria are selected from the rumens of cattle adapted to a high-grain diet and introduced to cattle entering a feedlot. This should augment indigenous populations of these bacteria, thereby preventing acute lactic acidosis, allowing a faster introduction to grain and improving the efficiency of starch utilization. This initial work was successful in identifying *Megasphaera elsdenii* YE34 as a promising lactic-acid-utilizing bacterium that was capable of rapidly colonizing the rumen of grain (barley)-fed cattle. However, the strain of *Butyrivibrio*

fibrisolvens selected as a non-lactic-acid-producing starch utilizer failed to colonize and was rapidly lost from the ecosystem. The cattle did efficiently utilize grain during this trial and this current study was aimed at the use of denaturing gradient gel electrophoresis (DGGE) to identify the dominant bacteria (presumptively starch utilizers) in these cattle, the isolation of these species for future probiotic use and confirmation of their selection to a dominant population, as the percentage of grain in the diet increased, by real-time TNA.

Materials and methods

Sample collection and DNA extraction

Samples of ruminal contents from eight rumen-cannulated steers that were rapidly introduced (over 6 days) to a high-grain diet (75% rolled barley), as previously reported (Klieve *et al.* 2003), were examined. The rumen fluid samples were collected throughout the experimental period, and they were centrifuged and bacterial pellets stored were frozen at −20°C until required for DNA extraction or to isolate bacteria (Klieve *et al.* 2003). DNA was extracted from the samples taken 9 and 14 days after grain feeding began. DNA extraction from ruminal fluid samples and from bacterial isolates was by physical disruption using a bead beater as described by Whitford *et al.* (1998).

PCR amplification and restriction enzyme digestion

The hypervariable V2/V3 region of the bacterial 16S rRNA gene was amplified from the total bacterial DNA extract from each rumen sample using the primers 341F-GC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer *et al.* 1993).

Methods for PCR amplification of 16S rRNA gene, restriction enzyme digestion with *Msp*I, *Cfo*I and *Hae*III (Roche, Mannheim, Germany) and analysis by manual assignment to groups based on all three digestion patterns have been reported previously (Ouwerkerk and Klieve 2001; Ouwerkerk *et al.* 2002).

Denaturing gradient gel electrophoresis

DGGE (DCode System; BioRad, Hercules, CA, USA) was used to separate the V2/V3 PCR products into a profile representing the diversity and abundance of bacteria present in the sample. PCR products were loaded onto a DGGE gel with 8% acrylamide and 30–60% formamide/urea gradient. A reference marker was constructed from pure cultures of *Escherichia coli* K13, *B. fibrisolvens* AR27, *Ruminococcus albus* AR67, *Prevotella ruminicola* AR29, *Selenomonas ruminantium* AR55 and *Bifidobacterium pseudolongum* subsp. *globosum* YE285. The V2/V3 PCR products from these bacteria formed a ladder that spanned the length of the gel. This ladder was loaded onto the gel to allow comparison of bands within and between gels. Gels were electrophoresed at 100 V for 18 h in 0·5× TAE (Tris–acetate, 0·04 M; EDTA, 0·001 M) buffer, at 60°C.

Following electrophoresis, the gel was silver-stained to visualize the DNA (Kocherginskaya *et al.* 2005).

Band selection, DNA purification and cloning

Following V2/V3 amplification of ruminal DNA, PCR amplicons were electrophoresed on DGGE gels for all animals on each day of sampling. From DGGE profiles of ruminal contents at 9 and 14 days after the start of grain feeding, a number of bands were selected that appeared to be dominant (dark staining) and common to many of the animals.

A sample of the DNA was obtained from selected bands by stabbing through bands with a sterile 1 ml autopipette tip and placing the excised plug into the PCR reaction mix and allowing to stand for 5 min. Following centrifugation of the reaction mix, the V2/V3 region was then re-amplified and run on a DGGE gel to confirm that the majority of amplicons were those targeted. Clone libraries of the V2/V3 PCR products of each band were produced using the TA Cloning Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Approximately 20 clones for each band that had been stabbed were selected and plasmid DNA containing a copy of the original product was extracted from the clones using the QIAprep Spin Miniprep Kit (QIAGEN, Doncaster, Vic., Australia). The V2/V3 insert was amplified from the plasmid and run on a DGGE gel alongside the DNA banding profile from the steer to confirm the clones contained the correct insert, i.e. the V2/V3 PCR product migrated to the same position as the band that the stab was taken from. The DNA sequence of the V2/V3 region was determined (see below) for five clones containing the correct insert from each band and a minimum of three identical sequences from different clones was deemed sufficient for assigning the sequence to a specific band.

DNA sequencing and analysis

Sequencing of PCR-amplified 16S rRNA genes was performed using the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit Version 3.1 with Amplitaq® DNA Polymerase FS and a model 373A DNA

sequencing system (PE Applied Biosystems, Inc., Foster City, CA, USA) at the Griffith University DNA Sequencing Facility (Brisbane, Qld, Australia). All procedures were performed according to the manufacturer's protocols. The primers used for sequencing the cloned V2/V3 bands were T3 or T7 (TOPO Cloning Kit; Invitrogen, Carlsbad, CA, USA) or for sequencing the full 16S rRNA gene, 27F, 530F, 1525R and 907R (Stackebrandt and Charfreitag 1990; Lane 1991) were used. Sequence fragments were assembled using ContigExpress from the Vector NTI Suite 6 package [Informax, Frederick, MD, USA (http://www.informaxinc.com)]. Assembled 16S rRNA gene sequences were compared with GenBank, EMBL and DBJJ nonredundant nucleotide databases using the Gapped BLAST database search program (Altschul *et al.* 1990) at the National Centre for Biotechnology Information (NCBI). Similar 16S rRNA gene sequences were included in the phylogenetic analysis and the sequences were aligned using ClustalW (Ludwig *et al.* 2004) and manually corrected using Genedoc. Preliminary analyses were performed using PAUP* neighbour-joining methods, with 1000 bootstrap replicates. For maximum likelihood (ML), the appropriate parameters were selected with the application 'ModelTest' version 3.7 (Posada and Crandall 1998) to test nucleotide alignments against 56 models of DNA evolution. The resultant negative log likelihood ($-\ln L$) scores and associated parameters were subjected to Akaike information criterion to determine which model best fitted the sequence data. The model of DNA evolution selected was GTR+I+G. These parameters were then specified in PAUP* 4·0b10 (Swofford 1998) to build the tree using tree-bisection-reconnection heuristics. The final value of -ln *L* for the ML trees is 6997·9578.

Nucleotide sequences have been deposited in the GenBank database under the accession numbers DQ882649 and DQ882650.

Bacterial isolation and confirmation of identity

From stored samples of the rumen contents from the barley-fed cattle, standard anaerobic microbiological methods (Hungate 1969; Holdeman *et al.* 1977; Ogimoto and Imai 1981) were used to isolate and culture species identified from sequencing DGGE DNA bands. A variety of anaerobic media [rumen-fluid-based medium (Klieve *et al.* 1989) supplemented with wheat starch, barley, maltose or cellobiose and glucose] were used. Hungate's (1969) roll tube method was used to isolate pure strains of bacteria. Samples of ruminal contents were thawed, mixed with 4·5 ml of dilution solution (Ogimoto and Imai 1981) and serially diluted at 10^{-2} increments to 10^{-8}. Aliquots (500 μl) of diluted sample were injected through the septum of a Hungate tube containing 4·5 ml of molten agar (at 50°C), mixed and placed on a tube roller and allowed to solidify as a thin layer on the inner wall of the tube. Roll tubes were incubated at 39°C for 24 h. The procedures for the purification and general handling of anaerobic micro-organisms followed those reported by Holdeman et al. (1977) and Hungate (1966).

Only bacterial isolates with cell morphology consistent with the target bacteria were investigated further. Once isolated, DNA was extracted from the bacteria, and the 16S rRNA gene was amplified by PCR. Amplicons were digested with three restriction endonucleases [*Msp*I, *Cfo*I and *Hae*III (Roche, Mannheim, Germany)], as detailed above, to determine which isolates were genetically distinct. The 16S rRNA gene was then sequenced from a representative of each unique genotype. Of the bacteria whose sequence corresponded to the target species, further confirmation that they were the bacteria identified by DGGE was achieved by PCR amplification of the V2/V3 region of the 16S rRNA gene and by comparison on a DGGE gel with the position of the band(s) in the original bacterial profile from rumen contents. Bacterial isolates were stored according to the method of Teather (1982).

Enumeration of *Ruminococcus bromii* populations by real-time *Taq* nuclease assay

The development and application of a real-time *Taq* nuclease assay (TNA) for the enumeration of *R. bromii* was based on those developed for *M. elsdenii*, *B. fibrisolvens* and *Strep. bovis* (Ouwerkerk *et al.* 2002; Klieve *et al.* 2003). The development followed the same steps in terms of DNA extraction, PCR amplification, sequencing and sequence analysis of 16S rRNA genes, PCR calibration and verification of specificity. Primers and probes were selected from 16S rRNA gene sequence data using Primer Express Version 2 software (Applied Biosystems, Inc., Foster City, CA, USA) to fit the specifications of the RealMasterMix probe mix (Eppendorf, Hamburg, Germany). Identified primers and probe were checked for specificity using the program Probe Match version 2.1 (Maidak *et al.* 2000) against the Ribosomal Database Project and the BLAST program (Altschul *et al.* 1990) at the NCBI site against the GenBank database. The 5′–3′ sequences of the selected primers and probe were: YE282F1, ATACCGCA TGACATATGATTGT; YE282R1, GCTAATCAGACGCGA GTCCAT; and YE282P1 (probe), 6-FAM-ATGGCAGACA TATCA-MGB-BHQ.

Real-time TNA assays were run on a Rotor-Gene RG-3000 (Corbett Research, Australia) under the following conditions: one cycle at 94°C for 1 min; then 45 cycles at 94°C for 10 s, 66°C for 30 s; and a final cycle of 30°C for 1 min.

Quantitative standards containing known numbers of *R. bromii* YE282 were prepared by directly enumerating a pure culture of YE282 following sonication in a sonication bath to fragment the characteristic long chains. Cells were directly enumerated through triplicate counts using a Petroff-Hauser Bacteria Counter (Arthur H. Thomas Company, PA, USA), according to the manufacturer's instructions, at a magnification of 400× with an Olympus BH-2 microscope. Cells were diluted in TE buffer or pelleted rumen fluid re-suspended in TE buffer, to 1×10^{10} cells per ml and a log dilution series prepared down to 1×10^{2} cells per ml. Genomic DNA was isolated from 1 ml of these dilutions via bead beating, and the DNA was re-suspended in 500 μl sterile water.

The specificity of the TNA was evaluated using genomic DNA isolated from the following isolates: *Bacteroides fragilis* 683, *B. fibrisolvens* strains AR12, AR27, AR73, ATCC 19171 and YE44, *Clostridium butyricum* strains YE12 and YE15, *Cl. glycolicum* YE255, *E. coli* K13 (ATCC 15766), *E. coli* YE261, *Shigella flexneri* YE254, *Eubacterium ruminantium* AR2, *Eu. cellulosolvens* YE257, *Eu. rectale* YE131, *Fusobacterium necrophorum* AR4, *Lactobacillus* sp. YE07, YE08 and YE16, *P. ruminicola* subsp. *brevis* AR20, *P. ruminicola* AR29, *Prevotella* sp. YE139, *Ruminococcus flavefaciens* AR45, *R. productus/hansenii* YE168, *R. albus* AR67, *S. ruminantium* AR55, *Strep. bovis* AR25, SB15, YE01 and 2B, *Streptococcus intermedius* AR36 and *M. elsdenii* YE34. In addition, genomic DNA isolated from both the bovine and ovine rumen contents was used to evaluate assay specificity.

Results

DGGE band selection and cloning

The DNA banding profiles from a cross-section of ruminal samples from the cattle at either 9 or 14 days after they began grain (barley) feeding are presented in Fig. 1. It is evident that although there was considerable diversity between animals, some dominant bands (boxed in Fig. 1) were common to many of these animals and likely to represent the dominant species utilizing the barley diet. An exception was band W, which was bright in only some animals but because of this species' apparent dominance, particularly in steer 12 on day 9, it was also selected for sequencing.

DNA was excised from bands Q, V, W, X and Z from more than one animal (except for W) and five clones with V2/V3 inserts that migrated to the correct position for that band were sequenced. Clones of bands Q, V, W

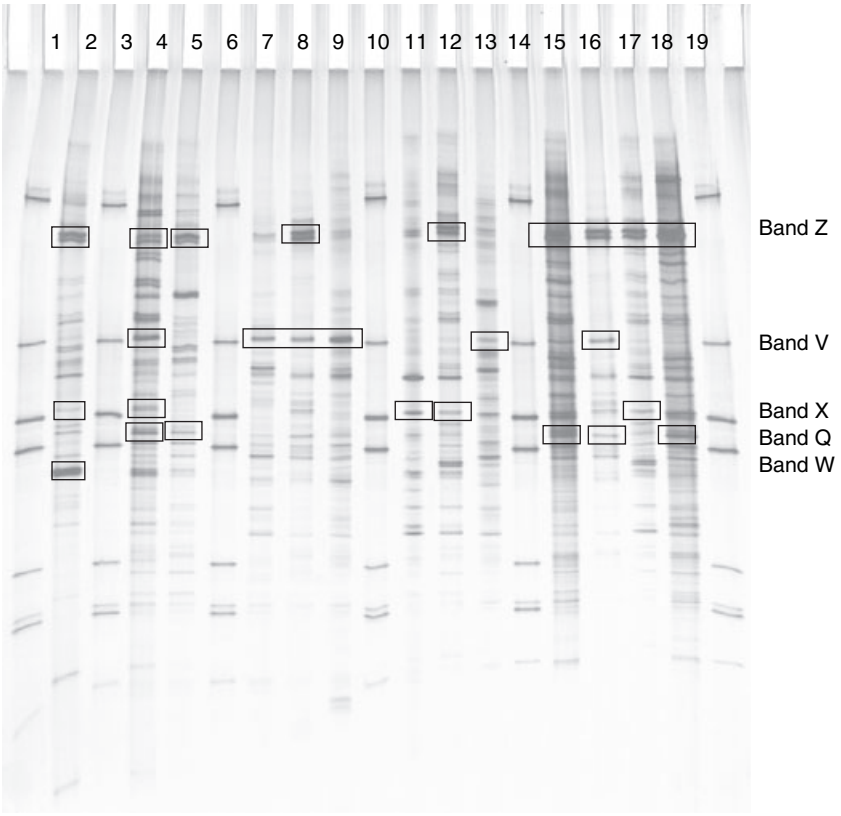

Figure 1 DGGE banding profiles from steers after being fed barley-based ration for 9 or 14 days. Lanes 1, 3, 6, 10, 14 and 19 are a reference marker; lane 2, steer 12 day 9; lane 4, steer 6 day 9; lane 5, steer 16 day 9; lane 7, steer 1 day 9; lane 8, steer 18 day 9; lane 9, steer 18 day 14; lane 11, steer 6 day 14; lane 12, steer 12 day 14; lane 13, steer 4 day 14; lane 15, steer 14 day 9; lane 16, steer 18 day 9; lane 17, steer 12 day 14; and lane 18, steer 14 day 14. Bands identified as dominant were labelled Q, V, W, X and Z. Where they occur in each profile is indicated by a box.

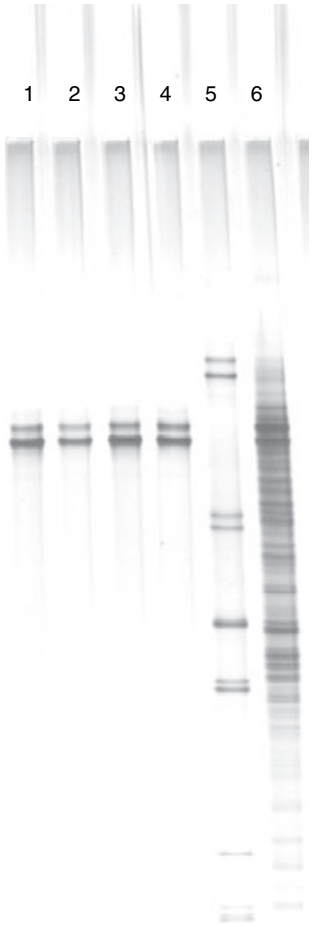

Figure 2 DGGE profile from steer 14 day 9 (lane 6) and DGGE profiles from four clones of band Z from steer 14 day 9 (lanes 1–4). Lane 5 is a reference marker.

and X gave individual V2/V3 bands when amplified and run on DGGE gels, as would be expected, but this was not the case with band Z. Clones from either the upper or the lower band in the doublet always resulted in the doublet reforming when amplified and run on DGGE (Fig. 2). Sometimes one band or the other appeared brighter but this was not consistent.

V2/V3 DNA sequence

The results from sequencing selected clones' V2/V3 insert are presented in Table 1. From DNA sequence comparisons, it was apparent that the bacterial species that appeared most dominant and represented by the doublet band Z was *R. bromii*. Although the V2/V3 region presented as a doublet on DGGE gels despite being cloned, there were no anomalies in the DNA sequence, suggesting that exactly the same DNA was present in both bands. Another dominant group of bacteria belonged to the genus *Prevotella* and was represented by a variety of species which were present in a number of different dominant bands.

Also evident was the fact that some single bands on DGGE gels, e.g. band Q, comprised DNA from a number of unrelated species. In the case of band Q, clones from the same band had DNA from three different species of bacteria. The mix of species found in this band was the same in both steers that had this band excised from their V2/V3 profile.

Bacterial isolation, identification and V2/V3 banding pattern

One hundred and nine colonies were selected and screened on cell morphology, 16S rRNA gene restriction digestion pattern and DNA sequence of the 16S rRNA gene (including the V2/V3 region) to determine whether they were the correct bacteria. Four bacteria isolated on maltose-supplemented medium were cocci with 16S rRNA sequence relating them closely to *R. bromii*. These isolates were initially designated *R. bromii* YE281, YE282, YE283 and YE284. Isolate YE281 grew as single cells and diplococci and was morphologically distinct from the remaining three strains which grew in extremely long chains (hundreds of cells in a single chain) (Fig. 3). The latter strains appeared identical to one another following digestion of the 16S rRNA gene by endonucleases (results not shown) and DNA sequencing, whereas YE281 was related (94% similarity) but distinct from the other isolates. The phylogenetic position of *R. bromii* YE282 and *Ruminococcus* sp. YE281 relative to each other and related bacterial species is shown in Fig. 4. The sequence of the V2/V3 region of YE282 was identical (100% match) to the sequence of band Z from the DGGE results. Further confirmation that isolates YE282–284 were responsible for band Z was obtained by running a DGGE gel with the V2/V3 region of the isolates alongside the corresponding V2/V3 profile from rumen contents of a steer (Fig. 5). The V2/V3 band from YE282 migrated to the same position as band Z, confirming that the YE282 group of isolates comprised this dominant band in the profile. The V2/V3 band (also a doublet) from YE281 was present in the steer's rumen bacterial profile but it did not appear to be a dominant population (Fig. 5).

Real-time TNA to enumerate *Ruminococcus bromii*

Calibration curves of the relationship between cell numbers and fluorescence at the cycle threshold (C_T), set at

Table 1 DNA sequence similarity of V2/V3 clones from dominant bands in DGGE gels to known bacteria in the GenBank database

Band	No. of steer profiles DNA excised from	No. of clones sequenced	Closest match by BLAST identification (% similarity)
Z	4	20	*Ruminococcus bromii* (99%)
V	3	15	*Prevotella aff. ruminicola* (96%)
X	2	10	*P. ruminicola* strain 223 (97%)
Q	2	10	*Prevotella dentalis* (94%)
			Anaerovibrio lipolytica DSM 3074(T) (92%)
			Prevotella bryantii (93%)
W	1	5	Butyrate-producing bacterium SM4 (97%)

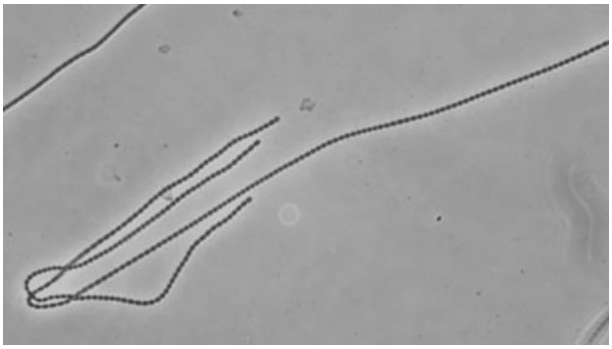

Figure 3 Morphology and growth habit of *Ruminococcus bromii* YE282.

a normalized fluorescence of 0·1, gave a straight-line relationship with a correlation coefficient approximating to unity. Standard curves for diluted DNA series from pure cultures of *R. bromii* YE282 and the dilution series of known numbers of this bacteria in samples of the rumen contents prior to DNA extraction were parallel, with the diluted DNA series giving C_T values approximately 3·5 cycles lower than the corresponding dilution in the rumen contents. It was assumed that, as with *M. elsdenii* (Ouwerkerk *et al.* 2002), the parallel relationship would be maintained at all bacterial densities.

In silico analysis of the target amplicon, incorporating both primers and probe, revealed high levels of specificity, with 100% sequence similarity to a single 16S rRNA gene from an uncultured bacterium clone from a pig gastrointestinal tract (GenBank accession AF371772; Leser *et al.* 2002). The unidentified clone displayed significant homology to the *R. bromii* 16S rRNA gene (1197 bases, or 98%, over 1220 bases), suggesting that it is likely a *R. bromii* strain. The forward primer and probe displayed sequence homology with one additional submission each; the reverse primer displayed sequence homology with eight additional submissions. The combination of primers and probe provided sufficient specificity to differentiate the target bacterium *R. bromii*.

From the specificity panel assays, no nonspecific amplification was detected following 40 cycles with any of the tested isolates or mixed species.

Sensitivity of the real-time TNA was evaluated using genomic DNA extracted from a log dilution series of *R. bromii* YE282 in the rumen fluid. The initial culture used to create the dilution series was enumerated by triplicated direct count using a Petroff-Hauser chamber. The real-time TNA was able to detect 10^6 *R. bromii* cell equivalents (RBCE) per ml of the original sample, or 10^3 RBCE/TNA reaction.

Results of the enumeration of *R. bromii* populations, in eight cattle before and during introduction to a barley diet (Klieve *et al.* 2003), are presented in Fig. 6. *Ruminococcus bromii* populations were below detectable limits in all eight cattle before the introduction of grain into the diet but by 3 days after the beginning of grain-feeding were at populations above 10^6 RBCE per ml in all animals and reached as high as 10^{10} RBCE per ml in some animals by 14 days after the introduction of barley into the diet.

Discussion

The combination of DGGE (to profile complex rumen bacterial populations), the sequencing of dominant bands (to identify and target for isolation, dominant bacteria) and real-time TNA (to confirm bacterial dominance) has been an effective process for obtaining dominant species from the rumen ecosystem.

It was clear from the DGGE profiles of individual steers that the bacterial profiles changed once cattle started feeding on a barley diet and that the presumptively dominant species (darkest bands) also changed. The individual steer profiles were not identical to one another but after feeding on barley for 9 and 14 days there were a number of presumptively dominant species that many had in common. In particular, two bands toward the top of the profile (indicating DNA of high AT content) were dense and commonly encountered. These bands were always of similar density to each other and could not be separated by cloning as cloning, one or the other, resulted in the re-appearance of both. In addition, DNA sequencing of

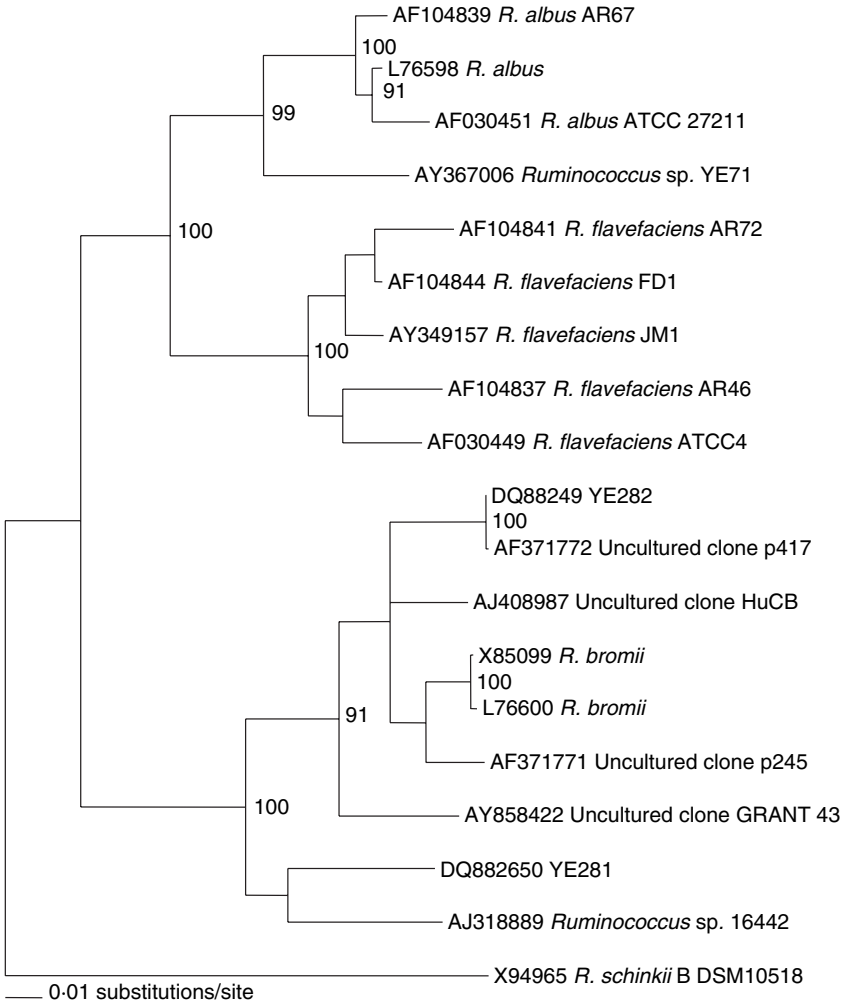

Figure 4 Phylogenetic dendrogram, based on 16S rRNA gene sequence data, indicating the positions of the bacterial isolates to related species and clones. The GenBank database accession numbers for known micro-organisms and cloned sequences are indicated.

this DNA showed no anomalies, suggesting that both bands had identical base sequences. The isolation and amplification of the V2/V3 region of the 16S rRNA gene of *R. bromii* YE282 confirmed that the doublet was from this species but why it continually appeared as a doublet was perplexing. Often multiple bands from a pure culture indicate multiple copies of the gene in the genome that vary from each other slightly in their DNA sequence (Peixoto *et al.* 2002). However, in these cases the individual copies of the gene are separated by cloning and this was not the case with the doublet attributed to *R. bromii* YE282 (and also *Ruminococcus* sp. YE281). It may be possible that the doublet results from the formation of a semi-stable secondary structure within the V2/V3 region of these bacteria.

The decision to select five clones for sequencing from a single band stab and attributing the band to a bacterial species only when at least three sequences were identical (in most cases all five were identical) was made to preclude the chance occurrence that a V2/V3 amplicon from a nondominant species that by coincidence migrated to the same position as the dominant species was cloned and sequenced. To further ensure that a given band could be attributed to a bacterial species with a high level of confidence, we tried to ensure that the sequence was obtained from apparently identical bands in the profiles from at least two steers. Band W was an exception to this rule as although it was present in one steer on high grain, it appeared particularly dominant in that animal.

In addition to multiple bands being attributed to a single bacterium, some apparently dominant bands were attributed to more than one species (band Q). The species that coincidently had V2/V3 amplicons with the same melting temperatures were consistently present but this does reflect that some apparently dominant 'species' were a mix of species and these bands cannot be used to infer dominance or abundance within the profile. This also supports the use of complementary technologies such as real-time TNA assays to confirm dominance in the

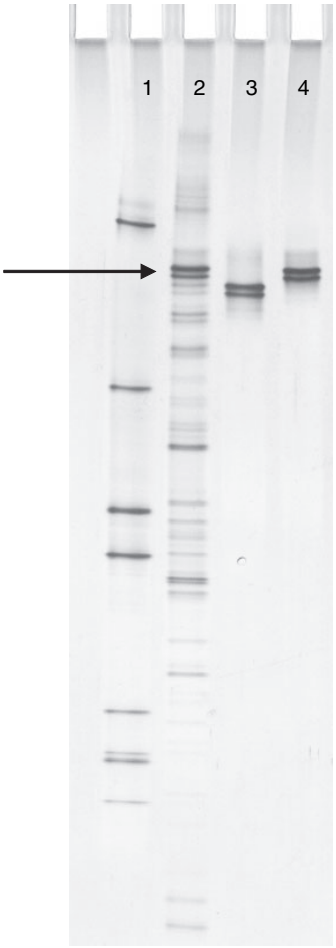

Figure 5 Comparison of migration of V2/V3 DNA from a steer (lane 2) showing the dominant band Z (indicated with an arrow) and the V2/V3 DNA from *Ruminococcus* sp. YE281 (lane 3) and *Ruminococcus bromii* YE282 (lane 4). Lane 1 is a reference marker.

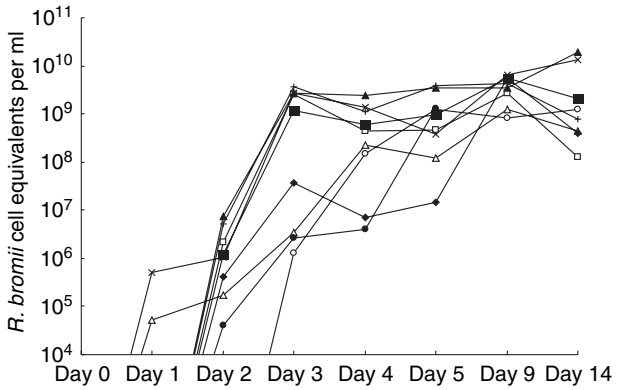

Figure 6 Population densities of *Ruminococcus bromii* in cattle after the introduction of barley into the diet (day 0). *R. bromii* was undetected in all animals prior to the introduction of grain. Steer numbers 1 (○), 4 (△), 6 (□), 11 (●), 12 (▲), 13 (■), 14 (×), 16 (+) and 18 (♦).

population. In the case of the *R. bromii* YE282 group of isolates, the complementarity of these techniques worked extremely well with the apparently dominant doublet attributable to *R. bromii* YE282 being confirmed as a dominant species in the ecosystem and at times represented up to 10^{10} RBCE per ml or approximately 10% of the total bacterial population (Hungate 1966).

The ability to deduce cell morphology from DNA sequence data was useful in ensuring isolation of the target species. The sequence of band Z indicated that *R. bromii* was a dominant species and therefore targeted for isolation. As the expected cellular morphology was that of a coccus, only colonies with this morphology were characterized. Initial isolation attempts selected the most common colony types at the most dilute extreme of the dilution series, on the basis that the dominant species would produce the most colonies. However, initial attempts at isolating *R. bromii* failed. Visual observation of the sample prior to dilution indicated that the majority of cocci present in the mixed culture grew in extremely long chains (hundreds of cells in a chain). Successful isolation was achieved by selecting and screening colonies from less diluted roll tubes. It became apparent that although *R. bromii* YE282 was numerically dominant on a per cell basis, its growth habit in culture was such that it produced far fewer colonies than would have normally been expected from cell numbers because each chain of 100 or so cells only produced a single colony.

Ruminococcus bromii is a culturable bacterium that has been isolated from ruminants and humans and it is regarded as primarily a starch digesting bacterium that has a significant role in the rumen (Stewart and Bryant 1988). However, the degree of dominance and population density on a barley diet has not been previously defined and without the aid of real-time TNA enumeration, a classical enumeration based on colony count would have dramatically underestimated numbers due to the growth habit of strains such as YE282. Furthermore, in the present study we have demonstrated that there is considerable genetic diversity among strains of *R. bromii* (Fig. 4) that had not previously been recognized.

The dominance of *R. bromii* YE282 was confirmed by real-time TNA and this species will now be investigated for inclusion as a probiotic inoculant along with *M. elsdenii* YE34 to increase the efficiency of starch utilization and decrease the incidence of lactic acidosis in barley-fed cattle.

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