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# Diversity of methanogens in ruminants in Queensland

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**Abstract.** Methane emissions from ruminant livestock represent a loss of carbon during feed conversion, which has implications for both animal productivity and the environment because this gas is considered to be one of the more potent forms of greenhouses gases contributing to global warming. Many strategies to reduce emissions are targeting the methanogens that inhabit the rumen, but such an approach can only be successful if it targets all the major groups of ruminant methanogens. Therefore, a thorough knowledge of the diversity of these microbes in different breeds of cattle and sheep, as well as in response to different diets, is required. A study was undertaken using the molecular techniques denaturing gradient gel electrophoresis, DNA cloning and DNA sequence analysis to define the extent of diversity among methanogens in ruminants, particularly *Bos indicus* cross cattle, on differing forages in Queensland. It was found that the diversity of methanogens in forage-fed cattle in Queensland was greater than in grain-fed cattle but there was little variability in methanogen community composition between cattle fed different forages. The species that dominate the rumen microbial communities of *B. indicus* cross cattle are from the genus *Methanobrevibacter*, although rumen-fluid inoculated digestors fed *Leucaena leucocephala* leaf were populated with *Methanosphaera*-like strains, with the *Methanobrevibacter*-like strains displaced. If ruminant methane emissions are to be reduced, then antimethanogen bioactives that target both broad groups of ruminant methanogens are most likely to be needed, and as a part of an integrated suite of approaches that redirect rumen fermentation towards other more useful end products.

**Additional keywords:** Archaea, pasture.

## Introduction

Global warming due to increases in the atmospheric concentration of gases such as carbon dioxide and methane is an important issue. The generation of methane from livestock industries, and particularly from ruminants, significantly contributes to the problem. Methane's global warming potential is 23 times greater than carbon dioxide and it has been calculated that 71% of Australia's anthropogenic agricultural methane emissions (or ~62.1 million tonnes of carbon dioxide equivalents annually) is from livestock (NGGIC 2007).

Methane is a by-product from the microbial digestion of plant material by cattle and sheep. Effectively, it is wasted feed material and energy that could otherwise be available for animal production. If methanogenesis were reduced in the rumen and the carbon was captured by other mechanisms, such as reductive acetogenesis, this would represent an energetic gain of 4–15% to the animal (Nollet *et al*. 1997; Joblin 1999). Ruminal methane is generated by a diverse group of microorganisms within the Domain Archaea. In order to develop control mechanisms to reduce methanogenesis, it is necessary to know what diversity of methanogens are present in cattle and sheep in typical production systems in Australia. It is known that few of the dominant methanogens in the rumen have been obtained in culture and that diversity changes with dietary change, and possibly, species and breed of ruminant (Whitford *et al*. 2001; Wright *et al*. 2006, 2007). To date, relatively little is known of the dominant methanogens in ruminants, particularly *Bos indicus* cattle in northern Australia. This paper uses culture-independent methods to analyse the dominant methanogens present in the rumen of cattle and sheep (and in *in vitro* fermentations initiated with rumen fluid) fed a variety of diets in northern Australia.

## Materials and methods

### Rumen samples

Twenty-two rumen fluid samples were collected from a variety of sources for this study including *B. indicus* cross cattle fed either Mitchell grass hay (2), rye grass hay (2), spear grass hay (2), Pangola grass hay (4), a barley-based (75%) feedlot ration (4) or a leucaena–grass pasture mix (2). Merino wethers fed lucerne pellets (2) or fresh cut kikuyu grass (2) and two samples of fluid from a fermentor receiving *Leucaena leucocephala* leaf as a feedstock were collected. Samples of rumen fluid were collected either *per os* using a stomach tube and vacuum pump, or through a rumen canula, as previously reported (Klieve *et al*. 1998, 2003). Rumen fluid (50–100 mL)

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was collected and strained through nylon gauze into an open-mouthed jar. From each sample, four 1-mL aliquots were placed in 1.5-mL Eppendorf tubes and centrifuged at 15 000*g* for 15 min. The supernatant fluid was discarded and pellets frozen and stored at −20°C.

### *DNA extraction, PCR amplification and denaturing gradient gel electrophoresis (DGGE)*

DNA extraction from the pelleted materials described above was by physical disruption using a bead beater, as described by Whitford *et al*. (1998). The DNA extracts were used as a template for nested PCR amplification of the region spanning the V2/V3 hypervariable loops of the methanogen 16S rRNA gene. Initially, partial 16S genes of Archaea were amplified from the total genomic DNA extracted from each rumen sample using the primers Arch 46F (Ovreås *et al*. 1997) and Arch 1017R (Barns *et al*. 1994). The amplification was optimised on a Hybaid PCR machine (Hybaid, Middlesex, UK) and consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, 47.3°C for 45 s, 72°C for 45 s, with a final elongation step consisting of 72°C for 5 min. The resulting PCR product was used as template in the second (nested) PCR with the primers Arch 344F-GC (Raskin *et al*. 1994) and Univ522R (Amann *et al*. 1995). The conditions consisted of a denaturation step of 94°C for 3 min; followed by 20 cycles of a 94°C for 30 s, 65°C (decreasing 0.5°C every cycle) for 30 s and 72°C for 30 s; the next 15 cycles consisted of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s followed by an elongation step of 72°C for 10 min. All PCR reactions were a total volume of 50 µL consisting of 10 µmol/L of each primer, 200 µmol/L dNTPs, and 1 U of FastStart Taq in the supplied buffer (Roche Diagnostics, Mannheim, Germany).

DGGE was performed on a BioRad DCode system (BioRad, Hercules, CA, USA) to separate the PCR products into a profile representing the diversity of methanogens present in the samples. PCR products were loaded onto a DGGE gel with 8% acrylamide and 30–60% formamide/urea gradient. Gels were electrophoresed at 100 V for 18 h in 0.5 × TAE (Tris-acetate, 0.04 mol/L; EDTA, 0.001 mol/L) buffer, at 60°C. Following electrophoresis, the gel was silver stained to visualise the DNA (Kocherginskaya *et al*. 2005) and images of the gels were obtained by scanning using a flatbed scanner.

### *Clone libraries*

Clone libraries of the primary PCR products of each sample were produced using the TA Cloning Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. 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 cloned insert was amplified from the plasmid and run on a DGGE gel alongside the DGGE banding profile from the animal to identify the band in the profile corresponding to the clone.

### *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 manufacturers' protocols. Gene sequences were compared with GenBank, EMBL, and DBJJ non-redundant nucleotide databases using the gapped basic local alignment search tool database search program (Altschul *et al*. 1990) at the National Centre for Biotechnology Information (Bethesda, MA, USA). Phylogenetic analysis followed the protocols described by Klieve *et al*. (2007).

## Results and discussion

The DGGE banding profiles of methanogen populations in the rumen of the animals examined are presented in Fig. 1. The profiles from *B. indicus* cross cattle being fed the various forages showed a diverse population of methanogens present with between six and eight bands visible (lanes 1–9 and 20–22). However, despite the large variation in diet quality and forage type (viz. leucaena–grass pasture mix cf. rye grass hay cf. spear grass hay) there was very little difference among the DGGE profiles produced from all these animals, suggesting that they all harboured similar methanogen communities. The rye grass-fed *B. indicus* cross animal did have a band present in the upper region of its DGGE profile, which migrated to the same level as band F found in the *L. leucocephala* leaf-fed fermenter samples but its identity needs to be confirmed by sequencing. Sheep fed forage (lucerne or kikuyu grass (lanes 12–15) had similar profiles to the *B. indicus* cross steers except that they had more bands in the upper regions of the gel, particularly those sheep fed lucerne (up to 12). This pattern is indicative of there being species present with a greater A : T base content in the 16S rRNA encoding gene (because the amplicons were denatured at a lower concentration of denaturant). Whether these differences can be attributed to host–animal differences with respect to eating behaviour and/or rumination, or whether legumes like lucerne provide greater amounts of pectins and other materials that can also support methanogen growth remains to be determined. Unlike the cattle and sheep on forage diets, the profiles from *B. indicus* cattle on grain (lanes 16–19) were different to those on forage and varied considerably between animals. An exception was animal 3665 (lane 17), which had a profile similar to some animals on pasture (lanes 20–21). The profiles were also simplified with the total number of bands in the profile reduced to three or four and only one or two main bands per animal. It would seem that grain feeding reduces the species diversity of methanogens in cattle and increases species specificity between individual cattle. Finally, the two leucaena fermentor samples produced methanogen profiles that were entirely different to all other samples and were restricted to three bright bands of high A : T content. It is suspected that the 100% leucaena diet fed to the fermentor and the conditions in terms of feeding rate and retention time, exerts a strong selection pressure on resident populations and those normally present with other forage diets are displaced.

A total of 86 clones were obtained from seven of the samples and 39 clones representing DGGE bands of interested were sequenced. The identity of species represented in the DGGE banding profiles and the genetic relationship between cloned

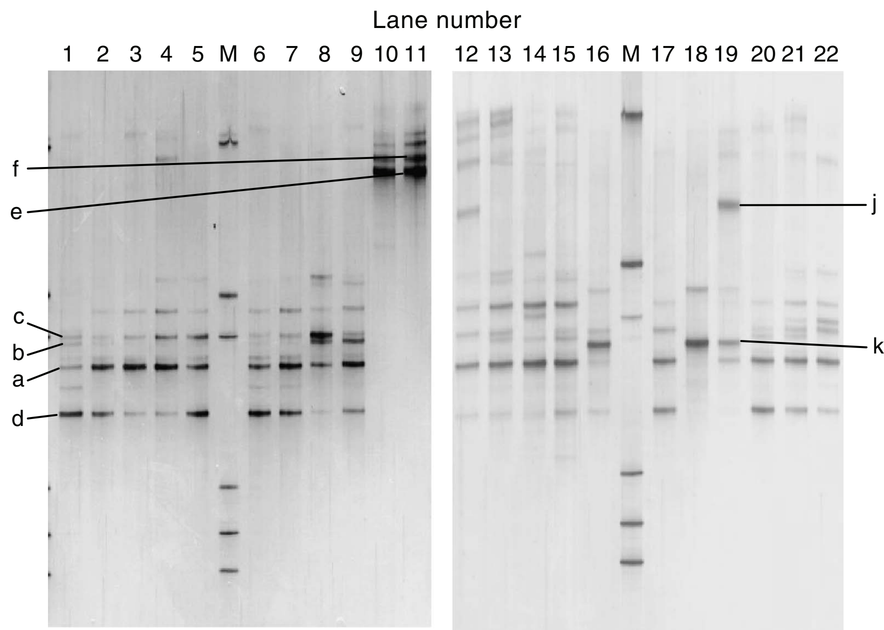


**Fig. 1.** Methanogen denaturing gradient gel electrophoresis profiles from *Bos indicus* cross steers fed Mitchell grass hay (lanes 1 and 2), rye grass hay (lanes 3 and 4), spear grass hay (lanes 5 and 20), Pangola grass hay (lanes 6, 7, 8 and 9), a barley-based feedlot ration (lanes 16, 17, 18 and 19) or leucaena (lanes 21 and 22); Merino wethers fed lucerne pellets (lanes 12 and 13) or fresh cut kikuyu grass (lanes 14 and 15);

methanogen sequences and their nearest cultured relatives is presented in Table 1. Despite the variety of cattle and sheep and the diets that were fed in this study, all of the gene sequences from the DGGE and clone libraries produced from these animals belonged within the genus *Methanobrevibacter* and were closely matched to cultured methanogens. Two clonal sequences were identical to previously isolated methanogens and the most distantly related sequence was still 97% similar to the sequence from a culturable methanogen. The dominance of *Methanobrevibacter* in the rumen of domestic ruminants is in accord with other recent studies in Western Australia and Canada (Wright *et al*. 2004, 2007) but contrasts with the only other report from Queensland (Wright *et al*. 2006), where *Methanobrevibacter* sequences accounted for 9% of total clones sequenced from the ruminal contents of adult sheep. From the samples examined in the present study, the only anomalies to the finding of Wright *et al*. (2006) were the sequences from the leucaena-fed fermentor where both sequences were most closely related to the human intestinal methanogen, *Methanosphaera stadtmanae* (Fricke *et al*. 2006). Sequences related to this species have also been identified previously from cattle, particularly in Canada (Whitford *et al*. 2001; Wright *et al*. 2007). Interestingly, *M. stadtmanae* is incapable of growth (and methane production) solely from carbon dioxide and hydrogen, using the methanol released during the fermentation of pectins as its primary carbon source for growth (Fricke *et al*. 2006). Whether these ‘unusual’ methanogens identified from the fermentors share

**Table 1. Nearest cultivable genetic relatives to cloned methanogen sequences represented in denaturing gradient gel electrophoresis (DGGE) banding profiles**

| DGGE band designation | No. of clones sequenced | No. of bases sequenced | Closest culturable relative (% similarity) | Reference |
|---|---|---|---|---|
| A | 6 | 950 | *Methanobrevibacter* sp. 1Y (99.5%) | Rea *et al*. (2007) |
| B | 4 | 955 | *Methanobrevibacter* sp. SM9 (98%) | Skillman *et al*. (2006) |
| C | 9 | 950 | *Methanobrevibacter* sp. Z8 (100%) | Wright and Pimm (2003) |
| D | 3 | 951 | *Methanobrevibacter* sp. ZA-10 (99%) | Wright and Pimm (2003) |
| E | 3 | 952 | *Methanosphaera stadtmanae* DSM3091 (96%) | Fricke *et al*. (2006) |
| F | 2 | 953 | *M. stadtmanae* DSM3091 (96%) | Fricke *et al*. (2006) |
| J | 4 | 952 | *Methanobrevibacter* sp. NT7 (97%) | Skillman *et al*. (2006) |
| K | 8 | 949 | *Methanobrevibacter* sp. AbM4 (100%) | Jarvis, Skillman, Stroempl, Naylor, Joblin and Moore (unpubl. data). GenBank accession AJ550156 |

similar characteristics remains to be determined. The results do suggest, however, that other types of methanogens can arise if the growth potential of the *Methanobrevibacter* types is negatively affected.

In conclusion, the diversity of methanogens in forage-fed *B. indicus* cross cattle in Queensland appears greater than in grain-fed cattle. There is little variability in methanogen community composition between cattle fed different forages but considerable variability between animals fed grain. Like humans, and ruminants in other environments, the species that dominate the ruminal ecosystem of *B. indicus* cross cattle are from the genus *Methanobrevibacter*. However, the results with the rumen-fluid inoculated fermentors revealed the presence of other, not-yet cultured types of methanogens, which become more noticeable when conditions do not favour the growth of the *Methanobrevibacter* types and (or), are more favourable for these unusual methanogens. Bioactives that target both groups of methanogens will need to be developed.

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