RESEARCH ARTICLE

Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane

Stuart E. Denman1, Nigel W. Tomkins2 & Christopher S. McSweeney1

1CSIRO Livestock Industries, Queensland Bioscience Precinct, St Lucia, Qld, Australia; and 2CSIRO Livestock Industries, JM Rendel Laboratory, Rockhampton, Qld, Australia

Correspondence: Stuart E. Denman, CSIRO Livestock Industries, Queensland Bioscience Precinct, 306 Carmody Road, St Lucia, Qld, 4067, Australia. Tel.: +61 7 322 14 2273; fax: +61 7 322 14 2900; e-mail: stuart.denman@csiro.au

Received 12 February 2007; revised 5 June 2007; accepted 23 August 2007. First published online November 2007.

DOI:10.1111/j.1574-6941.2007.00394.x

Editor: Michael Wagner

Keywords
rumen; methanogens; methyl coenzyme-M reductase; mcrA; bromochloromethane; qPCR.

Abstract

Methyl coenzyme-M reductase A (mcrA) clone libraries were generated from microbial DNA extracted from the rumen of cattle fed a roughage diet with and without supplementation of the antimethanogenic compound bromochloromethane. Bromochloromethane reduced total methane emissions by c. 30%, with a resultant increase in propionate and branched chain fatty acids. The mcrA clone libraries revealed that Methanobrevibacter spp. were the dominant species identified. A decrease in the incidence of Methanobrevibacter spp. from the clone library generated from bromochloromethane treatment was observed. In addition, a more diverse methanogenic population with representatives from Methanococcales, Methanomicrobiales and Methanosacinales orders was observed for the bromochloromethane library. Sequence data generated from these libraries aided in the design of an mcrA-targeted quantitative PCR (qPCR) assay. The reduction in methane production by bromochloromethane was associated with an average decrease of 34% in the number of methanogenic Archaea when monitored with this qPCR assay. Dissociation curve analysis of mcrA amplicons showed a clear difference in melting temperatures for Methanobrevibacter spp. (80–82°C) and all other methanogens (84–86°C). A decrease in the intensity of the Methanobrevibacter spp. specific peak and an increase for the other peak in the bromochloromethane-treated animals corresponded with the changes within the clone libraries.

Introduction

In Australia, the largest contributor to greenhouse gas emissions from the agriculture sector has been ruminant livestock. In 2003, livestock enteric fermentation was responsible for 62.7 Mt (CO2-equivalents) of Australia’s greenhouse gas emissions, or about 12% of net national emissions (Australian Greenhouse Office, 2005). Mitigation of methane, a significant greenhouse gas, has been recognized as an important international goal.

Methane production from enteric fermentation can be affected by a number of factors. Level of feed intake, forage processing, type of carbohydrate, addition of lipids and ionophores to the diet, changes in rumen microorganisms and level of animal productivity have been identified (Johnson & Johnson, 1995; Hironaka et al., 1996; McAllister et al., 1996).

An antimethanogen, containing bromochloromethane in an α-cyclodextrin matrix, has been developed and found to inhibit methane production when fed to ruminants (May et al., 1995; Tomkins & Hunter, 2004). Bromochloromethane is a halogenated methane analogue that is believed to inhibit methane production by reacting with reduced vitamin B12 and inhibiting the cobamide-dependent methyl transferase step of methanogenesis (Wood et al., 1968; Chalupa, 1977). This step is immediately before the terminal reductive reaction and is responsible for the synthesis of methyl coenzyme M (Wood et al., 1982).

Several groups have reported the monitoring of methanogen populations from environmental samples through
targeting of the 16S ribosomal gene (Takai & Horikoshi, 2000; Yu et al., 2005; Sawayama et al., 2006; Stewart et al., 2006). While researchers have traditionally used the 16S rRNA gene for phylogenetic diversity, many researches are now addressing the diversity of the methanogenic Archaea by studying sequence divergence within the methyl coenzyme-M reductase subunit A (mcrA) gene (Luenders et al., 2001; Luton et al., 2002; Hallam et al., 2003). Methyl coenzyme-M reductase is ubiquitous to methanogens and is crucial to the terminal step of methanogenesis where it is involved in the reduction of the methyl group bound to coenzyme-M. Within this study, it was aimed to analyse the methanogenic diversity within the rumen of cattle using the mcrA gene as the phylogenetic marker for both control and bromochloromethane-treated animals and, from this gathered information, to design quantitative PCR (qPCR) primers. Here, the design and use of qPCR primers are reported to monitor total methanogenic populations within the cattle rumen by targeting the mcrA gene. In addition, the use of dissociation curve analysis of the mcrA qPCR revealed differences between the amplicons from the predominant methanogens and from rumen methanogens.

Materials and methods

Animal trial

Six, rumen fistulated, Brahman-crossbred (Bos indicus) steers, with a mean initial live weight of 363 ± 4.7 kg (mean ± sem), were used in the experiment. The animals were maintained on long chopped Rhodes grass (Chloris gayana), ad libitum, and 1 kg day⁻¹ proprietary grain pellets (Custom beef pellet, min crude protein 14%, BetterBlend, Oakey, Qld) throughout the experiment under controlled animal house conditions for 97 days. All animal experiments were approved by the relevant institutional animal ethics and experimentation committee. Animals were offered half their roughage-based daily ration at 08:00 and 16:00 hours each day. For the first 63 days of the experiment, 100 g cotton seed meal was added twice daily to the basal diet of all animals and for the remaining 34 days an antimethanogen, bromochloromethane, was added to the cotton seed meal (CSM), was also fed CSM, twice daily, at 08:00 and 16:00 hours each day, in a dedicated feeder. Control animals were fed CSM, twice daily, without bromochloromethane. All steers consistently consumed the CSM within 1 h.

Individual daily ad lib intakes were ensured so that 0.5–2.0 kg of the feed offered remained uneaten after 24 h. Fifty days after the commencement of the experimental period, and 28 days after the addition of bromochloromethane to the diet, c. 500 mL of rumen digesta was collected before the first daily feed and 4, 8, 12 and 24 h thereafter, pooled from five different sites in the rumen.

Methane measurements

Fifty-five days after the commencement of the experimental period, and 28 days after the addition of bromochloromethane to the diet, methane production was measured using confinement-type closed-circuit respiration chambers similar to those described by Turner & Thornton (1966). The rate of methane production for each animal was derived from the rate of increase in methane concentration in the chamber over time. Chambers were sealed for periods of 45 min, followed by a period of 9–12 min when chambers were opened to prevent the build-up of CO₂ and to allow gas concentrations to return to baseline levels (McCrabb & Hunter, 1999). Methane concentration was determined every 3 min for a total of 45 min using an infrared gas analyser (Model 880A Rosemount Analytical). Individual rates of methane production were calculated as the mean rate of production per hour over a 12-h period. Methane production was expressed as L kg⁻¹ DM intake, MJ day⁻¹ and as a proportion of the calculated gross energy (GE) intake for the sampling period.

Sampling and analysis of rumen fluid

Rumen digesta was passed through a 1 mm × 1 mm sieve to remove coarse material and subsampled for the determination of volatile fatty acid (VFA) molar proportions, ruminal pH and total DNA extraction. Rumen pH was measured immediately and subsamples (50 mL) were stored at −70°C for later analysis of VFAs. VFA analysis was performed on centrifuged (12 000 g, 10 min) rumen fluid samples as described previously (Playne, 1985).

DNA extraction

Total genomic DNA was isolated from pure cultures of methanogens and from rumen samples using the FastDNA kit and FastPrep instrument (Q-BIO gene, Quebec, Canada) as described previously (Denman & McSweeney, 2006). For rumen samples a 1.5 mL aliquot was taken from the 500 mL rumen sample using a wide bore pipette so as to ensure a homogenous sample containing fluid and digesta. This was centrifuged at 12 000 g for 5 min and the supernatant was removed before DNA extraction.
Amplification, cloning and sequencing of mcrA genes from rumen samples

Rumen microbial DNA extracted from animals before dosing with bromochloromethane (control) and after dosing were used as templates in a PCR using mcrA forward primer 5'‐GGTGGTGTMGGATCAGCAYGWCAGC and mcrA reverse primer 5'‐TTCACTGRTAGTWWGGRTAGTT (Luton et al., 2002). Reactions were performed in 30 μL volumes with the addition of 2.5 mM MgCl₂ and 1 U of Platinum Taq (Invitrogen Carlsbad, California) using a Bio‐Rad iCycler thermal cycler under the following conditions: one cycle at 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 60 °C for 15 s and 68 °C for 1 min. The PCR products were analysed by running on a 2% agarose gels containing ethidium bromide. Amplicons were excised and gel purified through the use of a Qiagen gel extraction kit (Qiagen). Purified products were then cloned into the TA cloning vector pGEMTeasy (Promega) and transformed into Escherichia coli TOP10 electrocompetent cells (Invitrogen).

From the libraries, 50 clones from each library were chosen for sequencing. Sequencing of clones was performed using the BigDye cycle sequencing kit v3 (ABI) with the use of SP6 and T7 sequencing primers. The sequences have been deposited at GenBank under the accession numbers DQ192255–DQ192264 (CLI101–CLI110) and EF379242–EF379255 (CLI22–CLI35) from the control library and DQ192265–DQ192275 (CLI11–CLI21) and EF379256–EF379273 (CLI36–CLI53) for the bromochloromethane library.

Phylogenetic analysis

The sequences obtained in this study were imported into the ARB software environment for sequence database handling and data analysis (Ludwig et al., 2004) and aligned using the FASTALIGNER program to mcrA gene sequences downloaded from GenBank including recently described rumen methanogenic archaeal sequences (Tatsuoka et al., 2004). Aligned sequences were manually edited. Gene sequences were translated within ARB and amino acid alignments were then generated. Phylogenetic trees were constructed from the alignments, and bootstrap analysis using parsimony and neighbour-joining was performed using 100 replicates in PAUP* 4.0 (Swofford, 2002).

Distance matrix files were generated in ARB using the neighbour-joining method and imported into DOTUR (Schloss & Handelsman, 2005) for estimation of microbial diversity. Shannon diversity index, collector’s curves for richness observations (Chao1, ACE), were calculated using the furthest neighbour with an operational taxonomic units (OTU) cutoff of 20% for species level using DOTUR (Schloss & Handelsman, 2005).

In addition, Good's coverage estimation was calculated as \[ [1 – (n/N)] \times 100, \] where \( n \) is the number of singleton sequences and \( N \) is the total number of sequences for the analysed sample (Good, 1953). Evenness was calculated from the Shannon diversity index using the formula \( E = H/H_{\text{max}} \), where \( H_{\text{max}} = \ln S \) (Magurran, 1988).

qPCR primer design and analysis

Primers designed for the detection of methanogenic Archaea were targeted against the methyl coenzyme-M reductase (mcrA) gene. Multiple alignments of the mcrA gene-deduced amino acid sequences were used to identify conserved regions. The primers were then designed from multiple alignments of DNA sequences of relevant target sequences along with rumen methanogenic archaeal-specific sequences for these regions. The mcrA forward primer (qmrA-F) 5′-TTGTTGGATCGGATCGGCTGGGC and reverse primer (qmrA-R) 5′-GBARTTCGAWACGTAGATCCC targeted the GFGSYDL conserved amino acid sequence. Primers were analysed using primer express (Applied Biosystems) and designed for an optimal \( T_m \) of 60°C. Primers were then compared with sequences available at NCBI via a BLAST search to ascertain primer specificity (Altschul et al., 1990). qPCR assays were performed on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems). Assays were set up using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Optimization of assay conditions was performed for primer, template DNA and MgCl₂ concentrations. An optimal primer concentration of 300 nM, a final MgCl₂ concentration of 3 mM and a DNA template concentration of between 1 and 100 ng were used for each assay under the following cycle conditions: one cycle of 50 °C for 2 min and 95 °C for 2 min for initial denaturation, 40 cycles at 95 °C for 15 s and 60 °C for 1 min for primer annealing and product elongation. Fluorescence detection was performed at the end of each denaturation and extension step. Amplicon specificity was performed via dissociation curve analysis of PCR end products by increasing the temperature at a rate of 1 °C/30 sec from 60 to 95 °C. Total microbial rumen DNA from sampling time points was diluted 1:10 before use in quantitative real-time PCR.

Amplification efficiencies for the methanogenic Archaea qPCR assay were performed on plasmid DNA purified from clone CLI06 and CLI09 from the mcrA clone library generated from the rumen as described above and DNA from Methanobrevibacter ruminantium M1. Standard curves for absolute quantification of methanogens were performed using DNA from M. ruminantium M1 grown overnight in culture at 39 °C. Cultures were centrifuged and the cells were resuspended in clarified rumen fluid. The numbers of cells were determined using a Helber counting chamber (Weber et al., 2007).
Statistical analysis

Statistical analysis of data was performed by ANOVA, with differences determined by the method of least significant differences at the 5% level (P < 0.05). All statistical analyses were run with STATISTICA 6 (StatSoft Inc., Tulsa, Ok.).

Results

Methane and other biochemical measurements

Methane measurements obtained from animals after the twice-daily inclusion of bromochloromethane indicated a significant reduction in methanogenesis (Table 1) and a corresponding decrease in dietary gross energy lost as methane. Methane production throughout the 12-h sampling period (Fig. 1) also indicated a sustained response in suppressing methane production when bromochloromethane was included in the diet (Table 1). This equates to a total mean reduction in methane emission over 12 h of 29%. During the control period, methane emissions were observed to increase an additional 73% 2 h after feeding, while the bromochloromethane treatment resulted in a 33% reduction in methane emission (Fig. 1). This equated to a 59% reduction in methane emissions at 2 h after feeding for the animals when dosed with bromochloromethane with respect to the control period at the same time. Two hours after the second addition of bromochloromethane, there was again a 35% reduction in methane emissions for these animals, while in the control period there was a 12% increase. Dry matter intake and mean live weight gains were not affected by the addition of bromochloromethane to the diet. Rumen pH values ranged from 6.7 ± 0.08 to 7.1 ± 0.08 during the 12-h postfeeding sampling period and were not significantly different (P > 0.05) between experimental periods.

The total mean VFA concentration was unaffected by the treatments but the acetate:propionate ratio was significantly higher (P < 0.01) in the bromochloromethane-treated animals compared with the controls (Table 2). The branched chain VFs, iso-butyrate and iso-valerate as a percentage of total VFA were higher (P < 0.01) in the bromochloromethane treatment as was valerate.

mcrA clone library and phylogenetic analysis

Amplicons generated from primers targeting the mcrA gene were analysed from two samples, one designated control, which was taken from animals that had not been dosed with bromochloromethane, and then a second treatment sample was extracted from the same animals after administration of bromochloromethane twice daily. One hundred randomly selected clones from the libraries were studied to ascertain library diversity and coverage. The Shannon diversity index calculations revealed the bromochloromethane library to be significantly different (P < 0.05) and more diverse than the control with values of 1.25 ± 0.26 and 0.75 ± 0.24, respectively. The control library had a lower evenness value (0.54) compared with inhibited library (0.70), indicating an over-representation of certain clones. The coverage of the libraries was almost complete, with values of 98% and 100% for the control and bromochloromethane libraries, respectively. Collector’s curves for richness observations (Chao1, ACE) were calculated using the furthest neighbour with an OTU cutoff of 20% for species. Chao1 estimate rarefaction curves leveled off and suggested a reasonable estimate of the true richness (data not shown).

Table 1. Animal productivity and methane production in steers with and without the antimethanogenic compound bromochloromethane

<table>
<thead>
<tr>
<th>Animal productivity</th>
<th>Control</th>
<th>Bromochloromethane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter intake (kg day⁻¹)</td>
<td>5.7 ± 0.11a</td>
<td>5.8 ± 0.07a</td>
</tr>
<tr>
<td>Average daily gain (kg day⁻¹)</td>
<td>0.5 ± 0.03a</td>
<td>0.5 ± 0.04a</td>
</tr>
<tr>
<td>Methane production*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mL min⁻¹</td>
<td>166 ± 7.1a</td>
<td>118 ± 6.6a</td>
</tr>
<tr>
<td>L kg⁻¹ dry matter intake</td>
<td>24 ± 1.9a</td>
<td>16 ± 0.9a</td>
</tr>
<tr>
<td>GE loss as CH₄ (MJ day⁻¹)</td>
<td>4.7 ± 0.2a</td>
<td>3.3 ± 0.19b</td>
</tr>
<tr>
<td>CH₄ energy (% GEI)</td>
<td>6.0 ± 0.47a</td>
<td>3.9 ± 0.24a</td>
</tr>
</tbody>
</table>

*Direct measure, the methane production values – L kg⁻¹ DMI are based exclusively on individual intakes recorded over a 12 h period when the animals were physically in the respiration chambers, GE, gross energy; GEI, GE intake.

Means in the same row that do not share common superscripts are significantly different (P < 0.05) n = 6.
and 77% identity to CLI19 showed a high level of identity at 99.3% to each other mobile each other and with values similar to Methanobacteriales nant.

noted that 56% of the clones clustered within the predominant order observed for the control sample showed that 74% of the clones were from the Methanobacteriales order. Deduced amino acid sequence similarity searches revealed that the most closely related sequences were uncultured rumen isolates that were obtained from a recently published study (93.2–99.2% identity) (Tatsuoka et al., 2004). In addition to these clones, CLI01 identified most closely to the Methanosphaera stadtmanae (AJ584650) mrtA gene with 90.4% identity. Several other clones clustered closest to an uncultured euryarchaeote clone OS55 (AF414019) from a landfill site in Odcombe, Somerset, UK (Luton et al., 2002), with 84–85.5% identity.

The phylogenetic placement of the clones based on their deduced amino acid sequences is presented in Fig. 2. Phylogenetic trees were constructed for both the DNA sequences (data not shown) and the deduced amino acid sequences of the cloned mcrA genes along with sequences downloaded from GenBank including recently published rumen isolated mcrA clones (Tatsuoka et al., 2004). No major differences were observed in the clustering pattern between the constructed DNA and amino acid trees (data not shown).

The second clone library generated from the same animals after dosing with the antimethanogenic compound bromochloromethane was constructed and analysed in the same manner. Of the 50 randomly picked clones, it was noted that 56% of the clones clustered within the predominant Methanobacteriales order as observed for the control sample. This was reflected in the evenness calculations for this library with a value of 0.70 compared with 0.54 for the control analysis. The remaining clones were not placed within the Methanobacteriales order; these included three clones CLI16, 18 and 21 that were 98–99% similar to each other and with values similar to Methanomicrobium mobile in the order Methanomicrobiales. Clones CLI14 and CLI19 showed a high level of identity at 99.3% to each other and 77% identity to Methanosarcina bakeri within the Methanosacinales order. Another clone (CLI45) clustered with the mrtA clone CLI01 and had a sequence identity of 94%. The remaining clones from this library formed two clusters: one with clone CLI09 and the other with CLI25 from the control library. All these clones shared 84–85% identity to the uncultured euryarchaeote clone OS55, except for clone CLI40, which had only 76% identity (Fig. 2). Within these two clusters, the members ranged in identities of 92–100% to each other.

### qPCR primer design and validation

Primers for the monitoring of general methanogenic populations were designed based on conserved amino acid sequences around the catalytic regions of the mcrA gene. Multiple alignments using deduced amino acid sequences indicated two conserved stretches c. 40 amino acids apart that would correlate to a PCR product of around 140 bp.

The reverse primer was designed to target a conserved region within the catalytic site GFGGQR, where the numbering from the mcrA sequence for Methanothermobacter thermoautotrophicus F443 and Y444 is involved in the cofactor F430 interaction-binding site and G445 is one of the five methyl-modified amino acids identified in the active site. The forward primer targets the conserved sequence FGGSQR, where the methyl-modified amino acid Q400 is located (Ermel et al., 1997). An initial DNA multiple alignment containing all rumen associated and representatives of other methanogenic groups revealed a high level of conservation for the sequence regions corresponding to the identified amino acid sequences.

A degenerate primer set was synthesized and tested against the previously isolated mcrA rumen clones from this study and against total rumen microbial DNA. Conventional PCR using this primer set on total rumen microbial DNA resulted in a single amplicon corresponding to 140 bp.

Sequencing of 24 clones derived from this amplicon all resulted in mcrA sequences (data not shown). An identical product size was observed when templates derived from the CLI clones (rumen mcrA libraries) were used. The qPCR assay conditions were optimized, resulting in primer and MgCl2 concentrations of 300 nM and 3 mM, respectively. Amplification efficiencies for the general methanogenic primer set were investigated by examining a serial dilution series of total microbial rumen DNA template, Methanobrevibacter ruminantium M1, and DNA from clones CLI06 and CLI09. The general methanogenic primers targeting the mcrA gene exhibited an efficiency of 1.95 on rumen microbial DNA 1.94 on Methanobrevibacter ruminantium M1 and 1.97 on the DNA from clones CLI06 and CLI09.

Standard curves for absolute quantification of methanogens were performed using DNA from Methanobrevibacter ruminantium M1 grown overnight in culture at 39 °C.
Fig. 2. Phylogenetic placement of deduced amino acid sequences for the mcrA gene. GenBank accession numbers are presented in brackets for those sequences used. CU01-10 and CU22-35 represent sequences obtained from the control period; CU11-21 and CU36-53 represent sequences obtained from bromochloromethane treatment period. Annotations in brackets following the CU clones represent the number of similar clones found within the C, control and B, bromochloromethane treatments. Bootstrap values > 50% are displayed based on 100 replicates (neighbour joining on top and parsimony on bottom). Methanopyrus kandleri was used as an outgroup reference. Scale bar represents 0.1 amino acid substitutions per site.
Dissociation curve analysis for the general methanogenic archaeal primer set produced a dissociation curve with a single peak at 81 °C when *Methanobrevibacter ruminantium* M1 DNA was used as a template, while two peaks at 81 and 86 °C were observed when using total rumen microbial DNA as a template. CLI clones from the *Methanobacteriaceae* family all possessed a single dissociation curve between 80 and 82 °C, while all other CLI clones produced a single dissociation curve at 84–86 °C. The *mrtA* gene sequence clone CLI01 produced a single dissociation curve peak at 81 °C.

### Population monitoring within the rumen

Methanogenic archaeal populations in the control showed no significant change in numbers across the monitoring period and reached a peak of $1.34 \times 10^9$ at 8 h (Fig. 3). When animals were supplemented with bromochloromethane, the methanogenic archaeal population was 3.1-fold or 67.5% lower ($4.36 \times 10^8$, $P < 0.05$) than the controls at 8 h after feeding (Fig. 3). Across the entire monitored period, there was an average decrease of 34% for the total number of methanogens. Dissociation curve analysis of the amplicons revealed that in the control animals, two peaks were clearly identifiable at 81 and 86 °C. However, there was a reduction in the 81 °C dissociation peak for animals treated with bromochloromethane.

### Discussion

The use of an antimethanogenic compound (bromochloromethane) has proven to be a useful tool to perturb the rumen population of methanogenic Archaea. This aided in the development and evaluation of a quantitative real-time PCR assay, which was used to correlate changes in the numbers of methanogens with methane production. The disturbance of the methanogenic population with this inhibitor apparently resulted in an increased diversity of the Archaea populations in an *mcrA* library and the identification of minor populations that have only recently been described (Luton et al., 2002).

The use of bromochloromethane to reduce methanogenesis using similar dose rates has been reported previously (Tomkinds & Hunter, 2004). In the current study, the reduction in methane production and acetate:propionate ratio, and increase in the molar proportion of branched chain VFAs due to bromochloromethane treatment are consistent with those reported by McCrabb et al. (1997). A reduction in the acetate:propionate molar ratio in rumen fluid has been described as a common feature of several antimethanogens (Mohammed et al., 2004a, b; Mwenya et al., 2004). This indicates a concurrent reduction of methane formation and redirection of hydrogen from methane to reduced short-chain fatty acids.

**Fig. 3.** qPCR data representing methanogenic archaeal populations with respect to the percentage of total rumen microbial 16S rRNA gene. Points represent the mean of six animal samples for the control (■) and bromochloromethane treatment period (●); error bars show SEs of the mean. Time represents hours at which samples were collected after feeding.

**mcrA clone libraries**

The initial characterization of the two *mcrA* clonal libraries revealed that the majority of clones sequenced from the rumen environment in this study are found to be closely related and all tend to cluster closely to *Methanobrevibacter ruminantium* within the Methanobacteriaceae family. This is consistent with the high coverage estimate values and previous observations whether the studies have used the 16S ribosomal gene or the *mcrA* gene as a phylogenetic marker (Tajima et al., 2001; Wright et al., 2004b; Tatsuoka et al., 2004). A recently published study investigating methanogenic diversity in the rumen of cattle using the *mcrA* gene only identified Archaea that were closely related to *Methanobrevibacter ruminantium* (Tatsuoka et al., 2004). Their study used an alternative primer set published by Hales et al. (1996), ME1 and ME2, which has been shown to have reduced diversity coverage of the methanogenic lineages (Edwards et al., 1998; Lueders et al., 2001) and therefore may have restricted their ability to amplify more diverse targets. It is apparent, however, that the majority of cloned methanogen-related sequences from the rumen of cattle and sheep are grouping within the *Methanobacteriales* order. In addition to this, it is commonly observed that isolates that cluster closely to *Methanosphaera stadtmanae* are detected (Tajima et al., 2001; Whitford et al., 2001; Wright et al., 2004b). This was also observed in this study when using the *mcrA* primers of Luton et al. (2002). However, the sequence generated from this clone aligned with the *mrtA* gene that encodes for the α-subunit of the methyl reductase isoenzyme methyl coenzyme reductase II. Owing to the high homology between these genes, amplification of the isoenzyme gene will occur. However, the *mrtA* genes do group as a single cluster close to the Methanococcales *mcrA* and can therefore be easily distinguished (Luton
et al., 2002). Only the four clones encoding the mrtA were observed from the generated libraries and there was no evidence of the equivalent mcrA sequence from an *Methano¬sphaera stadtmanae*-like isolate in the data collected. The very low detection of mrtA genes from the sequenced clones using the primers of Luton et al. (2002) would suggest that either most of the methanogens within the rumen possess only the mcrA gene or that those methanogens that possess the mrtA gene are present as a minor population. *Methano¬thermobacter thermautotrophicus* carries and expresses both reductase homologues, and studies on this strain revealed that under high H2 concentrations or during the initial periods of the growth stage, the mrtA is expressed while later in the growth stage or under reduced H2 concentrations the *mcrA* gene is switched on (Bonacker et al., 1992; Pihl et al., 1994). If a similar mechanism for these genes is present in rumen methanogens and considering that the partial pressure of H2 is maintained at low levels within the rumen then perhaps the *mrtA* gene has become redundant within this system. A more extensive survey of rumen samples would be required because differences could exist between ruminant species fed roughage- or concentrate-based diets.

The clonal library generated from the bromochloromethane treatment period, where methanogen populations were reduced, revealed greater diversity through the observation of clonal isolates from most other methanogenic orders that may have been masked previously by the dominant methanogenic rumen populations. However, it could be interpreted that the dominant methanogens in the control were more sensitive to the bromochloromethane and other methanogenic orders that were less sensitive filled the niche vacated by the dominant methanogens. As bromochloromethane inhibits cobamide-dependent methanogenesis, it may also be possible that some of the populations detected after bromochloromethane treatment may possess a noncobamide-dependent pathway and are therefore not inhibited like the cobamide-dependent *Methanobrevibacter ruminantium*-like Archaea. Earlier studies investigating *Methanobacterium M.o.H* allude to a noncobamide dependent pathway when no evidence could be found of cobamide-containing proteins in extracts from this microorganism (Wolfe, 1971).

A previous study using 16S ribosomal clone libraries from the rumen (Tajima et al., 2001) identified the presence of a unique archaeal cluster more closely related to thermoacidophilic Archaea than other methanogenic isolates. A further independent study indicates that this group is an indigenous population within the rumen (Wright et al., 2004b). A similar cluster has also been identified in this study using the mcrA gene, containing rumen clones that group closely with an uncultured archaeal clone OS55. Many studies have now concluded that a similar branching order is conserved when using the 16S ribosomal gene or the deduced amino acid sequence of the *mcrA* gene for the purpose of inferring phylogeny (Lueders et al., 2001; Luton et al., 2002; Hallam et al., 2003). This distant cluster within the *mcrA* phylogenetic tree is observed to branch in a position similar to the unique cluster of 16S ribosomal gene sequences identified previously (Tajima et al., 2001; Wright et al., 2004b). However, as bootstrap values were not obtained for this branch order, at this stage, it is not possible to infer whether this cluster relates to that observed by Tajima et al. (2001). Isolation of representatives from this cluster will allow for a more thorough investigation.

**qPCR analysis of rumen methanogen population**

The methanogen qPCR primers used in this study were observed to only amplify mcrA sequences because all the cloned amplicons sequenced from rumen microbial DNA using the qPCR primer set were found to be highly homologous to previously sequenced *mcrA* gene sequences. However, it is important to note that when the *mrtA* clone CLI01 was used as a template for the qPCR primer set, a product was detected showing that these primers would also be capable of amplifying the *mrtA* gene. Although based on only minor representation of *mrtA* gene sequence in the clone libraries, it would seem that the *mcrA* gene is the predominant methyl reductase gene within the rumen and values calculated using the qPCR primers are likely to reflect the detection of this gene.

The administration of bromochloromethane was observed to elicit a rapid reduction in methane release from the rumen with a reduction of 59% within 2 h of dosing compared with the control period at the same time point (Fig. 1). However, it was not until 8 h after dosing that any significant decrease in methanogen population numbers was observed. Bromochloromethane is known to inhibit the cobamide-dependent methyl transferase step in the release of methane (Wood et al., 1968; Johnson et al., 1972); inhibition of this enzymatic step would immediately have an effect on methane emission levels, which is clearly observed here. The observed delay for a reduction in methanogen numbers would suggest that inhibition of methanogenesis affected the growth of organisms, which was reflected in a decline in numbers several hours after initial exposure to the highest concentration of the inhibitor. During normal ruminal flow dynamics, bromochloromethane will be removed from the rumen, and depending on the initial dose may fall below inhibitory concentrations. Hence, any targeted abatement strategy that is affected by ruminal flow dynamics must consider this observation.

Dissociation curve analysis of the qPCR amplicons revealed that there were two distinct peaks observed for animals on a control diet. The peak at c. 81°C was similar.
to that of the clonal isolates that cluster with Methanobrevibacter ruminantium, while the second peak present at around 86 °C corresponded to that of the isolates from the other methanogenic orders. Although these peaks were present at similar fluorescent intensities within control animals, they are not quantitative as they represent an ‘end point’ analysis of the PCR. The analysis of the clone library would suggest that these populations are not present in equal amounts especially when considering the disproportionate representation of the clones in the library from the control diet based on calculated evenness values. Dissociation curve analysis of the bromochloromethane-treated animals revealed a reduction in the fluorescence intensity of the 81 °C peak, but no reduction in the 86 °C peak. This observation was also reflected in libraries constructed from these samples, where only 52% of the clones were observed to be similar to the Methanobacteriales order and an increase in the evenness of the library. A change in the methanogen population diversity in response to an inhibitor has not been reported previously but should be considered as a possible consequence of manipulating this functional group through vaccine and chemical interventions as mitigation strategies. In previous attempts to vaccinate against methanogens, it was only the Methanobrevibacter spp. vaccine that significantly reduced methane emission (Wright et al., 2004a). This lends further support to the conclusion that these species are the predominant methane producers within the rumen. The design of primers to specifically target these subpopulations in the rumen are being designed so that the effect of antimethanogenic-mediated abatement techniques on these discrete populations can be determined quantitatively. In addition, primers that can distinguish between the Methanobacteriales order with a dissociation peak of 81 °C and the other groups with a dissociation peak of 86 °C could prove useful in truly determining the methanogen population shifts when subjected to treatment with bromochloromethane.

Previous studies have not specifically monitored the effect that such treatments elicit on the methanogenic populations within the rumen. The adoption of molecular techniques such as qPCR for monitoring specific populations within complex environments provides advantages such as rapidity and specificity over traditional microbiological techniques like microscopy and isolation.

Acknowledgements

Laurie Conlan, Dean Gibson and Karina Tane are thanked for their technical assistance in collecting the rumen samples. This work was partially supported by Meat Livestock Australia and the Australian Greenhouse Office.


