RESEARCH ARTICLE

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

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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 methanongens (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 (CO_2-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 B_{12} 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 (Lueders *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 *Methanobrevibacter* spp. and the other 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 $\pm$ 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, at a rate of 0.3 g/100 kg live weight, was added to the cotton seed meal. The antimethanogen was a halogenated aliphatic hydrocarbon, bromochloromethane, encapsulated in the α-cyclodextrin matrix (May *et al.*, 1995). Staff at the Australian Animal Health Laboratories (AAHL) prepared the antimethanogen complex, containing 10–12% bromochloromethane by weight for all experimental periods. The bromochloromethane formulation, mixed in 100 g cottonseed meal (CSM), was also fed 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* intake was 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 CO_2 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 $\times$ 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 $-70\,^{\circ}$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′-GGTGGTGTMGGATTCACACARTAYGCWACAGC and *mcrA* reverse primer 5′-TTCATTGCRTAGTTWGGRTAGTT (Luton *et al.*, 2002). Reactions were performed in 30 μL volumes with the addition of 2.5 mM $MgCl_2$ 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 (CLI01–CLI10) 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. Phylogentic 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 $E = H/H_{max}$, where $H_{max} = \ln S$ (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 (qmcrA-F) 5′-TTCGGTGGATCDCARAGRGC was designed to target the conserved amino acid sequence FGGSQR while the reverse primer (qmcra-R) 5′-GBARGTCGWAWCCGTAGAATCC targeted the GFYGYDL 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 $MgCl_2$ concentrations. An optimal primer concentration of 300 nM, a final $MgCl_2$ 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

Scientific Instruments, West Sussex, UK) at a × 400 magnification. DNA was then extracted from a known number of cells and used in a 6-log dilution series (10^9–10^4 cells)

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.

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

	Experimental period	
	Control	Bromochloromethane
Animal productivity		
Dry matter intake (kg day^{-1})	5.7 ± 0.11^a	5.8 ± 0.07^a
Average daily gain (kg day^{-1})	0.5 ± 0.03^a	0.5 ± 0.04^a
Methane production*		
mL min^{-1}	166 ± 7.1^a	118 ± 6.6^b
L kg^{-1} dry matter intake	24 ± 1.9^a	16 ± 0.9^b
GE loss as CH_4 (MJ day^{-1})	4.7 ± 0.2^a	3.3 ± 0.19^b
CH_4 energy (% GEI)	6.0 ± 0.47^a	3.9 ± 0.24^b

*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 ($P < 0.05$) $n = 6$.

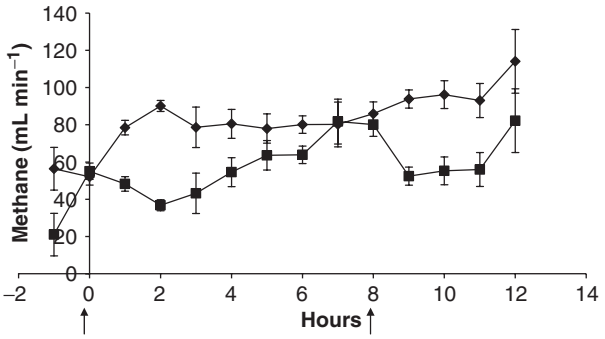

Fig. 1. Twelve-hour methane production for steers during the control (◆) and bromochloromethane treatment period (■). Arrows indicate twice-daily dose of bromochloromethane. Data are presented as the average methane production rate (mL min^{-1}) for each hour.

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 VFAs, 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 administrationof 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 overrepresentation 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 2. Volatile fatty acid ratios and molar proportions (%) in steers with or without the antimethanogenic compound bromochloromethane

	Experimental period	
	Control	Bromochloromethane
Total VFA (mM)	106.43 ± 1.96	106.81 ± 2.45
Acetate : propionate	4.69 ± .06[a]	4.45 ± 0.05[b]
VFA (% total VFA)		
Acetate	79.59 ± 1.51	77.97 ± 1.71
Propionate	16.99 ± 0.34	17.59 ± 0.49
N-butyrate	7.61 ± 0.19	8.04 ± 0.37
Iso-butyrate	0.65 ± 0.03[a]	0.90 ± 0.02[b]
N-valerate	0.67 ± 0.02[a]	1.02 ± 0.05[b]
Iso-valerate	0.93 ± 0.04[a]	1.29 ± 0.05[b]

Values with a different superscript in the same row are significantly different ($P < 0.01$).

Examination of clones from 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 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 GFYGYDL where the numbering from the *mcrA* sequence for *Methanothermobacter thermoautotrophicus* F443 and Y444 is involved in the cofactor F_{430} 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 (Ermler *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 $MgCl_2$ 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.

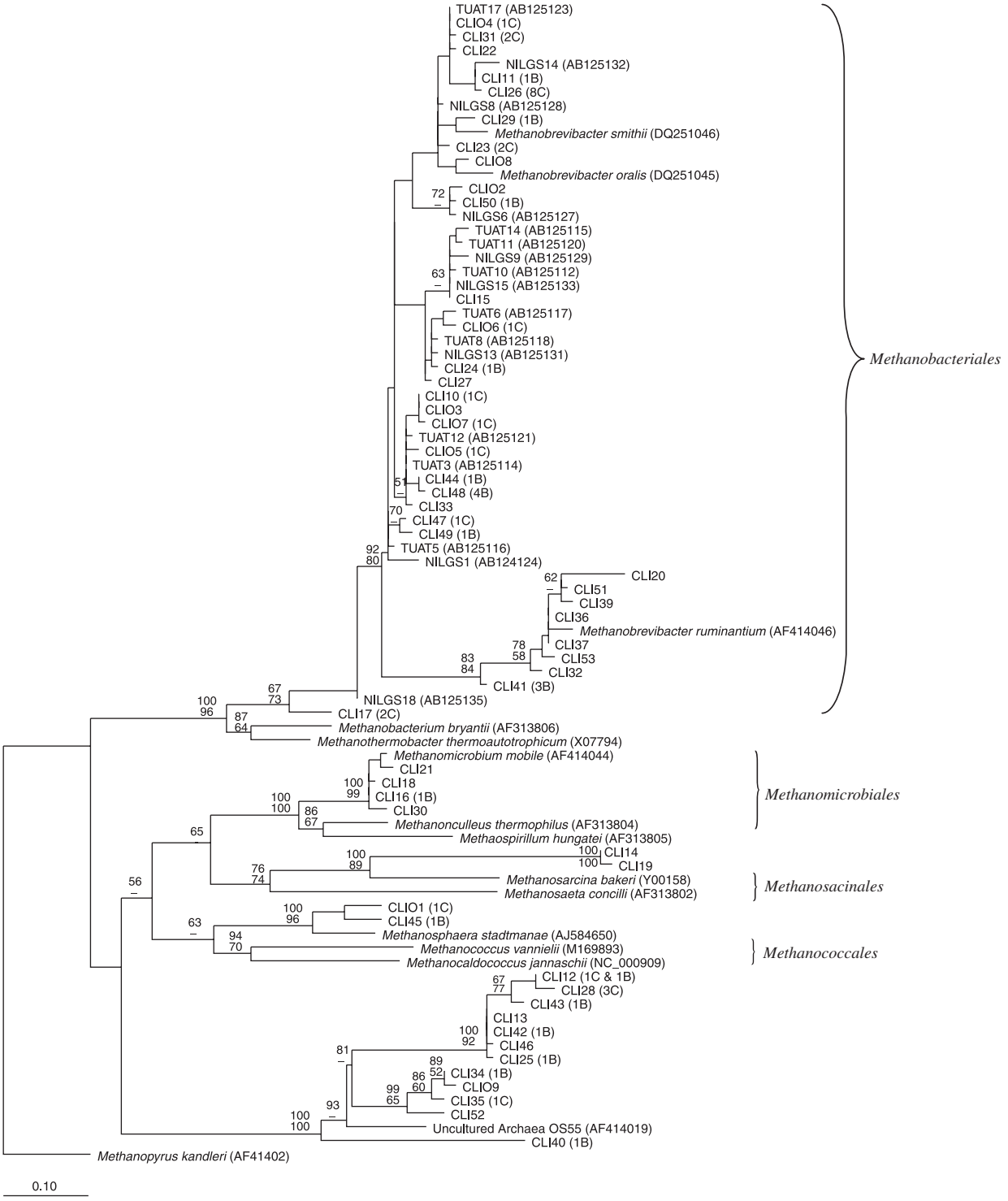

Fig. 2. Phylogenetic placement of deduced amino acid sequences for the *mcrA* gene. GenBank accession numbers are presented in brackets for those sequences used. CLI01-10 and CLI22-35 represent sequences obtained from the control period; CLI11-21 and CLI36-53 represent sequences obtained from bromochloromethane treatment period. Annotations in brackets following the CLI 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 1.34×10^9 at 8 h (Fig. 3). When animals were supplemented with bromochloromethane, the methanogenic archaeal population was 3.1-fold or 67.5% lower (4.36×10^8, $P < 0.05$) 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 (Tomkins & 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.

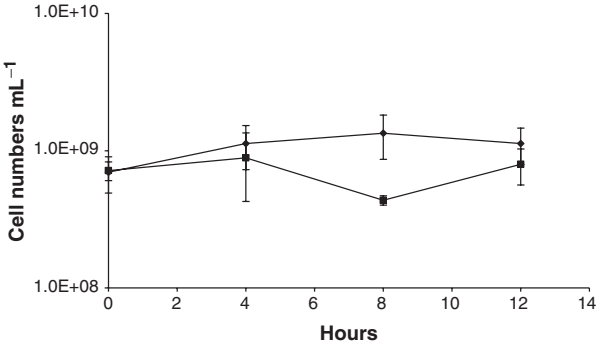

Fig. 3. qPCR data representing methanogenic archaeal populations with respect to the percentage of total rumen microbial 16S rRNA gene. Points represent the mean of six animal samples for the control (◆) and bromochloromethane treatment period (■); error bars show SEs of the mean. Time represents hours 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 *Methanosphaera 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. *Methanothermobacter thermautotrophicus* carries and expresses both reductase homologues, and studies on this strain revealed that under high H_2 concentrations or during the initial periods of the growth stage, the *mrtA* is expressed while later in the growth stage or under reduced H_2 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 H_2 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.

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