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Diversity of Prokaryotes in the Rumen of Steers Fed a Diet Supplemented with or without Bromochloromethane, an Anti-methanogenic Compound

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Abstract

The effect of bromochloromethane-cyclodextrin (BCM-CD) on the microbial community structure in the rumen of steers was examined in this study. Two animals were fed a diet supplemented with BCM-CD (1.0 g/100 kg body weight/day) for two weeks, while two other animals were fed a basal diet during the same experimental period to serve as controls. Two 16S rRNA gene clone libraries were constructed from the rumen fluid of both the control and BCM-treated animals. DNA sequences from the libraries were searched for homology. The statistical differences between both libraries were examined by LIBSHUFF analysis. The DNA sequences of each library were taxonomically classified and the proportion was calculated. A total of 107 (control) and 110 (BCM-treated) clones were analyzed, and the Chao-1 species estimate was 1.6-fold higher in BCM-treated samples. LIBSHUFF comparison showed a statistically significant difference between the two clone libraries. No methanogenic archaeal clone was detected in the BCM-treated clone library. Within *Firmicutes*, the proportion of *Lachnospiraceae* clones in the BCM-treated library was 4.5-fold higher than that in the control library. The proportion of *Prevotellaceae* clones was 1.7-fold higher in the BCM-treated library than in the control library. These results suggest that BCM-CD indirectly affects eubacterial community in the rumen.

Discipline: Animal Science
Additional key words: methanogenic archaea, methanogenesis, microbial community, rumen

Introduction

Dietary components ingested by ruminants undergo microbial digestion and subsequent fermentation in the rumen. Short-chain fatty acids, H_2, and CO_2 are produced as fermentation end-products. Methane is produced by methanogenic archaea from H_2 and CO_2 in the rumen, and then emitted by eructation into the atmosphere. The CH_4 emitted from ruminants contributes to approximately 15% of the total atmospheric methane emissions (Takahashi et al. 2005). Therefore, ruminant livestock are major contributors to global warming. Moreover, methanogenesis in the rumen causes a 2-12% loss of energy by the host animals (Czerkawski 1969). Mitigation of methane emissions from the rumen is therefore necessary.

The effects of various anti-methanogenic compounds have been investigated to understand the mechanisms involved in suppressing methane production. Bromochloromethane (BCM) is among the strongest methane inhibitors used in experimental methane inhibition. Bromochloromethane, encapsulated in a cyclodextrin matrix (BCM-CD), is often used experimentally as an anti-methanogenic agent (Abecia et al. 2012, Denman et al. 2015, McCrabb et al. 1997, Mitsumori et al. 2014). The supplementation of BCM-CD influences the molecular diversity of homoacetogens in the rumen of the animals (Mitsumori et al. 2014). Denman et al. (2007) investigated the quantity and diversity of methanogens in the rumen of Brahman-crossbred steers fed a diet supplemented with BCM-CD. The methanogen population was lower in the BCM-treated animals than in the control 8 h after feeding. The diversity of methanogens significantly decreased with the supplementation of

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BCM-CD. Abecia et al. (2012) surveyed the effect of BCM-CD on the methanogenesis, rumen fermentation, lactating performance, and milk-fatty acid of lactating dairy goats. They found that the administration of BCM-CD did not affect the population density of total bacteria, protozoa, and methanogens. Mitsumori et al. (2012) fed goats a diet containing a low, mid-, and high doses of BCM-CD. They monitored the microbial community in the rumen using denaturing gradient gel electrophoresis and real-time PCR assay. Populations of *Fibrobacter succinogenes* and some groups of *Prevotella* increased in a dose-dependent manner. More recently, Denman et al. (2015) examined the microbial community structure and metagenome in the rumen of goats fed a diet supplemented with BCM-CD using next-generation sequencing analysis. Thus, information on microbial community structures in the rumen of animals fed diets supplemented with BCM-CD is still limited. In this study, the microbial community structure of prokaryotes in the rumen of steers fed a diet with or without BCM-CD was analyzed using a 16S ribosomal RNA gene clone library.

Materials and methods

1. Animals and sampling

The experiments were described in our previous work (Itabashi et al. 1999). Briefly, four Holstein steers (with average body weight of 572 kg) were fed whole crop sorghum silage as a basal diet at a maintenance level, twice daily at 09:00 and 16:00 hours. The anti-methanogenic compound BCM-CD was used. Two of the animals were fed a diet supplemented with BCM-CD (1.0 g/100 kg body weight/day) for two weeks, while the other two animals were fed the basal diet during the same experimental period to serve as controls. Rumen content was collected on the last day of each experimental period via the oral cavity using a flexible stainless-steel stomach tube. The same rumen contents were used as described in Itabashi et al., 1999. The rumen contents were strained through two layers of surgical gauze and stored at −80°C until DNA extraction.

2. Extraction of DNA and construction of clone libraries

The total DNA from the rumen contents of the control samples and BCM-CD supplemented samples was extracted using the bead-beating method and purified according to Tajima et al. (2001). Concentration of the DNA was adjusted to 15 ng μL^{-1}.

The DNA samples from the individual animals were pooled together for each treatment. The combined DNA was used as a template for constructing the clone library. The clone library was constructed as described by Matsui et al. (2010). Forward (530F; 5′-GTGCCAGCMGCCGCGG-3′) and reverse (1392R; 5′-ACGGGCGGTGTGTRC-3′) primers were used to amplify 16S ribosomal RNA gene fragments with M (A and C) and R (A and G) representing degeneracy. The following parameters were used for amplification: an initial denaturation step at 95°C for 2 min; followed by 15 thermal cycles consisting of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min; and a final extension step at 72°C for 10 min. PCR products were electrophoresed on a 1% agarose gel in a Tris-acetate EDTA buffer and visualized with ethidium bromide staining. The PCR products were cloned with a TA Cloning Kit (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol. Positive clones were randomly selected.

3. DNA sequence and phylogenetic analysis

The cloned DNA fragments in the positive clones were sequenced using the BigDye® Terminator v.3 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on the ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). The DNA sequences were searched for homology with the BLAST program (Altschul et al. 1997). Chimeric artifacts of 16S rDNA by PCR were checked with CHECK_CHIMERA, an online program of the Ribosomal Database Project (RDP-II) (Cole et al. 2003), and then removed from the analysis. For the operational taxonomic unit (OTU) assignment, calculations of Chao-1 and the Shannon-Wiener index were carried out with the mothur program (Scholoss et al. 2009) based on a 98% sequence similarity criterion. Each sequence was classified by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 128; http://www.arb-silva.de) using blastn (version 2.2.30; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings. The proportion of the classified sequence was calculated by SILVAngs 1.3 (Quast et al. 2013).

4. Nucleotide accession number

All nucleic acid sequences obtained in this study were deposited in the DDBJ, EMBL, and GenBank databases under accession numbers LC341705-LC341811 for the control animals and LC341812-LC34121 for the animals treated with BCM.

Results

The effects of BCM-CD supplementation on ruminal parameters have been published in Itabashi et al. (1999). The supplementation of animal feed with BCM-CD decreased methane production by 95% and considerably

increased H_2 production *in vivo*. Moreover, the molar proportion of propionate in the rumen fluid increased with BCM treatment.

1. Diversity indices of 16S rDNA clone library

Table 1 summarizes the diversity indices of the two clone libraries. Here, 111 (control) and 116 (BCM-treated) clones were sequenced. Four and six chimera sequences were detected in the control and BCM-treated libraries, respectively, and then removed from further analysis. Thus, 107 (control) and 110 (BCM-treated) clones were analyzed (Table 1). The sequences were grouped into 82 and 88 OTUs in the control and BCM-treated libraries, respectively. Diversity, represented by the Shannon-Wiener index (*H′*), of the control and BCM-treated samples was similar. However, the Chao-1 species estimate was 1.6-fold higher in BCM-treated samples. The rarefaction curves of the clones of both libraries showed similar trends (Fig. 1).

2. LIBSHUFF comparison of the libraries

To determine whether BCM treatment affects a clone library, the two libraries were statistically compared using LIBSHUFF analysis (Table 2). Despite *H′* not being different, the LIBSHUFF comparison showed a statistically significant difference between both clone libraries ($P<0.05$).

3. Composition of the clone library

In the control library, five out of 107 clones showed ≥ 98% homology against known bacterial species (data not shown). In the BCM-treated library, nine out of 110 clones showed ≥ 98% homology against known bacterial species. No clone in either library could be classified as a known acetogen.

Table 3 shows the differences in microbial composition between the control and BCM-treated libraries. The clones were classified and the proportion against the total clone number was calculated.

Although the clone library from the control animals consisted of both eubacteria and methanogenic archaea, no methanogenic archaeal clone was detected in the

Table 1. Diversity indices of two clone libraries derived from the rumen of steers fed a diet with or without bromochloromethane

Item	Control	BCM-treated
Number of clones	107	110
Number of OTUs	82	88
Shannon-Wiener index	4.280	4.338
Chao-1 species estimate	249	405

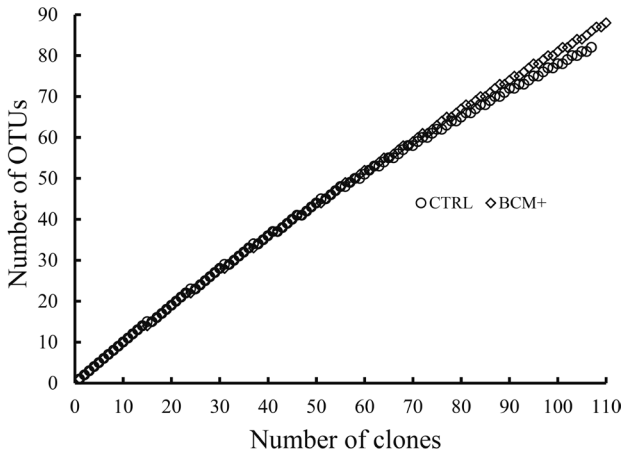

Fig. 1. Rarefaction curve of each clone library
CTRL: Control library; BCM+: BCM-treated library

Table 2. LIBSHUFF comparison of the control (X) and BCM supplementation (Y) libraries

Comparison	dCXYScore	*P*-value*	Significantly different
Control - BCM-treated	0.00240	0.0015	*yes*
BCM-treated - Control	0.00125	0.0223	

*Critical *P*-value is defined as a value＜0.025 in LIBSHUFF.

Table 3. Classification and proportion (%) of each clone

Phylum	Group	Control	BCM-treated
Archaea	Methanogen	5	0
Firmicutes	Ruminococcaceae	23	15
	Christensenellaceae	19	11
	Family XIII	5	0
	Lachnospiraceae	4	18
	Acidaminococcaceae	2	2
	Lactobacillales	0	2
	Others	1	1
	Subtotal	54	49
Bacteroidetes	Prevotellaceae	21	35
	Rikenellaceae	5	5
	BS11 gut group	4	2
	S24-7 group	3	3
	Others	1	0
	Subtotal	34	45
Mollicutes	RF9	4	2
Proteobacteria		2	0
Actinobacteria	Coriobacteriaceae	2	3
Others		1	2

BCM-treated clone library.

In both libraries, the majority of clones (88% for the control, 94% for the BCM-treated library) belonged to two phyla-*Firmicutes* and *Bacteroidetes*. The proportion of clones belonging to *Firmicutes* was slightly higher in the control library than in the BCM-treated library (54% *vs.* 49%). Within *Firmicutes*, the proportion of clones from *Lachnospiraceae* was 4.5-fold higher in the BCM-treated library than in the control library. Although no clones of the order Lactobacillales were detected in the control library, these clones were detected in the BCM-treated library. However, the proportion of clones from *Ruminococcaceae*, *Christensenellaceae*, and *Clostridiales* (Family XIII) were lower in the BCM-treated library than in the control library.

The proportion of clones from the phylum *Bacteroidetes* was higher in the BCM-treated library than in the control library (45% *vs.* 34%). The proportion of clones from the family *Prevotellaceae* was 1.7-fold higher in the BCM-treated library than in the control library. There was no change in the proportion of clones from *Rikenellaceae* and the S24-7 group of *Bacteroidetes* between both libraries.

Discussion

In the present study, the effects of the supplementation of a steer diet with BCM-CD on the diversity and community structure of rumen microbiota were examined. Although a small change in *H′* was observed, both the Chao-1 species richness and the proportion of *Prevotellaceae* clones increased. BCM is a halogenated methane analogue that can inhibit apparent methane production. This analogue reacts with reduced vitamin B_{12} to inhibit the cobamide-dependent methyltransferase step of methanogenesis (Bayaru et al. 2001, Wood, Kennedy & Wolfe 1968). BCM directly and specifically inhibits methanogens in the rumen (Itabashi et al. 1999). Therefore, changes in the microbial community structure could be an indirect effect of BCM. Administration of BCM also led to an increase in H_2 gas production and a fermentation shift to produce more propionate in the rumen (Itabashi et al. 1999). In the present study, propionate-producing *Prevotellaceae* clones increased proportionally with the administration of BCM-CD. Therefore, the shift in fermentation could be due to the increased microbial population of *Prevotellaceae*. Denman et al. (2015) demonstrated through metagenomic analysis that the abundance of reads associated with enzymes of the randomizing pathway from pyruvate was 2-fold higher in the rumen of goats treated with large doses of BCM (5 g/100 kg LW) than in the rumen of the control animals. The *Prevotellaceae* group has the highest representation of the gene for the propionate pathway (Denman et al. 2015). Mitsumori et al. (2012) observed that two of the *Prevotella* clusters increased in abundance at high BCM dosing levels by real-time PCR assay. Therefore, an increase in the proportion of *Prevotellaceae* clones observed in this present study is in agreement with these two publications.

Despite the proportion of Firmicutes clones decreasing in the BCM-treated library, the proportion of clones affiliated with *Lachnospiraceae* and *Lactobacillales* increased. Bacteria included in these groups can produce lactate as a fermentation product. Metabolic pathways that produce lactate from pyruvate can incorporate H_2. Excess H_2 resulting from the inhibition of methanogenesis might be used for the production of lactate. Furthermore, *Lachnospiraceae* contains ruminal acetogens, such as *Acetitomaculum ruminis*, *Blautia producta*, and *Blautia schinkii*. Although no known acetogens were detected in the BCM-treated library, 'possible' ruminal acetogens may be present. Therefore, an increased proportion of *Lachnospiraceae* clones may be due to an increased proportion of 'possible' ruminal acetogens.

The present study did not examine the populations of hydrogen-producing eukaryotes, anaerobic rumen fungi, and rumen protozoa. Therefore, further study should be conducted to analyze diversity and quantify fungi and protozoa.

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