Biodiversity and composition of methanogenic populations in the rumen of cows fed alfalfa hay or triticale straw

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Abstract

It is clear that methanogens are responsible for ruminal methane emissions, but quantitative information about the composition of the methanogenic community in the bovine rumen is still limited. The diversity and composition of rumen methanogens in cows fed either alfalfa hay or triticale straw were examined using a full-cycle rRNA approach. Quantitative fluorescence in situ hybridization undertaken applying oligonucleotide probes designed here identified five major methanogenic populations or groups in these animals: the Methanobrevibacter TMS group (consisting of Methanobrevibacter thaueri, Methanobrevibacter millerae and Methanobrevibacter smithii), Methanobrevibacter ruminantium-, Methanosphaera stadtmanae-, Methanomicrobium mobile-, and Methanimicrococcus-related methanogens. The TMS- and M. ruminantium-related methanogens accounted for on average 46% and 41% of the total methanogenic cells in liquid (Liq) and solid (Sol) phases of the rumen contents, respectively. Other prominent methanogens in the Liq and Sol phases included members of M. stadtmanae (15% and 33%), M. mobile (17% and 12%), and Methanimicrococcus (23% and 9%). The relative abundances of these methanogens in the community varied among individual animals and across diets. No clear differences in community composition could be observed with dietary change using cloning techniques. This study extends the known biodiversity levels of the methanogenic communities in the rumen of cows.

Introduction

Archaea constitute about 3–4% of the total microbial numbers in the rumen with the majority of this domain being methanogens (Lin et al., 1997; Sharp et al., 1998; Yanagita et al., 2000; Ziemer et al., 2000). Most ruminal methanogens utilize H2 or formate as substrates and derive energy from the reduction of CO2 to CH4. In the rumen, polymeric carbohydrates and proteins are degraded to volatile fatty acids, NH4+, CO2, and H2. Disposal of H2 through the reduction of CO2 to CH4 is essential to prevent the accumulation of reducing equivalents and the overall impedance of ruminal fermentation. Methane is a potent greenhouse gas with a global warming potential that is 25 times that of CO2 and emissions can account for 2–12% of the gross energy intake of the host (Ellis et al., 2007). Methanogenesis leads to a loss of dietary energy and contributes to climate change, which together emphasize that a better understanding of the microbiology of methanogenesis is essential for greenhouse gas mitigation and improvement of production efficiency in the livestock industry (Janssen & Kirs, 2008).

Eight methanogenic species have been isolated, cultured, and identified from the bovine rumen. They are Methanobrevibacter smithii, Methanobrevibacter ruminantium, Methanobrevibacter thaueri, Methanosphaera stadtmanae, Methanobacterium aarhusense, Methanobacterium formicicum, Methanosarcina barkeri, and Methanomicrobium mobile (Jarvis et al., 2000; Whitford et al., 2001; Nicholson et al., 2007; Wright et al., 2007). However, many of the methanogens identified through sequencing techniques have not been cultured in the laboratory, but
two genera, Methanobrevibacter and Methanomicrobium, and a clade that is distantly related to the genus Thermoplasma [also referred as the RCC cluster (Janssen & Kirs, 2008)] appear to account for the majority of rumen methanogens (Wright et al., 2007; Hook et al., 2009; King et al., 2011; Tymensen et al., 2012).

Most studies examining changes in the composition of ruminal methanogenic communities have used 16S rRNA gene clone library analyses. However, such data cannot be assessed quantitatively (Head et al., 1998) and only a few studies have attempted to quantify individual rumen methanogenic community members. Using reverse transcriptase qPCR, Zhou et al. (2009) reported that the size of ruminal methanogenic community was not affected in cattle in response to a low or high energy diet. With qPCR, Tymensen et al., 2012) showed that switching the diet from forage to high-grain resulted in a profound reduction in total RCC abundance and an increase in the relative abundance of free-living Methanobrevibacter spp. However, all such studies have been carried out at the whole community or genus level, and the composition of the methanogenic community at the species level is less clear.

In this study, the full-cycle rRNA approach (Amann et al., 1995) was used to investigate the composition of methanogenic populations in the liquid (Liq) and solid fraction (Sol) of the rumen digesta of cows fed a high-quality forage in the form of alfalfa hay or a low-quality forage in the form of triticale straw. 16S rRNA gene clone libraries were constructed using pooled DNA templates from rumen DNA of individual animals fed the same or different diet(s). Oligonucleotide 16S rRNA gene-targeted probes were designed against sequences retrieved from clone libraries. Quantitative fluorescence in situ hybridization (qFISH) was used to estimate the relative abundances of individual members of the methanogenic populations to investigate changes among individual animals and diets.

Materials and methods

Sample collection and fractionation

Sample collection and fractionation of the rumen digesta were carried out as described previously (Kong et al., 2010a). Briefly, four mature rumen-cannulated dry Holstein cows were used in this crossover study. In the first 1-month period, two cows were fed alfalfa hay plus 2 kg of a dry cow protein/mineral supplement, while the other two were fed triticale straw plus 2 kg of supplement. Rumen samples were obtained in the last week of this period and then the diets were switched at the start of the second 1-month period. The second set of rumen samples were collected in the last week of this second period. Before the cattle were fed, rumen digesta was collected from three sites (reticulum, dorsal, and ventral sac) within the rumen, thoroughly mixed and a subsample was placed in 200-mL air-tight centrifuge tubes and immediately transported to the laboratory. Before DNA extraction, rumen samples from each animal were fractioned into liquid, feed particle loosely associated and feed particle adherent fractions as described by Kong et al. (2010a).

FISH sample fixation and FISH probing

Samples for FISH were prepared as described by Kong et al. (2010b). Briefly, digesta samples were collected from the rumen as described above. On-site, the mixture (100 mL) was then transferred to a heavy-wall 250-mL beaker and squeezed using a Bodum (Bodum Inc., Triegen, Switzerland) coffee maker plunger. The extruded liquid samples were fixed in paraformaldehyde (PFA, 4% final concentration) for FISH probing of planktonic methanogens (i.e. those in the Liq phase). The remaining liquid was discarded, and the squeezed particulate samples containing particle-associated cells were also fixed in 4% PFA. After 3 h fixation, these particulate samples together with 4% PFA solutions were ‘stomached’ (Stomacher 400 Circulator, Seaward, UK) at 230 r.p.m. for 6 min. Stomached samples were then transferred into clean 250-mL beakers and squeezed again. Filtrates were centrifuged (5000 g) to recover cells, which were then washed three times with 10 mL 1× PBS buffer, resuspended in 50% ethanol (ca. 8–10 g L\(^{-1}\) dry weight) and stored at \(-20^\circ\)C until FISH probing of methanogens associated with particulate digesta was executed (i.e. methanogens in the Sol phase).

FISH was carried out according to Amann (1995) on glass cover slips coated with gelatin (Kong et al., 2010a) according the procedure described by Xia et al. (2012). Compositions of the methanogen communities in the Liq and Sol were screened with previously described oligonucleotide probes targeting methanogens at the family or order level. Those used were MB1174 (Raskin et al., 1994) for members of the Methanobacteriales, MS1414 (Raskin et al., 1994) for Methanosarcinaceae, MX825 (Raskin et al., 1994) for Methanosetaeaceae and MG1200b (Crocetti et al., 2006) for Methanomicrobiales. The probe ARC915 (Stahl & Amann, 1991) was used to estimate the total numbers of Archaea in each sample. Hybridization conditions and optimal formamide concentrations for these probes are listed in probeBase (Loy et al., 2003). 4′,6-diamidino-2-phenylindole (DAPI) staining of biomass samples to determine total cell numbers was carried out after FISH probing. DAPI (100 μL, 0.003 mg mL\(^{-1}\)) was
added to air-dried FISH glass cover slips, which were then kept at room temperature for 10 min before being gently washed by dipping each slide in distilled water 3–5 times. Slides were air-dried and examined microscopically.

**DNA extraction, PCR amplification, clone library construction, and sequencing**

Total DNA was extracted from each of the three isolated fractions from rumen digesta for each individual animal and pooled in equal molar amounts for PCR amplification as described previously (Kong et al., 2010b). Clone libraries were then constructed for each individual animal for each of the two diets fed. The sequence of FISH probe MB1174 (Raskin et al., 1994; Crocetti et al., 2006) was used to make a reverse PCR primer (renamed as Met1174r: 5′-CAG GCC TAA CAC ATG CAA GTC-3′), and the universal methanogenic primer Met86f (5′-GCT CAG TAA CAC GTG G-3′) (Wright & Pimm, 2003) was used as a forward primer to amplify 16S rRNA genes of the methanogenic community using AmpliTaq polymerase Gold (Applied Biosystems, Carlsbad, CA).

The PCR cycle used consisted of a 5 min denaturation period at 95 °C followed by 25 cycles of 95 °C 1 min, 55 °C 1 min and then 72 °C 1.5 min. After 25 cycles, an 8 min extension period at 72 °C was added. Clone libraries were built using a TOPO TA Cloning Kit (Invitrogen Canada Inc., Burlington, ON, Canada) with electrocompetent cells following the protocol provided by the manufacturer. Individual clones were picked with a Qpix II (Genetix USA Inc., MA) into 96-well plates (Genetix). Sequencing of these was carried out by Polymorphic (Polymorphic DNA Technologies, Inc., CA) using an ABI3730XL Genetic Analyzer with a 50 cm capillary array.

**Phylogenetic and biodiversity analyses**

Partial 16S rRNA gene sequences were assembled using SEQUENCER 4.5 (Gene Code Cooperation) and screened using the Chimera check program provided in RDP-II (Cole et al., 2005) and Bellerophon (Huber et al., 2004). For phylogenetic analysis, 16S rRNA gene sequences were pooled and submitted to the CLASSIFIER tool provided in RDP-II. Trees in Figs 1–3 were built with ARB (Ludwig et al., 2004) using the neighbor-joining technique. Bootstrap values were calculated with MEGA 4 with the model Kumura 2-Parameter (Tamura et al., 2007). Biodiversity index calculations including OTU recognitions, Shannon index values, and Chao 1 and Libshuff estimations were carried out using MOthur (version 1.10.2 http://www.mothur.org) following the procedures provided. The distance files used in all calculations were prepared with PHYLIP 4.0 (http://evolution.genetics.washington.edu/phylip/phylip-web.html), and employing the model Kumura 2-Parameter. Phylogenetic identification of OTUs was performed using the BLAST search engine against the NCBI nucleotide sequence database (Altschul et al., 1997).

**Design and specification of oligonucleotide probes**

All 16S rRNA gene-targeted probes were designed using ARB software against a database consisting of 16S rRNA gene sequences retrieved from the Silva database (Pruesse et al., 2007) and from the clone libraries constructed in this study (Figs 1–3). The names, specificities, and formamide concentrations of the newly designed probes are listed in Fig. 4 together with the clones used to validate each of these probes. Their specificities were assessed using Clone-FISH, carried out according to Schramm et al. (2002). The plasmid and host cell pair used was pGEM-T and JM103 (DE3), respectively (Promega, Fisher Scientific, Toronto, ON, Canada). Briefly, clones with zero or one mismatch were inserted into the plasmid pGEMT equipped with a T7 RNA polymerase, and cloned into JM109 (DE3) competent cells. These cells were incubated at 37 °C with isopropylthio-β-galactoside (IPTG) (1 mM) and chloramphenicol (170 mg L⁻¹) before being fixed in PFA (4%) for FISH probing. The highest formamide concentration (tested in 5% step-wise increases) at which a clear fluorescent signal was observed after FISH probing with competent cells with zero mismatches, but not with competent cells with one mismatch was selected as the optimal PFA concentration.

**Microscopy and enumeration of methanogens hybridized in FISH**

FISH and DAPI images were captured with a Leica epifluorescence microscope (Leica DM6000B) equipped with a Leica DFC500 camera and visualized and captured using the Cy3 and DAPI filters, respectively, after samples had been mounted in CITIfluor (Citifluor Ltd, London, UK). The percentages of cells hybridizing with each probe were calculated as percentages of the total number of cells hybridizing with the probe ARC915 (designed for total Archaea) or stained with DAPI (detecting all microbial cells) in the same microscopic field. All enumerations were carried out on digital images using IMAGE (Abramoff et al., 2004) following the procedures described by Kong et al. (2010a). At least 60 sets (FISH image with Cy3 labeled probe and DAPI image) of images taken with the 100× objective lens from three different glass cover slips (20 from each) were used to estimate percentages of targeted Archaea in the Liq or
Fig. 1. Distance tree (neighbor-joining) built using Archaeal 16S rRNA gene sequences obtained in this study (names in bold) and their close relatives. The oligonucleotide probes designed to match target sites in these clones are also shown. The bootstrap values (only those > 50% are shown) were calculated based on 1000 re-samplings. Scale bar represents 1 substitution per 10 nucleotides.
Sol samples from each cow fed each specified diet. The mean values and standard deviation (Fig. 6) were calculated based on the three mean values obtained from each of the 20 sets of images derived from one glass cover. Between 2000 and 4000 DAPI, positive cells were visualized for each image.

Fig. 2. (a, b) Distance tree (neighbor-joining) subtrees of Fig. 1 built using Archaegal 16S rRNA gene sequences obtained in this study (names in bold) and their close relatives. The oligonucleotide probes designed to match target sites in these clones are also shown. The bootstrap values (only those > 50% are shown) were calculated based on 1000 re-samplings. The scale bars represent 1 substitution per 20 nucleotides.

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Fig. 3. (a, b) Distance tree (neighbor-joining) subtrees of Fig. 1 built using Archaeal 16S rRNA gene sequences obtained in this study (names in bold) and their close relatives. The oligonucleotide probes designed to match target sites in these clones are also shown. The bootstrap values (only those > 50% are shown) were calculated based on 1000 re-samplings. The scale bars represent 1 substitution per 20 nucleotides.
The 16S rRNA gene sequences obtained in this study have been deposited in the GenBank database under accession numbers JQ179497–JQ179858.

### Results

#### 16S rRNA gene clone library analyses

To examine the composition of methanogen communities in these rumen samples, a set of already available oligonucleotide FISH probes targeting methanogens at the family or order level (as described earlier) was first applied. The MB1174 probe designed for Methanobacteriales hybridized to cells in all samples examined, and more than half (50–60%) of the Archaea hybridized with the archaeal probe ARC915 (Fig. 5a). The MS1414, MX825, and MG1200b probes designed for members of the Methanosarcinaceae, Methanosaetaceae, and most Methanomicrobiales, respectively, each hybridized with a small fraction (<5%) of the cells that hybridized with the ARC915 probe (data not shown). Therefore, the sequence of probe MB1174 was chosen as a reverse primer in combination with the universal forward primer Met86f to amplify the 16S rRNA genes of the methanogens using PCR. In total, eight clone libraries were constructed from the pooled templates from three fractions (liquid, feed particle loosely associated and feed particle adherent fractions) for each of the individual animals fed the same or different diet(s). A total of 361 methanogenic clones (ca. 1100 bp) were successfully retrieved after excluding any bacterial clones (25 clones) and archaeal clones closely related to Thermoplasmatales (75 clones).

#### Identification of methanogenic populations

The 361 methanogenic clones were classified using CLAS-SIFIER provided in RDP-II. They were all members of the phylum Euryarchaeota, belonging to genera Methanobrevibacter (314 clones) and Methanosphaera (19 clones) in the Methanobacteriaceae, Methanimicrococcus (one clone) in the Methanosarcinaceae and Methanomicrobiaceae (seven clones) in the Methanomicrobiaceae, and unclassified Methanobacteriaceae (two clones) and unclassified Euryarchaeota (18 clones). Mothur analysis revealed that these clones comprised 23 OTUs at a distance of 0.03 (Table 1). Of these, 15 OTUs contained representatives of previously cultivated species: Methanobrevibacter strain NT7 (represented by OTUs 1, 13, and 16), M. stadtmanae (represented by OTU 2), M. ruminantium (represented by OTU 3), M. thaueri (represented by OTU 5), Methanobrevibacter millerae (represented by OTUs 6 and 11), Methanobrevibacter gottschalkii (represented by OTU 7), Methanobrevibacter strain Z8 (represented by OTUs 12 and 14), Methanobrevibacter strain SM9 (represented by OTU 15), M. mobile (represented by OTU 15), and Methanobrevibacter strain Y1 (represented by OTU 17). Others consisted largely of unclassifiable 16S rRNA gene sequences of Euryarchaeota (represented by OTUs 4, 8, 10, and 20), Methanobrevibacter (OTUs 18 and 19), and a few Methanosphaera (OTU 21) and Methanimicrococcus (OTU 23).

#### Community biodiversity analysis

The differences in the composition of the ruminal methanogenic communities between any two cows fed the same diet or different diets were estimated statistically using S-Libshuff in Mothur (Table 2). No clear pattern was
observed with regard to the effects of the individual animals or diet on the composition of methanogenic communities. At a distance of 0.03, between 7 and 13 methanogenic OTUs could be detected in a single animal. The species richness values of methanogenic populations in different animals using a Chao 1 estimation were between 7 and 18, and the Shannon index values calculated for the methanogenic populations in different animals fell between 1.23 and 2.18.

qFISH of methanogenic populations in the rumen of cows fed alfalfa hay or triticale straw

Five FISH probes were designed against the methanogenic 16S rRNA gene sequences obtained in this study (Fig. 1) and their close relatives. The probe Mbr830 was designed to target *M. thaueri*, *M. millerae*, *M. smithii*, and their related clones, which are referred to here as the ‘thaueri-millerae-smithii’ (TMS) group. The probes Mbr626, Msp541, Mmi1123, and Mmi1042 were designed to target *M. ruminantium*, *M. stadtmanae*-related, *M. mobile*, and *Methanimicrococcus*-related clones respectively. In all the samples examined, the probe Mbr830 hybridized with large coci (Fig. 5b), probe Mbr626 hybridized with small coci or coccobacilli (Fig. 5c), probe Msp541 with large coci of variable sizes (Fig. 5d), probe Mmi1123 with large coci (Fig. 5e), and probe Mmi1042 with small coci or coccobacilli of variable sizes (Fig. 5f). The relative abundances of these probe-defined methanogenic populations were determined as shown in Fig. 6.

The sums of the percentages of the methanogenic populations hybridizing with these probes varied among individual animals and ranged between 1.9% and 3.7% of the total cell numbers (cells stained with DAPI) determined in the Liq and 3.1–4.8% in the Sol phases. These values are within the ranges detected with probe ARC915 in the same samples (i.e. 2–5% of the total cell numbers). The average percentages of these probe-defined methanogenic populations in the Liq and Sol phase of the rumen of cattle fed with alfalfa hay were 2.5 (±0.6)% and 4.4 (±0.6)% respectively, which were similar to those [2.7 (±1.0)% and 3.8 (±0.5)%, respectively] detected in the rumen of cattle fed triticale straw.

FISH probing was used to investigate the relative abundances of these probe-defined methanogenic populations. Their percentages varied widely in the Liq or Sol phases in the rumen of the same cattle fed different diets, among individual cattle fed the same diet and across individual cattle fed different diets. Except cow #2 fed alfalfa hay, members of the TMS group were detected in the Liq and
Sol phases of all other cows with a large range [18–58% and 14–58%, respectively, of the total methanogens (total sum of percentages of the methanogens detected using five FISH probes)]. Methanobrevibacter ruminantium-related methanogens were detected in the Liq and Sol phases of all cows except those from cow #2 fed triticale straw and the Liq of cow #4 fed triticale straw, and accounted for 16–53% and 6–53% of the total methanogens in the Liq and Sol phases, respectively, of other individual cows. Methanosphaera stadtmanae-related methanogens were detected in all samples except those in the Sol phase of cows #1–3 fed triticale straw and accounted for 16–34% and 12–50% of total methanogens in the Liq and Sol phases, respectively. Methanomicrobium mobile-related methanogens were detected in the Liq and Sol phases of all cows except in cow 1 fed triticale straw and accounted for 6–33% and 6–41% of total methanogens in the Liq and Sol phases, respectively.

Methanimicrococcus-related methanogens were detected in all samples except in the Sol phase of cows #2 and #3 fed triticale straw and accounted for 7–42% and 3–21% of the total methanogens in the Liq and Sol phases, respectively.

All the probe-defined methanogenic populations detected in the ruminal Liq phases were also detected in the Sol phases of the same animals except in cows #2 and #3 where the M. stadtmanae-related methanogens were only detected in the Sol phases, and in cow #4 fed triticale straw where M. ruminantium-related methanogens were only detected in the Sol phase. When the percentages of the individual probe-defined methanogenic populations in the Liq and Sol phases in the rumen of cows fed alfalfa hay were compared with those in the Liq and Sol phases in the rumen of same animals fed triticale straw, they were highly variable, and no clear trend was observed with regard to the type of forage fed. The same results were found when the percentages of the individual probe-defined methanogenic populations in the Liq and Sol phases in the rumen of cows fed alfalfa hay were compared to those in the rumen of different cows fed triticale straw.

**Discussion**

In this study, most of the methanogens in the rumen of the cows fed with two different diets were identified using a full-cycle rRNA approach. They belonged to at least five methanogenic genera or populations. Their relative abundances in the community varied among individual animals and across diets. The composition of these methanogenic communities is more diverse than known.

This study describes the first published systematic high-resolution FISH study on the composition of the ruminal methanogenic communities in cows fed with different diets. Most (up to 80%) of the Archaea in the rumen of cows fed alfalfa hay or triticale straw could be
Table 2. Biodiversity index, distribution, and similarity of methanogenic populations in rumen digesta of animals fed either alfalfa hay or triticale straw

<table>
<thead>
<tr>
<th>Libraries*</th>
<th>Number of sequences</th>
<th>Number of sequences</th>
<th>Species richness</th>
<th>OTUs shared (%)</th>
<th>Shannon index</th>
<th>P value† (XY, YX)</th>
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<td>43 vs. 52</td>
<td>8 vs. 7</td>
<td>13 vs. 7</td>
<td>5/8</td>
<td>5/7</td>
<td>1.23 vs. 1.23</td>
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<tr>
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<td>43 vs. 40</td>
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<td>13 vs. 11</td>
<td>6/8</td>
<td>6/11</td>
<td>1.23 vs. 1.52</td>
</tr>
<tr>
<td>C4AL vs. C3TR</td>
<td>43 vs. 52</td>
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<tr>
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</tr>
<tr>
<td>AL vs. TR</td>
<td>167 vs. 194</td>
<td>22 vs. 17</td>
<td>25 vs. 19</td>
<td>16/22</td>
<td>16/17</td>
<td>3.18 vs. 2.89</td>
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</table>

* C1–C4 represent the four cows used in this study. The AL and TR represent the rumen microbial communities fed with alfalfa hay (AL) and triticale straw (TR), respectively.
† XY represents the number of OTUs in the first library (as indicated in column 1) shared with the second library. YX represents the number of OTUs in the second library shared with the first library.
‡ XY represents the P values obtained when the first library (as indicated in column 1) is compared with the second library. YX represents P values obtained when the second library is compared with the first library.

Identified using the five probes designed here (data not shown). In total, members of genera *Methanobrevibacter* and *Methanomicrobiurn* constituted on average 63% and 53% of the total methanogens in the Liq and Sol phases, respectively, a result which is in agreement with results from cloning studies conducted by others (Wright et al., 2007; Hook et al., 2009; King et al., 2011; Tymensen et al., 2012), where members of these two genera and the RCC clade accounted for the majority of rumen methanogens identified. Moreover, half of each of total *Methanobrebiacter* cells in the Liq and Sol phases emerged as *M. ruminantium*–related methanogens, a finding that suggests that this group plays an important role in ruminal methanogenesis. However, in addition to the *Methanobrebiacter* and *Methanomicrobiurn* methanogens related to *M. stadtmanae* and *Methanimicrococcus blatticola* are also important members of these communities, and account on average for 37% and 47% of the total methanogens in the Liq and Sol phases, respectively.

*Methanobrebiacter*-related methanogens comprised about half of the total methanogen cells in the rumen communities examined here, lower than the percentages reported by Sharp et al. (1998) in a Holstein cow (89.3%) and a model rumen broth (99.2%). On the other hand, percentages of *M. stadtmanae*-related methanogens detected here (16–34%) and 12–50% of total methanogens in the Liq and Sol, respectively) are generally higher than those (6–12% of total methanogens) detected by Zhou et al. (2009) using qRT-PCR in the Liq phase of the rumen of steers fed a mixture of oat grain (74%) and
grass hay (20%). While members of *Methanobrevibacter* sp. strain AbM4 were detected in rumen communities of these steers (4–8% of total methanogens), this strain was not identified in clone libraries retrieved from our samples. Moreover, *M. aarhusense*, *M. formicicum*, and *M. barkeri*, all of which have been isolated from bovine rumen samples, could not be detected in our study. It is still not clear whether this is because they are not present in these cattle or that they were not recovered during cloning because of the relatively small number of cattle sampled, the clones sequenced or biases of DNA extraction and/or PCR amplification procedures.

To our knowledge, this is the first report that *M. blatticola*-related methanogens are present in high relative abundances in the bovine rumen. However, in this study, only one such clone (C4TRD08) was retrieved with the MB1174 reverse PCR primer designed to target the *Methanobacteriales*. FISH performed showed that on average, 23% and 9% of the methanogens in the Liq and Sol phases, respectively, were members closely related to *M. blatticola*. Whitford et al. (2001) and Sundset et al. (2009) also retrieved *M. blatticola*-related 16S rRNA gene clones from cattle and reindeer rumen, but made no effort to quantify them *in situ*. This would suggest that the role and importance of *M. blatticola*-related methanogens in ruminal methanogenesis have been underestimated in past studies.

The primer pair MB1174r and Met87f was applied successfully for high-resolution cloning analysis of members of the *Methanobacteriales*. Many universal primer pairs have been used for PCR amplification of 16S rRNA genes of ruminal methanogens (Skillman et al., 2006; Zhou et al., 2011). However, the coverage of these is limited, and different primer pairs will amplify fragments from different methanogenic populations in the same sample (Skillman et al., 2006; Zhou et al., 2011). In this study, the FISH probe MB1174 (designed for *Methanobacteriales*) hybridized with more than half the methanogens in all rumen samples examined. When its sequence was used as a reverse primer together with the universal methanogen forward primer Met87f, a high-resolution cloning analysis of members of the *Methanobacteriales* was achievable. *Methanobrevibacter* clones constituted the majority (87%) of the clones retrieved (314 of 361 clones), and 16 *Methanobrevibacter* OTUs were recognized. Fourteen of these shared > 97% sequence similarity to nine *Methanobrevibacter* isolates cultivated previously in the laboratory (Table 1). Of these, four have been validly described and others share < 97% sequence similarity with all other available methanogen clones, suggesting they represent putative new species. The *Methanobrevibacter* detection rate is markedly higher than that suggested by the four OTUs (97% cutoff) generated by Wright et al. (2007) from the rumen of cattle (241 clones sequenced) with the Met86f and Met1340r primer pair, or the eight OTUs (98% cutoff) reported by Hook et al. (2009) from 166 clones from dairy cows using the same primer pair. Furthermore, the OTU detection rate is substantially higher than the four OTUs (97% cutoff) reported by Tymensen et al. (2012) after analyzing 100 clones of free-living methanogens and those associated with protozoa from the rumen of black Angus heifers with the primer
pair Met86f and Met915r. In all these studies, Mothur was used to estimate the number of OTUs. The high Methanobrevibacter species detection rate obtained in the present study is considered to arise from using the novel Met86f and Met1174r primer pair together with fractionating the rumen digesta samples before preparation of DNA templates for PCR amplification.

Seventy-five clones belonging to the RCC clade were retrieved in the clone libraries constructed in this study. They were not included in our phylogenetic analysis, and no FISH probes were designed for them because their roles in rumen are still not clear. Paul et al. (2012) reported (after this manuscript was submitted) that Thermoplasmatales-related Archaea in termite guts and other environments are methanogens. Their relative abundance in the methanogen communities will be examined in future research.

No clear pattern was apparent regarding the effect of diet or individual host cow on the composition of methanogenic communities after cloning analysis, and the similarity in methanogen biodiversity in all cows regardless of diet suggests that neither had a marked effect on ruminal methanogen community composition. This is an outcome in sharp contrast to that of our earlier study using only clone library analyses, where population compositions of the bacterial communities in these ruminal digesta samples changed markedly among different hosts and diets (Kong et al., 2010a). It supports the view that the diverse chemoorganoheterotrophic bacterial populations in the rumen are more sensitive to the nature of the organic substrates presented for fermentation than are the methanogens that utilize a relatively narrow spectrum of energy sources (Janssen & Kirs, 2008). The methanogens identified here including Methanobrevibacter, Methanosphaera, Methanomicrobium, and Methanimicrococcus use mainly $\text{H}_2$ or formate as their energy sources (Liu & Whitman, 2008).

In agreement with the cloning data, statistically significant differences in percentage abundances were not found for most of the FISH probe-defined populations examined here (data not shown), either in response to the different diets or among individual cows, although the percentages of the five FISH probe-defined populations varied markedly among cattle fed the same or different forages, or with the same individual host fed different diets. All support the view that the composition of the methanogenic populations in the rumen fed alfalfa hay is not substantially different from that fed triticale straw. In contrast, Tymensen et al. (2012), using qPCR, found that relative abundance of the Methanobrevibacter spp. in black Angus heifers changed when diet was switched from a forage-based to a high-grain diet, where changes in nutrient sources and the flow of reducing equivalents would be expected to be dramatic.

In conclusion, the diversity and population composition of methanogenic communities, in particular, the Methanobrevibacter populations, in the rumen of cattle fed alfalfa hay or triticale straw have been revealed here at a high-resolution level. Populations related to the Methanobrevibacter TMS group and M. ruminantium accounted for approximately half of the total methanogens identified, while the remaining members consisted of M. stadtmanae, M. mobile, and M. blatticola. The composition of the methanogenic community and the relative abundances of individual methanogen populations were not affected markedly by changing the diet from alfalfa hay to triticale straw and vice versa. The composition of the methanogen community is shown by methodological improvements to be more diverse than previously suspected.

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Authors’ contribution

Y.H.K. and Y.X. contributed equally to this study.

References


