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Rumen Methanogenic Ecology under Different Diets and Cattle Feed Efficiency

by

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DEDICATION

I dedicate this thesis to my loving parents. Without their love, support, encouragement, and patience, I would never go through this long journey.

ABSTRACT

Ruminal methanogenesis is a microbial fermentive process conducted by methanogens, releasing methane (CH_4) gas through eruction, and resulting in a dietary energy loss to the host animals and a contribution to greenhouse gas emissions by the agricultural industry. However, the association amongst methanogenic ecology, host feed efficiency, and host enteric CH_4 production is not clear. The overall objective of this research was to investigate the potential linkage among these sectors, and thus four studies were performed. Study 1 and Study 2 were conducted to investigate the correlation between cattle's feed efficiency and methanogenic ecology under growing and finishing diets. The composition of the methanogenic community varied significantly between the two diets, and the associations between methanogenic phylotypes and host's feed efficiency differed between the two diets. When animals were fed growing diet, *Methanobrevibacter* sp. AbM4 and *Methanosphaera stadtmanae* were more prevalent in inefficient animals; while under a finishing diet, multiple unidentified species were more common in inefficient animals. In Study 3, the correlation between methanogenic ecology and host CH_4 production were studied in dairy cows, and the dietary effect on such correlation was also analyzed. Phylotypes resembling methanogenic archaeon CH1270 and *Mbb. gottschalkii* strain HO tended to be related to host's CH_4 production, but the total methanogen population was not related to the amount of CH_4 yield. In Study 4, host effect on ruminal methanogenic community and its adaptation to dietary treatments was examined in beef heifers. The unique microbiota of each animal and the distinctive responses to the dietary treatments within individuals indicate that the

animal-to animal variation may be the main cause leading to the inconsistency of host response to dietary or environmental changes. Therefore, individual variation should be taken into account when studying ruminal microbial ecology. In summary, this research revealed that biodiversity of methanognic community rather than then total methanogen density plays an important role in affecting host feed efficiency, determining host's enteric CH_4 production, and adapting to different dietary conditions. Furthermore, host is an essential factor determining its symbiotic relationship with methanogens.

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1.0 LITERATURE REVIEW[1]

1.1 Overview of Rumen Methanogenesis

Ruminants have developed a microbial symbiosis to digest fibre in ingested feeds (Dehority, 1997). Along with the ruminal fermentation, hydrogen (H_2) is produced as the result of the carbohydrate decomposition. The accumulation of H_2 would interfere further microbial metabolism (Sharp et al., 1998). Methanogenesis is one of the important means to remove H_2 from the rumen, thus reducing the partial H_2 pressure, maintaining the optimal microbial fermentation, and supporting the complete digestion of ingested fibre (Zinder, 1993; Sharp et al., 1998). The end production of methanogenesis is the methane (CH_4) gas.

Enteric CH_4 is the predominant greenhouse gas generated from ruminant livestock systems. CH_4 is a gas that has 25 times global warming potential that of carbon dioxide (CO_2). The atmospheric lifetime of CH_4 is 12 years. Thus the accumulation of CH_4 in the atmosphere is harmful to the global environment (Intergovernmental Panel on Climate Change, 2001). Globally, more than 50% of the CH_4 emissions are from agriculture sector, especially from the livestock industry (Environment Canada, 2011). Enteric CH_4 takes up nearly 90% of the total CH_4 production of ruminants (Murray et al., 1976). The annual CH_4 release from ruminants is as much as 86 Tg, among which beef cattle are responsible for about 55.9 Tg with dairy cattle accounting for around 18.9 Tg (McMichael et al.,

[1] A part of this section has been published. Zhou M., T.A. McAllister, and L.L. Guan. 2011. Molecular identification of rumen methanogens: Technologies, advances and prospects. Anim. Feed Sci.Tech. 166-167: 76-86.

2007). As the worldwide market requirement for meat and milk is growing, the annual emission of enteric CH_4 is increasing.

Besides of its influence on animal production, this process also has detrimental effects on the animals, although methanogenesis is favored by ruminal microbes. It is estimated that the energy loss derived from ruminal methanogenesis ranges from 2-12% (Johnson and Johnson, 1995). The total energy loss rate depends on the type of diet and animal. Furthermore, the CH_4 production and energy loss also vary significantly among individual animals even if they are raised under similar conditions.

1.2 Cattle Feed Efficiency and Methanogenesis

1.2.1 Definition and Measures of Cattle Feed Efficiency

It is estimated that about 55 to 75% of the total cost of beef cattle production is related to feed (NRC, 2000; Arthur et al., 2001). Minimizing feeding cost and improving feed efficiency of cattle would increase the profitability of beef production (Herd et al., 2003). Cattle feed efficiency, describing the efficiency of cattle converting feeding material into animal weight gain, is an important indicator for cattle production (Lamb & Maddock, 2009). Cattle with higher feed efficiency are desirable within the beef industry.

Traditional measures of cattle feed efficiency simply compare the ratio between feed consumption amount and growth, including grain:feed ratio (G:F) or its reverse form termed feed conversion ratio (FCR) and partial efficiency of growth (PEG) (Carstens and Tedeschi, 2006). Residual feed intake (RFI) is an

alternative proposed by Koch et al. (1963), measuring the difference between the actual feed intake and its expected feed requirement to maintain the same production. Cattle with low RFI consume less feed yet performed the same as others, and as such are considered to be more efficient.

RFI is moderately heritable (Herd and Bishop, 2000; Arthur et al., 2001; Crews et al., 2003) and phenotypically independent (Lamb and Maddock, 2009). Thus it is more widely accepted as an appropriate and accurate index that indicates an animal's feed efficiency.

1.2.2 Factors Affecting RFI

Since RFI has been recently proposed as one measurement of efficiency that has been gradually accepted by the industry, the following sections will review this concept. Feed efficiency of cattle can be affected by multiple factors. As proposed by Herd et al. (2004), there are five major factors that contribute to the variation of RFI.

1. Feed intake of the animals: increasing feed intake results in larger amount of energy required for digestion, and thereby increasing maintenance requirement. The feeding behavior of cattle has been ascribed to be an important factor contributing to RFI variance. Feeding pattern, feeding time, eating rate, and number of eating sessions directly affect an individual's feed intake, and thus further affect RFI (Richardson, 2003; Robinson and Oddy, 2004; Dobos and Herd, 2008; Durunna et al., 2011b).

2. Digestion and energy source: differences in digestion processes and substrate availability may lead to variance in the efficiency of energy utilization. Richardson et al. (1996) found that the variance in dry matter intake (DMI) accounted for ~14% divergence in feed intake between high-RFI and low-RFI cattle. Variance of amino acid supply to the host is partly due to the variation in efficiency of ruminal microbial protein production (Kahn et al., 2000). Lower digestibility and energy supply would results in lower feed efficiency.

3. Animal anabolism and catabolism: metabolic processes contributing to heat production are responsible for the differences in feed efficiency. For example, differences in carcass composition were correlated to RFI variance for beef steers, where progeny steers of low-RFI parents had less carcass fat and more carcass protein than those of high-RFI parents (Richardson et al., 2001). Kolath et al. (2006) found that mitochondrial respiration increased in beef steers with low RFI and electrons flux through the electron transport chain was hindered in high RFI steers.

4. Animal activity: more activity of animal would lead to larger amount of heat production, hence decrease metabolic efficiency. Energy expenditure in the form of animal activity has been reported to have a phenotypic correlation of 0.32 for RFI and counted for ~10% of the observed RFI variance in cattle (Richardson et al., 1999). Herd et al. (2004) calculated the energy cost of movements such as feeding, ruminating, and locomotion, and found approximately 5% increase in feed energy intake for low (high RFI) as compared to high efficient (low RFI) animals.

5. Thermoregulation: evaporative heat loss is the principal route of energy loss in the ruminants (Blaxter, 1962). Although no studies have examined the relationship between respiration rate and RFI in cattle, animals with slower respiration frequency would be expected to have lower RFI value (Herd and Arthur, 2008).

Besides the above, more elements have been reported to be associated with feed efficiency in cattle. Sherman et al. (2008) reported 6 single-nucleotide polymorphisms (SNPs) which were correlated to RFI in feedlot cattle in Canada. Mujibi et al. (2010) reported that seasonality effects such as temperature, wind speed and humidity affected cattle feed intake and RFI. Durunna et al. (2011a) examined cattle's feed efficiency under two different diets and found a low RFI-ranking correlation of the animals under grower versus finisher diet.

1.2.3 Methanogenesis and Cattle's Feed Efficiency

Generally, cattle displayed considerable variation in their metabolic energy use and partitioning and hence variance in feed efficiency. Dietary energy loss in the form of enteric CH_4, feces, and urine is one of the major causes related to such variation (Basarab et al., 2003). Johnson and Johnson (1995) claimed that enteric CH_4 emission is affected by the level of cattle's feed intake. The release of CH_4 represented a loss of dietary energy for dairy cattle, range-cattle, and feedlot cattle ranging from 5.5-9.0%, 6.0-7.5%, and 3.5-6.5%, respectively (Johnson and Ward, 1996). In addition to the type of host, the dietary energy loss also varies according to geographical locations, feed quality, feed intake, feed composition,

and feed processing (Johnson and Ward, 1996). Nkrumah et al. (2006) have compared the enteric CH_4 production among three groups of cattle with low-RFI, medium-RFI, and high-RFI, respectively, and found that cattle with low-RFI produced much lower CH_4 than high- or medium-RFI cattle. Hegarty et al. (2007) examined a larger group of cattle and further confirmed that cattle selected with lower RFI reduced their enteric CH_4.

1.3 Rumen Methanogens

1.3.1 Characteristics of Methanogens

Methanogens are distinguished from other microbes by their cell wall components and unique membrane lipids. Methanogen cell walls consist of pseudomurein and surface layer proteins and lack peptidoglycan, which is a common component of bacterial cell walls. Membrane lipids in methanogens link the alkyl chains to the glycerol by phytane or biphytane, rather than ester-linked fatty acyl glycerol derivatives. Methanogens also have distinctive 16S rRNA gene sequences compared to other microorganisms. Finally, methanogens possess specific cofactors and coenzymes such as F_{420}, methanopterin and coenzyme M, enzymes that are involved in methanogenesis (Baker, 1999; De Rosa and Gambacorta, 1988; Jones et al., 1987; Kletzin, 2007; Woese et al., 1990).

Methanogens grow at redox potentials below -300 mV (Stewart and Bryant, 1988), and at an optimal pH range of 6–8 (Jones et al., 1987). They are strictly anaerobic, obligate methane producers that derive most of their energies

through methanogenesis and provide oxidized reducing factors (e.g., NAD^+) to other microbial metabolic pathways (Hungate et al., 1970; Wolin, 1979).

Methanogens have been found in a wide variety of anaerobic habitats, including rumen, lower intestinal tract of mammals, gut of termites, sewage, anaerobic digesters, landfills, rice paddies, freshwater sediments, marine sediments, geothermal systems, and heartwood of trees (Liu and Whitman, 2008).

1.3.2 Phylogeny of Rumen Methanogens

Methanogens belong to the domain *Archaea*, phylum *Euryarchaeota* (Balch et al. 1979), and are subdivided into five orders (Ferry and Kastead, 2007). Methanogens are abundant in the rumen. However, the fastidious nutritional requirement and sensitivity to oxygen make the in vitro cultivation of methanogens difficult. Thus, only a few species have been isolated and cultured (Table 1.1).

Molecular methods have been further used to define the diversity of rumen methanogens. Based on the available global data set, the majority of ruminal methanogenic community is present in three genes-level groups: *Methanobrevibacter*, *Methanomicrobium*, and rumen cluster C (RCC) (Janssen and Kirs, 2008). Additionally, unculturable species belonging to Methanobacteriaceae and Methanosarcinaceae families also exist in the rumen at lower densities (Sundset et al., 2008). The composition of methanogenic community varies among studies. This can be attributed to the host, type of diet, environment, animal health, animal genotype, animal age, DNA extraction

Table 1.1 Cultured methanogens from ruminants.

Species	Host
Methanobacterium formicicum	Bovine; ovine
Methanobacterium bryantii	Bovine
Methanobrevibacter ruminantium	Bovine; ovine; corvine
Methanobrevibacter smithii	Ovine
Methanobrevibacter olleyae	Bovine; ovine
Methanobrevibacter millerae	Bovine; ovine
Methanomicrobium mobile	bovine
Methanosarcina barkeri	Bovine, caprine
Methanoculleus olentangyi	Cervine

Summarized from Jarvis et al., 2000; Joblin, 2005; Rea et al., 2007; Janssen and Kirs, 2008

methods, and the chosen PCR primers (McSweeny et al., 2007).

In addition, methanogens that have ecto- and endo-symbiosis with protozoa are also commonly discovered in the ruminal community (Finlay et al., 1994). The same as the classification of the entire ruminal methanogenic communities, the protozoa-associated methanogens also fall into the three groups, the genera *Methanobrevibacter*, *Methanomicrobium*, and the RCC clade (Janssen and Kirs, 2008). However, the abundance of each group varied notably in different studies. Chagan et al. (1999) reported that phylotypes belonging to *M. ruminantium* are abundant from sheep; *Methanomicrobium* sp. dominated the community of sheep in the study of Regensbogenova et al. (2004b); Ohene-Adjei et al. (2007) found that members of RCC are predominant in the rumen of ovine. Since all the above experiments only studied selected protozoa, the results can be strongly biased. The limited information regarding to the protozoa-associated methanogen is not sufficient to verify the idea that protozoa-associated methanogens are distinct from free-living methanogens.

1.3.3 Substrate Range of Methanogenesis

The organic substances in the ingested feed include cell wall polymers, starch and proteins, which methanogens are not able to digest. Instead, a group of other microorganisms, including bacteria, protozoa, and fungi, ferment these compounds and provide the actual substrates for methanogenesis, which provides thermodynamically favorable conditions for consistent ruminal microbial degradation (Zinder, 1993). Although methanogens are diverse, they can only

derive energy from a very limited range of substrates. The major substrates are restricted into three types: CO_2, methyl-group containing compounds and acetate (Hedderich and Whitman, 2006).

The first type of methanogenesis substrate is CO_2. Most methanogens can reduce CO_2 to CH_4 with H_2 as the electron donor. In some cases, methanogens can also use formate as the electron donor. In addition, secondary alcohols such as 2-propanol, 2-butanol, cyclopentanol, and ethanol can be used by some species (Hedderich and Whitman, 2006). The second type of methanogenesis substrate is the methyl-group containing compounds, including methanol, monomethylamine, dimethylamine, trimethylamine, tetramethylammonium, methylsulfide, and methanethiol. Electrons used for reducing these substrates arise from the oxidation of another methyl group within these compounds (Hedderich and Whitman, 2006). The third type of methanogenesis substrate is acetate. Electrons for acetate reduction are derived from the oxidation of the carboxyl carbon on acetate (Hedderich and Whitman, 2006). As the passage rate of rumen contents is greater than the growth rate of acetate-utilizing methanogens, usage of acetate is very rare for ruminal methanogens (Wolin, 1979).

Methanogens belong to different phylogenetic groups utilize different substrate for methanogenesis (Table 1.2). Among the methanogens identified in the rumen, species belonging to *Methanobacterum* mainly utilize the CO_2/H_2 pathway in methanogenesis; whereas species of *Methanosarcina* utilize methylamines, methanol or acetate to produce methane (Patterson and Hespell,

Table 1.2 Summary of methanogenesis reactions and involved organisms.

Methanogenesis substrate	Reaction	Organisms
CO_2	$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	Most methanogens
	$4\ HCOOH \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	Many hydrogenotrophic methanogens
	$CO_2 + 4$ isopropanol $\rightarrow CH_4 + 4$ acetone $+ 2\ H_2O$	Some hydrogenotrophic methanogens
	$4\ CO + 2\ H_2O \rightarrow CH_4 + 3\ CO_2$	*Methanothermobacter* and *Methanosarcina*
CH_3-	$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	*Methanosarcina* and other methylotrophic methanogens
	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	*Methanomicrococcus blatticola* and *Methanosphaera*
	$2\ (CH_3)_2\text{-}S + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ H_2S$	Some methylotrophic methanogens
	$4\ CH_3\text{-}NH_2 + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_3$	Some methylotrophic methanogens
	$2\ (CH_3)_2\text{-}NH + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ NH_3$	Some methylotrophic methanogens
	$4\ (CH_3)_3\text{-}N + 6\ H_2O \rightarrow 9\ CH_4 + 3\ CO_2 + 4\ NH_3$	Some methylotrophic methanogens
	$4\ CH_3NH_3Cl + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_4Cl$	Some methylotrophic methanogens
CH_3COOH	$CH_3COOH \rightarrow CH_4 + CO_2$	*Methanosarcina* and *Methanosaeta*

Adapted and modified from Liu and Whitman (2008).

1979; Rowe et al., 1979). An exception is *Methanosphaera stadtmanae*, which is restricted to using methanol and H_2 for methanogenesis because of its unique genome sequence that lacks the open reading frame to encode enzymes involed in other pathways (Fricke et al., 2006).

1.3.4 Biochemistry of Methanogenesis

Depending on the type of substrates, three different pathways are involved in the conversion of these three substrates into the methane precursor, methyl-coenzyme M (CH_3-S-CoM). These pathways are generally classified as CO_2 reduction pathway, C_1 compound conversion pathway, and acetate fermentation pathway (Hedderich and Whitman, 2006).

In the CO_2 reduction pathway, CO_2 is firstly fixed to methanofuran (MFR), generating formyl-MFR as the first intermediate. Then the formyl group is transferred to tetrahydromethanopterin (H_4MPT) and sequentially reduced to methenyl, methylenyl, and finally a methyl group. Afterwards, the methyl group is transferred to coenzyme M (HS-CoM) to form CH_3-S-CoM (Figure 1.1 A).

In C_1 compound conversion pathway, the methyl group is transferred to HS-CoM by methyltransferase. The methyltransferase system for each substrate is unique, specific for methanol (Mta), monomethylamine (Mtm), dimethylamine (Mtb), trimethylamine (Mtt), tetramethylammonium (Mtq), and methylthiols (Mts) (Hedderich and Whitman, 2006). Each system is composed of two methyltransferase, MT1 and MT2. The methyl group is transferred to corrinoid

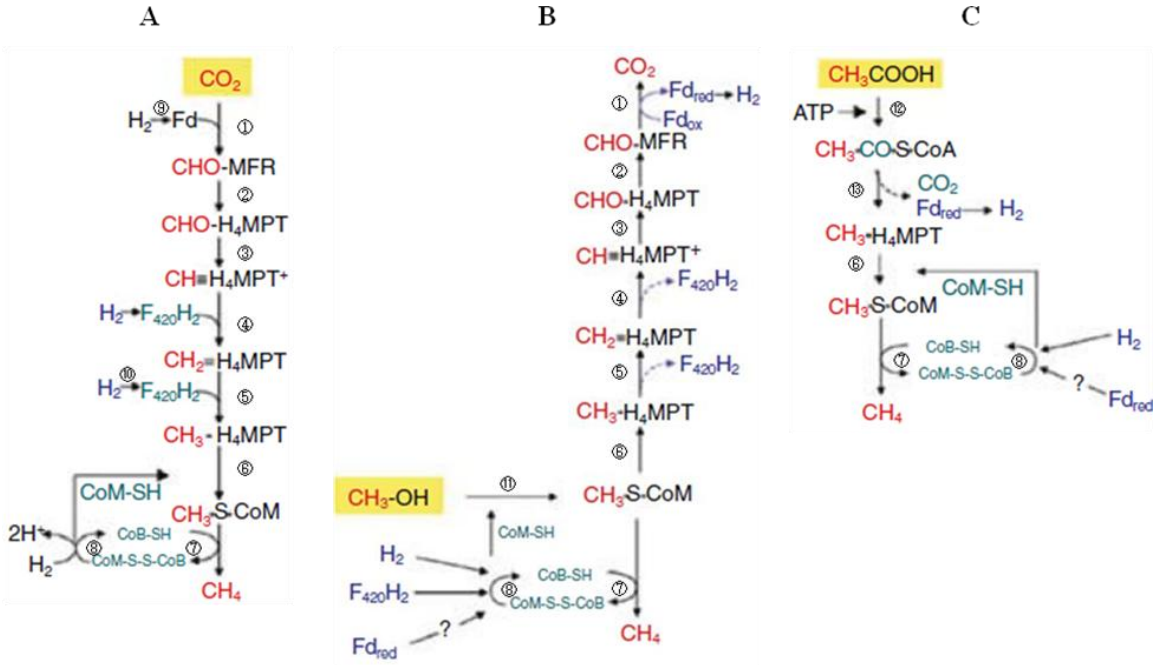

Figure 1.1 Methanogenesis pathways. (A) CO_2 reduction. (B) Methyl-group conversion. (C) Acetate fermentation. Abbreviations: CHO-FMR, *N*-formylmethanofuran; CHO-H_4MPT, N^5-formyltetrahydromethanopterin; CH=H_4MPT$^+$, N^5,N^{10}-methenyl-tetrahydromethanopterin; CH_2=H_4MPT, N^5,N^{10}-methylene-tetrahydromethanopterin; and CH_3-H_4MPT, N^5-methyl-tetrahydromethanopterin; Fd_{red}, ferredoxin (reduced form); Fd_{ox}, ferredoxin (oxidized form); $F_{420}H_2$, coenzyme F_{420} (reduced form); H_4MPT, tetrahydromethanopterin; CoM-SH, coenzyme M; CoB-SH, coenzyme B; CoM-S-S-CoB, CoM CoB heterodisulfide; CoA-SH, coenzyme A. Enzymes involved: 1. formyl-MFR dehydrogenase (Fmd); 2. formyl-MFR:H_4MPT formyltransferase (Ftr); 3. methenyl-H_4MPT cyclohydrolase (Mch); 4. methylene-H_4MPT dehydrogenase (Hmd); 5. methylene-H_4MPT reductase (Mer); 6. methyl-H_4MPT:HS-CoM methyltransferase (Mtr); 7. methyl-CoM reductase (Mcr); 8. heterodisulfide reductase (Hdr);9. energyconserving hydrogenase (Ech); 10. F_{420}-reducing hydrogenases; 11. methyltransferase; 12. acetate kinase (AK)-phosphotransacetylase (PTA) system in *Methanosarcina*; AMP-forming acetyl-CoA synthetase in *Methanosaeta*; 13. CO dehydrogenase/acetyl-CoA synthase (CODH/ACS). Adapted and modified from Hedderich and Whitman (2006) and Liu and Whitman (2008).

cofactor by MT1. Afterwards, MT2 converts the intermediate to CH_3-S-CoM (Figure 1.1 B).

In the acetate fermentation pathway, acetate is firstly phosphorylated to acetyl-CoA, followed by aceticlastic reaction where the carboxyl carbon (C-1) is oxidized to CO_2 and the methyl carbon (C-2) is reduced to CH_3-S-CoM (Figure 1.1 C).

The final steps of the three methanogenesis pathways occur through a common series of reactions. Catalyzed by methyl-coenzyme M reductase (Mcr), CH_3-S-CoM and coenzyme B (CoB-SH) are converted into to CH_4 and heterodisulfide (CoM-S-S-CoB), coupled with ATP synthesis (Thauer, 1998).

1.3.5 Gene Regulation of Methanogenesis

Genes involved in methanogenesis can be regulated by substrate availability. In the CO_2 reduction pathway, H_2 level determines the expression of several genes (Ferry and Kastead, 2007). For instance, under H_2 excess, genes encoding H_2-dependent methenyl-H_4MPT reductase and isozyme II of methyl-CoM reductase (MRII) are transcribed in *Methanothermobacterium thermautotrophicus* (Morgan, et al., 1997); while in *Methanothermobacter marburgensis*, genes encoding $F_{420}H_2$-dependent methylene-H_4MPT dehydrogenase are expressed (Afting et al., 2000). When H_2 becomes limited, expression of formate dehydrogenase is up-regulated in *Methanobacterium thermautotrophicum* Z-245 (Nolling and Reeve, 1997) and *Methanococcus maripaludis* (Wood et al., 2003). In the C_1 compound conversion pathway,

up-regulation of methanol-specific methyltransferases MT1 and MT2 was commonly observed in *Methanosarcina* species when methanol was provided to the cell culture, reflecting their preference for methanol over acetate for growth (Hovey et al., 2005; Li et al., 2005). The proposed mechanism of such regulation is that the synthesis of methyltranserases in acetate-grown cells facilitates their transition from acetate to methanol as a substrate (Ding et al., 2002). In the acetate fermentation pathway, the expression level of the Cdh complex (involved in transformation from CH_3CO-S-CoA to CH_3-H_4MPT) operon is down-regulated 54 folds in *Methanosarcina acetivorans* grown on methanol as compared to acetate (Apolinario et al., 2005). A similar response to the substrate alteration from methanol to acetate has also been found in *Methanosarcina thermophila*, in which the gene encoding ferredoxin (an electron receptor from the Cdh complex) is down-regulated (Clements and Ferry, 1992).

Trace element availability is another factor affecting gene expression in methanogens. The activities of the enzymes containing transition metals in their active site depend on the accessibility to the metal ions. One example is the isoenzymes of formylmethanofuran dehydrogenase (Fmd) in *Methanobacterium thermoautotrophicum*, in which the tungsten formylmethanofuran is consistently formed while molybdenum formylmethanofuran is only formed when molybdenum is available in the culture (Hochheimer et al., 1996). Nickel (Ni) is another essential trace element for methanogenesis. When *Methanothermobacter marburgensis* is cultivated under Ni-limited condition, a 6-fold and a 4-fold increase in activities of H_2-forming N^5,N^{10}-methylene-H_4MPT dehydrogenase

(Hmd) and coenzyme F_{420}-dependent N^5,N^{10}-methylene-H_4MPT dehydrogenase (Mtd) have been observed, respectively. When the cells are grown under Ni-sufficient condition, a 180-fold lower activity of coenzyme F_{420}-reducing hydrogenase (Frh) has been reported (Hedderich and Whitman, 2006).

To date, gene regulation has been studied primarily in pure cultures and these same responses have not been confirmed in the rumen ecosystem. Whether the rumen methanogens exhibit similar gene regulation patterns while integrated into the overall microbial ecosystem is unknown.

1.3.6 Factors Affecting Rumen Methanogens

Differences in diets, such as the type of grain or fibre and dietary supplements, can alter the rumen methanogenic ecology. Jeyanathan et al. (2011) compared the methanogen communities of four cattle under three different diets (summer pasture, winter pasture, and silage) and found that different diets resulted in different PCR-DGGE patterns of the methanogens within the same animal. Yu et al. (2008) found that *Msp. stadtmanae* was stimulated while *Mbb.* sp. AbM4 was inhibited when sheep were supplemented with tallow (animal fat). Guo et al. (2008) proposed that adding tea saponins may suppress protozoa and as such further depress methanogens.

Rumen pH changes may also play a role in changing the methanogenic activities. Van Kessel and Russell (1996) studied the effects of pH on the ruminal methanogen population and proposed that a pH below 6.0 suppressed the methanogen activity, but did not kill them. Hook et al. (2011) investigated the

effect of low ruminal pH induced by subacute ruminal acidosis (SARA) and observed that methanogen populations declined with decreasing ruminal pH.

As a large group of methanogens are protozoa-associated, changes in protozoa community such as defaunation may lead to changes in rumen methanogens. Mosoni et al. (2011) examined the methanogenic community in long-term defaunated sheep that produced less CH_4 and proposed that although methane production was reduced, the density and diversity of methanogens remain unchanged.

1.4 Molecular Technologies Applied to Study Rumen Methanogens

1.4.1 PCR-Based Methods for Detection of Methanogens

1.4.1.1 PCR Amplification

For molecular identification, PCR amplification is the first essential step to enrich the DNA of microbial cells that are present in low numbers. Since methanogens possess unique 16S rRNA gene sequences, and produce CH_4, both 16S rRNA genes and genes coding enzymes that are unique to methanogens have been utilized to distinguish them from other microorganisms. Early experiments to obtain PCR amplicons of methanogen specific genes from different environments included amplification of the methyl-coenzyme M reductase (*mcr*I) gene of the family *Methanosarcinaceae* (Springer et al., 1995) and the 16S rRNA gene from methanogens isolated from blanket bog peat samples (Hales et al., 1996). Presently, the 16S rRNA gene and methyl-coenzyme M reductase

α-subunit gene (*mcr*A) gene are the principal targets that are amplified and used to characterize methanogens from environmental samples.

The 16S rRNA gene is conserved among prokaryotic microbial species such as bacteria and archaea; with sequence differences widely used for taxonomy identification. Many primers have been designed to specifically amplify the 16S rRNA gene of methanogens (Table 1.3). Although PCR amplification can provide abundant products, by-products such as primer dimers and non-specific amplicons can be generated leading to false positive results. The choice of primers can affect the extent to which the composition of the methanogenic population can be defined. As described by Skillman et al. (2006), two separate primer pairs identified different predominant phylotypes within the same sample with the primer pair 21f/958r amplifying mostly *M. stadtmanae*-like sequences, while the primer pair Arch f364/Arch r1386 amplified mainly *Methanobrevibacter* sequences. Furthermore, clone sequences close to *Thermoplasma* species (Wright et al., 2006) and *Aciduliprofundum boonei* (Sundset et al., 2009) have been identified using a universal archaeal primer, confirming that the rumen archaeal community may include species other than methanogens. Our recent study on sequencing 16S rDNA clone libraries using universal archaeal primer pair Met86F/Met915R identified a few sequences belonging to bacterial 16S rRNA gene (23 out of 972 clones, Zhou et al., 2009) including *Lactobacillus acidophilus* and *Atopobium oviles*. Future attempts to incorporate other methanogen specific genes (e.g., *mcr*A, methyl-coenzyme M reductase α-subunit gene which catalyzes the last step of methanogenesis; *fwd*, tungsten formylmethanofuran

Table 1.3 Universal/Archaea-specific 16S rRNA gene primers.

Primer	Sequence (5' to 3')	*Methanobrevibacter*	*Methanobacterium*	*Methanosphaera*	*Methanoculleus*	*Methanomicrobium*	*Methanosarcina*	*Methanococcus*	Reference
Ar1000f	AGTCAGGCAACGAGCGAGA	+	+	+	+	+	-	-	Yanagita et al., 2000
Ar 1500r	GGTTACCTTGTTACGACTT	+	+	+	+	+	+	-	Yanagita et al., 2000
1Af	TCYGKTTGATCCYGSCRGAG	+	+	+	+	+	+	+	Whitford et al., 2001
1100 Ar	TGGGTCTCGCTCGTTG	+	+	+	+	+	+	-	Whitford et al., 2001
Arch f364	CCTACGGGRBGCAGCAGG	+	+	+	+	+	+	+	Skillman et al., 2004
Arch r1386	GCGGTGTGTGCAAGGAGC	+	+	+	+	+	+	+	Skillman et al., 2004
D30f	ATTCCGGTTGATCCTGC	-	-	-	+	-	-	-	Tajima et al., 2001
D33r	TCGCGCCTGCGCCCCGT	-	-	-	-	-	-	-	Tajima et al., 2001
0025e f	CTGGTTGATCCTGCCAG	+	+	+	+	+	+	-	Tajima et al., 2001
1492r	GGTTACCTTGTTACGACTT	+	+	+	+	+	+	-	Tajima et al., 2001
Met 86f	GCTCAGTAACACGTGG	+	+	+	+	+	+	+	Wright et al., 2004
Met 1340r	CGGTGTGTGCAAGGAG	+	+	+	+	+	+	+	Wright et al., 2004
Arch f	TTCCGGTTGATCCYGCCGGA	-	+	+	+	+	+	+	Shin et al., 2004
Arch r	YCCGGCGTTGAMTCCAATT	+	+	+	+	+	+	+	Shin et al., 2004
A109f	ACKGCTCAGTAACACGT	+	+	+	+	+	+	+	Whitehead and Cotta, 1999
21f	TTCCGGTTGATCCYGCCGGA	-	+	+	+	+	+	+	DeLong, 1992
958r	YCCGGCGTTGAMTCCAATT	+	+	+	+	+	+	+	DeLong, 1992
Arch 69f	TAAGCCATGCAAGTCGACG	-	-	-	-	-	-	-	Tokura et al., 1999
146f	GGSATAACCYCGGGAAACTCC	-	-	-	-	-	-	-	Marchesi et al., 2001
1324r	GCGAGTTACAGCCCWCRA	+	+	+	-	-	-	+	Marchesi et al., 2001
519r	GWATTACCGCGGCKGCTG	+	+	+	+	+	+	+	Embley et al., 1992
380r	TTTCGCGCCTGCTGC	+	+	+	+	+	+	+	Embley et al., 1992
A2Fa	TTCCGGTTGATCCYGCCRGA	-	+	+	+	-	+	+	Barns et al., 1994
A348r	CCCCRTAGGGCCYGG	+	+	+	+	+	+	+	Baker et al., 2003
A329r	TGTCTCAGGTTCCATCTCCG	+	+	+	-	-	+	-	Yu et al., 2008
A24f	TCYGKTTGATCCYGSCRGA	+	+	+	+	+	+	+	Baker et al., 2003
ARC344f	ACGGGGYGCAGCAGGCGCGA	+	+	+	+	+	+	+	Bano et al., 2004

A357f	CCCTACGGGGCGCAGCAG	+	+	+	+	+	+	+	Yu et al., 2008
A693r	GGATTACARGATTTC	+	+	+	+	-	+	-	Yu et al., 2008
ARC915r	GTGCTCCCCCGCCAATTCCT	+	+	+	+	+	+	+	Pinar et al., 2001
UNI-b-rev	GACGGGCGGTGTGTRCAA	+	+	+	+	+	+	+	Kleikemper et al., 2005
A1040f	GAGAGGWGGTGCATGGCC	+	+	+	+	+	+	+	Baker et al., 2003
A1204r	TTMGGGGCATRCIKACCT	+	+	+	+	+	+	+	Baker et al., 2003
Met448F	GGTGCCAGCCGCCGC	+	+	+	+	+	+	-	Wright and Pimm, 2003
Met1027F	GTCAGGCAACGAGCGAGACC	+	+	+	+	+	+	-	Wright and Pimm, 2003
Met448R	GCGGCGGCTGGCACC	+	+	+	+	+	+	-	Wright and Pimm, 2003
Met1027R	GGTCTCGCTCGTTGCC	+	+	+	+	+	+	-	Wright and Pimm, 2003
Met83F	ACKGCTCAGTAACAC	+	+	+	+	+	+	+	Wright and Pimm, 2003
Arch r934	GTGCTCCCCCGCCAATTC	+	+	+	+	+	+	+	Skillman et al., 2004
Arch f331	GAGATGGAACCTGAGACAAG	+	+	+	-	+	-	-	Skillman et al., 2004
Arch f2	TTCYGGTTGATCCYGCCRGA	-	+	+	+	+	+	+	Skillman et al., 2004
MSr r859	TCGCTTCACGGCTTCCCTG	-	+	-	+	-	+	-	Skillman et al., 2004
Mcc r	WASTVGCAACATAGGGCACGG	-	-	-	-	-	-	+	Skillman et al., 2004
fMbb1	CTCCGCAATGTGAGAAATCG	+	-	+	-	-	-	-	Skillman et al., 2004
fMbium	CGTTCGTAGCCGGCYTGA	+	+	+	-	-	-	-	Skillman et al., 2004
ArcF915	AGGAATTGGCGGGGGAGCAC	+	+	+	+	+	+	+	van Hoek et al., 2000
ArcR1326	TGTGTGCAAGGAGCAGGGAC	+	+	+	+	+	+	+	van Hoek et al., 2000
1392R	ACGGGCGGTGTGTRC	+	+	+	+	+	+	+	Amann et al., 1995
M301F	TACGGGTTGTGAGAGCAAGA	+	+	+	+	-	+	-	Tokura et al., 1999
M915R	TGCTCCCCCGCCAATTCCT	+	+	+	+	+	+	+	Tokura et al., 1999
uniMet1-F	CCGGAGATGGAACCTGAGAC	+	+	+	-	+	+	-	Zhou et al., 2009
uniMet1-R	CGGTCTTGCCCAGCTCTTATTC	+	+	+	-	+	+	-	Zhou et al., 2009

All primers were chosen from previous rumen studies and submitted to the 'probe match' option at Ribosomal Database Project (http://rdp.cme.msu.edu/) to check the matching taxonomic groups. Targeted groups were they phyla that have been reported in previous reports: the first three phyla were common species and the last four phyla were reported from rare cases. '+' represents matches found; '-' represents matches not found.

dehydrogenase gene, which catalyzes the conversion from CO_2 to formyl-methanofuran; and *ftr*, formylmethanofuran: tetrahydromethanopterin formyltransferase gene, which catalyzes transformation from formylmethanofuran to methyl- tetrahydromethanopterin) instead of 16S rRNA gene may improve the specificity of the procedure for methanogens.

An alternate to the 16S rRNA gene for phylogenic analysis, is the *mcr*A gene. It has been reported that biodiversities of methanogens displayed by these two genes were similar (Luton et al., 2002), thus validating application of *mcr*A gene as a PCR target to study the ecology of rumen methanogens. Tatsuoka et al. (2004) were the first to report a phylogenetic analysis of this gene from the bovine rumen, and suggested that there may be unidentified methanogens participating in ruminal methanogenesis. However, the available sequences of *mcr*A gene (Table 1.4) are very limited in the database, and the primers designed based on these sequences may result in inefficient amplification. Since available primers may not be applicable to all samples, there is a requirement to design more primers to ensure adequate coverage and amplification of methanogens.

1.4.1.2 Quantitative Real-Time PCR (qRT-PCR)

Although PCR amplification is a rapid method to detect methanogens, it is not quantitative. In contrast, qRT-PCR is an approach developed to provide quantitative measurement of a target from the early phase of PCR amplification. The detection threshold of qRT-PCR is termed the threshold cycle (Ct), which is the point where the amplification curve surpasses the threshold line and enters an

Table 1.4 Universal/Archaea-specific *mcr*A gene primers.

Primer	Sequence (5' to 3')	Reference
ME1	GCMATGCARATHGGWATGTC	Hales et al., 1996
mcrA forward	GGTGGTGTMGGATTCACACARTAYGCWACAGC	Luton et al., 2002
mcrA reverse	TTCATTGCRTAGTTWGGRTAGTT	Luton et al., 2002
ME1	GCMATGCARATHGGWATGTC	Hales et al., 1996
ME2	TCATKGCRTAGTTDGGRTAGT	Hales et al., 1996
AOM39_F	GCTGTGTAGCAGGAGAGTCA	Hallam et al., 2003
AOM40_R	GATTATCAGGTCACGCTCAC	Hallam et al., 2003
q*mcr*A-F	TTCGGTGGATCDCARAGRGC	Denman et al., 2007
*qmcr*A-R	GBARGTCGWAWCCGTAGAATCC	Denman et al., 2007
MrtA_for	AAACAATCAACCACGCACTC	Scanlan et al., 2008
MrtA_rev	GTGAGCCCAATCGAAGGA	Scanlan et al., 2008
mlas	GGTGGTGTMGGDTTCACMCARTA	Steinberg and Regan, 2008
mcrA-rev	CGTTCATBGCGTAGTTVGGRTAGT	Steinberg and Regan, 2008
MLf	GGTGGTGTMGGATTCACACARTAYGCWACAGC	Juottonen et al., 2006
MLr	TTCATTGCRTAGTTWGGRTAGTT	Juottonen et al., 2006

exponential phase. As a result, qRT-PCR can measure the relative density of target molecules by comparing the Ct value with a reference, or measure the absolute quantity of the targeted fragments by reference to an external standard. This method has been utilized to quantify the abundance of the archaeal community (Ohene-Adjei et al., 2008; Hook et al., 2009; Zhou et al., 2009, 2010) and to compare specific members among cattle fed different diets, and with different feed efficiencies (Zhou et al., 2009). An applicable perspective of this technique will be determination of adaptive variations in ruminal methanogens in response to changes in diet composition. For example, our previous study compared copy numbers of the 16S rRNA genes of total methanogens in cattle fed two different diets (Zhou et al., 2010). Additionally, due to its high sensitivity, it has also been used to measure low numbers of archaea associated with digesta from the small intestine of dairy cows (Frey et al., 2009).

Limitations of qRT-PCR include the high assay cost, low throughput and inability to detect two or more targets within a single sample when using intercalating dye based assays. A recently developed multi-plex qRT-PCR assay has shown some reliability to target multiple microbial targets simultaneously (Friedrich and Lenke, 2006). This assay may be utilized to measure two specific groups of methanogens within a sample, or to enumerate two or more functional genes involved in methanogenesis at the same time, but such an application has yet to be reported. Another limitation could be that the copy number of the target gene can differ among targeted methanogens. For example, the genome of *M. smithii* contains two copies of 16S rRNA gene, while that of *M. stadtmanae*

possesses four (Klappenbach et al., 2001). In order to estimate the absolute number of methanogens present in a sample, an adjustment factor that considers the copy number in the cells must be considered. A recent study conducted by Steinberg and Regan (2009) quantified total methanogens and different methanogen phylogenetic groups of environmental samples by targeting the *mcr*A gene. This provides an alternative target for rumen studies and may overcome the limitation of targeting the 16S rRNA gene. In addition, the primer design for qRT-PCR strongly relies on existing sequences in databases. Availability of more methanogen-related gene sequences may provide more candidate genes to target methanogens.

1.4.2 PCR-Based Molecular Typing of Rumen Methanogens

Molecular fingerprinting methods have been applied to study the ecology of rumen microbes (Nicholson et al., 2007). The most commonly used molecular typing techniques are PCR-denaturing gradient gel electrophoresis/temporal temperature gradient gel electrophoresis (PCR-DGGE/TTGE) and terminal restriction fragment length polymorphism (T-RFLP).

1.4.2.1 PCR-Denaturing/Temperature Gradient Gel Electrophoresis (DGGE/TTGE)

PCR-DGGE/TTGE is a molecular typing method which allows rapid separation of equal sized amplicons with different sequences at different denaturant/temperature gradients (Tzeneva et al., 2008). The compositional

diversity of the methanogenic community can be visualized through this technique by comparing the various band patterns, where each band represents a microbial phylotype. PCR-DGGE/TTGE is a suitable method to profile methanogens (Nicholson et al., 2007), and it is capable of targeting members of this community even when they are present in low numbers. Many studies have used this method to compare the methanogenic communities among animals and to study alterations in the methanogenic community in the rumen. For example, Nicholson et al. (2007) utilized PCR-TTGE to examine rumen methanogens of cattle and sheep and reported that uncultured methanogens and *Methanobrevibacter* sp. may be the predominant species in ruminants. Yu et al. (2008) reported that fat supplements altered PCR-DGGE profiles of methanogens, increasing the presence of *Msp. stadtmanae* and decreasing that of *Mbb.* sp. AbM4. Zhou et al. (2010) observed a strong shift in the PCR-DGGE profiles of rumen methanogens from one that consisted primarily of *Mbb. ruminantium* in cattle fed a growing diet to a mixture of different species in cattle fed a finishing diet. Comparing samples with distinct profiles can be used to rapidly select unique bands that can be subjected to further analysis in order to characterize shifts in the methanogen population. Excision, cloning and sequencing the bands can be used to identify individual species. Theoretically, this level of differentiation can be achieved in bands that differ only in a single base pair. For example, in our previous study, a band representing *Mbb. smithii* SM9 was only in cattle fed a finishing diet and not in those fed a growing diet (Zhou et al., 2010). Interpretation of PCR-DGGE/TTGE profiles becomes more difficult if

multiple bands represent the same species. Since most methanogens possess more than one copy of the 16S rRNA gene, it is impossible to distinguish whether the multiple bands arise from the same or different genomes. In addition, PCR-DGGE fails to provide quantitative information of each phylotype.

1.4.2.2 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP is an alternative DNA based microbial profiling method which uses restriction digestion enzymes to generate fingerprinting information of targeted PCR fragments from environmental samples. It has been used to characterize microbial communities in environments such as termite guts, enriched sludge from bioreactors, activated sludge from aeration tanks, and aquifer sand (Liu et al., 1997). It has also been used to define microbial community dynamics in the gastrointestinal tracts of pigs (Leser et al., 2000), mice (Kibe et al., 2004), and humans (Jernberg et al., 2005), and it has been utilized to assess microbial diversity in the rumen and small intestine samples of dairy cattle (Frey et al., 2009). Adaptation of microbial communities to selective pressures can be assessed by analyzing cluster patterns. As with other PCR-based technologies, T-RFLP may underestimate the true microbial diversity as only limited bands are resolved with each gel, and some products may co-migrate to the same position (Fierer and Jackson, 2006). Following T-RFLP, sequencing of clone libraries is often required to better describe overall microbial populations. An expansion of this technology will be incorporation of a statistical analysis such as Bray-Curtis coefficient to compare similarity among the examined patterns and

non-metric multi-dimensional scaling to describe observed patterns (Rees et al., 2004).

As discussed by authors of various studies comparing these two technologies, both PCR-DGGE/TTGE and T-RFLP allow monitoring of many samples simultaneously (Muyzer et al., 1993; Moeseneder et al., 1999) and are capable of defining biodiversity indices among samples (Simpson et al., 1999). The difference between these technologies is that PCR-DGGE/TTGE separates bands by melting behavior, whereas T-RFLP separates fragments by size (Moeseneder et al., 1999). An increasing number of sequences have been obtained and found not to be affiliated with any known methanogen species, thus it is likely that rumen methanogen community is more complex than currently proposed. As discussed by Podar et al. (2007), minor components within the microbial community may also play important ecological roles in the rumen, suggesting that a greater effort is required to characterize those methanogens that represent only a small fraction of the methanogen community.

1.4.2.3 DNA Clone Library Analysis for Methanogenic Communities

DNA clone library analysis can be used to obtain a pool of homologues to analyze the diversity and phylogenic relationships among microbial community members. This method is usually applied to commonly expressed genes in targeted samples, such as 16S rRNA gene and *mcr*A gene for methanogens (Table 1.5). Currently, 16S rRNA gene library analysis is the most widely used method to determine the identity of each constituent within a microbial ecosystem. The

Table 1.5 Summary of molecular technologies applied for rumen microbial studies.

Methods	Common targets	Advantages	Limitations
PCR	particular genes	enrichment of molecular abundance for further analysis	amplification errors; no quantitative measurement
qRT-PCR	particular genes	enumeration of targeting genes; identification of minor microbial consortia	requirement of external standard
FISH	particular genes	in vivo observation of distribution; relative abundance estimation	requirement of fresh samples; disturbance by F_{420} autofluorescence of methanogens
Gene clone libraries	particular genes	investigation of the microbial composition	bias owing to primer selection; negligence of minor proportions
PCR-DGGE/TTGE	particular genes	observation of microbial composition and pattern shift	no quantitative measurement; askew estimation caused by targeting fragments
T-RFLP	particular genes	detection of microbial composition	no quantitative measurement; underestimation of biodiversity
Whole genome sequencing	total DNA of a single species	insights into all genes; prediction and explanation of phenotypic traits	limitation of available methanogen isolates and cultures
Metagenomics	total DNA of the entire sample	identification of genes from all organisms including the undiscovered ones; prediction of unknown metabolism	high cost; intensive efforts for data organization and interpretation

cloned partial or full length16S rRNA genes can be sequenced and compared with a reference data set as a means of identifying the microorganisms that the sequences are associated with. Using this method, rumen archaeal communities have been analyzed (Tokura et al., 1999; Tajima et al., 2001; Regensbogenova et al., 2004a; Shin et al., 2004; Wright et al., 2004, 2006, 2007, 2008; Skillman et al., 2006; Ohene-Adjei et al., 2007) with the majority of rumen methanogens being classified into three genus-level groups (*Methanobrevibacter* spp. *Methanomicrobium* spp., and RCC clade), as summarized by Janssen and Kirs (2008). This technique is more rapid than culturing, and it can identify many more methanogens through sequences associated with the 16S rRNA gene available in the database. However, species with extremely low numbers may be overlooked by this approach as only a limited number of clones are usually selected for sequence analysis. Therefore, bioinformatic tools such as Mothur program (Schloss et al., 2009) are required to determine whether the number of sequences obtained is sufficient to cover the community and to represent the diversity of the target population. Moreover, the 16S rRNA gene is not involved in methanogenesis pathways and not directly associated with the biochemistry of CH_4 production. It is only until recently that Popova et al. (2011) conducted a study measuring methanogen activities of rumen samples. Despite limitations, clone library analysis is a rapid and efficient technology to characterize rumen methanogens.

With development of an understanding of the methanogenesis pathway, more methanogen specific genes have been identified, providing alternatives for

gene library construction and phylogenetic analysis of methanogens. As mentioned above, the *mcr*A gene has been widely applied as an alternate to the 16S rRNA gene for phylogenic analysis. Denman et al. (2007) have utilized *mcr*A gene as a marker to identify effects of the anti-methanogenic compound bromochloromethane on ruminal methanogens. The cloned *mcr*A gene libraries revealed that, in the absence of this analogue, most detected phylotypes were *Methanobrevibacter* spp., whereas with the bromochloromethane *Methanobrevibacter* spp. were scarce and representatives from *Methanococcales*, *Methanomicrobiales* and *Methanosarcinales* predominated, a result that was confirmed with 16S rRNA gene clone libraries (Denman et al., 2007). The limitation of this technique is its target range. According to Fricke et al. (2006), *Msp. stadtmanae*, a common member of ruminal methanogen community, possesses the methyl-coenzyme M reductase II subunit A (*mrt*A) gene, which codes for a function similar to that of the *mcr*A gene. In Denman's study (2007), utilization of *mcr*A gene primers also amplified four *mrt*A genes in *Msp. stadtmanae*. The similarity between *mrt*A gene of *Msp. stadtmanae* and *mcr*A gene of *Mbb. ruminantium* and *Mbb. smithii* was ~75%, and the encoding amino acid sequences had ~80% identity. As *Msp. stadtmanae* does not possess *mcr*A gene, alignment results suggest that *mrt*A gene is a homolog of the *mcr*A gene in *Methanobrevibacter* spp. Since *Msp. stadtmanae* has restricted nutritional requirement for growth (Denman et al., 2007), and may be associated with feed efficiency of the host (Zhou et al., 2009), the relative prevalence of these genes may be important in identifying the proportions of these species in the rumen.

Since the *mcr*A gene or its homolog *mrt*A is part of the methanogenesis pathway, studies focusing on it may provide insights into the diversity and function of the methanogenic community (Popova et al., 2011). Future applications to link expression of this gene to methanogen diversity and density may provide insight into relationships between the composition of the methanogen community and enteric CH_4 emissions.

1.4.3 DNA Directed Studies of Methanogenic Populations

1.4.3.1 Fluorescence in situ Hybridization (FISH)

FISH is a procedure where fluorescent probes are used to hybridize with specific genes enabling the distribution of targeted population within a microbial community to be investigated. It is estimated that by combining three fluorochromes, up to 7 distinct groups of organisms can be identified at one time (Amann et al., 1996). The phylogenic identity, morphology, number and spatial arrangements of microorganisms can be reflected by observing the fluorescence emissions from the probes (Amann et al., 1995). By using this approach, the targeted methanogen can be visually identified *in vivo* at defined sampling times. Sharp et al. (1998) utilized probes to target different components within the rumen microbial community and compared the archaeal abundance among protozoal fraction, rumen fluid and within a continuously fed in vitro isolated rumen culture. Soliva et al. (2003) utilized FISH to study effects of a mixture of lauric and myristic acid on rumen methanogens, and successfully identified methanogens of the order *Methanococcaceae,* which were previously not reported

in PCR-based analysis. The most updated application of FISH in the study of rumen microbial communities by Kong et al. (2010) has quantified different groups of bacteria (e.g., *Bacteriodetes*, *Firmicutes*, and *Proteobacteria*) as well as methanogens, and found that the abundance of methanogens was stable when cows were fed a diet with or without flaxseed.

There are some limitations to FISH as instant analysis is required after sample fixation and probes may lack the sensitivity or specificity to hybridize with target cells. Furthermore, methanogens contain autofluorescent F_{420} and plant residues may also be fluorescent and, as a result dyes, involved in FISH analyses must be carefully selected to avoid interference from non-target sources (Amann et al., 1990). Some other deficiencies of this technique in studying rumen methanogens include the requirement of mechanical disturbance of the cell wall to enable probes to interact with intracellular DNA (Bottari et al., 2006) and the inability of probes to differentiate between live and dead microbial cells. Improved probe design and use of highly sensitive fluorescent detectors should overcome some of these limitations in the future.

1.4.4 Next-Generation Sequencing

While the dominant Sanger method has been used for over 30 years, new high throughput sequencing technologies are causing a fundamental shift in molecular biology. These new methods are referred to as next generation sequencing (NGS), including systems such as Roche/454, Illumina/Solexa, Applied Biosystems/SOLiD, Helicos BioSciences, and Ion Torrent. These

methods enable rapid characterization of targeted sequences and cost much less than traditional Sanger sequencing (Metzker, 2009). NGS has the potential to provide new insight into the entire metagenome of the rumen environment, including genes that are present within this microbial community at very low levels.

1.4.4.1 Whole Genome Sequencing (WGS)

De novo assemblies of microbial genomes have been accomplished with NGS technologies. Currently, 6 genome sequences belonging to four species are available for the family *Methanobacteriaceae*. Among them, *Mbb. ruminantium* is usually found to be the predominant species in the rumen, whereas *Mbb. smithii* and *Msp. stadtmanae* are common but less abundant. The genome sizes of these three species are 2.9, 1.8, and 1.7 Mb, respectively, with all species having a GC content of ~30% (Fricke et al., 2006; Samuel et al., 2007; Leahy et al., 2010). *Mbb. ruminantium* is the first methanogen from the rumen to have a completely assembled genome sequence. The *Mbb. ruminantium* genome appears to contain more diversity of genes encoding surface adhesion-like proteins than either *Mbb. smithii* or *Msp. stadtmanae*. The surface adhesion-like proteins of *Mbb. ruminantium* contain a cell anchoring domain. The successful discovery of these proteins has provided candidate targets on the cell surface for vaccine development (Leahy et al., 2010). The unique cell wall architecture of methanogens also makes it less likely that vaccines developed against cell surface proteins will exhibit cross reactivity to other members of the rumen microflora.

A more thorough understanding of the complete genome of methanogens could also pave the road to novel CH_4 mitigation technologies such as gene silencing. With this approach, artificial small RNA could be synthesized to interfere with expression of genes involved in methanogenesis. As more methanogen genomes are sequenced, genes encoding these proteins could serve as specific indicators of the types of methanogen within the rumen ecosystem.

1.4.4.2 High Throughput Microbial Diversity Detection

This technique has been recently widely applied to study microbial diversity in the gastrointestinal tract at a phylogenetic level by targeting the SSU of rRNA gene (Andersson et al., 2008; Dethlefsen et al., 2008). The procedure enables rapid screening of the gut microbiota, and thus provides a preliminary picture of the microbial ecology of the target sample. It is also utilized to generate statistically relevant links between microbial ecology and gastrointestinal tract disorders (Zoetendal et al., 2008). As the methodology and statistical approaches to this procedure are refined, it could also be applied to study of rumen methanogens. However, the technique cannot interpret the underlying mechanisms responsible for how the microbial community influences host responses, highlighting the need to continue to explore the rumen ecosystem from a functional perspective.

1.4.4.3 Function-Driven Metagenomics

This technology is commonly utilized to reveal the genetic potential of an environmental microbial community by screening out novel enzymes or probable activities from sequencing the total microbial genomes within this environment. By analyzing the data, it is possible to predict encoded proteins and metabolic pathways of the entire microbial community, as well as provide insight into microbial host interactions. Metagenomic analysis is particularly powerful as it provides information of the metabolic activities of all members of the microbial community, including those that were previously unculturable. Examples of this approach include observation of structural differences between amylase- and cyclodextrinase-like enzymes among different bacteria such as *Bacillus* spp. and *Lactobacillus* spp. (Ferrer et al., 2007), discovery of a novel laccase that contains distinct laccase motifs from the genus *Bacteroides* (Beloqui et al., 2006), isolation of novel hydrolases sequences from rumen microbes in dairy cows (Ferrer et al., 2005), and analysis of virulence associated and antibiotic resistance genes of microbial populations in cattle feces (Durso et al., 2011).

Next generation sequencing is a robust technology that generates an abundance of data that is not obtainable with other approaches, but the small fragment reads (mostly ~100–150 nt, up to 400 nt) requires sophisticated approaches to genome analysis. Data processing and handling involved in gene prediction have been described by Kunin et al. (2008). The schematic workflow of the data processing at Joint Genome Institute (JGI) is in Figure 1.2. Briefly, marker genes such as 16S rRNA gene are selected to prescreen the microbial

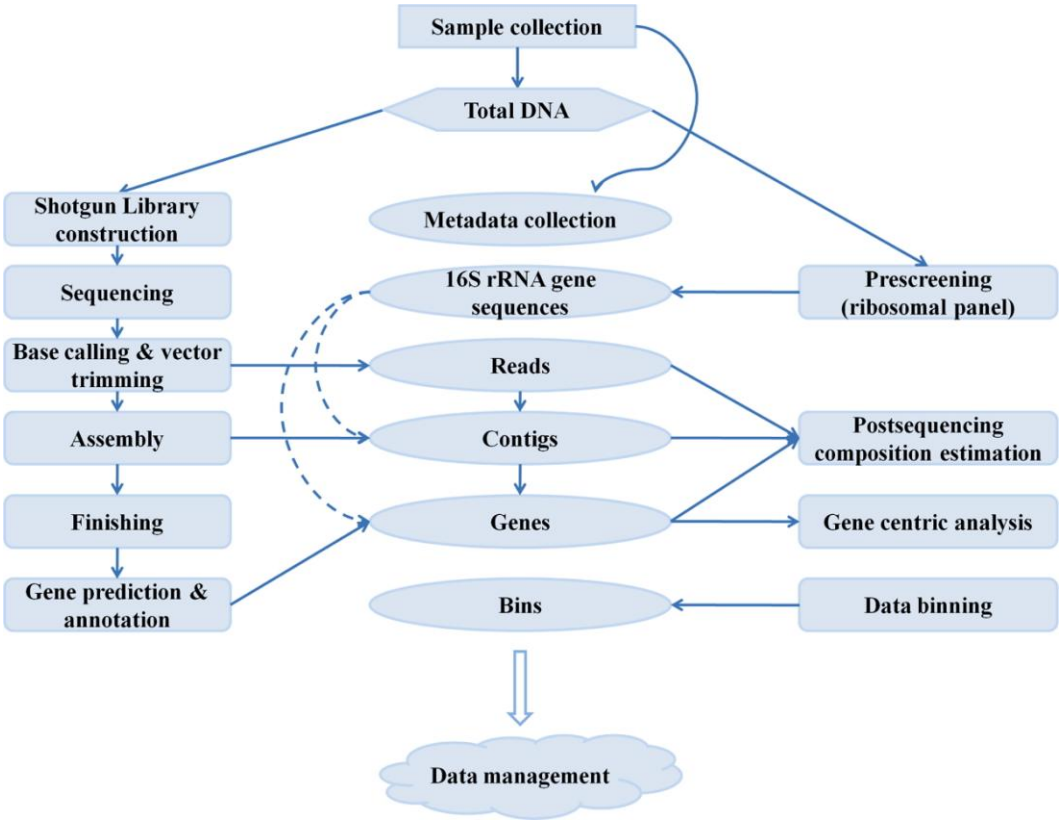

Figure 1.2 Workflow of metagenomic analysis of bacterial and archaeal communities at the JGI. Adapted from Zhou et al. (2011).

composition of collected samples. Then shotgun clone libraries are prepared using different average fragment sizes. The vector sequences are removed from the reads and the trimmed sequences are subjected to assemble and generate contigs that are formed into scaffolds. The follow up genome closure and verification are dependent on sample type. Gene prediction is then conducted to identify protein and RNA sequences that may be encoded from the assembled sequences. Genes are annotated by either homology searches or context interpretation. After gene prediction and annotation, a projection of the composition of the post-sequencing community is generated and assessed for biases that may have arisen in prescreening. Redundant sequences, sequence chimeras, and misassembled contigs are removed and, upon final verification, the complete sequence is made publicly available. Although data processing is precisely designed, it is still impossible to sequence every single sample collected from a large scale animal study. As the rumen ecosystem is dynamic, the sampling time and sampling location may influence the nature of the community observed. Prescreening animals and determining the proper samples for metagenomic analysis is a key part of the procedure. Despite large time and labor requirements, metagenomics is a powerful tool for prediction of the function and role of presently undefined members within the rumen microbial community.

1.4.5 Future Application of Molecular Techniques

The knowledge of methanogens is still limited compared to those of other ruminal microbes. Most studies have been conducted using rumen fluid- or

solids-associated methanogens with work on rumen epithelium or protozoa associated methanogens being limited. Tokura et al. (1999) examined methanogens associated with rumen ciliates using oligonucleotideprobe hybridization, and found that *Mbb. smithii* was the major species associated with protozoa, but did not determine if they were ecto- or endo-symbionts. Shin et al. (2004) examined methanogens from the bovine rumen epithelium and found that most of the epithelium associated methanogens belonged to *Methanomicrobium. mobile*, whereas Pei et al. (2010) have reported a more diverse archaeal community on the rumen epithelium of Jinnan cattle in China. However, these studies have not indicated whether these methanogens were directly attached to the epithelium or associated with other microbes within epithelial biofilms. FISH analysis may be helpful in studying the spatial association of methanogens with protozoa and the rumen epithelium. Metagenomics may also provide insights into methanogen protozoa interactions and the function of those attached to epithelial tissue. Furthermore, using other 'omics' technologies, such as metatranscriptomics, metaproteomics and metabolomics, could provide an integrated understanding of the role and function of methanogens within the rumen microbial community.

1.5 Summary

Strategies targeted at enhancing ruminant performance and controlling and minimizing agricultural GHG emissions by altering ruminal methanogenesis rely heavily on the understanding of the ecology and metabolism of ruminal

methanogens. Although many trials and experiments have been conducted, the ecology of rumen methanogens, their response to environmental changes, their activities, their interaction with other microorganisms, and their impact on host CH_4 production are not well understood up till now.

We hypothesize that the ruminal methanogenic ecology determines host CH_4 production and thereafter affects host feed efficiency and performance. The long-term goals are to understand the relationship amongst ruminal methanogens, the rest ruminal microbiota, and ruminant biology, to explore how different factors regulate this association, and to redirect the ruminal microbial community to produce less GHG and enhance host meat and milk production. The current project is a pilot study to achieve the long-term goal. One of the main objectives of the current study is to investigate the linkage between the ruminal methanogenic ecology and cattle feed efficiency traits and CH_4 emission, aiming to verify such linkage under different conditions. This study also aims to understand the mechanism how methanogenic ecology affects cattle's methane production. Results from these studies will contribute to improvement of animal production and control of enteric CH_4 emission.

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2.0 ASSESSMENT OF THE MICROBIAL ECOLOGY OF RUMINAL METHANOGENS IN CATTLE WITH DIFFERENT FEED EFFICIENCIES[2]

2.1 Introduction

Microbial fermentation and ruminal nutrient absorption are key steps in the energy metabolism of cattle. The microbiota in the rumen is highly associated with diet, age, antibiotic use, and health of host animals (Stewart et al., 1997). Different types of symbiotic anaerobic microorganisms, including bacteria, archaea, ciliated protozoa, and fungi, inhabit the rumen (Kamra, 2005), interact with each other, and play important roles in affecting the host's performance. The microbial-host relationships are highly complex and varied, ranging from mutually beneficial cooperation to competition (Hungate, 1984). Among ruminal microbes, bacteria ferment the feed into short-chain (C_1 to C_5) fatty acids, amino acids, H_2, and CO_2, etc. (Mackie et al., 2000). To maintain the low hydrogen level in this habitat, hydrogen-utilizing microbes, such as methanogens, utilize H_2 and carbon substrates, mainly CO_2, acetate, or methanol, to generate methane gas and hence to reduce hydrogen pressure in the rumen (Hedderich and Whitman, 2006). However, this process causes a significant (2-12%) loss of dietary energy in the form of methane emission (Johnson and Johnson, 1995), which contributes to 13 to 19% of global greenhouse gas

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(Lassey et al., 1997), and is one of the significant agricultural "causative sectors" contributing to global warming (Joblin, 1996). Therefore, the energy loss and the consequent methane emission are both a nutritional and environmental concern to the livestock industry.

Archaeal methanogens are obligate anaerobes (Woese et al., 1990), and species of the order *Methanobacteriales* are the most common methanogens found in the rumen (Jarvis et al., 2000). Recent studies using culture-independent methods to investigate the methanogenic communities in the rumen, and have identified 21 different strains belonging to 13 species in sheep (Wright et al., 2004, 2006, 2008; Yu et al., 2008) and 13 different strains related to 8 species in cattle (Whitford et al., 2001; Nicholson et al., 2007; Wright et al., 2007). In addition, the identification of novel uncultured methanogens in the rumen (Tajima et al., 2001; Wright et al., 2004; Nicholson et al., 2007) suggests that the understanding of methanogenic ecology is limited. Cattle with higher feed efficiencies are reported to produce 20 to 30% less methane (Nkrumah et al., 2006; Hegarty et al., 2007). However, the linkage between rumen methanogenic composition and the host's feed efficiency and methane production has not been studied and reported.

As one of the indicators of feed efficiency in cattle, residual feed intake (RFI) measures the difference between an animal's actual feed intake and the expected feed requirements for growth (Archer et al., 1999; Basarab et al., 2003). Cattle with low RFI (L-RFI) are designated "efficient," while animals with high RFI (H-RFI) are designated "inefficient." A recent study reporting a correlation

between bacterial profiles and cattle RFI has suggested the probable linkage between rumen microbial ecology and feed efficiency in cattle (Guan et al., 2008). Therefore, we hypothesized that the structures and populations of methanogens may be also associated with RFI and methane gas production by the host. In this study, the compositions of methanogens in the rumens of cattle with different RFIs were compared by sequence analysis of the partial 16S rRNA genes (800 bp) generated from two constructed libraries, using pooled DNA from efficient (L-RFI) and inefficient (H-RFI) animals. The population of selected species in each steer was evaluated using quantitative real-time PCR (qRT-PCR) analysis, and the correlation between methanogenic structure/population and cattle RFI was investigated.

2.2 Materials and Methods

2.2.1 Animal Experiment and Rumen Sample Collection

Fifty-eight 10-month-old steers (Hereford crossed with Aberdeen Angus) were raised following the guidelines of the Canadian Council on Animal care (1993) in a feedlot at the Kinsella Research Station, University of Alberta, using a finishing diet described by Nkrumah et al. (2006). The animal protocol was approved by the Animal Care and Use Committee (Moore-2006-55), University of Alberta. Feed intake data were collected using the GrowSafe automated feeding system (GrowSafe Systems, Ltd., Airdrie, Alberta, Canada), a total mixed finishing composed of approximately 74% oats, 20% hay, and 6% feedlot supplement (32% crude protein beef supplement containing Rumensin [400

mg/kg of body weight] and 1.5% canola oil) (Basarab et al., 2003). The feed efficiencies of steers were ranked as inefficient (H-RFI [RFI of > 0.5]) or efficient (L-RFI [RFI of < -0.5]) on the basis of calculated RFI values as described by Nkrumah et al. (2006). In this study, the RFI values for the examined steers (n = 58) were ranked as L-RFI (-0.68 ± 0.04 kg/day) and H-RFI (0.65 ± 0.05 kg/day) groups ($P < 0.0001$). Rumen sampling was performed within 1 week after RFI evaluation. Ruminal fluid was collected within 3 h after feeding by inducing flexible plastic tubing into the rumen and using the suction created with a 50-ml syringe to remove the fluid from the tubing. For each animal, 50 to 100 ml of rumen fluid was collected twice and transferred into a separate sterilized container, immediately frozen with liquid nitrogen, and stored at 80°C until processing.

2.2.2 DNA Extraction

Total DNA was extracted from 58 rumen fluid samples by using the methods outlined by Guan et al. (2008). In brief, 0.5 ml of frozen rumen fluid was thawed on ice and washed with 4.5 ml of TN150 (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) buffer, followed by 30 s of vortexing and 5 min of centrifugation at 200 × g at 4°C. Then, 1 ml of supernatant was transferred to a new microcentrifuge tube containing 0.3 g autoclaved zirconium-silica beads (0.1-mm diameter), and the cells were lysed by physical disruption in a model 8 BioSpec mini-bead beater at 4,800 rpm for 3 min. The supernatant of each sample was collected, DNA extraction was then performed with phenol-chloroform-isoamyl

alcohol (25:24:1) extractions, and the DNA was precipitated with cold ethanol and dissolved in 20 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The concentration and quality of DNA were measured at A_{260} and A_{280} by using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.2.3 Construction of 16S rRNA Gene Libraries

Individual total DNA extracted from rumen fluid was diluted to a concentration of 50 ng/μl and was pooled by mixing 2 μl of each DNA sample from efficient animals (n = 29) (library 1) and inefficient animals (n = 29) (library 2) for library construction. The partial archaeal 16S rRNA gene (~800 bp) was amplified with the universal primer pair Met 86f/Met 915r (Table 2.1), using the following program: an initial denaturation for 5 min at 94°C; 30 cycles at 94°C for 30 s, 57°C for 30 s, and 68°C for 1 min; and a final elongation for 7 min at 68°C. The PCR solution (50 μl) contained 1 μl of 20 pmol of each primer, 1 μl of 10 mM deoxynucleoside triphosphate, 2.5 U of Taq polymerase (Invitrogen, Carlsbad, CA), 1 × PCR buffer, 1 μl of 50 mM $MgCl_2$, and 1 μl of pooled DNA template. Amplified PCR products were then cloned into the TOP10 vector (TOPO TA cloning kit; Invitrogen). Colonies with insertion were then selected on X-Gal (Sigma, St. Louis, MO) medium, and the plasmid DNA was extracted using a Millipore plasmid extraction kit (Millipore, Billerica, MA).

Table 2.1 Primers used in this study to target methanogen 16S rRNA genes.

Primer[a]	Sequence (5'to 3')	Reference
Met 86f	GCTCAGTAACACGTGG	Wright and Pimm, 2003
Met 915r	GTGCTCCCCCGCCAATTCCT	Watanabe et al., 2004
21F	TTCCGGTTGATCCYGCCGGA	Shin et al., 2004
1389-1406R	ACGGGCGGTGTGTGCAAG	Loy et al., 2002
Met 1340r	CGGTGTGTGCAAGGAG	Woese et al., 1990

[a] "f" designates the forward primer and "r" the reverse primer.

2.2.4 Sequencing and Phylogenetic Analysis

From libraries 1 and 2, 624 and 672 clones, respectively, were randomly selected and subjected to sequence analysis with an ABI 3730 sequencing system, using an ABI PRISM BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The sequence reaction was performed with 10 μl of solution containing 0.5 μl of BigDye, 3.2 pmol of M13 Forward (CGCCAGGGTTTTCCCAGTCACGAC) or M13 Reverse (TTCACACAGGAAACAGCTATGAC) primer, 2.0 μl of 5 × sequencing buffer, and 20 ng of plasmid DNA as the template. All sequences were subjected to BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches to determine the closest known taxon and were aligned using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/). The phylogenetic analysis was performed using the neighbor-joining method with the PHYLIP package (version 3.67, http://evolution.genetics.washington.edu/phylip.html). Bootstrap numbers obtained from 1,000 replicates were assigned beside the nodes to verify the clustering of the sequences.

2.2.5 Clone Library Analysis

The obtained libraries were then analyzed using the Mothur program (Mothur v1.3.0, http://www.mothur.org/wiki/Main_Page) by comparing operational taxonomic units (OTUs) on the basis of 97% similarity between sequences. Distance matrices were calculated by using the DNADIST program within the PHYLIP software package. Rarefaction analysis of library structure

was conducted based on the principle in the DOTUR program (Schloss et al., 2005). Diversity indices, such as the Shannon index, the Simpson index, and the Chao1 index, were used to measure the diversity of each library. Differences between the libraries were analyzed by comparing the levels of coverage of the samples, the similarities of community membership (Ochiai index), and the community structures (Bray-Curtis index) based on the principle in the ∫-LIBSHUFF program (Schloss et al., 2004). Community diversity was compared in a phylogenetic context, using the UniFrac significance test and the P test within UniFrac (Lozupone et al., 2006).

2.2.6 qRT-PCR Analysis

The populations of selected species were determined by calculating the copy numbers of 16S rRNA genes. Three pairs of primers (Table 2.2) were used to detect *Methanobrevibacter* sp. strain AbM4, *Methanosphaera stadtmanae*, and total methanogens in each rumen sample. Species-specific and universal primers were designed based on the alignment of the identified targeted species sequences and all sequences, respectively, in two libraries, and the conserved region was targeted by using the software package Primer Express 3.0 (Applied Biosystems, Foster City, CA). qRT-PCR was performed with SYBR green chemistry (Fast SYBR green master mix; Applied Biosystems), using the StepOnePlus real-time PCR system (Applied Biosystems) with a fast cycle, a melting curve section, and the following program: 95°C for 10 min, followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. For melting curve detection, the temperature was increased

Table 2.2 Primers used in this study for methanogen 16S rRNA gene qRT-PCR analysis.

Organism(s) targeted	Primer[a]	Sequence (5' to 3')	Product size (bp)
Mbb. sp. strain AbM4	AbM4-F	TTTAATAAGTCTCTGGTGAAATC	~160
	AbM4-R	AGATTCGTTCTAGTTAGACGC	
Msp. stadtmanae	Stad-F	CTTAACTATAAGAATTGCTGGAG	~150
	Stad-R	TTCGTTACTCACCGTCAAGATC	
Total methanogens	uniMet1-F	CCGGAGATGGAACCTGAGAC	~160
	uniMet1-R	CGGTCTTGCCCAGCTCTTATTC	

[a] "F" designates the forward primer and "R" the reverse primer. All sequences were determined in this study.

0.3°C every 20 s from 60°C to 95°C. The standard curves were constructed by using species-specific primers based on a serial dilution of plasmid DNA from clones identified as *Methanobrevibacter* sp. strain AbM4 and *Methanosphaera stadtmanae*. The copy numbers of each standard curve were calculated based on the formula $(NL \times A \times 10^{-9}) / (660 \times n)$, where NL is the Avogadro constant (6.02×10^{23} molecules per mol), A is the molecular weight of the molecule in standard, and n is the length of the amplicon (bp). The copy numbers of 16S rRNA genes of targeted methanogens per ml rumen fluid were calculated using the formula $(MQ \times C \times VD) / (S \times V)$, where MQ is the quantitative mean of the copy number, C is the DNA concentration of each sample, VD is the dilution volume of extracted DNA, S is the DNA amount (ng) subjected to analysis, and V is the rumen fluid volume subjected to DNA extraction. PCR efficiency (E) was calculated using the equation $E \times (10^{-1/\text{slope}\ -1}) \times 100$, and the data generated from reactions with more than 90% efficiency were used for further analysis.

2.2.7 Statistical Analysis

Copy numbers and proportions of specific methanogen species were obtained from each individual, and the mean value was used for statistical analysis. Student's t test was used to verify the difference in each targeted species of methanogen between L-RFI and H-RFI animals. A simple covariance mixed model was used to correlate methanogen population with volatile fatty acid production and RFI by using the SAS system (version 9.1; SAS Institute, Cary, NC). Significance was defined at P values of < 0.05.

2.2.8 Nucleotide Sequence Accession Numbers

The nucleotide sequences generated from this work have been deposited in GenBank under accession numbers FJ579097 to FJ580045.

2.3 Results

2.3.1 Comparison of Sequences Generated from 16S Clone Libraries

To identify methanogen profiles in the rumen, different combinations of reported universal methanogenic primers were used to amplify full or partial 16S rRNA gene products for library construction. But the attempt to generate a full 16S rRNA fragment with the combination of 21F (Shin et al., 2004) and 1389-1406R (Loy et al., 2002) as described by Ohene-Adjei et al. (2007) was not successful, although these primers successfully targeted total methanogens in the ovine rumen. The usages of Met 86f/Met 1340r as outlined by Wright and Pimm (2003) and the primer combination 21F/Met 1340r were not able to generate the PCR products from all animals. Only the primer pair Met 86f/Met 915r targeting a partial 16S rRNA gene product (~800 bp) was found to generate amplicons from all 58 rumen samples. Therefore, this primer pair was used to amplify the pooled rumen DNA for library construction.

In total, 482 and 490 sequences were obtained from library 1 (pooled L-RFI animals) and library 2 (pooled H-RFI animals), respectively. From the rumens of L-RFI animals (library 1), 478 out of 482 sequences were identified to

be methanogens, while 471 out of 490 sequences were identified to be methanogens from the rumen of H-RFI animals (library 2). The sequences identified to be nonmethanogens, 4 sequences from library 1 and 19 sequences from library 2, were found to belong to 13 bacterial phylotypes. Since up to 16 bp of the primer sequences matched with the same region of bacteria, it is not surprising that the universal methanogen primers could also amplify some groups of bacteria. These sequences were not included for methanogenic community analysis.

The taxonomy of each methanogen library was characterized first by determining the OTUs on the basis of 97% sequence similarity. In total, 31 unique OTUs were identified, with 22 OTUs from library 1 and 27 from library 2 (Table 2.3). Eighteen OTUs were found in both libraries (58.06% of total OTUs), while four and nine OTUs were found to be library 1- and library 2- specific, respectively (Figure 2.1). When the structures and diversities of the two libraries were compared, higher values for Shannon index, diversity, and richness were observed in library 2, revealing that the methanogenic community of library 2, consisting of H-RFI animals, was more diverse than that of library 1, consisting of L-RFI animals. The differences in OTUs between the libraries at 100% similarity in a phylogenetic context were significant, with P values of < 0.01 by both the P test (for transfer of lineages between libraries) and the UniFrac test (for evolutionary history shared between two libraries) in the UniFrac program (data not shown).

Table 2.3 Comparison of structure diversities of sequenced clones in library 1 and library 2[a].

Sample Source	No. of sequences	No.of OTUs	Shannon index	Diversity[b]	Richness[c]	Coverage (%)[d]	Similarity of community membership[e]	Similarity in community structure[f]
Library 1	478	22	0.9 (0.8-1.1)	0.42	23 (20-41)	100	0.7385	0.773
Library 2	471	27	1.5 (1.4-1.7)	0.84	32 (25-68)	96.2		

[a] Estimates of Shannon index, diversity, and richness are all based on 3% differences in nucleic acid sequence alignments. Values in parentheses are 95% confidence intervals as calculated by Mothur.

[b] Sample-size-independent estimate of diversity based on negative natural log transformation of Simpson's index values as calculated in Mothur.

[c] Chao1 values, a nonparametric estimate of species richness.

[d] Coverage values for distance = 0.01, as calculated by Mothur.

[e] Ochiai index describing the similarity between two communities.

[f] Bray-Curtis index describing the similarity between the structure of two communities.

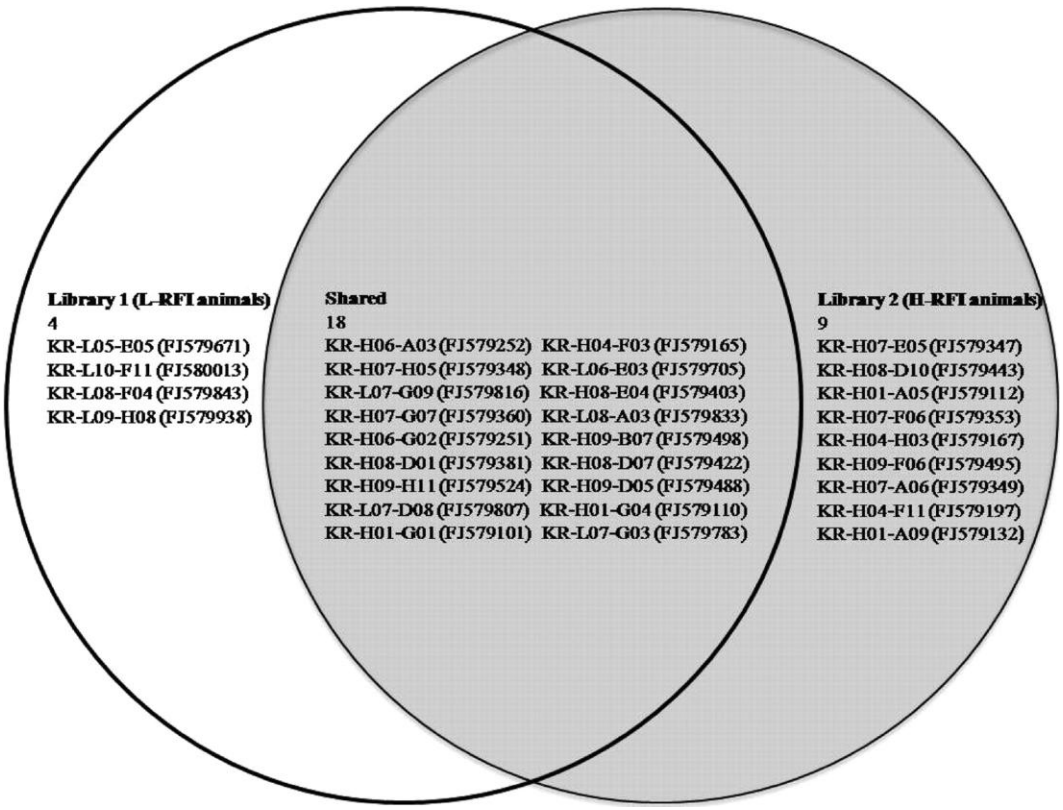

Figure 2.1 Diagram of OTUs identified by the Mothur program at the 97% similarity level within and between libraries 1 (L-RFI animals) and 2 (H-RFI animals). Representative OTUs are presented by the clone identification numbers, with GenBank accession numbers in parentheses.

2.3.2 Taxonomy Characterization of Methanogenic Ecology in the Rumen

To evaluate the identified difference in community structure between the two libraries, the taxonomies of all the OTUs were further investigated by a BLAST search based on an approach described by Ben-Dov et al. (2006). The following criteria were used to determine the taxonomy of each OTU: a ≥ 97% match between the clone sequence and the GenBank data was considered to represent strains within the species level, and 93 to 96% identity represented different species at the genus level. All the OTUs obtained in this study resembled seven strains within five known species: *Mbb. ruminantium*, *Mbb. thaueri*, *Mbb. smithii*, *Mbb. wolinii*, and *Msp. stadtmanae*.

Four hundred and twelve and 322 sequences in library 1 (L-RFI animals) and library 2 (H-RFI animals), respectively, were identical to *Mbb. ruminantium* NT7 (AJ009959), which was predominant in both groups of animals, but with different distributions: 89.2% of the total clones from the library 1 and 73.0% of the total clones from library 2 (Figure 2.2). The distributions of other species also varied between L-RFI and H-RFI animals. For example, for L-RFI animals, five sequences resembled *Mbb.* sp. strain AbM4 and eight sequences resembled *Msp. stadtmanae*, accounting for 1.0% and 1.7% of the total sequences, respectively (Figure 2.2). For H-RFI cattle, 53 sequences resembled *Mbb.* sp. strain AbM4 and 27 sequences resembled *Msp. stadtmanae*, representing 10.8% and 5.7% of the total sequences, respectively (Figure 2.2). *Mbb. wolinii*-like *Mbb.* sp. strain AbM4 sequences have not previously

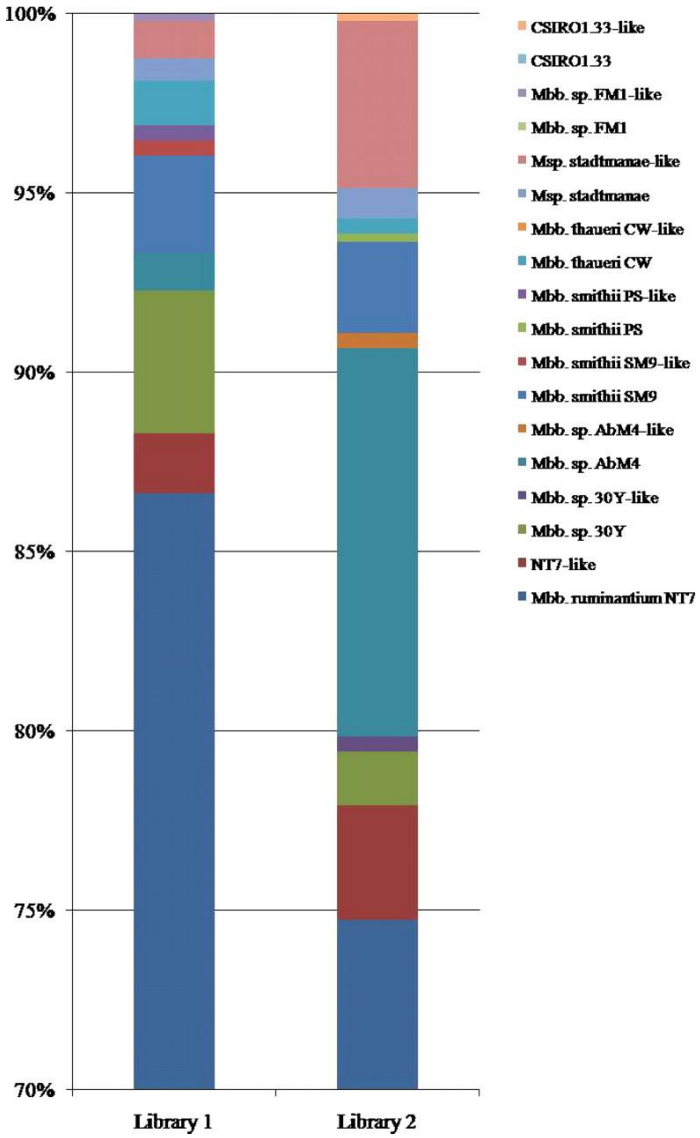

Figure 2.2 Distribution of methanogenic species on the basis of their sequences, classified as methanogens from library 1 (L-RFI animals) and library 2 (H-RFI animals). NT7, *Mbb. ruminantium* NT7; 30Y, *Mbb.* sp. strain 30Y; AbM4, *Mbb.* sp. strain AbM4; SM9, *M. smithii* SM9; PS, *Mbb. smithii* PS; CW, *Mbb. thaueri* CW; FM1, *Mbb.* sp. strain FM1; CSIRO1.33, *Methanobacteriales* archaeon CSIRO1.33 clone. The *y* axis shows that the percentages of >70% for more than 70% of the sequences were *Mbb. ruminantium* NT7 sequences in both libraries.

been reported to occur in the bovine rumen. In addition, the distributions of different strains varied between the two groups of animals (data not shown).

Furthermore, variation of methanogens at the genotype level in the two libraries was observed. For example, numerous genotypes in the sequences identified as *Mbb. ruminantium* NT7 were observed to have high levels of diversity of single-nucleotide polymorphisms (SNPs). For instance, for the sequences with 99% identity with the *Mbb. ruminantium* NT7 strain, 197 sequences in library 1 and 163 sequences in library 2 belonged to 264 genotypes. Figure 3 shows the alignment of six sequences with 99% identity with *Mbb. ruminantium* NT7 strain with SNPs observed in six representative locations (Figure 2.3). When the association between the genotypes and cattle RFI was analyzed, some genotypes were detected only in L-RFI animals, while some were identified only in H-RFI animals. For example, clones KR-L06-H10, KR-L08-E10, and KR-L06-C11 were identified only in library 1 (L-RFI animals), and clones KR-H06-H03 and KR-H11- B04 were identified only in library 2 (H-RFI animals). Some genotypes, for example, clones KR-H11-H06 (FJ579567) and KR-H11-D04 (FJ579552), were identified in both groups of animals.

Eight putative methanogens were identified at the genus level on the basis of sequences with 93 to 96% identity with the closest species. These OTUs may represent unidentified ruminal methanogens. Among them, the sequences similar to *Mbb. ruminantium* NT7-like and *Msp. stadtmanae*-like OTUs were detected in both L-RFI and H-RFI animals, while *Mbb. smithii* SM9-like, *Mbb. smithii* PS-like, and *Mbb.* sp. strain FM1-like OTUs were detected only in L-RFI

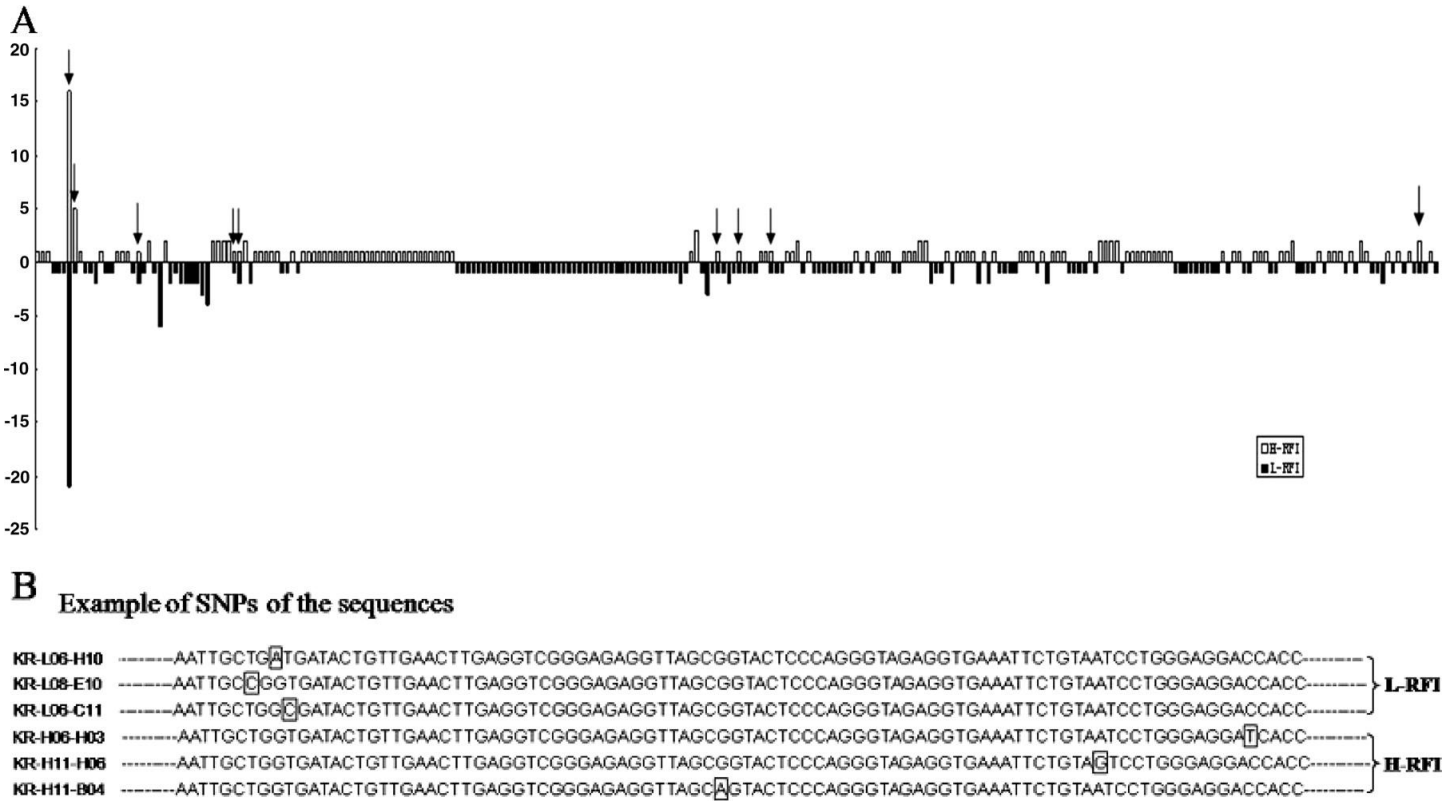

Figure 2.3 (A) Genotype analysis of all sequences with 99% identity with the *Mbb. ruminantium* NT7 strain. The bars indicate the number of sequences of each genotype in the 16S rRNA library generated from L-RFI and H-RFI animals. The arrows point out the genotypes that existed in both L-RFI and H-RFI animals. (B) Example of SNPs shown in the sequences belonging to this category. The position with a square represents the nucleotide position with SNPs. The base with a square indicates the particular SNPs of each sequence.

animals. *Mbb.* sp. strain 30Y-like, *Mbb. wolinii*-like, and *Methanobacteriales*-like OTUs were detected only in H-RFI animals (Figure 2.2).

Phylogenetic analysis of the sequenced 16S rRNA libraries was performed based on the representative OTU sequences generated from the Mothur program and the typical methanogen species. As shown in Figure 2.4, the major sequences clustered with their closest classification (the clone identification numbers are shown). Almost all major branches contained sequences from both L-RFI and H-RFI animals, with only one exception, KRH01-A09. The methanogens detected in L-RFI animals and H-RFI animals did not differ greatly at the species level. However, some sequences with low levels of identity with the known species did not cluster with the closest species, as shown in the tree. For example, OTU KR-L10-F11, with *Mbb. ruminantium* NT7 as the closest known species, was grouped with *Mbb. smithii* instead of *Mbb. ruminantium*. This confirmed our classification in which the genus-like sequences with ≥ 97% identities may represent new species within the genus *Methanobrevibacter*.

2.3.3 Comparison of Methanogen Populations between L-RFI and H-RFI Animals

The populations of total methanogens, *Mbb.* sp. strain AbM4, and *Msp. stadtmanae* were selected for qRT-PCR analysis to investigate these populations in 58 animals and the correlations with RFI. The mean total

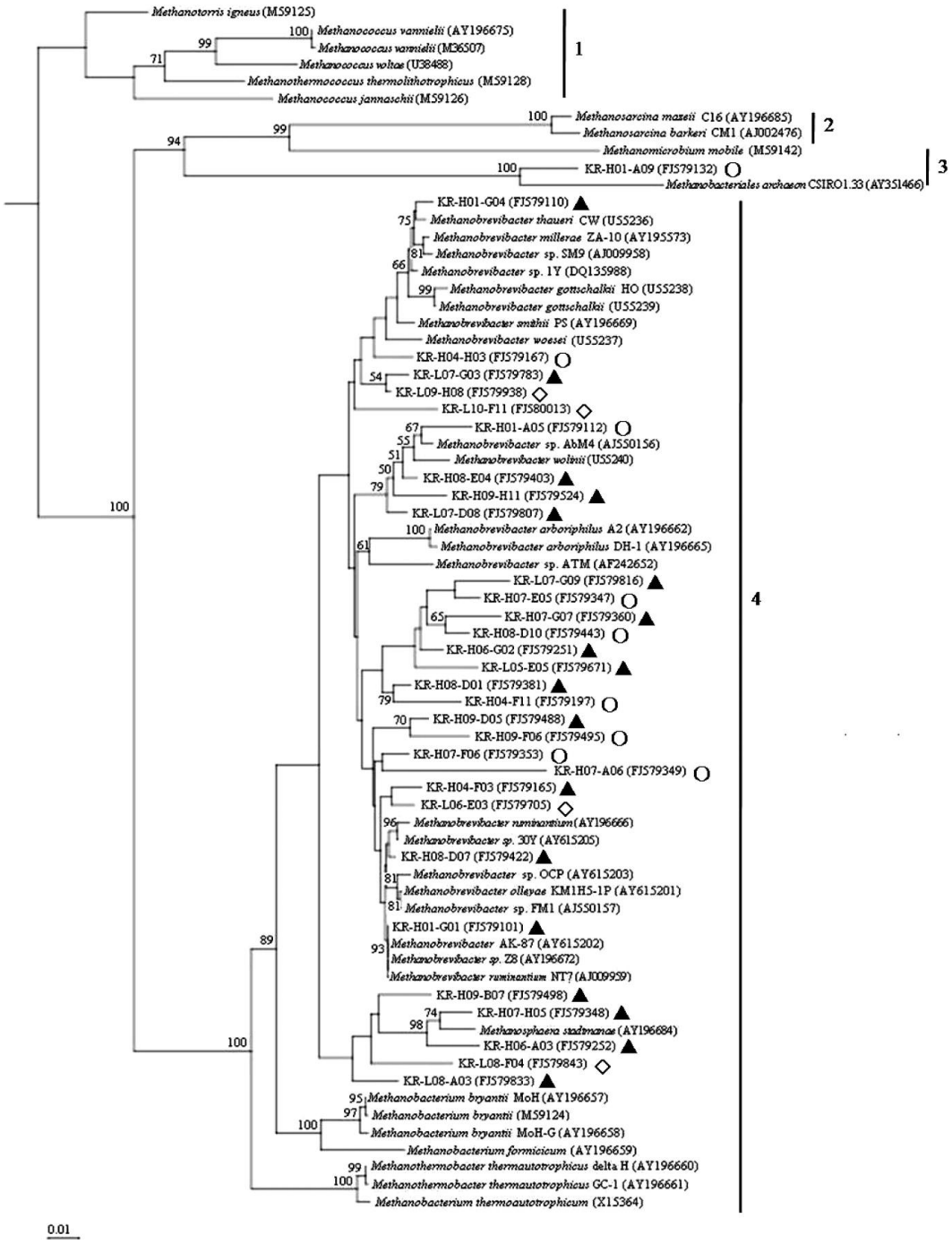

Figure 2.4 Phylogenetic analysis of methanogen partial 16S rRNA sequences obtained in this study. Representative sequences were generated by the Mothur program at a 3% difference level. GenBank sequences are identified by accession number. Bootstrap values (>50%) from 1,000 replications are indicated on the tree. 1, *Methanococcales*; 2, *Methanosarcinales*; 3, *Methanomicrobiales*; 4, *Methanobacteriales*; ▲, representative OTUs appearing in both libraries; ◇, representative OTUs appearing only in library 1 (L-RFI animals); ○, representative OTUs appearing only in library 2 (H-RFI animals).

Table 2.4 Comparison of copy numbers of targeted methanogen 16S rRNA genes in L-RFI and H-RFI animals.

	L-RFI (n = 29)	H-RFI (n = 29)	P	L-RFI (n = 29)	H-RFI (n = 29)	P	Amplification efficiency (%)
	(copies/ml)			Proportion of methanogen (%)			
Total methanogen	2.12×10^{7}	2.52×10^{7}	0.162	100	100	-	93.55
Methanosphaera stadtmanae	1.33×10^{6}	2.48×10^{6}	0.032	9.6	18.4	0.024	94.46
Methanobrevibacter sp. AbM4	5.69×10^{5}	2.20×10^{6}	0.021	3.0	6.7	0.018	94.30

methanogen populations in L-RFI and H-RFI animals were 2.12×10^7 cells/ml and 2.52×10^7 cells/ml, respectively (Table 2.4), confirming the similar quantitiesof the methanogens as previously reported (Morvan et al., 1996; Saengkerdsub et al., 2007). The proportions and absolute copy numbers of 16S rRNA genes of *Msp. stadtmanae* and *Mbb.* sp. strain AbM4 were significantly lower ($P < 0.05$) in L-RFI animals than in H-RFI animals (Table 2.4). No significant difference between the two groups was observed for total methanogen population ($P = 0.16$).

Statistical covariation analysis was performed for population of targeted species, total methanogen population, and RFI. The *Mbb.* sp. strain AbM4 and *Msp. stadtmanae* populations were positively correlated with total methanogens ($P = 0.033$ and 0.011, respectively). The total methanogen, *Mbb.* sp. strain AbM4, and *Msp. stadtmanae* populations were not linearly correlated with RFI ranking (P ranged from 0.17 to 0.69).

2.4 Discussion

Previous studies have identified eight methanogenic species in the bovine rumen: *Mbb. ruminantium*, *Mbb. thaueri*, *Mbb. smithii*, *Msp. stadtmanae*, *Methanomicrobium mobile*, *Methanobacterium aarhusense*, *Methanobacterium formicicum*, and *Methanosarcina barkeri* (Jarvis et al., 2000; Whitford et al., 2001; Nicholson et al., 2007; Wright et al., 2007). In this study, we identified *Mbb. ruminantium*, *Mbb. thaueri*, *Mbb. smithii*, *Mbb. wolinii*, and *Msp. stadtmanae* in the beef cattle that were examined. This concurs with previous

studies showing that species belonging to *Methanobrevibacter* are the predominant methanogens in the rumen (Tokura et al., 1999; Skillman et al., 2004, 2006; Wright et al., 2004; 2008). Contrasting with the results from previous studies, *Methanobacterium aarhusense*, *Methanomicrobium mobile*, *Methanobacterium formicicum*, and *Methanosarcina barkeri* were not detected in our study. This may be due to differences in many aspects, such as sampling procedures, types of rumen samples, DNA extraction methods, primers used, pooling approaches for construction of libraries, diets, animal hosts, and geographic regions. Previous studies have shown that the primers used for PCR amplification could affect the taxonomy identification of predominance of methanogens in the rumen. For example, Skillman and coworkers reported that using two different sets of primers revealed differences in methanogen predominance in rumen samples: 21f/958r amplified mainly *Msp. stadtmanae*-like sequences, whereas Arch f364/Arch r1386 generated mainly *Methanobrevibacter* sequences (Skillman et al., 2006). Our results for sequencing and qRT-PCR analysis showed that *Msp. stadtmanae* copy numbers accounted for < 20% of the total methanogens (Figure 2.2 and Table 2.4), confirming the observation that *Methanobrevibacter* is the dominant genus in the rumen. In addition, the characterization of unidentified methanogen groups (the genus-like sequences) (Figure 2.2) supports the suggestion that a significant population of uncultured methanogens may inhabit the rumen (Nicholson et al., 2007). Since fewer than 700 clones were sequenced from each library, species with smaller populations might not be detected. Further experiments using more clones may improve the

identification of sequences representing species with lower population densities. In addition, since we pooled DNA from each rumen sample for amplicons for library construction, this may reduce the amplification of the rare species. Pooling the amplicon from each rumen sample for library construction may also improve the identification of numbers of species.

Sequence analysis of methanogenic structures showed that the methanogen communities in the L-RFI and H-RFI animals differed at the species, strain, and genotype levels (Figure 2.2 and 2.3). The identification of 148 and 125 genotypes with sequences 99% identical to *Mbb.* sp. strain NT7 in L-RFI and H-RFI animals, respectively, with only 9 genotypes conserved between the two groups of animals, revealed that very diverse genotypes of methanogens were represented in the rumen. It is not surprising that high numbers of genotypes of this particular strain were found, since the sequences were generated from the DNA pooled from samples derived from 29 individuals. Similarly, a study of identification of *Escherichia coli* in cows showed 240 different subtypes in 24 animals (Jenkins et al., 2003). Our data, in combination with those from the *E. coli* study, suggest that the variation of genotypes in methanogens may result from microbial mutation/adaptation to the specific host environment. Our discovery of the large portion of genotypes of methanogens indicates that the key members of the ruminal "methanogenbiome" are more complicated to define and are influenced by the host animal. Given that the genotypes were associated with RFI (Figure 2.3), it may be suggested that the difference shown in genotypes of methanogens could also influence the metabolic energy traits of the host, leading

to a variance in feed efficiency among the host animals. Future studies are needed to determine whether differences in genotype are associated with differences in methane production between L-RFI and H-RFI animals. Furthermore, the genotypes of a particular species may differ in each individual on account of many other factors, such as ruminal pH, the structures of other microbes (e.g., bacteria and protozoa), and the fermentation parameters in the rumen. Higher degrees of diversity at the species and genus levels have been reported for other microorganisms, such as bacteria (> 40 species) and protozoa (15 different genera), compared to what was found for methanogens (8 species) (Kamra, 2005), in the rumen. However, it is not clear whether the genotypes of bacteria and protozoa could also be associated with the host animals and the methanogen structure in the rumen. Further studies correlating methanogen diversity to that of other microbes, including bacteria and protozoa, may lead to the discovery of the roles of microbial-microbial interactions in feed efficiency in the host.

The unique combination of ruminal microbiota in each animal may have important roles in the host's nutrition uptake and energy metabolism, phenotypes that are usually regulated by the genetics, diet, and environment of the host. Host breed was found to have influence on ruminal bacterial structure and association between bacteria and cattle RFI within the breed (Guan et al., 2008), implying that methanogen structure may also be associated with host genetic variation. Diet is known to be another key factor that influences the microflora in the rumen. The impact of diet on methanogen profiles in the ovine rumen has been confirmed by identification of higher levels of methanogen diversity in pasture-grazing animals

than in animals fed with oaten hay (Wright et al., 2004). Diet has also been reported to influence methane gas production and population changes for particular methanogens. Recent studies in which fat was added to the feed showed reduced methane production in the rumen (Zinn et al., 1996; Machmuller et al., 2003). A preliminary study by Yu et al. (2008) revealed that dietary tallow might stimulate *Msp. stadtmanae* but inhibit *Mbb.* sp. strain AbM4 (Yu et al., 2008). Future studies of change in methanogenic structure in response to diet at the strain and/or genotype level for each animal will be essential. Furthermore, the environment may also contribute to differences in microbial diversity. It is not surprising that different species of methanogens were identified in the animals examined in our study, since different methanogens have been reported to occur in cattle raised in Canada (Whitford et al., 2001; Wright et al., 2007) and New Zealand (Nicholson et al., 2007). Cattle with higher feed efficiencies have been reported to produce less methane (Hegarty et al., 2007). The total methanogen abundance in L-RFI and H-RFI animals did not differ (Table 2.4), indicating that the quantity of total methanogens may not be vital for feed efficiency traits and may be associated with differences in methane yield. The methanogenic structures (species, strains, and genotypes) and populations of particular species/strains/genotypes may be associated with feed efficiency in cattle. The identification of higher populations of *Msp. stadtmanae* and *Mbb.* sp. strain AbM4 in H-RFI animals (Table 2.4) suggests a probable difference in methane production pathways in these inefficient animals. These two species were chosen because (i) their sequences were distributed at significantly different proportions

between the two libraries (Figure 2.2), (ii) *Mbb.* sp. strain AbM4 was identified for the first time in cattle in this study, and (iii) *Msp. stadtmanae* has been well studied for its methane production pathways. *Msp. stadtmanae* generates methane only by reduction of methanol with H_2 (Miller and Wolin, 1985). This species lacks the carbon monoxide dehydrogenase or acetyl-coenzyme A decarbonylase complex required for acetate substrate or acetyl-coenzyme A synthesis from substrates like CO_2 and a methyl group (Fricke et al., 2006). The population of *Mbb.* sp. strain AbM4 was negatively correlated with acetate concentration ($P < 0.01$) (unpublished data), indicating that acetate may be the substrate in the methanogenesis pathway of *Mbb.* sp. strain AbM4. However, more studies are required to verify such speculated mechanisms associated with methane yield and feed efficiency in the host. It has been shown that different strains of microorganisms of the same species could have distinct metabolic capacities and surface properties (Gill et al., 2006; Walker, 2007). Hence, the difference in methanogenesis substrates may be due to the results of the strain variation as we identified above. Thus, the strain level divergence of methanogens cannot be ignored, and the investigation of profiles of methanogen should include the strain variation.

2.5 Conclusion

This study demonstrated differences in methanogen ecology between rumens of beef cattle with different feed efficiencies with a growing diet. The methanogen communities were found to be different at the genus, species, strain,

and genotype levels between efficient and inefficient animals. The cattle's feed efficiency was also correlated with the population of a particular species but not with the total quantity of methanogens. *Msp. stadtmanae* and *Mbb.* sp. strain AbM4 were found to have larger amounts and proportions of 16S rRNA genes in inefficient (H-RFI) animals, suggesting that organic-substrate-based methane biosynthesis pathways may be the cause of the low feed efficiency. Future studies for linking the methanogenic structure with the methane gas yield from cattle with different RFIs will be performed to verify and elucidate the different mechanisms of methanogenesis in the animals with higher feed efficiencies. This is the first study reporting the probable association between the "methanogenic biome" and feed efficiency in cattle. Our study of the linkage between the microbial ecology of methanogens and feed efficiency in cattle will allow better understanding of the gut microbiome and its impact on host physiology.

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3.0 CHARACTERIZATION OF VARIATION IN RUMEN METHANOGENIC COMMUNITIES UNDER DIFFERENT DIETARY AND HOST FEED EFFICIENCY CONDITIONS, AS DETERMINED BY PCR-DENATURING GRADIENT GEL ELECTROPHORESIS ANALYSIS[3]

3.1 Introduction

Ruminal methanogens use methanogenesis pathways to maintain low hydrogen partial pressure and to facilitate digestion in the rumen by converting hydrogen into methane gas (Zinder, 1993; Stewart et al., 1997). However, although it is necessary, this process also has adverse effects because the released methane represents a significant loss of dietary energy for the host animal (Johnson and Johnson, 1995) and it constitutes a large proportion of the agricultural greenhouse gas emitted (Intergovernmental Panel on Climate Change, 2001; Environment Canada, 2004). Many studies to obtain a better understanding of rumen methanogens have been conducted in order to improve the efficiency of ruminal function and to mitigate methane release. Assessments by both cultivation-dependent and cultivation-independent methods have found that members of the genus *Methanobrevibacter* account for the majority of the methanogens in the rumens of sheep and cattle (Miller et al., 1986; Sharp et al., 1998; Tokura et al., 1999; Whitford et al., 2001; Irbis and Ushida, 2004; Skillman

[3] A version of this section has been published. Zhou M., E. Hernandez-Sanabria, and L.L. Guan. 2010. Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. Appl. Environ. Micro. 76: 3776-3786.

et al., 2004, 2006; Wright et al., 2007, 2008). In addition, *Methanosphaera stadtmanae, Methanobacterium* species, and *Methanosarcina barkeri* have also been found in some studies (Wolin et al., 1997; Jarvis et al., 2000). Although the phylogenetic positions of the methanogens in the rumen are diverse, these organisms utilize only three major pathways for methanogenesis: the CO_2 reduction pathway, the C_1 compound (e.g., methanol and methylamine) conversion pathway, and the acetate fermentation pathway. Each methanogen species has a substrate preference, and most methanogens can use only one or two substrates (Zinder, 1993).

Previous studies of rumen methanogens focused primarily on determining the methanogen species composition in different samples and developing strategies to reduce the methane yield from ruminants. Recently, there has been a strong desire to understand the impact of methanogens on host biology. Two primary studies found that feedlot beef cattle with higher feed efficiency (designated "efficient" animals) produced about 20% less methane gas than animals with lower feed efficiency (designated "inefficient" animals) (Nkrumah et al., 2006; Hegarty et al., 2007). The methanogenic communities of efficient and inefficient animals fed a growing diet have been compared, and divergence between the two communities has been reported (Zhou et al., 2009). However, it is not clear how the methanogens in the rumen of cattle change when the animals are fed a different diet.

The aims of this study were to describe the methanogenic communities in 56 steers with different feed efficiencies that were fed two distinct diets (a

growing diet and a finishing diet) and to understand how methanogenic communities change in response to diet modification using PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and sequence analysis. Multivariate analysis was used to analyze the association of PCR-DGGE bands with the daily dry matter intake (DMI), average daily gain (ADG), feed conversion ratio (FCR), and residual feed intake (RFI). Methanogens that were associated with diet and with host feed efficiency were identified. In addition, the methanogen population of each rumen sample was examined by quantitative real-time PCR (qRT-PCR), and the results for different RFI groups and both diets were compared.

3.2 Materials and Methods

3.2.1 Animal Experiment and Sample Collection

All experimental procedures were approved by the Animal Care and Use Committee for Livestock at University of Alberta. The steers involved in this study (n = 56; 10 months old; Hereford × Aberdeen Angus) were raised following the guidelines of the Canadian Council on Animal Care (Ottawa, Ontario, Canada) at the Kinsella Research Station, University of Alberta. Initially, the animals were fed a growing feedlot diet (74% oats, 20% hay, and 6% feedlot supplement) for 90 days, and the RFI was measured using the GrowSafe system (GrowSafe Systems Ltd., Airdrie, Alberta, Canada). Ruminal fluid samples were collected from the 56 steers on the same day before feeding and within 1 week after evaluation of the RFI by inserting a flexible plastic tube into the rumen and transferring the fluid into sterile 200-ml containers. Approximately 100 ml of

rumen fluid was obtained from each animal, immediately frozen in dry ice, and stored at -80°C until the next processing step was performed. The animals were then fed a finishing density feedlot diet (28.3% oats, 56.7% barley, 10% alfalfa pellets, and 5% feedlot supplement) for 90 days. The same RFI measurement and sample collection procedures were performed using the methods described above. In this study, animals were also classified for each variable using the following criteria: animals with a value greater than the mean plus 0.5 standard deviation (SD) were placed in the H group, while animals with a value between the mean minus 0.5 SD and the mean plus 0.5 SD were placed in the M group and animals with a value less than the mean minus 0.5 SD were placed in the L group. Thus, all of the animals were first classified using RFI values and were placed in the H-RFI (n = 20; 0.76 ± 0.05 kg/day), M-RFI (n = 14; 0.14 ± 0.11 kg/day), and L-RFI (n = 22; -0.75 ± 0.05 kg/day) ($P < 0.0001$) groups and fed the growing diet. After the diet was changed, all of the animals were reclassified based on the new RFI values, as follows: H-RFI group (n = 14, 0.94 ± 0.11 kg/day); M-RFI group (n = 23, 0.02 ± 0.05 kg/day); and L-RFI group (n = 19, -1.25 ± 0.11 kg/day) ($P < 0.0001$). Additionally, data for the daily dry matter intake (DMI), average daily gain (ADG), and feed conversion ratio (FCR) were also evaluated and used for both trials described above.

3.2.2 DNA Extraction

Total DNA was extracted from 56 rumen fluid samples using the methods described by Guan et al. (2008). Briefly, 0.5 ml of frozen rumen fluid

was thawed on ice and washed with 4.5 ml of TN150 buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl). The liquid was vortexed for 30 s and then centrifuged at 200 × *g* at 4°C for 5 min. After this, 1 ml of the supernatant was transferred to a new microcentrifuge tube containing 0.3 g of autoclaved zirconium-silica beads (diameter, 0.1 mm). The cells were lysed by physical disruption with a BioSpec Mini Bead-Beater-8 at 4,800 rpm for 3 min. The supernatant obtained from each sample was then transferred to a new sterile tube for phenol-chloroform-isoamyl alcohol (25:24:1) extraction. The extracted DNA was precipitated with cold ethanol and resuspended in 20 µl of nuclease-free water. The concentration and quality of DNA were determined at A_{260} nm and A_{280} nm using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

3.2.3 PCR Amplification of Methanogenic 16S rRNA Genes

Total DNA was extracted from each rumen fluid sample, diluted to obtain a concentration of 50 ng/µl, and used as a template in PCRs. The universal primer pair Met 86f/Met 915r (Table 3.1) was used for the initial PCR to amplify a partial 16S rRNA gene fragment (~800 bp) using the following program: initial denaturation for 5 min at 94°C; 30 cycles of 94°C for 30 s, 57°C for 30 s, and 68°C for 60 s; and final elongation for 7 min at 68°C. The PCR solution (50 µl) contained 20 pmol of each primer, each deoxynucleoside triphosphate at a

Table 3.1 Primers used in this study to target methanogen 16S rRNA genes.

Primer[a]	Sequence (5' to 3')	Reference
Met 86f	GCTCAGTAACACGTGG	(Wright and Pimm, 2003)
Met 915r	GTGCTCCCCCGCCAATTCCT	(Watanabe et al., 2004)
GC-ARC344f[b]	ACGGGGYGCAGCAGGCGCGA	(Bano et al., 2004)
519r	GWA TTA CCG CGG CKG CTG	(Bano et al., 2004)
uniMet1-F	CCGGAGATGGAACCTGAGAC	(Zhou et al., 2009)
uniMet1-R	CGGTCTTGCCCAGCTCTTATTC	(Zhou et al., 2009)

[a]f designates forward primer and r reverse primer.

[b]Primer with a 40-bp GC clamp

(CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG)

concentration of 0.2 mM, 2.5 U *Taq* polymerase (Invitrogen, Carlsbad, CA), 1 × PCR buffer, 100 mM $MgCl_2$, and 50 ng of DNA template. The PCR products were then used as templates for nested PCR amplification using the universal primer pair GCARC344f/519r (with a GC clamp added to the 5' end of ARC344f), which targeted the V3 region of the 16S rRNA gene (Bano et al., 2004) (Table 3.1). Since different PCR primer sets generate different amplicons and thus influence the observed diversity of a community, proper primers have to be used in ecological studies. As reported by Yu et al. (2008), the V3 region of the 16S rRNA gene is the preferred target in PCR-DGGE analysis when ruminal archaeal communities are profiled. Therefore, primers targeting the V3 region of the 16S rRNA gene were used for ruminal methanogenic community profiling in this study. The amplification conditions were as follows: denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 s, 56.5°C for 30 s, and 72°C for 30 s; and final elongation for 7 min at 72°C.

3.2.4 PCR-DGGE Analysis of Methanogens

The nested PCR products were subjected to DGGE using the DCode universal mutation detection system (Bio-Rad Laboratories, Inc., Hercules, CA). PCR amplicons were separated using a 6% polyacrylamide gel in 1× TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA) with a 35 to 45% linear denaturing gradient. The gel was run at 60°C and 150 V for 4 h and stained with 300 ml of 0.1% (vol/vol) ethidium bromide for 15 min. Then the gel was washed with 400 ml water for 30 min and photographed by using UV

transillumination. To effectively assign the band positions for each gel, a common ladder was included in each gel as an internal control. The ladder was generated by mixing the amplicons of plasmid DNA obtained in our previous 16S rRNA sequence analysis (Zhou et al., 2009). As shown in all PCR-DGGE gels (see Figure 3.1 to 3.3), the ladder (from top to bottom) included two bands that resembled *Methanosphaera stadtmanae* (bands *Msp. s.*-1, *Msp. s.*-2), a band that resembled a *Methanobacteriales* archaeon CSIRO1.33 clone band (band Arch.), a *Methanobrevibacter ruminantium* NT7 band (band *Mbb. r.*), a *Methanobrevibacter* sp. AbM4 band (band *Mbb.* AbM4), a *Methanobrevibacter smithii* band (band *Mbb. s.*), and a *Methanobrevibacter olleyae* band (band *Mbb. o.*). In addition, two other PCR by-product bands were identified; the sequence of one of these bands was unknown (band un.), and another band resembled a *Methanobrevibacter gottschalkii* band (band *Mbb. g.*). The DGGE band patterns obtained were analyzed using the BioNumerics software (version 5.1; Applied Maths, Inc., Austin, TX). Since the clustering analysis of the PCR-DGGE patterns could be affected by various factors, such as position bias in gels, band assignment, and different settings in the BioNumerics software, the optimal position tolerance and optimization setting were calculated using the tolerance and optimization analysis program supplied with the BioNumerics software package to ensure that band patterns were better matched. The similarity of the DGGE profiles was calculated using the average Dice similarity coefficient (D_{sc}) index and the 0.32% optimization and 0.48% position tolerance settings based on the program analysis described above.

3.2.5 Cloning and Sequence Analysis of PCR-DGGE Fragments

A total of 28 distinct bands were excised aseptically from the gels and transferred to diffusion buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA [pH 8.0], 0.1% SDS). DNA fragments were extracted using a QIAEX II gel extraction kit (Qiagen Sciences, MD) and the polyacrylamide gel extraction protocol. The extraction products were reamplified using the ARC344f/519r primers without a GC clamp as described above. The fresh PCR products were then cloned into the TOP10 vector (TOPO TA cloning kit; Invitrogen, Carlsbad, CA) using the manufacturer's chemical transformation procedures and were screened using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Sigma, St. Louis, MO) medium containing ampicillin. Colonies with insertions (white colonies) were randomly selected and used for extraction of plasmid DNA with a Millipore plasmid extraction kit (Millipore, Billerica, MA). A sequencing reaction was performed with a 10 μl solution containing 0.5 μl of BigDye solution, 3.2 pmol of M13 Forward primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3'), 1 × sequencing buffer, and 20 ng of plasmid DNA as the template using the ABI 3730 sequencing system and an ABI PRISM BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The sequence compositions for the animals were compared using the UniFrac online comparison tool (Lozupone et al., 2006).

3.2.6 qRT-PCR Analysis

The total methanogen population in each ruminal sample was determined by determining 16S rRNA gene copy numbers. A universal primer pair targeting methanogens was used as described in a previous study (Wright and Pimm, 2003). qRT-PCR was performed with the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) using SYBR green master mixture (Fast SYBR green master mixture; Applied Biosystems, Foster City, CA) with a fast cycle and melting curve and the following program: 95°C for 10 min, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. At the melting curve detection stage, the temperature was increased from 60°C to 95°C at a rate of 0.3°C every 20 s. Standard curves were constructed using serial dilutions of plasmid DNA from a clone identified as *Methanobrevibacter* sp. AbM4. The copy numbers for each standard curve were calculated based on the following equation: copy number = (NL × A × 10^{-9}) / (660 × n), where NL is the Avogadro constant (6.02×10^{23} molecules per mol), A is the molecular weight of the molecule in the standard, and n is the length of the amplicon (in bp). The copy number of a targeted methanogen 16S rRNA gene per ml of rumen fluid was calculated using the following equation: copy number = (QM × C × DV) / (S × V), where QM is the quantitative mean copy number, C is the DNA concentration of the sample, DV is the dilution volume of extracted DNA, S is the amount of DNA (in ng) subjected to analysis, and V is the rumen fluid volume used for DNA extraction. The PCR efficiency (E) was calculated using the following equation: $E = [10^{-1/\text{slope}} - 1] \times$

100%. The data generated for reactions with efficiencies between 90 and 110% were used for further analysis.

3.2.7 Statistical Analysis

Acetate concentration and feed efficiency data used in this study were obtained previously (Hernandez-Sanabria et al., 2010). All statistical analyses were performed using SAS (SAS System, version 9.2; SAS Institute, Cary, NC). The band pattern for each rumen was first analyzed using SAS and a categorical model to identify the effects of different factors on the band distribution. When the animals were classified using category variables (e.g., diet), all samples were taken into account. In contrast, when the impact of numerical variables (e.g., DMI, FCR, acetate concentration, etc.) on the band patterns was tested, animals were placed in different classes based on the following criteria: animals with a value greater than the mean value plus 0.5 standard deviation were placed in the high group (H group), and animals with a value less than the mean value minus 0.5 standard deviation were placed in the low group (L group). Only the H and L groups were used for the analysis. When the impact of RFI on methanogenic patterns was examined, only the L-RFI and H-RFI groups were used.

In the correlation analysis, PCR-DGGE patterns were converted to categorical data, and the metabolite measurements and qRT-PCR measurements were used as numerical variables. All statistical analyses were performed by using SAS (SAS System, version 9.1; SAS Institute, Cary, NC). A mixed model was used to test the differences among the RFI values and the possible interactions

between indexes. A model was used to identify covariation for all of the numeric measurements. Principal component analyses (PCA) and categorical models were used to measure the linkage between DGGE profiles and metabolic data. A P value of < 0.05 was considered statistically significant.

3.3 Results

3.3.1 Comparison of Methanogenic PCR-DGGE Profiles

PCR-DGGE profiles were obtained for samples and were first compared using each diet. For the growing diet, 24 methanogen DGGE bands were identified (Figure 3.1A); the predominant band for most of the samples was at the location of the *Mbb. ruminantium* NT7 band in the ladder, while the other bands were much less intense. The band patterns for the L-RFI group tended to group together and were separate from those for the H-RFI group, while the band patterns for the M-RFI group were more likely to group with the band patterns of either the L-RFI or H-RFI group instead of generating a distinct cluster. The overall average D_{sc} for the DGGE patterns for the growing diet was 56.4%, and the average D_{sc} values for the H-RFI group, the M-RFI group, and the L-RFI group were 59.3%, 59.5%, and 65.4%, respectively.

For the finishing diet, 22 distinct bands were identified (Figure 3.1B). Unlike the PCR-DGGE profiles obtained for the growing diet, the predominant bands for the animals were different. In general, there was only one predominant band for most animals in the L-RFI group, which corresponded to the

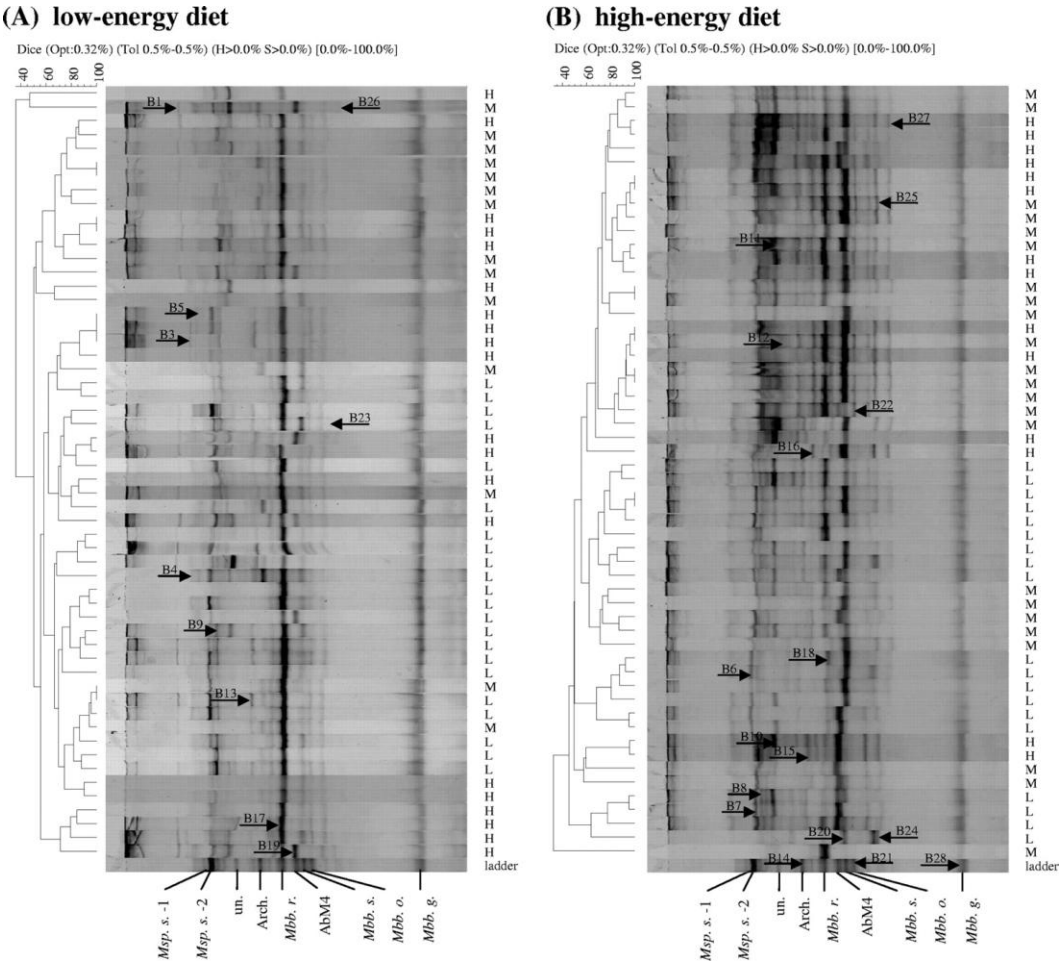

Figure 3.1 Methanogenic PCR-DGGE profiles generated using ruminal fluid from 56 animals and primers GC-ARC344f and 519r (35 to 45% DGGE). The indices for the clustering analysis are indicated at the top for each comparison. Opt, optimization (original setting, 0.32%); Tol, position tolerance, expressed as a rounded-up value (0.5%); H and S, minimum height and minimum surface, respectively (0% used for the comparison); 0.0%-100%, indicating the entire length of each lane. RFI was a variable used to identify the cattle's feed efficiency (1). The RFI groups are indicated on the right (H, H-RFI; M, M-RFI; L, L-RFI). The comparison of the PCR-DGGE profiles was generated by using BioNumerics software (as described in the text). The band pattern for the major bands assigned to species or strains in the ladder is shown at the bottom. *Msp. s.*-1 and *Msp. s.*-2, *Methanosphaera stadtmanae*; Arch., *Methanobacteriales* archaeon clone CSIRO1.33; *Mbb. r.*, *Methanobrevibacter ruminantium* NT7; AbM4, *Methanobrevibacter* sp. AbM4; *Mbb. s.*, *Methanobrevibacter smithii*; *Mbb. o.*, *Methanobrevibacter olleyae*; *Mbb. g.*, *Methanobrevibacter gottschalkii* HO. The 28 distinct bands are indicated by arrows (B, band). (A) PCR-DGGE profiles for animals fed the growing diet. (B) PCR-DGGE profiles for animals switched to the finishing diet.

band representing *Mbb. smithii* or *Mbb.* sp. AbM4 in the ladder. However, for most animals belonging to the M-RFI and H-RFI groups there were multiple prominent bands corresponding to the *Mbb. smithii* and *Mbb. ruminantium* NT7 bands. The clustering of the band patterns for this diet was similar to that for the growing diet. The overall level of similarity of all profiles was 56.9%, and the levels of similarity of the band patterns for the three groups were 70.3% for the H-RFI group, 62.0% for the M-RFI group, and 56.7% for the L-RFI group.

All of the PCR-DGGE profiles were also compared for each RFI group. As shown in Figure 3.2, all three comparisons generated the same clustering pattern with two large clusters depending on the diet. The levels of similarity of the band patterns were 51.4% for the L-RFI group, 52.6% for the M-RFI group, and 55.4% for the H-RFI group.

3.3.2 Sequence Analysis of Methanogenic PCR-DGGE Bands

A total of 28 distinct PCR-DGGE bands were identified for the entire set of samples. To characterize the taxonomic relationships of the bands, all of the bands were cloned and sequenced, and 20 bands were successfully identified (Table 3.2). Of the 20 bands identified, 17 generated a single reading sequence, while 3 bands generated multiple reading sequences. Sequencing bias was eliminated by performing DGGE again and excluding the redundant sequences from the analysis.

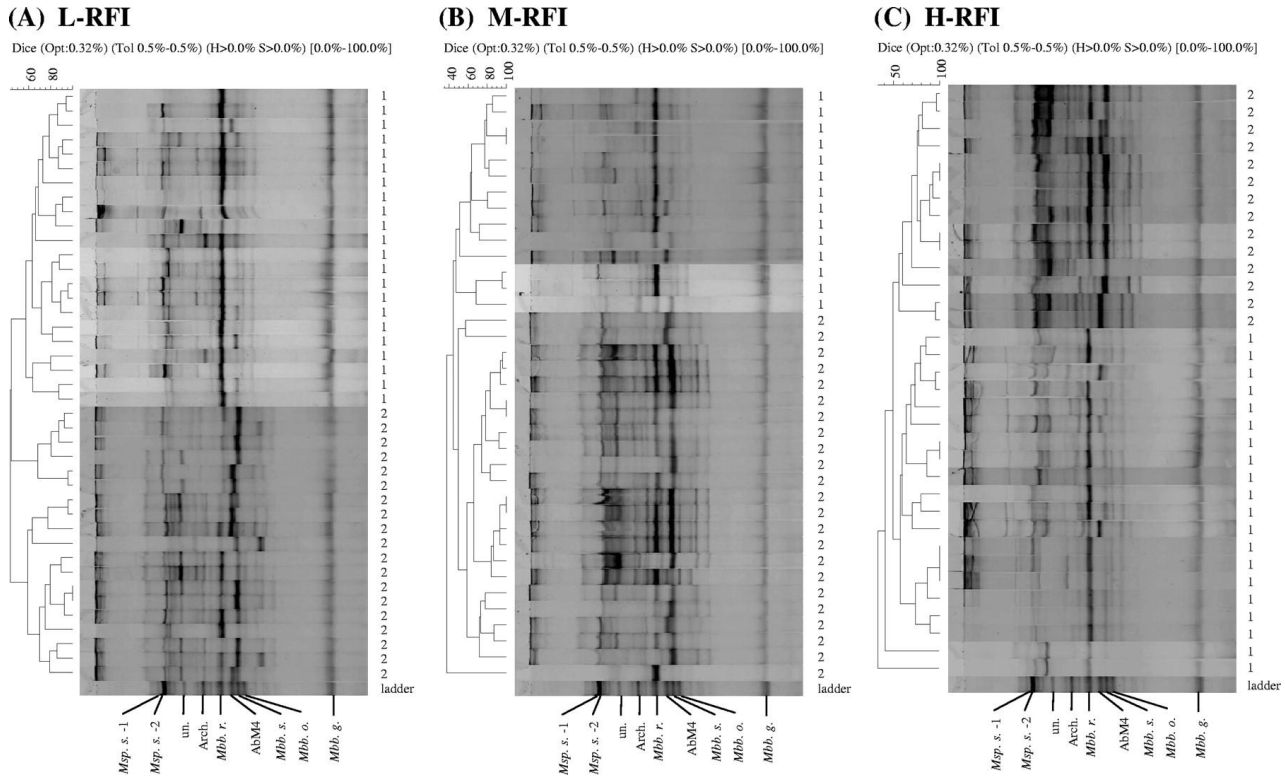

Figure 3.2 Comparison of methanogenic PCR-DGGE profiles for different RFI groups. The indices for the clustering analysis are indicated at the top for each comparison. Opt, optimization (original setting, 0.32%); Tol, position tolerance, expressed as a rounded-up value (0.5%); H and S, minimum height and minimum surface, respectively (0% used for the comparison); 0.0%-100%, indicating the entire length of each lane. The numbers on the right indicate the diets used (1, growing diet; 2, finishing diet). (A) Animals from the L-RFI group. (B) Animals from the M-RFI group. (C) Animals from the H-RFI group. The ladder shows the positions of the major bands (see the legend to Figure 3.1).

Table 3.2 Sequence identification of the PCR-DGGE bands.

PCR-DGGE Band	Closest species (GenBank Accession No.)	Similarity (%)
3	*Methanobrevibacter thaueri* strain CW (U55236)	100
5	*Methanobrevibacter gottschalkii* strain HO (U55238)	94
6	*Methanosphaera stadtmanae* (AY196684)	96
7	*Methanosphaera stadtmanae* (AY196684)	96
8	*Methanobrevibacter gottschalkii* strain HO (U55238)	94
9	*Methanogenic archaeon* SRmetG36 (EU413657)	99
10	*Methanobrevibacter ruminantium* strain NT7 (AJ009959)	94
11	*Methanobrevibacter* sp. AbM4 (AJ550156)	94
13	*Methanobrevibacter gottschalkii* strain HO (U55238)	94
14	*Methanobacteriales archaeon* clone CSIRO1.33 (AY351466)	96
16	*Methanobrevibacter gottschalkii* strain HO (U55238)	93
17	*Methanobrevibacter ruminantium* strain NT7 (AJ009959)	100
18	*Methanobrevibacter smithii* PS (U55233)	100
19	*Methanobrevibacter* sp. AbM4 (AJ550156)	100
20	*Methanobrevibacter smithii* (AY196669)	99
21	*Methanobrevibacter olleyae* (AY615201)	99
22	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	100
24	*Methanobrevibacter* sp. AbM4 (AJ550156)	99
27	*Methanobrevibacter smithii* SM9 (AJ009958)	99
28	*Methanobrevibacter gottschalkii* strain HO (U55238)	92

The sequences obtained from the 20 PCR-DGGE bands represented seven different known species and two methanogen clones, including *Mbb. thaueri* strain CW (band 3), *Mbb. smithii* strain PS (band 18), *Mbb. smithii* (band 20), *Mbb. olleyae* strain KM1H5-1P (band 21), *Mbb. smithii* ATCC 35061 (band 22), and *Mbb. smithii* SM9 (band 27). Methanogenic archaeon SRmetG36 (band 9) and *Methanobacteriales* archaeon clone CSIRO1.33 (band 14) were represented by a single phylotype; *Msp. stadtmanae* (bands 6 and 7) and *Mbb. ruminantium* NT7 (bands 10 and 17) were represented by two phylotypes; *Mbb.* sp. AbM4 was represented by three phylotypes (bands 11, 19, and 24); and *Mbb. gottschalkii* strain HO was represented by five phylotypes (bands 5, 8, 13, 16, and 28).

3.3.3 Changes in PCR-DGGE Band Patterns in Response to Changes in Host Feed Efficiency

To investigate potential associations between the methanogenic PCR-DGGE profiles and host feed efficiency and to examine the influence of the two diets on the microbial community, the band patterns generated for the H- and L-RFI groups were further compared. As shown in Figure 3.3A, for the animals that were in the L-RFI group when both diets were used, 15 common bands were found. However, for the animals that switched from the L-RFI group to the H-

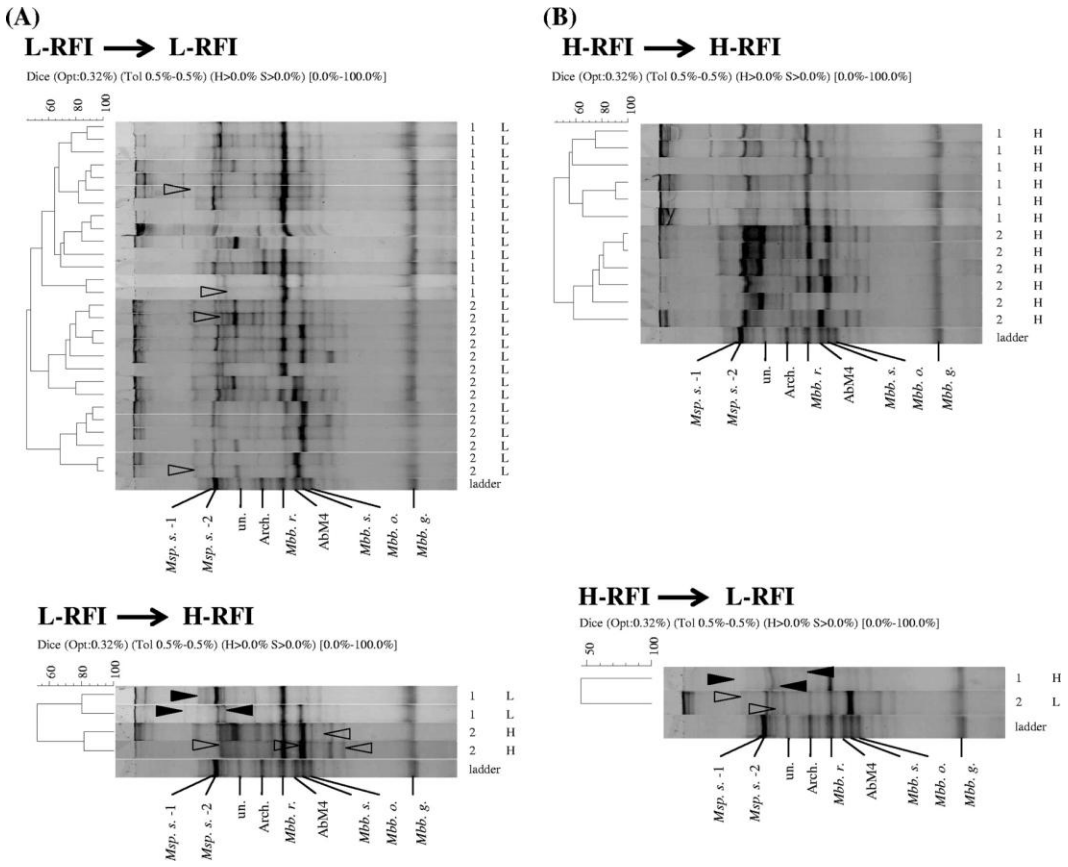

Figure 3.3 Comparison of methanogenic PCR-DGGE profiles for samples grouped based on RFI conditions. The indices for the clustering analysis are indicated at the top for each comparison. Opt, optimization (original setting, 0.32%); Tol, position tolerance, expressed as a rounded-up value (0.5%); H and S, minimum height and minimum surface, respectively (0% used for the comparison); 0.0%-100%, indicating the entire length of each lane. The numbers on the right indicate the diets used (1, growing diet; 2, finishing diet). The letters on the right indicate the RFI groups (H, H-RFI; M, M-RFI; L, L-RFI). The triangles indicate the bands that shifted for the two sets of samples (described in the text). (A) PCR-DGGE profiles for animals in the L-RFI group when the first diet was used. (B) PCR-DGGE profiles for animals in the H-RFI group when the first diet was used. For an explanation of other abbreviations, see the legend to Figure 3.1.

RFI group, three new bands were identified: band18 (*Mbb. smithii* PS), band 24 (*Mbb.* sp. AbM4), and band 27 *Mbb. smithii* SM9). Also, the intensities of two bands, bands 1 and 9 corresponding to methanogenic archaeon clone SRmetG36 bands, decreased. Figure 3.3B shows the 15 bands shared by the animals that were in the H-RFI group when both diets were used; for the animals that changed from the H-RFI group to the L-RFI group, band 3 (*Mbb. thaueri* strain CW) appeared, whereas bands 1 and 9 (methanogenic archaeon clone SRmetG36) vanished. The changes in the band pattern for the M-RFI group were also compared, and no specific band was identified for this group of animals when they switched from the L-RFI or H-RFI group and vice versa.

In addition, all of the PCR-DGGE band patterns were analyzed further to determine the impact of diet. When the 28 bands were examined, bands 1, 5 (*Mbb. gottschalkii* strain HO), and 9 (methanogenic archaeon clone SRmetG36) were found in the rumen samples only when the growing diet was used, while band 24 (*Mbb.* sp. AbM4) and band 27 (*Mbb. smithii* SM9) were observed in the rumen samples only when the finishing diet was used.

3.3.4 Associations between PCR-DGGE Patterns, Host Feed Efficiency, and Changes in Diet

To determine the frequency of the presence of the bands and to evaluate whether diet or host RFI group influenced the band distribution for the population examined, a multivariate analysis was performed. Diet-specific bands were identified for both diets (Figure 3.4A). Bands 1, 2, 4, 5 (*Mbb. gottschalkii* strain

HO), 23, and 26 were distinctively linked to the growing diet (band 1 was found in about 80% of the samples, while the other bands were found in less than 10% of the animals), whereas bands 18 (*Mbb. smithii* PS), 24 (*Mbb.* sp. AbM4), 25, and 27 (*Mbb. smithii* SM9) were found to be associated with the finishing diet (bands 24 and 27 were both detected in about 80% of the samples).

For the RFI groups, certain trends for band distribution were identified (Figure 3.4B). More than 10 bands were observed for most of the animals in both the H- and L-RFI groups (bands 3, 7, 8, 11, 13, 16, 17, 20, 21, 22, and 28), while the frequency of another set of bands was low in both RFI groups (bands 5, 12, 18, 19, and 25). Also, band 6 (*Msp. stadtmanae*) was detected more frequently for L-RFI group animals, and band 10 (*Mbb. ruminantium* strain NT7) was more likely to appear for H-RFI group animals. Additionally, five bands were found to be RFI group specific; bands 2, 4, and 23 were observed only for L-RFI group animals, and bands 15 and 18 (*Methanobrevibacter smithii* PS) were observed only for H-RFI animals. However, the abundance of each of these four bands was relatively low; band 15 was the only band that was identified for more than 20% of the entire population. Furthermore, samples from neither the H-RFI group animals nor the L-RFI group animals produced band 23 or 26; these two bands were detected only for animals in the M-RFI group.

In addition to the comparisons based on diet and RFI group, all of the PCR-DGGE band patterns were also compared for other indexes related to ruminal fermentation, such as DMI, ADG, FCR, and acetate concentration. In general, more than one-half of the bands were observed for either the H group

orthe L group for each measurement (Figure 3.4C to 3.4F). One of these four parameters, DMI, tended to be related to four bands; band 15 was observed only for an H-DMI animal, band 6 (*Msp. stadtmanae*) was found to be more prevalent for L-DMI animals, and band 10 (*Mbb. ruminantium* NT7) and band 27 (*Mbb. smithii* SM9) were more prevalent for H-DMI animals. The band distribution for the FCR revealed that bands 2, 4, 5 (*Mbb. gotschalkii* HO), and 26 were found only for L-FCR animals (at low frequencies), while band 15 was found only for H-FCR animals. The band distributions for ADG and acetate concentration were similar to each other, and in particular, bands 23 and 26 were found for only one group. The significance of each grouping was tested, and the band distribution was found to be significantly different only for the RFI groups ($P < 0.0001$) and the DMI groups ($P < 0.0001$).

3.3.5 Comparison of Total Methanogen Populations

The results for the total methanogen population were compared for the two diets and different RFI groups (Table 3.3). When animals were fed the growing diet, the sizes of the total methanogen populations in the L-RFI and H-RFI group animals were 2.12×10^7 cells/ml and 2.52×10^7 cells/ml, respectively (Wright et al., 2008). When animals were fed the finishing diet, the sizes of the total methanogen populations in the L-RFI and H-RFI group animals were 2.15×10^7 cells/ml and 2.18×10^7 cells/ml, respectively. The total methanogen population did not change in response to different diets ($P > 0.05$),

and no difference between the L-RFI group animals and the H-RFI group animals was detected ($P > 0.05$).

3.4 Discussion

Methane is an undesirable end product of ruminal fermentation because it is a greenhouse gas that has adverse effects and because it causes a notable loss of energy in cattle. Understanding the ecology of methanogens in animals with different feed efficiencies and/or different diets can help elucidate the role of methanogens in ruminal methanogenesis and the mechanisms of this process. Methanogens have fastidious nutritional requirements, and thus the availability of culturable ruminal methanogens is limited. PCR-DGGE analysis is a useful culture-independent tool for identifying the microbial components in diverse environmental samples and for observing adaptation of microbial communities to various conditions. In this study, 56 animals fed two different diets and with different feed efficiencies were used for PCR-DGGE analysis. A comparison of the results allowed detection of a complex methanogenic microbiota under various conditions that could be used to elucidate methanogenic ecological changes that may be associated with different diets and different levels of host performance.

Table 3.3 Comparison of copy numbers of targeted methanogen 16S rRNA genes in L-RFI and H-RFI animals between growing and finishing diet.

	L-RFI (copies/ml) [a]	P	H-RFI (copies/ml) [a]	P	Amplification efficiency (%)
growing diet[b]	$(2.12 \pm 0.29)\times10^7$	0.96	$(2.52 \pm 0.29)\times10^7$	0.76	93.5-108.5
finishing diet	$(2.15 \pm 0.50)\times10^7$		$(2.18 \pm 1.10)\times10^7$		

[a] Values were represented by Mean value ± SE.

[b] Values of growing diet were published by Zhou et al. (2009).

Consistent with the results of previous 16S rRNA gene sequencing analyses (Miller et al., 1986; Tokura et al., 1999; Jarvis et al., 2000; Tajima et al., 2001; Skillman et al., 2006; Wright et al., 2007; Zhou et al., 2009), the predominant members of the ruminal methanogenic community found in our PCR-DGGE analysis were *Mbb.* species. In particular, the predominant band for the samples from animals fed the growing diet was a *Mbb. ruminantium*, in accordance with a previous report (Zhou et al., 2009). The PCR-DGGE profiles of most animals contained multiple bands resembling *Mbb. gottschalkii* strain HO bands (Figure 3.4). Although it was not identified in the previous 16S rRNA library analysis, its appearance in the current study and elsewhere (Skillman et al., 2004; 2006) suggesting that this species may be common in the rumen under various diet conditions. The difference between the 16S rRNA library and the PCR-DGGE analysis data for this species may have been due to its low abundance in the rumen of the animals examined. Since PCR-DGGE can detect microbial components that comprise as little as 1% of the total population, rare species may be more likely to be discovered by analysis of the PCR-DGGE bands for each animal. The bands detected with a low frequency indicate the complexity in individuals and demonstrate the challenges when ruminal microbial communities are identified and compared.

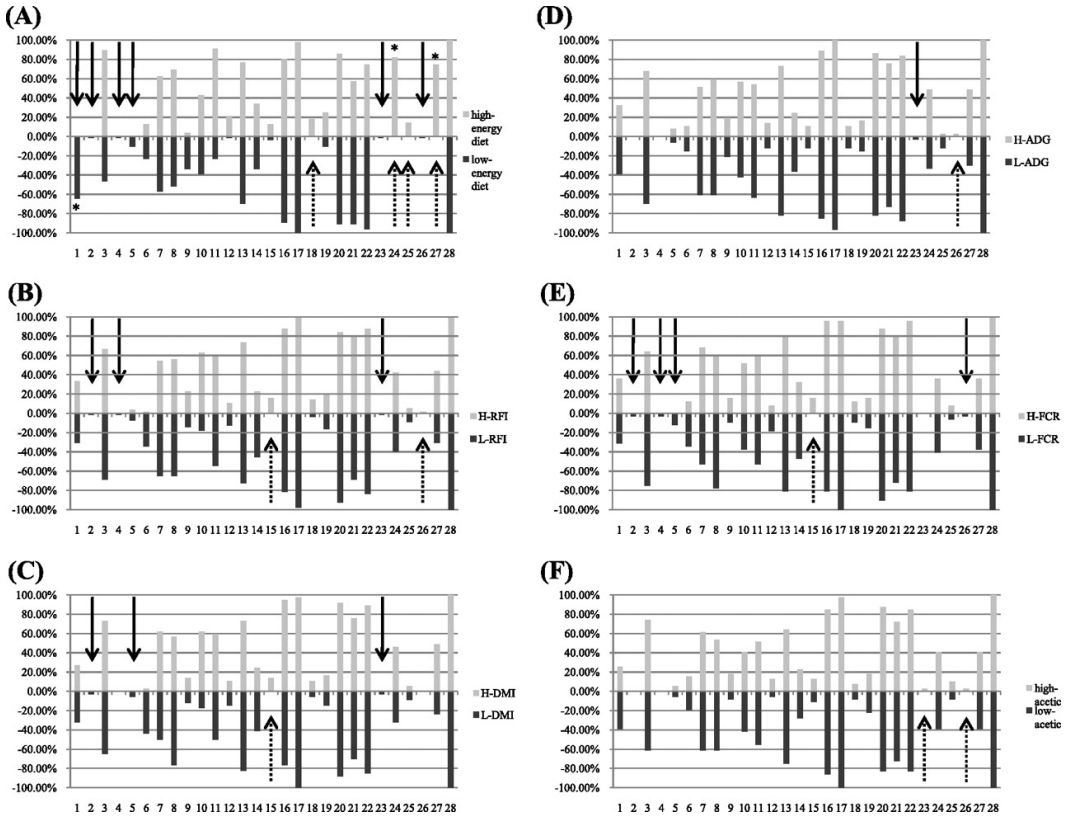

Figure 3.4 Band plot of the PCR-DGGE band frequencies. The percentages indicate the frequencies of appearance of the bands. The numbers indicate the bands from the top to the bottom of the gel. The solid arrows indicate L-group-specific bands; the dotted arrows indicate the H-group-specific bands. Group-specific bands for which the level of appearance was more than 20% are indicated by an asterisk. (A) Band plot for different diets. (B) Band plot for different RFI groups. (C) Band plot for different DMI groups. (D) Band plot for different ADG groups. (E) Band plot for different FCR groups. (F) Band plot for different acetate concentration groups.

Similar to the findings of our previous study (Zhou et al., 2009), various strains and/or genotypes of the same species were identified. It was not surprising to find multiple DGGE bands representing the same species. For example, five bands were found for *Mbb. gottschalkii* strain HO and two bands were found for *Msp. stadtmanae* (Table 3.2). This may have been due to microbial adaptation to different host animals. An alternative explanation is that the multiple bands may have been a result of amplification of a heteroduplex of the 16S rRNA gene. PCR-DGGE band patterns were compared using several tools. The sequence compositions of samples were compared using UniFrac (Lozupone et al., 2006) based on a 97% similarity cutoff. No difference at the species level was found between the groups of animals for any classification (data not shown), indicating that the divergence in the methanogenic community tended to be at the strain or genotype level rather than at the species level.

The importance of starin variance was noticeable in other microbial species. One example is the bacteria species *Escherichia coli*, which composes of hundreds of different strains, including pathogenic ones (e.g. strain O157:H7, O104:H4) and probiotic ones (e.g. strain Nissle 1917). However, the research on methanogens was not sufficient to elucidate the functional variation of different strains. Since our data revealed the strain-level variance of methanogens within the rumen microbiota of cattle with different feed efficiency, further studies are required to reveal the functions and roles of these different methanogen strains within the rumen.

As shown in Figure 3.4, the very prevalent bands may represent the core species, which are commonly found in the majority of the animals despite changes in diet, while the less prevalent bands may represent species that adapt to host conditions or particular diets. The observed change in the predominant methanogen population when the two different diets were used may have been due to a substrate utilization preference for methanogenesis by the phylotypes of the methanogens present. For instance, *Mbb. ruminantium*, the predominant species detected in numerous rumen samples, produces methane by utilizing CO_2 as the substrate (Miller et al., 1986), and *Mbb. smithii* PS utilizes CO_2-H_2 and/or formate for methanogenesis (Samuel et al., 2007) but also contains enzymes involved in the methanol-ethanol pathways (Berk and Thauer, 1997; Fricke et al., 2006). *Mbb.* sp. AbM4 was recently found in the bovine rumen (Zhou et al., 2009), but its substrate preference for methanogenesis pathways is unknown. In this study, two bands (bands 11 and 19) representing *Mbb.* sp. AbM4 were found when both diets were used, while band 24 was found only when the finishing diet was used. Sequence mutations were found for these three bands (Figure 3.5), suggesting that the band 24 phylotype may preferentially inhabit the rumen of cattle fed the finishing diet. Another example is the bands representing *Mbb. smithii*, bands 20 and 22, which represent two different strains of *Mbb. smithii* identified when both diets were used. However, in another case, bands 18 and 27 were present only when the finishing diet was fed, showing that there was a difference in the band distribution at the strain level. Accordingly, it can be speculated that diet has an impact on the methanogenic community structure in

Figure 3.5 Alignment of *Methanobrevibacter* sp. AbM4-associated bands (bands 11, 19, and 24). Mutations are indicated by boxes.

the rumen, resulting in selection of methanogens that have particular methanogenesis pathways. This supports our hypothesis that the differences at the strain or genotype level of methanogens may play an important role in differences in methane production and hence contribute to the variations in the energy lost in host animals.

Potential correlations between the PCR-DGGE bands and some phenotypic data were analyzed by using PCA and multivariate analysis. PCA showed that band 10 was more likely to be associated with RFI and DMI when the finishing diet was used and with RFI and FCR when the growing diet was used (data not shown). This band was classified as a *Mbb. ruminantium* strain NT7, but the level of identity to this species was low (94%). As shown in Figure 3.6, this band did not cluster with band 17 corresponding to a *Mbb. ruminantium* strain NT7 band; instead, it was more closely related to bands corresponding to *Msp.* sepcies. Since *Mbb. ruminantium* strain NT7 and *Msp.* species have different substrate utilization profiles for methanogenesis, the phylotype represented by band 10 may utilize substrates more similar to the substrates utilized by *Msp.* species than to the substrates utilized by *Mbb.* species. Moreover, the distribution of band 10 showed that a higher proportion of the animals in the H-RFI group than in the L-RFI group produced this band. This suggests that this phylotype may prefer H-RFI group animals over L-RFI group animals and that a possible substrate difference may be the reason for this preference. Therefore, strain and/or genotype diversity should not be neglected when the impact of methanogens on bovine rumen performance is considered. For instance, *Mbb.*

gottschalkii strain HO was represented by five different bands (bands 5, 8, 13, 17, and 28), each with a different strain sequence type. Only one of these bands, band 5, was found to be associated with the L-DMI group (Figure 3.4B). *Mbb. gottschalkii* has been reported to form a clade with *Mbb. thaueri* and *Mbb. millerae* and to occur in the rumen of lambs (Janssen and Kirs, 2008), and it has also been identified in the rumen of feedlot cattle (Wright et al., 2007). This species utilizes a CO_2-H_2 methanogenic pathway and requires acetate for growth (Miller and Lin, 2002). Further understanding of this rumen species and the methanogenic pathways utilized by it may help explain its distribution based on the DMI classification.

As indicated in our previous study, the total methanogen populations of animals with high and low feed efficiencies were not different when a growing diet was used (Zhou et al., 2009). Similarly, no significant difference in the total methanogen populations was detected when the diet was switched from a growing diet to a finishing diet. Comparable results were reported by Hook et al. (2009), who found that after a long period of monensin supplementation, the quantity of methanogens did not change significantly. In both studies, the periods after the diet was changed were long enough for the host animal to acclimate to the new conditions; thus, the total population of methanogens adapted to the fishining diet so that the level was the same as the original level. Accordingly, the total ruminal methanogen population may not be the key factor that affects methane production. The observed differences in the methanogenic communities with different methanogenesis pathways may be a fundamental characteristic of the rumen

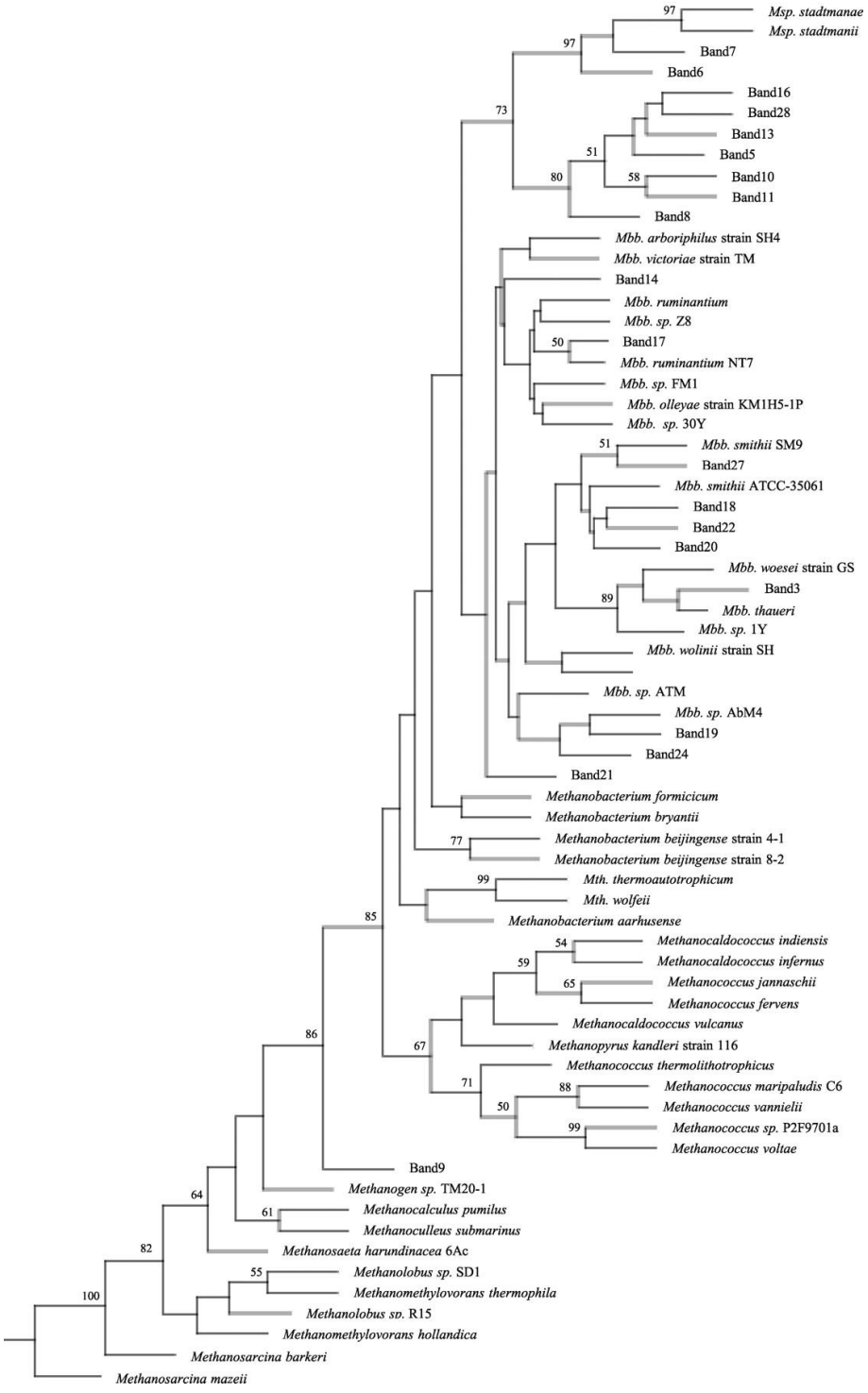

Figure 3.6 Phylogenic analysis of methanogen partial 16S rRNA sequences obtained from PCR-DGGE bands and identified species. Bootstrap values of 50% based on 100 replications are indicated at the nodes. *Msp., Methanosphaera*; *Mbb., Methanobrevibacter*; *Mth., Methanothermobacter*.

ecosystem when different feeding strategies are used, as well as of individuals.

In addition, band 9, which resembled the methanogenic archaeon clone SRmetE18 (accession no. EU413577), was detected for animals in all RFI groups (H-RFI, L-RFI, and M-RFI) when the growing diet was used, as well as for animals in the M-RFI group (n = 2) when the finishing diet was used. This clone was described in a study of Svalbard reindeer, in which it clustered with other ruminal archaeal clones and acidophilic archaea, forming a new phylogenetic clade (Sundset et al., 2009). However, the known archaeal species most closely related to the SRmetE18 clone were *Aciduliprofundum boonei* and *Thermogymnomonas acidicola*, both of which are thermophilic and hence unlikely to be ruminal species. Consequently, the physiology of the species which clone SRmetE18 represented could not be predicted. The identification of a PCR-DGGE band having a sequence similar to a sequence of this clone suggests that there may be archaea other than methanogens in the bovine rumen that have not been reported previously, and the functions of these archaea should be determined.

This study was a preliminary study which showed that there was a change in the ruminal methanogenic community when the diet was changed from a growing diet to a finishing diet. We are performing an experiment with multiple sampling points and a diet swap design to further confirm the impact of diet on ruminal methanogenic ecology and to investigate methanogen adaptation in response to diet modification. In the rumen, methanogens rely on bacteria, protozoa, and fungi to provide digestive products for methanogenesis. Therefore,

the variation in the methanogen community may also be related to these other microbial components. For example, some rumen methanogens have been reported to be associated with protozoa (Stumm et al., 1982) and to account for 37% of the total ruminal methane production (Finlay et al., 1994). Thus, our results may underestimate the complexity of the methanogen community and the interaction of methanogens with other ruminal microbes. As a result, further studies identifying protozoan-associated methanogens are necessary to determine the effect of the associations on rumen methanogen ecology, host feed efficiency, and methane production. Additionally, the results of PCR-DGGE and sequence analysis obtained in this study could have been biased by the quality of the DNA, PCR amplification, and limitations of the sequence information in the database. Use of other technologies, such as multiplex qRT-PCR assays, should increase the spectrum and quantity of the target methanogens detected and should help identify low-abundance species that we were unable to identify in this study.

3.5 Conclusion

The methanogenic community varied in the rumens of steers with different feed efficiencies that were fed different diets. The ruminal methanogenic structure was found to correlate strongly with diet, and it may be associated with RFI in beef cattle. This is the first study to report a link between the ruminal methanogenic community profile, host metabolic variables, and host feed efficiency. Methanogens were reported to interact with bacteria (Ley et al., 2006; Turnbaugh et al., 2008), however their roles in the gut and their impacts to the

host remained unclear. Our study provides a model for investigating the interactions between methanogens and hosts, as well as the interactions with other microorganisms, and for elucidating how these interactions could be impacted by nutrients in the gut. The demonstrated variation in the methanogenic community in individuals at the strain or genotypic level indicates the importance of the microbial adaptation relationship with the host and its impact on animal performance. Advanced technologies and further studies are required to obtain a worldwide perspective of ruminal microbiome for cattle and to generate a reference database for prediction of methane production, as well as animal performance.

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4.0 RELATIONSHIP BETWEEN RUMEN METHANOGENS AND METHANE PRODUCTION IN DAIRY COWS FED DIETS SUPPLEMENTED WITH A FEED ENZYME ADDITIVE[4]

4.1 Introduction

Annual global emission of methane (CH_4) from dairy cows is approximately 18.9 Tg (McMichael et al., 2007) and it represents a loss of 5.5-9.0% of dietary gross energy (Johnson and Ward, 1996). Reducing CH_4 production in dairy cows should improve productivity if it is not accomplished at the expense of lowering the digestibility of the diet. To date, various CH_4 mitigation methods have been applied (McAllister et al., 1996; Martin et al., 2010), such as defaunation (Ushida et al., 1997), dietary inclusion of monensin (Van Nevel and Demeyer, 1977), redirecting reducing equivalents to alternate acceptors (Johnson and Johnson, 1995), and stimulation of methanogen competitors such as acetogens (Leedle and Greening, 1988). However, many of these approaches are negated through microbial adaptation shortly after being applied and as a result long-term effective CH_4 mitigation methods have yet to be identified.

Dairy cows, as with all ruminants, have a symbiotic relationship with the microorganisms responsible for the fermentation of plant fibre with CH_4 being

[4] A version of this section has been published. Zhou M., Y.-H. Chung, K.A. Beauchemin, L. Holtshausen, M. Oba, T.A. McAllister, and L.L. Guan. 2011. Relationship between rumen methanogens and methane production in dairy cows fed diets supplemented with a feed enzyme additive. J. Appl. Micro. 111: 1148-1158.

one of the end products of this process. Methanogenesis is conducted by methanogens, the major archaeal members in the rumen which utilize hydrogen to reduce carbon dioxide to CH_4. As proposed by Martin et al. (2010), successful mitigation practices must account for the rumen microbiota. Thus strengthening our knowledge of the relationship between ruminal CH_4 production and the ecology of ruminal methanogens has considerable merit.

Owing to their fastidious nature, only seven species of methanogens have been isolated from the rumen: *Methanobacterium bryantii*, *Methanobacterium formicicum*, *Methanobrevibacter millerae*, *Methanobrevibacter olleyae*, *Methanobrevibacter ruminantium*, *Methanomicrobium mobile*, and *Methanosarcina barkeri* (Oppermann et al., 1957; Paynter and Hungate, 1968; Miller et al., 1986; Jarvis et al., 2000; Rea et al., 2007). Culture-independent molecular methods have shown a more diverse ruminal methanogen community in dairy cows, with the majority of the populations being represented by *Methanobrevibacter* or *Methanosphaera* spp. (Tajima et al., 2001; Whitford et al., 2001; Tatsuoka et al., 2004; Skillman et al., 2006). Members of the methanogen community have also been shown to vary among diets and with different feeding frequencies (Morvan et al., 1996; Wright et al., 2004; Saengkerdsub et al., 2007; Yu et al., 2008; Zhou et al., 2010). However, the selective pressures that give rise to these changes in the methanogen community remain largely undefined.

Several experiments to determine the mode of action and the efficacy of exogenous fibrolytic enzymes in dairy cows have been conducted, as summarized by Beauchemin et al. (2003). In many cases, exogenous enzymes improve ruminal

fibre digestibility (Rode et al., 1999), feed efficiency (Arriola et al., 2011) and increase milk production (Lewis et al., 1999; Yang et al., 2000). The impacts of exogenous enzymes on digestion are multi-faceted, and increasing the activity of endogenous microbes within the gastrointestinal tract is one proposed mode of action (Dawson and Tricarico, 2007). Recently, Holtshausen and colleagues (2011) reported increased feed conversion efficiency for milk production by supplementing dairy cows' diets with an exogenous fibrolytic enzyme additive. Further work (Chung et al., 2011) showed that the addition of this enzyme mixture to a total mixed ration (TMR) increased enteric CH_4 production in lactating dairy cows, suggesting that this enzyme additive may have altered the activity of rumen methanogens. Therefore, we hypothesize that methanogenic ecology is associated with enteric CH_4 production of the cows; supplementation of a dairy cow diet with this exogenous fibrolytic enzyme additive induces numerical and/or structural shifts in ruminal methanogens and their association with CH_4 production.

4.2 Materials and Methods

4.2.1 Animal Experimentation

The experimental procedures were approved by the Animal Care and Use Committee at the University of Alberta and the study was conducted at the Dairy Research and Technology Centre (University of Alberta, Edmonton, Alberta, Canada). Nine ruminally cannulated lactating Holstein cows (79 ± 43 days-in-milk and 37.1 ± 6.1 kg milk/d) were used in a replicated 3 × 3 Latin

Square design with three dietary treatments and three 21-d experimental periods. Within each experimental period, enteric CH_4 production was measured from individual cows from day 18 to 20 using the sulphur hexafluoride (SF_6) tracer gas technique with halters and polyvinyl chloride yokes (Johnson et al., 1994) as described by McGinn et al. (2009). Rumen digesta samples were collected before (day 15) and after (day 19) the period of CH_4 gas collection.

Cows were fed once daily with a total mix ration (TMR) consisting of barley silage (206 g/kg DM), alfalfa silage (206 g/kg dry matter; DM), alfalfa hay (108 g/kg DM), barley grain (147 g/kg DM), and corn grain (149 g/kg DM). The exogenous enzyme product, Econase RDE (AB Vista, Marlborough, Wiltshire, UK) contained endoglucanase (EC3.2.1.4; 722 nmol/μl) and xylanase (EC 3.2.1.8; 2604 nmol/μl) activities, with activities determined at 39°C and pH 6.0 using low viscosity carboxymethyl cellulose (catalog no. C-5678; Sigma Chemical Co., St Louis, MO, USA) and birchwood xylan (Sigma Chemical Co., catalog no. X-0502) as substrates (10 mg/ml in 0.1 mol/l citrate phosphate buffer, pH 6.0), following the procedures outlined by Holtshausen et al. (2011). The enzyme product was mixed with 4 L of water and added to the TMR at a low- (0.5 ml enzyme/kg TMR DM; Low-enzyme treatment) and high- (1.0 ml enzyme/kg TMR DM; High-enzyme treatment) dose 1 h prior to feeding. An equal amount of water without the enzyme was added to the control treatment (4 liter added to 100 kg TMR DM). The experimental diets were fed to the cows for *ad libitum* intake once daily at 0800h. Cows were milked in their stalls twice daily at 0400 and 1400 h. This study was conducted at the same time as that reported by

Holtshausen et al. (2011) and used the same basal TMR. Details of ingredients and chemical composition of the basal TMR and animal housing and care are reported in Holtshausen et al. (2011).

Within each experimental period, enteric CH_4 production was measured from individual cows from day 16 to 18 using the sulphur hexafluoride (SF_6) tracer gas technique with halters and polyvinyl chloride yokes (Johnson et al., 1994) as described by McGinn et al. (2009). Rumen digesta samples were collected before (day 15) and after (day 19) the collection for CH_4 gas at 0, 6 and 12 h after feeding.

Rumen digesta samples were collected through the rumen cannula, and a composite sample was collected from 5 different locations within the rumen, mixed and squeezed through two layers of polyester monofilament fabric (Pecap 7-255/47, mesh opening-355 μm; Tetko Inc., Scarborough, ON, Canada). Aliquots of the filtrate were frozen immediately on dry ice and stored at -80°C until further molecular analyses.

4.2.2 DNA Extraction and PCR Amplification

Total DNA was extracted from the strained filtrates based on the protocol described by Guan et al. (2008) with modification. In brief, frozen filtrates were thawed on ice and 2 ml of the thawed ruminal filtrate was transferred to a sterile tube and washed with 4.0 ml of TN150 buffer (10 mmol/l Tris-HCl [pH8.0], 150 mmol/l NaCl). The mixture was vortexed for 30 s and centrifuged at 200 g for 5 min at 4°C, and then 1 ml of the supernatant was transferred to a microcentrifuge

tube containing 0.3 g of autoclaved zirconium-silica beads (0.1 mm diameter). Microbial cells were lysed by physical disruption in a Mini Bead-Beater-8 (BioSpec, Bartlesville, OK, USA) at maximum speed (2800 oscillations/min) for 3 min. The supernatant of each sample was then extracted with a mixture of phenol-chloroform-isoamyl alcohol (25:24:1). Extracted DNA was precipitated with 500 µl of cold ethanol and resuspended in 40 µl of nuclease-free water. The concentration and quality of DNA were measured at A_{260nm} and A_{280nm} using a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA).

Methanogenic 16S rRNA genes were amplified from 50 ng of each DNA sample. A primary PCR was conducted using a universal primer pair Met 86f (5'-GCTCAGTAACACGTGG-3', Wright and Pimm, 2003) / Met 915r (5'-GTGCTCCCCCGCCAATTCCT-3', Watanabe et al., 2004) following the conditions described by Zhou et al. (2009). The amplified products were then subjected to a nested PCR amplification using primers GC-ARC344f (5'-ACGGGGGGACGGGGYGCAGCAGGCGCGA-3') / 519r (5'-GWATTACCGCGGCKGCTG-3') (Bano et al., 2004) with a 40-bp GC-clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC, Yu et al., 2008) added at the 5' end of ARC344f, using the amplification conditions described by Zhou et al. (2010).

4.2.3 PCR-DGGE Analysis of Methanogens

The nested PCR products were pooled by cow (over the 3 sampling times within each sampling day) for each period and used for DGGE analysis using a

DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR fragments were separated in 6% polyacrylamide gel with 1 × TAE buffer (40 mmol Tris-base; 20 mmol glacial acetic acid; 1 mmol EDTA) under a 35 to 45% linear gradient of denaturant (100% denaturing gradient contains 7 mol/l urea and 40% deionized formamide). In each gel, a previously developed ladder consisting of seven bands representing known phytlotypes was included (Zhou et al., 2010). The gel was run at 60°C and 150V for 4 h and then it was stained with 300 ml ethidium bromide (EB) solution (300 μg of EB) for 15 min, destained with 400 ml water for 30 min, and photographed using UV transillumination. The DGGE band patterns were interpreted and clustering analyses were completed using BioNumerics software (version 5.1, Applied Maths, Inc., Austin, TX, USA). Optimal position tolerance and optimization settings for this particular experiment were calculated using the 'tolerance and optimization analysis' program, and the similarity of the DGGE profiles was analyzed from the average Dice's similarity coefficient (D_{sc}) index with 0.50% optimization and 0.32% position tolerance.

All distinct bands generated in PCR-DGGE profiles were excised from the gels for cloning and sequence analysis. The isolated PCR-DGGE bands were stored in diffusion buffer (0.5 mol/l ammonium acetate; 10 mmol/l magnesium acetate; 1 mmol/l EDTA, pH8.0; 0.1% SDS) and purified using a QIAEX® II Gel Extraction Kit (QIAGEN Sciences, MD, USA) following the manual. The purified DNA fragments were reamplified using the ARC344f/519r primers and products were checked on agarose gel electrophoresis prior to cloning. The

confirmed PCR products were cloned into a TOP10 vector (TOPO TA Cloning kit, Invitrogen, Carlsbad, CA, USA) using chemical transformation followed by colony screening using S-Gal (Sigma, St. Louis, MO, USA) medium containing ampicillin (50 µg/ml). For the isolated DNA from each band, three to five colonies with insertions (white colonies) were randomly selected and subjected for plasmid DNA extraction using Millipore Plasmid Extraction Kit (Millipore, Billerica, MA, USA). Each of the plasmids was reamplified with GC-ARC344f/519r primers and rerun the DGGE gel to verify the migration of clone and the original bands. The clone containing an insert with the same migration location as the original band was then subjected to sequencing.

The sequencing reaction (10 µl) contained 3.2 pmol of M13 forward or reverse primer, 20 ng plasmid DNA, 1 × sequencing buffer and 0.5 µl BigDye solution (ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA, USA). Sequence signals were collected using an ABI 3730 sequencing system (Applied Biosystems). Vector and primer sequences were removed and the trimmed sequences were compared with the Genbank database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Reference sequences were obtained from Genbank and phylogenic analysis among the collected sequences and references were conducted using the neighbor-joining method within the PHYLIP package (version 3.69; http://evolution.genetics.washington.edu/phylip.html).

4.2.4 qRT-PCR

The total methanogen population was estimated for each individual sample by measuring copy numbers of the 16S rRNA gene using universal primer pairs from a previous study (Zhou et al., 2009). The measurement was conducted with a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using a Fast SYBR® Green Master Mix kit (Applied Biosystems); the reaction was conducted under a fast cycle running method with melt curve section included. Standard curves were constructed by serially diluting the plasmid DNA containing *Mbb.* sp. AbM4 insert (7.0×10^6 to 7.0×10^2 copies). The copy numbers of each standard curve were calculated based on the following equation: $(NL \times A \times 10^{-9}) / (660 \times n)$, where NL was the Avogadro constant (6.02×10^{23} molecules/mol), A was the molecular weight of the standard DNA molecule and n was the length of the amplicon (bp). The copy numbers of 16S rRNA genes of targeted methanogens per ml of rumen fluid were calculated using the following equation: $(QM \times C \times DV) / (S \times V)$, where QM was the quantitative mean of the copy number, C was the DNA concentration of each sample, DV was dilution volume of extracted DNA, S was the DNA amount (ng) subjected to analysis and V was rumen fluid volume (2 ml) subjected to DNA extraction. The amplification efficiency was calculated using the equation: $E = (10^{-1/slope} - 1) \times 100\%$ and only the data generated from reactions with efficiency between 90 to 110% were used for further analysis.

4.2.5 Statistical Analysis

All statistical analyses were performed using SAS (SAS System version 9.2, SAS Institute, Cary, NC). The band pattern for each rumen sample was analyzed using a categorical model to identify the effects of diet and period. The presence or absence of each DGGE band was converted to a 1/0 numerical format. The correlation between CH_4 yield (g/d) and each DGGE band was analyzed using a point biserial correlation coefficient model with a significant correlation set at a P value of < 0.05. The association among DGGE bands was analyzed using a Chi-square model by estimating Phi coefficient with significant association determined using a P value of < 0.05.

All estimates of methanogen 16S rRNA gene copy numbers were transformed to a log10 scale prior to analysis and then analyzed using a mixed model that included time point and treatment as fixed effects and animal as random effect. A linear correlation was conducted to estimate the relationship between copy numbers and rumen CH_4 yield, in which CH_4 yield was the dependent variable and copy number the independent variable. Significance was indicated with a P value of < 0.05.

4.3 Results

4.3.1 PCR-DGGE Profiling of Methanogens

To eliminate possible biases caused by sampling location within the rumen and sampling time relative to feeding, the original samples were collected

from five different locations and three time points related to the feeding time and pooled PCR products were used for all analyses. Similar profiles were observed for the PCR-DGGE band patterns generated from samples taken before and after CH_4 measurements (i.e., day 15 versus 19) (all D_{sc} > 90%, data not shown). Therefore, only the PCR-DGGE profiles for the samples collected after CH_4 measurement were further analyzed. The similarity analysis of the PCR-DGGE profiles showed that the average D_{sc} was 66.6% when comparing samples collected across cows, periods and enzyme treatments (Figure 4.1). Band patterns did not cluster according to dietary treatment, CH_4 yield (g/d), or period. Clustering patterns also did not appear to differ substantially among individual cows.

As shown in Figure 4.1, 26 distinctive bands were detected from all the PCR-DGGE profiles. Profiles from each cow exhibited multiple predominant DGGE bands that were largely common to all individuals. In most animals, the predominant bands migrated to the same position as the bands representing *Msp. stadtmanae* (Band 7), *Mbb. smithii* (Band 22), and *Mbb. gottschalkii* (Band 26). Besides, profiles from some individuals exhibited predominant bands (e.g., Band 14, Band 24, etc.) that did not coincide with those included in the reference ladder.

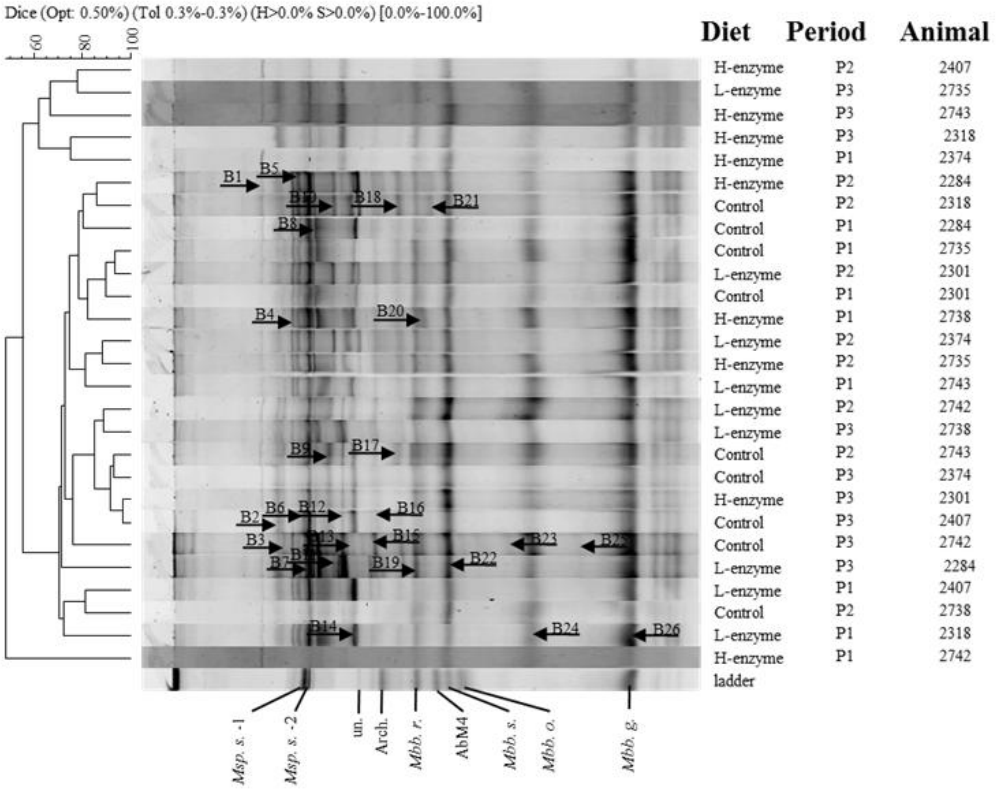

Figure 4.1 Methanogenic PCR-DGGE profiles generated from ruminal fluid from 9 animals using primers GC-ARC344f and 519r (35-45% DGGE). Treatment, period, and animal ID are listed beside each lane. Bands indicated by arrows with band ID number are the ones subjected for cloning and sequence analysis.

4.3.2 Sequence Analysis of the Methanogenic PCR-DGGE Bands

An attempt was made to clone all 26 bands visualized in the gel, including the PCR-DGGE bands that matched the positions with those from the reference ladder, with 20 bands being successfully cloned and sequenced. The taxonomy of the sequences was determined under the following criteria: with a similarity ≥ 97% for a species-level identification and similarity < 97% were considered as the taxon-like sequences. Among the sequenced 20 bands, 12 bands were classified as five known species and two clones while 8 bands were linked to two known species and two clones (Table 4.1).

A phylogenic tree was constructed with the sequences of 20 DGGE bands and reference sequences obtained from GenBank database (Figure 4.2) to verify the sequence similarity to the known taxons and to classify all the obtained bands. The bands clustered with the identified phylotypes into three major groups: Group I represented *Methanobrevibacter* sp., Group II represented *Methanosphaera* sp., and Group III represented an unknown taxonomy.

4.3.3 Association between the Methanogen Diversity and CH_4 Yield

The presence of two DGGE bands, Band 21 (correlation coefficient = -0.39, $P < 0.05$) and Band 13 (correlation coefficient = -0.35, $P = 0.08$), tended to be negatively correlated with CH_4 yield. In contrast, Band 14 (*M. gottschalkii* strain HO) tended to be positively associated with CH_4 yield (correlation

Table 4.1 Sequence identification of the PCR-DGGE bands.

PCR-DGGE Band[a]	Nearest taxon (GenBank Accession No.)	Similarity (%)
6	*Methanosphaera stadtmanae* (AY196684)	95
7	*Methanosphaera stadtmanae* (AY196684)	96
8	Methanogenic archaeon CIRG-GM02 (FJ951431)	90
9	*Methanosphaera* sp. R6 (AB022186)	97
10	Methanogenic archaeon CH1270 (DQ445723)	93
11	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	98
12	*Methanosphaera* sp. R6 (AB022186)	97
13	Methanogenic archaeon CH1270 (DQ445723)	90
14	*Methanobrevibacter gottschalkii* strain HO (U55238)	98
15	*Methanobrevibacter gottschalkii* strain HO (U55238)	94
16	*Methanosphaera* sp. R6 (AB022186)	97
17	Methanogenic archaeon CH1270 (DQ445723)	90
18	Methanogenic archaeon CIRG-GM02 (FJ951431)	97
19	*Methanobrevibacter ruminantium* M1 (CP001719)	99
20	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	99
21	Methanogenic archaeon CH1270 (DQ445723)	92
22	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	100
23	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	99
24	*Methanobrevibacter millerae* SM9 (AJ009958)	100
25	*Methanobrevibacter ruminantium* M1 (CP001719)	98

[a] Band 1-5 and Band 26 were not successfully cloned and sequenced.

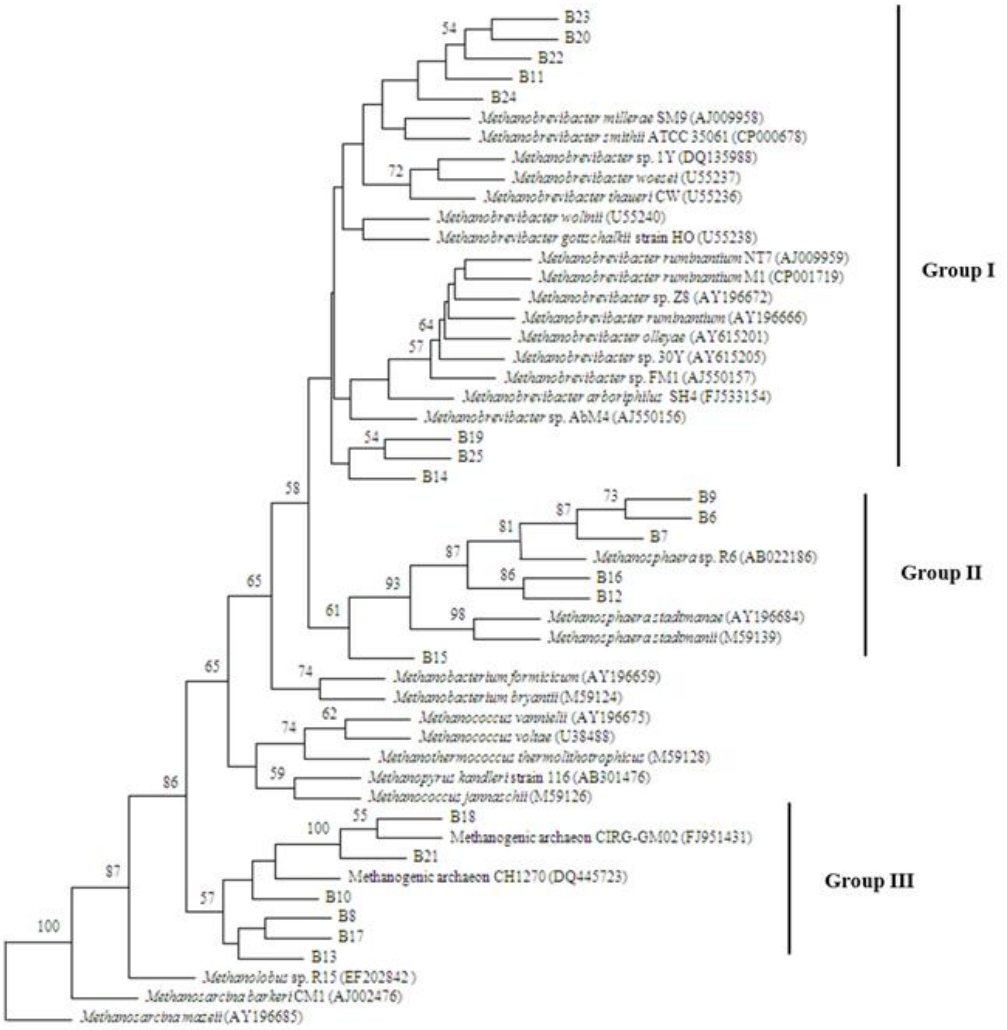

Figure 4.2 Phylogenic analysis of sequences identified from PCR-DGGE bands. The bands were represented in the form 'B' following with the number. References sequences were indicated by taxon names and Genebank accession number. Bootstrap values were calculated from 100 replications and the values larger than 50% were indicated next to the nodes.

coefficient = 0.33, P = 0.10). Additionally, the presence/absence of some bands showed strong correlation with each other. For example, Band 16 (*Msp.* sp. R6-like) and Band 18 (Methanogenic archaeon CIRG-GM02-like) occurred concurrently (P < 0.01), while Band 14 (*Mbb. gottschalkii* strain HO-like) and Band 25 (*Mbb. ruminantium* M1) occurred singly (P < 0.01).

Among the 26 bands detected, 19 bands were associated with all three dietary treatments; whereas the presence of Band 15 (*Mbb. gottschalkii* strain HO-like, P < 0.05) was strongly affected by treatment and Band 18 (Methanogenic archaeon CIRG-GM02, P = 0.09) tended to be affected by treatment. In addition, Band 13 (Methanogenic archaeon CH1270-like) was only present when animals received the Low-enzyme diet; Band 20 (*Mbb. smithii* ATCC35061) was only detected in animals fed the High-enzyme diet; Band 17 (Methanogenic archaeon CH1270-like) only appeared when enzyme was added; and Band 3 and Band 10 (Methanogenic archaeon CH1270-like) disappeared in animals fed the High-enzyme diet. Besides, the presence frequencies of Band 8 (Methanogenic archaeon CIRG-GM02-like) decreased as enzyme dose increased (Table 4.2).

4.3.4 Comparison of Copy Numbers within Methanogen Populations

The total methanogen 16S RNA gene copy numbers (log10 transformation) were compared among sampling time points and enzyme treatments (Table 4.3). Methanogen densities were not altered by enzyme treatments or sampling time. Methanogen density was averaged over the three

Table 4.2 Presence/Absence of PCR-DGGE bands under each diet.

PCR-DGGE bands	Control	Low-enzyme	High-enzyme	P
1	+++[a]	+++	+++	NS[c]
2	+++	+++	+++	NS
3	+	+	-[b]	NS
4	+++	+++	++	NS
5	++	+++	++	NS
6	+	+	+	NS
7	+++	+++	+++	NS
8	+++	++	+	NS
9	+	+	++	NS
10	+	+	-	NS
11	++	+	+	NS
12	+	+	+	NS
13	-	+	-	NS
14	++	++	+++	NS
15	+++	+++	++	0.04
16	+++	+++	++	NS
17	-	+	+	NS
18	+++	+++	++	0.09
19	+++	+++	+++	NS
20	-	-	+	NS
21	+	-	+	NS
22	+++	+++	+++	NS
23	+	-	+	NS
24	+++	+++	+++	NS
25	++	++	++	NS
26	+++	+++	+++	NS

[a] '+' represents presence in 1-3 animals; '++' represents presence in 4-6 animals; '+++' represents presence in 7-9 animals.

[b] '-' represents absence.

[c] 'NS', non-significant.

Table 4.3 Total methanogen density (copy numbers of 16S rRNA gene/ml).

Treatment	Sampling time after feeding (h)			SEM	P
	0	6	12		
Control	8.52[a]	8.28	8.45	0.19	0.66
Low-enzyme	8.38	8.29	8.65	0.19	0.38
High-enzyme	8.48	8.57	8.60	0.10	0.70
SEM	0.13	0.16	0.20		
P	0.72	0.39	0.75		

[a] Numbers were demonstrated using the log10 conversion of the absolute copy numbers/ml of samples.

sampling time points and examined for a relationship with CH_4 production. Neither absolute copy number of the 16S rRNA gene nor the log10 conversion of the copy numbers was correlated with CH_4 yield (P = 0.37 and P = 0.69, respectively).

4.4 Discussion

Most rumen methanogens have not been isolated and cultured, and culture-dependent methods do not provide a quantitative measurement of the overall ruminal methanogen community in cattle. Consequently, culture-independent molecular methods have been widely applied to quantify and characterize microbial communities, particularly to detect community shifts in response to different dietary treatments. Molecular methods were used in the present study to monitor changes in the methanogenic communities in the rumen of dairy cows fed diets supplemented with a fibrolytic enzyme additive. Consistent with previous PCR-DGGE profiling analyses of the rumen ecosystem, most of the obtained sequences from the DGGE bands belonged to the genera *Mbb.* or *Msp.* (Yu et al., 2008; Hook et al., 2009; Zhou et al., 2010). Among all the DGGE-representing phylotypes, *Mbb. gottschalkii*, *Mbb. ruminantium*, *Msp. stadtmanae*, and *Mbb. smithii* were species commonly reported in various studies from beef cattle and dairy cows by either culture (Miller et al., 1986; Miller and Lin, 2002) or molecular-based methods (e.g. Wright et al., 2007, Zhou et al., 2010). The wide spread distribution of these four species in ruminants suggests that they may be part of the core species of the rumen methanogen community.

In contrast, some of the phylotypes described in our study have only been occasionally identified in the methanogen community. For example, Methanogenic archaeon CIRG-GM02 was found in the rumen of goats (Gupta and Chaudhary, 2007), and *Msp.* sp. R6 was reported to be associated with a rumen ciliate isolated from sheep (Tokura et al., 1999). These phylotypes have yet to be cultured and therefore their physiology is largely unknown. However, given that DNA associated with these methanogens is infrequently isolated from the rumen, it would appear that under most circumstances they represent a minority within the rumen methanogen community. The extent to which mitigation practices or geographic location alters the predominance of individual species within the methanogen population remains to be determined.

Additionally, four of the isolated DGGE bands had sequences resembling Methanogenic archaeon CH1270, which is a methanogen previously identified in the cecum of chickens (Saengkerdsub et al., 2007). Although the sequence similarity between CH1270 and our clones was limited to 90-92%, phylogenic analysis showed that these phylotypes were distinct from the known *Mbb.* or *Msp.* species but form the cluster with Methanogenic archaeon CIRG-GM02 (Figure 4.2). Since CIRG-GM02 was also identified in the rumen of goats (Gupta and Chaudhary, 2009), it can be speculated that phylotypes belonging to this cluster may represent a distinctive group of rumen methanogens that has yet to be characterized. By comparing the current phylogenic tree with previous analysis (Janssen and Kris, 2008), this group of methanogens are likely to group with

Methanosarcina species and *Methanomicrococcus* species rather than the rumen cluster C species.

Similar to our previous report (Zhou et al., 2010), different phylotypes belonging to the same species were identified from this study. For example, two bands were identified as *Msp. stadtmanae*, three were associated with *Msp.* sp. R6, and four were linked to Methanogenic archaeon CH1270. According to Klappenbach et al. (2001), methanogens can possess from 1-4 copies of 16S rRNA gene within their individual genome. Therefore, the multiple 16S rRNA gene phylotypes representing the same taxon can be either the paralogs within a single genome or the orthologs in different genomes. However, the current analyses are unable to classify whether the sequences representing the same taxon originate from a single genome or multiple genomes.

The diversity of phylotypes may represent different CH_4 synthesis pathways of the methanogens or species diversification in response to host environment. The presence/absence of particular phylotypes and their prevalence among animals under different feeding conditions may suggest their adaptability to changes in the rumen environment or their roles in affecting host feed efficiency (Zhou et al., 2009). As described above, the distribution of four DGGE bands was strongly affected by treatment, and two bands were only present under a single dietary condition. This may be due to the different physiology of each species or their preference to the substrates for methanogenesis. However, among these diet-related phylotypes, only the species represented by Band 20 (*Mbb. smithii* ATCC 35061) and Band 25 (*Mbb. ruminantium* M1) are well studied with

their completed genome sequence information (Samuel et al., 2007, Leahy et al., 2010). The availability of methanogen genome sequences is limited and it is impossible to predict the physiology and/or methanogenesis pathways of each observed taxa at this stage. Thus, further details about other phylotypes and the physiology of each phylotype identified in the rumen are required to provide the proper interpretation of their relationships to different treatments.

Overall shifts in methanogen structure in response to enzyme supplementation were not as evident as those observed from our previous study, in which the methanogen community was characterized by the single predominant species *Mbb. ruminantium* under a growing diet and multiple predominant species under a finishing diet (Zhou et al., 2010), Minor shifts in the PCR-DGGE profiles with enzyme supplementation were also observed. For example, Band 20 appeared while Band 3 and Band 10 disappeared when a high dose of Econase RDE was applied. However, such changes only occurred in very few samples and were not statistically significant, thus the phylotypes represented by these bands were not considered to be treatment-associated. In contrast, although being identified under both control and treatment groups, the proportion of Band 15 (*Mbb. gottschalkii* HO-like) decreased ($P < 0.05$), and the proportion of Band 18 (Methanogenic archaeon CIRG-GM02) tended ($P = 0.09$) to decrease with increasing enzyme dosage. As only the frequency of two bands was shown to be correlated to treatment, it was not surprising that no diet-related clusters of the PCR-DGGE profiles were displayed (Figure 4.1). This result may be due to the fact that in the present study the composition of the diets (except the enzyme) was

identical and thus the structure of the methanogen community remained stable. Thus, the enzyme supplement did not strongly impact the observed methanogen community structure.

Moreover, no difference in methanogen 16S RNA copy number was detected among the dietary treatments (Table 4.3). It has been reported that lower rumen pH and the lower acetate/propionate ratio can alter the ruminal methanogenic ecology (Van Kessel and Russell, 1996; Russell, 1998). Since the ruminal VFA and pH were not affected by the enzyme treatments in the present study, it is not surprising to observe stable methanogen densities between treatment group and control group (Chung et al., 2011). In addition, the fibrolytic enzyme supplement can improve diet digestibility (Beauchemin et al., 2003). Future study to link all rumen fermentation features including bacteria, fermentation parameters, and passage rates to the data obtained from this study may help to elucidate the mechanisms how the enzyme additive altered the diversity but not the density of the methanogen, as well as the possible linkage to the changes in CH_4 yield.

The host enteric CH_4 yield, expressed as total daily produced amount (g/head/d; control: 471; Low-enzyme: 505, High-enzyme: 545; $P = 0.10$) or the daily production corrected for dry matter intake (g/kg DMI; control: 19.3, Low-enzyme treatment: 20.8; High-enzyme treatment: 21.7; $P = 0.04$), increased with increasing dose rate of enzyme in the diet (Chung et al., 2011). In a companion study with similar dietary treatments, we observed a linear improvement in feed conversion efficiency with increasing dose of enzyme when

the same TMRs were feed to lactating dairy cows (Holtshausen et al., 2011). Improved feed conversion efficiency would suggest that the enzyme treatment improved fibre digestion, which should have increased total H_2 production and increased the amount of substrate available for methanogenesis. As we observed changes in the detectable PCR-DGGE profiles, it could be speculated that the activity of individual species rather than the total number of methanogens has the greatest effect on CH_4 production. The proposed mechanism of the adjustment in ruminal methanogen community may be that the shift of proportions of each methanogen species instead of increasing or decreasing the total methanogen population was sufficient for the entire methanogenic community to adapt to the enzyme supplement. The correlation of CH_4 yield with Bands 13 (Methanogenic archaeon CH1270-like), 14 (*Mbb. gottschalkii*), and 21 (Methanogenic archaeon CH1270-like) represents such a structural shift, but further qRT-PCR analysis should be applied to verify the quantitative change of these species. However, different species of methanogens have different copy numbers of 16S rRNA gene, with each methanogen genome containing two to four copies of the gene (Ribosomal RNA Operon Copy Number Database, Klappenbach et al., 2001). Future studies that include other markers related to CH_4 metabolism such as the *mcr*A gene may give a more accurate reflection of the impact of dietary treatments on methanogenic activity.

In addition, the microbial diversity showed animal-to-animal variation among cows fed the same diet, indicating that the microbial adaptation within each animal host appears to differ. As observed in many studies, the nature of the

host affects the type and density of microbes inhabiting the rumen even when cows/cattle are fed identical diets (Weimer et al., 1999; Martinez et al., 2010). The conclusions from our study must be verified in subsequent work because of two major weaknesses. Only a small number of animals were used in this study and the rumen fluid samples were examined, and the CH_4 data represent the average daily amount of gas emitted over a 3-day period. To accurately determine the relationship between CH_4 production and the methanogen community it might be necessary to conduct whole animal chamber measurements so that CH_4 data coincide more directly with rumen sampling times and to investigate the rumen digesta including the fluid and solids as a whole. In addition, it would be helpful to use a larger sample size. Each animal has a different capacity of hosting ruminal microbiota (Martinez et al., 2010). Thus, the use of more animals may increase the statistical power to derive conclusions that are presently masked by variability among hosts. Moreover, the animal experiment followed a Latin square design, which is helpful because it accounts for animal variability. However, carry-over effects on the microbial profiles of each cow due to the relatively short period length cannot be discounted. Consequently, to observe how each phylotype changed in response to enzyme addition, it may have been better to incorporate a control group without any treatment throughout the whole experiment in addition to treatment groups supplemented with incremental dosages of enzyme.

4.5 Conclusion

Supplementing the diet of lactating dairy cows with exogenous fibrolytic enzymes did not dramatically influence the total population and species composition of the ruminal methanogenic community. However, the distribution of PCR-DGGE bands representing *M. gottschalkii* strain HO and Methanogenic archaeon CIRG-GM02 tended to differ among enzyme doses. Bands representing unknown species close to Methanogenic archaeon CH1270 and *M. gottschalkii* strain HO tended to play a role in affecting the host's CH_4 production, but the total methanogen population was not related to daily CH_4 yield. These results demonstrate that the particular species, rather than the entire methanogenic population, may respond to dietary changes and that these changes may influence enteric CH_4 emissions. Advanced technologies and further analyses of the methanogenic activity will help attain a better understanding of the role of each methanogenic species on the ruminal ecosystem and their effects on host animal CH_4 production.

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5.0 COMPARISON OF ANIMAL AND DIETARY EFFECTS ON RUMINAL METHANOGENS AND THEIR ASSOCIATION WITH PROTOZOA IN BEEF CATTLE[5]

5.1 Introduction

Reducing enteric methane (CH_4) production from ruminants has been a continued objective as it increases available energy to the host and reduces greenhouse gas emissions (Beauchemin and McGinn, 2008). Various dietary strategies have been proposed to reduce enteric CH_4 emissions without compromising animal performance, but long-term effective methods have yet to be developed (Granger and Beauchemin, 2011). Since ruminal methanogenesis arises from anaerobic fermentation, characterizing the ecology of methanogens and differences among individual host animals could be pivotal to the design of effective CH_4 mitigation strategies. Most studies have focused on the influence of diet (Whitford *et al.*, 2001; Skillman *et al.*, 2004; Wright *et al.*, 2007) and transition between diets (Yu *et al.*, 2008; Zhou *et al.*, 2010) on the ecology of ruminal methanogens. Although all methanogenic phylotypes can be classified into three distinctive clusters, *Methanobrevibacter* sp., *Methanomicrobium* sp., and rumen cluster C (RCC) (Janssen and Kirs, 2008), the biodiversity and the relative abundance of each cluster differs across studies (Whitford *et al.*, 2001; Skillman *et al.*, 2004; Wright *et al.*, 2007).

[5] A version of this section has been submitted to FEMS Micro. Ecol.

In addition to dietary factors, properties of the host are also a factor that influences the symbiotic microbiota (Khachatryan *et al.*, 2008; Benson *et al.*, 2010). Previous studies reported that the ecology of ruminal methanogens was more similar within the same host species than among different host species (Jeyanathan *et al.*, 2011). King *et al.* (2011) compared the methanogen population of two dairy cow breeds (Jersey and Holstein) and found that the methanogen biodiversity was more similar within than between breeds. Weimer *et al.* (2008) showed that the variance in three species of cellulolytic bacteria in the rumen of dairy cows was influenced more by the host than the presence or absence of monensin in the diet. Durso *et al.* (2010) compared the fecal microbial diversity in six cross-bred beef heifers of similar age and body weight fed a mixed ration of corn silage, alfalfa hay, corn grain and liquid supplement, and reported animal-to-animal variation that was independent of breed, gender, diet or age.

The effectiveness of CH_4 mitigation practices such as the addition of fat to the diet has been shown to differ markedly among studies. For example, in the studies by Holter *et al.* (1992), Jordan *et al.* (2006), and Grainger *et al.* (2010), supplemental fat resulted in a long-lasting (> 10 weeks) decline in enteric CH_4 production; while Woodward *et al.* (2006) and Moate *et al.* (2011) found that the fat mediated reduction in CH_4 production did not persist. The non-consistent results of CH_4 mitigation studies may be due to the differences in several factors such as fat sources, dietary formula, and intake. In addition, variance in symbiotic methanogens within animals may also be responsible for the observed differences in response to CH_4 mitigation practices.

The objective of the current study was to identify and compare the ruminal methanogenic community and their shifts in response by the host animal to diets containing different types of dried distillers' grains with solubles (DDGS) using molecular identification methods.

5.2 Materials and Methods

5.2.1 Animal Experiment, CH_4 Measurement, and Sampling

Four ruminally cannulated crossbreed heifers were selected from a larger group of animals (*n*=16) used in a study by Hünerberg et *al.* (2010). The experiment was conducted at the beef cattle research facility located at Agriculture and Agri-Food Canada's Lethbridge Research Centre. The experimental protocol received institutional approval and was carried out in accordance to the guidelines of Canadian Council on Animal Care (1993). In order to facilitate the CH_4 measurements all animals were paired such that each pair of heifers had similar total body weight and received the same dietary treatment.

The experiment included four periods, each of which lasted for 21 days. To avoid carry over effects and assess the ruminal microbiota before any inclusion of DDGS in the diet, the selected heifers (*n*=4; 386.0±31.7 kg) were firstly fed a DDGS free control diet containing 55% whole crop barley silage, 35% barley grain, 5% canola meal and 5% supplement on a dry matter (DM) basis in the first period. Three DDGS treatment diets, which formulated by replacing a portion of the barley grain and canola meal in the control diet with corn-based DDGS

(CDDGS; 10.0% fat, DM basis), wheat-based DDGS (WDDGS, 4.1% fat, DM basis), or WDDGS + corn oil (9.5% fat, DM basis), were offered sequentially during the latter three periods. Corn oil was added to the WDDGS diet to achieve a fat level that was similar to the CDDGS diet. All diets were offered *ad libitum* once daily as a total mixed ration.

Based on the assumption that pairs of heifers with similar body weight will have similar feed intakes, the animals were paired based on the body weight directly before the start of the trial. Each pair of two animals (44 and 170; 294 and 360) were housed in the same environmental chamber to measure enteric CH_4 emissions over 4 days (d 18 - d 21) within each period as described by Hünerberg *et al.* (2010).

Rumen contents were collected from three different sites (reticulum, dorsal and ventral sac) within the rumen on d 14 at 0, 2 , 6, 12 and 24 h after feeding (outside chamber) and on d 19 to d 21 just prior to feeding (inside chamber, 0 h). Rumen content samples were immediately frozen in liquid nitrogen and stored at -80°C until further analyses.

5.2.2 DNA Extraction and PCR Amplification of Methanogen 16S rRNA Genes

Total DNA was extracted from each sample of rumen contents following the bead beating method (Guan *et al.*, 2008). Briefly, frozen rumen contents were washed with TN150 (10 m*M* Tris-HCl [pH 8.0], 150 m*M* NaCl) buffer. The pellet was lysed by beads beating with ~0.3 g of zirconium beads (0.1 mm diameter)

using a BioSpec Mini Bead-Beater-8 (BioSpec, Bartlesville, OK, USA) at 4,800 rpm for 3 min. Supernatant was collected and subjected to phenol-chloroform-isoamyl ethanol (25:24:1) extraction. Cold ethanol was used to precipitate DNA and the pellet was resuspended in 20 µl of nuclease-free water.

Methanogen 16S rRNA gene fragments were amplified using 50 ng of each DNA sample using primer pair ARC344f (5'-ACGGGGYGCAGCAGGCGCGA-3') / 519r (5'-GWATTACCGCGGCKGCTG-3') (Bano *et al.*, 2004) with a GC-clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG, Yu *et al.*, 2008) added to the 5'-end of ARC344f. The reaction and amplification condition were as described by Zhou *et al.* (2010).

5.2.3 PCR-DGGE Analysis of Methanogenic Community

The PCR products were subject to denaturing gradient gel electrophoresis (DGGE) analysis using aDCode^{TM} Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A ladder developed in a previous study (Zhou *et al.*, 2010) was loaded in each gel to indicate the position of separated bands and as an internal reference for comparison of profiles among different gels. The DGGE running conditions and photographing procedures were identical to those of Zhou *et al.* (2010). Because the DGGE profiles of samples taken at different time points (0, 2, 6, 12 and 24 h) on the same day did not differ from each other, PCR products from the 5 samples collected on d 14 were pooled by period and run together with d 19 - d 21 samples. The band patterns were

interpreted and analyzed using BioNumerics software (version 6.1, Applied Maths, Inc., Austin, TX, USA) with 0.625% position tolerance and 0.5% optimization. The similarity of the DGGE profiles was assessed by the average Dice's similarity coefficient (D_{sc}) index. The clustering analyses of the DGGE patterns were performed using the unweighted-pair group method with a mathematical averages (UPGMA) clustering algorithm and multidimensional scale (MDS) plotting. Analysis of similarity (ANOSIM) within Global *R* statistics (Clark, 1993) was run to verify the clustering of the DGGE patterns, where numbers close to 1 indicate distinction and numbers close to 0 indicate resemblance.

5.2.4 qRT-PCR

To validate the findings by Yu *et al*. (2008), the densities of total methanogens, *Methanobrevibacter* sp. AbM4, and *Methanosphaera stadtmanae* were estimated by measuring copy numbers of the 16S rRNA gene using universal primer pairs uniMet1-f (5'-CCGGAGATGGAACCTGAGAC-3') / uniMet1-r (5'- CGGTCTTGCCCAGCTCTTATTC-3'), AbM4-F (5'-TTTAATAAGTCTCTGGTGAAATC-3') / AbM4-R (5'-AGATTCGTTCTAGTTAGACGC-3'), and Stad-F (5'-CTTAACTATAAGAATTGCTGGAG-3') / Stad-R (5'-TTCGTTACTCACCGTCAAGATC-3') (Zhou *et al.* 2009). The reaction was conducted using a Fast SYBR® Green Master Mix kit (Applied Biosystems, Foster City, CA, USA), and fluorescence was measured with a StepOnePlus™

Real-Time PCR System (Applied Biosystems) under a fast cycle running method with a melt curve section included. Standard curves were constructed by serial diluting plasmid DNAs containing 16S rRNA genes of *Methanobrevibacter* sp. AbM4 (*Mbb.* sp. AbM4) with copy numbers between 7.0×10^6 and 7.0×10^2 and *Methanosphaera stadtmanae* (*Msp. stadtmanae*) with copy numbers between 4.18×10^6 and 4.18×10^2. The copy numbers of 16S rRNA genes of targeted methanogens per g of rumen content were calculated based on the equation described by Zhou *et al.* (2009). The PCR efficiency was calculated using the equation: $E= (10^{-1/slope} -1) \times 100\%$.

5.2.5 Protozoa Enumeration

For enumeration of protozoa, rumen content samples collected on d 14 (0, 2, 6, 12 and 24 h after feeding) were squeezed through two layers of polyester screen (pore size 355 μm). Filtrated fluid (5 ml) was combined with an equal volume of methyl green-formalin-saline (MFS) solution. The mixture was stored in darkness at room temperature. Protozoa were enumerated using a light microscope (model Primo Star, Zeiss, Germany) and Neubauer Improved Bright-Line counting chamber (Hausser Scientific, Horsham, PA).

5.2.6 Statistical Analysis

Statistical analyses were performed using SAS (SAS System version 9.2, SAS Institute, Cary, NC). The presence/absence of each DGGE band was converted to a 1/0 numerical format. The DGGE pattern for each sample was

analyzed using a PROC CATMOD model to identify the effects of diet and host on band distribution. Two-way contingency tables of cross classifications containing the frequencies of the bands per category (diet/animal) were obtained using PROC FREQ. Effects were declared significant at $P < 0.05$, and trends were discussed at $P < 0.10$.

To evaluate the effect of diet or animal on methanogen and protozoa densities, methanogen 16S rRNA gene copy numbers and total protozoa counts were transformed to a log10 scale prior to further analysis. Time effect on the protozoa and methanogen population were first analyzed for the d 14 data using PROC MIXED model, with sampling time as a fixed efect and animal and treatment as random effects. Because sampling time did not affect these measurements, the data from different sampling times were considered as repeated measurements for d 14. The animal effect for all variables was analyzed using a PROC MIXED procedure with animal as a fixed effect. Housing effect on the microbial populations was analyzed for each individual heifer using PROC MIXED model with housing condition (outside or inside the chamber) as a fixed effect. The microbial densities among treatments were compared using a PROC ANOVA model. A PROC CORR model was used to estimate the correlations between total methanogen, *Mbb.* sp. AbM4 and *Msp. stadtmanae* populations and proportion, and protozoa densities.

5.3 Results

5.3.1 PCR-DGGE Profiling of Methanogens

In total, 19 different DGGE bands were obtained from the entire sample set, which represented 19 different detectable phylotypes of rumen methanogens. The similarity of the obtained DGGE profiles were firstly analyzed using the UPGMA method, and the overall D_{sc} was 56.9% (Fig. 5.1). As shown in the dendrogram, the DGGE profiles clustered according to animal but not diet.

5.3.2 Housing Effect on Methanogen Abundance

A significant housing effect of having the animals in or out of environmental chambers was observed in that the total methanogens and *Mbb.* sp. AbM4 in the rumen was higher in, as opposed to out, of the chamber (Table 5.1). Animal 294 was the only individual in which this response was not observed for *Msp. stadtmanae.*

5.3.3 Dietary Effect on Methanogen Diversity and Methanogen/Protozoa Abundance

To investigate the dietary effect on the methanogen DGGE profiles and the abundance of methanogens and protozoa, all data were analyzed diet-wise. The D_{sc} of the DGGE profiles for the four experimental diets (Control, CDDGS, WDDGS and WDDG + oil) were low at 60.0%, 56.8%, 56.0% and 65.2%, respectively. The generated MDS plots of the DGGE profiles did not cluster

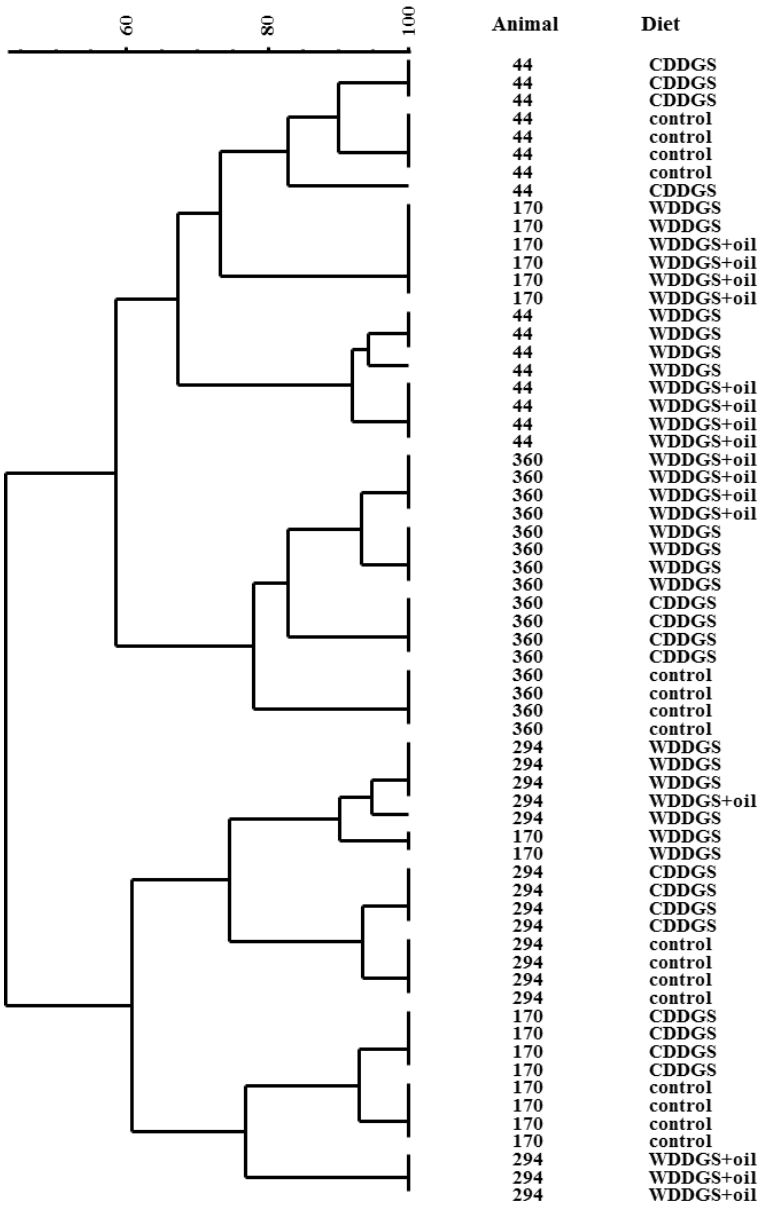

Figure 5.1 Clustering of methanogen PCR-DGGE profiles from all the samples using primers GC-ARC344f and 519r (35-45% DGGE). Animal and treatment were listed. Cluster analysis was conducted using UPGMA method, with optimization of 0.5% and position tolerance of 0.625%.

Table 5.1 Housing effect on total methanogens, *Methanobrevibacter* sp. AbM4, and *Methanosphaera stadtmanae* densities of each individual animal.

Animal	44			170			294			360		
Variables	Out (n=5) [a]	In (n=3) [a]	*P*	Out (n=5)	In (n=3)	*P*	Out (n=5)	In (n=3)	*P*	Out (n=5)	In (n=3)	*P*
Total methanogen	8.25[b]	8.58	***	8.59	8.97	**	8.35	8.71	***	8.23	8.53	***
Mbb. AbM4	7.86	8.20	***	8.14	8.61	***	8.01	8.29	***	7.65	8.06	***
Msp. stadtmanae	6.19	6.65	**	6.25	6.61	**	6.52	6.73	NS	6.46	6.75	***

[a] In: inside chamber; Out: outside chamber.

[b] log10 transformation.

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; NS non-significant.

according to diets (Fig. 5.2). In addition, the ANOSIM analysis did not indicate significance of the overall DGGE patterns among the diets (R = 0.02, $P = 0.19$).

The presence/absence of each DGGE band differed slightly among the four diets (Table 5.2). Among the 19 bands, 11 bands presented at similar frequencies, whereas 6 bands (Band 5, Band 8, Band 9, Band 15, Band 16, and Band 17) were different among diets ($P < 0.05$) and 2 bands (Band 1 and Band7) tended to be different among diets ($P < 0.10$).

The abundance of protozoa and total methanogens were different for the four diets, while the population of *Mbb.* sp. AbM4, and *Msp. stadtmanae* were similar among diets (Table 5.3).

5.3.4 Animal Effect on Methanogen Diversity and Methanogen/Protozoa Abundance

The PCR-DGGE profiles of methanogens and the population of methanogen and protozoa were then analyzed animal-wise. The D_{sc} of the DGGE profiles for animal 44, 170, 294, and 360 were higher than those of diets, being 82.0%, 78.1%, 75.1%, and 85.7%, respectively. The MDS plots of the DGGE profiles showed four clear clusters, each of which were generated from the same animal (Fig. 5.3). The clustering of the DGGE patterns was further verified by ANOSIM, as the R value among individuals was 0.87 ($P = 0.001$).

The presence/absence of all the 19 DGGE bands was significantly different among the four heifers. As indicated in Table 5.4, all of the methanogen phylotypes distributed unevenly among the animals.

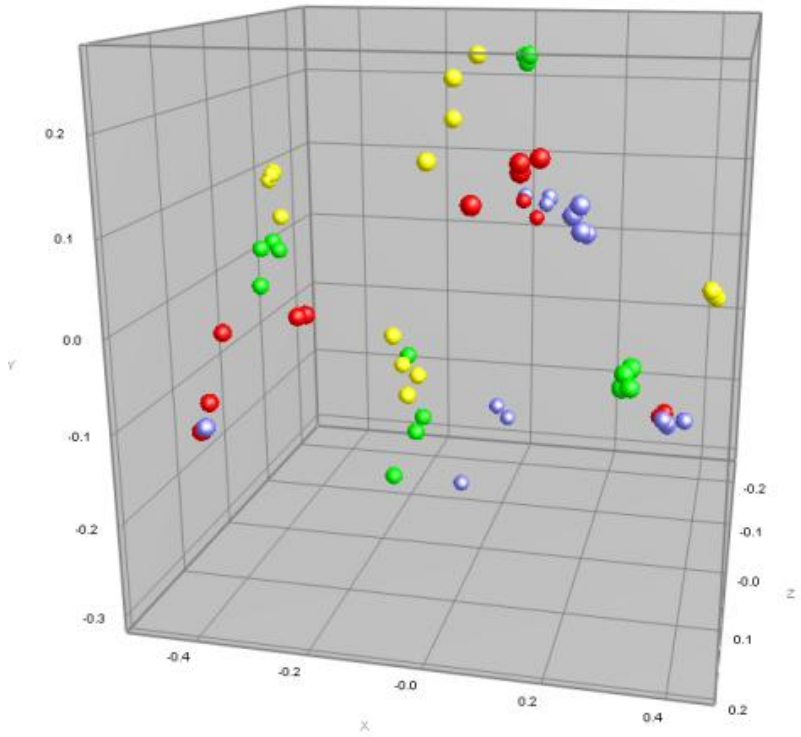

Figure 5.2 Diet-wise MDS plot of the overall PCR-DGGE profiles. Control, red; CDDGS, green; WDDGS, purple; WDDGS+oil, yellow.

Table 5.2 Diet-wise comparisons of band presence among the entire sample set.

Band	Diet				*P*
	Control[a] (%)	CDDGS (%)	WDDGS (%)	WDDGS+oil (%)	
B1	50	56	88	56	*
B2	50	50	50	50	NS
B3	25	19	13	25	NS
B4	25	25	38	6	NS
B5	75	25	69	50	**
B6	25	50	63	50	NS
B7	25	25	0	19	*
B8	75	50	38	94	***
B9	75	75	25	25	***
B10	50	50	38	25	NS
B11	50	50	63	75	NS
B12	25	25	25	6	NS
B13	50	50	50	69	NS
B14	25	25	38	50	NS
B15	50	75	38	25	**
B16	100	75	56	50	***
B17	75	100	100	100	***
B18	75	75	50	50	NS
B19	75	75	75	94	NS

[a] Control: control diet; CDDGS: corn DDGS diet; WDDGS: wheat DDGS diet; WDDGS+oil: wheat DDGS plus corn oil diet.

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; NS non-significant.

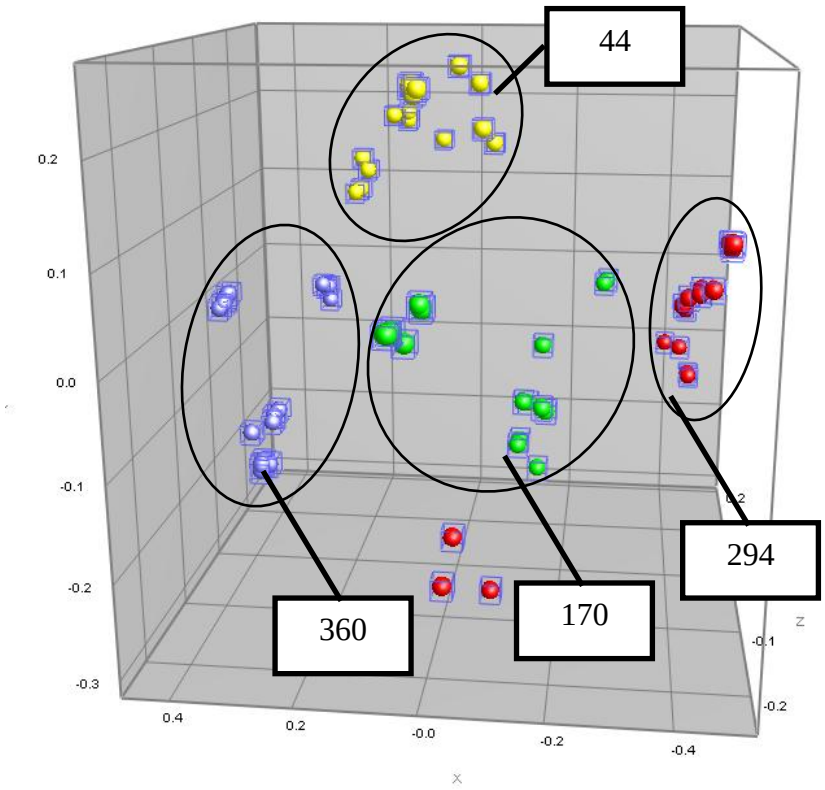

Figure 5.3 MDS plot of the PCR-DGGE profiles of each individual animal. Plotting of the samples was under treatment-wise classification: ctrl, red; CDDGS, green; WDDGS, purple; WDDGS+oil, yellow. Dots representing identical profiles were overlapped by each other (e.g., WDDGS of Animal 360).

Table 5.3 Diet effect on total methanogens, *Methanobrevibacter* sp. AbM4, and *Methanosphaera stadtmanae* densities.

Variables	Control[a]	CDDGS	WDDGS	WDDGS+oil	*P*
Protozoa	5.85[b]	5.51	5.43	5.50	***
Total methanogen	8.68	8.44	8.46	8.36	***
Mbb. sp. AbM4	8.19	7.97	8.07	8.00	NS
Msp. stadtmanae	6.45	6.37	6.39	6.52	NS

[a] Control: control diet; CDDGS: corn DDGS diet; WDDGS: wheat DDGS diet; WDDGS+oil: wheat DDGS plus corn oil diet.

[b] log10 transformation.

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; NS non-significant.

Table 5.4 Animal-wise comparisons of band presence among the entire sample set.

Band	Animal				*P*
	44 (%)	170 (%)	294 (%)	360 (%)	
B1	56	13	81	100	***
B2	0	100	100	0	***
B3	44	38	0	0	***
B4	0	13	81	0	***
B5	6	63	75	75	***
B6	75	63	25	25	***
B7	0	25	44	0	***
B8	50	88	19	100	***
B9	100	50	0	50	***
B10	0	63	100	0	***
B11	100	38	0	100	***
B12	0	0	81	0	***
B13	100	100	19	0	***
B14	0	38	0	100	***
B15	50	38	100	0	***
B16	100	100	56	25	***
B17	100	100	100	75	***
B18	50	40	40	0	***
B19	100	100	19	100	***

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; NS non-significant.

Similar to the results for methanogen diversity, the abundance of protozoa, total methanogen, *Mbb.* sp. AbM4, and *Msp. stadtmanae* were all significantly different among the four heifers (Table 5.5).

5.3.5 Adaptation of Methanogenic Community and Protozoa Population to Diets within Individuals

As the diversity and microbial population varied dramatically among individual animal, the adaptation of the methanogenic community and protozoa population to the four diets were analyzed within each animal. As shown in the MDS plots (Fig. 5.4), the PCR-DGGE patterns of the same diet were closer to each other than among diets. The profile variance among the diets was verified by the ANOSIM analysis (Table 5.6).

In each animal, the methanogen phylotypes responded to the four diets uniquely. As shown in Table 5.7, the DGGE bands that were affected by the diets were different among animals. For example, in Animal 44 and 360, Band 7 was not detected; in Animal 170, Band 7 was only found for the control diet; in Animal 294, Band 7 was found for CDDGS and WDDGS+oil diets. Band 12 was only detected for Animal 294.

The abundance of methanogens and protozoa changed along with the diet adaptation in a different way within each animal. As shown in Table 5.8, all of the measured variables were significantly affected by diet for Animal 44, whereas for

Table 5.5 Animal effect on total methanogen, *Methanobrevibacter* sp. AbM4, and *Methanosphaera stadtmanae* densities.

Variables	Animal 44	Animal 170	Animal 294	Animal 360	*P*
Protozoa	5.81[a]	5.51	5.55	5.41	***
Total methanogen	8.38	8.73	8.48	8.34	***
Mbb. sp. AbM4	7.99	8.32	8.11	7.81	***
Msp. stadtmanae	6.37	6.38	6.60	6.57	**

[a] log10 transformation

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; NS non-significant.

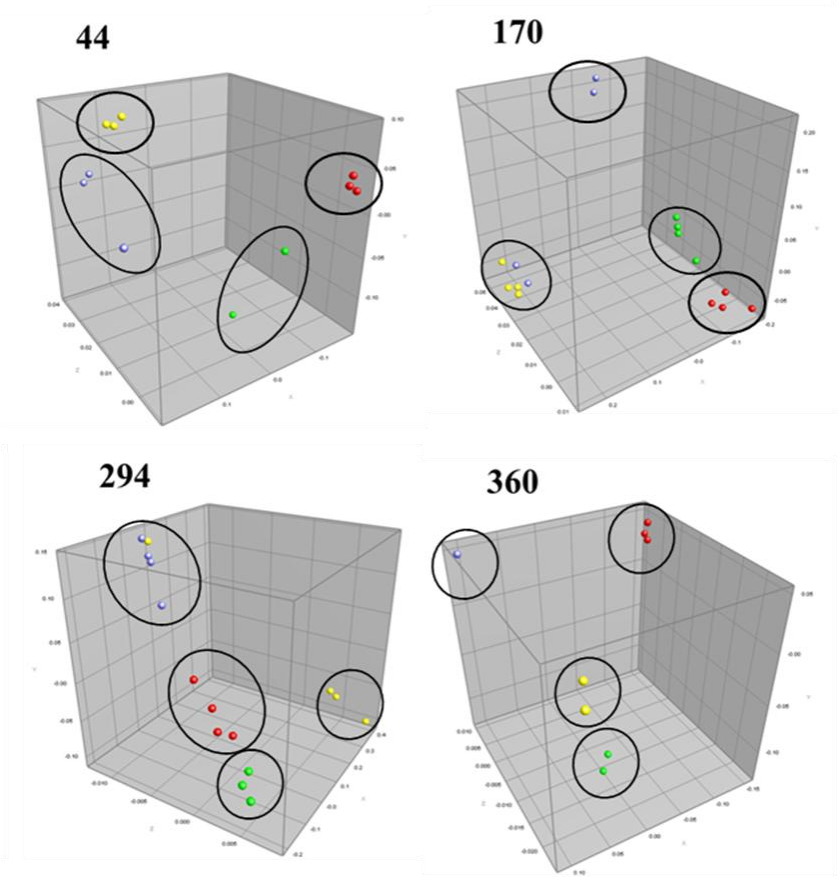

Figure 5.4 MDS plot of the PCR-DGGE profiles of each individual animal. Plotting of the samples was under diet-wise classification: Control, red; CDDGS, green; WDDGS, purple; WDDGS+oil, yellow. Dots representing identical profiles overlap (e.g., WDDGS of Animal 360). Circles indicated clusters.

Table 5.6 Analysis of similarity (ANOSIM) for treatment pairwise comparison of DGGE profiles from each individual animal.

Comparison	44		170		294		360	
	R[a]	*P*	R	*P*	R	*P*	R	*P*
Control-CDDGS[b]	0.813	**	1	**	1	**	1	**
Control-WDDGS	1	**	0.333	**	0.875	**	1	**
Control-WDDGS	1	**	1	**	0.875	**	1	**
CDDGS-WDDGS	1	**	0.333	**	1	**	1	**
CDDGS-WDDGS+oil	1	**	1	**	0.875	**	1	**
WDDGS-WDDGS+oil	0.813	**	0.167	NS	0.531	NS	1	**

[a] An R value > 0.75 indicates clearly different groups; an R value > 0.5 indicates separated groups but with some overlapping between groups; an R value < 0.25 indicates almost no difference between groups.

[b] Control: control diet; CDDGS: corn DDGS diet; WDDGS: wheat DDGS diet; WDDGS+oil: wheat DDGS plus corn oil diet.

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; NS non-significant.

Table 5.7 Diet-wise comparisons of band presence among animals.

Band	44				*P*	170				*P*	294				*P*	360				*P*
	Control[a] (%)	CDDGS (%)	WDDGS (%)	WDDGS+oil (%)		Control (%)	CDDGS (%)	WDDGS (%)	WDDGS+oil (%)		Control (%)	CDDGS (%)	WDDGS (%)	WDDGS+oil (%)		Control (%)	CDDGS (%)	WDDGS (%)	WDDGS+oil (%)	
1	0	25	100	100	***	0	0	50	0	*	100	100	100	25	**	100	100	100	100	NS
2	0	0	0	0	NS	100	100	100	100	NS	100	100	100	100	NS	0	0	0	0	NS
3	100	75	0	0	***	0	0	50	100	***	0	0	0	0	NS	0	0	0	0	NS
4	0	0	0	0	NS	0	0	50	0	*	100	100	100	25	**	0	0	0	0	NS
5	0	0	25	0	NS	100	100	50	0	***	100	0	100	100	***	100	0	100	100	***
6	0	100	100	100	***	0	100	50	100	***	100	0	0	0	***	0	0	100	0	***
7	0	0	0	0	NS	100	0	0	0	***	0	100	0	75	***	0	0	0	0	NS
8	100	0	0	100	***	100	100	50	100	*	0	0	0	75	**	100	100	100	100	NS
9	100	100	100	100	NS	100	100	0	0	***	0	0	0	0	NS	100	100	0	0	***
10	0	0	0	0	NS	100	100	50	0	*	100	100	100	100	NS	0	0	0	0	NS
11	100	100	100	100	NS	0	0	50	100	*	100	100	100	100	NS	100	100	100	100	NS
12	0	0	0	0	NS	0	0	0	0	NS	100	100	100	25	**	0	0	0	0	NS
13	100	100	100	100	NS	100	100	100	100	NS	0	0	0	75	**	0	0	0	0	NS
14	0	0	0	0	NS	0	0	50	100	***	0	0	0	0	NS	100	100	100	100	NS
15	100	100	0	0	***	0	100	50	0	***	100	100	100	100	NS	0	0	0	0	NS
16	100	100	100	100	NS	100	100	100	100	NS	100	100	25	0	***	100	0	0	0	***
17	100	100	100	100	NS	100	100	100	100	NS	100	100	100	100	NS	0	100	100	100	***
18	100	100	0	0	***	100	100	100	100	NS	100	100	100	100	NS	0	0	0	0	NS
19	100	100	100	100	NS	100	100	100	100	NS	0	0	0	75	**	100	100	100	100	NS

[a] Control: control diet; CDDGS: corn DDGS diet; WDDGS: wheat DDGS diet; WDDGS+oil: wheat DDGS plus corn oil diet.

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; NS non-significant.

Table 5.8 Effect of diet on protozoa, total methanogens, *Methanobrevibacter* sp. AbM4, and *Methanosphaera stadtmanae* densities of each individual animal.

Animal	Variables	Control[$]	CDDGS	WDDGS	WDDGS+oil	SEM	*P*
Animal 44	Protozoa	6.10[a]	5.73[b]	5.70[b]	5.70[b]	0.08	***
	Total methanogen	8.48[a]	8.33[b]	8.07[b]	8.13[b]	0.10	**
	Mbb. sp. AbM4	8.04[a]	7.70[b]	7.83[b]	7.89[b]	0.03	**
	AbM4 proportion (%)	37.11[a]	29.14[a]	58.90[b]	58.63[b]	6.70	**
	Msp. stadtmanae	6.41[a]	6.40[a]	5.36[b]	6.60[a]	0.14	***
	Msp. stadtmanae proportion (%)	1.00[a]	1.50[a]	0.23[a]	3.04[b]	0.36	***
Animal 170	Protozoa	5.78[a]	5.65[a]	5.11[b]	5.50[a]	0.05	***
	Total methanogen	8.97	8.48	8.66	8.25	0.18	NS
	Mbb. sp. AbM4	8.50[a]	8.07[b]	8.20[a]	7.79[b]	0.11	***
	AbM4 proportion (%)	37.55	44.40	39.54	41.84	11.35	NS
	Msp. stadtmanae	6.14[a]	6.35[b]	6.54[b]	5.98[a]	0.10	***
	Msp. stadtmanae proportion (%)	0.17	0.99	1.00	0.69	0.29	NS
Animal 294	Protozoa	5.81[a]	5.41[b]	5.39[b]	5.61[b]	0.07	***
	Total methanogen	8.39	8.39	8.47	8.14	0.11	NS
	Mbb. sp. AbM4	7.94	8.06	8.18	7.84	0.08	NS
	AbM4 proportion (%)	39.16	48.36	54.48	52.25	7.39	NS
	Msp. stadtmanae	6.62[a]	6.49[a]	6.68[a]	6.31[b]	0.09	**
	Msp. stadtmanae proportion (%)	1.87	1.29	1.70	1.48	0.25	NS
Animal 360	Protozoa	5.70[a]	5.27[b]	5.49[b]	5.17[b]	0.09	***
	Total methanogen	8.40	7.98	8.27	8.25	0.17	NS
	Mbb. sp. AbM4	7.92[a]	7.41[b]	7.57[b]	7.72[b]	0.10	**
	AbM4 proportion (%)	40.21	28.27	22.20	31.32	6.67	NS
	Msp. stadtmanae	6.29	6.45	6.54	6.56	0.11	NS
	Msp. stadtmanae proportion (%)	1.06[a]	3.14[b]	2.02[b]	2.20[b]	0.41	**

[a], [b]: means with the same letter were similar to each other.

[$] Control: control diet; CDDGS: corn DDGS diet; WDDGS: wheat DDGS diet; WDDGS+oil: wheat DDGS plus corn oil diet.

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; NS non-significant.

Animal 294, only protozoa counts and *Msp. stadtmanae* population changed among diets.

Some microbial sectors changed in a conserved way for all animals. In general, when DDGS was added to the diets, protozoa counts decreased in all of the animals; total methanogen and *Mbb.* sp. AbM4 reduced in three out of four animals (except Animal 294); *Msp. stadtmanae* population either increased or decreased compared to the control diet, depending on animal.

5.3.6 Correlation between Methanogens and Protozoa Population

In all four animals, *Mbb.* sp. AbM4 population was positively correlated to the total methanogen population, and the proportions of *Mbb.* sp. AbM4 and *Msp. stadtmanae* were positively correlated to each other. In addition, in three out of four animals, *Mbb.* sp. AbM4 population was positive correlated to protozoa counts (Table 5.9).

5.4 Discussion

The success of CH_4 mitigation strategies largely depends on the understanding of methanogens, including ecology, activities, their relationship with other rumen microorganisms and the host animal. Understanding the interactions among these factors and their impact on the methanogenic community is critical to the successful adoption of CH_4 mitigation technologies by the livestock industry.

Table 5.9 Correlation between microbial sectors within each individual animal.

Association	Animal 44	Animal 170	Animal 294	Animal 360
Total methanogen – *Mbb.* sp. AbM4	0.66[a]***	0.78***	0.82***	0.45*
Mbb. sp. AbM4% - *Msp.stadtmanae*%	0.72***	0.68***	0.73***	0.63**
Mbb. sp. AbM4 - protozoa	0.47*	0.56*	-0.18[NS]	0.53*

[a]: correlation coefficient.

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; NS non-significant.

Previous studies have reported that different diets can change the diversity of ruminal methanogenic community. For example, changing the diet composition of beef cattle from primarily hay (growing diet) to grain (finishing diet) was reported to reshape the composition of methanogens (Zhou *et al.*, 2010). Adding animal tallow to the diets fed to sheep stimulated *Msp. stadtmanae* but depressed *Mbb.* sp. AbM4 (Yu *et al.*, 2008). Supplementing the diet of dairy cows with fibrolytic enzymes affected the presence of phylotypes close to *Mbb.gottschalkii* strain HO and methanogenic archaeon CIRG-GM02 (Zhou *et al.*, 2011). However, the extent of methanogenic diversity changes was different among these studies, and the responses to the diets were not consistent in all the animals within study. Therefore, the current study was conducted to investigate the effect of DDGS diets on the methanogens and how such effect differed among host animals.

In general, utilizing CDDGS in ruminant diets has been shown to reduce CH_4 production, with this response being attributed to the elevated fat content of DDGS (McGinn *et al.*, 2009). The fat in DDGS is mainly composted of long-chain unsaturated fatty acids, predominantly linoleic acid (C18:2) and oleic acid (C18:1) (Jenkins and Lock, 2008; McKeown et al., 2010). As discussed by McAllister et al. (1996), long-chain fatty acids are directly toxic to methanogens, protozoa and cellulolytic bacteria, depressing ruminal fibre digestion and reducing acetate and butyrate production. Biohydrogenation of unsaturated fatty acids also competes with methanogens for hydrogen (Rasmussen and Harrison, 2011). The degree of CH_4 reduction has been reported to be affected by the fat concentration

in the diet. McGinn *et al.* (2009) reported a ~25% decrease of CH_4 (% of gross energy intake) from cattle fed CDDGS (~12% DM of fat) when added to the diet to supply 5% of DM as fat compared to a control diet containing only 2% of DM as fat. Hünerberg *et al.* (2010) compared diets containing DDGS (supplying 4-6% dietary fat compared to 2% dietary fat for the control) and found that CDDGS or WDDGS plus oil (~10% DM of fat) reduced CH_4 (% of gross energy intake) by ~20%, while adding WDDGS (~5% DM of fat) reduced CH_4 (% of gross energy intake) by ~15% compared with the control diet containing no DDGS. However, there is little information regarding changes in methanogenic communities in response to different types of DDGS diets.

As DDGS diets could change the enteric CH_4 production of the animals, it can be speculated that these diets also alter the methanogenic ecology. Therefore, the diet effect on the methanogenic ecology and protozoa population was firstly analyzed. According to our results, when the methanogen DDGE patterns of the entire sample set were compared from a diet perspective, no distinction was observed on the composition of the methanogenic community ($P>0.05$). When a comparison was conducted for each methanogenic phylotype, the presence/absence of six phylotypes was affected by DDGS diets, and two phylotypes tended to be affected by DDGS diets, suggesting that diet slightly affected the methanogenic composition. In addition, protozoa count and total methanogen population differed among the four diets. As enteric CH_4 production was lowered by the DDGS diets (Hünerberg *et al.*, 2010), it can be speculated that

DDGS diets suppressed CH_4 by changing the diversity and abundance of methanogens as well as by reducing hydrogen-supplying protozoa.

In contrast to the moderate effects of diet, the host strongly affected DGGE patterns ($P<0.01$), and the presence/absence of all phylotypes differed among animals. This suggests that host-related factors are very important in terms of shaping the ruminal methanogenic community in response to different types of DDGS. It is possible that in this study the dietary effect was masked or outcompeted by the significant host effect. Thus, to investigate how methanogenic communities adapted to the three DDGS diets, diet-wise comparisons were conducted separately for each individual animal.

When the animal effect was removed, a dietary effect on the methanogenic ecology was observed. Each animal hosted a unique combination of methanogenic phylotypes when different types of DDGS were fed. Besides, each methanogenic phylotype appeared to respond to the DDGS diets differently within each host. Unlike the fat-level-dependent changes observed for CH_4 production, the alteration in the presence of each methanogen phylotype was fat-independent. For example, in Animal 44, Band 3 was observed in all samples for the control diet, and in 75% samples for the CDDGS diet, while for the WDDGS and WDDGS+oil diets, it was not observed. In contrast, in Animal 170, Band 3 was not detected for the control and CDDGS diets, but it appeared in 50% of the samples for the WDDGS diet and in all samples for the WDDGS+oil diet. This result suggests that the same phylotype may display varied characteristics depending on host animals. Moreover, the protozoa counts, total

methanogen population, as well as population and proportions of *Mbb.* sp. AbM4 and *Msp. stadtmanae* changed according to the four diets uniquely within each heifer (Table 6). The variation in the microbial adaptation to the four diets within each animal may be due to the differences in the rumen environments within each animal, including characteristics of rumen size, pH, and passage rate. These host-related factors may be important in shaping the symbiotic methanogens and protozoa and regulating their adaptation to diet.

In the rumen, some methanogens are physically associated with protozoa (Finlay *et al.*, 1994), but the protozoa-associated methanogen species have not been well studied. In a preliminary study, Ohene-Adjei *et al.* (2007) inoculated the rumen of sheep with different protozoa species and found that inoculation with *Isotricha* and *Dasytricha* resulted in identification of archaeal clones affiliated with *Mbb. smithii*, while in *Entodinium* inoculated or protozoa-free sheep the methanogenic phylotypes were affiliated with unknown species. Tóthová *et al.* (2008) identified a methanogenic phylotype that was affiliated with the rumen protozoa, *Entodinium caudatum*, but the taxonomic information of the methanogen was unknown since it clustered with unidentified species. In the current study, *Mbb.* sp. AbM4 population was positively correlated with protozoa count. However, whether *Mbb.* sp. AbM4 is directly associated with protozoa or not is unknown. Further experiments are required to verify the existence of such a correlation.

In addition to diet and animal effects, housing conditions also had a significant influence on the population of methanogens. As shown in Table 1, the

methanogen population increased when cattle were placed inside chambers, but the reason for this alteration is unknown. Housing cattle inside the environmental chamber may have caused stress to the animals altering normal animal behavior and feed intake (McGinn *et al.*, 2004). Thus, rumen samples collected under the less artificial housing conditions outside the environmental chambers may have differed from those collected in the chamber. Therefore, we suggest that in the future studies, baseline samples that were collected before applying any facilities to the animals should be included to provide more accurate measurements of microbial sector.

5.5 Conclusion

This is the first study undertaking a detailed comparison of the individual variance of the ruminal methanogenic communities within cattle and to identify the unique microbial adaptation to different diets. The diversity of methanogens and the abundance of protozoa and methanogens varied among heifers and diets, and the adaptation of ruminal methanogenic ecology to the three DDGS diets was unique within each animal. The animal effect was stronger in shaping the methanogenic community in the current study, suggesting that individual animal variance should be taken into account in future studies. Understanding how each methanogen interacts with other microbes and within host animals may help to explain the uniqueness of microbial adaptation to different diets, so as to improve the efficiency in redirecting the ruminal methanogneic community.

5.6 References

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6.0 GENERAL DISCUSSION

6.1 Association between Rumen Methanogenic Ecology and Host Feed Efficiency

Cattle with different RFI produce different amounts of CH_4 (Nkrumah et al., 2006; Hegarty et al., 2007) and display varied ruminal bacterial ecology (Guan et al., 2008; Hernandez-Sanabria et al., 2010). Therefore, the primary hypothesis of the current research project was that cattle with different feed efficiency host different ruminal methanogens in the rumen. Study 1 was conducted to compare the methanogenic community between high and low RFI animals fed a growing diet. The diversities of the methanogenic communities between two groups of cattle were significantly different. Although both groups of animals shared some methanogen phylotypes, there were certain phylotypes that specifically affiliated with either high or low RFI cattle. Total methanogenic population was similar among all the animals, but quantitative variation was observed in two particular species, *Mbb*. sp. AbM4 and *Msp. stadtmanae*. Different substrate preference of these two species suggests that their metabolism may influence host's feed efficiency, but the exact mechanism is unknown.

Although two methanogen species were found potentially related to cattle's RFI, Study 1 only investigated the ruminal methanogenic community of cattle fed a single diet type. It was not clear whether the same correlation between cattle's RFI and specific methanogen species existed or not when the cattle were fed different diets. Thus, Study 2 was conducted to investigate the dietary impact on the association between methanogenic ecology and host RFI. As observed in

Study 2, the biodiversity of the observed methanogens changed significantly when cattle were fed a finishing diet compared to the growing diet. Regardless of dietary composition, animals with different RFI still hosted specific methanogen phylotypes in addition to the core species observed under each specific diet. The observed PCR-DGGE patterns were more similar between animals within the same RFI group. But the phylotypes that were potentially associated with RFI were different across the two diets. The total methanogen abundance was similar among different groups of cattle, which was consistent with Study 1. This suggests that the composition of the methanogenic community rather than the total number of methanogens may be more important in affecting host's feed efficiency.

Each microbial species possesses a unique combination of characteristics; their specific preference in substrate utilization, fermentation products, and growth yield are all different from one another (Weimer, 1996). It can be speculated that although methanogens all utilize primary digestion products as substrates to produce CH_4, the efficiency of substrate utilization, the conversion pathways, and the production rate could be different among different species. In addition, different methanogen species also displayed distinctive cell structure and physiology (Ferry and Kastead, 2007). As a result, the adaptation to environmental changes could be different among each methanogen species, and the ones that play a "causative" role in changing host enteric CH_4 and host feed efficiency could be different.

Although data was not shown above, primary analyses on the relationships between methanogen abundance and the metabolites (total VFA and each type of VFA) were conducted. The total methanogen density was not correlated to any of the metabolite measurements, suggesting that although the composition of the methanogenic community was different among animals and diets, the overall VFA consumption was relatively stable. On contrast, proportion of *Mbb.* sp. AbM4 positively correlated to butyrate proportion and negatively correlated to acetate proportion. This further supports our assumption that the total methanogen population is not an important factor contributing to the variance of the host animals; instead, particular species of methanogens may be associated with the metabolic characteristics and performance of the host animals. As such, future studies combining all of the metabolic measurements and microbial ecology data (including bacteria, methanogen, and protozoa) may be helpful to elucidate the mechanism how ruminal microbiota contribute to the variance of rumen digestion and thereafter influence host performance.

6.2 Relationship between Methanogenic Ecology and CH_4 Production

Although methanogenic ecology has been examined in many studies, the relationship between methanogenic ecology and host enteric CH_4 production has not been well described. In Study 3, we studied the association between methanogenic ecology and host CH_4 production and found that two phylotypes related to Methanogenic archaeon CH1270 tended to be negatively correlated, while one phylotype related to *Mbb. gottschalkii* strain HO tended to be positively

correlated with CH_4 yield. Since this is the only report defining the association between particular methanogen phylotypes and host CH_4 production, whether these phylotypes also play a role in dairy cattle fed a different diet or whether the same case would happen in beef cattle is unknown.

On the contrary, the density of methanogens was neither correlated with absolute CH_4 yield (Study 3, Zhou *et al.*, 2011) nor with CH_4 expressed as percentage of GEI (Study 4). Liu *et al.* (2011) examined the methanogen population and CH_4 production potential in natural wetland and Dong *et al.* (1997) measured the CH_4 production and methanogen abundance within an artificial rumen system. In both of the two studies, no linear relationships were found between methanogen population and CH_4 production. In rice fields, methanogen population was also not correlated with the CH_4 emission (Joulian *et al.*, 1997). All the results showed the same fact that the number of methanogens does not directly influence the CH_4 production. We speculated that the diversity and the activities of the methanogens may be more important in affecting the CH_4 emission.

6.3 Dietary Factors Affecting Rumen Methanogenic Ecology and Its Association with Cattle Feed Efficiency and Enteric CH_4 Production

Different microbial species have different requirements for nutrients and have varied digestibility of different diet ingredients. Thus, it is not surprising that changes in diet have led to alterations in ruminal microbial ecology. Dietary effects on ruminal methanogens have been demonstrated in several studies (Guo

et al., 2008; Yu et al., 2008; Jeyanathan et al., 2011). In general, there are two major types of dietary changes: changes of the fibre/grain type and addition of dietary supplements. The effects of both types of dietary changes on the ruminal methanogenic community were examined in the current project.

In Study 2, the types of fibre of the two diets were different. The growing diet contained 74% oats, 20% hay, and 6% feedlot supplement; while the finishing diet contained 28.3% oats, 56.7% barley, 10% alfalfa pellets, and 5% feedlot supplement. In this study, the different fibre types of the two diets have led to a transition in the methanogenic ecology. Under the growing diet, *Mbb. ruminantium* was the solely predominant species; under finishing diet, each animal hosted several predominant species. As the composition of methanogenic community changed, it was not surprising to see the RFI-associated phylotypes change between the two diets.

In Study 3, an enzyme that enhance ruminal fibre digestibility was added at different doses to the diet. Owing to the identical basal diet, the observed PCR-DGGE patterns of methanogens were similar among control, low-enzyme, and high-enzyme diet, with only slight changes being observed when enzyme was added. Among all the 26 observed phylotypes, only three were found to be affected by the enzyme supplement. The result of this study suggests that the fibrolytic enzyme may improve rumen digestibility by altering the microbial activity rather than reshaping the microbiota.

In Study 4, DDGS was added to the diet to replace barley grain, increasing the crude fat content of the diet formula. When DDGS was included, both the

abundance and the biodiversity of the methanogenic community changed. The fat components within DDGS may influence methanogens by having direct toxic effect on the methanogens and/or reducing protozoa numbers and thus further reducing numbers of methanogens. As different methanogenic phylotypes were found to have different responses to the DDGS treatments, it can be speculated that each methanogen species has varied adaptive mechanism to the increase of fat along with the DDGS supplementation.

Based on the above, different dietary strategies have different effects on the ruminal methanogenic ecology. The whole formula of the diet seemed to be the biggest driven force shaping the methanogenic community. As shown in the three studies, the change in Study 2 was most obvious, where the community shifted from single-species-predominant to multi-species-predominant. Increasing cattle digestibility by adding fibrolytic enzyme did not dramatically affect the total population and species composition of the ruminal methanogenic community. When fat was included in the diet, numbers of both methanogens and protozoa were reduced and the observed PCR-DGGE profiles of methanogens were altered.

6.4 Identification of Individual Variation in Rumen Methanogenic Community

In the symbiosis relationship between the host and the gut microbiota, the host displayed a unique environment and exerts selective pressure on inhabiting microorganisms. A good example explaining the individual variance of the gut

microbiota was the study by Stewart *et al.* (2005) on humen identical twins, fraternal twins, and unrelated pairs. In their study, although identical twins hosted the most similar gut microbiota compared to the other two groups, the detected bacterial profiles still displayed dissimilarity between each other even when they were fed identical diets. In addition, the gut microbiota of fraternal twins was more different, and even more divergent in unrelated pairs. Therefore, taking individual variation into account would be more appropriate to study the symbiotic microbial ecology within the gastrointestinal tract and its adaptation to environmental changes.

In Study 4, we have examined four animals subjected to the same maintenance condition and dietary transition respectively. In this experiment, we found that the ruminal methanogenic community was composed of different detectable phylotypes from each cow. In addition, while the animals were subjected to dietary transition, the microbial adaptation within each individual showed unique alteration patterns. These results were in agreement with the results from Benson *et al.* (2010) who explored host genetic effects on gut microbiota composition and the results from Durso *et al.* (2010) who identified animal-to-animal variation in fecal microbial diversity among beef cattle. The discrepancy of the ruminal methanogenic and bacterial community reported in the above studies may be one of the main reasons leading to the inconsistency of animal production, host CH_4 production, as well as the efficiency of the CH_4 mitigation methods applied to them. Emphasizing individual variance will provide a more precise option to monitor the effects and efficacy of all the trials. Although

the significant individual variation was observed from many studies, the mechanisms of host effect on gut microbial diversity are not clear. We speculated that host genetics may play a role to impact on the colonization of the community and its adaptation to the changes in environment. Future studies to dissect the variation of the host genotypes may supply the evidence to our speculations.

6.5 Comparison of Dietray and Animal Effect on Ruminal Methanogens

As discussed above, both diet and host animal can impact on the ruminal methanogenic ecology. Which factor is stronger seemed to vary among experiments. Based on our results, the strongest dietary impact was observed in Study 2, in which the types of fibre and grain were completely different between growing and finishing diet. In this study, the clustering of the PCR-DGGE patterns was predominantly in accordance with diet. Besides, as the steers used in this study originated from three different sire breeds (Angus, Charolais, and Angus × Charolais), the difference on methanogen composition of each animal under the same diet was observed as expected. On contrast, animal effect was more noticeable in Study 4. In this study, the PCR-DGGE patterns were completely different among the patterns of all animals through the dietary transition. Since all the heifers used in this study were cross-bred animals, it was not surprising to observe a huge variation of the ruminal methanogens among individuals. When animal effect was removed, dietary effect on the methanogenic ecology was shown. When the three types of DDGS were used to replace a part of the control diet, the methanogenic community adapted to the dietary changes and

displayed different diversities under the four diets. In Study 3, the basic composition of the three diets was identical, and the only difference among the three diets was the dose of the fibrolytic enzyme. Therefore, the variation among the diets may not be huge enough to induce a fundamental change in the composition and quantity of methanogens. Additionally, the cows were the same breed (Holstein), and the genetic variance of these animals may not be big as the other two studies. As a result, no significant animal effect was observed in this study. According to the above, dietary effect and animal effect should be taken into account at the same time when analyzing the ruminal methanogens.

6.6 Future Directions

To our best knowledge, this is the first study aiming to link cattle's feed efficiency and CH_4 production to ruminal methanogenic ecology, and to investigate individual variance of the ruminal methanogenic community and its adaptation to dietary transition in details. However, there are limitations of the present study. Firstly, although the potential correlation between methanogenic ecology and cattle's feed efficiency was identified under two dietary conditions as reported in Study 1 and 2, neither of these two studies measured the CH_4 production from the animals. Therefore, we were not able to reveal the linkage between methanogenic communities and host enteric CH_4 production. Further, without the CH_4 measurement, it was not possible to verify whether the two species can influence host CH_4 yield or not. Secondly, the Latin Square experimental design utilized in Study 3 made the analysis of the microbial

ecology complicated. Such design made the microbial profile analysis very challenging due to the lack of method to remove the cross contamination between each diet transition. Since there is no statistical model to analyze the entire DGGE pattern under the Latin Square design, we only conducted the analysis for each phylotype respectively. A more precise statistical analysis model needs to be developed for similar studies and the samples before any treatment need to be analyzed to define the cross contamination effect. Additionally, the numbers of animal used in the current study was small. To make the measurements more representative and more statically powerful, increasing animal number in the future studies would be an ideal option.

Regardless of the limitations, the current study has contributed fundamental knowledge regarding to rumen methanogens. Results from the four studies have pointed out a key finding that the composition of the methanogenic community is more important than the total abundance which may be associated with host's CH_4 production and feed efficiency. Since the knowledge about methanogens is very limited and only very few species have been isolated and subjected to genome sequencing analysis, more studies are required to completely understand how methanogens function in the rumen and how they interact with host animals and other microorganisms. As such, the future directions are listed below.

1. The PCR-DGGE analysis can serve as a rapid and cheap method to conduct preliminary selection of the samples. After screening, candidate samples with specific combinations of methanogens can be subjected to metagenomic

analysis using next sequencing technology to get a more complete and precise description of the composition and function of the entire ruminal microbiota. Unlike other microbial groups within the rumen, methanogens are largely unknown. Metagenomics may be a robust tool to facilitate in revealing all the members of methanogens and the unidentified functional genes within the samples. By knowing all the species and functional genes, the potential interactions and functions of methanogens may be predicted which may explain the mechanisms of effect of diversity rather than population on feed efficiency and enteric CH4 production.

2. As all the animal experiments in the current study were conducted as separate trials, it was difficult to bring all the potential linkages determined in each experiment together. Collecting a complete sample set for microbial study, measuring all the indices within the host and the rumen samples, as well as clarifying host genetic information would be favorable to provide a complete framework of the complex association among all the factors.

3. Validation of the detected and/or predicted interactions among different microbial groups and associations with host phenotypes is required. Transcriptomics and proteomics analysis are necessary to confirm the microbial-microbial and microbial-host interactions, as well as the actual activities of the microbial members within the rumen. Measuring activities of the microbes, such as the expression of functional genes and the activities of the enzymes involved in methanogenesis may provide support for interpreting how methanogenesis affects host energy availability and supply more options in

manipulating the methanogenic communities. In addition, the researches on methanogens should be combined with the studies on bacteria, protozoa, and host metabolism, so as to get a complete understanding about the impact of ruminal microbiota on ruminants.

6.7 Implications

In summary, this was the first study to identify the association among ruminal methanogenic ecology, enteric CH_4 production, and host feed efficiency under different diets. This was also the first study to conduct a detailed comparison between diet effect and host effect on methanogenic ecology. Based on our results, the composition is more important than the density of ruminal methanogens contributing to the variance of host's performance. Our findings contribute fundamental knowledge to understand ruminal microbial ecology and provide information for CH_4 mitigation method development in cattle, so as can help to reduce feeding cost and improve animal production.

6.8 References

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