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**Investigation of Rumen Methanogens in New Zealand
Livestock**

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Abstract

Methane emitted by farmed ruminants contributes 30.3% to New Zealand's anthropogenic greenhouse gas inventory. Methanogens living in the rumen produce methane from H₂ and CO₂ as a byproduct of feed fermentation. The use of vaccines and small molecule inhibitors against the methanogens are promising methods to reduce methane emissions from extensively-grazed ruminants in New Zealand. Knowledge of the methanogens present in New Zealand ruminants is an important first step for successful vaccine and inhibitor development to target all methanogens.

In this study, the methanogen diversity of farmed ruminants (sheep [*Ovis aries*], cattle [*Bos taurus*] and red deer [*Cervus elaphus*]) was investigated using molecular ecological techniques. Ruminants fed different diets had largely similar rumen methanogen communities. The major methanogen groups identified were from the *Methanobrevibacter ruminantium* clade (*Mbb. ruminantium* and closely-related species), *Methanobrevibacter gottschalkii* clade (*Mbb. gottschalkii* and closely-related species), *Methanosphaera* spp., and the putative methanogens belonging to the group designated Rumen Cluster C. A total of 37.5 - 57% of 16S rRNA genes in the rumen of a group of cows originated from members of Rumen Cluster C. Chloroform treatment of cows increased the abundance of Rumen Cluster C to 82% - 93% of archaeal 16S rRNA genes. In parallel, a total of 22% of *mcrA* genes belonged to an unassigned group of archaea, and chloroform treatment increased the unassigned group of archaea to 92% of all *mcrA* genes. This suggested that Rumen Cluster C archaea contain the gene *mcrA*.

No members of the Rumen Cluster C group have previously been cultured, and currently there is no reported rumen isolate of *Methanosphaera* spp. A strain of *Methanosphaera* sp. was isolated from a sheep rumen and initial characterization suggests that this may be a new species. Three enrichment cultures were obtained containing members of Rumen Cluster C as the only archaea. Initial studies of these enrichment cultures showed that these three isolates were from three different sub-groups of Rumen Cluster C and that they produced methane.

The investigation of methanogen diversity in New Zealand farmed ruminants and isolation of previously uncultured rumen methanogens reported here in this thesis will

significantly aid the development of methane reduction strategies for farmed ruminants in New Zealand.

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Non-standard abbreviations

BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
C	cow
CoM	coenzyme M
D	red deer
DGGE	denaturing gradient gel electrophoresis
DM	dry matter
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DMSO	dimethyl sulfoxide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EM	electron microscopy
FISH	fluorescence <i>in situ</i> hybridization
GHG	greenhouse gas
h	hour
HPLC	high-performance liquid chromatography
IR	infra-red
LB	Luria-Bertani
Ltd	Limited
M	molar
Mbb	<i>Methanobrevibacter</i>
MCR	methyl coenzyme reductase
min	minutes
MOPS	3-(N-morpholino) propanesulfonic acid
MPN	most probable number
PCR	polymerase chain reaction
qPCR	quantitative real-time polymerase chain reaction
RCC	Rumen Cluster C
RF	rumen fluid
RNA	ribonucleic acid
RODS	relative one-dimensional surface

rpm	revolutions per minute
S	sheep
SRB	sulphate reducing bacteria
subs	substrate
TGGE	temperature gradient gel electrophoresis
U	unit
UV	ultraviolet
v/v	volume per volume
VFA	volatile fatty acid
vit	vitamins
w/v	weight per volume
w/w	weight per weight
YE	yeastextract

Chapter 1

Literature Review

1.1 Introduction

The changing composition of Earth's atmosphere has become an important climatic issue over the last few decades, as this change seems to have caused the observed increase in atmospheric temperature (Moss *et al.*, 2000). It has been predicted that atmospheric temperature could increase between 1°C to 6°C during the twenty-first century (IPCC, 2007). This temperature rise is mainly due to the increase in concentration of greenhouse gases (CO₂, CH₄, N₂O, chlorofluorocarbons [CFCs]) in the atmosphere. Greenhouse gases (GHG) in the atmosphere are essential for life on Earth, as they trap the solar infrared (IR) radiation from the sun and re-emit IR radiation at lower frequencies with a high absorptive power (Moss *et al.*, 2000). This makes the Earth's surface warmer, and without them the Earth would be permanently frozen (Moss *et al.*, 2000). However, their concentrations in the atmosphere should not be allowed to rise beyond a certain threshold limit, as this causes the average global temperature to rise. The increasing global temperature has many consequences for life on Earth, such as a rise in global mean sea level due to the thermal expansion of the oceans and melting ice/ice-packs in the Arctic and Antarctic regions, effect on water supplies, new distributions of deserts and wet areas in the world, and changes in distribution of diseases of animals, human and plants (Moss *et al.*, 2000). Among the GHG, carbon dioxide (CO₂), methane (CH₄) and nitrous oxide (N₂O) are the most important gases, as human activities cause increases in their concentrations in the atmosphere (IPCC, 2007).

To stabilise GHG concentrations below critical levels in the atmosphere, the Climate Change Convention was signed by 188 nations, including New Zealand, in 1994 (United Nations, 1997). The main objective of the Climate Change Convention is to achieve stabilisation of GHG concentrations in the atmosphere at a level that would prevent dangerous anthropogenic (caused by humans) interference with the climate. The Kyoto Protocol, which describes the detailed commitments and actions of these countries regarding climate change, was adopted by many of these countries in 1997.

New Zealand ratified the Kyoto protocol in 2002 and it came into action in 2005 (Ministry of Environment, 2009).

New Zealand has an unusual emissions profile for a developed country. Greenhouse gas emissions from the agricultural sector dominate the New Zealand national GHG emissions inventory, contributing 46.6% in 2008. In contrast, emissions from agriculture account for typically around 11% of total emissions in other developed countries (Ministry of Environment, 2009). The sources of GHG emission from agriculture in New Zealand were mainly enteric methane (CH₄) emissions from ruminant farm animals (65.1%) and nitrous oxide emissions from agricultural soils (32.7%). These two gases contributed when calculated as CO₂-equivalents 30.3% and 15.2%, respectively, to the New Zealand total emissions (Ministry of Environment, 2009). Methane has a global warming potential 21 times that of CO₂ and is therefore an important GHG (IPCC, 2007).

Methane is a by-product of digestion in ruminants and is produced mainly in the reticulo-rumen. The reticulo-rumen, often called simply the rumen, is the primary location for microbial fermentation of plant material, containing a microbial population made up of bacteria, archaea (methanogens), protozoa, fungi and phage. Rumen microorganisms function via complex interactions, which are essential to sustain their population and activity. The ingested feed is fermented by bacteria, protozoa and fungi into short chain fatty acids which are then used by the host as its energy source. One by-product of this fermentation process is H₂ and this is used by rumen methanogens to produce CH₄. By utilizing this H₂, methanogens play a key role in decreasing the partial pressure of H₂ in rumen, making the fermentation process more efficient and continuous (Wolin *et al.*, 1997). Unfortunately, they also contribute to global warming. Approximately 95.5% of the methane emitted by ruminants is produced in the rumen and exhaled via the nose and mouth (Wright *et al.*, 2004a).

There have been several techniques proposed by international and New Zealand researchers to reduce rumen methanogenesis (Baker, 1999; Beauchemin *et al.*, 2008; Clemens & Ahlgrim, 2001; Eckard *et al.*, 2010; Martin *et al.*, 2010; McAllister & Newbold, 2008; Shibata & Terada, 2010; Wedlock *et al.*, 2010), but most of these techniques are only possible with housed ruminant animals. The main feeding system employed for raising New Zealand ruminants is extensive grazing. Thus, any methane-

abatement strategy should not be suitable to this type of feeding system. Two possible options being investigated to reduce methane emission from New Zealand ruminants are to use vaccines and small-molecule inhibitors to decrease the number and activity of methanogens in the rumen. (Buddle *et al.*, 2010; Wedlock *et al.*, 2010). Attempts to vaccinate animals with crude vaccine of whole methanogens have not always successfully reduced methane production *in vivo* (Williams *et al.*, 2009; Wright *et al.*, 2004a). The difficulty in producing an effective vaccine targeting a number of methanogens was the presumed reason for this observation. Thus, the development of a vaccine or small-molecule inhibitors, which would be broadly effective against the entire range of ruminal methanogens, is a major challenge. It is therefore necessary to understand the diversity of methanogens present in the rumen, their specific roles, and their interactions with other microorganisms within the rumen. A recent study that sequenced and analysed the entire genome of *Methanobrevibacter ruminantium* has revealed more insights into methanogens (Leahy *et al.*, 2010). Despite this recent advance, more genome sequencing of methanogens that are important in New Zealand ruminants is still needed for the successful development of a vaccine or small-molecule inhibitors.

This literature review investigates the present knowledge of diversity of rumen methanogens and their interactions with other microorganisms. Isolation and molecular-ecological techniques used to study rumen methanogen diversity are briefly discussed with their merits and short-comings. Finally, potential methane reduction techniques which could be used for the grazing ruminants are discussed. The research objectives of this thesis were formulated based on current knowledge on rumen methanogen diversity and the knowledge still needed for developing successful methane reduction strategies for New Zealand ruminants.

1.2 Rumen methanogens

Methanogens belong to the domain Archaea, which is comprised of two major kingdoms, the Crenarchaeota and the Euryarchaeota (Woese *et al.*, 1990). Recently a third kingdom, Thaumarchaeota has been proposed (Brochier-Armanet *et al.*, 2008). Methanogens, which belong to the kingdom Euryarchaeota, are the only identified microbes belonging to the domain Archaea that have been identified as living in the

rumen. Methanogens are a distinct phenotypic group of archaea which produce methane as a major catabolic product (Stewart *et al.*, 1997) and are obligate anaerobes. Small ribosomal subunit RNA sequences from members of the kingdom Crenarchaeota have been found in some 16S rRNA gene clone libraries constructed from rumen samples (Shin *et al.*, 2004; Skillman *et al.*, 2006). However, additional studies are needed to demonstrate that they are not simply transients, as they could easily reach the rumen through feeding.

Methanogens become established in the rumen soon after birth (Fonty *et al.*, 1987; Morvan *et al.*, 1994) and they have been estimated to comprise 0.5-3.0% of the microbial population in the fully developed rumen (Janssen & Kirs, 2008; Lin *et al.*, 1997; Yanagita *et al.*, 2000). The methanogens in the rumen are found in different fractions: in rumen fluid, attached to particulate material, and associated with the rumen epithelium (Pei *et al.*, 2010; Shin *et al.*, 2004). They can also be attached to the internal and external surfaces of protozoa (Finlay *et al.*, 1994; Ohene-Adjei *et al.*, 2007; Regensbogenova *et al.*, 2004; Tokura *et al.*, 1999; Wolin *et al.*, 1997). There is evidence to suggest that the diversity of methanogens between these different fractions can vary (Pei *et al.*, 2010; Shin *et al.*, 2004). The methanogens associated with these different fractions can be expected to have different growth rates, since they will be removed from the rumen at different rates (Janssen & Kirs, 2008).

1.3 Rumen methanogenesis

In the rumen, formation of methane (methanogenesis) is the major pathway for hydrogen elimination, which is one of the major end products of fermentation of feed by bacteria, protozoa and fungi. The reactions involved in methane production in the rumen are the sole energy-generating mechanism of methanogens (Rouviere & Wolfe, 1988). Biochemically, methanogenesis is a complex process and many coenzymes are involved in this process (Whitman *et al.*, 1991). Some of the coenzymes involved in the process of methanogenesis are almost totally unique to methanogens, e.g. coenzyme M, methanofuran, HS-coenzyme B, tetrahydromethanopterin and deazaflavin F₄₂₀ (Rouviere & Wolfe, 1988). One key intermediate product of methanogenesis is methyl-coenzyme M, which forms in the terminal part of the process. This intermediate product is reduced by methyl-coenzyme M-reductase, and CH₄ is formed. The last step produces

energy for the next cycle, and is a very important step for the continuation of methanogenesis (Rouviere & Wolfe, 1988).

The pathway of methanogenesis varies depending on the substrates (Rouviere & Wolfe, 1988). Due to this, methanogens can be divided into three nutritional categories (Garcia *et al.*, 2000). The hydrogenotrophic methanogens (e.g. *Methanobrevibacter* spp.) oxidise H₂ and reduce CO₂. Members of this category can also utilize formate, certain alcohols and their metabolism has the highest potential for energy conservation during methanogenesis (Table 1.1). The methylotrophs (e.g. *Methanosphaera* spp. and *Methanomicrococcus* spp.) utilize methyl compounds such as methylamine or methanol (Garcia *et al.*, 2000). *Methanosphaera* spp. can only utilize methanol (Miller & Wolin, 1985), but *Methanimicrococcus* spp. can utilize both methanol and methylamine (Sprenger *et al.*, 2000). The potential for energy conservation during methanogenesis by this group of methanogens is lower than the hydrogenotrophic methanogens (Table 1.1). Acetotrophic methanogens (e.g. *Methanosarcina* spp.) utilize acetate to form methane. The potential for energy conservation in this pathway is the lowest among the methanogenic substrates (Table 1.1).

Table 1.1. Reaction and standard changes in free energies for methanogenesis (Garcia *et al.*, 2000)

Reaction	$\Delta G_0'$ (kJ/mol CH ₄)
4 H ₂ + CO ₂ → CH ₄ + 2 H ₂ O	-135.6
4 Formate → CH ₄ + 3 CO ₂ + 2 H ₂ O	-130.1
2 Ethanol + CO ₂ → CH ₄ + 2 Acetate	-116.3
Methanol + H ₂ → CH ₄ + H ₂ O	-112.5
4 Methanol → 3 CH ₄ + CO ₂ + 2 H ₂ O	-104.9
4 Methylamine + 2 H ₂ O → 3 CH ₄ + CO ₂ + 4 NH ₄ ⁺	-75.0
4 Trimethylamine + 6 H ₂ O → 9 CH ₄ + 3 CO ₂ + 4 NH ₄ ⁺	-74.3
2 Dimethylsulfide + 2 H ₂ O → 3 CH ₄ + CO ₂ + H ₂ S	-73.8
2 Dimethylamine + 2 H ₂ O → 3 CH ₄ + CO ₂ + 2 NH ₄ ⁺	-73.2
4 2-Propanol + CO ₂ → CH ₄ + 4 Acetone + 2 H ₂ O	-36.5
Acetate → CH ₄ + CO ₂	-31.0

In the rumen, H₂ utilizing methanogens predominate and most of the CH₄ that is formed is derived from this substrate (Hungate, 1967). Formate is also an important electron donor used by many rumen hydrogenotrophic methanogens (Hungate *et al.*, 1970). Methanol and methylamine produced in the rumen are utilized by methylotrophic methanogens, and the contribution of these substrates to methanogenesis is likely to be small (Janssen & Kirs, 2008). Methanogens that utilize acetate have been suggested to grow too slowly on this substrate to remain in the rumen (Zinder, 1993). Acetotrophic methanogens are thus indicated to be rare in the rumen (Janssen & Kirs, 2008). However, some methanogens can utilize more than one substrates: for example *Methanosarcina* spp. are generalists that can utilize most of the methanogenic substrates including methyl groups, acetate and H₂ (Liu & Whitman, 2008).

Only a few species of methanogens have been isolated from rumen thus far. However, knowledge of this ecosystem is rapidly accumulating with the help of culture-independent techniques. The isolation and molecular-ecological techniques that are used in the study of rumen methanogen diversity are briefly discussed in the following sections.

1.4 Techniques for culturing methanogens

Isolation of microorganisms is an important step in the process of understanding the biology and ecology of microbial species. However, and unfortunately, the majority of microbes in natural environments typically fail to grow under laboratory conditions. Methanogens are strict anaerobes, which makes them particularly difficult to cultivate. In addition, some methanogens require long incubation periods for growth and some methanogens are difficult to separate from their syntrophic partners (Bryant *et al.*, 1967). The development of anaerobic cultivation techniques has allowed a number of species of methanogens to be isolated and characterized over the last six decades. The majority of these species have been isolated by the traditional anaerobic method described by Hungate (1950).

Initially, roll tubes were used to isolate methanogens in or on agar spread over the inner surface of Hungate tubes (Paynter & Hungate, 1968; Smith & Hungate, 1958). Until 1975 this roll tube method was commonly used and is popular even today. Some

studies used serum bottles instead of roll tubes to isolate methanogen colonies (Miller & Wolin, 1974). However, there are difficulties encountered in observing and picking the isolated colonies from tubes or serum bottles. To counter this, an agar plate method was described for the cultivation and identification of methanogens, which was more convenient than the roll tube method (Edwards & McBride, 1975). In this method, an anaerobic glove box with an inner chamber was employed for incubation of inoculated agar plates. Later, this technique was modified by storing inoculated plates in sealed incubation cylinders that could be transferred outside the anaerobic chamber where the gas atmosphere within the cylinder was exchanged and pressurized and the cylinders then incubated at any desired temperature (Balch & Wolfe, 1976). Subsequently, the agar bottle plate method was described to isolate methanogens (Hermann *et al.*, 1986), but this method has not been widely used for methanogen isolation.

Recently Kenters *et al.* (2010) isolated a large number of rumen bacteria in a single experiment using liquid media by a simple cultivation method. In this approach, the inoculum was diluted to introduce ≤ 1 culturable cells into a tube (or other culture vessel). A significant number of cultures can be obtained that are derived from single culturable cells by this method (Button *et al.*, 1993). This exact approach has not been used for rumen methanogen isolation thus far, and is a potential technique to isolate methanogens from rumen.

It has been found that traditional cultivation methods are laborious, time consuming, selective and biased for the growth of specific microorganisms (Zengler *et al.*, 2002). The artificial conditions provided in the media often have high substrate concentrations or lack specific nutrients required for growth. Recently, new techniques have been developed to better simulate the natural conditions of the environment where the microbes live. It has been shown, if the chemical components are provided as in their natural environment, previously uncultivable microorganisms could be grown in pure culture (Connon & Giovannoni, 2002).

The diffusion chamber method is one of the newer methods that simulates the organism's natural setting (Kaeberlein *et al.*, 2002). In this approach, microorganisms are placed in a diffusion chamber which is separated from the organism's natural environment by 0.03 μm pore-size membranes. This chamber is incubated in the natural habitat of the target organism. The membrane allows naturally occurring growth

components including nutrients and possible signalling components to diffuse to the cells and removes the metabolic products from the cells. It has been found that the diversity of the diffusion-chamber-derived strains of bacteria in an environmental sample exceeded that obtained from standard petri dishes (Bollmann *et al.*, 2007).

A high-throughput cultivation method based on the combination of a single cell encapsulation procedure with flow cytometry has been developed that enables cells to grow with nutrients that are present at environmental concentrations (Zengler *et al.*, 2002). In this method all the encapsulated cells from the environment are cultured together in a single vessel. This proximity simulates to some extent the natural environment; because the pore size of the encapsulation gels is large, it allows the exchange of metabolites and other molecules such as signaling molecules. In addition, this method is applicable to samples from various environments such as sea water, soil and alkaline lake sediment (Zengler *et al.*, 2002). However, none of these new methods have been used to isolate rumen methanogens thus far, mainly because of the difficulties in adapting them for use with extremely oxygen-sensitive anaerobes.

A co-culture method was recently used to isolate methanogens from rice field soil (Sakai *et al.*, 2007). The authors found that the Rice Cluster 1 (RC-1) methanogens, one of the uncultured methanogen groups found in rice field soil, could be cultivated by co-cultivation using syntrophs as a H₂ provider (Sakai *et al.*, 2007). The conventional method using a H₂/CO₂ gas mixture as a substrate has typically yielded only fast growing methanogens, whereas this method enabled the isolation of RC-1 methanogens, which are adapted to a low-H₂ habitat. The syntrophic propionate-oxidizing H₂-producing bacterium *Syntrophobacter fumaroxidans* supplied the H₂ at very low concentrations to the RC-1 methanogens. This finding strongly indicated that the co-culture method could work for isolation of some fastidious methanogens that may otherwise escape conventional isolation strategies. It is, however, doubtful that slow-growing methanogens could maintain themselves in the rumen.

Another important factor relating to isolation techniques is the media used for the isolation. So far, the media used for the isolation of rumen methanogens have been based on the methods of Hungate (Hungate, 1950). To isolate new methanogens, media should be reformulated and unrecognized growth substrates should be identified. A

large majority of methanogens may escape the isolation as they are not able to survive laboratory cultivation due to incomplete knowledge of their growth requirements.

Isolating novel rumen methanogens is an important task, as it would provide more insights into their properties. To develop effective methane mitigation techniques, characteristics of representative rumen methanogen species, including low abundance methanogens, should be known. A number of methanogen species have been identified in the rumen by non-culture molecular methods such as 16S rRNA and *mcrA* gene clone libraries (explained in detail in Section 1.5). These methanogens should be isolated for further studies.

1.5 Molecular ecology techniques used to identify methanogens

Methanogens are difficult organisms to cultivate. The use of molecular ecology techniques circumvents some of the limitations of cultivation approach. Most molecular techniques use nucleic acids extracted directly from the environment. Marker genes in this nucleic acid pool are used to survey the microbial diversity in the selected environment. The most frequently used marker gene is the gene encoding the 16S rRNA (small subunit rRNA) of prokaryotes. The 16S rRNA gene has functional regions that are highly conserved among all prokaryotes, as well as regions that are semi-conserved at the phylum level or are highly diverse among closely related genera (Ludwig & Schleifer, 1994; Woese 1987). Another gene that has been used as a marker for methanogens is the gene encoding methyl-coenzyme M reductase (*mcrA*) (Luton *et al.*, 2002). Methyl-coenzyme M reductase (MCR) is one of the key enzymes of methanogenesis, which catalyzes the final step in methanogenesis and is present in all methanogens (Friedrich, 2005). Recently, the *rpoB* gene, which encodes the β subunit of RNA polymerase, was used to study the abundance of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* in human stool samples (Dridi *et al.*, 2010).

The retrieval of 16S rRNA gene sequence information lays the background for the development of most of the molecular techniques. Some of these molecular methods that are involved in the analysis of the different aspects of the methanogen community in the rumen are 16S rRNA gene clone libraries (Table 1.2), fluorescence *in situ* hybridization (FISH) (Soliva *et al.*, 2004; Yanagita *et al.*, 2000), RNA-targeted DNA probes (Lin *et al.*, 1997; Sharp *et al.*, 1998), temporal temperature gradient gel

electrophoresis (TTGE)/denaturing gradient gel electrophoresis (DGGE) (Cheng *et al.*, 2009; Hook *et al.*, 2009; Nicholson *et al.*, 2007; Ohene-Adjei *et al.*, 2008; Ouwerkerk *et al.*, 2008) and quantitative real-time PCR (Hook *et al.*, 2009; Sundset *et al.*, 2009a; Williams *et al.*, 2009). Some of these, such as quantitative real-time PCR and clone library analysis have been performed using the *mcrA* gene as well (Tatsuoka *et al.*, 2004; Denman *et al.*, 2007). New high-throughput sequencing methodologies such as 454-pyrosequencing are becoming popular and are expected to increase the knowledge of the complex rumen ecosystem (Krober *et al.*, 2009; Zhang *et al.*, 2009). These different molecular techniques are described briefly in the following sections.

1.5.1 16S rRNA gene clone libraries

Clone libraries (16S rRNA gene) are prepared using DNA isolated from rumen samples using commercially available kits (Shin *et al.*, 2004), bead-beating techniques (Williams *et al.*, 2009), freezing-thawing methods (Tajima *et al.*, 2001b) and/or enzymatic lysis. To retrieve methanogen sequences for generation of 16S rRNA gene clone libraries, PCR amplification is performed with archaeal-specific primers. The PCR amplified 16S rRNA gene products are used to generate clone libraries by cloning into an appropriate vector followed by sequencing of the cloned fragments. Phylogenetic identities of these sequences are confirmed by comparative analysis with other sequences available in GenBank and other databases. The method has for the last 15 years been the most commonly used method to identify microbial diversity in an environment.

Despite the great utility of 16S rRNA gene based clone libraries, some limitations to the method have been documented. Microbial diversity obtained through this method is dependent on the efficiency of the previous steps such as sample handling methods, DNA extraction methods and the primers used. Also, for a reliable outcome, a large number of clones have to be sequenced, which is time consuming, tedious and expensive. Recently, new deep sequencing methods (pyrosequencing) have been developed which allows a large number of sequences to be collected efficiently and inexpensively (based on a per base basis) (Jäger *et al.*, 2009; Kim *et al.*, 2008; Krober *et al.*, 2009; Schluter *et al.*, 2008; Zhang *et al.*, 2009). This approach also eliminates the use of a cloning vector. For example, one run of the 454-Roche genomic sequencer

system has been reported to produce 400,000 sequences of 100-400 bp in length and the cost of pyrosequencing on a per-base basis is 30 times less than the conventional sequencing method (Hugenholtz & Tyson, 2008). However, the lengths of individual reads of DNA sequence obtained from this method are currently limited to 300-500 nucleotides, but are increasing rapidly.

1.5.2 *mcrA* gene clone libraries

Methyl-coenzyme M reductase (MCR) is almost unique to methanogens, with the only other organisms possessing it having been found to be marine anaerobic methane oxidisers (Hallam *et al.*, 2003). In methane oxidisers, MCR is thought to be involved in reverse methanogenesis (Hallam *et al.*, 2004; Kruger *et al.*, 2003). Among methanogens, two forms of MCR exist: MCR-I which is present in all methanogens, and its isoenzyme MCR-II, which is additionally present only in members of the order *Methanococcales* and *Methanobacteriales* (Lueders *et al.*, 2001). However, *Methanosphaera* spp., which belong to the order *Methanobacteriales*, only possess MCR-II and the gene responsible for this isoenzyme is termed *mrtA* (Fricke *et al.*, 2006).

Genes encoding the α subunit of MCR, *mcrA*, are evolutionarily highly conserved due to functional constraints on the catalytic activity of MCR (Hallam *et al.*, 2003). Thus, *mcrA* genes (gene responsible for MCR-I) can be used as a phylogenetic tool for the specific detection and the identification of methanogens, because the phylogeny of the *mcrA* and 16S rRNA genes from the recognized orders of methanogens have been clearly shown to be strongly similar (Luton *et al.*, 2002; Tatsuoka *et al.*, 2004). Although several studies have used *mcrA* gene sequences to analyse the diversity of the methanogenic community in rice field soil (Conrad *et al.*, 2006; Friedrich, 2005; Lueders *et al.*, 2001), there have been only a few studies published on rumen methanogens (Denman *et al.*, 2007; Tatsuoka *et al.*, 2004).

One potential problem in using the *mcrA* gene for detection of methanogens is the high degree of DNA sequence conservation between *mcrA* and *mrtA* genes. This means that detection based on primers for PCR designed for conserved region of *mcrA*, is likely to detect *mrtA* sequences as well (Luton *et al.*, 2002). However, careful analysis of phylogenetic relationships of *mcrA/mrtA* clones may help to avoid this problem. In

some cases it is important to amplify the *mrtA* genes. For example, as mentioned above, *Methanosphaera* spp. only possess the *mrtA* gene (Fricke *et al.*, 2006). Similar to 16S rRNA gene clone libraries, this method can also be affected by sample handling methods, DNA extraction methods and the primers used for the PCR.

1.5.3 DNA probe / fluorescence *in situ* hybridization (FISH)

DNA probe/FISH is often used for detection as well as quantifying the relative abundance of specific groups of microorganisms in environmental samples (Giovannoni *et al.*, 1996). The spatial distribution of methanogens in the rumen also can be identified by this technique (Lin *et al.*, 1997; Sharp *et al.*, 1998; Yanagita *et al.*, 2000). Probes are designed based on the 16S rRNA gene sequence information unique to the target group of organisms. These probes can be designed to target broad or narrow phylogenetic groups (Amann & Ludwig, 2000).

For quantitative studies using DNA probes, RNA or DNA is immobilized on membranes. Known amounts of standard are immobilized on the same membrane. The membrane is then hybridized with a particular oligonucleotide probe under optimized hybridization and post-hybridization conditions. The amount of probe bound to its target in the samples can be quantified by comparison to the standard (Amann & Ludwig, 2000). FISH detects microbial cells by using fluorescently-labelled probes that penetrate whole fixed cell membranes and hybridize specifically to intracellular rRNA within intact cells without altering their morphology. Bound probe can be directly visualized using epifluorescence microscopy, allowing identification of the cells that have bound the probe.

Probe permeability into the target cell is one of the important factors that determine the validity of the enumeration using FISH. Some researchers have reported difficulties with probe permeability when using the FISH technique on members of family *Methanobacteriaceae* (Sekiguchi *et al.*, 1999; Tokura *et al.*, 2000; Yanagita *et al.*, 2000). The distinctive cell walls of this family, containing pseudomurein, may be the reason for this probe impermeability. This problem can be solved to some extent by using the freeze-and-thaw method (Sekiguchi *et al.*, 1999), but this can damage the morphologies of the cells and is a laborious technique. Treatment with pseudomurein endoisopeptidase (Pei) was successfully used in FISH to clearly visualize the

methanogens from the family *Methanobacteriaceae* and found to be the most effective method for improving the permeability of the cells (Nakamura *et al.*, 2006). However, the optimum concentration of the Pei enzyme varies with the species and spatial distribution of the organism within the environment.

Other limitations associated with using rRNA targeted oligonucleotide probes for *in situ* studies are autofluorescence (methanogens) and low level of sensitivity as a result of starving or slow growing microbes that contain low ribosome content and so might not be detected (Amann *et al.*, 1995).

1.5.4 Denaturing gradient gel electrophoresis (DGGE)/Temperature gradient gel electrophoresis (TGGE)

Denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) has allowed the study of the structure and dynamics of microbial communities in environmental samples. This method examines microbial diversity based upon electrophoresis of PCR-amplified 16S rRNA fragments. One of the primers used in this PCR typically has a GC-rich clamp (40 bp) to prevent complete dissociation of the DNA under strong temperature or denaturing conditions (Muyzer *et al.*, 1993). When the PCR products migrate through the polyacrylamide gel containing a linear gradient of denaturant (a mixture of urea and formamide) or a linear temperature gradient, they change their migration speed in the gel based on their melting behaviour, causing them to separate based on their sequence composition. The DNA bands can then be visualized using a DNA stain. The banding pattern that emerges represents the diversity of the rRNA gene sequences present in that sample. The intensity of an individual band is a semi-quantitative measure for the relative abundance of the sequence in that particular sample. Since individual bands can be excised and sequenced, the identity of the methanogens present in the sample can be determined without cultivation. Theoretically, each band on the gel represents one type of sequence or one microbial species (Muyzer *et al.*, 1993). Commercially available software can be used to analyse the banding patterns of the gels (e.g the BioNumerics software package, Applied Maths, Sint-Martens-Latem, Belgium).

DGGE/TGGE techniques have been widely used to investigate bacterial diversity (Klieve *et al.*, 2007; Kocherginskaya *et al.*, 2001; Tajima *et al.*, 2001a) and archaeal

diversity in the rumen (Cheng *et al.*, 2009; Hook *et al.*, 2009; Nicholson *et al.*, 2007; Ohene-Adjei *et al.*, 2008; Ouwerkerk *et al.*, 2008; Yu *et al.*, 2008). These techniques are mostly used for comparison purposes and allowing the simultaneous analysis of multiple samples, making it possible to follow community changes over time and space.

There are some reported limitations using DGGE/TGGE. For example, DGGE banding patterns are dependent on the efficiency of the previous steps. Sample handling methods, DNA extraction methods and the PCR can affect the banding pattern of the DGGE gels (Wintzingerode *et al.*, 1997). There is also a limit to the length of fragments used in DGGE. The fragment length is often <500 bp, which limits the associated sequence information (Myers, 1985). An obvious limitation was observed in bacterial DGGE when samples from highly variable communities were used. DGGE will detect only the predominant species, as there are limitations to the number of different fragments visualised in the gel (Muyzer *et al.*, 1993). The other problems reported using the DGGE technique are co-migration of DNA fragments (Muyzer & Smalla, 1998) and complications resulting from the multiple rRNA operons in some organisms (Nübel *et al.*, 1996). Also, multiple bands for one sequence type was observed when degenerate primers were used (Kowalchuk *et al.*, 1997).

1.5.5 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) is a widely used technique for determining the abundance of microorganisms of interest in environmental samples. The principle of quantitative PCR is similar to conventional PCR. The difference is that in qPCR a fluorescent dye is used to measure relative fluorescence, which increases with each amplification and it is measured continuously. A detectable increase in fluorescence is obtained when the amplification becomes exponential and this cycle is assigned as the threshold cycle. This threshold cycle is compared with a standard curve made from a dilution series of samples containing known numbers of the target microorganisms or estimated copy numbers of the amplicon of interest, and then it is used to estimate the number of target organisms from the environmental samples (Denman & McSweeney, 2005).

There are a number of limitations in this method, which need to be taken to be eliminated or minimized. First, the results of qPCR can be affected by sample handling

and DNA extraction methods (Bergmann *et al.*, 2010). The DNA extracted from rumen samples can often contain PCR inhibitors (Krause *et al.*, 1999; 2001). These inhibitors can affect the efficiency of amplification. Therefore, PCR efficiency is estimated for each sample in this technique and only data from efficient reactions are used for the final calculations (Liu & Saint, 2002; Pfaffl, 2000; Tichopad *et al.*, 2003; 2004).

Selection of primers is also an important issue in qPCR. Primers should be specific to the target organisms to avoid overestimation of abundance by amplifying non-specific sequences. If primers are designed for a large group of microorganisms (e.g. total Archaea) care must be taken to cover all phylogenetic clades within that group. Also, the efficiency of amplification should be similar for all species within that group of microorganisms. The importance of primer design for studying the rumen has been highlighted in a study performed using archaeal primers (Skillman *et al.*, 2006).

Both *mcrA* and 16S rRNA genes have been used to quantify the number of methanogens in the rumen (Denman *et al.*, 2007; Hook *et al.*, 2009; Sundset *et al.*, 2009a; 2009b; Williams *et al.*, 2009). However, using the *mcrA* gene primers can give incorrect estimations of the methanogens, as these primers amplify both *mcrA* and *mrtA* genes (Luton *et al.*, 2002). Some methanogen groups possess both these genes, while others have only one (Lueders *et al.*, 2001). Furthermore, the *mcrA* gene primers are highly degenerate which reduce the efficiency of amplification and increase amplification of non-target genes (Luton *et al.*, 2002).

1.6 Cultured methanogens from ruminants

Culturable methanogen species typically represent only a small proportion of the total number of methanogen species that are present in most ecosystems. Culture-based techniques that have been used thus far to isolate, enumerate and characterize microbes in the rumen, account for only 10-20% of the rumen microbial population (McSweeney *et al.*, 2007). Using culture-based techniques, only 9 species of methanogens have been isolated thus far from the rumen. These are *Methanosarcina barkeri* (Beijer, 1952), *Methanobacterium formicicum* (Oppermann *et al.*, 1957), *Methanobrevibacter ruminantium* (Smith & Hungate, 1958), *Methanomicrobium mobile* (Paynter & Hungate, 1968), *Methanobacterium bryantii* (Joblin, 2005), *Methanoculleus olentangyi* (Joblin, 2005), *Methanobrevibacter smithii* (Joblin, 2005), *Methanobrevibacter millerae*

(Rea *et al.*, 2007) and *Methanobrevibacter olleyae* (Rea *et al.*, 2007). Among the above mentioned species, *Methanomicrobium mobile* and *Methanoculleus olentangyi* belong to the order *Methanomicrobiales* and *Methanosarcina barkeri* belongs to the order *Methanosarcinales*. All of the other 6 species belong to the order *Methanobacteriales*. A wider diversity of methanogens has been isolated from other gastrointestinal tract systems (Whitman *et al.*, 1991).

Isolation of the first pure culture of a ruminal methanogen, an acetate-utilizing *Methanosarcina* sp., was reported from the rumen of a fistulated goat in 1952 (Beijer, 1952). However, classification or characterization of that isolate has not been reported. Even though isolation of *Methanosarcina* spp. from the rumen has been reported in several studies (Genthner *et al.*, 1981; McInerney *et al.*, 1981; Patterson & Hespell, 1979) after the initial discovery, the first fully described pure culture of *Methanosarcina barkeri* from a ruminant was reported from goat faeces (Mukhopadhyay *et al.*, 1991). The same species was isolated later from the rumen of a grazing cow (Jarvis *et al.*, 2000).

The first fully described methanogen from the rumen was *Methanobrevibacter ruminantium*, isolated in 1958 from the bovine rumen (Smith & Hungate, 1958). It was initially named *Methanobacterium ruminantium*. Later studies showed several species of *Methanobrevibacter* in the rumen. The isolation of coenzyme M-producing and coenzyme M-requiring *Methanobrevibacter* species has been reported in two studies (Lovley *et al.*, 1984; Miller *et al.*, 1986b). However, in both studies no phylogenetic analyses were conducted to determine the taxonomic position of these organisms.

In 1968, *Methanomicrobium mobile* (originally named *Methanobacterium mobilis*) was isolated from the rumen of Holstein heifers (Paynter & Hungate, 1968). The name of this species was derived from the motile nature of the organism. A similar organism was later isolated from the rumen of a grazing cow (Jarvis *et al.*, 2000).

Recently two formate-utilizing *Methanobrevibacter* species were isolated from an ovine and a bovine rumen (Rea *et al.*, 2007). The 16S rRNA gene sequence analyses of these species revealed two novel species: *Methanobrevibacter olleyae* and *Methanobrevibacter millerae*, respectively. The isolation of *Methanobacterium formicicum* has been reported in two studies (Oppermann *et al.*, 1957; Jarvis *et al.*,

2000). The isolation of other methanogen species such as *Methanobacterium bryantii*, *Methanoculleus olentangyi* and *Methanobrevibacter smithii* were reported by Joblin (2005). However, detailed phylogenetic data and their characterization have not been published yet.

The above mentioned methanogens were isolated from the rumen of the ruminants except the first fully described *Methanosarcina barkeri*, which was isolated from goat faeces. Some of the methanogen species found in the rumen (Section 1.7) have been isolated from ruminant (cow and sheep) and various other animal's faeces (horse, goose, pig and rat) (Miller *et al.*, 1986a). Based on morphology, Gram reaction, physiological characteristics and DNA base ratios, these faecal methanogens belonged to *Methanobrevibacter* species (Miller *et al.*, 1986a). The phylogenetic relationships of these faecal methanogens was studied later using 16S rRNA gene sequence analysis and genomic DNA re-association and they were confirmed as *Methanobrevibacter* species (Lin & Miller, 1998). The detailed study and characterization of these isolates were reported in 2002 and three novel species were proposed (Miller & Lin, 2002): *Methanobrevibacter thaueri* from cow faeces, *Methanobrevibacter wolinii* from sheep faeces, and *Methanobrevibacter gottschalkii* from horse faeces.

A number of other methanogen species have been identified in the rumen by molecular ecology techniques (Section 1.7). However, isolation of these species has not been successful thus far. Furthermore, there are some unidentified archaeal groups in the rumen. The methanogen species which have been identified by molecular ecology techniques and the unidentified archaeal groups found in the rumen are discussed in the following sections.

1.7 Methanogens identified through molecular ecology techniques

Culture-independent molecular ecology techniques typically reveal greater methanogen diversity than culture-dependent methods. Molecular methods provide the opportunity to survey the full spectrum of microbial diversity of the selected environment, as in the absence of selective medium, culture-based enumeration of low abundance microbes is difficult (Sharp *et al.*, 1998). A more direct phylogenetic approach is based on the comparative analysis of 16S rRNA gene, and this approach has allowed researchers to study the diversity of methanogens fairly extensively. Most of

the published studies performed thus far on rumen methanogens have used 16S rRNA gene-based clone libraries.

A recent phylogenetic analysis performed using the 16S rRNA gene sequences from 14 studies has provided a global picture of rumen methanogen diversity (Janssen & Kirs, 2008). According to this analysis, the majority of the rumen methanogens are from three major groups: *Methanobrevibacter* (61.6%), *Methanomicrobium* (14.9%) and the uncultured archaea labelled as Rumen Cluster C or RCC (15.8%). Within the genus *Methanobrevibacter*, the sequences fall into two major clades: the *Methanobrevibacter gottschalkii* clade and the *Methanobrevibacter ruminantium* clade. The *Mbb. gottschalkii* clade comprises the larger part of the *Methanobrevibacter*-related sequences (33.6% of rumen archaea), and includes the species *Mbb. gottschalkii*, *Mbb. thaueri* and *Mbb. millerae*. The *Mbb. ruminantium* clade contains the species *Mbb. ruminantium* and *Mbb. olleyae* and contributes 27.3% of the total rumen archaea (Janssen & Kirs, 2008). Data from only 14 studies were used in this analysis. There have been several studies performed after this global analysis, but these new data seem only to confirm this global picture. Studies using 16S rRNA libraries to survey rumen archaeal communities and the archaeal groups identified are summarized in Table 1.2.

16S rRNA gene clone libraries constructed from rumen samples cover a number of ruminant species fed different diets and in different countries used different DNA extraction methods and PCR primers (Table 1.2). The above mentioned factors influence the interpretation of the resulting clone libraries. As such, the apparent methanogen diversity differences among these studies may have been due to diet or host species variation or it may simply have been due to methodological differences such as DNA extraction methods and the primers used.

Most of the published 16S rRNA clone libraries have been constructed using rumen samples obtained from cows and sheep. There have been two studies on reindeer in Norway (Sundset *et al.*, 2009a; 2009b) and one study in buffaloes in India (Chaudhary & Sirohi, 2009). There has also been a study conducted in foregut samples collected from wallabies (Evans *et al.*, 2009). Wallabies cannot be considered as ruminants, but their foregut is functionally similar to the rumen. The diet used in many of these studies was based on dried mixed rations and there have been few studies on grazing animals. A wide range of primers have been used to construct these 16S rRNA

gene clone libraries. The most widely used primers were Met86f and Met1340r (Wright & Pimm, 2003). Analysis has shown this primer pair to have a very low-level of mismatches against methanogens (Skillman *et al.*, 2006).

Most of the studies have shown that *Methanobrevibacter* spp. are the dominant methanogens, regardless of host species, diet or geographical differences (Table 1.2). The proportion of the two major clades of the *Methanobrevibacter* spp. (clade *Mbb. gottschalkii* and clade *Mbb. ruminantium*), however, varied greatly in each study. For examples, three clone libraries (Tajima *et al.*, 2001b; Shin *et al.*, 2004; Skillman *et al.*, 2006) did not detect any *Methanobrevibacter*-related sequences in their clone libraries. It was later reported that the primers used in these studies have mismatches against *Methanobrevibacter* species (Skillman *et al.*, 2006).

Methanogens from the family *Methanomicrobiaceae* have been identified as the dominant methanogens in some 16S rRNA gene clone libraries (Table 1.2). The FISH technique was used to enumerate *Methanomicrobium mobile* in the rumen of the sheep revealed that this species contributes 54% of the total methanogens (Yanagita *et al.*, 2000). In another comparative study among steers, cows and sheep, members from the family *Methanomicrobiaceae* were the dominant methanogens in the sheep rumen (Lin *et al.*, 1997). Unlike the other methanogen groups, the presence of *Methanomicrobium* spp. in the different 16S rRNA gene clone libraries has varied substantially from dominant to none (Table 1.2). Most of the recent studies performed using rumen samples from different ruminants have failed to identify this group of methanogens at a dominant level. One exception is a study which was performed on rumen samples from buffalo, which indicated this species to be the dominant methanogens (Chaudhary & Sirohi, 2009).

Another commonly occurring methanogen group in the rumen are *Methanosphaera* spp. This was first reported in cows fed mixed silage and concentrate in Canada (Whitford *et al.*, 2001). Later, methanogens belonging to this species were found in sheep in Western Australia (Wright *et al.*, 2004b), cows in New Zealand (Skillman *et al.*, 2006), Canada (Wright *et al.*, 2007; Hook *et al.*, 2009; Zhou *et al.*, 2009) and China (Pei *et al.*, 2008; 2009), reindeer in Norway (Sundset *et al.*, 2009) and also in the Australian Tammar wallaby foregut (Evans *et al.*, 2009). The presence of this species in a number of clone libraries suggests that they are common inhabitants of the

rumen, yet thus far there have been no reports of isolation of any pure cultures of this group from the rumen. Phylogenetic studies have suggested that there may be more than one species of *Methanosphaera* in the rumen (Whitford *et al.*, 2001; Skillman *et al.*, 2006). *Methanosphaera* species are incapable of growth solely from CO₂ and H₂. They probably use H₂ plus the methanol released during the fermentation of pectin substrates as their primary energy source for growth (Fricke *et al.*, 2006). This feature (methanol utilization), however, may not be true for the rumen strains. As such, isolation of pure cultures from the rumen will help to define the physiological and genetic traits of this group.

In addition to the above-mentioned common rumen methanogens, there are some groups of methanogens that have only been found in a few studies and at low abundance. For example, *Methanimicrococcus* spp. have been identified in a few studies (Sundset *et al.*, 2009a; 2009b; Tajima *et al.*, 2001b; Whitford *et al.*, 2001; Williams *et al.*, 2009; Wright *et al.*, 2007). Similarly, despite several pure cultures being available from the rumen (Oppermann *et al.*, 1957; Joblin, 2005), *Methanobacterium* spp., has only been found in a few clone libraries with very low abundance (Pei *et al.*, 2008; Shin *et al.*, 2004). The genus *Methanosarcina* includes versatile methanogens that can utilize a wide range of substrates. Interestingly, however, the presence of this group in the rumen has not been observed in any of the published clone libraries thus far. Pure cultures of this group have been isolated from the rumen of both goats and cow (Beijer, 1952; Jarvis *et al.*, 2000). *Methanosarcina* spp. grow relatively slower than other methanogens (Janssen & Kirs, 2008). The relatively high flow rate through the rumen may be one of the reasons for the absence of this species in the rumen (Liu & Whitman, 2008).

The above mentioned minor groups of methanogens cannot be neglected in the effort to reduce methane emissions from ruminants. If vaccination and metabolic inhibitors target only dominant rumen methanogens, the minor groups of methanogens may occupy the vacated niches. Any methane mitigation techniques targeting rumen methanogens should include these groups as well.

1.8 Unidentified archaea in the rumen

An important group of archaea found in considerable numbers in the rumen is the unidentified group, Rumen Cluster C or RCC (Janssen & Kirs, 2008). The term Rumen Cluster C was introduced for this group of archaeal 16S rRNA gene sequences by Janssen & Kirs (2008). This name was derived based on the phylogenetic position of these rumen-derived sequences within Rice Cluster C as defined by Kemnitz *et al.*, (2005). The physiology of this group has not yet been demonstrated. In some published studies this group was the dominant group of total methanogens (Sundset *et al.*, 2009b; Tajima *et al.*, 2001b; Wright *et al.*, 2006; 2007). This group has no closely related cultured isolates and is only distantly related to aerobic thermoacidophilic archaea. The closest cultured relatives of this group are *Thermoplasma acidophilum* and *Picrophilus oshimae* (Kemnitz *et al.*, 2005), with physiologies unsuited to the rumen. Members of RCC must have different physiologies.

The RCC group has been found in most of the clone libraries prepared thus far using different primers and regardless of host species, diet given or geographical region (Table 1.2). A recent study conducted in Australia using grazing sheep revealed the largest group of RCC sequences ever identified (80% of the total clones analysed) from the rumen (Wright *et al.*, 2006). Later, this group was found to be the dominant group in cows fed with a potato-based finishing diet (50% of the total clones analysed) in Canada (Wright *et al.*, 2007) and 48.5% in grazing reindeer in Norway (Sundset *et al.*, 2009). In the recent study on the Tammar wallaby, the RCC group dominated the foregut sample (91.7%) collected during summer (Evans *et al.*, 2009). Also, the RCC group has been suggested to be the dominant group associated with protozoa (Ohene-Adjei *et al.*, 2007), as 73% of the total sequences which were associated with protozoa were affiliated with the RCC group. However, only selected protozoa were used in this latter study, and the abundance of the individual protozoa species and the number of archaeal cells in each protozoon was not taken into account.

Sequences from the RCC group display a wide variation that can be classified as a number of distinct species and perhaps different genera (Janssen & Kirs, 2008). The physiology of the RCC group is not yet known. However, some studies suggest that they may contain the *mcrA* gene and that they may be methanogens (Denman *et al.*, 2007; Evans *et al.*, 2009). No studies have confirmed these suggestions. Since the RCC

group has been found in different ruminant species fed different diets and from different geographical locations, there is a need to know more about this group.

Two other groups of unidentified Archaea that have been reported in clone libraries prepared from rumen samples were the Qld26 group and the rumen Crenarchaeota group. The physiologies of these groups are not yet known. The close association of the Qld26 group with *Methanobrevibacter* spp., *Methanobacterium* spp., and *Methanosphaera* spp. suggests that they are likely to be hydrogen-utilizing methanogens (Janssen & Kirs, 2008). Only two studies have reported the sequences of Crenarchaeota (Shin *et al.*, 2004; Skillman *et al.*, 2006) in their clone libraries. Both of these studies used the same primers for their clone library construction. Detection of these groups in more studies would help substantiate whether they are transients or not.

Table 1.2. Details (animals, diets, DNA extraction methods, archaeal primers and identified archaea) of 16S rRNA gene clone library-based surveys of archaea in rumen samples. In each clone library the dominant archaeal group identified is marked with asterisk (*).

Mbb. ruminantium and *Mbb. gottschalkii* refer to the wider clade grouping (Section 1.7). Primer sequences are given in Table 1.3.

Animal and Country	Diet	DNA extraction method	Primers	Methanogen groups	Reference
Sheep Japan	Mixed ration (hay and commercial formula)	FastRNA kit and bead-beating method	Ar1000F & Ar1500r	<i>Mbb. gottschalkii</i> * <i>Mbb. ruminantium</i> <i>Methanomicrobium</i> spp.	Yanagita <i>et al.</i> , 2000
Cow Canada	Mixed ration (hay, haylage, silage and concentrate)	Bead-beating method	1Af & 1100Ar	<i>Mbb. ruminantium</i> * <i>Methanosphaera</i> spp. <i>Methanomicrococcus</i> spp.	Whitford <i>et al.</i> , 2001
Cow Japan	Mixed ration (hay and concentrate)	Combination of freeze-thawing and phenol-chloroform method	D30 & D33	RCC* <i>Methanomicrobium</i> spp.	Tajima <i>et al.</i> , 2001b
Cow Japan	Mixed ration (hay and concentrate)	Combination of freeze-thawing and phenol-chloroform method	0025ef & 1492r	<i>Methanomicrobium</i> spp.* RCC <i>Mbb. ruminantium</i> <i>Mbb. gottschalkii</i> <i>Methanomicrococcus</i> spp.	Tajima <i>et al.</i> , 2001b
Sheep Australia	Grazing	Cetyltrimethylammonium bromide protocol	Met86f & Met1340r	<i>Mbb. gottschalkii</i> * <i>Mbb. ruminantium</i> <i>Methanosphaera</i> spp. RCC	Wright <i>et al.</i> , 2004b
Sheep Australia	Formulated feed (oaten hay, grain, barley, etc.)	Cetyltrimethylammonium bromide protocol	Met86f & Met1340r	<i>Mbb. gottschalkii</i> * <i>Mbb. ruminantium</i> <i>Methanosphaera</i> spp.	Wright <i>et al.</i> , 2004b
Sheep Australia	Pellets (lucerne hay, grain, molasses)	Cetyltrimethylammonium bromide protocol	Met86f & Met1340r	<i>Mbb. gottschalkii</i> * <i>Mbb. ruminantium</i>	Wright <i>et al.</i> , 2004b
Cow Korea	Mixed ration (rice hull: concentrate 4:1)	G-spin TM Genomic DNA extraction kit (iNtRON Biotechnology, Korea)	21f & 958r	<i>Methanomicrobium</i> spp.* Qld26 group <i>Methanobacterium</i> spp. Crenarchaeota	Shin <i>et al.</i> , 2004

Animal and Country	Diet	DNA extraction method	Primers	Methanogen groups	Reference
Sheep Australia	Rhodes grass hay	Cetyltrimethylammonium bromide protocol	Met86F & Met1340R	RCC* <i>Mbb. gottschalkii</i> <i>Methanomicrobium</i> spp. Qld26 group	Wright <i>et al.</i> , 2006
Cow New Zealand	Grazing-ryegrass/clover	Phenol-chloroform method combined with mechanical disruption (with glass beads)	Arch364f & Arch1386r	<i>Mbb. ruminantium</i> * <i>Mbb. gottschalkii</i> <i>Methanosphaera</i> spp.	Skillman <i>et al.</i> , 2006
Cow New Zealand	Grazing-ryegrass/clover	Phenol-chloroform method combined with mechanical disruption (with glass beads)	21f & 958r	<i>Methanosphaera</i> spp.* Crenarchaeota	Skillman <i>et al.</i> , 2006
Cow Canada	Corn-based diet	Glass milk protocol	Met86F & 1340R	<i>Mbb. ruminantium</i> * RCC <i>Mbb. gottschalkii</i> <i>Methanosphaera</i> spp. <i>Methanomicrococcus</i> spp.	Wright <i>et al.</i> , 2007
Cow Canada	Potato by-products finishing diet	Glass milk protocol	Met86F & 1340R	RCC * <i>Mbb. gottschalkii</i> <i>Mbb. ruminantium</i>	Wright <i>et al.</i> , 2007
Sheep Venezuela	Mixed ration (alfalfa pellets, Bermuda hay)	Qiagen DNeasy Plant kit	Met86F & Met1340R	<i>Mbb. gottschalkii</i> * <i>Mbb. ruminantium</i> Qld26 group	Wright <i>et al.</i> , 2008
Jinnan Cattle China	Not mentioned	Phenol-chloroform/bead beating method	Archf364 & 1386r	<i>Methanobrevibacter</i> *	Pei <i>et al.</i> , 2008
Jinnan Cattle China	Not mentioned	Phenol-chloroform/bead beating method	1Af & 1100Ar	<i>Methanobacterium</i> spp. <i>Methanosphaera</i> spp.	Pei <i>et al.</i> , 2008
Jinnan Cattle China	Not mentioned	Phenol-chloroform/bead beating method	Met86F & Met1340R	<i>Methanobrevibacter</i> * <i>Methanomicrobium</i> spp. <i>Methanosphaera</i> spp. <i>Methanobacterium</i> spp. RCC	Pei <i>et al.</i> , 2008

Animal and Country	Diet	DNA extraction method	Primers	Methanogen groups	Reference
Reindeer Norway	Summer pasture	Glass milk protocol	Met86F & Met1340R	<i>Mbb. gottschalkii</i> * <i>Mbb. ruminantium</i> <i>Methanimicrococcus</i> spp. RCC	Sundset <i>et al.</i> , 2009a
Cow Canada	Mixed ration (60:40 forage to concentrate ration and soyhulls)	Glass milk protocol	Met86F & Met1340R	<i>Mbb. gottschalkii</i> * <i>Mbb. ruminantium</i> <i>Methanosphaera</i> spp.	Hook <i>et al.</i> , 2009
Buffalo India	Mixed ration (60:40 forage to concentrate ration)	DNA isolation kit (Chromas Biotech Pvt. Ltd., India)	Met86F & Met1340R	<i>Methanomicrobium</i> spp.* RCC	Chaudhary & Sirohi, 2009
Sheep Australia	Oaten hay-based ration	Phenol-chloroform/bead beating method	Met86F & Met1340R	<i>Mbb. gottschalkii</i> * RCC <i>Mbb. ruminantium</i> <i>Methanomicrobium</i> spp. <i>Methanosphaera</i> spp. <i>Methanimicrococcus</i> spp.	Williams <i>et al.</i> , 2009
Reindeer Norway	Late autumn pasture and late winter pasture	Power Soil™ DNA isolation Kit	Met86F & Met1340R	RCC* <i>Mbb. ruminantium</i> <i>Mbb. gottschalkii</i> <i>Methanosphaera</i> spp. <i>Methanomicrobium</i> spp. <i>Methanimicrococcus</i> spp.	Sundset <i>et al.</i> , 2009b
Jinnan Cattle China	Corn meal, cottonseed meal, whole corn stalk and wheat stalk	Bead-beating method, on rumen fluid only	Met86F & Met1340R	<i>Methanobrevibacter</i> * <i>Methanobacterium</i> spp. <i>Methanosphaera</i> spp. <i>Methanomicrobium</i> spp. RCC	Pei <i>et al.</i> , 2010
Jinnan Cattle China	Corn meal, cottonseed meal, whole corn stalk and wheat stalk	Bead-beating method, on rumen solid only	Met86F & Met1340R	<i>Methanobrevibacter</i> * <i>Methanobacterium</i> spp. <i>Methanosphaera</i> spp. RCC	Pei <i>et al.</i> , 2010

Animal and Country	Diet	DNA extraction method	Primers	Methanogen groups	Reference
Jinnan Cattle China	Corn meal, cottonseed meal, whole corn stalk and wheat stalk	Bead-beating method, On rumen epithelium samples	Met86F & Met1340R	<i>Methanobrevibacter</i> * <i>Methanobacterium</i> spp. <i>Methanosphaera</i> spp. RCC	Pei <i>et al.</i> , 2010
Cow Canada	Mixed ration (74% oats, 20% hay, 6% feedlot supplement)	Bead-beating method	Met86F & Met915R	<i>Mbb. ruminantium</i> * <i>Mbb. gottschalkii</i> <i>Methanosphaera</i> spp.	Zhou <i>et al.</i> , 2010

Table 1.3. Details of the archaeal 16S rRNA gene primers used to construct clone libraries from rumen samples in the studies reported in Table 1.2.

Primer	Sequence (5' to 3')
Ar1000F	AGTCAGGCAACGAGCGAGA
Ar1500r	GGTTACCTTGTTACGACTT
1Af	TCYGKTTGATCCYGSCRAG
1100Ar	TGGGTCTCGCTCGTTG
D30	ATTCCGGTTGATCCTGC
D33	TCGCGCCTGCGCCCCGT
0025ef	CTGGTTGATCCTGCCAG
1492r	GGTTACCTTGTTACGACTT
Met86f	GCTCAGTAACAC GTGG
Met1340r	CGGTGTGTGCAAGGAG
21f	TTCCGGTTGATCCYGCCG GA
958r	YCCGGCGTTGAMTCCAATT
Met915R	GTGCTCCCCCGCCAATTCCT

1.9 Interactions between methanogens and other rumen microbes

The rumen harbours a large number of different species of bacteria, archaea, protozoa and fungi. Interactions between the different groups are essential to sustain their populations and activity in the rumen. However, these interactions are not fully understood. In the effort to reduce methane emissions from ruminants, an understanding of the different interactions among rumen microbes will be useful. There are no reports on methanogen-methanogen interactions in the rumen yet. Methanogen genomes, including *Mbb. ruminantium*, have phage genes, suggesting that methanogen phage may represent a mechanism for affecting rumen methanogen ecology (Leahy *et al.*, 2010). The interactions between methanogens and bacteria have been described, with the collaboration between H₂-producing bacteria and methanogens being called “interspecies hydrogen transfer” (Wolin *et al.*, 1997). However, there may be a competitive relationship between methanogens and hydrogen-utilizing homoacetogenic bacteria which are not prominent in rumen (Le Van *et al.*, 1998). Methanogens and fungi have also been reported to show an “interspecies hydrogen transfer” collaboration (Orpin, 1975). The interactions between methanogens and protozoa have been reported to be synergistic (Williams & Coleman, 1997).

1.9.1 Bacterial-methanogen interactions

The rumen contains H₂-producing and H₂-utilizing bacteria. Cellulolytic and fermentative bacteria are the main H₂-producing bacteria and these are found in high numbers (Wolin *et al.*, 1997). Much of the primary attack on the feed ingested by the ruminants is mediated by bacteria. For the continued efficient fermentation process by bacteria, the H₂ released as a by-product of feed fermentation should be continually removed from the rumen. Methanogens living in the rumen perform this function by producing methane from this released H₂. This phenomenon is called as “interspecies H₂ transfer”. This relationship between H₂-producing bacteria and H₂-utilizing methanogens is one of the most prominent interactions in the rumen. Whilst this relationship might appear to be well-characterized, the number and variety of bacteria thought to participate in them continues to grow (Krause & Russell, 1996). Studies performed on bacterial 16S rRNA genes in the rumen of different ruminants has revealed a vast diversity of bacterial genera and species that have not been characterized, largely because there are no cultured representatives (Brulc *et al.*, 2009; Edwards *et al.*, 2004; Firkins & Yu, 2006; Kocherginskaya *et al.*, 2001; Koike *et al.*, 2003; Tajima *et al.*, 2007).

Increasing concern about global warming has stimulated the search for non-methanogenic sinks for the H₂ produced during the fermentation process. It may be possible to use H₂-utilizing bacteria as an alternative to methanogens to reduce methane emission from the rumen. Sulfate reducing bacteria (SRB), homoacetogenic bacteria (homoacetogens) and fumarate reducers are the main H₂-utilizing bacteria in the rumen. Homoacetogens use H₂ to reduce CO₂ to form acetate, SRB reduce sulphate to sulphide (Morvan *et al.*, 1996) and fumarate reducers reduce fumarate to succinate (Asanuma & Hino, 2000). However, in the rumen, these H₂-utilizing bacteria have to compete with methanogens for H₂, and methanogenesis appears to be the dominant mechanism in the rumen microbial ecosystem (Chaban *et al.*, 2006). One potential way to reduce methanogenesis is to promote the activity of H₂-utilizing bacteria in the rumen.

Acetate formation (acetogenesis) is a competitive pathway to methane production but current data suggests that it is insignificant in the rumen (Fonty *et al.*, 2007; Le Van

et al., 1998). Conversion of H₂ and CO₂ into methane is thermodynamically more favourable and yields more energy than conversion of H₂ and CO₂ into acetate (Le Van *et al.*, 1998; Nollet *et al.*, 1997a). However, the loss of feed energy and emission of methane to the atmosphere by the production of methane could be minimized, if H₂/CO₂ is used to form acetate (2CO₂ + 4H₂ → CH₃COOH + 2H₂O) instead of CH₄. This acetogenesis could be beneficial to ruminants because it yields acetate, a nutrient for ruminants rather than CH₄ (Joblin, 1999).

Homoacetogens have been found in ruminants from undetectable to 1.2×10^9 per gram of rumen contents and are established in the rumen before the methanogens (Doré *et al.*, 1995; Leedle & Greening, 1988; Le Van *et al.*, 1998). They are among the first species to colonise the rumens of lambs and are quite abundant in newborn lambs (Morvan *et al.*, 1996). The colonization and establishment of homoacetogens has been reported to be independent of other microbes, but when methanogens appeared, the homoacetogenic bacterial population decreased rapidly to below the detection threshold (Morvan *et al.*, 1994). The correlation between numbers of homoacetogenic bacteria and methanogens is negative (Doré *et al.*, 1995). Homoacetogens are outcompeted by methanogens as acetate formation yield less energy from oxidation of H₂ compared to CH₄ formation. In addition, they have a lower affinity and higher threshold level for H₂ the substrate (Cord-Ruwisch *et al.*, 1988; Le Van *et al.*, 1998; Weimer, 1998).

Rumen homoacetogen populations can be affected by diet, age of the animal and time of sampling (Doré *et al.*, 1995; Leedle & Greening, 1988; Le Van *et al.*, 1998). *In vitro* studies have shown that homoacetogenesis is stimulated after selective inhibition of methanogens (Le Van *et al.*, 1998; Nollet *et al.*, 1998) and later it was shown that ruminal methanogens are not essential for effective fermentation in the rumen as they can be replaced by homoacetogens (Fonty *et al.*, 2007). The use of yeasts as ruminal feed additives (live or dead) stimulated homoacetogenic species even in the presence of methanogens *in vitro* (Chaucheyras *et al.*, 1995). However, many attempts to increase the reductive acetogenesis process by increasing the number of homoacetogens or addition of non-rumen isolates have not been successful yet in *in vitro* or *in vivo* trials (Demeyer *et al.*, 1996; Le Van *et al.*, 1998; Lopez *et al.*, 1999; Nollet *et al.*, 1997a;1998). They may still fulfil the role of H₂ utilization if methanogens are

successfully inhibited, and the rumen does contain many potential homoacetogens (Henderson *et al.*, 2010).

Another H₂-utilizing bacterial group is the fumarate reducers. *In vitro* studies have shown that addition of sodium fumarate can decrease methane production (Asanuma *et al.*, 1999; Lopez *et al.*, 1999). According to those studies, the bacteria such as *Fibrobacter succinogens*, *Selenomonas ruminantium* ssp. *ruminantium*, *Selenomonas ruminantium* ssp. *lactilytica*, *Veillonella parvula* and *Wollinella succinogenes* oxidize H₂ by using fumarate as a final electron acceptor. This suggests that these bacteria could compete with methanogens for H₂. However, the affinity of these bacteria to H₂ was lower than it was for methanogens (Asanuma *et al.*, 1999). Succinate, the product of fumarate reduction, is decarboxylated to propionate, a valuable animal nutrient (Wolin *et al.*, 1997). Interestingly, higher than the expected quantities of propionate were observed when fumarate was added to the rumen of lambs (Fonty *et al.*, 2007). This suggested that fumarate metabolism in the rumen does not necessarily involve consumption of hydrogen gas but that it may simply be fermented. More studies are needed to check the potential to use of this group of bacteria as H₂-consumers.

Competitive and cooperative relationships between methanogens and sulfate reducing bacteria (SRB) have been found in anaerobic environments. In anaerobic environments, in which sulfate is not limiting, SRB generally compete with methanogens for common substrates (e.g H₂, formate and acetate). A cooperative relationship between methanogens and SRB can exist and is another example of an 'interspecies hydrogen transfer', since some SRB are capable of growth in the absence of sulfate and produce H₂. The rumen contains SRB and they were identified in the rumen by the third day after birth (Morvan *et al.*, 1994). No apparent competition was observed between SRB and methanogens. Sulfate cannot be added at a significant level in the rumen as an alternative to ruminal methanogenesis, due to the toxicity of sulphides for other ruminal micro-organisms and for the host animal (Morvan *et al.*, 1996).

1.9.2 Protozoal-methanogen interactions

Rumen protozoa represent a substantial part of the rumen microbial population, contributing up to 50% of the total microbial biomass (Williams & Coleman, 1997).

The ciliate protozoa in the rumen have been classified into two groups depending on their morphological characteristics: holotrich and entodiniomorphid protozoa (Williams & Coleman, 1997). One study suggests that protozoa are only found in rumen fluid and associated with rumen solids and not with the rumen epithelium (Shin *et al.*, 2004). Methanogens have a synergistic relationship with protozoa. Hydrogen production by rumen protozoa via fermentation provides a substrate for methanogens, and the protozoa benefit from hydrogen removal, since hydrogen is inhibitory to their metabolism (Sharp *et al.*, 1998). In addition, protozoa can also provide some advantage to methanogens by quenching oxygen through their oxygen-tolerant hydrogenosomes, or acting as a vehicle for retention of slower-growing methanogens in the rumen (Williams & Coleman, 1997). A 10-fold increase in the apparent oxygen tolerance of methanogenesis by *Methanosarcina barkeri* has been reported when rumen holotrich protozoa were present at the stated densities (Hillman *et al.*, 1988).

Methanogens form exo- (Tokura *et al.*, 1997; Vogels *et al.*, 1980) and endo- (Finlay *et al.*, 1994) symbiotic relationships with protozoa. It has been estimated that the proportion of methanogens in methanogen-containing protozoa is 1-2% of the total protozoan volume (Finlay *et al.*, 1994; Sharp *et al.*, 1998). Almost all rumen ciliate species support some methanogens on their external surface or internal organelles (Finlay *et al.*, 1994; Tokura *et al.*, 1999). Also, the numbers of methanogens attached per protozoa may vary depending on the species of protozoa (Tokura *et al.*, 1999). Physiologically, free-living and ciliate-associated methanogens display differences (Tokura *et al.*, 1997). In addition, among ciliate associated methanogens, endo- and exo-symbiotic methanogens may also differ. However, additional studies are needed to substantiate these statements.

The number of methanogens attached per protozoa can be affected by the diet and the time after feeding (Tokura *et al.*, 1997; Vogels *et al.*, 1980). It has been observed that the number of methanogens associated with ciliates increased after feeding and then declined to the initial level (Tokura *et al.*, 1997). The change occurred mainly due to the change in extra-cellularly associated methanogens. Endo-symbiotic methanogens were not affected by feeding. By the most probable number (MPN) technique, it was estimated that 10^3 - 10^4 methanogens were associated per rumen ciliate before feeding, with this decreasing to 1-10 methanogens after feeding (Tokura *et al.*, 1997). This may

be due to the number of methanogens attached to the outside of protozoal cells being modulated by the hydrogen partial pressure of the surrounding microenvironment (Stumm *et al.*, 1982). Also, methanogens associated with ciliates before and after feeding have different preferences for electron donors (Tokura *et al.*, 1997).

Colonization of methanogens on or in ciliates might also depend on the species of ciliate and methanogens involved (Chagan *et al.*, 1999). For example, the physical structure of the ciliates may affect the attachment of methanogens. Fine body striation is correlated with low frequency of association and coarse surface structure is correlated with a high frequency of association between ciliates and methanogens (Vogels *et al.*, 1980). Furthermore, creases and folds in the ciliate pellicle are locations frequently colonized by methanogens. Interestingly, the apparent rates of methane production of ruminal ciliates associated with methanogens were not uniform throughout the ciliate species tested by Ushida and Jouany (1996).

The majority of studies have reported methanogens from the family *Methanobacteriaceae* as the dominant methanogens associated with protozoa in the rumen (Chagan *et al.*, 1999; Irbis & Ushida, 2004; Sharp *et al.*, 1998; Tokura *et al.*, 1999). However, *Methanomicrobium* spp. (Regensbogenova *et al.*, 2004) and archaea of the RCC group (Ohene-Adjei *et al.*, 2007) have in some cases been the dominant protozoa-associated archaea. A recent review (Janssen & Kirs, 2008) noted that all of these studies were strongly biased as they only used selected protozoa, without considering the total protozoan population and different species of protozoa present in rumen samples.

There are few studies concerning endo-symbiotic methanogens in rumen ciliates, which were first reported in 1994 (Finlay *et al.*, 1994). On average, the most abundant ciliates *Entodinuim spp.* had 96 methanogens and *Dasytricha ruminantium* had 520 methanogens per ciliate (endosymbiotically). Endo-symbiotic methanogens have been estimated to generate 37% of rumen methane emissions (Finlay *et al.*, 1994). In some of the later studies, endo-symbiotic methanogens were termed as “ciliate-associated methanogens”, because methanogens may be engulfed by ciliates possibly originating from free-living methanogens (Irbis & Ushida, 2004).

1.9.3 Fungal-methanogen interactions

Rumen fungi are responsible for the degradation of ingested plant biomass together with rumen bacteria and protozoa and are thought to be one of the primary colonizers of plant material in the rumen. Therefore, the collaboration between H₂-producing fungi and methanogens represents another example of interspecies hydrogen transfer. There have been several studies investigating the interactions between rumen fungi and methanogens (Marvin-Sikkema *et al.*, 1993; Mountfort *et al.*, 1982; Stewart & Richardson, 1989). Rumens of lambs were colonized by substantial numbers of anaerobic fungi within the first two weeks of life, even when the lambs were separated from other sheep soon after birth (Fonty *et al.*, 1987).

The ability of co-cultures of methanogenic archaea with anaerobic fungi to enhance the fibre digesting capabilities of the fungi has been well documented (Teunissen *et al.*, 1992). Cellulose degradation by fungi with a methanogen in co-culture was rapid and greater than the rates obtained for H₂-utilizing bacterial-fungi co-cultures (Mountfort *et al.*, 1982). It has been shown that interspecies H₂ transfer between the cellulolytic H₂-producing anaerobic fungus *Neocallimastix frontalis* and methanogens resulted in increased CO₂ and acetate formation and decreased ethanol and lactate production. Methanogens maintain low H₂ levels in these co-cultures. As such, the production of H₂ is facilitated. This causes a shift in the products away from lactate, succinate and ethanol (Bauchop & Mountfort, 1981; Mountfort *et al.*, 1982). In the presence of *Methanobacterium bryantii* or *Methanobrevibacter smithii*, a 15-25% increase in the rate of cellulose degradation was observed compared with a pure culture of fungi (Marvin-Sikkema *et al.*, 1990). In addition, in the same study, when methanogens were used as the H₂-consuming organisms in the co-culture, a shift in fermentation pattern to more acetate and fewer other products was found. Most of the above studies were done *in vitro*. More *in vivo* studies are warranted in this topic.

1.10 Methods for controlling rumen methanogenesis

Methods for reducing methane emissions from ruminant livestock include using homoacetogenesis as an alternative pathway to methanogenesis (Denman *et al.*, 2007);

defaunation techniques to remove ciliate protozoan (Krumholz *et al.*, 1983; Stumm *et al.*, 1982); addition of alternative electron acceptors (Callaway *et al.*, 1997; Kalmokoff *et al.*, 1996; Sauer *et al.*, 1998); using vaccines or small-molecule inhibitors against methanogens (Wedlock *et al.*, 2010; Williams *et al.*, 2009; Wright *et al.*, 2004a); using plant extracts (Bodas *et al.*, 2008; Busquet *et al.*, 2005); and animal breeding (Chagunda *et al.*, 2009). However, most of these techniques have failed, or met with only limited success (McAllister & Newbold, 2008). Furthermore, most of these techniques are only suitable for housed ruminant animals, and currently there are no effective technologies for pasture grazed animals which is the main feeding system in New Zealand. Those are suitable for grazing ruminants, such as vaccines or inhibitors dosed from slow-release intra-ruminal devices, have not been successfully developed for on farm use. The following describes some of the possible strategies that have been investigated to reduce methane emissions from grazing ruminants.

1.10.1 Increasing productivity

This is possibly one of the most promising and cost-effective way to reduce methane emissions from ruminants. This can be achieved by improving feed quality, livestock management and genetic potential of ruminants. This approach helps to reduce the number of animals and reduce methane emission per unit of animal products, effectively increasing productivity per unit of methane emitted.

It is well established that increasing the level of concentrate in the diet leads to a reduction in methane emissions as a proportion of energy intake or by unit of animal product (Sauvant & Giger-Reverdin, 2007). However, feeding a high-concentrate diet is impractical in New Zealand. Also, feeding more concentrate might not be a good option overall to reduce atmospheric climate change as producing more concentrate feed may require higher fossil fuel use and is competitive with human food production. Therefore, increasing the contents of soluble sugars in forage plants could be an alternative approach. This is associated with an increase in feed intake, higher rates of ruminal fermentation and accelerated feed turnover, which result a shift of volatile fatty acids production from acetate towards propionate (Martin *et al.*, 2010). As a result, the relative proportion of ruminal hydrogen sources declines and hydrogen sinks increase.

This leads to lower methane production. However, high-sugar grasses need to be agronomically viable, which is not the case to-date.

Another way to increase productivity is animal breeding. Breeding for increased productivity reduces methane emission by increasing the proportion of feed energy used for production purposes while diluting the maintenance requirements (Chagunda *et al.*, 2009). However, productivity increases also require the use of high quality feeds. Residual feed intake (RFI), a measure of net feed efficiency, is a moderately heritable trait (Herd & Arthur, 2009; Herd & Bishop, 2000). Nkrumah *et al.* (2006) found that low RFI animals (efficient animals) emit up to 28% less methane than high RFI counterparts. Thus, selection for low RFI animals may have an effect on methane emissions. However, more research is needed on this subject. Interestingly, in a recent study performed on high and low efficient cows, methanogens composition also varied between these two groups (Zhou *et al.*, 2010).

1.10.2 Vaccination against methanogens

Using vaccines against methanogens is potentially one of the most promising methods to reduce methanogenesis in grazing animals. This approach would have the benefit of using the animal's immune system to produce antibodies against ruminal methanogens, instead of using chemicals, drugs, or antibiotics that may be potentially harmful to the animal or the environment. In addition, this approach is likely to be cost-effective. The vaccination approach may work well for rumen methanogens as there have been reports of success of vaccination against the rumen bacterium *Streptococcus bovis* (Gill *et al.*, 2000; Shu *et al.*, 2001).

Wright *et al.* (2004a) found that immunization of sheep with a mixed whole-cell preparation made from three *Mbb. ruminantium* strains (1Y, AK87 and ZA-10) reduced methane production (per kg DM intake) by 7.7%. However, this study targeted probably less than 20% of the methanogens in the rumen. More recently, Williams *et al.* (2009) found that a broad-spectrum vaccine was needed to be successful in the rumen. Using a vaccine based on a mixture of five major methanogens (*Methanobrevibacter* strains AK87 and 1Y, *Methanobrevibacter millerae* ZA10^T, *Methanomicrobium mobile* BP^T and *Methanosphaera stadtmanae* MCB-3^T), Williams *et al.* (2009) observed that methanogen diversity was significantly changed after the vaccination, although methane

output was not significantly reduced in the experimental animals. Thus, the vaccine may have altered the composition of the methanogen populations. For successful production of a vaccine against methanogens, the complete methanogen diversity in rumen, the complementary/competitive interactions among the rumen methanogens, and rumen processes must be thoroughly understood.

Recent sequencing of the *Methanobrevibacter ruminantium* genome has provided timely information regarding the targets which may be used in vaccine preparation (Leahy *et al.*, 2010). In a recent review, Buddle *et al.* (2010) described two approaches of vaccine preparation which are still at the research level, using genome sequence information of *Methanobrevibacter ruminantium*. The first approach is the utilization of the information obtained from the genome sequence to identify suitable targets for methanogen vaccines. Genes encoding these target proteins would be expressed in a heterologous system and the purified proteins used as antigens to vaccinate sheep. The second approach, which has shown some success at the *in vitro* level, was to generate antisera in sheep using sub-cellular fractions from *Methanobrevibacter ruminantium* (Wedlock *et al.*, 2010). These antisera have been tested with *in vitro* cultures of *Methanobrevibacter ruminantium*. This research is continuing.

For vaccination or small-molecule inhibitors to be successful, the full diversity of methanogens should be well known, as once the dominant methanogens have been knocked out, the vacant niches could be re-occupied fairly quickly by minor species. Producing vaccines that target all rumen methanogens and that do not affect other rumen microbes is challenging. In addition, there are potential limitations to this method as the vaccines may need to be re-applied continually to prevent the re-colonization and adaptation to the vaccines by methanogens.

1.10.3 Defaunation techniques

Elimination of rumen protozoa offers an opportunity to reduce rumen methane production as it decreases the amount of H₂ transferred from protozoa to methanogens. Also, methanogens attached exo- or endo-symbiotically to protozoa can be also removed by this process (Hegarty, 1999). An average reduction of 13% in methane emissions has been suggested following a defaunation technique used by Hegarty (1999). The lower methane emission in defaunated animals was maintained for more

than 2 years indicating that the changes induced are stable (Morgavi *et al.*, 2008). As such, it may be a potential technique to reduce methane emissions from grazing ruminants. However, reduction in emissions is not systematic as recently reported by Hegarty *et al.* (2008).

There have been several methods proposed to eliminate protozoa from the rumen and four of those methods stand out as being potentially applicable to grazing ruminants. These are generation of protozoa-free herds by rearing in isolation (Marcin & Sudekum, 2009; Williams & Coleman, 1997), nutrition-based defaunation can be achieved by short-term feeding of a high grain diet, which cause pH drop and kills protozoa (Hegarty, 1999), chemical defaunation, usually with detergents (Bird *et al.*, 2008; Hegarty *et al.*, 2008), and vaccination against protozoa (Williams *et al.*, 2008).

Vaccination against protozoa using whole fixed protozoal cells, however, did not significantly decrease protozoal numbers (Williams *et al.*, 2008). According to the authors, the vaccine could possibly be improved by using specific protozoal antigens. However, nothing is known about methanogen-protozoal interactions. Interestingly, the recent identification of adhesin-like proteins in *Methanobrevibacter ruminantium* genome may prove to be helpful in developing an effective vaccine (Leahy *et al.*, 2010). There are a number of research strategies progressing on this topic and in the future there may be an effective method to eliminate the protozoa without any deleterious effect to the animal and environment.

1.10.4 Other approaches

In addition to the above mentioned strategies, other techniques are also under investigation. For example, animal breeding for low methane emission may be a viable option, as some animals can show low methane emissions compared to others (Clark *et al.*, 2005; Pinares-Patiño *et al.*, 2003). If this is a stable and heritable trait, this may be useful in New Zealand systems.

Secondary plant components (e.g. tannins and saponins) have shown potential to decrease methane emissions (Animut *et al.*, 2008; Guo *et al.*, 2008; Lila *et al.*, 2005). Feeding fats and oils can also reduce methane emissions significantly (Beauchemin *et al.*, 1998). However, these methods are better suited to grazing animals, and would be

associated with significant costs when applying them to low cost grazing systems. A combination of different techniques may be useful for sustainable methane reduction without affecting animal productivity. With the advances of metagenomic studies and other technologies our understanding of methanogens and methanogenesis will be broadened, which should lead to more ways to reduce methanogenesis from the rumen in the future.

1.11 Conclusions

Understanding methanogen diversity in the rumen is important in the effort towards reducing methane emissions from ruminants. The analysis of gene sequences obtained from different studies performed on rumen samples using molecular-based studies have shown methanogens belonging to *Methanobrevibacter* spp., *Methanosphaera* spp., *Methanomicrobium* spp., *Methanimicrococcus* spp. and the RCC group to be the common methanogens residing in the rumen (Table 1.2). However, the prevalence of these different species in different studies has varied greatly. This may be due to methodological differences or may truly be due to biological factors such as host species, diet, location of the animals and age. To compare the different studies the differences related to methods (DNA extraction method, sample handling method and PCR related biases) should be eliminated or minimized. One of the most crucial factors that can affect the methanogen diversity determined from environmental samples is the primers used for the PCR and the DNA extraction methods employed. Choosing a suitable primer pair is very important to obtain an accurate determination of methanogen diversity from environmental samples and the extracted DNA should contain the DNA from all the targeted species.

There have not been many studies conducted to compare archaeal diversity in the rumens of different species or ruminants fed different diets to find out the effect of diet or species on methanogen diversity. It is fairly well established that diet plays an important role in rumen bacterial diversity (Klieve *et al.*, 2007; Kocherginskaya *et al.*, 2001; Tajima *et al.*, 2001a). Furthermore, bacteria detected in a 16S rRNA gene clone library of reindeer was different from the bacteria found in domestic animals such as sheep and cow (Sundset *et al.*, 2007). However, the effect of diet or host on rumen methanogen diversity is expected to be minimal as they use H₂ released by the process

of fermentation of feed. Furthermore, the rumen environment (T , pH, CO_2 , H_2 and salts) is fairly constant over time. However, the information available at present concerning the effects of diet/host on rumen methanogens is insufficient. Therefore, more comparison studies without methodological differences are needed on this aspect.

Although molecular-based studies give some indication of physiology of micro-organisms in a given environment, the isolation of the microbes is required to understand full physiology and potential of that particular organism in that environment. The genome sequencing of rumen methanogens provides more information about the organisms and help to identify targets that can be used for vaccine or small-molecule inhibitor production. Although genome sequencing does not require a pure culture, the isolation of a pure culture will make the genome sequencing much easier and facilitate subsequent confirmation of any physiological features identified in the genome. Although some rumen methanogens have been isolated already, there are some abundant and common rumen methanogens such as *Methanosphaera* spp., *Methanimicrococcus* spp., and members of the *Mbb. gottschalkii* and *Mbb. ruminantium* clades have not been isolated thus far from the rumen. Close relatives of some of these methanogens have been isolated from non-rumen environments. However, isolation of rumen strains will enhance our understanding of these organisms as rumen strains may not have the same characteristics as their non-rumen relatives.

Unidentified and uncultured rumen archaea have been consistently found in the rumen. For the development of broad spectrum vaccines, these groups should be identified and isolated. The important group in this category is archaea belonging to the RCC group. This group is represented in most of the clone libraries constructed regardless of species, diet or location of animals. In some cases they are the dominant group in the rumen. Nothing is known about this group, although some authors have suggested that they may be methanogens (Denman *et al.*, 2007; Evans *et al.*, 2009). Additional studies are needed on this group in relation to their physiology and possible isolation.

The objectives of my research were developed to answer some of the key questions raised in the literature review. The important knowledge presently needed for the development successful methane reduction strategy from New Zealand ruminants was considered. The major objectives of my study are given below.

1.12 Objectives of the thesis

- To investigate the methanogen diversity in the rumens of farmed New Zealand ruminants fed different diets.
- To isolate the previously uncultured rumen methanogens which are important in New Zealand ruminants, so that, they can be used to identify common targets in the preparation of vaccines or small-molecule inhibitors.

This thesis comprises seven chapters to fulfil the above mentioned objectives. The results chapters, Chapter 3 to Chapter 7, are partly written as manuscripts for future submission. Therefore, the introduction and discussion parts of these chapters are relatively independent of other chapters. As a result, there is some repetition in the introduction and discussion parts of the results chapters.

Chapter 1: Review of literature to understand the rumen environment, rumen microbes, methanogens which have been isolated or identified thus far, the interrelationships among these microbes and the potential methods to reduce the methane production from grazing ruminants. Knowledge gaps were identified from this review and my research objectives were formulated to find answers to some of these gaps.

Chapter 2: The materials and methods used to fulfil the objectives.

Chapter 3: Some published 16S rRNA and *mcrA* gene primers were selected and brief analysis was performed to select suitable primer pairs for the molecular ecology studies that were conducted in the following chapters.

Chapter 4: The archaeal diversity in the rumens of New Zealand farmed-ruminants (cows, sheep and red deer) fed different diets was assessed using DGGE and the findings were validated using clone library construction and quantitative real-time PCR.

Chapter 5: The physiology of the Rumen Cluster C group, which was identified as one of the important archaeal groups in New Zealand ruminants, was investigated. This study was performed to confirm the RCC group as methanogens.

Chapter 6: Cultivation experiments were set-up to isolate methanogens which have not been isolated before from the rumen. The RCC group and *Methanosphaera* spp. were targeted in these experiments.

Chapter 7: Initial characterization was performed for the pure isolate which was obtained in Chapter 6.

Chapter 8: General discussion and concluding remarks with recommendations for future research.

Chapter 2

Materials and Methods

2.1 Use of animals

The use of animals in this thesis was approved by the AgResearch Grasslands Animal Ethics Committee and complied with the AgResearch Code of Ethical Conduct for the Use of Animals in Research, Testing and Teaching, as prescribed in the Animal Welfare Act of 1999 and its amendments.

2.2 Rumen sample collection and processing

Samples of whole rumen contents consisting of fluid and solids (approximately 200 g) were collected from each animal via rumen fistulae or at slaughtering. The details of rumen samples collected in this study are given in Table 2.1. The pasture used in this study consisted of a perennial rye grass (*Lolium perenne*) and white clover (*Trifolium repens*) mix. The silage was made from lucerne (*Medicago sativa*) (ChaffHage; The Great Hage Company, Reporoa, New Zealand). Pasture-grazed animals were on that diet for at least one month and the silage-fed animals were adapted to their feed for 15 days prior to sample collection and fed twice daily, at 08:00 hours and 16:00 hours at 1.2 times their estimated energy requirements for maintenance (Swainson *et al.*, 2008). Samples from silage-fed animals were collected at different time intervals after feeding (2, 4, 6 and 8 h) and the other samples were collected only once, usually 2 h after feeding. Rumen samples from sheep fed a concentrate-based diet (containing 30% [w/w] maize grain, 15% [w/w] barley grain, 15% [w/w] lucerne meal, 15% [w/w] palm kernel extract, 10% [w/w] molasses, rumen-protected fat, vitamin/mineral premixes and fish oil) were collected from a second flock of sheep. Rumen samples were also collected from two further flocks of sheep fed pasture during autumn 2008 and fed willow (*Salix* sp.) fodder during the autumn 2008 (Ramírez-Restrepo *et al.*, 2010). All animals had unlimited access to water at all times. Samples were taken from different parts of the rumen, mixed, and then quickly frozen at -80°C and subsequently lyophilized. The samples were then homogenized (1-2 min) in a 100 W household coffee grinder (Russell Hobbs, Mordialloc, Victoria, Australia) and stored at -80°C until DNA was extracted.

In addition, samples collected from cows which were treated with chloroform (identification numbers C1, C3, C7) and control cows (identification numbers C2, C4, C6), have also been analysed in this study. These cows were fistulated mature non-lactating dairy cows (Friesian-Jersey cross) and they were fed with fixed 8.4 kg DM per day of lucerne (*Medicago sativa*) silage (ChaffHage, The Great Hage Company) and barley meal. The barley meal consisted of 77.6% barley (w/w), 7.8% palm kernel meal (w/w), 11.7% soybean meal (w/w), 1.15% dicalcium phosphate (w/w), 0.8% magnesium oxide (w/w), 0.8% salt (w/w), and 0.15% trace mineral and vitamin mix (w/w) (Nutritech, Auckland, New Zealand). Feed was provided with 40% of the diet being given in the morning and 60% in the afternoon. All animals had unlimited access to water at all times. The control group (3 cows) was drenched with 30 ml of sunflower oil and the treatment group (3 cows) was drenched with 30 ml of sunflower oil containing 1.5 ml of chloroform for 42 days. Drenching occurred every day in the morning (10:00 to 11:00 hours) for 42 days. Rumen samples were collected from each of the cows via the rumen fistula 1 to 2 h after the morning feed from the day before the treatment and continued to 42 days. These samples were processed and stored at -80°C as described above.

The samples for the cultivation experiments were collected from one grazing sheep (S4) on different days via rumen fistulae. This sheep was on pasture during the entire collection period. Rumen samples were collected from this sheep after 2 h of withdrawing from pasture. A glass container flushed with CO₂ was used to collect the samples. Collected samples were immediately brought to the laboratory for further processing (e.g. blending and inoculation). All processing and transfers were carried out under an O₂-free CO₂ atmosphere. Gases were passed over copper filings at 350°C to remove traces of oxygen.

The cows and sheep used in this study were kept and maintained at AgResearch, Grasslands, Palmerston North, or Ruakura, Hamilton, New Zealand, except for sheep S9-S18, which were kept at Riverside dryland farm, Wairarapa, New Zealand. The red deer used in this study were kept and maintained at Massey University, Palmerston North, New Zealand.

Table 2.1. The details of the animals and rumen sample collection methods used in this study. The “chapters” refer to the parts of this thesis in which studies on each group of animals are described.

Ruminant group & location	Details of animals	ID No	Diet (s)	Sampling method	Chapters
Cows AgResearch, Palmerston North	Non-lactating, Friesian-Jersey cross dairy cows Live weight 550 ± 30 kg Age 6 ± 0 years	C1- C5	Silage, summer pasture, winter pasture	Fistula	4
Cows AgResearch, Palmerston North	Non-lactating, Friesian-Jersey cross dairy cows Live weight 573 ± 30 kg Age 6 ± 0 years	C1-C4, C6, C7	Silage plus barley meal, and drenching with sunflower oil (C2, C4, C6), or sunflower oil containing CHCl_3 (C1, C3, C7)	Fistula	3 and 5
Red deer Massey University, Palmerston North	Castrated red deer stags Live weight 144 ± 25 kg Age 6 ± 4 years	D1-D4	Silage, summer pasture, winter pasture	Fistula	4
Sheep –Flock 1 AgResearch, Palmerston North	Romney wether sheep Live weight 55 ± 13 kg Age 3 ± 2 years	S1-S4	Silage, summer pasture, winter pasture	Fistula	4, 6 and 7
Sheep- Flock 2 AgResearch, Ruakura	Romney wether sheep Live weight 44 ± 6 kg Age 6 months	S5-S8	Concentrate-based diet	Slaughter	4
Sheep- Flock 3a Riverside dryland farm, Wairarapa	Suffolk–Romney cross ewe Live weight 41 ± 1 kg Age 1 year	S9-S13	Autumn pasture	Slaughter	4
Sheep- Flock 3b Riverside dryland farm, Wairarapa	Suffolk–Romney cross ewe Live weight 38 ± 1 Age 1 year	S14-S18	Willow	Slaughter	4

2.3 DNA extraction

2.3.1 DNA extraction from the rumen samples

Total DNA was extracted from 100 mg of ground freeze-dried rumen sample using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.3.2 DNA extraction from cultures

Three different methods were used to extract the DNA from cultures.

- a. DNA was extracted from pure cultures with the FastDNA kit and FastPrep instrument (Qbiogene, Carlsbad, CA, USA). Pure cultures of methanogens were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) or were from the AgResearch culture collection. These pure cultures were grown in the appropriate media before DNA extraction and were pelleted by centrifugation to get a pellet size of 50-100 mg. DNA extraction was then carried out according to manufacturer's instructions.
- b. The phenol-chloroform method described by Lueders *et al.* (2004) was used to extract the DNA from pure culture of isolate ISO3-F5. This DNA was used to determine the mol% of G+C content of the DNA. Briefly, cells were disrupted by combined beat-beating (45 s at 6.5 m/s) and phenol-chloroform-isoamyl alcohol treatment. DNA was then precipitated with 30% (v/v) polyethylene glycol, washed with 70% (v/v) ice-cold ethanol, and resuspended in DNA-free water (Section 2.4).
- c. DNA was extracted from mixed cultures (from cultivation experiments) using Chelex resin. Briefly, cells were harvested from 2 ml of live culture by centrifugation at 14,000 g for 5 min. The supernatant was removed and 500 µl of DNA-free water added. The pellet was re-suspended and then harvested again by centrifugation at 14,000 g for 5 min. DNA was extracted from the suspension of the pellet in 200 µl of 6% (w/v) Chelex 100 (InstaGene Matrix; Bio-Rad Laboratories Inc., Hercules, CA, USA) by heating at 56°C for 30 min, then mixing by vortexing for 10 s, followed by boiling at 100°C for 8 min and again mixing by vortexing for

10 s. Cell debris was removed from the suspension by centrifugation at 12,000 *g* for 3 min.

2.4 Preparation of DNA-free water

DNA-free water was prepared by filtering distilled water through a 0.2- μ m pore size sterile filter, autoclaving the water, then irradiating 2-ml aliquots in 2-ml polypropylene tubes with UV light (254 nm, 6 W) at a distance of 100 mm for 4 h.

2.5 Preparation of Luria Bertani (LB) medium

LB medium was prepared by mixing Bacto-tryptone (10 g/l), yeast extract (5 g/l) and NaCl (10 g/l) in distilled water. For the solid media bacteriological agar (15 g/l) was added to this mixture. The components were mixed together and sterilized by autoclaving for 20 min at 121°C. For the solid media the autoclaved mixture was transferred to the water bath maintained at 55°C. The ampicillin solution (50 mg/l w/v) was then added to the mixture when it was cooled (agar medium at 55°C). The broth medium was transferred into sterilized screw-capped bottles and stored at 4°C. The agar medium was spread onto disposable sterilized Petri plates and stored at 4°C after drying.

2.6 Polymerase chain reactions (PCR)

All PCR amplifications were performed in Hybaid Px2 thermal cyclers (ThermoElectron, Milford, MA, USA) using reagents from Roche (Roche, Auckland, New Zealand). DNA-free water was prepared according to the method described above (Section 2.4) and the primers were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). Partial fragments of 16S rRNA gene of Archaea, Bacteria and RCC and the *mcrA* gene of methanogens were amplified in this study. “Colony PCR” was performed to screen the plasmid inserts from the cloning.

Each PCR (50 μ l) contained 10 \times reaction buffer, MgCl₂ (optimized for each primer), 0.5 μ M of each primer, 200 μ M of each deoxyribonucleotide triphosphate, 2.5 U of *Taq* DNA polymerase (Roche), 25 ng of template DNA or a small amount of a colony (for colony PCR), and DNA-free water (Section 2.4). Each set of PCR contained a negative control to avoid false positive reactions caused by contaminating DNA in PCR reagents. The presence of a product of the expected size was considered a positive result. Oligonucleotide primers used in this study are listed in Tables 2.2, 2.3, 2.4, and 2.5, along with the sequences of primers, optimum Mg²⁺, and the details of the thermal cycling conditions.

2.7 Agarose gel electrophoresis

Amplified 16S rRNA or *mcrA* gene products were analyzed by agarose gel electrophoresis. Agarose gels (1.5% w/v) were made up in 1 \times TAE buffer which consisted of 40 mM tris(hydroxymethyl)aminomethane, 65 mM acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA) adjusted to pH 8.0 with NaOH. Amplified PCR products were loaded into the well with 20% (v/v) Orange G loading buffer (0.1 mM EDTA, 40% [v/v] glycerol, 0.15% [w/v] Orange G [Sigma-Aldrich, St Louis, USA]). TAE agarose gels were run in 1 \times TAE buffer at 100 V for 45 min. 1Kb⁺ marker (Invitrogen) was included in at least one lane of every gel. DNA stained with SYBR safe DNA gel stain (Invitrogen) was visualized using UV trans-illumination and photographed using a Gel Logic 200 imaging system (Eastman Kodak, New York, NY, USA).

2.8 Purification of PCR products

PCR products were purified using a Wizard SV gel and PCR clean-up system (Promega, Alexandria, NSW, Australia) according to the manufacturer's instructions.

2.9 Purification of plasmids

Escherichia coli cultures with plasmid inserts were grown overnight in 5 ml of LB broth medium (Section 2.5) containing ampicillin (50 μ g/ml). Then the plasmid purification was performed using QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions.

Table 2.2. Primers used for the PCR amplification to construct 16S rRNA gene clone libraries (Bacteria, Archaea and RCC) and to identify the presence of these organisms in sample/s or cultures

Primer pair	Target gene and fragment size	Sequence (5'-3') ^a	Mg ²⁺ (mM)	Thermal cycling conditions	References
519f	16S rRNA Bacteria (≈ 388 bp)	CAG CMG CCG CGG TAA NWC	1.5	94°C for 4 min	Stubner <i>et al.</i> , 2004
907r		CCG TCA ATT CMT TTR AGT T		94°C for 30 s 52°C for 30 s 72°C for 1 min 72°C for 7 min	
BAC27f	16S rRNA Bacteria (≈ 1465 bp)	GAG TTT GAT CMT GGC TCA G	2.5	94°C for 4 min	Lane, 1991
BAC1492r		GGY TAC CTT GTT ACG ACT T		94°C for 1 min 52°C for 1 min 72°C for 1 min 72°C for 7 min	
Met86f	16S rRNA	GCT CAG TAA CAC GTG G	1.5	94°C for 3 min	Wright & Pimm, 2003
Met1340r	Archaea (≈ 1254 bp)	CGG TGT GTG CAA GGA G		94°C for 30 s 58°C for 30 s 72°C for 1 min 30 s 72°C for 7 min	

Primer pair	Target gene and fragment size	Sequence (5'-3') ^a	Mg ²⁺ (mM)	Thermal cycling conditions	References
ARC8f ARC1510r	16S rRNA Archaea (≈ 1502 bp)	TCY GKT TGA TCC YGS C GGN YAC CTT GTT ACG ACT T	1.5	95°C for 3 min 95°C for 30 s 59°C for 30 s 72°C for 1 min 72°C for 7 min	This study 35 cycles
1Af 1100Ar	16S rRNA Archaea (≈ 1099 bp)	TCY GKT TGA TCC YGS CRG AG TGG GTC TCG CTC GTT G	2.5	94°C for 4 min 94°C for 30 s 57°C for 30 s 72°C for 1 min 72°C for 7 min	Whitford <i>et al.</i> , 2001 35 cycles
915af 1386r	16S rRNA Archaea (≈ 471 bp)	AGG AAT TGG CGG GGG AGC AC GCG GTG TGT GCA AGG AGC	1.5	95°C for 2 min 95°C for 15 s 59°C for 30 s 72°C for 1 min 72°C for 7 min	Watanabe <i>et al.</i> , 2004 Skillman <i>et al.</i> , 2004 35 cycles

Primer pair	Target gene and fragment size	Sequence (5'-3') ^a	Mg ²⁺ (mM)	Thermal cycling conditions	References
Met630f Met803r	16S rRNA Archaea (≈ 173 bp)	GGA TTA GAT ACC CSG GTA GT GTT GAR TCC AAT TAA ACC GCA	1.5	94°C for 4 min 94°C for 30 s 52°C for 30 s 72°C for 1 min 72°C for 7 min	Sundset <i>et al.</i> , 2009a
Ar109f Ar915r	16S rRNA Archaea (≈ 806 bp)	ACK GCT CAG TAA CAC GT GTG CTC CCC CGC CAA TTC CT	1.5	94°C for 4 min 94°C for 30 s 57°C for 30 s 72°C for 1 min 72°C for 7 min	Grosskopf <i>et al.</i> , 1998 Stahl & Amann, 1991
Ar109f 1386r	16S rRNA Archaea (≈ 1277 bp)	ACK GCT CAG TAA CAC GT GCG GTG TGT GCA AGG AGC	2.0	94°C for 4 min 94°C for 30 s 58°C for 30 s 72°C for 1 min 72°C for 7 min	Grosskopf <i>et al.</i> , 1998 Skillman <i>et al.</i> , 2004

Primer pair	Target gene and fragment size	Sequence (5'-3') ^a	Mg ²⁺ (mM)	Thermal cycling conditions	References
RCC762f	16S rRNA RCC	GAC GAA GCC CTG GGT C	1.5	94°C for 2 min	This study
RCC1099r	(≈ 337 bp)	GAG GGT CTC GTT CGT TAT		94°C for 30 s	
				56°C for 1 min 60 s	
				72°C for 1 min	
				72°C for 7 min	

^a Nonstandard symbols: **K**, equimolar G or T; **M**, equimolar A or C; **N**, equimolar G or A or T or C; **W**, equimolar A or T; **R**, equimolar A or G; **D**, equimolar G or A or T; **H**, equimolar C or A or T; **Y**, equimolar C or T; **S**, equimolar C or G .

Table 2.3. Primers used for the PCR amplification to construct *mcrA* gene clone libraries (methanogens) and to identify the presence of methanogens in samples or cultures

Primer pair	Sequence (5'-3') ^a	Mg ²⁺ (mM)	Thermal cycling conditions	References
ML1	GGT GGT GTM GGA TTC ACA CAR TAY GCW ACA GC	1.5	94°C for 3 min	Luton <i>et al.</i> , 2002
ML2 (≈ 470 bp)	TTC ATT GCR TAG TTW GGR TAG TT		94°C for 30 s 52°C for 15 s 72°C for 1 min 72°C for 7 min	
MCRf	TAY GAY CAR ATH TGG YT ^a	2.0	94°C for 4 min	Springer <i>et al.</i> , 1995
MCRr (≈ 490 bp)	ACR TTC ATN GCR TAR TT		94°C for 30 s 53°C for 30 s 72°C for 30 s 72°C for 7 min	
MCRmf	TWY GAY CAR NTN TGG YT ^a	2.0	94°C for 4 min	This study
MCRr (≈ 490 bp)	ACR TTC ATN GCR TAR TT		94°C for 45 s 57°C for 45 s 72°C for 45 s 72°C for 7 min	

Primer pair	Sequence (5'-3') ^a	Mg ²⁺ (mM)	Thermal cycling conditions	References
ME1	GCM ATG CAR ATH GGW ATG TC	1.5	94°C for 4 min	Tatsuoka <i>et al.</i> , 2004
ME2 (≈ 760 bp)	TCA TKG CRT AGT TDG GRT AGT		94°C for 30 s 56°C for 30 s 72°C for 1 min 72°C for 7 min	35 cycles

^a Nonstandard symbols: **K**, equimolar G or T; **M**, equimolar A or C; **N**, equimolar G or A or T or C; **W**, equimolar A or T; **R**, equimolar A or G; **D**, equimolar G or A or T; **H**, equimolar C or A or T; **Y**, equimolar C or T; **S**, equimolar C or G.

Table 2.4. Primers used for the amplification of inserts of pCR 2.1 TOPO vector

Primer pair	Sequence (5'-3')	Mg ²⁺ (mM)	Thermal cycling conditions	References
GEM2987f	CCC AGT CAC GAC GTT GTA AAA CG	2	94°C for 2 min	O'Farrell & Janssen, 1999
TOP168r	ATG TTG TGT GGA ATT GTG AGC GG		94°C for 15 s 56°C for 30 s 72°C for 1 min 72°C for 7 min	35 cycles Osborne <i>et al.</i> , 2005

Table 2.5. Primers used to obtain PCR products for analysis by denaturing gradient gel electrophoresis (Bacteria, Archaea and RCC)

Primer pair	Target gene and fragment size	Sequence (5'-3') ^a	Mg ²⁺ (mM)	Thermal cycling conditions	References
338f ^a 518r	16S rRNA Bacteria (≈180 bp)	ACT CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	2	95°C for 3 min 95°C for 30 s } 10 cycles 62°C for 30 s } (-0.5°C/cycle) 72°C for 30 s 95°C for 30 s } 57°C for 30 s } 26 cycles 72°C for 30 s 72°C for 30 min	Muyzer <i>et al.</i> , 1993
915af ^a 1386r	16S rRNA Archaea (≈471 bp)	AGG AAT TGG CGG GGG AGC AC GCG GTG TGT GCA AGG AGC	1.5	95°C for 2 min 95°C for 15 s } 59°C for 30 s } 25 cycles 72°C for 1 min 72°C for 30 min	Watanabe <i>et al.</i> , 2004 Skillman <i>et al.</i> , 2004

Primer pair	Target gene and fragment size	Sequence (5'-3') ^a	Mg ²⁺ (mM)	Thermal cycling conditions	References
RCC762f ^a RCC1099r	RCC	GAC GAA GCC CTG GGT C GAG GGT CTC GTT CGT TAT	1.5	94°C for 2 min 94°C for 30 s 56°C for 1 min 72°C for 1 min 72°C for 30 min	This study

^a Each forward primer included an additional 40-bp GC-rich sequence segment (CGCCCGCCGCGCGCGGGCGGGCGGGG GCA CGG GGG G) at the 5' end.

2.10 Extraction and purification of DNA from agarose gels

DNA fragments were excised from agarose gels with a clean, sharp scalpel. The DNA was extracted from this gel slice and purified using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions.

2.11 DNA quantification

DNA was quantified using the Quant-iT dsDNA BR Assay Kit on a Qubit fluorometer (Invitrogen) or using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) according to the manufacturer's instructions.

2.12 Cloning

Purified PCR products were ligated into the pCR 2.1 TOPO TA cloning vector (Invitrogen) according to manufacturer's instructions, and then transferred into TOP10 chemically competent *Escherichia coli* (one shot TOP10 cells, Invitrogen), according to the manufacturer's instructions. Transformed cells were then plated onto LB agar containing ampicillin (50 mg/l) and X-gal (40 mg/l) and incubated overnight at 37°C. Clones containing an insert were initially identified by their white colony colour. Clones were randomly selected for sequencing. Selected colonies were grown again on a LB plate (with ampicillin and without X-gal) overnight. In some cases, DNA was extracted by boiling the colonies for 10 min. PCR was performed using the DNA extracted from colonies or with a small amount of cell material (0.5-1 mg) with the primer pair GEM2987 and TOP168r (Table 2.4), and sent for sequencing.

2.13 Sequencing and chimera detection

PCR products were sent for sequencing either to the Allan Wilson Centre Genome Sequencing Service (Massey University, Palmerston North, New Zealand) or Macrogen Inc. (Seoul, Republic of Korea). Results of sequences were obtained as ABI files and the sequences were edited using MEGA 4.0 (Tamura *et al.*, 2007). The online chimeric detection program Bellerophon (Huber *et al.*, 2004) was used to identify chimeric sequences. These chimeric sequences were excluded from the subsequent analysis.

2.14 Phylogenetic analyses

All analyses were implemented in MEGA 4.0 (Tamura *et al.*, 2007). Nucleotide sequences were globally aligned with ClustalW using the IUB DNA matrix and a transition weight of 0.5. Phylogenetic trees for 16S rRNA gene sequences were constructed using the Neighbor-joining tree inference method (Saitou & Nei, 1987) with the Jukes-Cantor substitution model (Jukes & Cantor, 1969) assuming uniform rates among sites and using the complete deletion of gaps option. Bootstrap analyses were performed based on 1000 resamplings. The 16S rRNA gene sequences from *Methanocaldococcus jannaschii* JAL-1 (GenBank accession number M59126), *Methanococcus vannielii* SB (M36507), and *Methanothermococcus thermolithotrophicus* SN-1 (M59128) were used as out-groups for archaeal 16S rRNA gene phylogenetic trees.

Deduced amino acid sequences were used to construct *mcrA* gene phylogenetic trees. Amino acid sequences of *mcrA* gene clones and reference sequences were aligned with ClustalW and phylogenetic trees were constructed using the Neighbor-joining tree inference method with the Jones-Taylor-Thornton (JTT) matrix amino acid substitution model (Jones *et al.*, 1992) using the complete deletion of gaps option. Bootstrap values were generated from 1000 samplings for these trees. The *mcrA* gene sequence from *Methanopyrus kandleri* AV19 (AF414042) was used as the out-group for *mcrA* gene phylogenetic trees.

2.15 Analysis of clone libraries and the phylogenetic trees

The Shannon diversity (Shannon & Weaver, 1963) and Simpson's indices (Simpson, 1949) were estimated using the proportions of different methanogen groups in each clone library by version 2.01 of the PAST (PAleontological STatistics) software package (<http://folk.uio.no/ohammer/past>) (Hammer *et al.*, 2001). The Shannon diversity index increases with richness and evenness of sequence distribution. A higher value of the Simpson's index indicates greater dominance of one or few abundant group of sequences. Using the same software Shannon diversity *t* tests were also employed to compare methanogen diversities between two samples.

2.16. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed to determine the diversities of the Archaea, Bacteria and RCC in rumen samples. The purified PCR products were treated with mung bean nuclease to remove interfering single-stranded DNA products, as described by Kocherginskaya *et al.* (2001). The reaction mixture containing 15 µl of purified PCR products, 3 µl of 10× mung bean nuclease buffer (pH 5.0), 1.7 U of mung bean nuclease (Promega) in 1 µl of water, and 30 µl water, and was incubated at 37°C for 10 min. The reaction was stopped by adding 10 µl of DGGE loading dye (0.05% [w/v] bromophenol blue, 0.05% [w/v] xylene cyanol, 70% [w/v] glycerol, in water, pH 8.0) and immediate chilling on ice.

The reference standard for archaeal DGGE was created by combining equal quantities of PCR products generated using the primers GC-915af and 1386r from DNA extracted from pure cultures of methanogens obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), or from the AgResearch culture collection. These cultures, with their culture collection accession numbers (DSM) or the GenBank accession numbers of their 16S rRNA gene sequences, were *Methanobrevibacter thaueri* strain CW (DSM 11995), *Methanobrevibacter gottschalkii* strain HO (DSM 11977), *Methanobrevibacter ruminantium* strain M1 (DSM 1093), *Methanobrevibacter ruminantium* strain 31A (GenBank HM624055), *Methanospirillum hungatei* strain JF1 (DSM 864), *Methanobrevibacter smithii* strain PS (DSM 861), *Methanosarcina barkeri* strain CM1 (GenBank AJ002476), and *Methanobacterium formicicum* strain BRM9 (GenBank X99138). For the bacterial and RCC DGGE, standard markers (Marker II for RCC DGGE and Marker IV for bacterial DGGE) purchased from Japan (Nippongene, Japan) was used as constant position markers in all gels.

DGGE was performed by using a CBS scientific DGGE/TTGE gel system (C.B.S. Scientific Company, Del Mar, CA, USA). Polyacrylamide gels (6% [w/v]) were prepared and electrophoresed with 1× TAE buffer, which consisted of 40 mM tris(hydroxymethyl)aminomethane, 65 mM acetic acid, 1 mM ethylenediaminetetraacetic acid, adjusted to pH 8.0 with NaOH. The gels for separating PCR products contained a gradient of denaturant that increased in the direction of electrophoresis, with a 30% [v/v] to 55% [v/v] gradient for total archaeal products, a

40% [v/v] to 60% [v/v] gradient for total bacterial products, and a 15% [v/v] to 70% [v/v] for products amplified from RCC. The 100% denaturant solution contained 40% (v/v) formamide and 7.0 M urea. Approximately 300 ng of purified and mung bean nuclease-treated PCR products were loaded into each well. Electrophoresis was performed at 150 V (constant voltage) and 60°C (constant temperature) for 6 h. The gels were stained with 3.0 µl of SYBR Gold (Invitrogen) in 600 ml of water for 20 min and de-stained in 600 ml of water for 30 min. The gels were viewed using UV trans-illumination and photographed using a Gel Logic 200 imaging system (Eastman Kodak, New York, NY, USA).

2.17 DNA extraction from DGGE gels

To extract DNA from DGGE bands, selected bands were cut from the gel using a clean, sharp scalpel and transferred to a 1.5-ml microcentrifuge tube. DNA was eluted from polyacrylamide gel slices according to the method described by Etokebe and Spurkland (2000). Gel slices were each washed by incubation in 50 µl of water for 15 min at room temperature. The water was then removed and discarded. DNA was recovered by the addition of 50 µl of DNA-free water to each tube, followed by incubation overnight at 4°C in 1.5-ml tubes. The tubes were vortexed for 5 s, centrifuged for 1 min at 10,000 g, and the supernatants transferred to clean tubes. The DNA fragments in the supernatants were cloned as described above. Positive clones were re-amplified with the respective DGGE primers, and DGGE was performed to confirm the position of migration. At least three clones from each band were sequenced.

2.18 Analysis of DGGE gels

DGGE fingerprints were analyzed with the BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium). Each profile, consisting of a digitized image from the gel and representing one lane containing one separated PCR product, was normalized relative to the bands in the lane containing the reference standard. Similarities between the fingerprints were quantified using the Pearson correlation coefficient between each pair of profiles, and these values were represented graphically using the Unweighted Pair Group Method of Arithmetic Averages (UPGMA) method.

2.19 Quantitative real-time PCR (qPCR)

Abundances of 16S rRNA genes from the Archaea, Bacteria and members of RCC in rumen samples were quantified using a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Concorde, NSW, Australia) and amplicon detection by SYBR Green fluorescence (LightCycler 480 SYBR Green I Master Kit or LightCycler FastStart DNA Master SYBR Green I Kit: Roche).

2.19.1. qPCR of total archaea

Abundances of 16S rRNA genes from total archaea in rumen samples were quantified using the LightCycler 480 SYBR Green I Master Kit (Roche). External standards for archaea were prepared by mixing plasmids containing 16S rRNA gene fragments amplified using the primers 915af plus 1386r from pure cultures of *Methanobrevibacter ruminantium* strain M1 (DSM 1093), *Methanomicrobium mobile* strain BP (DSM 1539) and *Methanobacterium bryantii* strain M.o.H (DSM 863). Plasmids inserts were quantified with the Quant-iT dsDNA BR Assay Kit on a Qubit fluorometer (Invitrogen), and diluted 10-fold in series to produce 5 standards from 10^3 to 10^7 copies per reaction in duplicate for use in the archaeal qPCR. Reactions were set up in a Gene-Disc 100 (Corbett Life Science) and sealed with permanent adhesive film (Corbett Life Science).

Each reaction (20 μ l) contained 10 μ l of LightCycler 480 Master Mix (Roche), 10 pmol of each primer, 4 μ g of bovine serum albumin (Invitrogen) and 5 μ l of DNA. DNA amplification was performed in 3 different dilutions of the template (1:10, 1:100 and 1:200) each in duplicate, to give 6 replicates for each sample. Archaeal 16S rRNA genes were amplified using the primers 915af and 1386r. Amplification commenced with a hot start of 95°C for 10 min, followed by 50 cycles of 95°C for 10 s, 59°C for 5 s, and extension at 72°C for 10 s. After each run, melting curves between 72°C and 98°C were evaluated for each PCR to confirm the absence of nonspecific signals. Quantitative PCR efficiency was checked with the software LinRegPCR (Ruijter *et al.*, 2009). The copy numbers of 16S rRNA genes of targeted groups with the efficient reactions were calculated based on the dilutions made and the DNA yield per g of dry rumen contents.

2.19.2. qPCR of Rumen Cluster C

Abundances of 16S rRNA genes from the RCC group in rumen samples were also quantified using the LightCycler 480 SYBR Green I Master Kit (Roche). External standards for RCC were prepared by mixing equal quantities of three plasmids containing 16S rRNA gene fragments amplified by the RCC-specific primers (762f and 1099r) from rumen contents from cow C2 fed silage (NZCRCC007 [GenBank accession number HM624062], NZCRCC011 [HM624066], and NZCRCC014 [HM624069]). Plasmids inserts were quantified with the Quant-iT dsDNA BR Assay Kit on a Qubit fluorometer (Invitrogen), and diluted 10-fold in series to produce 5 standards from 10^3 to 10^7 copies per reaction in duplicate for use in the RCC qPCR. Reactions were set up in a Gene-Disc 100 (Corbett Life Science) and sealed with permanent adhesive film (Corbett Life Science).

Reaction mixture and the amplification cycles of RCC qPCR were similar to the archaeal qPCR except the annealing temperature which was 56°C for RCC. The primers used for the RCC qPCR were 762f and 1099r. DNA amplification was performed in 3 different dilutions of the template (1:10, 1:100 and 1:200) each in duplicate, to give 6 replicates for each sample. Quantitative PCR efficiency analysis and calculation of numbers of 16S rRNA genes of RCC were performed as described for total archaea quantification.

2.19.3 qPCR of total bacteria

Bacterial 16S rRNA genes were amplified using the primers 519f plus 907r using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche) to quantify the total bacteria in the rumen samples. A mixture of 3 plasmids containing 16S rRNA gene fragments (BAC15, BAC18 and BAC23) amplified with the bacteria-specific primers 27f and 1492r from rumen contents of sheep S4 fed winter pasture were used as the external standards for quantification of bacterial 16S rRNA genes. Gene sequences of these plasmids are given in Appendix 1. Plasmid inserts were quantified with the Quant-iT dsDNA BR Assay Kit on a Qubit fluorometer (Invitrogen), and diluted 10-fold in

series to produce 5 standards from 10^3 to 10^7 copies per reaction in duplicate for use in the bacterial qPCR. Reactions were set up in a Gene-Disc 100 (Corbett Life Science) and sealed with permanent adhesive film (Corbett Life Science).

Each reaction contained, in a total volume of 20 μ l, 2 μ l of Light Cycler Mix, 1 μ M of each primer (519f and 907r), $MgCl_2$ to final concentrations of 4 mM, 4 μ g bovine serum albumin (Invitrogen), and 2 μ l of standard or DNA template. Each original template DNA was measured in 3 different dilutions (1:100, 1:200 and 1:1000). The thermal protocol for quantitative PCR amplification and detection was 10 min of initial denaturation (94°C) followed by 50 amplification cycles (30 s at 94°C; 5 s at 52°C; 10 s at 72°C). After each run, melting curves between 72 and 95°C were evaluated for PCR reactions to confirm the absence of nonspecific signals. Quantitative PCR efficiency analysis and calculation of numbers of 16S rRNA genes of bacteria were performed as described for total archaea quantification.

2.20 Fluorescence *in situ* hybridisation (FISH)

The fluorescently labelled oligonucleotide probes targeting bacteria (EUB338; Sigma), Archaea (ARC915; Sigma) and RCC (RCC771; Invitrogen) used in FISH are shown in Table 2.6.

Table 2.6. 16S rRNA gene-targeted fluorescently-labelled oligonucleotide probes used in this study.

Probe	Label	Sequence (5'-3')	Target organism	Reference
EUB338	CY3	GCTGCCTCCCGTAGGAGT	Bacteria	Amann <i>et al.</i> , 1990
ARC915	FITC	GTGCTCCCCCGCCAATTCCT	Archaea	Stahl & Amann, 1991
RCC771	CY3, Alexa 488	CTAATCCCGTTTGCGACCCAG	RCC	This study

Cells were fixed with 4% (w/v) paraformaldehyde for 3 h at 4°C, washed once with PBS (130 mM NaCl, 10 mM sodium phosphate buffer: pH 7.2), and stored in PBS/ethanol (1:1, v/v) at -20°C. The fixed cells were spotted on a well of a Teflon-coated slide (Erie Scientific Company, New Hampshire, USA), and allowed to air dry. After dehydration in 50, 70 and 100% ethanol (3 min each), 10 ml of pre-warmed (46°C) hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl [pH 7.2], 0.01% sodium dodecyl sulphate [SDS], and 10% formamide) was prepared and 8 µl of hybridization buffer was spotted onto the fixed cells on the slide. Remaining hybridization buffer was poured on to the paper towel which was folded into a rectangle and placed into a 50-ml Falcon tube. For each well 25 ng of each probe was added and mixed carefully. The slide was placed into the 50-ml Falcon tube, where hybridization buffer was poured, and incubated for 2 h at 46°C. After hybridization, the slide was washed with pre-warmed (48°C) 50 ml washing buffer (0.45 M NaCl, 20 mM Tris/HCl, 0.01% SDS) for 20 min at 48°C. The slide was rinsed with ice-cold distilled water, air dried, and mounted with Vectashield anti-fading solution (Vector Laboratories Inc., Burlingame, CA). Fluorescence was observed in appropriate channels using the DM2500 microscope (Leica Microsystems, Wetzlar, Germany) and the images captured digitally using Leica Application suite software.

2.21 Cultivation experiments of methanogens

Four cultivation experiments were conducted and the procedures were similar with slight variations among these experiments. Forty grams of rumen contents from the sheep (S4) were blended in 360 ml of RM02 medium (Section 2.22.3) under anaerobic conditions using a Waring blender (Waring Products Inc., Torrington, CT, USA) with a glass chamber and tight-fitting rubber lid. The glass vessel was gassed with CO₂ prior to use, and during and subsequent manipulations. Using a 16-gauge hypodermic needle and a sterile plastic syringe, this blended rumen samples were transferred from the blender, in to serum vial containing RM02 medium under a gas phase of 100% CO₂ and sealed with a blue butyl rubber stopper and an aluminum closure (Bellco, Vineland, NJ,

USA). Further serial dilutions were then made, using sterile syringes and needles, to serum vials containing RM02 medium, to produce suspensions with different amounts of rumen contents. These dilutions were used to inoculate the culture tubes containing either RM02 medium or BY⁺ medium (Section 2.22.2) supplemented with substrates and other additives dependent on the experiment. These inoculated culture tubes were incubated at 39°C in the dark with shaking (200 rpm).

2.22 Media

2.22.1. BY medium

BY medium was prepared by mixing solution A (17% [v/v]), solution B (17% [v/v]), rumen fluid (30% [v/v]), NaHCO₃ (5 g/l [w/v]), yeast extract (2 g/l [w/v]), 5 drops of resazurin (0.1% [w/v]) and L-cysteine-HCl (0.5 g/l [w/v]). All components except L-cysteine-HCl were mixed thoroughly. This solution was boiled under O₂-free 100% CO₂ and cooled in an ice bath while it was bubbled with 100% CO₂. Once the solution was cooled, L-cysteine-HCl was added. This solution was then dispensed into Hungate tubes (16 mm dia., 125 mm long; BellCo Glass, Vineland, NJ, USA) while being gassed with 100% CO₂, at 9.5 ml of medium per tube, and the tubes were capped with black butyl rubber seals and perforated plastic caps, with a headspace of 100% CO₂. These tubes were sterilized by autoclaving for 20 min at 121°C. Before use, the tubes containing the medium were stored in the dark for at least 24 h. Salt solution A was prepared by mixing NaCl (6 g/l [w/v]), KH₂PO₄ (3 g/l [w/v]), (NH₄)₂SO₄ (1.5 g/l [w/v]), CaCl₂·2H₂O (0.79 g/l [w/v]) and MgSO₄·7H₂O (1.2 g/l [w/v]) in distilled water. Salt solution B was prepared by dissolving K₂HPO₄·3H₂O (7.86 g/l [w/v]) in distilled water.

Rumen fluid which was used in BY media was collected from fistulated cows that had been fed pasture hay for 48 h after being fed on a rye-grass clove pasture. Feed was withheld from the animal after 4:00 P.M and rumen contents collected the next day

at about 10:00 A.M. The material was filtered through a double layer of cotton cheesecloth with a mesh size of approximately 1 mm (Stockinette; Cirtex Industries Ltd., Thames, New Zealand) and then fine particle material removed from the filtrate by centrifugation at 10,000 *g* for 20 min. The supernatant (rumen fluid) was stored frozen at -20°C. The rumen fluid was thawed before use, and any new precipitates that formed were removed by centrifugation at 12,000 *g* for 15 min.

2.22.2. Modified BY medium (BY⁺)

BY⁺ was prepared in the same way as BY, except that 1 ml of selenite-tungstate solution (Tschech & Pfennig, 1984) was added per 1000 ml when all the components were mixed before boiling under O₂-free 100% CO₂.

Selenite-tungstate solution was prepared by adding 0.5 g NaOH, 3 mg of Na₂SeO₃·5H₂O and 4 mg of Na₂WO₄·2H₂O (w/v) to 1.0 l of distilled water and autoclaved in 50 ml aliquots in 100 ml screw-capped bottles.

2.22.3 RM02 medium

This medium is a bicarbonate-buffered mineral medium supplemented with vitamins. The mineral salt solution contained (per litre medium) 1.4 g of KH₂PO₄, 0.6 g of (NH₄)₂ SO₄, 1.5 g of KCl, 1 ml trace element solution SL10 (Widdel *et al.*, 1983), 1 ml of selenite-tungstate solution (Tschech & Pfennig, 1984), and 4 drops of 0.1% (w/v) resazurin solution. The mineral salt solution was mixed and then boiled under O₂-free 100% CO₂, before being cooled in an ice bath while it was bubbled with 100% CO₂. Once the mineral salts solution was cooled, 4.2 g of NaHCO₃ and 0.5 g of L-cysteine-HCl were added per litre. The cooled and supplemented mineral salts solution was then dispensed into Hungate tubes while being gassed with 100% CO₂, at 9.5 ml of medium per tube, and the tubes capped with black butyl rubber seals and perforated plastic caps,

with a headspace of 100% CO₂. These tubes were sterilized by autoclaving for 20 min at 121°C. Before use, the tubes were stored in the dark for at least 24 h. When required, 0.5 ml of rumen fluid-yeast extract-vitamin mixture (Section 2.23.1) was added per 9.5 ml of RM02.

2.23 Media additives

2.23.1. Rumen fluid-yeast extract-vitamin mixture

Base rumen fluid (Section 2.22.1) was bubbled with 100% N₂ for 10 min, before being autoclaved in partly filled serum vials closed with blue butyl rubber stoppers and aluminium caps (20 mm diameter; BellCo) under 100% N₂ for 15 min to inactivate viruses. The anoxic conditions were used to limit the formation of unwanted oxidation processes during autoclaving. The autoclaved rumen fluid was then stirred under air, and 1.63 g of MgCl₂·6H₂O and 1.18 g of CaCl₂·2H₂O was added per 100 ml. This formed a heavy precipitate, which was removed by centrifugation at 30,000 g at 4°C for 1 h. This supernatant was designated as clarified rumen fluid. To each 100 ml of clarified rumen fluid, 2 g of yeast extract were added. This was well mixed and then bubbled with N₂ gas for 15 min, before being transferred to a N₂-flushed sterile serum vial sealed with a blue butyl rubber stopper and an aluminium cap (Bellco) through a 0.22 µm pore size sterile filter (Millipore Corp., Bedford, MA, USA) using a sterile syringe and needle. Two ml of vitamin 10 concentrate was then added per 100 ml of preparation by syringe and needle.

2.23.2 RFgenV

For 100 ml of clarified rumen fluid, 0.36 g D-glucose, 0.34 g D-cellobiose, 0.3 g D-xylose, 0.3 g L-arabinose, 0.88 ml Na L-lactate syrup (50%), 2 g casamino acids, 2 g Bacto-peptone and 2 g of yeast extract were added and mixed well. This solution was bubbled with N₂ gas for 15 min and transferred to a N₂-flushed sterile serum vial through a 0.22-µm pore size filter using a sterile syringe and needle.

2.23.3 Vitamin 10 concentrate

Vitamin 10 concentrate contained 40 mg of 4-aminobenzoate, 10 mg of D-(+)-biotin, 100 mg of nicotinic acid, 50 mg of hemicalcium D-(+)-pantothenate, 150 mg of pyridoxine hydrochloride, 100 mg of thiamine chloride hydrochloride, 50 mg of cyanocobalamin, 30 mg of D,L-6,8-thioctic acid, 30 mg of riboflavin and 10 mg of folic acid in 1000 ml of distilled water. After preparation, the solution was well mixed and then bubbled with N₂ gas for 15 min, before being transferred to a sealed N₂-flushed sterile serum vial (BellCo) through a 0.22- μ m pore size sterile filter (Millipore) using a sterile syringe and needle.

2.23.4 Trace element solution (SL10)

The following components were added to 1 l of distilled water and autoclaved in 25 ml aliquots in 50 ml screw-capped bottles: 10 ml of 25% HCl, 1.5 g of FeCl₂·H₂O, 190 mg of CoCl₂·6H₂O, 100 mg of MnCl₂·4H₂O, 70 mg of ZnCl₂, 6 mg of H₃BO₃, 36 mg of Na₂MoO₄·2H₂O, 24 mg of NiCl₂·6H₂O and 2 mg of CuCl₂·2H₂O.

2.23.5 Volatile fatty acid solution

The following components were mixed well and pH was adjusted to 7.5 with NaOH: 17 ml of acetic acid (3 M), 6 ml of propionic acid (0.8 M), 4 ml of butyric acid (0.4 M), 1 ml of isobutyric acid (0.1 M), 1 ml of valeric acid (0.1 M), 1 ml of isovaleric acid (0.1 M) and 1 ml of D,L- α -methylbutyric acid (0.1 M). The volume of this mixture was brought up to 100 ml with distilled water. This mixture was then dispensed to Hungate tubes and autoclaved.

2.23.6 Substrate solutions

All of the substrates (Na formate, Na acetate and methanol) which were used for the cultivation experiments were added to the clarified rumen fluid (Section 2.22.1) prior to addition to the RM02 media. These substrates were prepared in distilled water to add to the BY⁺ media. The substrates used for the characterization studies were prepared individually or in different combinations.

The substrates for RM02 media were prepared by adding the relevant substrate/substrates to 100 ml rumen fluid along with 2 g yeast extract (2% [w/v]). This mixture was bubbled with N₂ gas for 15 min then transferred to N₂-flushed sterile serum

vial (BellCo) through a 0.22- μ m pore size Millex GP sterile filter (Millipore) using a sterile syringe and needle. For 100 ml preparation 2 ml vitamin-10 concentrate was also added through a 0.22- μ m pore size Millex GP sterile filter using a sterile syringe and needle. For each tube containing 9.5 ml of media, 0.5 ml of the substrate solution was added.

Substrates for the BY⁺ medium and for the characterization experiments were prepared by mixing substrate/substrates with distilled water at relevant concentrations. The solutions were boiled under O₂-free 100% CO₂ and cooled in an ice bath while being bubbled with 100% CO₂. Once the solutions were cooled, they were then dispensed into serum vials (Bellco) while being gassed with 100% CO₂, and the tubes capped with blue butyl rubber stoppers and an aluminium caps. These serum vials were sterilized by autoclaving for 20 min at 121°C and stored in the dark. For the substrates containing methanol, ethanol or isopropanol, these were added after the solution boiled under O₂-free 100% and cooled down and the containers covered with aluminium foil while bubbling with CO₂, to stop the evaporation of these substances.

2.24 Gram staining

Gram staining was performed according to Hucker's modification of the Gram stain method (Gephart *et al.*, 1981). Cultures were transferred to a glass microscope slide and air dried. The air dried cultures were fixed by passing them briefly through the flame without exposing the dried cells to the flame. Crystal violet staining reagent (2% [w/v] crystal violet, 20% [v/v] ethanol, 0.8% [w/v] ammonium oxalate) was added to the cells and washed off with tap water after 1 min. Gram's iodine solution (0.33% [w/v] iodine, 0.67% [w/v] potassium iodide) was then added to the cells and was washed off by tap water after 1 min. The cells were washed by 95% (v/v) of ethanol for 10 s and drained well. Safranin solution (2.5% [w/v] safranin O in 95% [v/v] ethanol) was used as the counter stain for 30 s and the slides washed with tap water. After draining the water and lightly blotted dry with a paper towel, the slide was air dried and the cells observed at 1000 \times magnification using a DM2500 microscope (Leica Microsystems, Wetzlar, Germany).

2.25 Phase contrast microscopy

Agarose-coated slides were prepared and then a small amount of culture added to the slides as described by Pfennig & Wagener (1986). Phase contrast and fluorescent photomicrographs were taken using a DM2500 microscope (Leica Microsystems, Wetzlar, Germany) with a 100× oil phase contrast objective, and the images captured digitally using Leica Application suite software.

2.26 Electron microscopy

Electron microscopic examination (Philips CM10 Transmission Electron Microscope with SIS Morada high-resolution digital imaging, Netherlands) was carried out at the Manawatu Microscopy and Imaging Centre (MMIC), Massey University, Palmerston North, New Zealand.

2.26.1. Negative staining

Cells were concentrated by centrifuging (4500 g, 5 min) and a drop of concentrated cells placed on formvar-coated copper grids and left for 2-6 min. The grids were drained with filter paper. A drop of 2% uranyl acetate (in water) was put on parafilm and the grids were placed on that and left for 6-10 min. The grids were drained well again and allowed to dry. The whole cells were then observed by electron microscopy at 60 Kv.

2.26.2. Transmission electron microscopy (TEM)

Thin sections of cells were prepared for transmission electron microscopy by pelleting by centrifugation (4500 g, 5 min) and then fixing the cell pellet with 3% (v/v) glutaraldehyde in 0.1 M Na and K phosphate buffer (pH 7.2) and letting it stand for 2 h at room temperature. This suspension was harvested by centrifugation (4500 g, 4 min) and drained well. To prevent the cell pellets falling apart during further processing, 3 drops of 20% (w/v) bovine serum albumin (BSA) was added to the pellet. This mixture was centrifuged again for 4 min at 4500 g. The BSA was then coagulated by adding 1 drop of 25% (w/v) glutaraldehyde (in 0.1 M Na and K phosphate buffer) to the top of pellet and the excess BSA discarded. The pellets were then cut into thin pieces and fixed again with 3% (v/v) glutaraldehyde in 0.1 M Na and K phosphate buffer (pH 7.2) to ensure all of the BSA was coagulated. The cells were rinsed three times (5, 10 and 15

min) in Na and K phosphate buffer and fixed in 1% (w/v) osmium tetroxide (OSO₄) in the same buffer for 1 h at room temperature. The cells were again rinsed three times (5, 10 and 15 min) in a fresh Na and K phosphate buffer before being dehydrated in increasing concentrations of acetone consisting 25% (v/v), 50% (v/v), 75% (v/v), 95% (v/v), 100% (v/v), 100% (v/v) and 100% (v/v) for 15 min at each step. Following dehydration, the cells were infiltrated with 50/50 acetone/resin (ProSciTech, Qld, Australia) (v/v) on a stirrer overnight to allow the acetone to evaporate slowly. Cells were then embedded in fresh resin (100%) for 8 h, overnight and then 8 h on a stirrer. The resin was polymerized in an oven at 60°C for 48 h. The embedded cells in the blocks were sectioned with a diamond knife on an Ultra-microtome (Leica Microsystems, Wetzlar, Germany). These ultra-thin sections were collected onto copper grids and double stained with saturated uranyl acetate in 50% (v/v) ethanol for 4 min and lead citrate for 4 min. The stained sections were observed by electron microscopy at 60 kV.

2.27 G+C mol % analysis of DNA using an HPLC method

The mol% of G+C of the DNA of isolate ISO3-F5 was obtained using a high-performance liquid chromatography (HPLC) method. Briefly, 3 µg of DNA was digested using 3 µl of DNA degradase (Zymo Research Corp, CA, USA) containing 7.5 µl of 10× DNA degradation reaction buffer at 37°C for 3 h in an orbital incubator (Stuart Scientific SI50, USA) at 100 rpm. Digested samples were then pipetted into an Ultra free MC HV spin column (0.45 µm: Millipore: ThermoFisher scientific) and centrifuged for 5 min at 100× g. DNA concentrations of the samples were re-quantified using a Nanodrop and the volumes adjusted to 75 µl using DNA-free water.

Samples (75 µl) were pipetted into 250 µl glass flat-bottomed HPLC vial inserts and loaded into 8 mm amber vials. The column was Luna 5 µ C8 10 nm, 150 × 4.6 mm (Grace Davison Discovery Sciences, Auckland, New Zealand), and the column temperature was maintained at 6°C (CTO10AS vp Shimadzu column oven, Shimadzu Oceania Ltd). The flow rate was 1 ml per min, with 50 µl injection volume. The HPLC used was a Shimadzu LC10vp, with a LC10AV UV/Vis detector. The concentrations of G+C were then calculated using standard curves.

Standard curves were generated by the following method. Known standards were measured and standard curves generated to ensure consistency across batches. Commercially available nucleotide standards (Millennium Science, New Zealand) for deoxythymidine monophosphate (dTMP), deoxycytosine monophosphate (dCMP), deoxyadenosine monophosphate (dAMP) and deoxyguanosine monophosphate (dGMP) were made up to concentrations of 0.0015, 0.015 and 0.15 mmol/L, spanning the expected concentration ranges of the samples. Each nucleotide standard was run individually to ensure retention times were the same as those observed in previous HPLC runs. A mixed sample of all four nucleotides was then used to generate calibration curves for each sample batch.

2.28 Measuring methanogen growth

2.28.1. Gas chromatography

Methanogen growth was monitored by measuring methane concentrations in the headspace of tubes by gas chromatography. Head space gas (0.3 ml) from the culture tube was taken using a sterile syringe and needle and injected into an Aerograph 660 (Varian Associates, Palo Alto, CA, USA) fitted with a Porapak Q 80/100 mesh column (Waters Corporation, Milford, MA, USA) and a thermal conductivity detector operated at 100°C. The column was operated at room temperature with N₂ as the carrier gas at 12 cm³/min.

2.28.2. Spectrophotometer

Growth of methanogens was followed by measuring the culture density at 600 nm by inserting the culture tubes directly into an Ultrospec 1100 pro UV/Vis spectrophotometer (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

2.29 Long term preservation of cultures

2.29.1. Preservation with dimethyl sulfoxide (DMSO)

DMSO solution contained salt solution A (Section 2.22.1) (17% [v/v]), salt solution B (Section 2.22.1) (17% [v/v]), distilled water (16% [v/v]), dimethyl sulfoxide

(50% [v/v]), NaHCO₃ (0.5 g/100 ml [w/v]), 2 drops of resazurin solution (0.1% [w/v]) and 0.05 g of L-cysteine·HCl (0.05 g/100 ml [w/v]). All the components except L-cysteine·HCl were mixed thoroughly. This solution was boiled under O₂-free 100% CO₂ and cooled in an ice bath while it was bubbled with 100% CO₂. Once the solution was cooled, the L-cysteine·HCl was added. This solution was then dispensed into Hungate tubes while being gassed with 100% CO₂, and the tubes capped with black butyl rubber seals and perforated plastic caps, with a headspace of 100% CO₂. These tubes were sterilized by autoclaving for 20 min at 115°C. This anaerobic dimethyl sulphoxide solution was injected into the viable culture to a final concentration of 10% (v/v) (5% DMSO in final mixture), mixed well and the tubes pressurised with H₂/CO₂. This culture was kept at 4°C for at least 4 h and transferred to an -80°C freezer.

2.29.2. Preservation with glycerol

The glycerol solution for preserving cultures was prepared anaerobically by adding 30 ml of glycerol, 30 ml of rumen fluid (Section 2.22.1), 17 ml of salt solution A (Section 2.22.1), 17 ml of salt solution B (Section 2.22.1), 0.5 g of NaHCO₃, 1 drop of rezazurin (0.1% [w/v]) and 0.05 g of L-cysteine·HCl. This solution was dispensed into Hungate tubes (10 ml/tube) and autoclaved for 20 min at 121°C. To preserve cultures of anaerobes, 3 ml of this glycerol mixture was added anaerobically to 10 ml of live culture. This was then frozen at -20°C for 4 h and transferred to -80°C.

Clones of *Escherichia coli* were selected and grown in LB broths overnight and sterile glycerol added to 30% (v/v). These cultures were frozen at -20°C for 4 h and then transferred to a -80°C freezer.

2.30 Re-generating frozen cultures

Frozen cultures of methanogens were warmed quickly to 39°C and inoculated (0.5 and 1 ml) into 10 ml of appropriate media (BY, BY⁺ or RM02). These inoculated tubes were pressurised with H₂/CO₂ and incubated at 39°C with shaking.

Clones of *Escherichia coli* were regenerated by streaking them onto pre-warmed (37°C) LB plate or adding small amount of clones into pre-warmed (37°C) LB broth media. The LB plate/LB broth was then kept at 37°C overnight.

Chapter 3

Selection of primers for the molecular-based studies of rumen methanogens

3.1 Introduction

The diversity of the microbial community in the rumen environment has become a great interest in the last few years as the methane produced in the rumen is a potent greenhouse gas. Methane contributed 30.3% to New Zealand's anthropogenic greenhouse gas total emission in 2008 (Ministry for the Environment, 2009). Ninety six percentage (96%) of this methane was generated by enteric fermentation, nearly all of this from the rumens of farmed ruminants. The methanogens, which account for a minor proportion (0.5-3.0%) of the total rumen microbes, produce this methane as a part of their energy metabolism (Hedderich & Whitman, 2006; Wolin *et al.*, 1997). Formation of methane is the major way to eliminate the H₂, which is the by-product of feed fermentation in the rumen. Thus, methanogens in the rumen play a major part in improving the efficiency of the conversion of complex carbohydrates to fermentable sugars which is in part due to the effective disposal of H₂, and they also contribute substantially to the GHG emission.

Cultivation methods to study the rumen methanogens are not always possible as this group of microbes are nutritionally and culturably fastidious (Chaban *et al.*, 2006; Garcia *et al.*, 2000). Due to this constraint, to explore the diversity of methanogens in the rumen, culture-independent molecular approaches have become indispensable. Molecular techniques use the nucleic acids extracted directly from the environment. Subsequently, the marker genes, such as 16S rRNA and *mcrA* genes are used to determine the methanogen diversity in that environment. The 16S rRNA gene has functional regions that are highly conserved as well as regions that are semi-conserved at the phylum level or highly diverse even among closely related genera which help to determine the phylogenetic relatedness of organisms (Ludwig & Schleifer, 1994; Woese, 1987). To study the methanogens in the environmental samples, archaeal 16S rRNA gene phylogenetic markers are usually used. This is because designing 16S rRNA gene markers that are specific to methanogens is quite difficult due to their high phylogenetic diversity (Banning *et al.*, 2005).

The other important target for the methanogens, the *mcrA* gene, is the gene encoding α subunit of methyl-coenzyme M reductase (MCR) (Hales *et al.*, 1996; Luton *et al.*, 2002; Springer *et al.*, 1995). Methyl-coenzyme M reductase is one of the key enzymes of methanogenesis, which catalyzes the final step of methane formation and is unique to methanogens (Friedrich, 2005), except for methane oxidising Archaea (Hallam *et al.*, 2003). Studies conducted on methanogen diversity in landfills using 16S rRNA and *mcrA* gene fragments have confirmed that both phylogenies are similar and that the *mcrA* gene can be used as a phylogenetic marker to study methanogens (Luton *et al.*, 2002). The 16S rRNA gene markers used in the study of rumen methanogens are common to all Archaea, but the *mcrA* gene marker is specific to methanogens.

One potential concern in using these targets for PCR-based molecular techniques is the possibility of PCR bias. One factor due to the primers themselves stands out as being very important for faithful amplification of the original populations. PCR bias can lead to the preferential amplification of certain templates (PCR selection) (Polz & Cavanaugh, 1998; von Wintzingerode *et al.*, 1997), the lack of amplification of certain groups (Skillman *et al.*, 2006; Pei *et al.*, 2008) and different amplification efficiencies for different environmental samples (Juottonen *et al.*, 2006). There have been a number of archaeal 16S rRNA and methanogen *mcrA* gene primers designed to target different regions of these genes to study rumen methanogens (Table 1.2). However, it's not sure whether the resultant PCR products by these primers represent the original samples. A few studies have compared different primers targeting the 16S rRNA and *mcrA* genes for the suitability to represent the methanogen diversity of selected environment (Friedrich, 2005; Juottonen *et al.*, 2006; Pei *et al.*, 2008; Skillman *et al.*, 2006; Tajima *et al.*, 2001). Despite this progress, more studies are still required to check the primer suitability for environmental samples to study the methanogen diversity.

In this thesis, methanogen diversity of New Zealand farmed-ruminants was determined using molecular techniques, and then cultivation techniques used to isolate previously uncultured rumen methanogens. Although there are other factors (e.g. DNA extraction methods, PCR inhibitors) that can also affect the determination of methanogen diversity in environmental samples, the selection of suitable primers was considered to be an important first step in this thesis. There were a number of 16S rRNA and *mcrA* gene primers published which targeted different regions of the

respective genes. However, in this study only a few 16S rRNA and *mcrA* gene primers were selected and checked for their suitability to use on the rumen sample. Small clone libraries were constructed using a single rumen sample with selected primers, and the different groups of methanogens identified and their relative proportions were considered in choosing suitable primers for molecular ecological studies of rumen methanogens to use in this thesis.

3.2 Materials and Methods

3.2.1 Sample collection

Whole rumen contents consisting of fluids and solids were collected from a fistulated mature non-lactating dairy cow (C2). This cow was fed with 8.4 kg DM per day of lucerne (*Medicago sativa*) silage (CHaffHage) and barley meal (Section 2.2). It had unlimited access to water at all times. The sample was collected via the rumen fistula 2 h after the morning feed and was immediately frozen at -80°C. This frozen sample was then freeze-dried, homogenized and stored at -80°C until DNA was extracted.

3.2.2 Total DNA extraction and PCR

Total DNA from the collected rumen sample was extracted from 100 mg of ground freeze-dried rumen sample, using a QIAamp DNA stool mini kit (Qiagen) according to the manufacturer's protocol and stored at -20°C until its usage (Section 2.3.1). PCR was performed with selected primers (16S rRNA and *mcrA* gene) using this extracted DNA and clone libraries were constructed using the PCR products. DNA was also extracted from pure cultures of methanogens (*Methanobrevibacter ruminantium* DSM 1093, *Methanospirillum hungatei* DSM 864, *Methanobrevibacter* sp. SM9, *Methanosarcina barkeri* CM1, *Methanobacterium formicicum* BRM9, *Methanobrevibacter* sp. NT7, *Methanomicrococcus blatticola* DSM 13328, *Methanobrevibacter thaueri* DSM 11995 and *Methanobrevibacter* sp. 31A) to validate the newly designed or modified primers and, to include the sequences of these organisms into the phylogenetic trees as reference organisms. DNA from pure cultures was extracted using the FastDNA kit and the FastPrep instrument (QBiogene) according to the manufacturer's protocol (Section 2.3.2a).

All of the PCR amplifications were performed in Hybaid Px2 thermal cyclers (ThermoElectron) (Section 2.6) and the resulting PCR products analyzed using agarose gel (1.5% [wt/vol]) electrophoresis (Section 2.7).

3.2.3 Primers used for the construction of clone libraries

Two newly designed archaeal 16S rRNA gene primers (ARC8f and ARC1510r) and one modified *mcrA* gene primer (MCRmf) were validated and checked for the suitability for the amplification of methanogens from the rumen sample. MCRmf was modified from the published primer MCRf (Springer *et al.*, 1995). The other primers used in this study were selected from published studies. The primers used to construct 16S rRNA and *mcrA* gene clone libraries in the comparison study are shown in Table 3.1. More details of these primers are given in Tables 2.2 and 2.3 in Chapter 2.

Table 3.1. Primer pairs used to construct clone libraries for the comparison study.

Name of the primer pair	Primers
16S rRNA gene	
P1	109f/ 915r
P2	630f / 803r
P3	1af / 1100r
P4	109f /1386r
P5	915af /1386r
P6	86f /1340r
<i>mcrA</i> gene	
M-P1	ML1 /ML2
M-P2	ME1 /ME2
M-P3	MCRf /MCRr
M-P4	MCRmf/ MCRr

3.2.4 Clone library construction, chimera detection and phylogeny

The PCR products were ligated into the pCR 2.1 TOPO TA cloning vector (Invitrogen) according to manufacturer's instructions, and then transferred into TOP10

chemically competent *Escherichia coli* (Invitrogen), according to the manufacturer's instructions (Section 2.12). Clones were randomly selected and sequencing was performed either by the Allan Wilson Centre Genome Sequencing Service (Massey University, New Zealand) or Macrogen Inc. (Seoul, Republic of Korea). Results of sequences were obtained as ABI files and then the sequences were edited using MEGA 4.0 (Tamura *et al.*, 2007). The online chimera detection program Bellerophon (Huber *et al.*, 2004) was used to identify chimeric sequences.

Phylogenetic analyses of the sequences of partial fragments of 16S rRNA and *mcrA* genes were performed using MEGA 4.0 software (Section 2.14). Nucleotide sequences were used for the construction of 16S rRNA gene phylogenetic trees and deduced amino acid sequences were used for the construction of *mcrA* gene phylogenetic trees.

3.2.5 Analysis of clone libraries and the phylogenetic trees

The Shannon diversity (Shannon & Weaver, 1963) and Simpson's indices (Simpson, 1949) were estimated using the proportions of different methanogens groups in each clone library by Version 2.01 of the PAST (PAleontological STatistics) software package (<http://folk.uio.no/ohammer/past>) (Hammer *et al.*, 2001). With the same software package, the Shannon diversity *t* test was also employed to compare the differences between clone libraries constructed using different primer pairs.

3.2.6 Nucleotide sequences of clones and cultures

Details of the clone sequences obtained from each of these 16S rRNA and *mcrA* gene clone libraries and the sequences obtained from pure cultures of methanogens are given in Appendix 1.

3.3 Results

3.3.1 Variations in the archaeal community structure in the clone libraries from different 16S rRNA gene primer pairs

The newly designed archaeal primers ARC8f and ARC1510r were validated using the DNA obtained from pure cultures of different methanogens (Table 3.2) and the rumen sample collected from sheep fed winter grass. During the validation process DNA from pure cultures of methanogens were successfully amplified by this primer pair. However, when DNA from the rumen sample was used the resultant product contained some bacterial sequences. As such, this primer pair was omitted from the comparison study as it was not specific to Archaea. However, it can still be used to obtain long fragments (1500 bp) of 16S rRNA gene from pure cultures of Archaea.

Table 3.2. Pure cultures that were used to validate the primer pair ARC8f and ARC1510r.

Pure cultures	Amplification of correct size
<i>Methanobrevibacter ruminantium</i> DSM 1093	Yes
<i>Methanospirillum hungatei</i> DSM 864	Yes
<i>Methanobrevibacter</i> sp. SM9	Yes
<i>Methanosarcina barkeri</i> CM1	Yes
<i>Methanobacterium formicicum</i> BRM9	Yes

Six different published archaeal 16S rRNA gene primer pairs were ultimately used in the comparison study (Table 3.1). The number of chimeric sequences detected by each of this primer pair is given in Table 3.3. The major archaeal groups identified by these primer pairs in the present comparison study and their percentage contributions are given in Figure 3.1. The five archaeal groups identified by these clone libraries were the *Methanobrevibacter gottschalkii* group, the *Methanobrevibacter ruminantium* group, the *Methanosphaera* spp. group, the *Methanomicrococcus* spp. group and the Rumen Cluster C (RCC) group. The RCC group consists of uncultured Archaea that have frequently been identified in several rumen clone libraries.

Table 3.3. The number of chimeric sequences detected in each of the primer pair used to construct clone libraries in this chapter.

Primer pair	Total sequences	Number (percentage) of chimeric sequences
16S rRNA gene		
P1 (109f/ 915r)	50	3 (6%)
P2 (630f / 803r)	64	5 (8%)
P3 (1af / 1100r)	70	9 (13%)
P4 (109f /1386r)	55	8 (15%)
P5 (915af /1386r)	50	4 (8%)
P6 (86f /1340r)	51	2 (4%)
<i>mcrA</i> gene		
M-P1(ML1 /ML2)	51	0
M-P2 (ME1 /ME2)	57	8 (14%)
M-P3 (MCRf /MCRr)	68	0

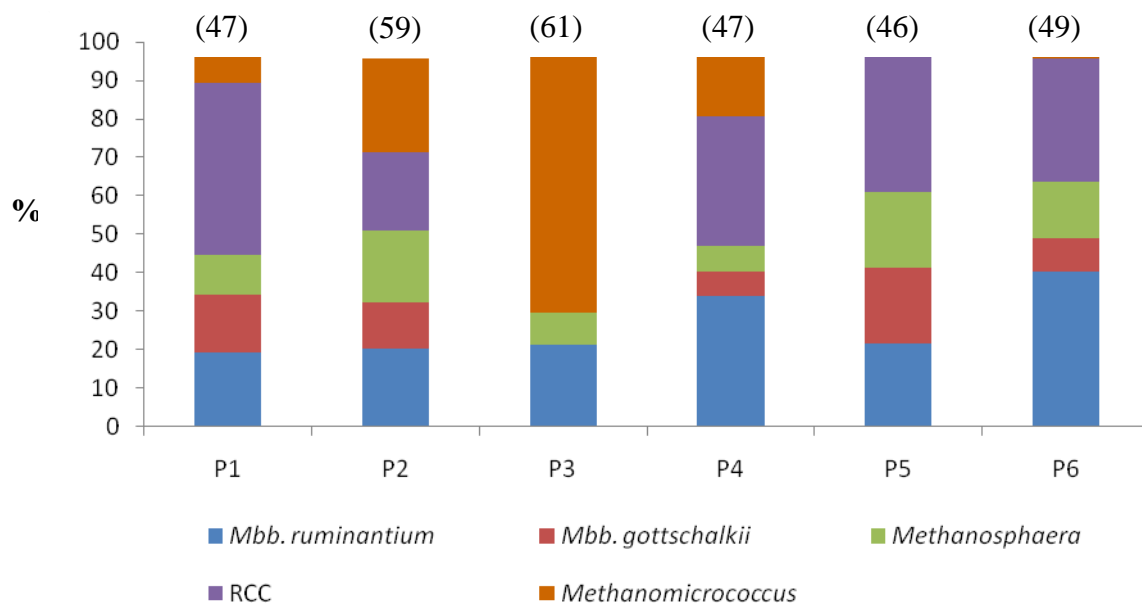


Figure 3.1. The percentages of different archaeal groups identified by six different 16S rRNA gene primer pairs (P1-P6) from the rumen sample obtained from a cow (C2) fed with lucerne silage and barley meal. The total number of clones sequenced using each primer pair is given at the top of each bar within parentheses. Chimeric sequences (Table 3.3) were excluded.

The percentage contribution of the 5 archaeal groups in the different clone libraries (different primer pairs) varied considerably ($P = 7.04 \times 10^{-18}$ in χ^2 -test for differences). Since the number of clones per each clone library was small (about 50 clones), some variations in these clone libraries can be expected. However, according to Figure 3.1 some of the primer pairs showed very high variations (e.g, P3). Since the same DNA was used for all the clone libraries constructed, the major factor that could have caused these variations may be bias related to the primer pair used in the PCR. The primer pairs used in this comparison study targeted different regions in the 16S rRNA gene and the fragment lengths of the resultant PCR products were also different. Primer pairs P1 (≈ 805 bp), P3 (≈ 1099 bp), P4 (≈ 1277 bp) and P6 (≈ 1254 bp) amplified longer fragments, while the primer pairs P2 (≈ 172 bp) and P5 (≈ 471 bp) amplified shorter fragments of the 16S rRNA gene. Short PCR products are optimal for some of the molecular-based techniques such as real-time PCR and DGGE.

Primer pairs P1, P2, P4 and P6 detected sequences from all of the five archaeal groups mentioned before. Primer pair P5 did not amplify any sequences from *Methanimicrococcus* spp. and primer pair P3 did not amplify any sequences from the *Methanobrevibacter gottschalkii* or RCC groups in this study. Primer pair P3 showed more variation in number and types of different groups obtained compared to the other primer pairs. The clone library of primer pair P3 was dominated by *Methanomicrococcus* spp. (70.5%), while in other clone libraries the percentage of this group varied considerably from 4.3% (P6) to 28.8% (P2).

All of the six primer pairs detected the sequences from *Methanobrevibacter ruminantium* group and it varied from 19.2-40.4% in these clone libraries. The percentage contributions of this group in the clone libraries with primer pairs P1, P2, P3 and P5 were similar (19.2-21.7%), while P4 (34%) and P6 (40.4%) showed slightly higher percentages. As mentioned before, since the number of clones per clone library was small, some variations in the percentage contribution can be expected. The other archaeal group that was detected by all primer pairs was *Methanosphaera* spp., and this group varied from 6.4% to 19.6%. The percentage contribution of this group in the clone libraries generated with primer pairs P1, P2, P5 and P6 were similar (10.6-19.6%) with P3 (8.2%) and P4 (6.4%) showing lower values. The other 3 groups of methanogens such as *Mbb. gottschalkii* (6.4-19.6%), RCC (20.3-44.7%) and the

Methanimicrococcus spp. (4.3-70.5%) groups were not detected by one or more of the primer pairs. The *Methanimicrococcus* spp. were not amplified by primer pair P5 while both RCC and *Mbb. gottschalkii* groups were not amplified by the primer pair P3.

Two widely used diversity indices (Shannon diversity and Simpson's diversity indices) were employed to examine the choice of primer pair on the detection of archaeal diversity in the rumen (Figure 3.2). The diversities were in the same range for P1, P4, P5 and P6 libraries. The primer pair P2 showed slightly higher values for both indices and P3 showed the lowest value for both indices.

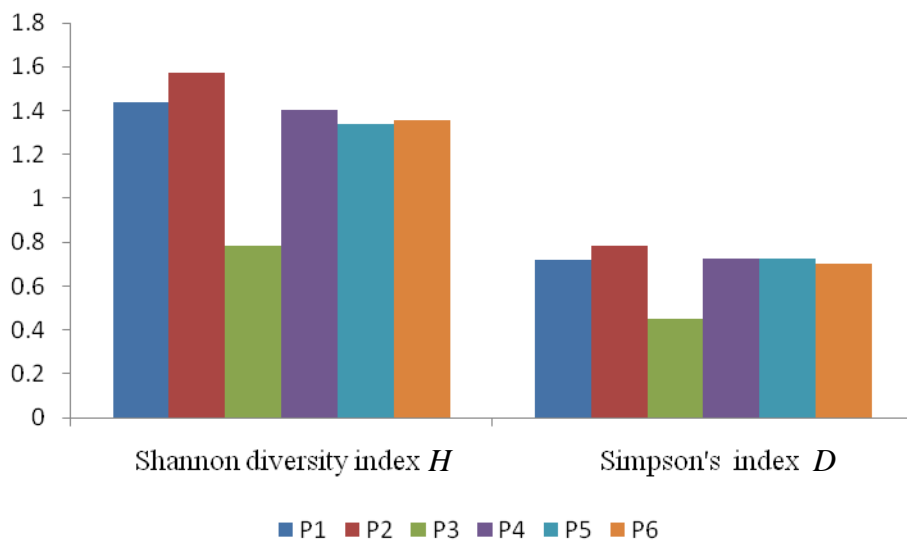


Figure 3.2. Diversity indices of rumen archaeal clone libraries constructed with six 16S rRNA gene primer pairs. Shannon diversity index and Simpson's diversity index were calculated from the number of clones per different archaeal groups in these clone libraries.

A Shannon diversity t test was employed to check the differences in their diversities between different primer pairs. Accordingly, the diversity of the clone library by primer pair P3 was different ($P < 0.00001$) from the diversity obtained by all other primer pairs (P1, P2, P4, P5 and P6). This was obvious as this primer pair detected only 3 groups of archaea and the clone library was dominated by the sequences of *Methanimicrococcus* species. The diversity of the clone library of primer pair P2 also

differed ($P < 0.05$) from the clone libraries of primer pairs P5 and P6. This difference was mainly due to the contribution of *Methanimicrococcus* sequences in these clone libraries. A very small percentage of *Methanimicrococcus* spp. was amplified by primer pair P6 (4.3%) and no *Methanimicrococcus* spp. were amplified by P5. Significantly, P3 amplified a considerable number of *Methanimicrococcus* species (28.8%). Overall, the presence of *Methanimicrococcus* species at different levels in different clone libraries caused more differences among clone libraries than the other archaeal groups.

3.3.2 Variation in the methanogen community structure in the clone libraries based on different *mcrA* gene primer pairs

Four different *mcrA* gene primers were used in this study (Table 3.1). The modified primer MCRmf and MCRr (M-P4) was validated using pure cultures of methanogens (*Methanospirillum hungatei* DSM 864, *Methanosarcina barkeri* CM1, *Methanobacterium formicicum* BRM9 and *Methanobrevibacter ruminantium* DSM 1093). When this modified primer pair was used to amplify the rumen samples, unidentified sequences which are partially similar to *mrtA* genes (74% similarity of *mrtA* sequence of *Methanosphaera stadtmanae*) were obtained. These sequences were highly similar to each other and distantly clustered with *mrtA* sequences of *Methanosphaera stadtmanae* and *Methanobacterium formicicum* (Figure 3.14). Occasionally a few *mcrA* sequences were also obtained with this primer pair (Table 3.3). Due to the dominance of these unidentifiable sequences, the modified primer pair M-P4 was omitted from the comparison study.

Table 3.4. The rumen samples used to validate the primer pair MCRmf and MCRr and the groups of sequences amplified

Rumen samples	Unidentified sequences	<i>Methanimicrococcus</i> species.	<i>Methanomicrobium</i> species	Total
Sheep (S4) Winter pasture	68 (98.5%)	0	1(1.5%)	69
Cow (C2) Silage	13 (92.9%)	1 (7.1%)	0	14

The different methanogen groups identified in the clone libraries constructed by three different published *mcrA* primer pairs are shown in Figure 3.3.

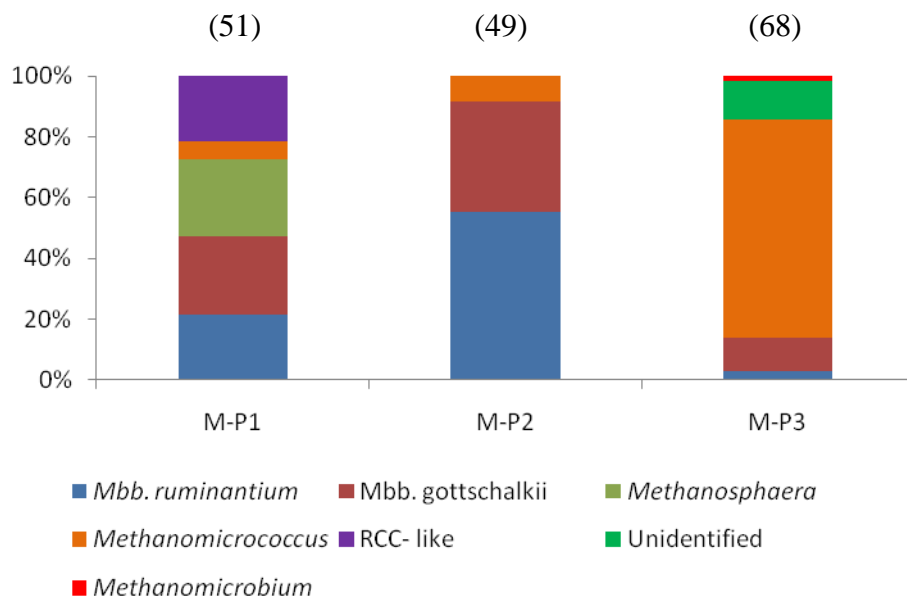


Figure 3.3. The percentages of different methanogen groups identified by three different *mcrA* gene primer pairs (M-P1 to M-P3) from the rumen samples obtained from a cow (C2) fed with lucerne silage and barley meal. The total number of clones in each primer pair is given at the top of each bar within parentheses.

All of the five methanogen groups identified by 16S rRNA gene primers were identified by the *mcrA* primers. The percentage contribution of different methanogen groups in the different clone libraries (different primer pairs) varied considerably ($P = 1.15 \times 10^{-28}$ in χ^2 -test for differences). A group of sequences amplified by primer pair M-P1 did not have any cultured relatives, but there were many clones in GenBank with similarity to these sequences. These sequences were assumed to be from the RCC group (RCC-like). Further studies were performed to confirm that these sequences are from the RCC group (Chapter 5). Only the primer pair M-P1 amplified RCC-like sequences and the percentage contribution of this group was comparable to the RCC group contribution in 16S rRNA gene clone libraries. The primer pair M-P1 amplified all five groups, which were amplified by 16S rRNA gene primers. Interestingly, the other two primers failed to amplify one or more groups of methanogens. The primer pair M-P2 amplified only three groups of methanogens. However, the primer pair M-P3 amplified five groups of methanogens and included a group of sequences which were

only partially similar to the *mrtA* gene. There were no clone sequences in GenBank with similarity to these unidentified sequences. These sequences clustered together and were highly similar to each other. In the clone library constructed with primer pair M-P3, the sequences contributed 13% of the total sequences. The clone library of M-P4 consisted exclusively from these sequences apart from one sequence from *Methanimicrococcus* spp. (Table 3.3).

The contribution of different methanogen groups in different *mcrA* gene clone libraries varied highly. The only methanogen group amplified by all four *mcrA* gene primer pairs (including the modified primer pair M-P4) was *Methanimicrococcus* species. However, its contribution to each clone library used in the comparison study varied from 5.8% to 71.9%. *mcrA* gene sequences belonging to the *Mbb. ruminantium* group (3.6 - 55.1%) and *Mbb. gottschalkii* group (12.5 - 36.7%) were also amplified by all three primer pairs (M-P1, M-P2 and M-P3). However, the percentage contribution of these groups in different clone libraries varied highly. The percentage contribution of different methanogens groups in the clone library with primer pair M-P1 was comparable to most of the 16S rRNA gene clone libraries. However, the clone library created by M-P2 was dominated by sequences from the *Mbb. ruminantium* and *Mbb. gottschalkii* groups. In contrast, the clone library created by primer pair M-P3 had these two groups at a very low percentage and this clone library was dominated by *Methanimicrococcus* sequences. Interestingly, one sequence which belongs to *Methanomicrobium* was amplified by M-P3. The sequences of *Methanomicrobium* species were not amplified by any other *mcrA* or 16S rRNA gene primer pairs. In addition, all of the sequences obtained from the *Methanosphaera* spp. group were found to belong to *mrtA* gene sequences which was only amplified by primer pair M-P1 (25.5%).

The diversity indices (Shannon and Simpson's diversity indices) of *mcrA* gene clone libraries from three different published *mcrA* gene primer pairs are shown in Figure 3.4. Both indices were high for the clone library of primer pair M-P1 and low for the clone library of primer pair M-P3. Primer pair M-P3 amplified four groups of methanogens but was dominated by *Methanimicrococcus* species. According to the Shannon diversity *t* test, the clone library from M-P1 was different ($P < 0.05$) from

clone libraries of M-P2 and M-P3. Only the clone library of M-P1 was comparable to the clone libraries constructed with the 16S rRNA gene (P1, P2, P4, P5 and P6).

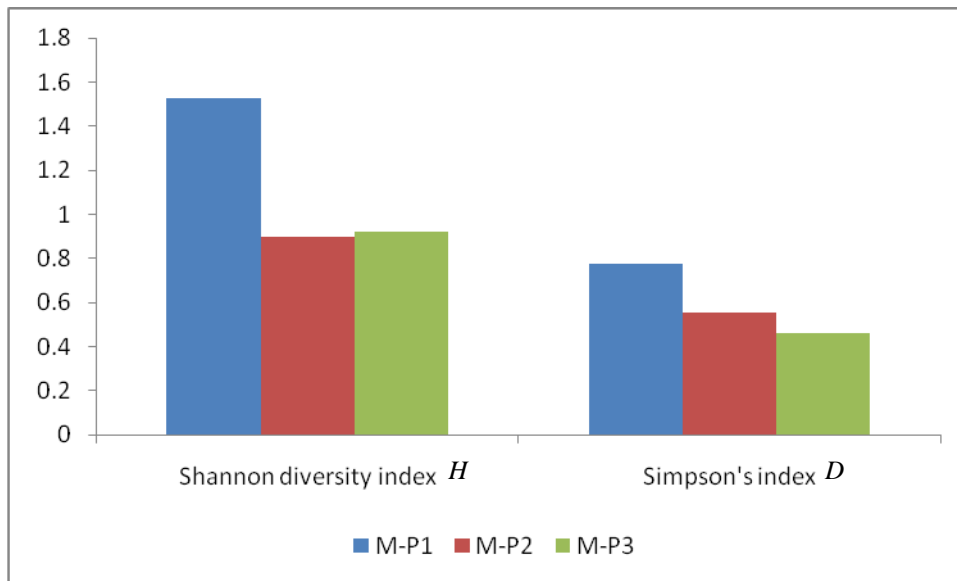


Figure 3.4. Diversity of the methanogen community in the rumen sample detected by three *mcrA* gene primer pairs. Shannon diversity index and Simpson's diversity index were calculated from contribution of this group in clone libraries.

3.3.3 Phylogenetic analysis of 16S rRNA partial genes

The clone sequences obtained with different 16S rRNA gene primer pairs were separately used to construct phylogenetic trees. Nucleotide sequences were used to construct these 16S rRNA gene phylogenetic trees (Figures 3.5-3.10).

In 16S rRNA gene clone libraries the five groups identified, clustered with expected reference sequences. The sequences belonged to the RCC group clustered away from other known methanogens and the subgroups within this group have high bootstrap values. This suggests that there may be multiple genera and species within this group. Only one 16S rRNA gene primer pair (1Af/1100Ar) did not amplify any sequences from this group, however, in all the other clone libraries this group was one of the dominant groups. More studies are warranted on this group and isolating a member from this group would be very helpful in understanding its characteristics and role in the rumen.

The sequences belonged to the *Methaniomicrococcus* species showed little variation, and they clustered away from the isolated *Methanimicrococcus blatticola* (Sprenger *et al.*, 2000). This suggests that the rumen *Methanimicrococcus* species are different from the isolated one.

The other three groups identified by the 16S rRNA gene primers in this comparison study (*Mbb. ruminantium*, *Mbb. gottschalkii* and *Methanosphaera* spp.) belong to the order *Methanobacteriales* and there were considerable variations found in the sequences within these groups. There has already been a number of rumen species identified within the *Mbb. ruminantium* and *Mbb. gottschalkii* groups. The sequences within these groups affiliated with the different cultured species such as *Methanobrevibacter* sp. NT7, *Methanobrevibacter* sp. SM9, *Mbb. ruminantium*, *Mbb. smithii*, *Mbb. thaueri*, *Mbb. gottschalkii* and *Mbb. millerae*. The sequences related to *Mbb. wolinii* were not amplified by most of the primers. Only one sequence clustered with the *Mbb. wolinii* reference sequence and this was amplified by primer pair P5.

There have been no *Methanosphaera* species isolated from rumen thus far, and only few sequences in this study clustered with the cultured human strain of *Methanosphaera stadtmanae*. Most of the clone sequences clustered separately from cultured *Methanosphaera stadtmanae*, and they showed high bootstrap values suggesting that rumen *Methanosphaera* species were different from isolated human strain. Also different subgroups with high bootstrap values suggest that there may be more than one species of *Methanosphaera* in the rumen.

3.3.4 Phylogenetic analysis of *mcrA* genes

The gene sequences obtained by different *mcrA* gene primer pairs were used to construct the phylogenetic trees from deduced amino acid sequences (Figure 3.11-3.14). The deeply branching *Methanopyrus kandleri* DSM 6324 (AF414042) was used as the out-group for these trees. All of the different groups identified by 16S rRNA gene primers were identified by *mcrA* gene primers and they clustered with the respective reference sequences. Since all the *mcrA* reference sequences were not available in the GenBank at the time of this study, some of the sequences were determined using pure cultures of methanogens such as *Methanobrevibacter* sp. SM9, *Methanobrevibacter* sp. NT7, *Methanobrevibacter* sp. 31A, *Methanobrevibacter thaueri* DSM11955 and

Methanimicrococcus blatticola DSM 13328. Details of the *mcrA* gene sequences of the pure cultures are shown in Appendix 1.

In the phylogenetic tree constructed using the sequences obtained from primer pair M-P1, all five groups which were identified in the 16S rRNA gene libraries clustered together with their reference sequences. The group of sequences that clustered separately from known methanogens were assumed to be the RCC group. This group has several sub-groups with high bootstrap values suggesting that it consists of different genera or species. This similar feature was observed in the 16S rRNA gene sequences of the RCC group. There were some sequences that clustered with *mrtA* gene sequences of *Methanosphaera stadtmanae*, and they are assumed to be from *Methanosphaera* species. None of the *mrtA* sequences from our sample clustered with cultured *Methanosphaera* species suggesting that rumen *Methanosphaera* species are different from the cultured human strain. Subgroups of *Methanosphaera* spp. have high bootstrap values, which suggests that more than one species of *Methanosphaera* exists in the rumen. This observation is consistent with the observation of 16S rRNA gene sequences of *Methanosphaera* species. Similarly, the *mcrA* gene sequences of rumen *Methanimicrococcus* species clustered separately from cultured *Methanimicrococcus* species suggesting that rumen *Methanimicrococcus* species are also different from cultured species.

The phylogenetic analysis of sequences from primer pair M-P2 found three groups of methanogens which clustered with their reference sequences accordingly (Figure 3.12). No *mrtA* sequences were amplified by this primer pair. In addition, there were no sequences representing uncultured groups when using this primer pair.

Interestingly, there was one sequence from *Methanomicrobium* spp. among the sequences of the primer pair M-P3 and this sequence clustered with the *mcrA* sequence of *Methanomicrobium mobile* (Figure 3.13). There were some unidentifiable sequences (not the RCC-like sequences) amplified by M-P3 (Figure 3.13). These sequences dominated the clone library constructed by the primer pair M-P4 (Figure 3.14). These sequences are distantly clustered with *mrtA* sequences of known methanogens. Unfortunately, there are not many *mrtA* sequences available in the GenBank yet. As such, not much information was obtained about these sequences. Their phylogenetic affiliation remains uncertain.

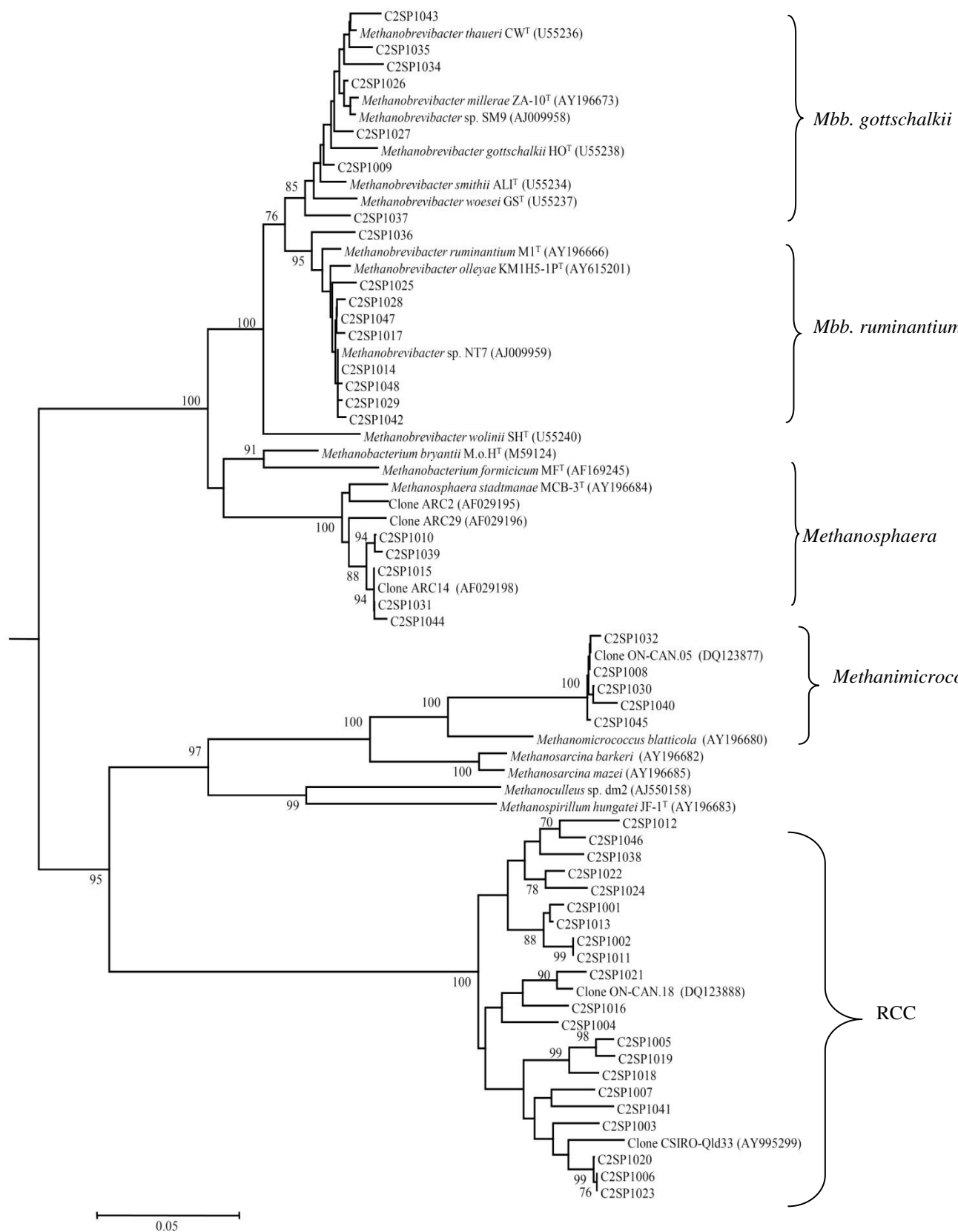


Figure 3.5. Inferred phylogenetic relationships between partial 16S rRNA genes (C2SP1001- C2SP1048) obtained by the primer pair 109f and 915r (P1) from the rumen sample of cow (C2) fed with lucerne silage and barley meal and reference 16S rRNA gene sequences. In the parentheses are the GenBank accessions numbers for reference sequences. The superscript ^T designates a type strain. The tree was constructed from an alignment of 760 nucleotide positions. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates 0.05 nucleotide substitutions per nucleotide position. The 16S rRNA genes of *Methanocaldococcus jannaschii* (GenBank accession M59126), *Methanococcus vannielii* (M36507) and *Methanothermococcus thermolithotrophicus* (M59128) were used as out-group sequences, and are not shown in the figure.

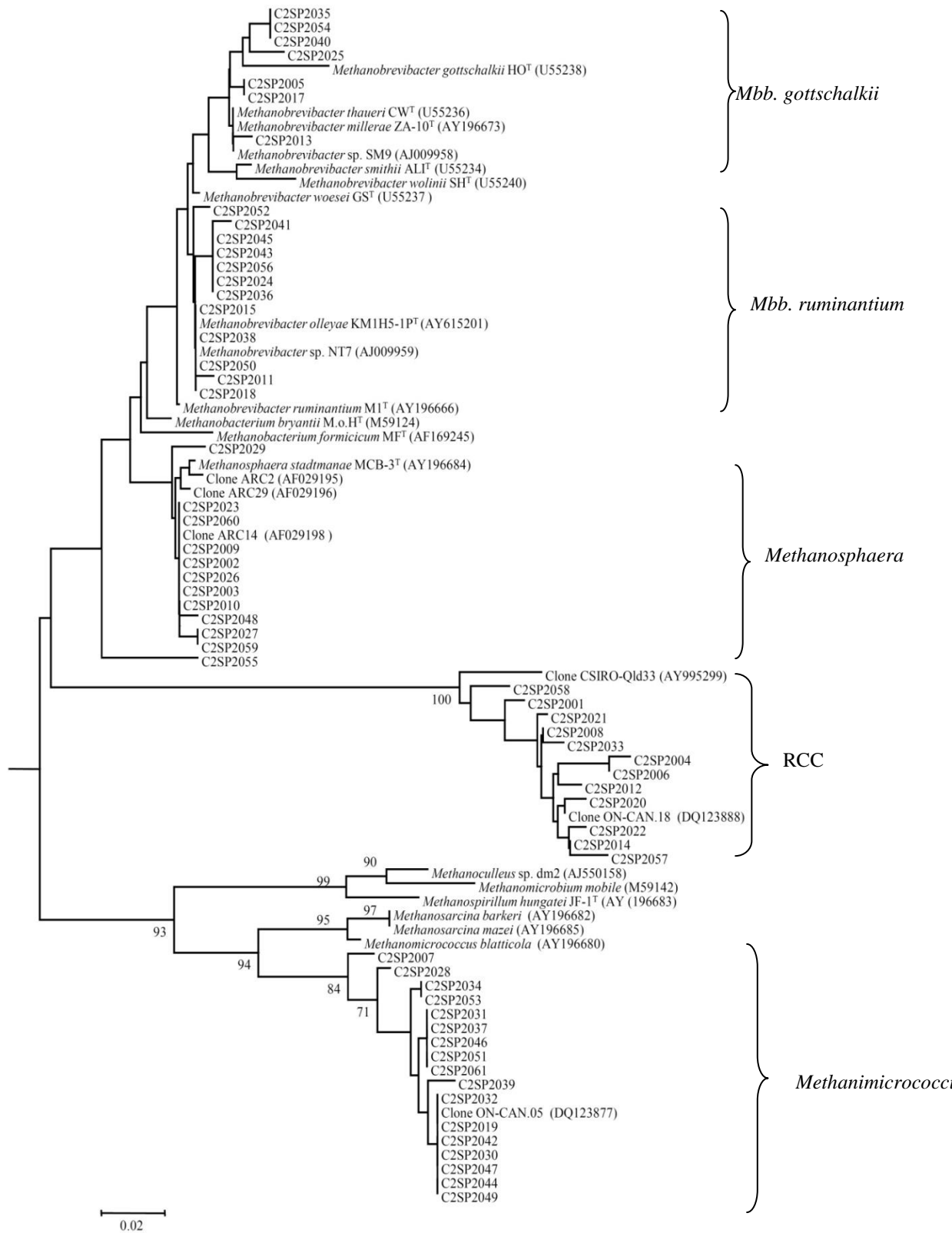


Figure 3.6. Inferred phylogenetic relationships between partial 16S rRNA genes (C2SP2001- C2SP2059) obtained by the primer pair 630f and 803r (P2) from the rumen sample of cow (C2) fed with lucerne silage and barley meal and reference 16S rRNA gene sequences. In the parentheses are the GenBank accessions numbers for reference sequences. The superscript ^T designates a type strain. The tree was constructed from an alignment of 185 nucleotide positions. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates 0.02 nucleotide substitutions per nucleotide position. The 16S rRNA genes of *Methanocaldococcus jannaschii* (GenBank accession M59126), *Methanococcus vannielii* (M36507) and *Methanothermococcus thermolithotrophicus* (M59128) were used as out-group sequences, and are not shown in the figure.

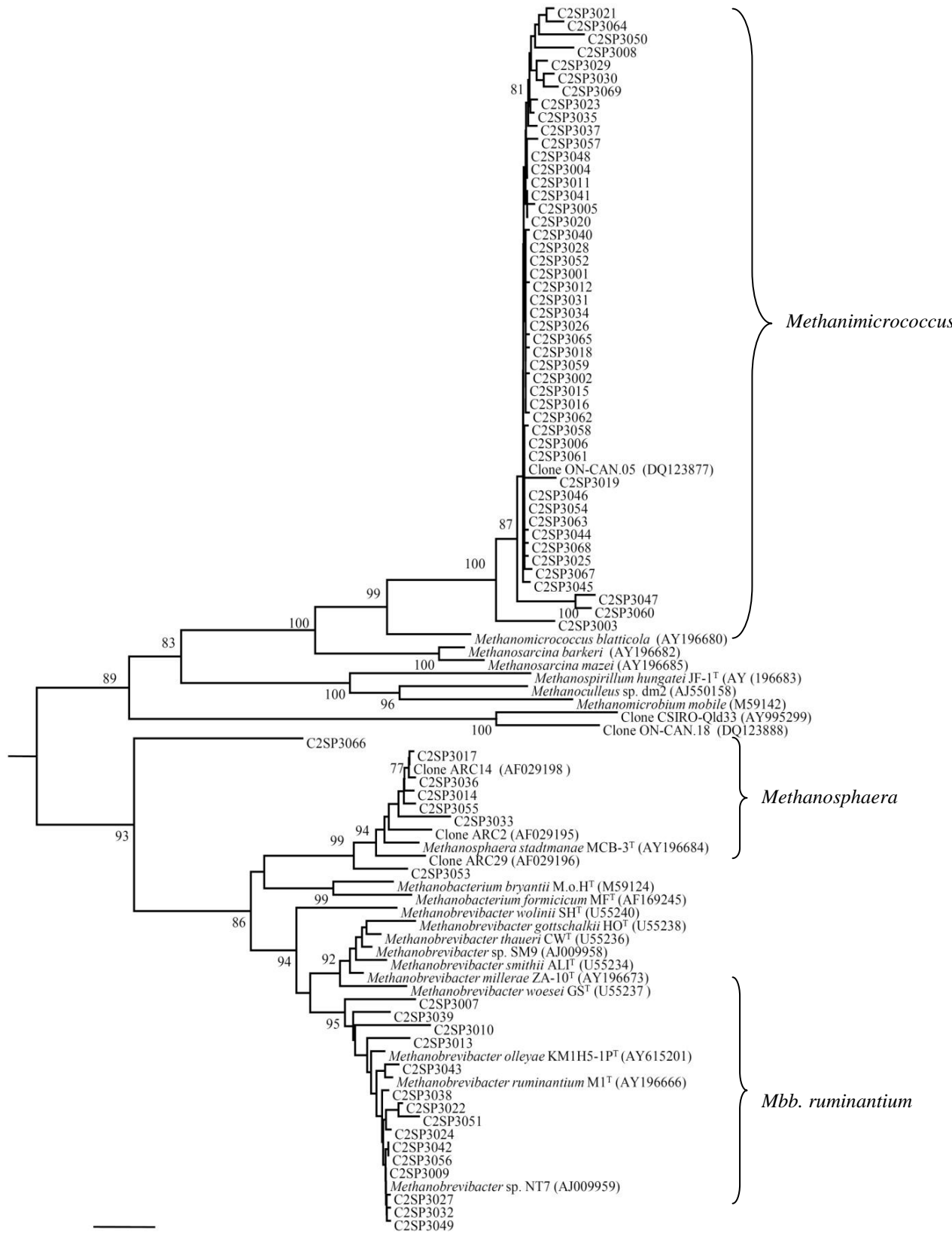


Figure 3.7. Inferred phylogenetic relationships between partial 16S rRNA genes (C2SP3001- C2SP3061) obtained by the primer pair 1af and 1100r (P3) from the rumen sample of cow (C2) fed with lucerne silage and barley meal and reference 16S rRNA gene sequences. In the parentheses are the GenBank accessions numbers for reference sequences. The superscript ^T designates a type strain. The tree was constructed from an alignment of 1022 nucleotide positions. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates 0.02 nucleotide substitutions per nucleotide position. The 16S rRNA genes of *Methanocaldococcus jannaschii* (GenBank accession M59126), *Methanococcus vannielii* (M36507) and *Methanothermococcus thermolithotrophicus* (M59128) were used as out-group sequences, and are not shown in the figure.

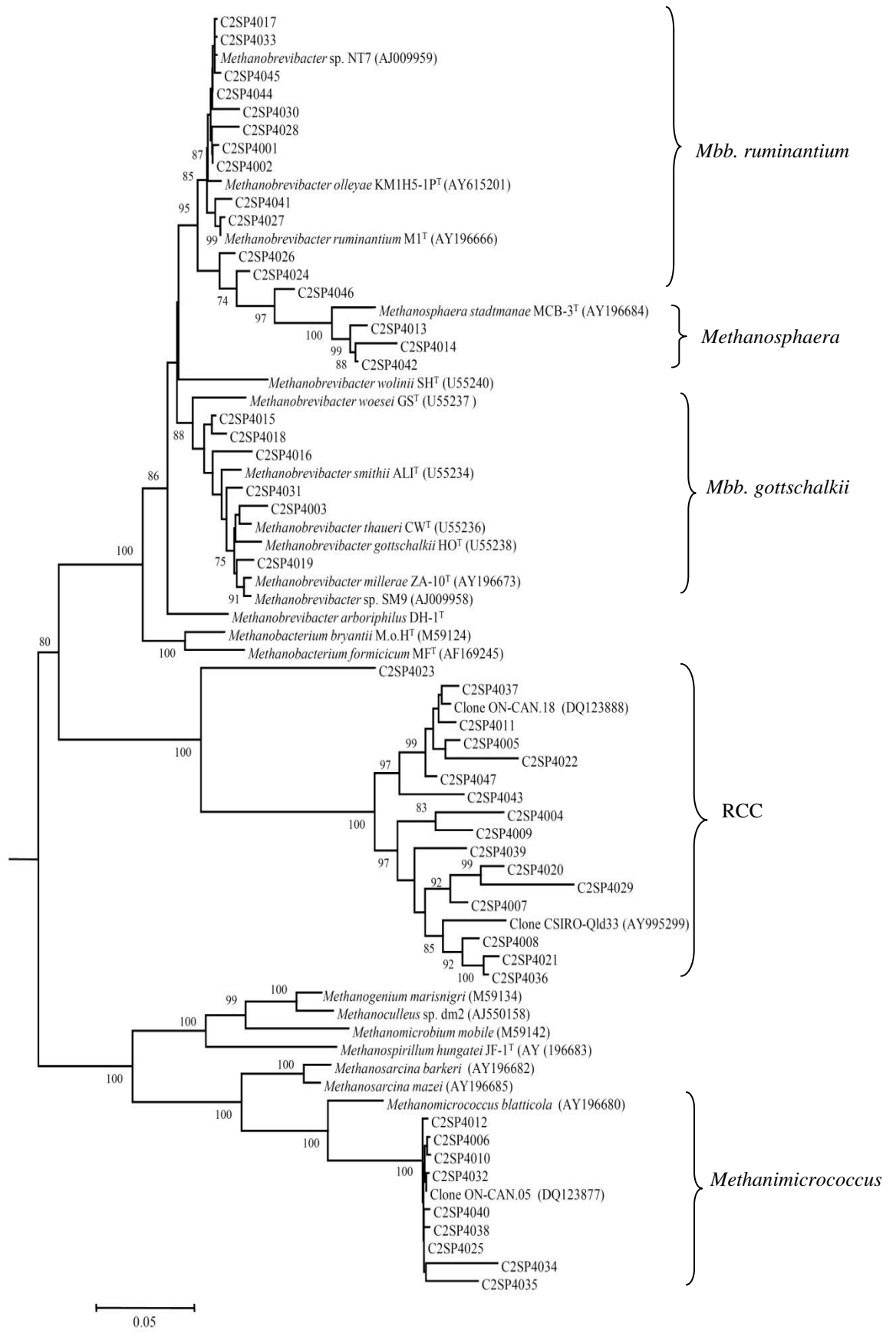


Figure 3.8. Inferred phylogenetic relationships between partial 16S rRNA genes (C2SP4001- C2SP4047) obtained by the primer pair 109f and 1386r (P4) from the rumen sample of cow (C2) fed with lucerne silage and barley meal and reference 16S rRNA gene sequences. In the parentheses are the GenBank accessions numbers for reference sequences. The superscript ^T designates a type strain. The tree was constructed from an alignment of 1180 nucleotide positions. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates 0.05 nucleotide substitutions per nucleotide position. The 16S rRNA genes of *Methanocaldococcus jannaschii* (GenBank accession M59126), *Methanococcus vanniellii* (M36507) and *Methanothermococcus thermolithotrophicus* (M59128) were used as out-group sequences, and are not shown in the figure.

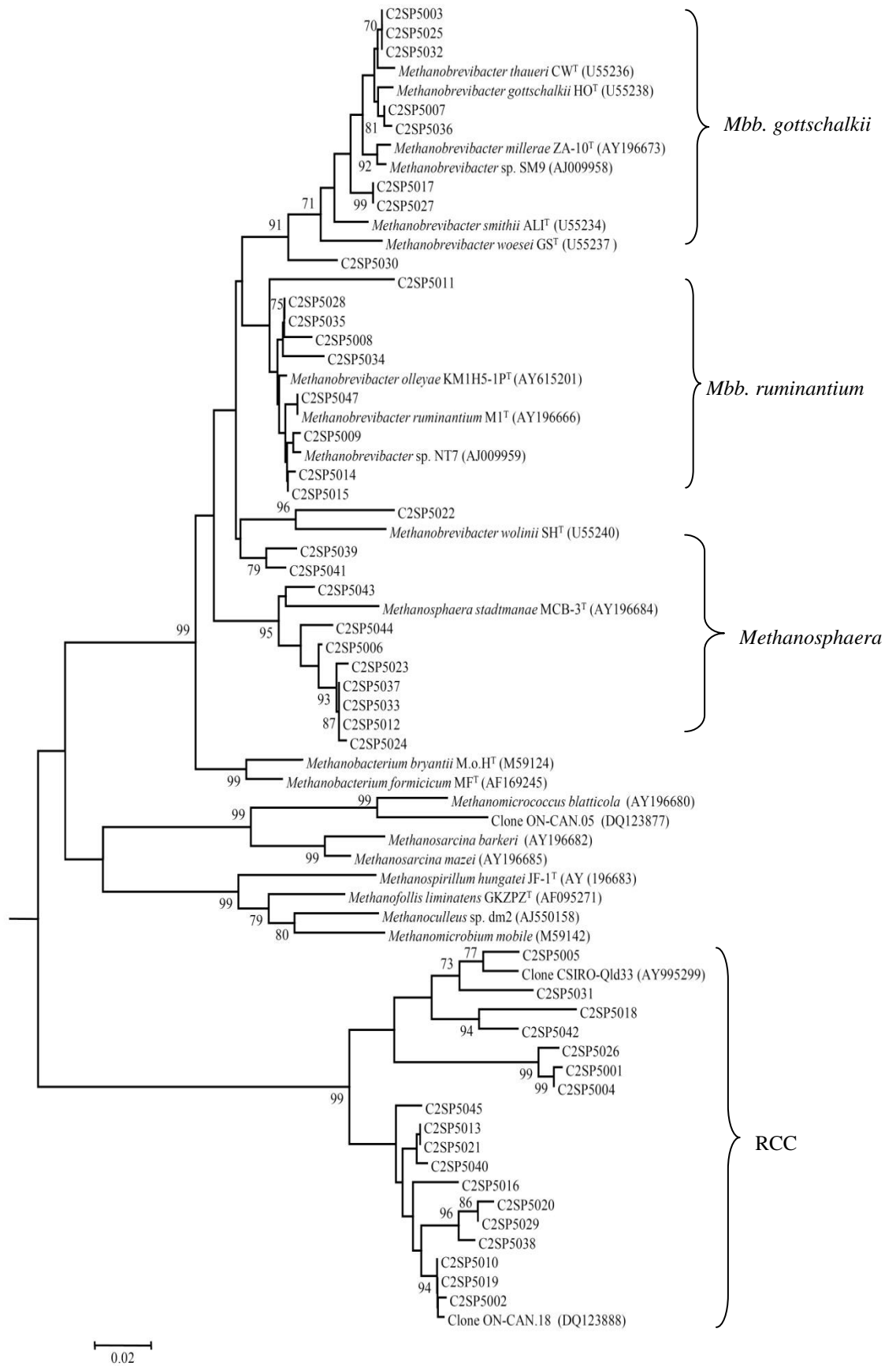


Figure 3.9. Inferred phylogenetic relationships between partial 16S rRNA genes (C2SP5001- C2SP5046) obtained by the primer pair 915af and 1386r (P5) from the rumen sample of cow (C2) fed with lucerne silage and barley meal and reference 16S rRNA gene sequences. In the parentheses are the GenBank accessions numbers for reference sequences. The superscript ^T designates a type strain. The tree was constructed from an alignment of 445 nucleotide positions. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates 0.02 nucleotide substitutions per nucleotide position. The 16S rRNA genes of *Methanocaldococcus jannaschii* (GenBank accession M59126), *Methanococcus vannielii* (M36507) and *Methanothermococcus thermolithotrophicus* (M59128) were used as out-group sequences, and are not shown in the figure.

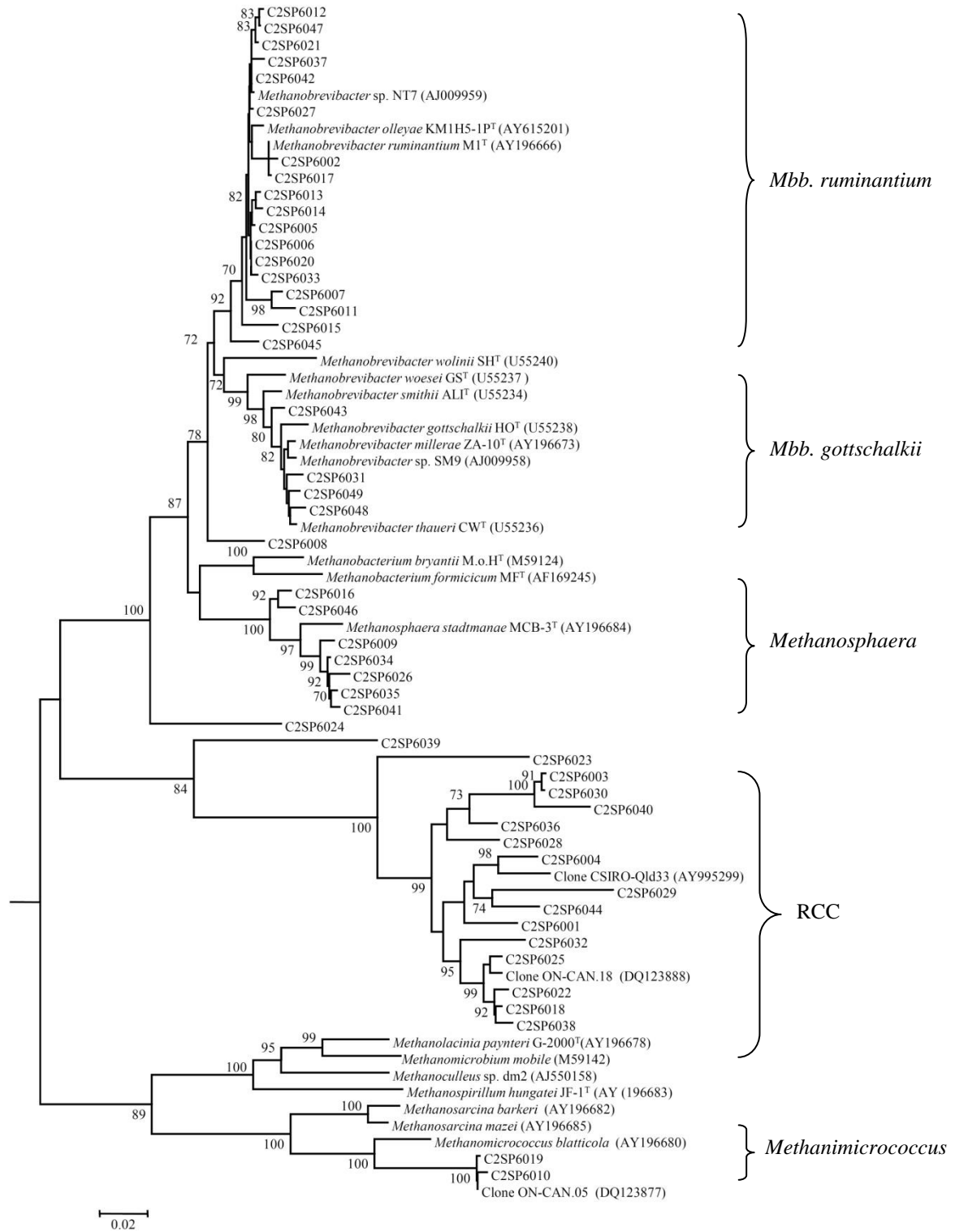


Figure 3.10 Inferred phylogenetic relationships between partial 16S rRNA genes (C2SP6001- C2SP6049) obtained by the primer pair 86f and 1340r (P6) from the rumen sample of cow (C2) fed with lucerne silage and barley meal and reference 16S rRNA gene sequences. In the parentheses are the GenBank accessions numbers for reference sequences. The superscript ^T designates a type strain. The tree was constructed from an alignment of 1185 nucleotide positions. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates 0.02 nucleotide substitutions per nucleotide position. The 16S rRNA genes of *Methanocaldococcus jannaschii* (GenBank accession M59126), *Methanococcus vannielii* (M36507) and *Methanothermococcus thermolithotrophicus* (M59128) were used as out-group sequences, and are not shown in the figure.

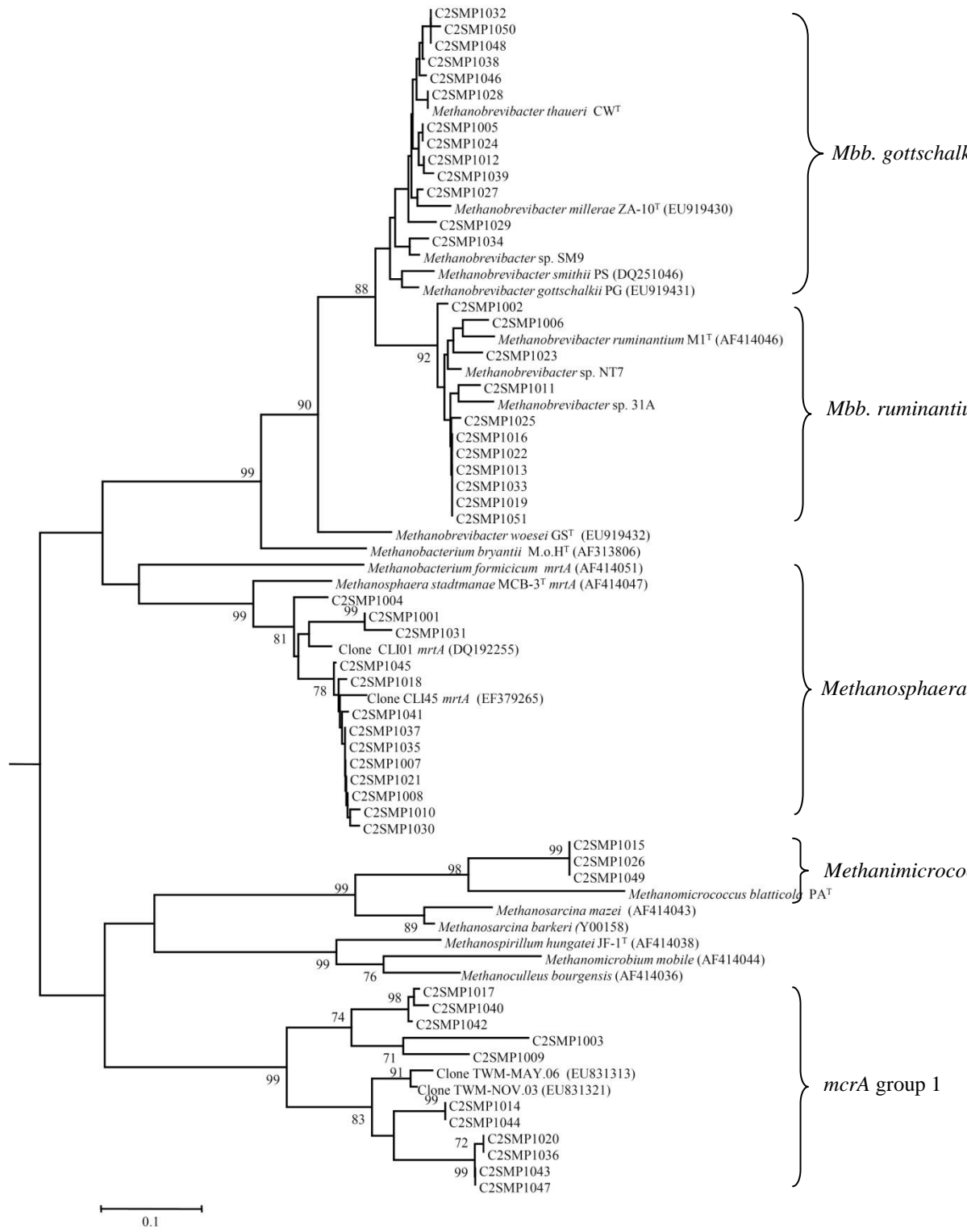


Figure 3.11. Inferred phylogenetic relationships between deduced amino acid sequences of *mcrA* genes (C2SMP1001- C2SMP1051) obtained by the primer pair ML1 and ML2 (MP1) from the rumen sample of cow (C2) fed with lucerne silage and barley meal and reference *mcrA* gene amino acid sequences. In the parentheses are the GenBank accession numbers for reference sequences. The superscript ^T designates a type strain. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates 10% sequence divergence. The *mcrA* genes of *Methanopyrus kandleri* (GenBank accession AF 414042) was used as out-group sequence, and are not shown in the figure.

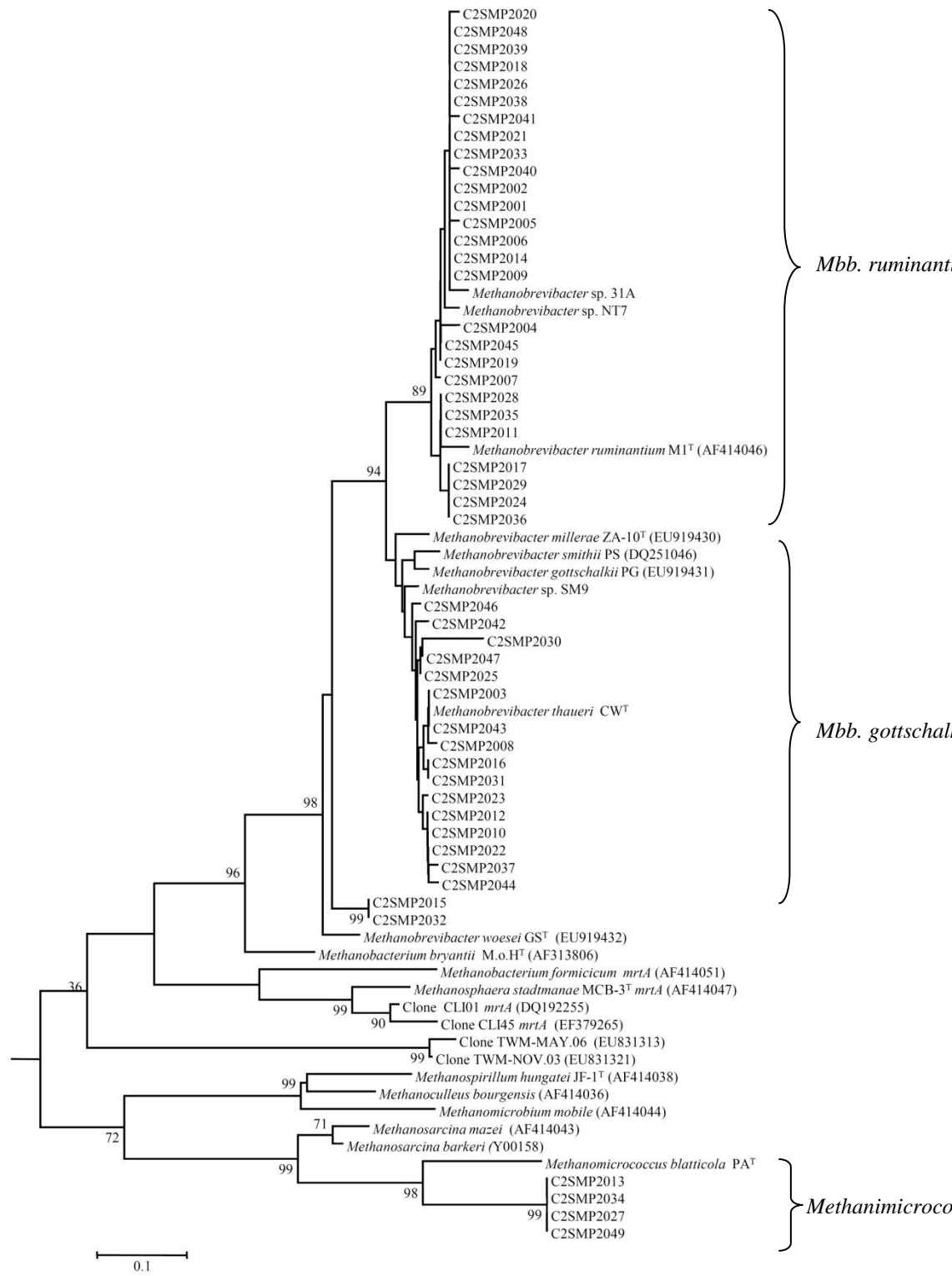


Figure 3.12. Inferred phylogenetic relationships between deduced amino acid sequences of *mcrA* genes (C2SMP2001- C2SMP2049) obtained by the primer pair ME1 and ME2 (MP2) from the rumen sample of cow (C2) fed with lucerne silage and barley meal and reference *mcrA* gene amino acid sequences. In the parentheses are the GenBank accessions numbers for reference sequences. The superscript ^T designates a type strain. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates 10% sequence divergence. The *mcrA* genes of *Methanopyrus kandleri* (GenBank accession AF 414042) was used as out-group sequence, and are not shown in the figure.

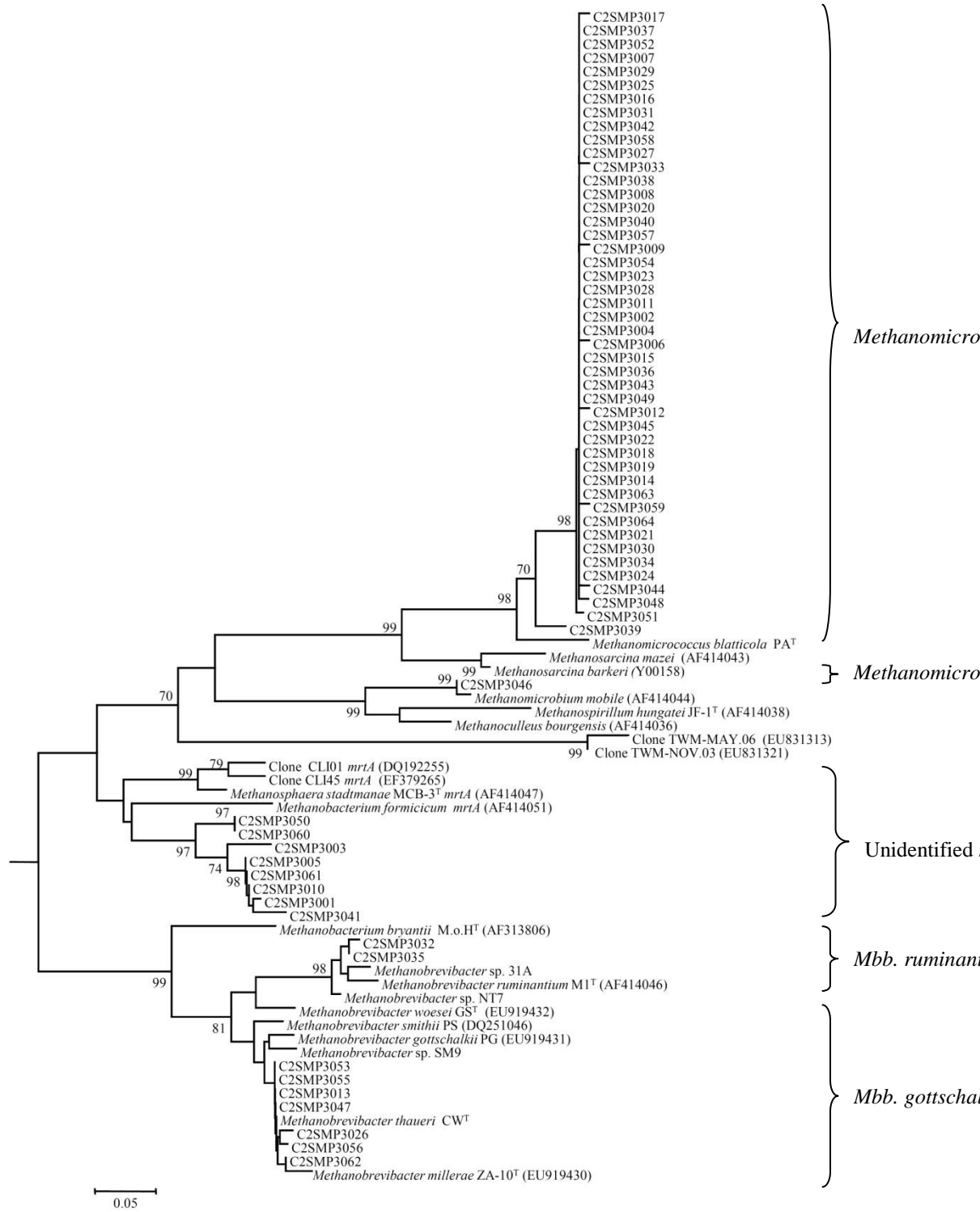


Figure 3.13. Inferred phylogenetic relationships between deduced amino acid sequences of *mcrA* genes (C2SMP3001- C2SMP3064) obtained by the primer pair MCRf and MCRr (MP3) from the rumen sample of cow (C2) fed with lucerne silage and barley meal and reference *mcrA* gene amino acid sequences. In the parentheses are the GenBank accessions numbers for reference sequences. The superscript ^T designates a type strain. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates 5% sequence divergence. The *mcrA* genes of *Methanopyrus kandleri* (GenBank accession AF 414042) was used as out-group sequence, and are not shown in the figure.

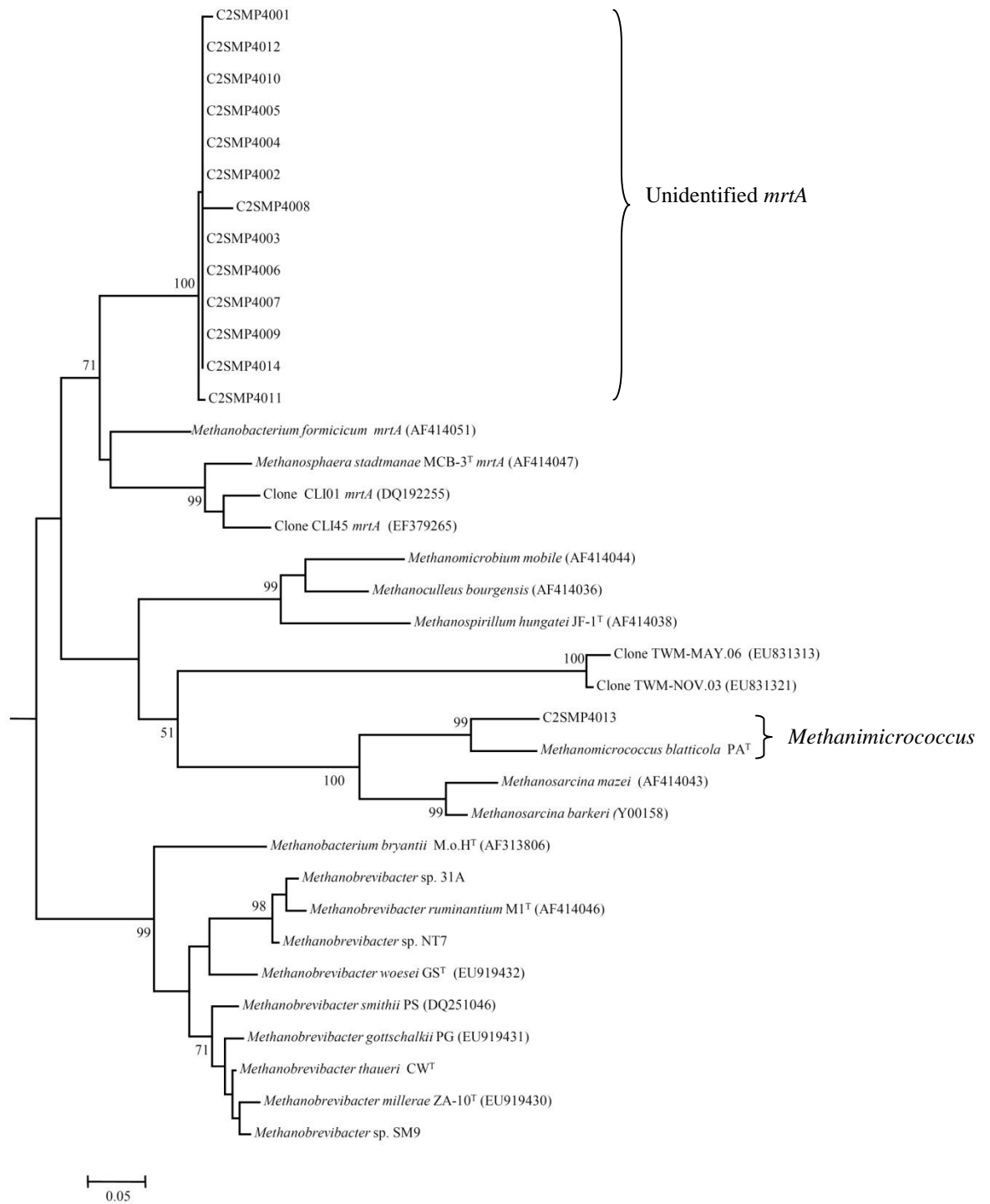


Figure 3.14. Inferred phylogenetic relationships between deduced amino acid sequences of *mcrA* genes (C2SMP4001- C2SMP4014) obtained by the primer pair MCRmf and MCRr (MP4) from the rumen sample of cow (C2) fed with lucerne silage and barley meal and reference *mcrA* gene amino acid sequences. In the parentheses are the GenBank accessions numbers for reference sequences. The superscript ^T designates a type strain. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates 10% sequence divergence. The *mcrA* genes of *Methanopyrus kandleri* (GenBank accession AF 414042) was used as out-group sequence, and are not shown in the figure.

3.4 Discussion

The use of molecular ecological techniques is indispensable in the study of rumen microbial diversity. Most molecular techniques analyse the nucleic acids extracted directly from rumen samples to study the microbial diversity. To determine the phylogenetic diversity, marker genes are selectively amplified from the DNA extracted from rumen sample and studied. One of the most frequently used markers to study the methanogen diversity, is the gene encoding the 16S rRNA (small subunit rRNA) (Janssen & Kirs, 2008). Another important marker, which is unique to methanogens (except methane oxidising archaea) is the gene encoding the α subunit of methyl-coenzyme reductase (Luton *et al.*, 2002; Springer *et al.*, 1995). These two marker genes are the most often used in molecular techniques to study methanogen diversity in the rumen. Recently the *rpoB* gene, which encodes the β subunit of RNA polymerase was used to study the abundance of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* in human stool samples. (Dridi *et al.*, 2010).

The 16S rRNA gene primers that were selected in this comparison study had been previously published and used by several researchers to study the archaeal diversity in environmental samples. Except for primer pairs P1 and P4, all of the other four primer pairs had already been used in the rumen samples to study methanogen community diversity (Table 1.2). The primer pair P1 has also been used to study archaeal diversity in environmental samples (Kemnitz *et al.*, 2005; Peng *et al.*, 2008). However, this primer pair has not been used on rumen samples thus far. The other primer pair 109f and 1386r (P4) has not been used to study the archaeal diversity in any environmental samples. However, the forward and reverse primers from this pair have been used with other combinations to study the methanogen diversity previously from environmental sample (Kemnitz *et al.*, 2005; Nicholson *et al.*, 2007; Skillman *et al.*, 2006).

The most often used primer pair to study the rumen methanogens community is P6 (Table 1.2). All rumen methanogens identified thus far, including the uncultured RCC group, are known to be amplified by this primer pair. Analysis performed in the study of Skillman *et al.*, (2004), showed that this primer pair possessed only a few mismatches against important rumen methanogens and has been suggested to be suitable to study the archaeal diversity in environmental samples. However, in the recent study performed in cows with different feed efficiency, this primer pair failed to

amplify archaeal DNA from some rumen samples (Zhou *et al.*, 2009). No other problems/limitations have been reported for this primer pair thus far.

The primer pair P2 has been used in DGGE analysis and qPCR studies of rumen methanogens in reindeer (Sundset *et al.*, 2009a). However, in the present study a few bacterial sequences were obtained in the clone library created by this primer pair. Recently, the amplification of bacterial sequences by this primer pair was also reported by Sundset *et al.* (2009b). Even though this particular primer pair amplified all of the known methanogen phylogenetic groups and had the highest Shannon and Simpson's diversity indices, it should be used under special conditions as this is not specific to Archaea.

The primer pair P3 has only been used in two studies to study the rumen methanogens (Pei *et al.*, 2008; Whitford *et al.*, 2001). Both of these studies showed reduced diversity of rumen methanogens and this primer pair failed to amplify sequences belonging to the *Mbb. gottschalkii* and RCC groups. In the present comparison study this primer pair amplified only three groups of Archaea: the *Mbb. ruminantium* clade, *Methanosphaera* spp. and *Methanomicrococcus* spp. and this clone library was dominated by *Methanomicrococcus* species (70.5%). This observation is unusual, as the studies performed thus far using rumen samples identified *Methanomicrococcus* species as a minor group. Preferential amplification of this group by the primer pair P3 may be the possible reason for this observation. The Shannon and Simpson's diversity indices were lowest for the clone library constructed using primer pair P3 and for all the other primer pairs the values were more or less similar. The Shannon diversity index increases with richness and evenness of sequence distribution. A higher value of the Simpson's index indicates greater dominance of one or few abundant group of sequences. Primer pair P3 only amplified three groups of methanogens from this rumen sample, whereas, other primer pairs typically amplified mostly five groups of methanogens.

The primer pair P5 which amplifies 450 bp amplicons was validated and successfully used in the Temperature Gradient Gel Electrophoresis (TTGE) analysis of the rumen sample (Nicholson *et al.*, 2007). Analysis showed that this primer pair covers most of the methanogens (Table 4.2). Some of the molecular techniques such as DGGE and qPCR require short PCR products. As such, this primer pair can be used in these

techniques. In the present study, this primer pair failed to amplify the sequences from *Methanomicrococcus* species. However, the same primer pair amplified the sequences of *Methanomicrococcus* in another study (Knight *et al.*, 2010). As such, absence of this species may be due to the small number of clones in the clone library and not the inability of the primers to detect this group.

In this comparison study, care has been taken to keep all factors constant except the primer pairs. As such, any variations observed must be due to the primers used. There have been many archaeal 16S rRNA gene primers published (Table 1.2). Only six primers were selected in this study and their suitability to amplify rumen sample was tested (Table 3.1). Compared to archaeal 16S rRNA gene primers, the number of published *mcrA* gene primers was low. Four *mcrA* gene primer pairs were selected in this study and one primer pair was a modified forward primer (Table 3.1).

The majority of 16S rRNA primers used in this comparison study showed either *Methanobrevibacter* spp. (*Mbb. ruminantium* and *Mbb. gottschalkii* clades) or RCC group as the dominant methanogens. This finding is in agreement with most of the studies performed on rumen methanogens. The majority of the studies have shown *Methanobrevibacter* spp. as the dominant methanogens regardless of diet, host or region (Table 1.2). In some cases, the rumen populations were dominated by the RCC group (Sundset *et al.*, 2009b; Wright *et al.*, 2006; 2007). In contrast, the clone library of the primer pair P3 (1Af/1100Ar) was dominated by *Methanomicrococcus* species (70.5%). The *Methanomicrococcus* spp. has not been identified as a dominant group in any of the studies performed in the rumen thus far (Table 1.2). Sequences belonging to this group have only been observed in a few studies (Sundset *et al.*, 2008a; Tajima *et al.*, 2001b; Whitford *et al.*, 2001; Wright *et al.*, 2007) and they were mostly present as a minor group.

The *mcrA* gene primer pairs used in our comparison study, except the modified primer pair M-P4, have been compared before for samples collected from peat land (Juottonen *et al.*, 2006). These primer pairs did not show any differences in the general community composition of that sample, but the identified communities were quantitatively different. The methanogen community in peat land is different from that in the rumen. Also, these *mcrA* primers were degenerate primers and the amplification efficiency remained poor. Furthermore, it was reported that these primers react

differently with different environmental samples (Juottonen *et al.*, 2006). Therefore, testing of the *mcrA* gene primers on diverse environmental samples is likely to be quite important. As such, we decided to test these primers on the rumen samples as there had not been any previous comparison studies performed with rumen samples.

Comparative phylogenetic studies have clearly indicated that the topologies of 16S rRNA gene-based and *mcrA* gene-based (or inferred amino acid sequences from *mcrA* genes) trees are similar (Lueders *et al.*, 2001; Springer *et al.*, 1995). The problem with *mcrA* primers is that a high degree of DNA conservation exists between the *mcrA* and *mrtA* genes (Luton *et al.*, 2002). This means that primers for *mcrA* gene can also amplify the *mrtA* gene. Members of the orders *Methanobacteriales* and *Methanococcales* contain both *mcrA* and *mrtA* genes which can be over-represented in the clone libraries. Fortunately, *mrtA* gene sequences clustered together and were readily distinguishable. Thus, careful analysis can overcome this problem. The genome analysis of *Methanosphaera stadtmanae* MCB-3^T showed no *mcrA* gene and only *mrtA* gene in their genome sequence (Fricke *et al.*, 2006). Due to this, amplification of the *mrtA* gene is also important for some methanogens. *Methanosphaera* spp. are common inhabitants of the rumen, and this group should be able to be amplified by *mcrA* primers. However, it might be premature to conclude that all *Methanosphaera* spp. possess only the *mrtA* gene. Furthermore, all the *Methanosphaera* spp. sequences obtained in our study clustered with *mrtA* sequences of *Methanosphaera stadtmanae*. More studies are needed to confirm that *Methanosphaera* species lack the *mcrA* gene.

The primer pairs M-P2 and M-P3 did not amplify all the groups which were identified by 16S rRNA gene primer pairs. The primer pair M-P2 has been used with rumen samples before and failed to cover all the methanogens groups (Tatsuoka *et al.*, 2004). Also, this primer pair failed to amplify some methanogens groups in rice field soil and the *mrtA* gene of family *Methanobacteriaceae* (Juottonen *et al.*, 2006). Absence of *Methanosphaera* spp. group in this clone library may be due to this bias. However, one advantage is that a 760 bp PCR product generates more phylogenetic information. The primer pair M-P3 also failed to amplify *Methanosphaera* spp. and the RCC-like group. Moreover, an M-P3 primer pair has the highest degree of degeneracy of the *mcrA* primer pairs which is apparently susceptible to different annealing temperatures (Lueders & Friedrich, 2003).

The primer pair M-P3 and the modified primer pair M-P4 amplified a group of sequences which were partially similar to the *mcrA/mrtA* genes. These sequences clustered together and did not show much variation. In the phylogenetic analysis (Figures 3.13 and 3.14) they clustered with *mrtA* sequences. High degeneracy of these primers may amplify this group of sequences non-specifically. These sequences were not used in the analysis performed in the present study. However, further analysis of these sequences is warranted.

In the global data analysis by Janssen and Kirs, (2008) the *Methanomicrobium* spp. were found to be one of the important group of methanogens. However, many of the recent studies performed in the rumen sample failed to identify this group (Table 1.2). Inclusion of sequences obtained from two studies in the global data analysis (Shin *et al.*, 2004; Tajima *et al.*, 2001b) where the *Methanomicrobium* sp. were found to be the dominant group of methanogens may be the reason for this high contribution. The primers used in these two studies were reported to have mismatches against *Methanobrevibacter* spp. and *Methanosphaera* spp. (Skillman *et al.*, 2006). Thus, application of these primers may be the reason for the dominance of *Methanomicrobium* spp. in these clone libraries. However, none of the 16S rRNA gene primers in the present comparison study amplified sequences from *Methanomicrobium* species. One sequence from this species was obtained by the *mcrA* primer pair M-P3. Only one rumen sample was used in this comparison study, as such, it is possible that they were present, but in such low numbers that they were undetected.

Among the different groups identified by the 16S rRNA gene primer pairs the RCC group showed high within-group sequence variation. This was seen in the phylogenetic trees constructed using these sequences. Similarly, the RCC-like sequences amplified by primer M-P1 also showed similar sequence variation. This suggested that the RCC group may possess a *mcrA* gene. Further studies were performed to confirm this suggestion (Chapter 5). RCC group is one of the dominant groups identified in this rumen sample. To successfully develop a methane reduction strategy it will be important to further study this group.

Based on the comparison described thus far in this study, four primers were chosen to study the rumen methanogens by molecular based techniques in this thesis. To obtain the 16S rRNA gene sequences of pure cultures, the primer pair 8f and 1510r

was used, which produces a 1500 bp PCR product. For 16S rRNA gene clone library construction the primer pair P1 (109f and 915r) was used. The analysis showed that this primer pair covers all the methanogens groups and is suitable for environmental samples. This primer pair produces 800 bp PCR products which provide sufficient phylogenetic information. For real-time PCR and DGGE analysis P5 (915aF and 1386r) were used which is sufficiently long for these methods and also covers most of the rumen methanogen groups. For the *mcrA* gene-based studies the primer pair M-P1 was used. This primer pair is less degenerate than other *mcrA* gene primer pairs. Also, it covers most of the methanogen groups and amplifies *mrtA* gene of *Methanosphaera* species and RCC-like sequences as well. Using *mcrA* gene primers for quantitative studies is not recommended (Friedrich, 2005) due to high degree of sequence conservation between the *mcrA* and *mrtA* genes, different *mcrA/mrtA* operon copy numbers and use of degenerate primers. In addition, degenerate primers are not suitable for DGGE techniques (Kowalchuk *et al.*, 1997).

Chapter 4

Archaeal community structure in the rumens of farmed sheep, cattle and red deer fed different diets

4.1 Introduction

Worldwide, ruminant-derived enteric methane emissions account for about one-quarter of all anthropogenic methane emissions, and are implicated as a driver of global climate change (Wuebbles & Hayhoe, 2002). In some countries, methane produced by ruminants can constitute the largest single source of anthropogenic greenhouse gas emissions. For example, enteric methane represented 32% of New Zealand's total anthropogenic greenhouse gas emissions in 2005 (Leslie *et al.*, 2008). Methane is produced by methanogenic archaea in the rumen, through their utilization of hydrogen formed during microbial fermentation of the ingested feed. Concerns about the possible environmental effects and associated economic burdens of ruminant methane emissions have led to an increased interest in manipulating methanogens and methanogenesis in the rumen (Leslie *et al.*, 2008; Buddle *et al.*, 2010; Martin *et al.*, 2010). This interest is not new, since methane has long been seen to represent a loss of feed energy for the ruminant animal (Czerkawski, 1969).

Knowledge of the methanogens present in the rumen could enable a more targeted manipulation of the rumen system (Leahy *et al.*, 2010). A number of studies have investigated the diversity of archaea in the rumen, based on the presence of 16S rRNA genes, which can be used to survey the microbial groups present in an environment. Janssen & Kirs (2008) analyzed the 16S rRNA genes reported from 14 studies, performed in different parts of the world, and provided a global picture of the archaeal diversity of the rumen. They found that the majority of the rumen archaea (92.3%) were from three genus level groups: the genus *Methanobrevibacter* (61.6%); the genus *Methanomicrobium* (14.9%); and a large group of uncultured archaea termed Rumen Cluster C, abbreviated here as RCC (15.8%). However, the proportions of these groups varied significantly among the different studies. These studies cannot be directly compared because sample processing methods and primers used for PCR-mediated amplification of the 16S rRNA genes were different. There are not many directly

comparable studies of archaeal diversity in the rumens of different species or animals on different diets. In one such study, using temporal temperature gradient gel electrophoresis, Nicholson *et al.* (2007) found no strong effect of host on rumen methanogen diversity in grazing sheep and cattle. In the studies based on clone libraries, archaea detected in reindeer in two different locations (Sundset *et al.*, 2009a; Sundset *et al.*, 2009b) were similar overall to those in cattle and sheep. Diet has been indicated to play an important role in rumen bacterial diversity (Kocherginskaya *et al.*, 2001; Tajima *et al.*, 2001a; Klieve *et al.*, 2007), but there is little information available concerning the effects of diet on the composition of the methanogen community in the rumen.

Analysis of the genome of *Methanobrevibacter ruminantium* has identified a number of proteins and pathways that could be targeted in methane mitigation efforts (Leahy *et al.*, 2010). However, interventions directly targeting methanogens by using vaccines or small-molecule inhibitors have to be effective against the full diversity of rumen methanogens present in different species of farmed ruminants and on different diets. Otherwise, populations of other species of methanogens may just increase to fill the vacated niche space. Effective intervention will require detailed knowledge of a wider range of rumen methanogens than is currently being investigated (Buddle *et al.*, 2010). In this study, we compared the diversity of archaea in rumen samples from cattle, sheep and red deer fed with different diets, and identified the dominant archaea present. We present an assessment of ruminal archaeal diversity of farmed ruminants (cattle, sheep and red deer) fed different diets. We analyzed the major archaeal groups present by using PCR to amplify 16S rRNA genes of archaea in the rumen, then performed denaturing gradient gel electrophoresis (DGGE) combined with gene sequencing to find out if and how the archaeal community varied with ruminant species and diet. We also sequenced PCR-amplified 16S rRNA genes and used quantitative real-time PCR to verify our findings.

4.2 Materials and methods

4.2.1 Use of animals

The use of animals was approved by the AgResearch Grasslands Animal Ethics Committee and complied with the AgResearch Code of Ethical Conduct for the Use of Animals in Research, Testing and Teaching, as prescribed in the Animal Welfare Act of 1999 and its amendments.

4.2.2 Sample collection

Samples of whole rumen contents consisting of fluid and solids (approximately 200 g) were collected via rumen fistulae from 4 Romney wether sheep, 5 non-lactating Friesian-Jersey cross dairy cows and 4 castrated red deer stags. These animals were grazed on pasture consisting of perennial rye grass (*Lolium perenne*) and white clover (*Trifolium repens*) in summer and winter, and fed lucerne (*Medicago sativa*) silage (ChaffHage; The Great Hage Company, Reporoa, New Zealand) in winter. Pasture-grazed animals were on that diet for at least one month. Silage-fed animals were adapted to their feed for 15 days prior to sample collection and fed twice daily, at 08:00 hours and 16:00 hours at 1.2 times their estimated energy requirements for maintenance (Swainson *et al.*, 2008). Samples from silage-fed animals were collected at different time intervals after feeding (2, 4, 6 and 8 h), but, unless noted otherwise, only the 2-hour samples were analysed. Other samples were collected only once. Rumen samples were also collected at slaughter from four Romney wether sheep fed a concentrate-based diet (containing 30% [w/w] maize grain, 15% [w/w] barley grain, 15% [w/w] lucerne [*Medicago sativa*] meal, 15% [w/w] palm kernel extract, 10% [w/w] molasses, plus rumen-protected fat, vitamin/mineral premixes and fish oil). Rumen samples were also collected at slaughter from five Suffolk–Romney cross ewe hoggets fed perennial rye grass/white clover pasture during autumn and five Suffolk–Romney cross ewe hoggets fed willow (*Salix* sp.) fodder during autumn (Ramírez-Restrepo *et al.*, 2010). All animals had unlimited access to water at all times. Samples were taken from different parts of the rumen, mixed, and then frozen immediately at -80°C and subsequently lyophilized. The samples were then homogenized (1-2 min) in a 100 W

household coffee grinder (Russell Hobbs, Mordialloc, Victoria, Australia) and stored at -80°C until DNA was extracted.

4.2.3 Total DNA extraction and PCR

Total DNA was extracted from 100 mg of ground freeze-dried rumen sample using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracted DNA was used as a template for PCR to amplify the 16S rRNA genes of total archaea, total bacteria, and the RCC group of archaea. The primers used in this study are listed in Table 4.1. Forward primers used to amplify 16S rRNA genes for DGGE analysis included a GC-clamp at the 5'-terminus (Table 4.1). All the PCR were performed in a Px2 thermal cycler (ThermoElectron, Milford, MA, USA) and the resultant PCR products analyzed using agarose gel (1.5% [w/v]) electrophoresis prior to DGGE or cloning.

The archaea-specific primers GC-915af and 1386r were used to amplify a 491-bp product covering the V6-V8 region of the 16S rRNA gene for DGGE. The PCR (50 µl) contained 5 µl of 10× reaction buffer without MgCl₂ (Roche, Auckland, New Zealand), 1.5 mM MgCl₂, 1 µM of each primer, 200 µM of each deoxyribonucleotide triphosphate, 2.5 U of Taq DNA polymerase (Roche), 25 ng of template DNA, and DNA-free water. DNA-free water was prepared by filtering distilled water through a 0.2-µm pore size sterile filter, autoclaving the water, then irradiating 2-ml aliquots in 2-ml polypropylene tubes with UV light (254 nm, 6 W) at a distance of 100 mm for 4 h. The amplification was done using the following cycling parameters: an initial denaturation for 2 min at 95°C; 25 cycles each of denaturation for 15 s at 95°C, annealing for 30 s at 59°C, and primer extension for 1 min at 72°C; and a final extension of 1 h at 72°C to overcome double-banding problems sometimes observed within DGGE (Janse *et al.*, 2004).

Archaeal 16S rRNA gene clone libraries were constructed from DNA fragments amplified using two primer pairs, 109f plus 915r and 915af plus 1386r, from DNA extracted from the sample obtained from sheep no. 4 (S4) grazed on winter pasture. The reaction mixture and the cycling parameters were those used for the amplification of archaeal 16S rRNA genes for DGGE, except that the final extension time used for both

these primer pairs was 7 min, and the annealing temperature with the primer pair 109f plus 915r was 57°C.

Part of the bacterial 16S rRNA gene (V2-V3 region) was amplified with the bacteria-specific primer pair GC-338f plus 518r (Muyzer *et al.*, 1993). The reaction mixture was the same as that used to amplify archaeal 16S rRNA genes above, and the amplification was performed using the following touchdown PCR protocol: an initial denaturing for 3 min at 95°C; 10 cycles each of denaturing for 30 s at 95°C, annealing for 30 s initially at 62°C and decreasing by 0.5°C per cycle, and primer extension for 30 s at 72°C; 26 cycles each of denaturing for 30 s at 95°C, annealing for 30 s at 57°C, and primer extension for 30 s at 72°C; and a final extension for 30 min at 72°C.

An alignment of 81 16S rRNA gene sequences assigned to RCC (Janssen and Kirs, 2008) was analysed for conserved regions. Two primers, 762f and 1099r (Table 4.1) were designed to amplify a region of the 16S rRNA gene of this group. In total, 88% of the sequences contained primer target regions with perfect matches to the primers, and 98% of the sequences contained primer target sites with no or only one mismatch. A clone library (24 sequences) was constructed using this primer pair to confirm that only 16S rRNA genes from RCC were amplified. The template DNA was extracted from a rumen sample from cow no. 2 (C2) fed silage. The reaction mixture for the PCR the same as used for amplification of total archaeal 16S rRNA genes. Amplification was performed with the following parameters: an initial denaturing for 2 min at 94°C; 30 cycles each of denaturing for 30 s at 94°C, annealing for 60 s at 56°C, and primer extension for 1 min at 72°C; and a final extension for 7 min at 72°C. For the PCR to amplify fragments for DGGE analysis, the primer pair GC-762f and 1099r was used, and the final extension time was increased to 1 hour.

Table 4.1. Primers used in this study to target 16S rRNA genes of total archaea, total bacteria, and archaea of the RCC group.

Target	Primers	Sequence (5'-3')	Reference
Archaea	109f	ACKGCTCAGTAACACGT	Großkopf <i>et al.</i> (1998)
	915af*	AGGAATTGGCGGGGAGCAC	Watanabe <i>et al.</i> (2004)
	915r	GTGCTCCCCCGCCAATTCCT	Watanabe <i>et al.</i> (2004)
	1386r	GCGGTGTGTGCAAGGAGC	Skillman <i>et al.</i> (2004)
Bacteria	27f	GAGTTTGATCMTGGCTCAG	modified from Lane (1991)
	338f*	ACTCCTACGGGAGGCAGCAG	Lane (1991)
	518r	ATTACCGCGGCTGCTGG	Muyzer <i>et al.</i> (1993)
	519f	CAGCMGCCGCGGTAAANWC	Lane (1991)
	907r	CCGTCAATTCMTTTRAGTTT	Lane (1991)
	1492r	GGYTACCTTGTTACGACTT	modified from Lane (1991)
	RCC	762f*	GACGAAGCCCTGGGTC
	1099r	GAGGGTCTCGTTCGTTAT	This study

*Where the primer name is prefaced by “GC-” in the text, a GC-clamp was added to the 5' terminus of the primer. The CG-clamp sequence was 5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGG-3'.

4.2.4 Denaturing gradient gel electrophoresis (DGGE)

PCR products were purified using a Wizard SV gel and PCR clean-up system (Promega, Alexandria, NSW, Australia) and quantified with a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Subsequently, the purified PCR products were treated with mung bean nuclease to remove interfering single-stranded DNA products, as described by Kocherginskaya *et al.* (2001). The reaction mixture containing 15 µl of purified PCR products, 3 µl of 10× mung bean nuclease buffer (pH 5.0), 1.7 U of mung bean nuclease (Promega) in 1 µl of water, and 30 µl water, was incubated at 37°C for 10 min. The reaction was stopped by adding 10 µl of DGGE loading dye (0.05% [w/v] bromophenol blue, 0.05% [w/v] xylene cyanol, 70% [w/v] glycerol, in water, pH 8.0) and immediate chilling on ice. The

reference standard for DGGE was created by combining equal quantities of PCR products generated using the primers GC-915af and 1386r from DNA extracted from pure cultures of methanogen obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), or from the AgResearch culture collection. These cultures, with their culture collection accession numbers (DSM) or the GenBank accession numbers of their 16S rRNA gene sequences and the band label a-h in Figure 1a were *Methanobrevibacter gottschalkii* strain HO (DSM 11977, band a), *Methanobrevibacter thaueri* strain CW (DSM 11995, band b), *Methanobrevibacter ruminantium* strain M1 (DSM 1093, band h), *Methanobrevibacter ruminantium* strain 31A (GenBank HM624055, band g), *Methanospirillum hungatei* strain JF1 (DSM 864, band d), *Methanobrevibacter smithii* strain PS (DSM 861, band c), *Methanosarcina barkeri* strain CM1 (GenBank AJ002476, band e), and *Methanobacterium formicicum* strain BRM9 (GenBank X99138, band f). The same reference standard mixture was used in all DGGE gels. DGGE was performed by using a CBS scientific DGGE/TTGE gel system (C.B.S. Scientific Company, Del Mar, CA, USA). Polyacrylamide gels (6% [w/v]) were prepared and electrophoresed with 1× TAE buffer, which consisted of 40 mM tris(hydroxymethyl)aminomethane, 65 mM acetic acid, 1 mM ethylenediaminetetraacetic acid, adjusted to pH 8.0 with NaOH. The gels for separating PCR products contained a gradient of denaturant that increased in the direction of electrophoresis, with a 30% [v/v] to 55% [v/v] gradient for total archaeal products, a 40% [v/v] to 60% [v/v] gradient for total bacterial products, and a 15% [v/v] to 70% [v/v] for products amplified from RCC. The 100% denaturant solution contained 40% (v/v) formamide and 7.0 M urea. Purified and mung bean nuclease-treated PCR products (300 ng) were loaded into each well. Electrophoresis was performed at 150 V (constant voltage) and 60°C (constant temperature) for 6 h. The gels were stained with 3.0 µl of SYBR Gold (10, 000× concentrate in DMSO; Invitrogen, Carlsbad, CA, USA) in 600 ml of water for 20 min and de-stained in 600 ml of water for 30 min. The gels were viewed using UV trans-illumination and photographed using a Gel Logic 200 imaging system (Eastman Kodak, New York, NY, USA).

To extract DNA from DGGE bands, selected bands were cut from the gel using a clean, sharp scalpel and transferred to a 1.5-ml microcentrifuge tube. DNA was eluted from polyacrylamide gel slices according to the method described by Etokebe and Spurkland (2000). Gel slices were each washed by incubation in 50 µl of water for 15

min at room temperature. The water was then removed and discarded. DNA was recovered by the addition of 50 µl of UV-treated water to each tube, followed by incubation overnight at 4°C in 1.5-ml tubes. The tubes were vortexed for 5 s, centrifuged for 1 min at 10,000 g, and the supernatants transferred to clean tubes. The DNA fragments in the supernatants were cloned as described below. Positive clones were re-amplified with the primers GC-915af and 1386r, and DGGE was performed to confirm the position of migration. At least three clones from each band were sequenced.

4.2.5 Cloning and sequencing

Libraries of 16S rRNA genes of total archaea, RCC, and bacteria, and from pure cultures of methanogens were prepared from PCR products produced as described above. PCR products were cloned by ligation into a plasmid vector (pCR 2.1; Invitrogen) and transformation into competent *Escherichia coli* TOP-10 cells, using a TOPO-TA cloning system (Invitrogen). Clones were randomly chosen and sequenced using the primers M13f (5'-CCCAGTCACGACGTTGTAAAACG-3') or M13r (5'-AGCGGATAACAATTTACACAGG-3') at the Allan Wilson Centre Genome Sequencing Service (Massey University, Palmerston North, New Zealand) or Macrogen Inc. (Seoul, Republic of Korea).

4.2.6 Analysis of electrophoresis patterns

DGGE fingerprints were analyzed with the BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium). Each profile, consisting of a digitized image from the gel and representing one lane containing one separated PCR product, was normalized relative to the bands in the lane containing the reference standard. This results in an output file, where the amount of DNA in any one band is reported as a relative intensity for that band within the total amount of DNA in all bands in that lane. Similarities between the fingerprints were quantified using the Pearson correlation coefficient between each pair of profiles, and these values were represented graphically using the Unweighted Pair Group Method of Arithmetic Averages (UPGMA) method. The Pearson correlation coefficient takes into consideration the presence and relative intensity of each band. To assess the significance of groupings, the distances between samples within a group were compared with distances between samples from different

groups in a *t*-test. Simpson's and Shannon's diversity indices were calculated from relative one-dimensional surface metrics for all bands within a lane as a measure of proportional abundance (Sait *et al.*, 2003).

4.2.7 Quantitative real-time PCR

Abundances of 16S rRNA genes from archaea, bacteria and members of RCC in rumen samples were quantified using a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Concorde, NSW, Australia) and amplicon detection by SYBR Green I fluorescence (LightCycler FastStart DNA Master SYBR Green I Kit; Roche). Reactions were set up in a Gene-Disc 100 (Corbett Life Science) and sealed with permanent adhesive film (Corbett Life Science). Each reaction contained, in a total volume of 20 μ l, 10 μ l SYBR green mix (Roche), 10 pmol of each primer, 4 μ g bovine serum albumin (Invitrogen), and 5 μ l of DNA. DNA amplification was performed on 3 different 10-fold dilutions of the template, each in duplicate, to give 6 replicates for each sample. Archaeal 16S rRNA genes were amplified using the primers 915af and 1386r at an annealing temperature of 59°C. Bacterial 16S rRNA genes were amplified using the primers 519f plus 907r (Stubner, 2004) at an annealing temperature of 52°C. RCC 16S rRNA genes were amplified using the primers 762f and 1099r at an annealing temperature of 56°C. Amplification commenced with a hot start of 95°C for 10 minutes, followed by 50 cycles of 95°C for 10 s, the appropriate annealing temperature for 5 s, and extension at 72°C for 10 s. After each run, melting curves between 72 and 98°C were evaluated for each PCR to confirm the absence of unspecific signals. Quantitative PCR efficiency was checked with the software LinRegPCR (Ruijter *et al.*, 2009). The copy numbers of 16S rRNA genes of targeted groups were calculated based on the dilutions made and the DNA yield per g of dry rumen contents.

External standards for archaea were prepared mixing equal quantities of plasmids containing 16S rRNA gene fragments amplified using the primers 915af plus 1386r from pure cultures of *Methanobrevibacter ruminantium* strain M1 (DSM 1093), *Methanomicrobium mobile* strain BP (DSM 1539) and *Methanobacterium bryantii* strain M.o.H (DSM 863). These isolates were obtained from the Deutsche Sammlung von Mikroorganismen. External standards for RCC were prepared by mixing equal quantities of three plasmids containing 16S rRNA gene fragments amplified by the

RCC-specific primers (762f and 1099r) from rumen contents from cow no. 2 (C2) fed silage (NZCRCC007 [GenBank accession number HM624062], NZCRCC011 [HM624066], and NZCRCC014 [HM624069]). A mixture of 3 plasmids containing 16S rRNA gene fragments amplified with the bacteria-specific primers 27f and 1492r from rumen contents of sheep no. 4 (S4) grazed winter pasture were used as the external standards for quantification of bacterial 16S rRNA genes. The plasmid copy numbers in the standards were calculated based on the plasmid concentration determined by the Quant-iT kit (Invitrogen). Appropriate external standards ranging from 1.0×10^3 to 1.0×10^7 copies/reaction were prepared and used with every quantitative PCR run to enumerate the archaea, RCC and bacteria in the extracted DNA from the rumen samples.

Differences in 16S rRNA gene abundances were analysed by ANOVA of log-transformed data, using GenStat 12th edition (VSN International, Hemel Hempstead, UK).

4.2.8 Phylogenetic analyses

All analyses were implemented in MEGA 4.0 (Tamura *et al.*, 2007). Nucleotide sequences were globally aligned with ClustalW using the IUB DNA matrix and a transition weight of 0.5. Phylogenetic trees were constructed using the neighbor-joining tree inference method (Saitou & Nei, 1987) with the Jukes-Cantor substitution model (Jukes & Cantor, 1969) assuming uniform rates among sites and using the complete deletion of gaps option. Bootstrap analyses were performed based on 1000 resamplings. The 16S rRNA gene sequences from *Methanocaldococcus jannaschii* JAL-1 (GenBank accession number M59126), *Methanococcus vannielii* SB (M36507), and *Methanothermococcus thermolithotrophicus* SN-1 (M59128) were used as out-groups.

4.2.9 Nucleotide sequence accession numbers

Nucleotide sequences from this study have been deposited in GenBank. Those from the DGGE bands have been deposited in the GenBank database under accession numbers GU329839 to GU329883. The 95 and 103 sequences of the cloned PCR-amplified archaeal 16S rRNA genes amplified from sheep no. 4 using two different primer sets, 915af plus 1386r and 109f plus 915r, have been deposited in GenBank

under accession numbers HM211304 to HM211398 and GU329736 to GU329838, respectively. The sequences of the 24 16S rRNA genes PCR-amplified from members of RCC from cow no. 2 using primers 762f plus 1099r have been deposited in the GenBank database under accession numbers HM624056 to HM624079.

4.3 Results

4.3.1 General archaeal community structure

Based on data from the literature (Table 4.2), we identified 16 primer binding sites used by previous researchers to amplify partial 16S rRNA genes from Archaea. Because we planned to use these primers to amplify fragments for DGGE analysis, we selected only regions that required no degeneracies in the matching oligonucleotides used as primers, since degeneracies can result in multiple bands being generated from a single target sequence (Kowalchuk *et al.* 1997). The match of these regions to 16S rRNA genes of Archaea was examined (Table 4.2). We chose to use primers 915af and 1386r (Table 4.1), which target sites 8 and 12, respectively (Table 4.2), because each primer matched $\geq 86\%$ of archaeal 16S rRNA gene sequences and because they had previously been validated and used successfully by Nicholson *et al.* (2007). No clade of rumen archaea identified by Janssen and Kirs (2008) was excluded using these primers.

We generated DGGE fingerprints of partial 16S rRNA gene fragments from the mixed archaeal communities in samples of rumen contents from the same 5 cattle, 4 sheep and 4 red deer fed summer pasture (Fig. 4.1a), winter pasture (Fig. 4.1b) and silage (Fig.4.1c). Overall, there were few major differences between the DGGE profiles produced from these animals, despite differences in the host species and diets. The visually dominant bands had a relative one-dimensional surface (RODS), as calculated by Gaussian fit, of ≥ 8.31 , i.e., each of these bands made up $\geq 8.31\%$ of the signal intensity within a profile. A dominant band was generated from samples from all animals on all diets, visible in the upper half of the gels (35-40% portion of the gradient), in a position equivalent to that of standard b (e.g., bands 1, 2, 15, and 17 in Fig. 4.1). A second dominant band was present just below it (e.g., bands 3, 5, and 6 in Fig. 4.1). Another group of obvious bands found in nearly all samples was present lower in the gels (45-50% portion of the gradient), close to the migratory position of standard h (e.g., bands 7, 11, 13, 16, 18, and 20 in Fig. 4.1).

To confirm these general findings, additional analyses were performed on the rumen contents from different flocks of sheep fed on different diets (Figs. 4.2 and 4.3). The DGGE fingerprints of the ruminal archaeal communities from these four different flocks of sheep from two different locations fed four different diets (winter pasture,

autumn pasture, willow, and a concentrate-based diet) were remarkably similar to each other, and to the other samples from sheep, cattle and red deer (Fig. 4.1). In addition, DGGE was performed with additional gels to compare directly the methanogen community fingerprints when the same animals were fed different diets (data analysis shown in Fig. 4.4). The patterns of dominant bands were similar in all cases with only some minor variations.

Table 4.2. Primer binding sites considered for targeting the archaeal 16S rRNA gene for DGGE analysis.

Site	Position *	Target sequence (5' - 3')	Number of sequences tested [†]	Match (%)	Reference
1	6-22	ATTCCGGTTGATCCTGC	339	49.9	Arahal <i>et al.</i> (1996)
2	8-25	TCCGGTTGATCCTGCCGG	726	59.6	Kolganova <i>et al.</i> (2002)
3	112-127	GCTCAGTAACACGTGG	1326	54.6	Großkopf <i>et al.</i> (1998)
4	334-348	TCCAGGCCCTACGGG	1326	37.5	Reysenbach & Pace (1995)
5	344-363	ACGGGGTGCAGCAGGCGCGA	1326	9.7	Casamayor <i>et al.</i> (2002)
6	781-797	AACCGGATTAGATACCC	1326	79.1	Ihara <i>et al.</i> (1997)
7	913-934	AAAGGAATTGGCGGGGAGCAC	1326	86.5	García-Martínez & Rodríguez-Valera (2000)
8	915-934	AGGAATTGGCGGGGAGCAC	1326	86.7	Casamayor <i>et al.</i> (2002)
9	1093-1111	AGTCAGGCAACGAGCGAGA	1326	49.7	Weisburg <i>et al.</i> (1991)
10	1100-1114	CAACGAGCGAGACCC	1326	53.8	Reysenbach & Pace (1995)
11	1100-1115	CAACGAGCGAGACCCA	1326	16.9	Embley <i>et al.</i> (1992)
12	1385-1402	GCTCCTTGCACACACCGC	1325	90.4	Skillman <i>et al.</i> (2004)
13	1386-1401	CTCCTTGCACACACCG	1325	90.8	Wright & Pimm (2003)
14	1482-1512	AAGTCGTAACAAGGTAGCCGT	562	71.2	Wood <i>et al.</i> (1998)
15	1491-1510	AGTCGTAACAAGGTAACC	562	13.3	Achenbach & Woese (1995)
16	1492-1510	AAGTCGTAACAAGGTAACC	562	13.3	Weisburg <i>et al.</i> (1991)

*Numbering according to the 16S RNA gene of *Escherichia coli* (Brosius *et al.*, 1978).

[†]Number of sequences with sequence information able to be compared.

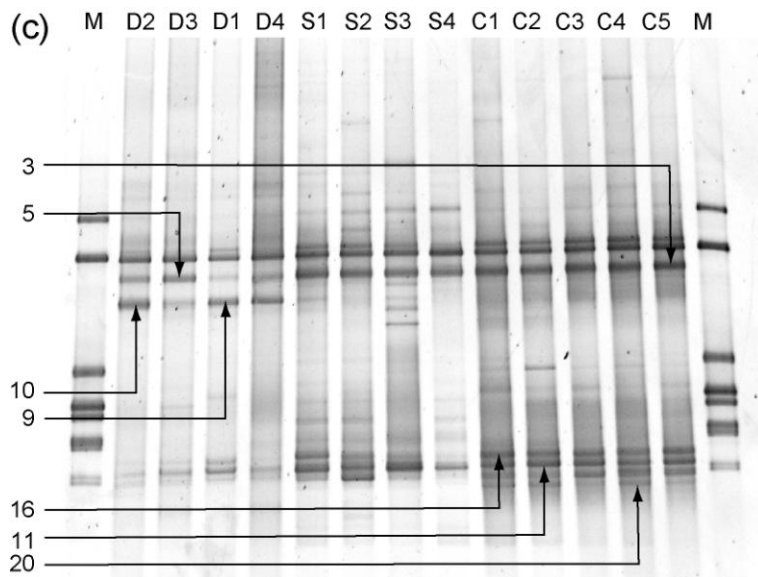
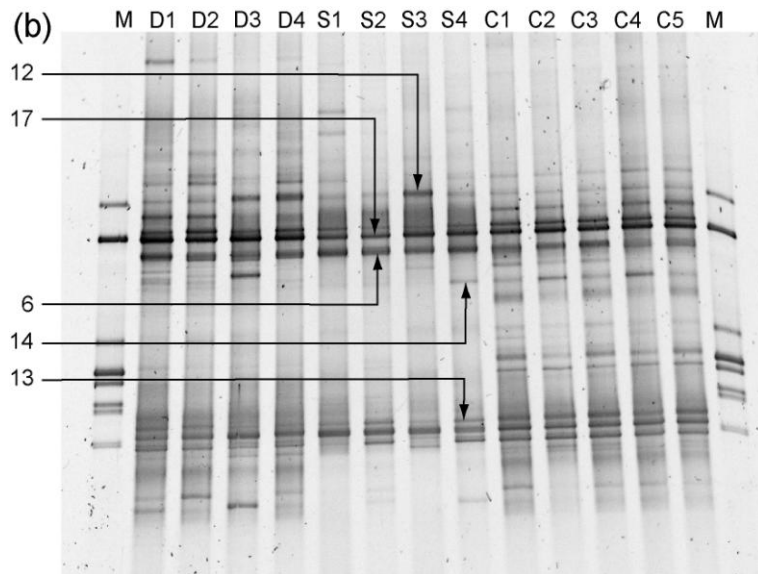
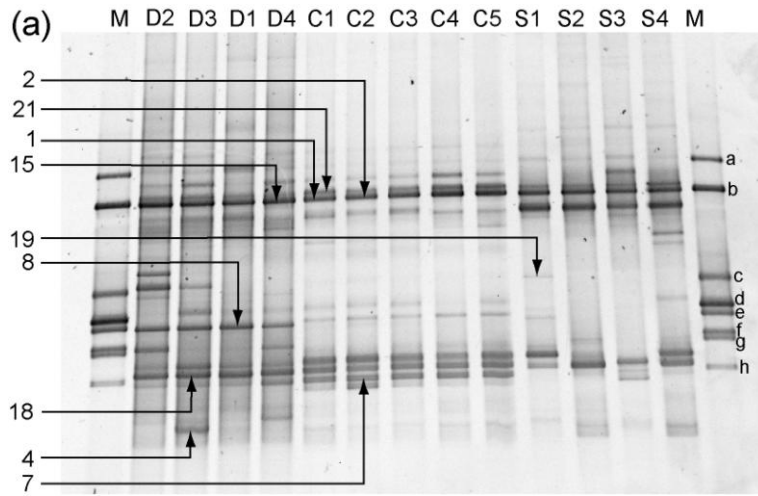


Figure 4.1. DGGE fingerprints of ruminal archaea in red deer (D1-D4), cattle (C1-C5) and sheep (S1-S4) fed (a) summer pasture, (b) winter pasture, and (c) silage. The labelled arrows indicate the bands that were excised and sequenced. The marker (M) consisted of amplicons of 16S rRNA genes from pure culture of methanogens, and are labelled a to h. The sample from sheep no. 4 fed winter pasture was used to generate a clone library of 16S rRNA gene sequences using a different primer combination (see text).

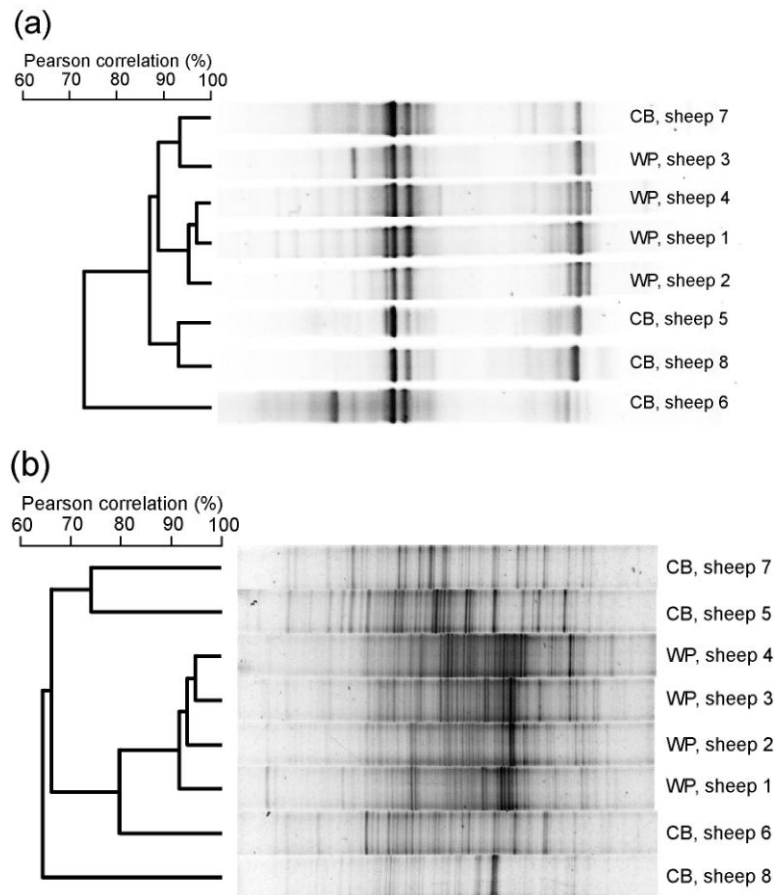


Figure 4.2. DGGE fingerprints of (a) ruminal archaea and (b) ruminal bacteria in two different flocks of sheep fed winter pasture (WP) or a concentrate-based diet (CB). The similarities of the fingerprints are indicated in the dendrograms to the left of each panel. Individual lanes are not in the same order as they were on the original gels, but those in each panel are from one gel. The gel origin is on the left.

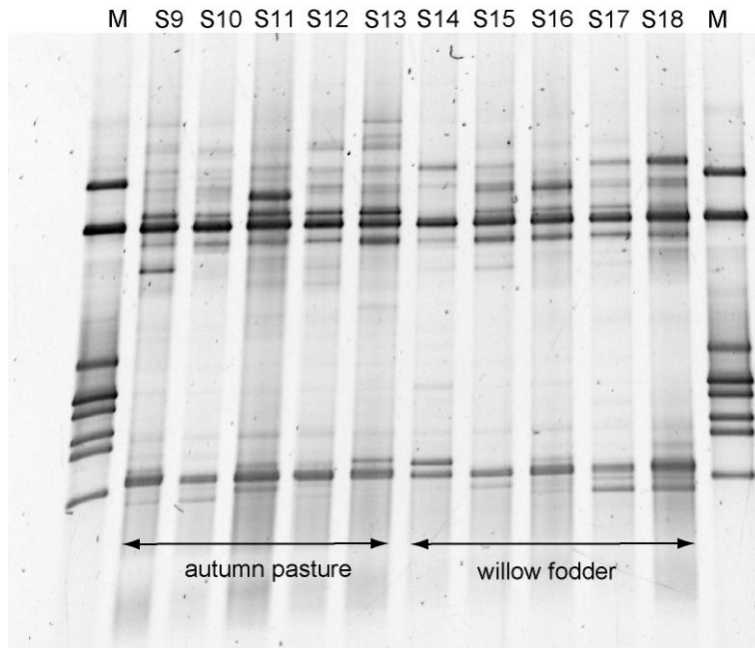


Figure 4.3. DGGE fingerprints of ruminal archaea in two different flocks of sheep fed autumn pasture (perennial rye grass/white clover mix) or willow fodder (*Salix* sp.). The markers (M) are the same as those in Figure 4.1.

4.3.2 Variations due to host, species or diet

In general, the DGGE fingerprints from cattle (6 to 9 bands) and red deer (6 to 11 bands) were made up of more bands than those from sheep (4 to 9 bands). However, patterns from red deer rumen samples showed a wide variation in the total number of bands present. In addition, samples from animals fed pasture (summer or winter) yielded comparatively more bands overall (6 to 12 bands) than samples from animals fed silage (4 to 8 bands). Only bands that had a RODS of ≥ 5 were considered here. There were a number of subtle differences in banding patterns, attributable to both minor bands (RODS 5.0 to 8.3) and dominant bands (RODS ≥ 8.31). For example, the fingerprints from samples from cattle fed summer and winter pasture had a minor band present near standards d and e in all of the animals (Fig. 4.1). These bands were absent in fingerprints from the same cattle when they were fed silage. A similar type of diet-based difference was obvious in the red deer. When the red deer were fed summer pasture, the patterns generated from all four individuals contained a dominant band close to the standard e. When they were fed silage, the patterns generated all had a dominant band close to the standard b. These two bands were largely absent when the red deer were fed pasture during winter. Such diet-based differences were not apparent in the sheep fed autumn pasture, winter pasture, summer pasture, silage, or a concentrate-based diet.

Cluster analysis (Figure 4.4) was performed to examine the potential effects of diet (summer pasture, winter pasture, and silage) on the archaeal community structure in the same cattle ($n=5$), sheep ($n=4$) and red deer ($n=4$). The three different ruminant species had different archaeal communities on all 3 diets tested: summer pasture (Fig. 4.4a; $P = 1.5 \times 10^{-19}$), winter pasture (Fig. 4.4b; $P = 9.8 \times 10^{-10}$), and silage (Figure 4.4c; $P = 2.8 \times 10^{-12}$). The banding patterns from the cattle directly reflected the diet, with all samples grouping by diet rather than individual animal (Figure 4.4d; $P = 3.5 \times 10^{-23}$). Samples from cattle fed summer pasture and winter pasture were more similar to each other than they were to samples from silage-fed cattle. Red deer showed a more complex pattern, with the highest variability in banding patterns. There was no clear separation between the archaeal communities in red deer fed silage and winter pasture, but samples from red deer fed summer pasture grouped coherently and with less variability. Overall, there was support for diet-based differences in archaeal community

structure in red deer (Figure 4.4e; $P = 0.004$). Samples from sheep were intermediate in variability, and showed no cohesive clustering by diet (Figure 4.4f; $P = 0.32$).

To assess the degree of short-term temporal variability, samples were collected 2, 4, 6, and 8 h after feeding silage to cattle, sheep and red deer. The archaeal communities in these samples were analyzed by DGGE using samples from one cow, one sheep and one red deer. The DGGE profiles at the different sampling times were all >95% similar to each other (Figure 4.5). Interestingly, the sample 2 h after feeding was the most different, and the other time points grouped in the same way for all three animals.

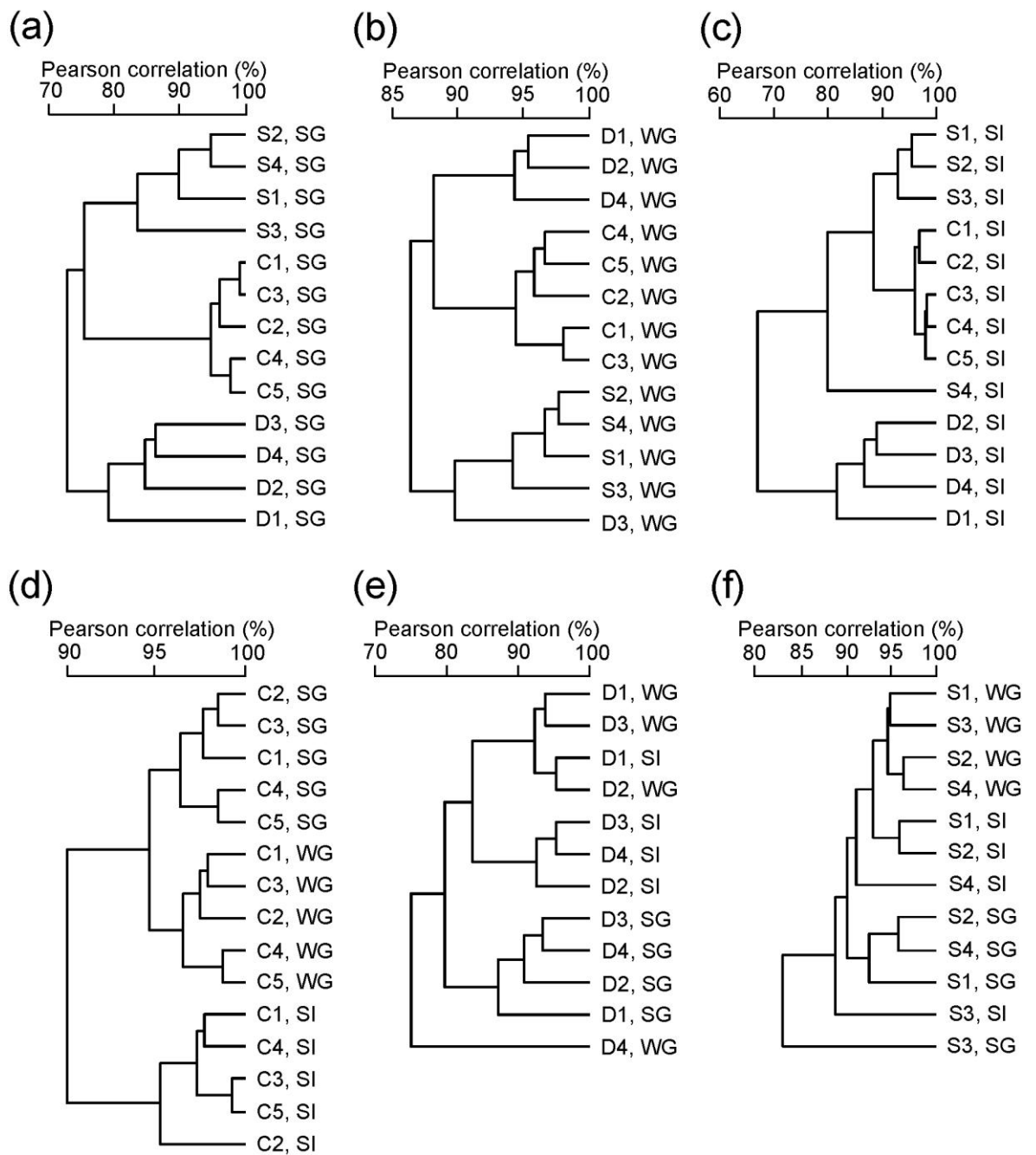


Figure 4.4. Similarities of DGGE profiles of ruminal archaea in the same group of cattle (C1 – C5), red deer (D1 – D4), and sheep (S1 – S4) fed summer pasture (SG), winter pasture (WG) and silage (SI). Each panel represents the similarities of band patterns of lanes on a separate DGGE gel. (a) Cattle, red deer, and sheep fed summer pasture. (b) Cattle, red deer, and sheep fed winter pasture. (c) Cattle, red deer, and sheep fed silage. (d) Cattle fed summer pasture, winter pasture, and silage. (e) Red deer fed summer pasture, winter pasture, and silage. (f) Sheep fed summer pasture, winter pasture, and silage.

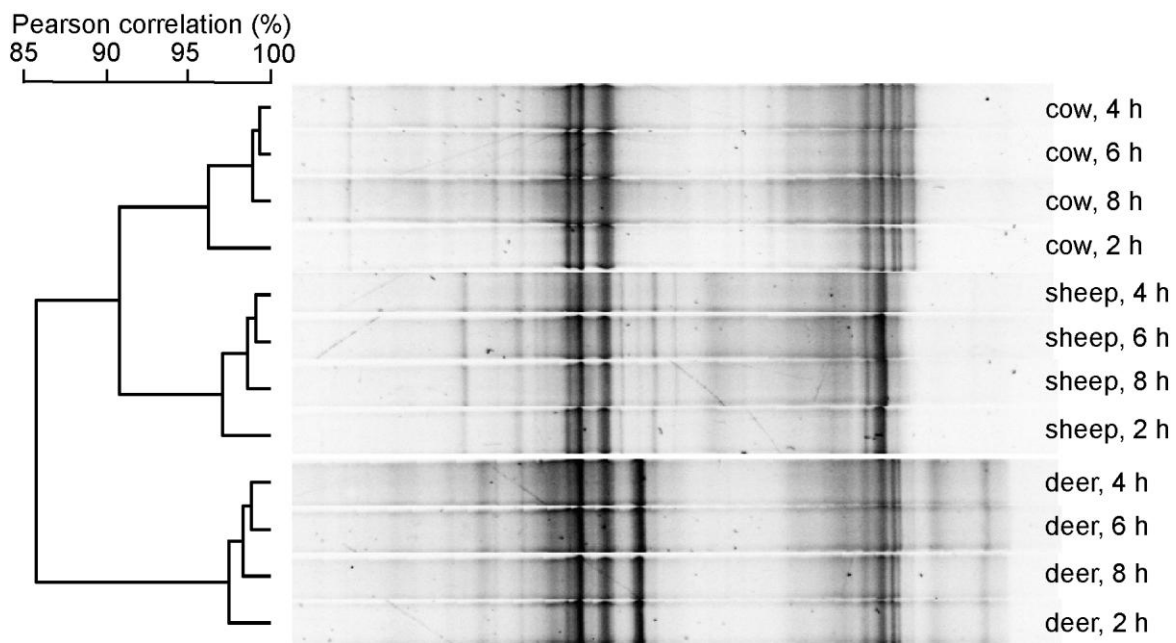


Figure 4.5. DGGE profiles showing stability of archaeal communities in a cow, a red deer, and a sheep, 2, 4, 6, and 8 hours after feeding silage. The similarities of the band patterns are indicated by the dendrogram to the left of the gel image. Individual lanes are not in the same order as they were on the original gel, but all are from the same gel. The gel origin is on the left.

4.3.3 Comparison with bacterial communities

To compare the degree of variation in the rumen archaeal community with that in the bacterial community, DGGE patterns of partial 16S rRNA genes from bacteria were generated from rumen samples collected from two different groups of sheep. One group was fed winter pasture and the second was fed a concentrate-based diet. The banding patterns in the DGGE profiles generated from bacterial 16S rRNA genes was considerably more complex than obtained from the archaeal 16S rRNA genes in the same samples (Figure 4.2). Both the bacterial ($P = 3.4 \times 10^{-4}$) and archaeal ($P = 0.04$) communities were more different between individuals on the concentrate-based diet than on the winter pasture diet.

We assessed if there was less variability between the archaeal communities of individual sheep than between the bacterial communities of the same individuals. The similarity of the archaeal and bacterial communities in all pairwise comparisons of 8 sheep (4 fed winter pasture and 4 fed concentrate-based diet, 28 pairwise comparisons) was determined. The bacterial communities of any pair of individuals were, on average, 18.9% less similar than were the methanogen communities of that same pair of sheep ($P = 8.3 \times 10^{-7}$, paired *t*-test, $n = 28$). The difference increased to 24.7% when the comparisons were limited to pairs of animals on different diets ($P = 1.1 \times 10^{-8}$, paired *t*-test, $n = 16$).

4.3.4 Identity of dominant archaea

To identify the dominant archaea present in the rumen, dominant bands (RODS ≥ 8.31) which appeared to be common to most of the animals were excised from DGGE gels and sequenced. In addition, some of the bands which were not common to all the animals were also excised to identify some of the archaea that represented the minor variations in community structure. A total of 86 sequences were obtained from clones prepared from the 21 bands selected (indicated by numbered arrows in Figure 4.1). A phylogenetic dendrogram was constructed to display the apparent relatedness of the sequences to each other and to reference sequences (Figure 4.6). Sequences from any one band displayed small differences, indicating that different variants were present. These may have arisen during the PCR, from different genes in the same genome, or

from different strains. However, the variations were small, and the sequences from any one band always fell into the same clade (Figure 4.6).

Overall, the sequences grouped into six different clades of archaea: relatives of *Methanobrevibacter ruminantium*, relatives of *Methanobrevibacter gottschalkii*, *Methanosphaera* spp., *Methanosarcina* spp., *Methanoculleus* spp., and RCC. Of these, bands identified as originating from the *Mbb. ruminantium* clade, the *Mbb. gottschalkii* clade, and from *Methanosphaera* spp. were common to all three animal species. The sequences affiliated with the *Mbb. ruminantium* clade, represented by up to four bands, were very similar to the 16S rRNA gene sequence from *Mbb. ruminantium* and *Mbb. olleyae*. The sequences that fell into the *Mbb. gottschalkii* clade were related to *Mbb. gottschalkii*, *Mbb. thaueri* and *Mbb. millerae*. The sequences affiliated with *Methanosphaera* spp. formed two clear sub-clusters supported by high bootstrap values, together with another lineage represented by only one sequence, suggesting that there was more than one species of *Methanosphaera* in these rumen samples. The RCC sequences grouped separately from the other five clades.

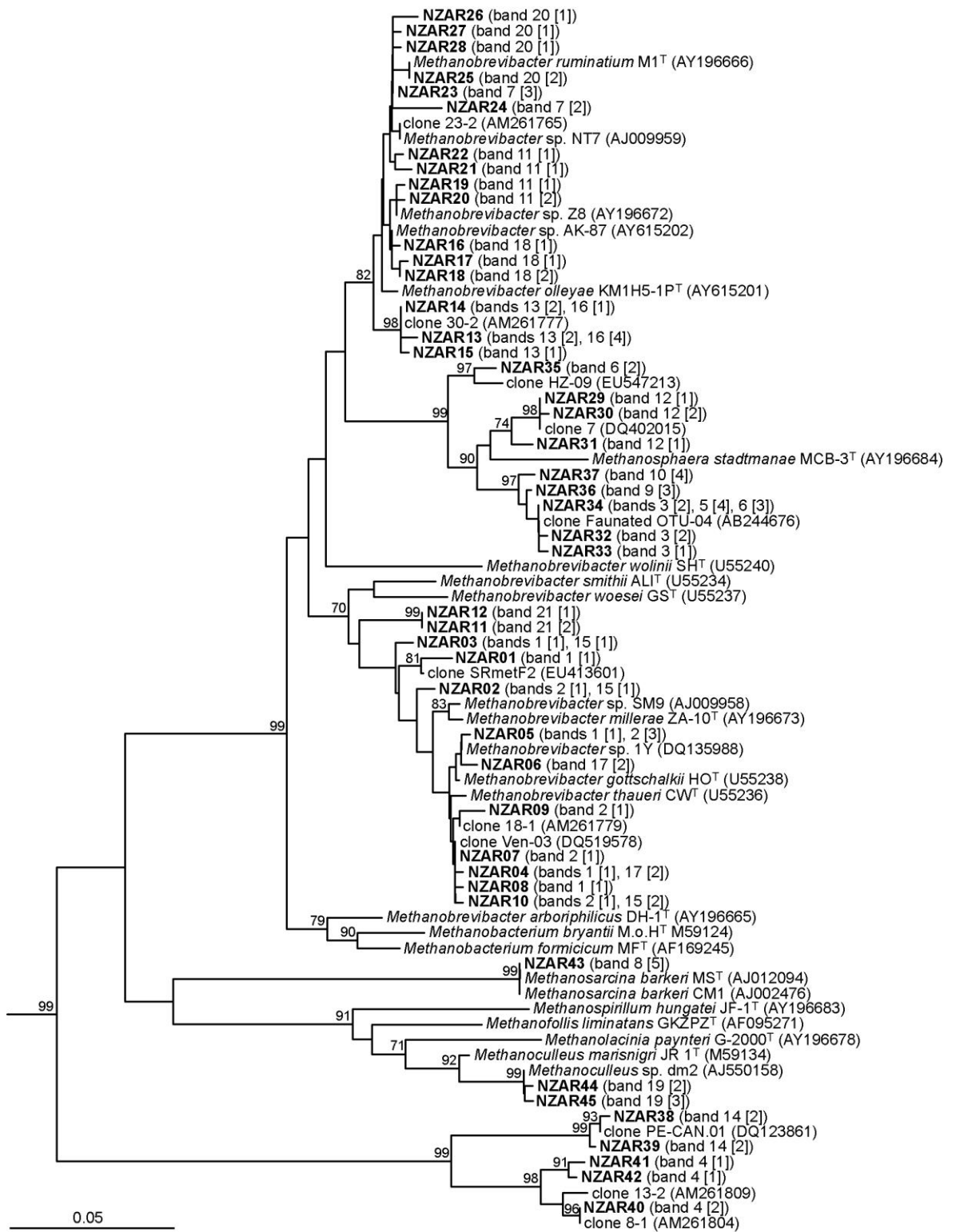


Figure 4.6. Inferred phylogenetic relationships between partial 16S rRNA genes of derived from excised DGGE bands (bolded) and reference 16S rRNA gene sequences. In the parentheses are the GenBank accessions numbers for reference sequences, or the band number and the number of clones (in brackets) from which the sequences were derived. The band numbers correspond to those in Figure 1. The superscript ^T designates a type strain. The tree was constructed from an alignment of 425 nucleotide positions. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates 0.05 nucleotide substitutions per nucleotide position. The 16S rRNA genes of *Methanocaldococcus jannaschii* (GenBank accession M59126), *Methanococcus vannielii* (M36507) and *Methanothermococcus thermolithotrophicus* (M59128) were used as outgroup sequences, and are not shown in the figure.

The identification of the species from which the bands observed in the DGGE profiles originated allowed an assessment of the dominant Archaea present in the rumen samples analyzed. The two dominant bands that migrated near standard b (e.g., bands 1, 2, 15, and 17, Figure 4.1) were both from members of the *Mbb. gottschalkii* clade, while the bands that migrated slightly further (e.g., bands 3, 5, and 6, Figure 4.1) were all affiliated with *Methanosphaera* spp. The groups of bands that migrated with standard h (e.g., bands 7, 11, 13, 16, 18, and 20, Figure 4.1) were all identified as members of the *Mbb. ruminantium* clade. These three clades are the dominant and common methanogens in New Zealand cattle, red deer and sheep fed different diets during different seasons.

The sequence of the band commonly found in all the red deer fed summer pasture, which migrated with standard g (e.g., band 8, Figure 4.1), identified it as originating from a *Methanosarcina* sp.. Another band, which was present in all the red deer fed silage (e.g., bands 9 and 10, Figure 4.1), originated from *Methanosphaera* spp. A further band formed from *Methanosphaera* spp. (e.g., band 12, Figure 4.1) was present at different intensities in nearly all animals on most diets. Although this band migrated distinctly from those formed by 16S rRNA genes generated from the other *Methanosphaera* spp., they did not form a distinct phylogenetic group. Other bands that were not common or dominant originated from members of the RCC clade (bands 4 and 14) and from a *Methanoculleus* sp. (band 19, Figure 4.1).

4.3.5 Diversity of RCC

We generated DGGE profiles using a primer set that amplified partial 16S rRNA genes of members of the RCC group, and compared the RCC community structure in sheep, cattle, and red deer red summer pasture (Figure 4.7). These were the same samples used to generate the total archaeal profiles shown in Figure 4.1a. The new analysis revealed a larger number of bands (mean = 22.8, S.D. = 3.5) than found using universal archaeal primers (mean = 11.2, S.D. = 2.6), indicative of a more even diversity ($P = 7.1 \times 10^{-8}$, paired *t*-test). Similarly, Simpson's and Shannon's diversity indices were greater for the profiles generated using RCC-targeted primers than using Archaea-targeted primers. The mean Simpson's diversity indices were 16.9 (S.D. = 3.1) and 9.1 (S.D. = 2.2) for RCC and Archaea, respectively ($P = 1.1 \times 10^{-6}$, paired *t*-test),

and the mean Shannon's diversity indices were 2.94 (S.D. = 0.17) and 2.26 (S.D. = 0.27) for RCC and archaea, respectively ($P = 1.6 \times 10^{-6}$, paired t -test).

The variation between the RCC communities of individual animals was greater among the samples from sheep than among the red deer or cattle (Figure 4.6). There was a clear grouping by ruminant species ($P = 6.1 \times 10^{-13}$). More bands ($P = 0.008$ to 0.024 for the different pairwise comparisons by ruminant species) were detected in the samples from the red deer (mean = 26.8, S.D. = 2.6) than in those from the cows (mean = 21.8, S.D. = 2.3) and sheep (mean = 20.3, S.D. = 1.7).

4.3.6 Validation of DGGE data

To compare the species composition of the archaeal community, as determined by DGGE, with another method, we selected one of the sheep (no. 4 in Figure 4.1b) fed winter pasture. Libraries of PCR-amplified 16S rRNA genes were prepared using two primer pairs, and randomly-selected PCR products were sequenced and the sequences placed phylogenetically by tree construction. The two primer sets, although amplifying different regions of the 16S rRNA gene, resulted in very similar library compositions (Table 4.3; $P = 0.11$ in χ^2 -test for differences). Three of the four major archaeal groups were the same ones that were also detected using DGGE, viz., the *Methanobrevibacter gottschalkii* clade, the *Methanobrevibacter ruminantium* clade, and *Methanosphaera* spp.. Both libraries contained sequences from the RCC group, making up 22 and 28% of the libraries. In addition, small numbers of sequences affiliated with other lineages were found (Table 4.3). The amount of sequence variation, measured as uncorrected sequence distances, was greatest ($\geq 5.3\%$) among the sequences affiliated with RCC, and lower ($\leq 3.1\%$) among sequences affiliated with the other major groups (Table 4.3).

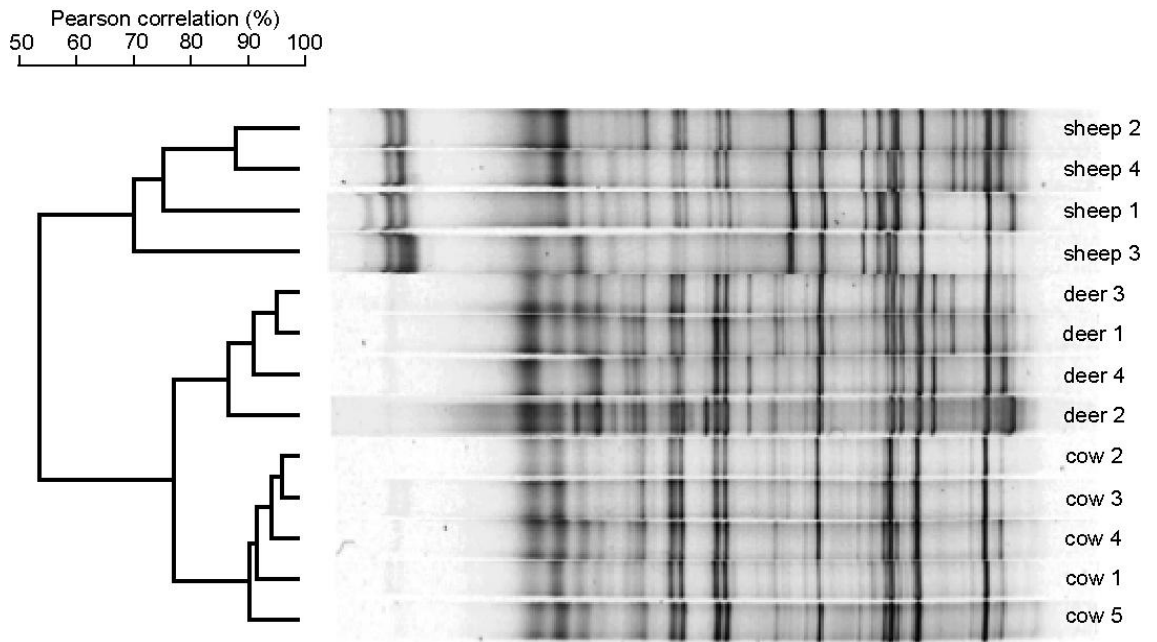


Figure 4.7. DGGE fingerprints of RCC in sheep, red deer, and cattle fed summer pasture. The similarities of the band patterns are indicated by the dendrogram to the left of the gel image. Individual lanes are not in the same order as they were on the original gel, but all are from the same gel. The gel origin is on the left.

Table 4.3. Abundance of different clades of archaea in libraries of PCR-amplified 16S rRNA genes from rumen contents of sheep no. 4 fed winter pasture. The differences between sequences were calculated from percentage identities between pairs of aligned sequences.

Primer pair	Measure	Archaeal group				
		<i>Mbb. gottschalkii</i> clade	<i>Mbb. ruminantium</i> clade	<i>Methanosphaera</i> spp.	RCC clade	Other clades
109f + 915r	No. of sequences	41	29	9	21	3*
	Mean sequence difference (\pm S.D.)	1.4 \pm 0.7%	2.1 \pm 1.3%	1.0 \pm 0.8%	5.3 \pm 3.3%	n.a.†
	Range of sequence differences	0 – 4.7%	0 – 6.7%	0 – 2.5%	0 – 10.5%	n.a.
915f + 1386r	No. of sequences	40	12	14	27	2‡
	Mean sequence difference (\pm S.D.)	1.8 \pm 1.5%	2.2 \pm 1.2%	3.1 \pm 1.9%	5.8 \pm 3.2%	n.a.
	Range of sequence differences	0 – 7.9%	0 – 5.0%	0 – 7.7%	0 – 10.8%	n.a.

*Two sequences only basally-affiliated with RCC, and one sequence from a *Methanobrevibacter* sp. outside the *Mbb. gottschalkii* and *Mbb. ruminantium* clades.

†n.a., not applicable.

‡One sequence only basally-affiliated with RCC, and one sequence from a *Methanosarcina* sp.

The abundance of 16S rRNA genes from total bacteria, total archaea, and from members of the RCC clade of archaea in samples taken from different sheep on a range of diets was measured using quantitative real-time PCR. Bacterial 16S rRNA gene numbers ranged from 1.0×10^{11} to 1.2×10^{12} (mean = 4.5×10^{11} , S.D. = 3.8×10^{11}) copies per g of dry rumen contents. Archaeal 16S rRNA genes were not as abundant, from 3.5×10^9 to 6.2×10^{10} (mean = 1.4×10^{10} , S.D. = 1.3×10^{10}) copies per g of dry rumen contents, and RCC 16S rRNA genes were less abundant again, from 1.3×10^9 to 7.2×10^9 (mean = 2.6×10^9 , S.D. = 1.2×10^9) copies per g of dry rumen contents. There was no detectable correlation between the number of bacterial and archaeal 16S rRNA genes in the samples ($r = 0.06$), but RCC numbers were moderately correlated with total archaeal numbers ($r = 0.64$). On average, archaeal 16S rRNA genes were present at only 5.1% of the abundance of bacterial 16S rRNA genes, and the data suggest that RCC made up an average of 26.5% of the archaeal pool. ANOVA of log-transformed data indicated that there were differences in archaeal ($P = 0.002$) and bacterial ($P < 0.001$) abundances between the different groups of sheep. Abundances of RCC groups did not appear to be significantly different ($P=0.84$) among the different groups of sheep.

Quantitative real-time PCR indicated that 16S rRNA genes from RCC made up 35.5% of the archaeal pool in sheep no. 4 fed winter pasture, in agreement with clone library estimates of 22 to 28% (Table 4.2).

4.4 Discussion

4.4.1 General archaeal community structure

DGGE is a useful method for examining gross differences between microbial communities in environmental samples. DGGE has previously been used to investigate bacterial diversity in the rumen (Kocherginskaya *et al.*, 2001; Tajima *et al.*, 2001a; Klieve *et al.*, 2007), and there have been some studies conducted on rumen archaea. The first application of a technique similar to DGGE to analyze the populations of rumen archaea was performed by Nicholson *et al.* (2007). They analyzed sequences of bands from Temporal Temperature Gradient Gel Electrophoresis profiles to provide a picture of the ruminal archaea in grazing cattle and sheep. Later, Ouwerkerk *et al.* (2008) and Cheng *et al.* (2009) used DGGE to examine the archaeal community structure in a number of rumens. Ohene-Adjei *et al.* (2008) used DGGE as a tool to study the effects of plant extracts on the diversity of archaea in lambs. Recently Hook *et al.* (2009) used this technique to identify the changes in archaeal communities when animals were supplemented with monensin.

In our study we compared the communities of archaea in rumen samples from cattle, sheep and red deer fed with different diets. The DGGE fingerprints of archaea from different species of ruminants (sheep, cattle, and red deer) fed different diets (autumn pasture, winter pasture, summer pasture, silage, a concentrate-based diet, and willow) were all similar, regardless of ruminant species or diet. Time after feeding also had very little impact on the community composition. The overall similarity of most of the profiles suggests that similar dominant archaeal populations were present in these animals, despite being from different individuals and different ruminant species or from animals fed different diets. Similar findings were reported by Ouwerkerk *et al.* (2008), who observed no major differences among DGGE patterns when they examined the archaeal communities of cattle and sheep fed a variety of forages (hay from different grass types, *Leucaena* sp.-grass mix, lucerne pellets and fresh grass). Ouwerkerk *et al.* (2008) did observe greater variation between archaeal communities in individuals fed a barley-based diet, and these patterns were different from those fed forages. The higher grain content (75% [w/w]) in these diets compared with ours (45% [w/w]) may have been the reason for this. High grain contents can result in lower ruminal pH values, which may adversely affect some methanogen species (Lana *et al.*, 1998).

4.4.2 Dominant archaea

Based on the 16S rRNA gene sequences of common dominant bands in our DGGE gels, we suggest that 3 groups of known methanogens dominate New Zealand ruminants: species affiliated with the *Methanobrevibacter gottschalkii* clade; species affiliated with the *Methanobrevibacter ruminantium* clade; and *Methanosphaera* spp. The overall dominance of *Methanobrevibacter* spp. is in agreement with previous clone library-based and TTGE/DGGE-based studies done on different species of ruminants fed with different diets all over the world (Janssen & Kirs, 2008; Pei *et al.*, 2008; Cheng *et al.*, 2009; Williams *et al.*, 2009; Zhou *et al.*, 2009).

Our finding of *Methanosphaera* spp. as a dominant group in New Zealand ruminants is consistent with the studies reported by Nicholson *et al.* (2007) and Skillman *et al.* (2006) on a more limited range of ruminants and diets. Phylogenetic analysis of sequences from our study showed two major clusters of *Methanosphaera* spp. One of these is affiliated with *Methanosphaera stadtmanae*, which was isolated from human feces, and the other cluster probably represents an as-yet uncultured species. *Methanosphaera* spp. have not yet been isolated from rumen, but their presence in the rumen has been implicated in several studies (Wright *et al.*, 2004; Wright *et al.*, 2006; Wright *et al.*, 2007; Pei *et al.*, 2008; Hook *et al.*, 2009). Whitford *et al.* (2001) reported the presence of *Methanosphaera* spp. in ruminants for the first time, and suggested that there may be more than one species of *Methanosphaera*. Comparative analysis of the sequences from our study also supports this statement. *Methanosphaera stadtmanae* is the only known member of the order *Methanobacteriales* that can generate methane only by the reduction of methanol with H₂. Rumen *Methanosphaera* spp. may require the methanol released during the fermentation of pectins as its primary carbon source for growth (Fricke *et al.*, 2006). Fresh forages rich in pectin may be the reason for this high occurrence of *Methanosphaera* spp. in New Zealand ruminants. However, all the phylotypes of *Methanosphaera* spp. may not display the same phenotype, and isolation of rumen *Methanosphaera* spp. will be required to provide more information of their gene complement and physiology.

Although members of the genus *Methanomicrobium* have been reported as one of the dominant groups of methanogens in rumen (Janssen & Kirs, 2008; Chaudhary & Sirohi, 2009), this has not been confirmed for New Zealand ruminants. No dominant

bands representing this group were found in any of the samples we analyzed. The genus *Methanomicrobium* belongs to the order *Methanomicrobiales* and two species of methanogens belonging to this order have been isolated from rumen: *Methanomicrobium mobile* (Paynter & Hungate, 1968) and *Methanoculleus* sp. (Joblin, 2005). We did find a band that originated from a *Methanoculleus* sp. in one sample, confirming their presence in at least some New Zealand ruminants.

4.4.3 Rumen Cluster C archaea

According to Janssen & Kirs (2008), the RCC group contributed 15.8% of total archaea in their global data set. RCC is a group of uncultured archaea found to be numerically abundant in some libraries of archaeal 16S rRNA genes (Tajima *et al.*, 2001*b*; Wright *et al.*, 2006; Nicholson *et al.*, 2007; Wright *et al.*, 2007; Williams *et al.*, 2009). They may not be universally present, as some studies have reported finding no evidence for the presence of members of this group (Skillman *et al.*, 2006; Wright *et al.*, 2008; Hook *et al.*, 2009; Zhou *et al.*, 2009). Our DGGE fingerprints of total archaeal communities did not reveal any common dominant bands of that group in all the rumen samples. However, we found 2 bands (bands 4 and 14) corresponding to that group, one in red deer grazed with summer pasture and one in sheep grazed with winter pasture. These two bands were from widely different places in the gel, and represented different sequence types. It is probable that there are other bands corresponding to this group present in various places in the gels, but that these were less intensely stained because of the low abundance of any one sequence type.

Libraries of PCR-amplified 16S rRNA genes prepared from a sample from one of the sheep (no. 4, fed with winter pasture) used in our study revealed that 22-28% of the sequences were from members of RCC. This was confirmed by the quantitative PCR analysis of 16S rRNA gene abundances in the same sample, which suggested that RCC made up 35.5% of archaeal 16S rRNA genes in that sample and an average of 26.5% of archaeal 16S rRNA gene sequences in sheep on a range of diets. The moderate correlation of RCC and total archaeal numbers indicates that they form a relatively constant part of the total archaeal community. Sequences falling into RCC displayed more sequence-to-sequence variation than did sequences falling into other groups. DGGE analysis of RCC 16S rRNA genes confirmed this, with a greater number of

bands detected than when analysing the total archaeal community. Since RCC is a subgroup of archaea, this indicates that there is a much greater diversity of archaea than is detectable using DGGE targeting total archaea, with each sequence type being too rare to be detected in PCR products dominated by the few very abundant archaeal groups. The analysis of a large number of sequences from multiple libraries (Janssen & Kirs, 2008) indicates that the RCC group displays considerable variation in sequence types that fall into a number of distinct lineages and may, therefore, represent a number of distinct species and genera. The abundance of any one sequence type is low, so members of this group are not readily observed by DGGE using domain-level primers. This highlights a limitation of the DGGE approach, which is useful for assessing differences in patterns of dominant components of the community. In the case of the rumen archaea, it was useful for assessing differences in patterns among the less diverse but major clades of known methanogens (*Methanobrevibacter* spp., *Methanosphaera* spp.), but could not detect changes among the more diverse but less abundant RCC clade.

We confirmed the presence of many different bands attributable to RCC by performing DGGE using primers targeting this group. RCC community patterns were different in sheep, cows, and red deer fed the same diet. Members of RCC may be methane-producing microbes (Nicholson *et al.*, 2007), but since their physiology is not known, it is not possible to speculate on the reason for these host-based differences. We have not examined diet-specific differences in RCC community composition.

4.4.4 Host and diet specific differences

Even though the general archaeal community composition was similar in all samples, we did find that the archaeal communities of the different ruminant species tested (cattle, sheep, and red deer) were distinctive, regardless of diet, indicating host-specific community structures. We also found that, within ruminant species, archaeal communities in cattle were different when different diets were fed, but that this was not as strong with red deer, and not detectable in sheep. While the major clades of archaea detected were similar in all samples, the differences were in minor parts of the communities, and subgroups within major clades. The amount of variation in the archaeal communities was less than in the bacterial communities of the same animals,

and there were fewer detectable bands on DGGE gels of archaeal 16S rRNA genes than of bacterial 16S rRNA genes.

Archaeal communities in the rumens of red deer had structures similar to those in cattle and sheep, with some minor differences. The dominant bands present in cattle and sheep samples were also common to red deer. However, compared to cattle and sheep, the individual variation among red deer rumen methanogen communities was greater. When they were fed with summer pasture, a *Methanosarcina* sp. was found as one of the dominant bands in all of the red deer. In addition, the samples from silage-fed red deer contained a *Methanosphaera* sp. of a different sequence type that resulted in a DGGE band distinct from those formed from other *Methanosphaera* spp. These two distinct bands were largely absent when these same red deer were fed winter pasture. It has been found that red deer exhibit strong seasonal cycles of rumen digestive function, with increased rumen retention time in summer (Freudenberger *et al.*, 1994). *Methanosarcina* spp. grow relatively slowly and have higher K_m values for H_2 than other methanogens (Zinder, 1993) and they would be washed out of the rumen unless the flow through the rumen is slowed. The unusual digestive physiology of red deer may also partly be the reason for the high variation in their banding patterns compared to cattle and sheep. To our knowledge, there have been no published studies on the rumen methanogens of red deer. Sundset *et al.* (2009a, 2009b) have reported studies on the diversity of rumen methanogens in Norwegian and Svalbard reindeer. They found the methanogens present in the reindeer were similar to the methanogens found in domesticated ruminants (cattle and sheep). Our study shows that the same is true of domesticated red deer.

Several studies have found host-species differences on the bacterial community of the rumen (Shi *et al.*, 2008; Sundset *et al.*, 2007). Effects of diet on changes on the diversity of bacterial species in the rumen have also been reported (Kocherginskaya *et al.*, 2001; Tajima *et al.*, 2001a). Different microbes act on the ingested feed during feed fermentation, and the species composition can be expected to be strongly influenced by the properties of the feed. Therefore, it is expected that there should be differences in the bacterial communities in rumens when animals are fed different diets. We found the bacterial community to be more complex than the archaeal community. We also found that the amount of variation between bacterial communities of animals fed different

diets, and between animals on the same diet, was significantly greater than variations between the archaeal communities. Most rumen methanogens grow with the H₂ produced during the fermentation of the ingested feed. The rumen environment (*T*, pH, CO₂, H₂ and salts) is relatively constant over time. This is probably the reason why the diversity of methanogens in the rumen of different ruminant species fed with different diets is relatively similar. The effect of diet was strongest in cattle, less so in red deer, and not detectable in sheep. It is interesting to speculate that the observation of effects of diet on cattle methanogen community relative to sheep and red deer is due to the fact that cattle rumens are much larger than those in either sheep or red deer. Cattle rumens may represent a more stable environment leading to less variability in methanogen community structure between individuals. Variation between individual sheep and red deer may be greater, masking the subtle diet effects.

All rumens sampled in this study contained remarkably similar archaeal communities, suggesting a common core of ruminal methanogen species. This is significant for efforts to develop methane mitigation strategies, as it limits the number of methanogen groups that need to be targeted to control the majority of methane producers. It is unclear, however, if the minor components of the methanogen community are specialists occupying narrow niches with limited capacity to contribute to total methane production, or if they will increase in abundance if the dominant groups are eliminated.

Note

A shorter version of this chapter has been published in *FEMS Microbiology Ecology*. This chapter therefore has more extensive methods and discussion sections than the other chapters.

Jeyanathan J, Kirs M, Ronimus RS, Hoskin SO & Janssen PH (2011) Methanogen community structure in the rumens of farmed sheep, cattle and red deer fed different diets. *FEMS Microbiol Ecol*, no. doi: 10.1111/j.1574-6941.2011.01056.

Chapter 5

Effects of rumen-administered chloroform on the abundance and diversity of *mcrA* and 16S rRNA genes of rumen archaea indicate the presence of as-yet uncultured methanogens

5.1 Introduction

Methanogenic archaea living in the rumen of ruminant animals produce methane (CH₄) as an end product of ruminal feed fermentation, primarily from the hydrogen (H₂) produced during the fermentation of feed by bacteria, fungi and protozoa. The environmental and economic concerns of methane production from ruminant animals have led to research to develop means of mitigating CH₄ production emanating from the rumen and to channel the carbon and energy to more volatile fatty acids (VFAs) and microbial biomass (McAllister & Newbold, 2008). Research has focused on reducing the amount of CH₄ formed in the rumen by feed manipulation (Fievez *et al.*, 2003; Guo *et al.*, 2008), broad-range inhibitors (Božić *et al.*, 2009; Hamilton *et al.*, 2010), animal selection (Chagunda *et al.*, 2009), interfering with H₂ transfer to the methanogens (Joblin, 1999), or by specifically inhibiting the methanogens themselves using vaccines and small-molecule inhibitors (Wedlock *et al.*, 2010; Williams *et al.*, 2009). Many of these techniques have limitations and met with only limited success (McAllister & Newbold, 2008). Vaccines and small-molecule inhibitors which target methanogens could be effective technologies to reduce methane emissions from New Zealand farmed ruminants, as they are relatively readily applied to pasture-grazed animals. To produce targeted vaccines and inhibitor-based technologies, identification of targets found only in rumen methanogens is essential. This requires knowledge of rumen methanogens.

A meta-analysis of rumen archaea suggested that most are known methanogens, but some archaeal lineages of unknown physiology were also present (Janssen & Kirs, 2008). The largest of these, designated Rumen Cluster C (RCC), made up about 16% of the global dataset (Janssen & Kirs, 2008), and up to 81% of the archaeal 16S rRNA genes detected in some rumen samples (Wright *et al.*, 2006). Unlike other known rumen methanogens, the contribution of RCC group to the total archaeal pool in various rumen samples has varied widely from highly dominant to completely absent (Table 1.2). In

New Zealand ruminants, RCC was one of the dominant groups of archaea and qPCR analysis of rumen samples obtained from sheep fed a range of diets indicated that, RCC made up an average of 26.5% of the archaeal pool (Chapter 4). However, the role of RCC in the rumen is not known yet.

Metabolic inhibitors are very useful to study microbial processes in a given environment. Chloroform (CHCl_3) is an inhibitor that selectively inhibits methanogens at low concentrations. Chloroform been used experimentally at low concentrations to specifically inhibit methanogens without changing the remaining overall microbial community structure (Achnich *et al.*, 1995; Chidthaisong & Conrad, 2000; Chin & Conrad, 1995; De Graaf *et al.*, 1996). The inhibitory action of CHCl_3 on rumen methanogens was accidentally found by Bauchop in 1967. Low concentrations of CHCl_3 inhibit cobamide dependent methyl-transfer reactions (Wood *et al.*, 1968; Kenealy & Zeikus, 1981) that normally lead to the formation of methane by methanogens. However, it appears that CHCl_3 also inhibits the methyl coenzyme M reductase, an enzyme diagnostic of methanogens that catalyzes the formation of CH_4 from methyl-coenzyme M (Gunsalus & Wolfe, 1978). Thus, there may be multiple modes of inhibition by CHCl_3 . Upon the CHCl_3 treatment, archaea that are methanogens should be inhibited and be eliminated from the rumen, while non-methanogenic archaea may be resistant.

There is no strong evidence to suggest that RCC are methanogens thus far. In this study CHCl_3 was added to the rumens of cows and the responses of the archaeal community were studied by monitoring the abundance and diversity of genes encoding for the 16S rRNA, and for the subunit of the methyl-coenzyme M reductase.

5.2 Materials and Methods

5.2.1 CHCl_3 treatment and sample collection

This experiment was carried out by Knight *et al.*, (2011) to study the effect of CHCl_3 on methane production. Samples of total rumen contents collected from this study was used in the present study for microbial analysis. Briefly, the rumen samples were collected from six mature fistulated non-lactating dairy cows (Friesian-Jersey cross), fed with a fixed 8.4 kg DM per day of lucerne silage and barley meal (Section

2.2). Feed was provided with 40% of the diet in the morning and 60% of the diet in the afternoon. These cows were divided into control and treatment groups based on their methane measurement obtained while being fed lucerne silage. The control group (C2, C4 and C6) was drenched orally with 30 ml sunflower oil and the treatment group (C1, C3 and C7) was drenched with 30 ml sunflower oil containing 1.5 ml of CHCl_3 each day morning (10:00 to 11:00 A.M.) for 42 days (resulting concentration of CHCl_3 in rumen ≈ 0.2 mM). Methane emissions were measured once a week, using the sulphur hexafluoride (SF_6) release method (Pinares-Patiño *et al.*, 2008). Rumen samples were collected from each of the cows via the rumen fistula 2 h after the morning feed (at $\sim 9:00$ A.M.) from the day before the start of drenching with CHCl_3 and, thereafter once a week for 6 weeks. Samples were immediately frozen at -80°C and subsequently lyophilized. The samples were then homogenized and stored at -80°C until DNA was extracted.

5.2.2 Total DNA extraction and PCR

Total DNA was extracted from 100 mg of ground freeze-dried rumen sample using the QIAamp DNA stool mini kit (Qiagen) according to the manufacturer's protocol, and stored at -20°C . This extracted DNA was used for the construction of 16S rRNA and *mcrA* gene clone libraries, quantitative real time PCR (qPCR), and denaturing gradient gel electrophoresis (DGGE). The primers used for the clone library construction, DGGE and qPCR are shown in Table 5.1. Details of the reaction mixtures and the thermal cycling conditions for these primers are shown in Tables 2.2, 2.3, 2.5 and in Section 2.19. PCR amplifications were performed in a Hybaid Px2 thermal cycler (ThermoElectron) and the qPCR amplifications were performed in Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science).

5.2.3 Clone library construction and phylogeny

Purified PCR products were ligated into the pCR 2.1 TOPO TA cloning vector and transferred into TOP10 chemically competent *Escherichia coli*. Transformed cells were then plated onto LB agar containing ampicillin and incubated overnight at 37°C (Section 2.12). Clones were randomly selected and sequenced (Section 2.13).

DNA extracted from all CHCl₃-treated and control cows were used for the construction of 16S rRNA gene clone libraries. Clone libraries of *mcrA* genes were constructed using DNA from one CHCl₃-treated cow (C3) and one control cow (C2). All libraries were constructed using DNA extracted from the samples collected on day 5 of the experiment (4 days after CHCl₃ treatment), when methane production was the lowest. Diversity indices (Shannon diversity and Simpson's indices) and Shannon diversity *t* test were conducted using the proportions of different methanogens groups in each clone library by Version 2.01 of the PAST (PAleontological STatistics) software package (Section 2.15).

Table 5.1. Primer pairs selected for qualitative (clone libraries, DGGE) and quantitative (qPCR) study of rumen microbes (Archaea, bacteria and RCC) in CHCl₃-treated (C1, C3 and C7) and control cows (C2, C4 and C6).

Method	Target gene	Primers	DNA used
Clone library	16S rRNA gene of archaea	109f/915r	C1, C2, C3, C4, C6, C7
	<i>mcrA</i> gene of methanogens	ML1/ML2	C2, C3
DGGE	16S rRNA gene of archaea	915af ^a /1386r	C1, C2, C3, C4, C6, C7
	16S rRNA gene of RCC	762f ^a /1099r	
qPCR	16S rRNA gene of archaea	915af/1386r	C1, C2, C3, C4, C6, C7
	16S rRNA gene of RCC	762f/1099r	
	16S rRNA gene of bacteria	519f/907r	

^aThese primers were tagged with a 40 bp long GC-rich sequence segment (CGCCCGCCGCGCGCGGGCGGGCGGGGCGGGGGCACGGGGGG) on the 5'-end for DGGE.

5.2.4 Phylogenetic analysis

Phylogenetic analyses of the sequences of partial fragments 16S rRNA and *mcrA* genes were performed using MEGA 4.0 software (Section 2.14). Nucleotide

sequences were used for the construction of 16S rRNA gene phylogenetic trees and deduced amino acid sequences were used for the construction of *mcrA* protein phylogenetic trees.

5.2.5 Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed for archaea and RCC using selected rumen samples obtained before and after the CHCl₃ treatment. The primer pairs used for the DGGE are shown in Table 5.1. DGGE was performed by using a CBS scientific DGGE/TTGE gel system (Section 2.16) and DGGE fingerprints were analyzed with the BioNumerics software package (Section 2.18).

5.2.6 Quantitative real-time PCR analysis

The primer pairs used for the qPCR are shown in Table 5.1. Abundances of 16S rRNA genes from Archaea, bacteria and members of RCC in selected rumen samples were quantified using a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science) and amplicon detection with SYBR Green (Section 2.19)

5.2.7 Sequences of 16S rRNA and *mcrA* gene clones

Details of the sequences obtained from the 16S rRNA and *mcrA* gene clone libraries from control and treated cows (Table 5.2) are shown in Appendix 1.

Table 5.2. The details of 16S rRNA and *mcrA* gene clones created from CHCl₃-treated and control cows.

Samples	CHCl ₃ -treatment	Target gene	Names of clones
C1	-	16S rRNA	C1SP1T001 to C1SP1T024
C3	-		C3SP1T001 to C3SP1T041
C7	-		C7SP1T001 to C7SP1T022
C2	+		C2SP1001 to C2SP1047
C4	+		C4SP1001 to C4SP1021
C6	+		C6SP1001 to C6SP1024
C2	+	<i>mcrA</i>	C2SMP1001 to C2SMP1051
C3	-		C3SMP1T001 to C3SMP1T048

5.3 Results

5.3.1 Methane production

CHCl₃ was introduced as an emulsion (1.5 ml in 30 ml of sunflower oil) into the rumens of 3 cows, while a group of 3 control cows received only the 30 ml of sunflower oil. Methane emissions from CHCl₃-treated and control cows are presented in Figure 5.1. Average methane emission from CHCl₃-treated cows was reduced from 146.12 g/day to 6.84 g/day after 4 days of CHCl₃ treatment. After day 5, the CH₄ emissions from CHCl₃-treated cows gradually increased, and by day 38 it had reached 76.23 g/day. There were some fluctuations observed in the average methane emission from control cows (108.76 - 185.00 g/day), however, it was not strongly reduced like for the CHCl₃-treated cows, which showed a decrease of approximately 95% after 4 days of treatment.

Statistical analysis was performed to compare the control and treated cows and it showed that the methane emissions did not differ significantly between CHCl₃-treated and control cows 13 and 2 days before the start of CHCl₃ treatment. The methane emission between these two groups differed significantly after 4 days ($P < 0.05$), 11 days ($P < 0.01$), 18 days ($P < 0.05$) and 25 days ($P < 0.05$) of the start of CHCl₃ addition. However, there were no significant differences observed in methane emission after 32 and 39 days of the start of CHCl₃ addition. Average methane emissions in CHCl₃-treated cows was lowest after 4 days of the CHCl₃ treatment. However, the difference between the CHCl₃-treated and control cows was highest ($P < 0.01$) after 11 days of start of CHCl₃ addition. A slight increase in the methane production in 2 control cows (C4 and C6) on day 12 compared to day 5 is the reason for this observation. There was a reduction in methane production on day 35 in all control cows, but there was no obvious reason for this decline.

5.3.2 Archaeal and bacterial community sizes

Changes in the number of bacteria, archaea and RCC in rumen samples collected during the experimental period from both control and treated cows were estimated using quantitative PCR of 16S rRNA gene numbers. The statistical analysis performed using both actual copy numbers of these organisms and log-transformed data from the actual

copy numbers were shown similar results. The results shown here were from the actual copy numbers of these organisms,

The number of bacterial 16S rRNA genes was not significantly different between CHCl₃-treated and control cows throughout the experimental period (Figure 5.2). This varied from 4.62×10^{10} to 2.56×10^{11} copies per g of dry rumen contents. This suggests that total bacteria were not affected by the CHCl₃ treatment. Methane production in control animals was reduced on day 35 due to some unknown reasons (Figure 5.1). Corresponding to that, the number of bacterial 16S rRNA genes was also reduced at that point, suggesting it was related to rumen digestion or feed intake.

Changes in the number of 16S rRNA genes of total archaea are shown in Figure 5.3. The number of archaeal 16S rRNA genes decreased rapidly after the addition of the CHCl₃, to about 7.9% of the starting number in 4 days, however, the archaeal 16S rRNA genes in control cows remained unchanged during the same period. The archaeal 16S rRNA genes remained low in CHCl₃-treated cows till 8 days after the start of treatment and then gradually increased over time, and eventually reached about 45% of that in the control animals in 35 days.

The statistical analysis showed that the numbers of archaeal 16S rRNA genes were not different between CHCl₃-treated and control cows before the start of CHCl₃ treatment. The numbers of 16S rRNA genes in CHCl₃-treated cows were significantly lower after 1 ($P < 0.01$), 4 ($P < 0.05$), 8 ($P < 0.05$), and 13 ($P < 0.01$) days of the CHCl₃ treatment compared to the control cows. The difference in archaeal 16S rRNA genes between CHCl₃-treated and control cows was not significant after 20 and 27 days of CHCl₃ treatment. Surprisingly this difference was again significant ($P < 0.05$) after 34 days of CHCl₃ treatment. The sudden reduction of 16S rRNA copy numbers in one of the CHCl₃-treated cow (C7) in day 35 was the reason for this difference.

The number of 16S rRNA genes from the RCC group showed a similar pattern to that for the 16S rRNA genes of total archaea (Figure 5.4). The number of RCC 16S rRNA genes in CHCl₃-treated cows decreased after the addition of the CHCl₃, to about 18% of the starting number in day 5. The number of RCC 16S rRNA genes remained low for 5-9 days after the CHCl₃ treatment and, similar to archaeal 16S rRNA gene numbers, they increased over time and reached 87.5% on average of the starting number

at day 35. In some CHCl₃-treated cows the number of 16S rRNA genes of the RCC group exceeded the initial number before the treatment. Thus, members of RCC may have adapted to the CHCl₃ treatment more quickly than the other rumen methanogens. The other possibility is that the members of CHCl₃-resistant groups of RCC may have filled the niches vacated by rumen methanogens or CHCl₃-sensitive RCC.

The statistical analysis showed that the numbers of RCC 16S rRNA genes were not different between CHCl₃-treated and control cows before the start of CHCl₃ treatment. The number of RCC 16S rRNA genes in CHCl₃-treated cows were significantly low after 4 ($P < 0.05$) and 8 ($P < 0.01$) days of the CHCl₃ treatment compared to the control cows. Similar to archaeal 16S rRNA genes, the difference between CHCl₃-treated and control cows was again significant ($P < 0.05$) after 34 days of CHCl₃ treatment.

The contributions of 16S rRNA genes of RCC to total archaeal 16S rRNA genes over the experimental period are presented in Figure 5.5. The percentage of RCC 16S rRNA genes in the CHCl₃-treated cows increased on average from 37% before the CHCl₃ treatment to 89% immediately after the treatment, and decreased again to 45% by day 35. In the control animals, the percentage of RCC 16S rRNA genes remained more or less similar over the experimental period. Interestingly, their percentage contribution to the total archaeal community in the control cows was higher when methane production and the number of bacterial 16S rRNA genes were reduced due to some unknown reasons on day 35.

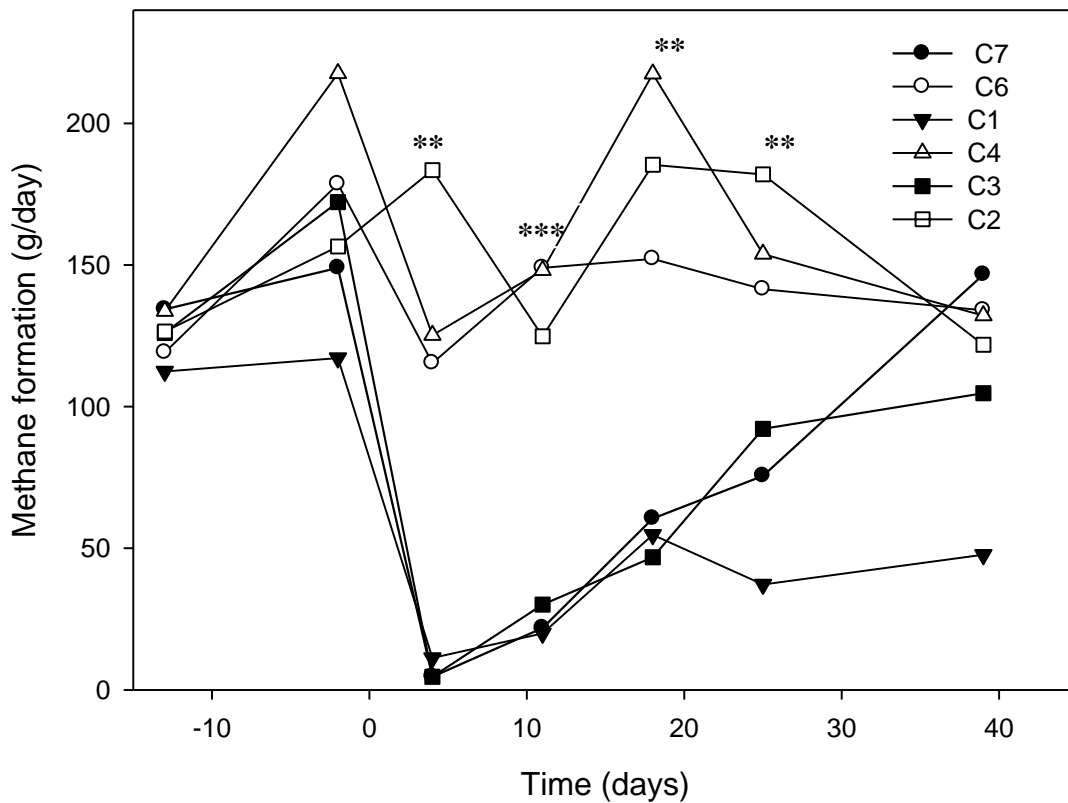


Figure 5.1. Methane emissions from control (C2, C4 and C6) and CHCl_3 -treated (C1, C3 and C7) cows over the experimental period. The filled symbols represent CHCl_3 -treated cows and open symbols represent control cows. CHCl_3 treatment was commenced on day 1. Data obtained from the CHCl_3 trial conducted by Knight T, Ronimus RS, Dey D, Tootill C, Naylor G, Evans P, Molano G, Smith A, Tavendale M, Pinares-Patiño CS and Clark H was used to create this graph. The significance of statistical differences (performed in this study) between control and CHCl_3 -treated cows are shown at each points with the symbols ** for $P < 0.05$ and *** for $P < 0.01$.

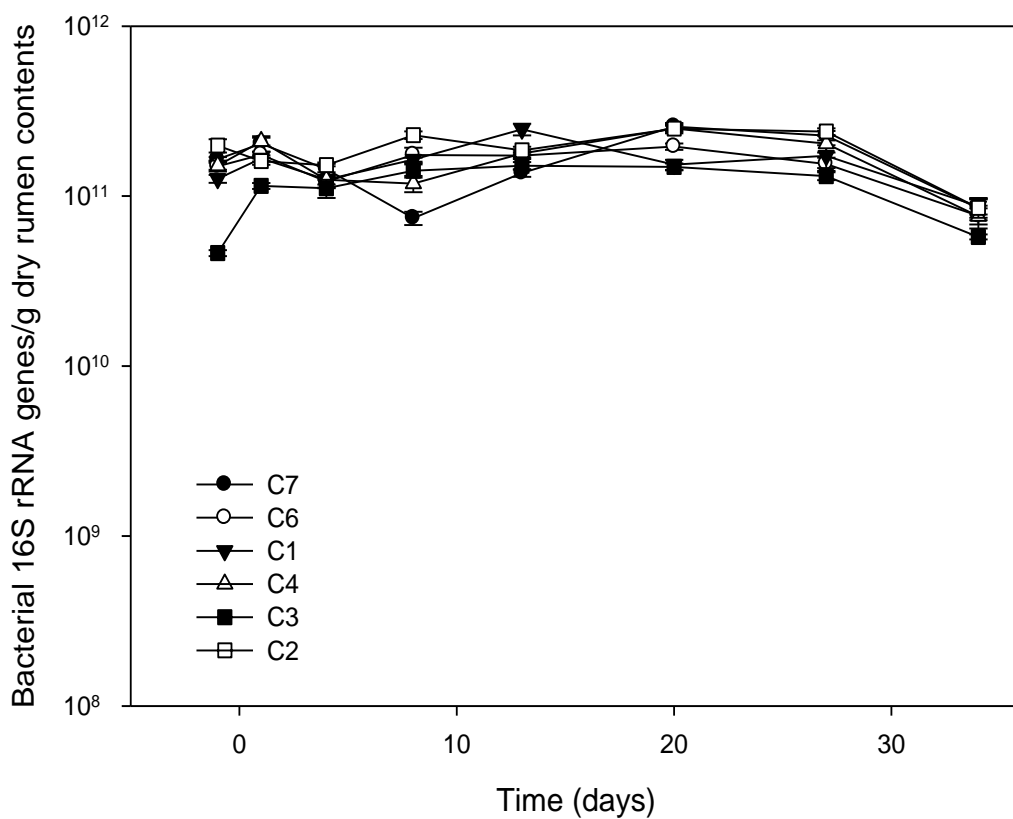


Figure 5.2. Changes in the number of bacterial 16S rRNA genes in control (C2, C4 and C6) and CHCl₃-treated (C1, C3 and C7) cows over the experimental period (before and after the CHCl₃ addition). CHCl₃ was added at day 0. The filled symbols represent CHCl₃-treated cows (CHCl₃) and the open symbols represent control cows. The symbols in the panel are means of 6 replicates, and the thin vertical bars in panel represent the standard error on either side of the mean.

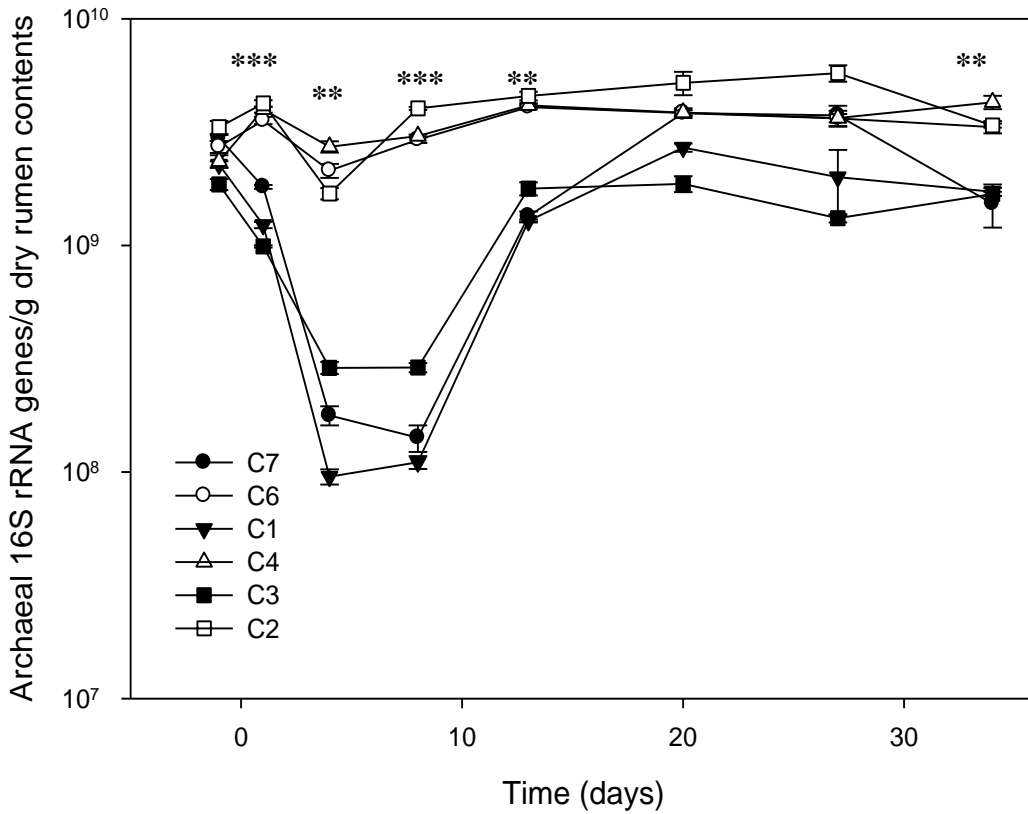


Figure 5.3. Changes in the number of archaeal 16S rRNA genes in control (C2, C4 and C6) and CHCl₃-treated (C1, C3 and C7) cows over the experimental period (before and after the CHCl₃ addition). CHCl₃ was added at day 0. The filled symbols represent CHCl₃-treated cows (CHCl₃) and the open symbols represent control cows. The symbols in panel are means of 6 replicates, and the thin vertical bars in panel represent the standard error on either side of the mean. The significance of statistical differences between control and CHCl₃-treated cows are shown at each points with the symbols ** for $P < 0.05$ and *** for $P < 0.01$.

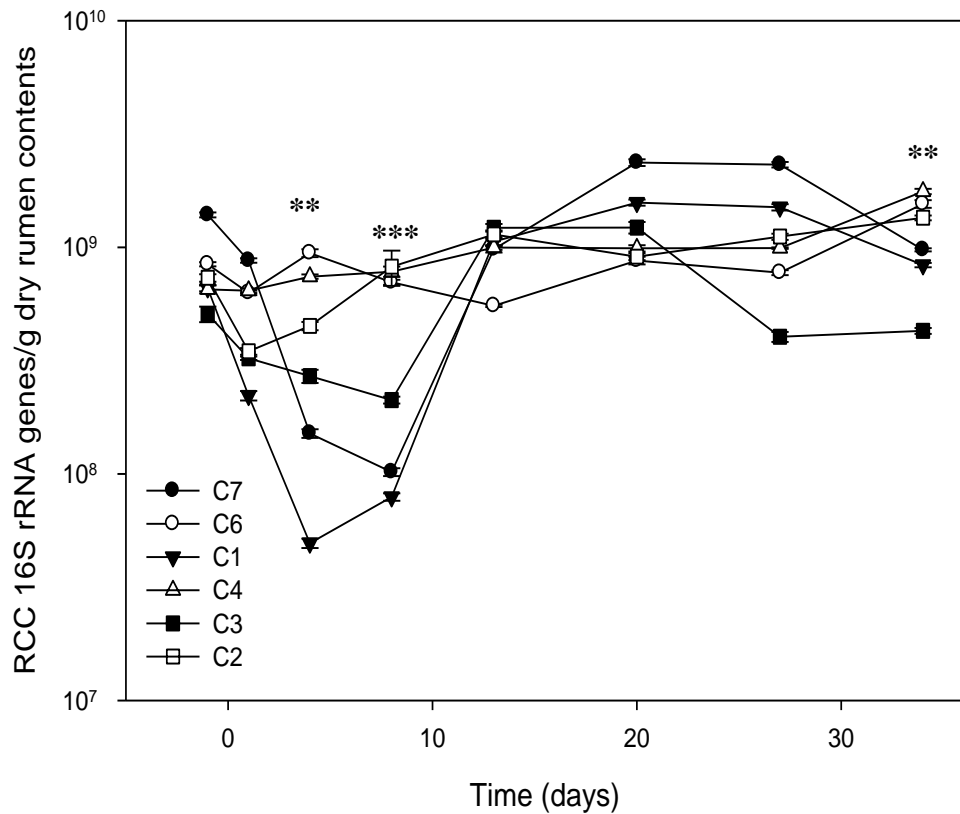


Figure 5.4. Changes in the number of RCC 16S rRNA genes in control (C2, C4 and C6) and CHCl₃-treated (C1, C3 and C7) cows over the experimental period (before and after the CHCl₃ addition). CHCl₃ was added at day 0. The filled symbols represent CHCl₃-treated cows (CHCl₃) and the open symbols represent control cows. The symbols in panel are means of 6 replicates, and the thin vertical bars in panel represent the standard error on either side of the mean. The significance of statistical differences between control and CHCl₃-treated cows are shown on each points with the symbols ** for $P < 0.05$ and *** for $P < 0.01$.

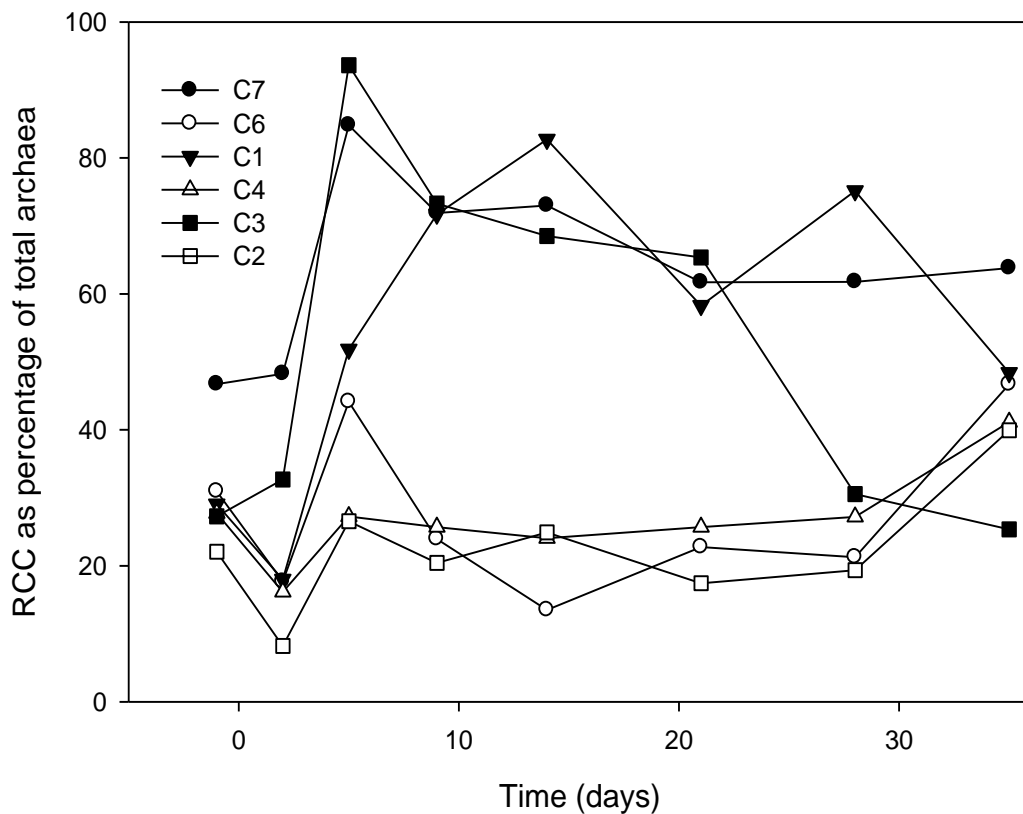


Figure 5.5. Changes in the percentages of RCC 16S rRNA genes in the total archaeal 16S rRNA genes in control (C2, C4 and C6) and CHCl₃-treated (C1, C3 and C7) cows over the experimental period (before and after the CHCl₃ addition). CHCl₃ was added at day 0. The filled symbols represent CHCl₃-treated cows (CHCl₃) and the open symbols represent control cows.

5.3.3 Total archaeal community structure

The archaeal communities in the rumens of the CHCl_3 -treated and control cows were analyzed by generating DGGE fingerprints of partial 16S rRNA genes (Figure 5.6). Samples collected when the CH_4 production was lowest (day 5) were used to check the effect of CHCl_3 treatment on the archaeal community. Archaeal DGGE showed a clear difference before and after the treatment in CHCl_3 -treated cows and no difference in control cows. All the major bands disappeared when the cows were treated with CHCl_3 . There were no significant differences in the numbers of bands between control cows and CHCl_3 -treated cows before the CHCl_3 treatment. After 4 days of CHCl_3 treatment the number of bands in CHCl_3 -treated cows was reduced significantly ($P < 0.01$) compared to the control cows. There were far fewer bands (3 bands on average) with little variation in intensity in the patterns generated from the CHCl_3 -treated cows on day 5.

The banding pattern before the CHCl_3 treatment of the CHCl_3 -treated cows and the control animals were similar to the banding pattern obtained in Chapter 4 where different ruminants (cattle, sheep and red deer) on various diets were compared. After the CHCl_3 treatment, a few weak bands were observed in the position where members of major methanogen groups such as the *Methanobrevibacter gottschalkii* clade, the *Methanobrevibacter ruminantium* clade and *Methanosphaera* spp. were found (Chapter 4). Most of these methanogens were eliminated after the CHCl_3 treatment, evidenced by very low methane production and the weak banding pattern of the archaeal DGGE. However, it appears that some of these methanogens were still present in small numbers in the CHCl_3 -treated cows after the CHCl_3 treatment. The effect of CHCl_3 on members of RCC cannot be checked by the archaeal DGGE as they are a very diverse group and less intensively stained bands not readily visible in the DGGE gels may represent this group (Chapter 4).

Analysis of the band patterns in the archaeal DGGE gel grouped the CHCl_3 -treated cows separately from the other cows, i.e. all the cows before the CHCl_3 treatment and control cows after the treatment (Figure 5.7). The overall similarity value of CHCl_3 -treated and control cows was 57.7%. The cluster of CHCl_3 -treated cows showed high similarity among themselves (93.7%) with only few bands and these bands were mostly present in similar positions in all lanes for the CHCl_3 -treated cows. All the

other cows clustered together with an overall similarity of 94.6%. This clearly shows that the banding pattern of DGGE gel was different after the CHCl_3 treatment for the CHCl_3 -treated cows and not for the control cows.

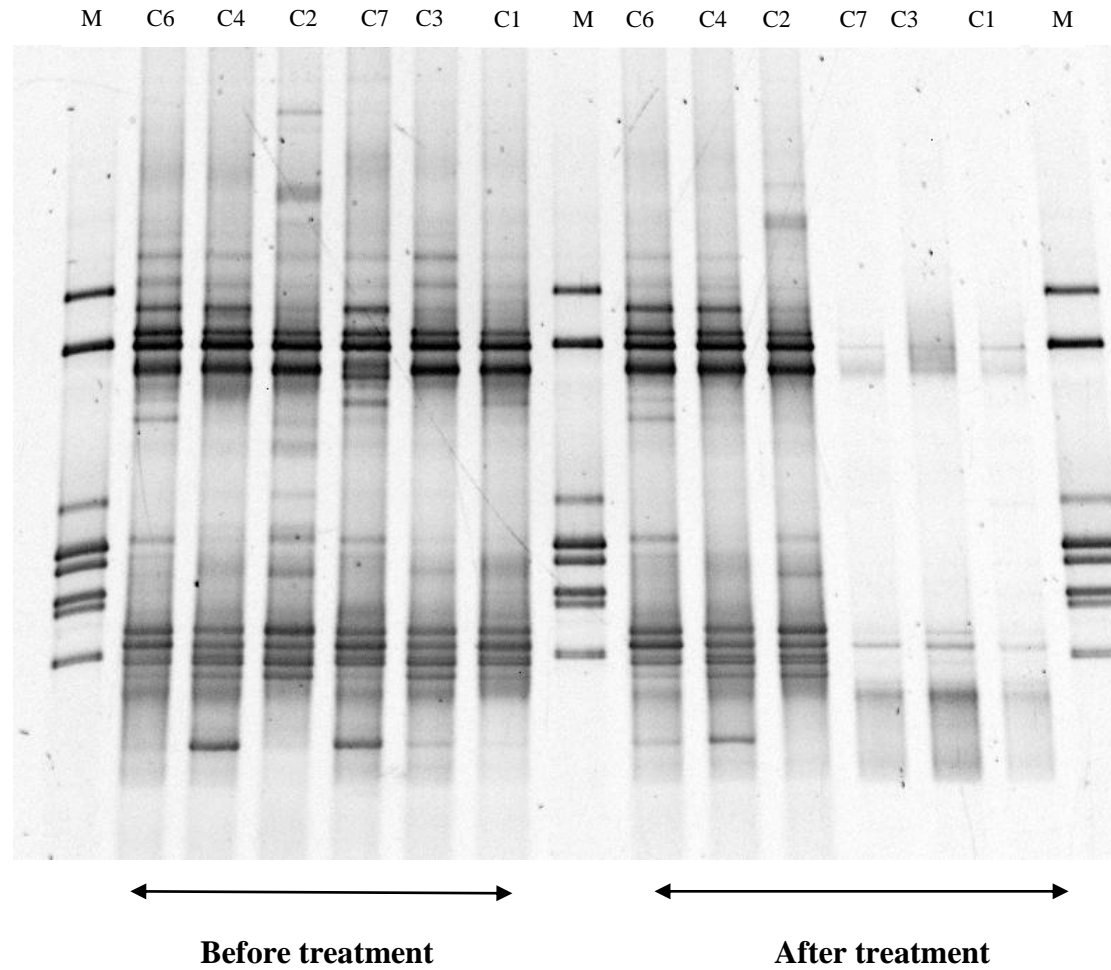


Figure 5.6. DGGE fingerprints of ruminal archaea in control cows (C2, C4 and C6) and CHCl₃-treated cows (C1, C3 and C7) before (1 day) and after (4 days) the CHCl₃ treatment. The marker (M) consisted of amplicons of 16S rRNA genes from pure culture of methanogens (Section 2.16).

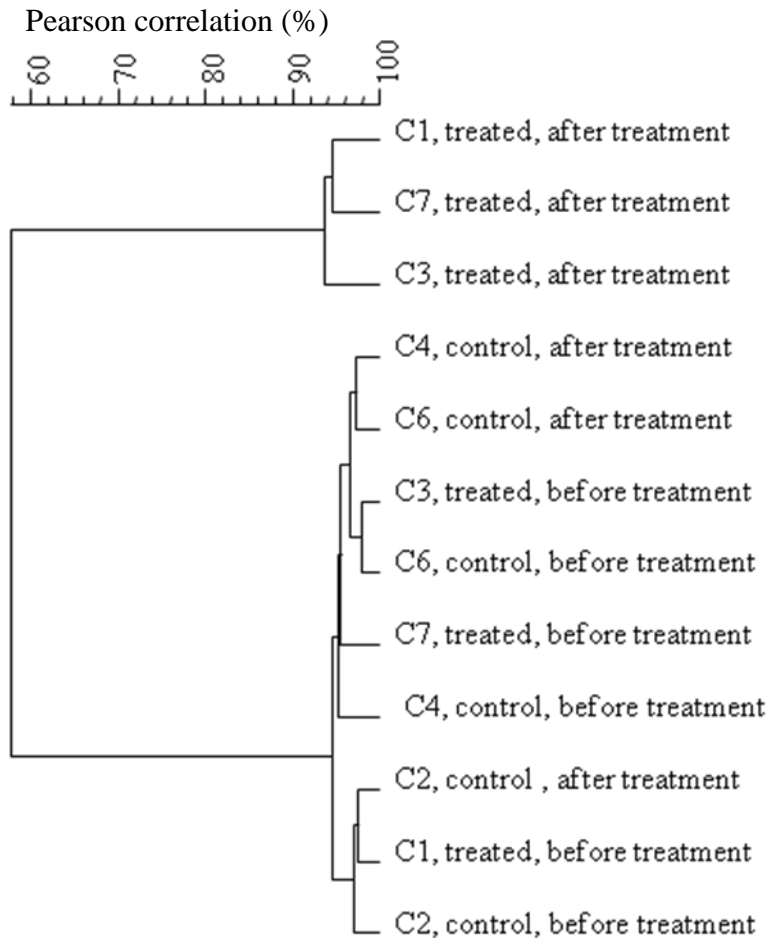


Figure 5.7. Similarities of DGGE profiles of ruminal archaea in the control and CHCl_3 -treated cows before (1 day) and after (4 days) the CHCl_3 treatment.

5.3.4 Total RCC community structure

The RCC group displays considerable variation in sequence types, which fall into a number of distinct lineages (Chapter 4). The abundance of any one sequence type is low, so members of this group are not readily observed by DGGE using domain-level primers (Chapter 4). The diversity of RCC present in the control and CHCl₃-treated cows was analyzed using a set of primers that amplified partial 16S rRNA genes only from members of RCC. The RCC community was also affected by the addition of CHCl₃ (Figure 5.8) but this effect was not as strong as the effect observed with total archaea. There were no significant difference in the number of bands between control cows and CHCl₃-treated cows before the CHCl₃ treatment. After 4 days of CHCl₃ treatment the number of bands in CHCl₃-treated cows reduced significantly ($P < 0.01$) compared to the control cows. Not all of the major bands were affected by CHCl₃ treatment. Some of the major bands were still present after the CHCl₃ treatment, suggesting that some RCC members were resistant to CHCl₃ at that concentration. On average, there were 17 bands before the CHCl₃ treatment, and this was reduced to 8 bands after the treatment. Therefore, CHCl₃ did affect some RCC. Furthermore, some new bands appeared after the CHCl₃ treatment, suggesting that some of the minor groups of RCC which were not affected by CHCl₃ treatment increased in numbers and filled the niches of the CHCl₃-sensitive RCC or other methanogens.

Analysis of the band patterns in the RCC DGGE gel showed that there is a treatment effect on cows after CHCl₃ administration into their rumen (Figure 5.9). All the CHCl₃-treated cows clustered together with a similarity value of 79.9%. All of the other cows (control cows before and after the treatment, treatment cows before the treatment) clustered together with a similarity value of 75.2%. The variation among the individual animals in RCC banding pattern was higher than the archaeal banding pattern before the treatment in both control and treatment groups. Since each cow had different groups of RCC, the effect of CHCl₃ treatment on RCC varied in each animal. Thus, even after the CHCl₃ treatment, the similarity value among the cows in the CHCl₃-treated group was low. There were some resistant groups of RCC in each cow after the CHCl₃ treatment and these groups were not always the same in all of the cows.

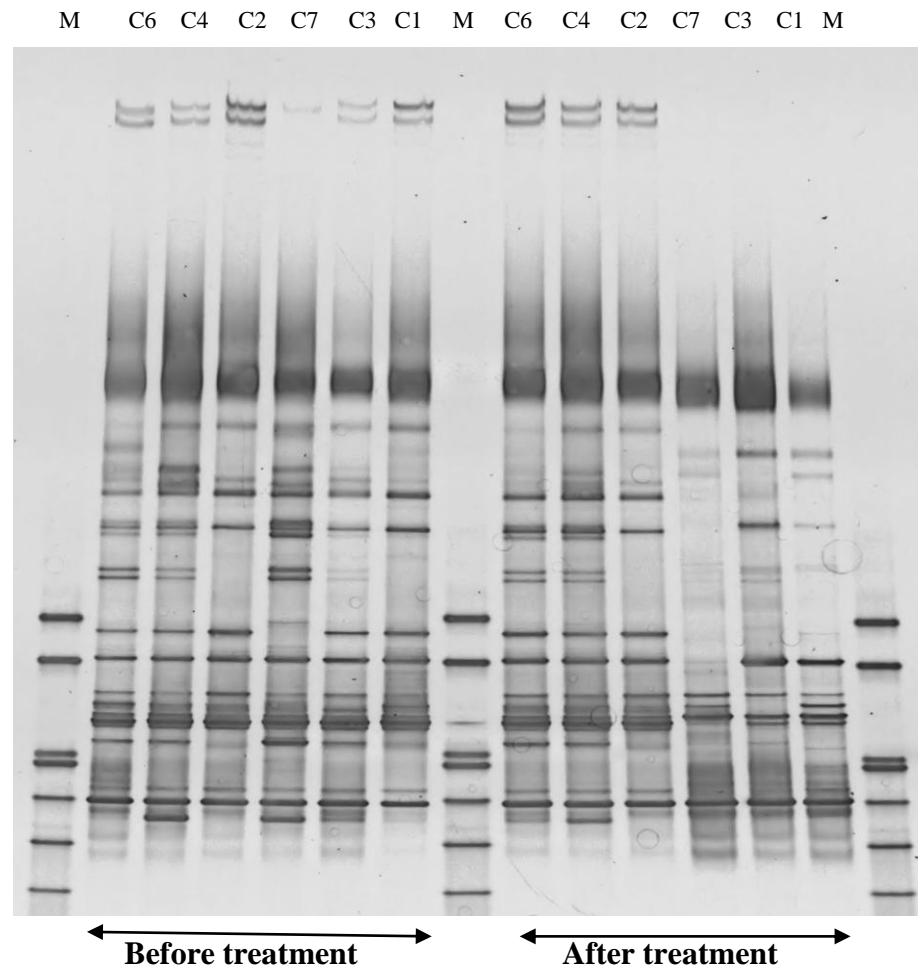


Figure 5.8. DGGE fingerprints of ruminal RCC in control cows (C2, C4 and C6) and CHCl₃-treated cows (C1, C3 and C7) before (1 day) and after (4 days) the CHCl₃ treatment. The marker (M) was Marker II from Nippongene (Japan).

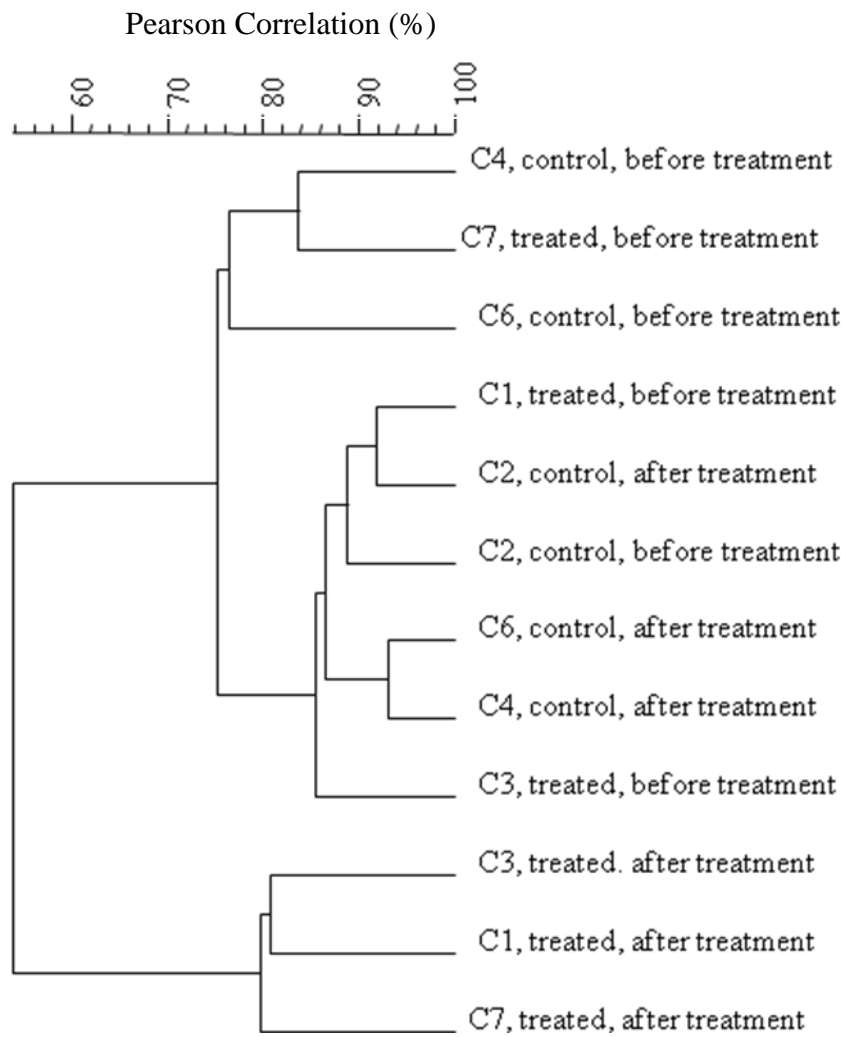


Figure 5.9. Similarities of DGGE profiles of ruminal RCC in the control and CHCl_3 -treated cows before (1 day) and after (4 days) the CHCl_3 treatment.

5.3.5 Gene-based analysis of archaeal community structure

Libraries of PCR-amplified partial 16S rRNA genes of archaea and *mcrA* genes of methanogens were prepared from DNA extracted from rumen contents from one (C3) of the CHCl₃-treated cow and from rumen contents from a control cow (C2), both collected at day 5 of the experiment, when CH₄ formation was lowest in the CHCl₃-treated cows (Figure 5.1).

The 16S rRNA gene library from the cow of control group (C2) revealed the presence of 5 groups of archaea: the *Methanobrevibacter ruminantium* clade, the *Methanobrevibacter gottschalkii* clade, *Methanosphaera* spp., *Methanimicrococcus* spp., and RCC (Table 5.3). The community structure in the CHCl₃-treated cow was quite different. Members of RCC dominated this library, accounting for 93% of the 16S rRNA gene sequences. A few sequences indicative of members of the *Methanobrevibacter ruminantium* clade and *Methanosphaera* species were also found. The archaeal diversity in the CHCl₃-treated cow was significantly different than the archaeal diversity in control animals ($P = 3.83 \times 10^{-7}$, Shannon diversity *t* test). Both Shannon diversity and Simpson's diversity indices were higher for the control cow (1.445 and 0.724, respectively) than the CHCl₃-treated cow (0.3083 and 0.138, respectively).

Parallel libraries of partial *mcrA* genes revealed a very similar pattern to that for 16S rRNA gene libraries. The *mcrA* gene library of the control cow revealed the presence of 5 groups of archaea: the *Methanobrevibacter ruminantium* clade, the *Methanobrevibacter gottschalkii* clade, *Methanosphaera* spp., *Methanimicrococcus* spp., and a group of *mcrA* genes (*mcrA* group 1 in the Table 5.3) that could not be assigned to known lineages of methanogens. In the CHCl₃-treated cow, the *mcrA* group 1 genes dominated the library, accounting for 92% of the *mcrA* gene sequences. There were few sequences from the *Methanobrevibacter ruminantium* clade, *Methanobrevibacter gottschalkii* clade or *Methanosphaera* spp.. The difference of *mcrA* gene sequences between CHCl₃-treated and control cows was significantly different ($P = 1.8 \times 10^{-27}$, Shannon diversity *t* test). Both Shannon diversity and Simpson's diversity indices were higher for control cow (1.525 and 0.774, respectively) than the treated cow (0.374 and 0.157, respectively).

Table 5.3. Abundance of different groups of archaea in libraries of PCR-amplified partial 16S rRNA and *mcrA* genes generated from DNA extracted from the rumen of a CHCl₃-treated cow (C3) and a cow from a control group (C2).

Archaeal group	CHCl ₃ -treated cow C3		Control cow C2	
	no. of 16S rRNA genes (%)	no. of <i>mcrA</i> genes (%)	no. of 16SrRNA genes (%)	no. of <i>mcrA</i> genes (%)
<i>Mbb. ruminantium</i> clade	2 (5)	1 (2)	9 (19)	11 (22)
<i>Mbb. gottschalkii</i> clade	0	1 (2)	8 (17)	13 (25)
<i>Methanosphaera</i> spp.	1 (2)	2 (4)	5 (10)	13 (25)
<i>Methanimicrococcus</i> spp.	0	0	5 (10)	3 (6)
Rumen Cluster C	38 (93)	N.A. ^a	21 (44)	N.A.
<i>mcrA</i> group 1	N.A.	44 (92)	N.A.	11 (22)

^a N.A., not able to be assigned using this gene.

To further confirm that CHCl₃ treatment had resulted in an increase in RCC in the 16S rRNA gene clone library, small clone libraries were constructed using DNA extracted from the remaining CHCl₃-treated (C1 and C7) and control cows (C4 and C6) (Table 5.4). The CHCl₃-treated cows showed dominance of the RCC group in their libraries (82% and 83%), while the RCC group in the control cows contributed only 37.5% and 57% of the total archaeal community. The mean Shannon diversity and Simpson's diversity indices were also higher in control cows (1.31 and 0.68, respectively) compared to CHCl₃-treated cows (0.55 and 0.30, respectively).

Table 5.4. Abundance of different groups of archaea in libraries of PCR-amplified partial 16S rRNA genes generated from DNA extracted from the rumen of CHCl₃-treated cows (C1 and C7) and control cows (C4 and C6).

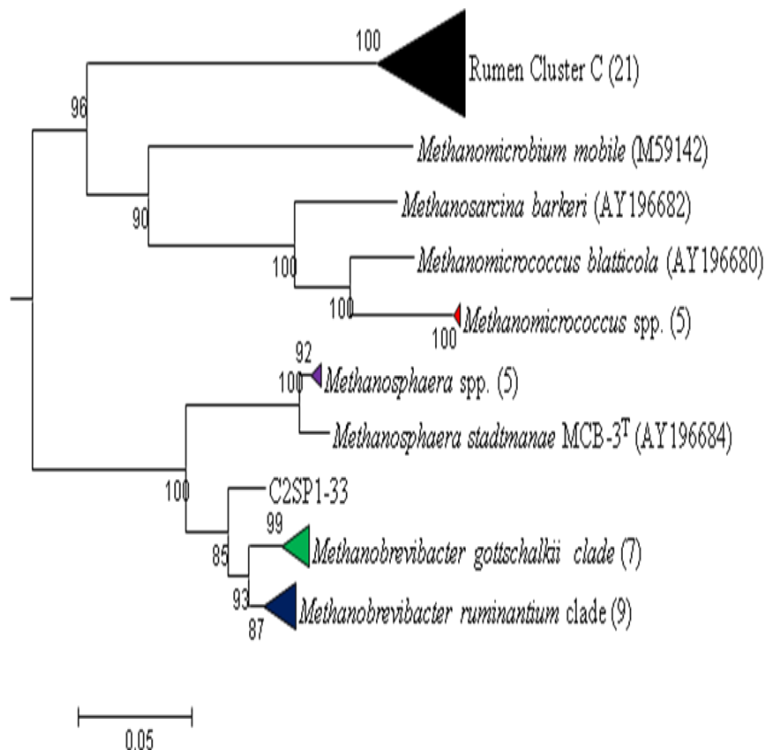
Archaeal group	CHCl ₃ -treated cows		Control group cows	
	C1 (%)	C7 (%)	C4 (%)	C6 (%)
<i>Mbb. ruminantium</i> clade	1 (4)	4 (18)	2 (9.5)	4 (16.5)
<i>Mbb. gottschalkii</i> clade	0	0	2 (9.5)	5 (21)
<i>Methanosphaera</i> spp.	1 (4)	0	2 (9.5)	6 (25)
<i>Methanomicrococcus</i> spp.	0	0	3 (14.5)	0
<i>Methanobacterium</i> spp.	2 (8)	0	0	0
Rumen Cluster C	20 (84)	18 (82)	12 (57)	9 (37.5)

Comparisons of 16S rRNA and *mcrA* genes from the control cow (C2) and CHCl₃-treated cow (C3) are shown as phylogenetic trees in Figures 5.10 and 5.11. The phylogenetic tree constructed using 16S rRNA gene PCR products revealed the presence of 5 groups of archaea (Table 5.3). A parallel phylogenetic tree constructed using *mcrA* gene PCR products also resulted the presence of 5 groups of methanogens in the same rumen sample.

Apart from the known methanogens of the *Methanobrevibacter ruminantium* clade, *Methanobrevibacter gottschalkii* clade, *Methanosphaera* spp., and *Methanomicrococcus* spp., the 16S rRNA gene library contained sequences of RCC, which contributed 44% of total archaea in control cow (C2). Similarly, the phylogenetic tree constructed using the *mcrA* gene revealed the presence of the above mentioned groups of known methanogens other than RCC. A group of *mcrA* genes, *mcrA* group 1 (22% of total methanogens), was also obtained (Table 5.3), which could not be assigned to any known methanogens. Considering the number and diversity of sequences of *mcrA* group 1, the sequences belonging to this group may be from RCC.

Comparison of the phylogenetic trees of 16S rRNA and *mcrA* genes from the CHCl₃-treated cow (C3) further confirmed that RCC contained the *mcrA* gene (Figure 5.11). A largest portion of the 16S rRNA gene clone library of the CHCl₃-treated cow was represented by the RCC (93%). Apart from the RCC sequences, there were few sequences from the *Methanobrevibacter ruminantium* clade and *Methanosphaera* spp. The parallel *mcrA* gene phylogenetic tree from the same CHCl₃-treated cow revealed the presence of a group of *mcrA* gene (*mcrA* group 1) that could not be assigned to any known methanogens, and that was dominant (92%). Apart from the *mcrA* group 1 sequences, there were a few sequences from the *Methanobrevibacter gottschalkii* clade, *Methanobrevibacter ruminantium* clade and *Methanosphaera* spp. The abundance of RCC sequences in 16S rRNA gene phylogenetic tree closely matched the abundance of *mcrA* group 1 gene in *mcrA* gene phylogenetic tree (93% vs. 92%). This strongly suggests that the members of RCC possess a *mcrA* gene.

(a)



(b)

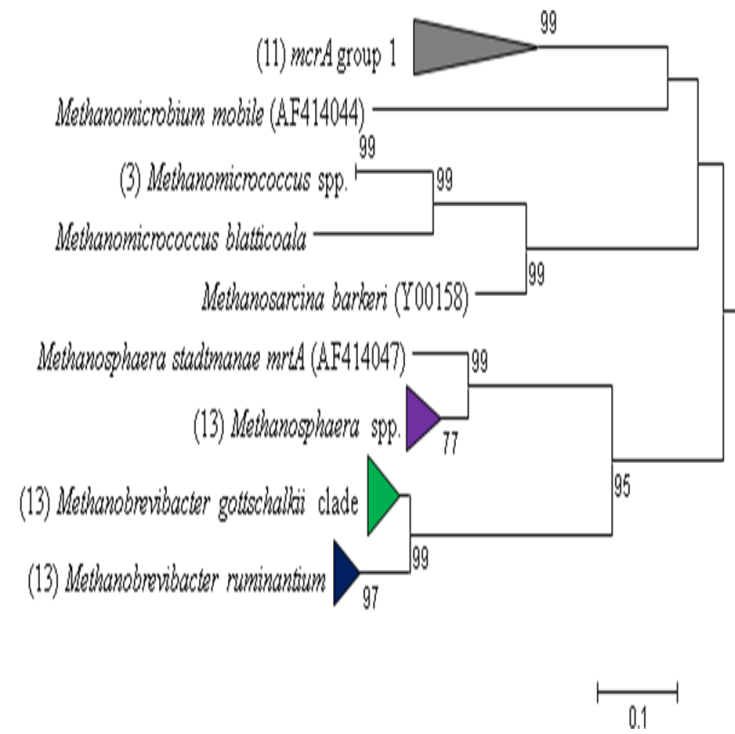
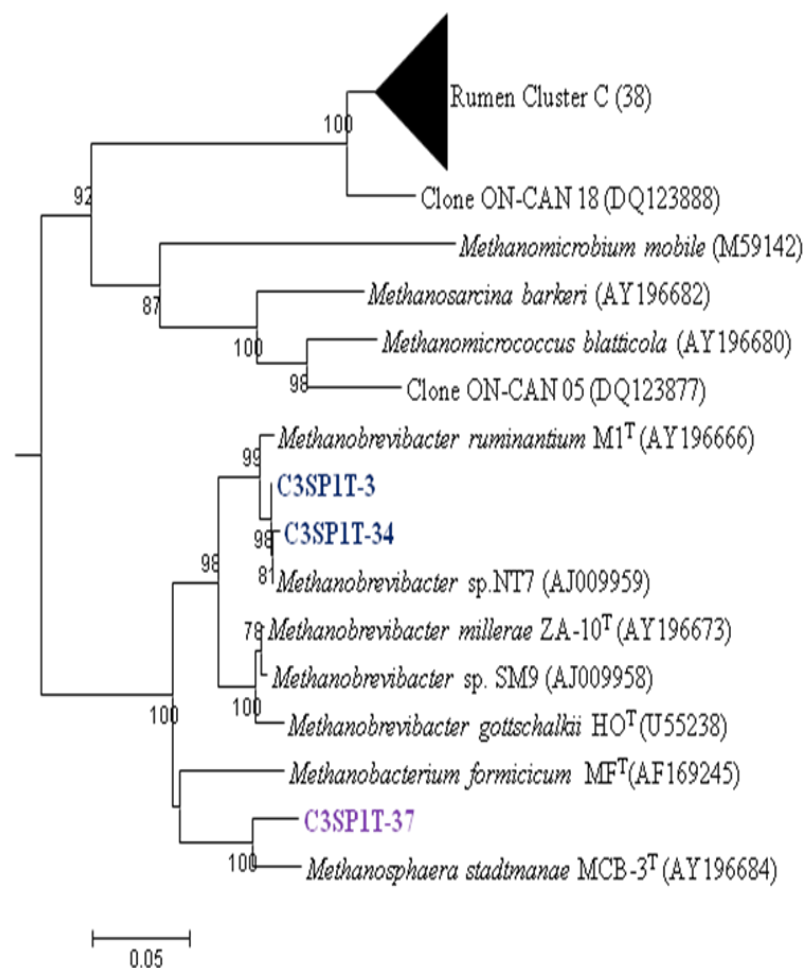


Figure 5.10. Comparison of 16S rRNA and *mcrA* gene phylogenetic trees of the control cow (C2). Nucleotide sequences were used to construct 16S rRNA gene phylogenetic tree (a) and deduced amino acid sequences were used for the construction of *mcrA* gene phylogenetic tree (b). The 16S rRNA gene sequences from *Methanocaldococcus jannaschii* JAL-1 (M59126), *Methanococcus vanniellii* SB (M36507), and *Methanothermococcus thermolithotrophicus* SN-1 (M59128) were used as out-groups for the archaeal 16S rRNA gene phylogenetic tree. The amino acid sequence of the *mcrA* gene from *Methanopyrus kandleri* AV19 (AF414042) was used as the out-group for the *mcrA* gene phylogenetic tree. In parentheses after the reference sequences are the GenBank accession numbers. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates nucleotide/amino acid substitutions per nucleotide/amino acid position. The triangles represent groups of similar sequences and the number of sequences within each triangle is given in parentheses. The coloured triangles and sequences are the ones found in this study and each colour represents a different group of Archaea (● *Mbb. gottschalkii* clade, ● *Mbb. ruminantium* clade, ● *Methanosphaera* spp., ● *Methanomicrococcus* spp., ● RCC group and ● *mcrA* group 1).

(a)



(b)

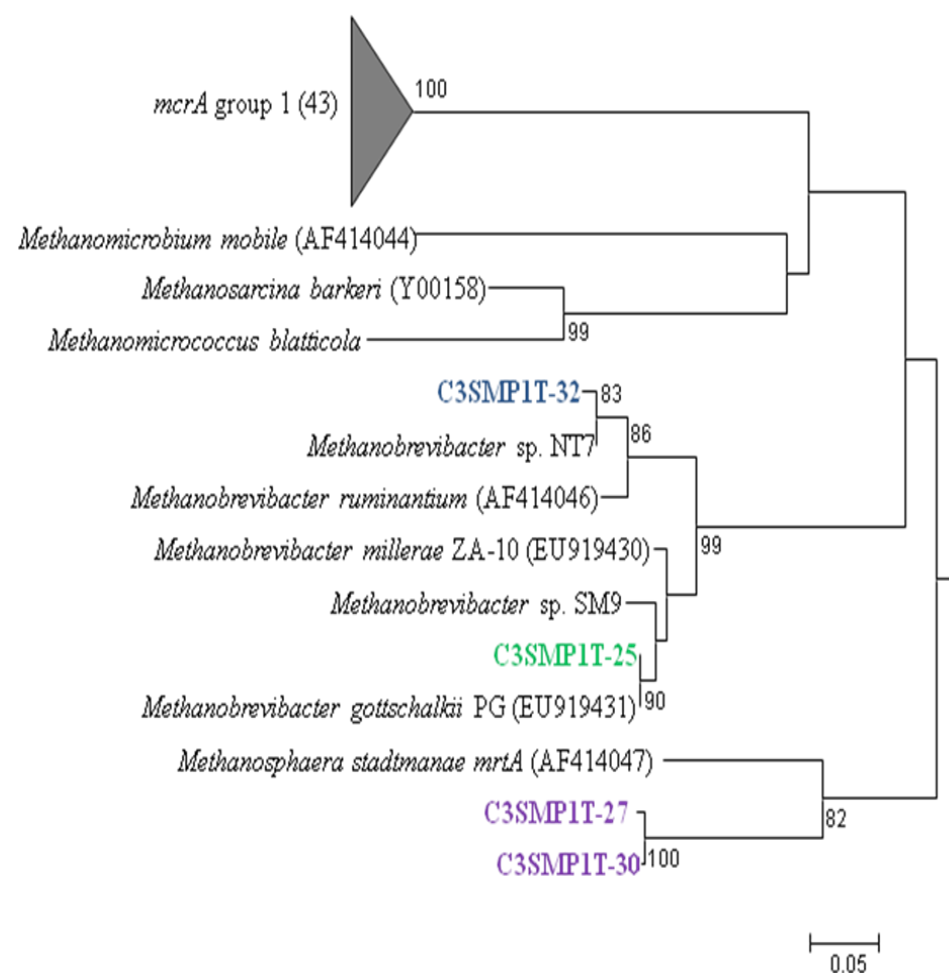


Figure 5.11. Comparison of 16S rRNA and *mcrA* gene phylogenetic trees of the CHCl₃-treated cow (C3). Nucleotide sequences were used to construct 16S rRNA gene phylogenetic tree (a) and deduced amino acid sequences were used for the construction of *mcrA* gene phylogenetic tree (b). The 16S rRNA gene sequences from *Methanocaldococcus jannaschii* JAL-1 (M59126), *Methanococcus vannielii* SB (M36507), and *Methanothermococcus thermolithotrophicus* SN-1 (M59128) were used as out-groups for the archaeal 16S rRNA gene phylogenetic tree. The amino acid sequence of the *mcrA* gene from *Methanopyrus kandleri* AV19 (AF414042) was used as the out-group for the *mcrA* gene phylogenetic tree. In parentheses after the reference sequences are the GenBank accession numbers. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates nucleotide/amino acid substitutions per nucleotide/amino acid position. The triangles represent groups of similar sequences and the number of sequences within each triangle is given in parentheses. The coloured triangles and sequences are the ones found in this study and, each colour represents different groups of Archaea (● *Mbb. gottschalkii* clade, ● *Mbb. ruminantium* clade, ● *Methanosphaera* spp., ● *Methanomicrococcus* spp., ● RCC group and ● *mcrA* group 1).

5.3.6 Analysis of RCC community structure

Detailed phylogenetic analysis of 16S rRNA gene sequences belonging to RCC revealed that the sequences from the control cows and CHCl₃-treated cows mostly clustered separately (Figure 5.12). In a 16S rRNA gene phylogenetic tree constructed using all sequences of RCC group (all the CHCl₃-treated and control cows), the sequences clustered into three major groups: a group which contained sequences mostly from CHCl₃-treated cows, a group which contained sequences mostly from control cows, and a third group which contained sequences exclusively from CHCl₃-treated cows. Therefore, there seem to be distinct groups of CHCl₃-sensitive and CHCl₃-insensitive RCC. Further evidence is needed to confirm this.

Phylogenetic analysis of *mcrA* group 1 sequences obtained from the control cow and the CHCl₃-treated cow showed that the two samples contained distinct gene groups (Figure 5.13). Similar to the 16S rRNA gene sequences of RCC group, the sequences from control cow and CHCl₃-treated cow clustered separately. One cluster consisted exclusively of sequences from the control cow and others consisted of sequences exclusively from the CHCl₃-treated cow. This also suggests that the sequences that belong to the *mcrA* group 1 may be from the RCC group and that there may be at least two different physiologies within the RCC group. However, there were variations observed in RCC banding pattern in DGGE analysis among individual cows. As such the different clustering between CHCl₃-treated and control cows may also be due to animal variations. More studies are needed to confirm this suggestion.

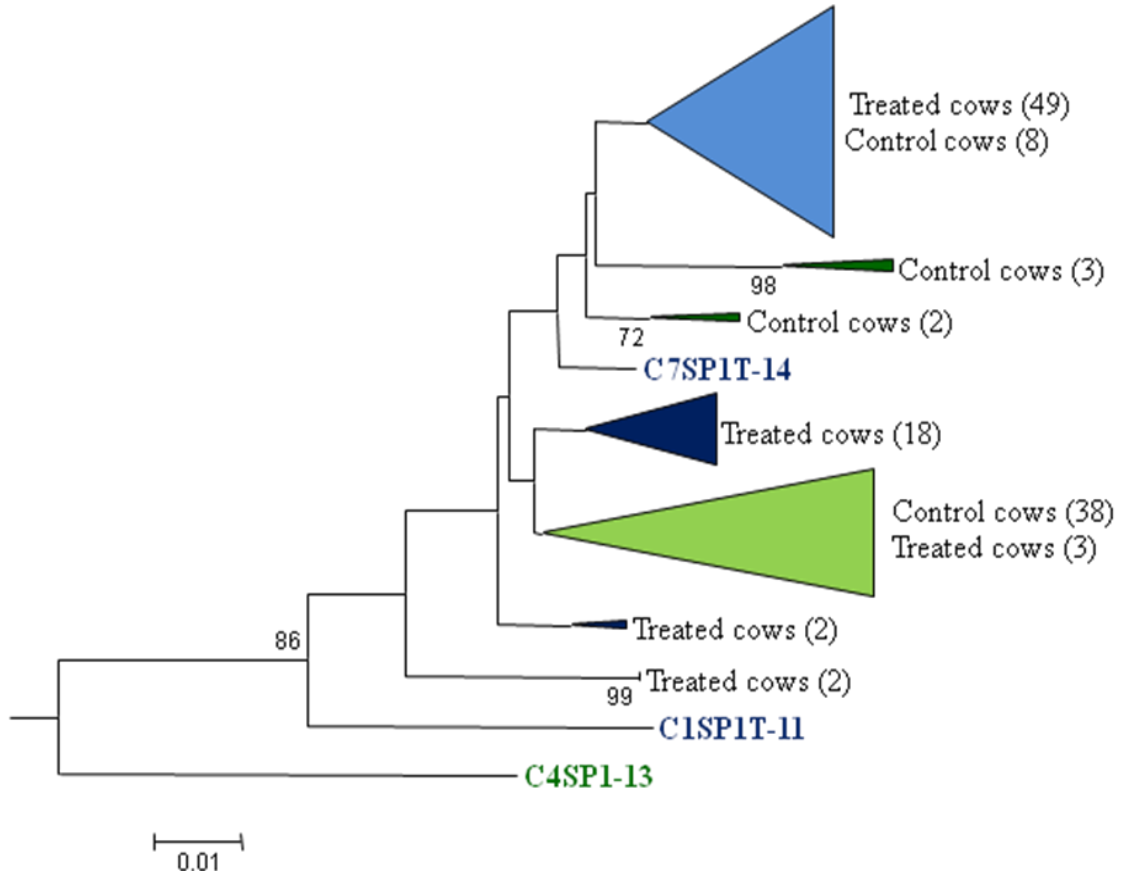


Figure 5.12. Phylogenetic tree of 16S rRNA gene sequences constructed using the sequences from RCC group obtained from CHCl_3 -treated (C1, C3 and C7) and control (C2, C4, C6) cows. The 16S rRNA gene sequences from *Methanocaldococcus jannaschii* JAL-1 (M59126), *Methanococcus vannielii* SB (M36507), and *Methanothermococcus thermolithotrophicus* SN-1 (M59128) were used as out-groups. The scale bar indicates nucleotide substitutions per nucleotide position. The triangles represent groups of similar sequences, and the number of sequences within each triangle is given in parentheses. Each colour represents a different group of RCC (● sequences exclusively from control cows, ● sequences mostly from control cows, ● sequences exclusively from CHCl_3 -treated cows and ● sequences mostly from CHCl_3 -treated cows).

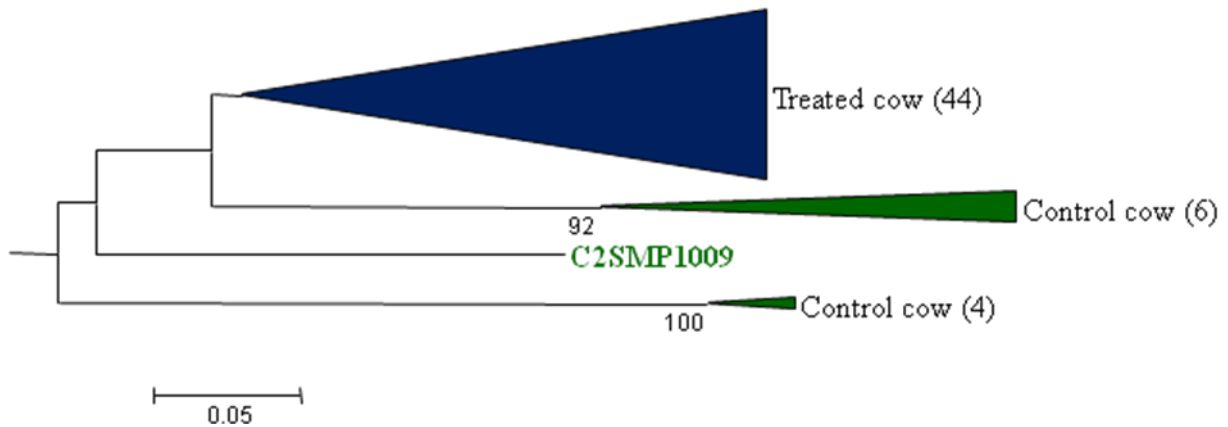


Figure 5.13. Phylogenetic tree of *mcrA* gene sequences, constructed using deduced amino acid sequences from the *mcrA* group 1 obtained from CHCl_3 -treated (C3) and control (C2) cows. The amino acid sequence of the *mcrA* gene from *Methanopyrus kandleri* AV19 (AF414042) was used as the out-group. The scale bar indicates amino acid substitutions per amino acid position. The triangles represent groups of similar sequences and the number of sequences within each triangle is given in parentheses. Each colour represents different groups within *mcrA* group 1 (● sequences exclusively from control cows, and ● sequences exclusively from CHCl_3 -treated cows).

5.4 Discussion

Rumen Cluster C or RCC is a group of archaea that has been found by many researchers as an important archaeal group in the rumen, regardless of diet, ruminant species or location (Table 1.2). In some instances, members of this group appear to outnumber the major known rumen methanogens such as *Methanobrevibacter* spp. (Sundset *et al.*, 2009b; Wright *et al.*, 2006). Furthermore, the RCC group contributed 73% of the total sequences which are associated with protozoa in a study which concentrated only on protozoa-associated archaea (Ohene-Adjei *et al.*, 2007). Members of the RCC group also dominated (91.7%) the foregut sample of a Tammer wallaby during summer but not winter (Evans *et al.*, 2009). There has been an indication that members from the RCC group may be present in the human intestinal gut as well (Mihajlovski *et al.*, 2008). In addition to the intestinal tract, members of the RCC group have been shown to be present in swine waste storage pit (Whitehead & cotta, 1999), biogas reactor systems (Klocke *et al.*, 2008) and leachate from landfill (Huang *et al.*, 2003).

The physiology of members of the RCC group is not yet known. Their closest phylogenetic relatives are members of a distantly branching clade of archaea including *Thermoplasma volcanium* and *Thermoplasma acidophilum*. Members of the genus *Thermoplasma* are acidophilic, and mesophilic to thermophilic. They are all aerobes, although *Thermoplasma* species can grow under anaerobic conditions by sulfur respiration and ferric iron reduction. Most of the *Thermoplasma* species grow heterotrophically on yeast extract (Darland *et al.*, 1970; Segerer *et al.*, 1988). However, members of the RCC group may not possess any of these characteristics, as their 16S rRNA genes are only 70% identical to those of members of the genus *Thermoplasma*. In the effort to produce vaccines and small-molecule inhibitors targeting methanogens, it is very important to know about their physiology and their role in methane production.

Low concentrations of CHCl_3 selectively inhibit methanogenic activity (Achnich *et al.*, 1995; Chidthaisong & Conrad, 2000; Chin & Conrad, 1995; De Graaf *et al.*, 1996; Hu & Chen, 2007; Hu *et al.*, 2009). However, it has been shown in some studies that there can be methanogen groups that are resistant to CHCl_3 at the concentrations normally used (Hu *et al.*, 2009). In a study performed using methanogenic granules, it was found that the methane producing system changed to a hydrogen producing system

after administering CHCl_3 at a low concentration. Clone library analysis in this particular study showed that *Methanosaeta concilii* was not affected by the CHCl_3 treatment. However, no significant amount of methane was produced after the CHCl_3 treatment, which clearly proved that methanogenic activity had been stopped. A similar pattern was observed in the experiment described in this chapter, where the methane production was reduced significantly after the addition of CHCl_3 (resulting concentration in rumen ≈ 0.2 mM) while some known methanogens and large group of uncultured archaea, RCC, were not completely eliminated by CHCl_3 . The residual methane produced in the CHCl_3 -treated cows at this point may have been from the known methanogens which were not completely inhibited by the CHCl_3 treatment, and/or from members of RCC group.

Comparison between 16S rRNA and *mcrA* gene clone libraries of control and CHCl_3 -treated cows (Table 5.3, Figures 5.10 and 5.11) strongly suggests that members of RCC contain a *mcrA* gene. In the control animal, there were 16S rRNA genes able to be assigned to RCC and a similar number of *mcrA* genes that could not be assigned to known methanogens and that formed a coherent cluster called *mcrA* group 1. In the CHCl_3 -treated animal, both the number of 16S rRNA genes affiliated with RCC and the number of *mcrA* genes falling in *mcrA* group 1 increased dramatically. This suggests that the 16S rRNA genes assigned to RCC, and the *mcrA* genes assigned to *mcrA* group 1, are derived from members of the same lineage of archaea. In a study performed using the antimethanogenic compound bromochloromethane, Denman *et al.*, (2007) suggested that members of the RCC group may contain a *mcrA* gene. Our study provides strong evidence indicating that members of RCC contain a *mcrA* gene.

The *mcrA* gene, which is the gene encoding the α subunit of methyl-coenzyme M reductase enzyme, one of the key enzymes of methanogenesis, can be used as a phylogenetic tool to study methanogens (Denman *et al.*, 2007; Luton *et al.*, 2002). All known methanogens contain this enzyme and it is the terminal enzyme in the methane producing pathway of methanogens. Methyl coenzyme-M reductase catalyses the reduction of a methyl group bound to coenzyme-M, with the concomitant release of methane (Rouviere & Wolfe, 1988). Since CHCl_3 is known to inhibit the methyl coenzyme M reductase, the resistance of some of the members of RCC group was not expected. However, there were also some of the members of RCC affected by CHCl_3 .

Identification of at least two different physiological groups, CHCl_3 -sensitive and CHCl_3 -insensitive groups in RCC, suggests that this group is not homogeneous.

The isolation of members of RCC group has still not been reported. This group may have strict growth requirements or may have obligate synergistic relationships with other microbes and may therefore be difficult to culture and isolate. Identification of *mcrA* genes in RCC makes this group an important target group to investigate in efforts to reduce methane formation in rumen. Further investigation is needed into what role RCC plays in rumen.

Chapter 6

Isolating Uncultured Rumen Methanogens

6.1 Introduction

Methanogen communities in New Zealand farmed ruminants (sheep, cattle and red deer) fed different diets (winter pasture, summer pasture, silage, autumn pasture, willow and concentrate-based diet) were found to be relatively similar (Chapter 4). The analysis performed using DGGE revealed that similar dominant archaeal populations are found in these animals. In the same study, DNA extracted from dominant bands showed that the dominant methanogens in these animals were mainly from three major groups: the *Methanobrevibacter gottschalkii* clade, the *Methanobrevibacter ruminantium* clade, and *Methanosphaera* spp. The clone library analysis of the selected rumen sample (sheep [S4] fed winter pasture) confirmed this finding. However, the clone library analysis also found large numbers of uncultured archaeal sequences that clustered together phylogenetically, designated as Rumen Cluster C or RCC. The sequences of the RCC group showed a high level of variation amongst themselves, so that they were not identified as dominant bands in the archaeal DGGE analysis (Chapter 4).

Two of the potentially practical methane reduction strategies for ruminant animals in New Zealand where the farming practice is mainly based on grazing are to use an anti-methanogen vaccine or small molecule inhibitors (Wedlock *et al.*, 2010). However, for these to be practical, there is a need to develop a vaccine or small molecule inhibitor that targets, if possible, all methanogens. Identifying candidate vaccine components, based on their importance to the growth of methanogens and/or methanogenesis and their cross-reactivity in various methanogens, is a key factor in developing a broad spectrum vaccine. Identification of such components is greatly assisted by genome-sequencing (Leahy *et al.*, 2010). Genome-sequencing and subsequent functional studies on candidate targets coded by the genomes are greatly simplified if pure cultures representing the diversity of rumen methanogens are available. There have been few methanogens isolated from rumen (Janssen & Kirs, 2008), and some of the methanogen species that have been identified in the rumen, have only been isolated from other

environments such as animal faeces (Miller & Lin, 2002), human faeces (Miller & Wolin, 1985) and the intestinal tract of monogastric animals (Biavati *et al.*, 1988). However, rumen strains may not share the same characteristics as their non-ruminant relatives. It is therefore important to isolate a wide diversity of strains of rumen methanogens.

Two species of the *Mbb. ruminantium* clade and one species of the *Mbb. gottschalkii* clade have been isolated from the rumen, named and characterized. The isolated rumen strains belonging to the *Mbb. ruminantium* clade are *Methanobrevibacter ruminantium* (Smith & Hungate, 1958) and *Methanobrevibacter olleyae* (Rea *et al.*, 2007). *Methanobrevibacter millerae*, which was isolated from an ovine rumen (Rea *et al.*, 2007), is the only fully characterised rumen methanogen belonging to the *Mbb. gottschalkii* clade. An isolate named SM9 (Joblin, 2005), from the *Mbb. gottschalkii* clade, has also been reported from the rumen. However, this isolate has not been characterized thus far. Two members of the *Mbb. gottschalkii* clade have been isolated from animal faeces: *Methanobrevibacter thaueri* from cow faeces and *Methanobrevibacter gottschalkii* from horse faeces (Miller & Lin, 2002). The other methanogen species isolated and characterized from the rumen thus far are from relatively minor groups such as *Methanobacterium* spp. (Jarvis *et al.*, 2000; Oppermann *et al.*, 1957), *Methanosarcina* spp. (Beijer, 1952; Jarvis *et al.*, 2000) and *Methanomicrobium mobile* (Jarvis *et al.*, 2000; Paynter & Hungate, 1968).

Methanosphaera spp. and members of the RCC group are common inhabitants of the rumen and have been found by many studies performed in different parts of the world (Table 1.2). Members of these two groups were also abundant in New Zealand ruminants (Chapter 4). Unfortunately, no members from these two groups have been isolated from the rumen thus far. Two species of the genus *Methanosphaera* have been isolated previously: *Methanosphaera stadtmanae* from human faeces (Miller & Wolin, 1985) and *Methanosphaera cuniculi* from rabbit rectum (Biavati *et al.*, 1988). A clone library analysis of rumen samples has suggested that there may be more than one species of *Methanosphaera* in the rumen (Chapter 3; Whitford *et al.*, 2001).

In contrast to other known dominant methanogen groups, very little is known about the RCC group. This group has been detected in many environments such as the rumen (Table 1.2), human gut (Mihajlovski *et al.*, 2008), swine waste storage pits

(Whitehead & Cotta, 1999), biogas reactor systems (Klocke *et al.*, 2008) and leachate from landfill (Huang *et al.*, 2003). Significantly, no members from this group have been isolated from any environmental samples thus far. Members of this group of archaea possess a *mcrA* gene (Chapter 5). However, no other physiological properties are currently known about this group.

An attempt was made to isolate previously uncultured methanogens, especially representatives of the RCC and *Methanosphaera* spp. groups, from the rumen of sheep using a simple cultivation strategy.

6.2 Materials and Methods

6.2.1 Sample collection

Samples for the isolation experiments were collected from a ruminally-fistulated wether sheep (Romney cross, 9 years old) fed rye-grass clover pasture *ad libitum* (Section 2.2). Collected sample was immediately brought to the laboratory for further processing. Samples were collected at four different occasions, each on different days (isolation experiments 1 - 4). Isolation experiments 1 and 2 (ISO1 and 2) were conducted to determine a suitable medium and blending time of rumen samples, and isolation experiments 3 and 4 (ISO3 and ISO4) were set up to isolate uncultured rumen methanogens.

6.2.2 Media and blending time

Two different media were used in these isolation experiments: modified BY (BY⁺) and RM02 media (Section 2.22). The media were prepared in Hungate tubes and sterilized by autoclaving for 20 min at 121°C. Before use, the tubes containing the medium were stored in the dark for at least 24 h. Substrates and other additives (Section 2.23) were added on the day of inoculation of rumen samples to the tubes.

The rumen contents collected from the sheep were blended with 360 ml of RM02 medium under anaerobic conditions for these isolation experiments. Two different blending times were chosen and they were designated as short and long in this chapter. The short blending time for blending the rumen sample on the HI setting was for 20 s in

a Waring blender (Waring Products Inc.). The long blending time was blended the rumen samples for four bursts of 20 s each, with an interval of 60 s between each burst.

6.2.3 Substrates used

For the initial isolation and maintenance of the cultures, the following substrate mixture was used (final concentrations): Na formate (60 mM), Na acetate (20 mM), methanol (20 mM) and H₂/CO₂ (4:1; 200 kPa). The preparation of substrate solutions for both media is given in Section 2.23.6.

6.2.4 Isolation experiments

All isolation experiments were conducted in Hungate tubes (16 mm dia., 125 mm long; BellCo Glass, Vineland, NJ, USA). Inoculated tubes were incubated at 39°C in the dark with shaking (200 rpm). No antibiotics were used in these experiments. Except for isolation experiment 4, all other experiments were not supplemented with coenzyme M. Methanogen growth was monitored by measuring methane in the headspace of the tubes by gas chromatography (Section 2.28.1). The major assumption taken for all the experiments was that the rumen typically contains 10⁹ methanogens per gram of rumen sample on a wet weight basis (Denman *et al.*, 2007).

6.2.4.1 Isolation Experiment 1 (ISO1)

This initial experiment was conducted to select a suitable medium and blending time for the isolation experiments. This experiment contained 4 different treatments.

Treatment 1: RM02 media and the short blending time (20 s at HI)

Treatment 2: BY⁺ media and the short blending time (20 s at HI)

Treatment 3: RM02 media and the long blending time [4 × (20 s at HI + 60 s off)]

Treatment 4: BY⁺ media and the long blending time [4 × (20 s at HI + 60 s off)]

For each treatment, a 10-fold dilution series of rumen sample was performed. The plan for the dilution and inoculation is given in Table 6.1. The same plan was followed for all the treatments in this experiment. The most probable number (MPN) counts were

calculated from the tubes with positive growth, estimated by the methane production and dilution factor (Hurley & Roscoe, 1983).

6.2.4.2 Isolation experiment 2 (ISO2)

This experiment was conducted to select a suitable medium for isolating RCC. Most of the known methanogens grow in both BY⁺ and RM02 media, but one of the target groups for isolation was the RCC group. Since no pure cultures were available from this group, selecting suitable media for this group was thought to be important. For isolation experiment 2 only the long blending time [4 × (20 s at HI + 60 s off)] was selected for both media. Sample collection, sample processing, media and substrate preparation were similar to ISO1, which reduced the number of treatments to two.

Treatment 1: RM02 media and the long blending time [4 × (20 s at HI + 60 s off)]

Treatment 2: BY⁺ media and the long blending time [4 × (20 s at HI + 60 s off)]

Similar to the initial experiment, this experiment was also conducted using a 10-fold dilution series of rumen sample. Serial dilutions of the rumen sample were performed according to the scheme outlined in Table 6.2. DNA from selected tubes (positive for methane) was extracted using Chelex resin (Section 2.3.2c) and total archaea and RCC counts were made by quantitative real time PCR (Section 2.19).

6.2.4.3 Isolation experiment 3 (ISO3)

This experiment was conducted to isolate previously uncultured rumen methanogens. Special emphasis was taken to isolate members from the RCC group. It was known that some of the RCC members are less affected by chloroform than are known methanogens (Chapter 5). Therefore, two treatments were used in this experiment: without chloroform (Treatment 1) and with 50 μM chloroform (Treatment 2). BY⁺ medium and a long blending time [4 × (20 s at the HI + 60 s off)] were used in both of these treatments. The dilution and inoculation scheme for these treatments are given in Table 6.3. Rumen sample collection, sample processing, and media and substrate preparations were the same as in the previous experiments. No antibiotics were used in this experiment.

Tubes from terminal methane-positive dilutions were sub-cultured into the same medium with the same substrates. Methane production was monitored by gas chromatography to check the growth of methanogens in those particular tubes. DNA was extracted from the successful subcultures using Chelex resin, and was used for the archaeal 16S rRNA gene PCR to identify the methanogens present. Cultures of interest were then purified with several methods in attempt to remove contaminating bacteria.

6.2.4.4 Isolation experiment 4 (ISO4)

The only differences in this experiment compared to ISO3 were that a short blending time (20 s at HI) was used, and co-enzyme M (final concentration of 10 μ M) was added to the media. No antibiotics or chloroform were used in this experiment. The dilutions and the experimental plans were given in Table 6.4. All the other methods (sample collection, sample processing, media and substrate preparations) were the same as in the previous experiments. Cultures which were interest were sub-cultured into the same medium containing the same substrates.

Table 6.1. Estimation of methanogen MPN and plan for isolation experiment 1 (ISO1)

Step	Series name	Dilution factor	Amount of material from previous step	Medium volume	Viable counts of methanogens/ml	Assumed counts of methanogens in Hungate tube if inoculated with 0.5 ml of dilution	Number of tubes inoculated
1		0	sample		1×10^9	5×10^8	
2		10	40 g	360 ml	1×10^8	5×10^7	
3		10	10 ml	90 ml	1×10^7	5×10^6	
4		10	10 ml	90 ml	1×10^6	5×10^5	
5	A	10	10 ml	90 ml	1×10^5	5×10^4	5
6	B	10	10 ml	90 ml	1×10^4	5000	5
7	C	10	10 ml	90 ml	1000	500	5
8	D	10	10 ml	90 ml	100	50	5
9	E	10	10 ml	90 ml	10	5	5
10	F	10	10 ml	90 ml	1	0.5	5
11	G	10	10 ml	90 ml	0.1	0.05	5
12	H	10	10 ml	90 ml	0.01	5×10^{-3}	5
13	I	10	10 ml	90ml	0.001	5×10^{-4}	5

Table 6.2. Estimation of methanogen MPN and plan for isolation experiment 2 (ISO2)

Step	Series Name	Dilution factor	Amount of material from previous step	of from Medium volume	Viab le counts of methanogens/ml	Assumed counts of methanogens in Hungate tube if inoculated with 0.5 ml of dilution	Number of tubes inoculated
1		0	sample		1×10^9	5×10^8	
2		10	40 g	360 ml	1×10^8	5×10^7	
3		10	10 ml	90 ml	1×10^7	5×10^6	
4		10	10 ml	90 ml	1×10^6	5×10^5	
5		10	10 ml	90 ml	1×10^5	5×10^4	
6		10	10 ml	90 ml	1×10^4	5000	
7	A	10	10 ml	90 ml	1000	500	10
8	B	10	10 ml	90 ml	100	50	10
9	C	10	10 ml	90 ml	10	5	10
10	D	10	10 ml	90 ml	1	0.5	10

Table 6.3. Estimation of methanogen MPN and plan for isolation experiment 3 (ISO3)

Steps	Series name	Dilution factor	Amount of material from previous step	Medium volume	Viable counts of methanogens/ml	Assumed counts of methanogens in Hungate tube if inoculated with 0.1 ml of dilution	Number of tubes inoculated
1		0	sample		1×10^9	1×10^8	
2		10	40 g	360 ml	1×10^8	1×10^7	
3		10	5 ml	45 ml	1×10^7	1×10^6	
4		10	5 ml	45 ml	1×10^6	1×10^5	
5		4	3 ml	9 ml	2.5×10^5	2.5×10^4	
6		4	3 ml	9 ml	6.25×10^4	6250	
7	A	4	3 ml	9 ml	1.56×10^4	1562.5	8
8	B	4	3 ml	9 ml	3910	390.63	8
9	C	4	3 ml	9 ml	977	97.66	8
10	D	4	3 ml	9 ml	244	24.41	8
11	E	4	3 ml	9 ml	61	6.10	8
12	F	4	3 ml	9 ml	15.3	1.53	8
13	G	4	3 ml	9 ml	3.8	0.38	8
14	H	4	3 ml	9 ml	0.95	0.09	8

Table 6.4. Estimation of methanogen MPN and plan for isolation experiment 4 (ISO4)

Step	Series name	Dilution factor	Amount of material from previous step	Medium volume	Viable counts of methanogens/ml	Assumed counts of methanogens in Hungate tube if inoculated with 0.1 ml of dilution	Number of tubes inoculated
1		0	Sample		1×10^9	1×10^8	
2		10	40 g	360 ml	1×10^8	1×10^7	
3		10	5 ml	45 ml	1×10^7	1×10^6	
4	A	10	5 ml	45 ml	1×10^6	1×10^5	
5	B	4	3 ml	9 ml	2.5×10^5	2.5×10^4	
6	C	4	3 ml	9 ml	6.25×10^4	6250	
7	D	4	3 ml	9 ml	1.56×10^4	1562.5	
8	E	4	3 ml	9 ml	3910	390.6	
9	F	4	3 ml	9 ml	977	97.7	60
10	G	4	3 ml	9 ml	244	24.4	60
11	H	4	3 ml	9 ml	61	6.1	100
12	I	4	3 ml	9 ml	15.3	1.5	100
13	J	4	3 ml	9 ml	3.8	0.4	100

6.2.5 DNA extraction and PCR

DNA was extracted from the culture tubes using Chelex resin. Extracted DNA was subjected to PCR amplification with archaeal 16S rRNA gene primers 915af/1386r or 86f/1340r (Table 2.2) and *mcrA* gene primers ML1/ML2 (Table 2.3) for the identification of methanogens present. Bacterial PCR, using the primer pair 27f and 1492r (Table 2.2), was performed on the DNA extracted from the tubes to confirm the purity of the culture and to identify the bacterial species present in the particular tubes. Amplified 16S rRNA gene products were analyzed by agarose gel (1.5% w/v) electrophoresis (Section 2.7).

6.2.6 Cloning and DNA sequencing

The PCR products were cloned into a plasmid vector (pCR 2.1; Invitrogen) and used to transform competent *Escherichia coli* TOP-10 cells using a TOPO-TA cloning system (Invitrogen) (Section 2.12). Positive clones were randomly selected and sequenced. Some of the sequencing was performed directly using PCR products without cloning. Sequencing was done either by Allan Wilson Centre Genome Sequencing Service (Massey University, Palmerston North, New Zealand) or Macrogen Inc. (Seoul, Republic of Korea). Results of sequences were obtained as ABI files and the sequences were edited using MEGA 4.0 (Tamura *et al.*, 2007). All sequences were subjected to BLAST search to determine the closest known taxon (Altschul *et al.*, 1990).

6.2.7 Phylogenetic analysis of methanogens isolated in this study

16S rRNA and *mcrA* genes trees were constructed to check the phylogenetic placement of new methanogens cultured in this study. The phylogenetic tree of 16S rRNA gene sequences were constructed using the neighbor-joining tree inference method with the Jukes-Cantor substitution model, assuming uniform rates among sites and using the complete deletion of gaps option implemented in Version 4 of the MEGA software package (Section 2.14). Deduced amino acid sequences were used to construct the *mcrA* gene phylogenetic tree. Amino acid sequences of *mcrA* gene clones and reference sequences were aligned with ClustalW and the phylogenetic tree constructed using the neighbor-joining tree inference method with the Jones-Taylor-Thornton

matrix amino acid substitution model using the complete deletion of gaps option (Section 2.14).

6.2.8 Quantitative real-time PCR analysis

Abundances of 16S rRNA genes of total archaea and members of RCC in selected culture tubes from isolation experiment 2 (ISO2) were quantified using a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science) and amplicon detection with SYBR Green (Section 2.19.1 and 2.19.2).

6.2.9 Microscopy

Live cultures were examined under phase contrast and epifluorescence microscopy for cell morphology and F₄₂₀ fluorescence using a DM2500 microscope (Leica Microsystems, Wetzlar, Germany), after placing aliquots on agarose-coated slides (Section 2.24).

6.2.10 Fluorescence *in situ* hybridization (FISH)

FISH (Section 2.20) was performed with enrichment culture ISO4-G11 using probes detailed in Table 2.6.

6.2.11 Eliminating bacteria

Since no antibiotics were used in these experiments, all the cultures were contaminated with bacteria. Several methods were tried to eliminate bacteria from these cultures. Some of these elimination methods were not successful. During this process of purification, many of the cultures lost their viability. The methods used to remove or kill the bacteria from the culture tubes are briefly explained below.

6.2.11.1 Dilution

The cultures were subjected to 10-fold serial dilutions. Methane-positive tubes from very high dilution steps were used for further processing. This will not remove all the bacteria, however, some bacteria can still be removed from the cultures.

6.2.11.2 Using antibiotics

Different antibiotics were tested individually and in combination. The antibiotics used were streptomycin (10 µg/ml), ampicillin (10 µg/ml), vancomycin (86.7 µg/ml) and bacitracin (10 µg/ml).

6.2.11.3 Heat treatment

Cultures were subjected to heat treatment by complete submersion in a water bath, immediately after inoculation, with various temperatures and time periods. For example, some cultures were treated at 50°C or 55°C for 10, 20 and 30 min.

6.2.11.4 Using lysozyme

Lysozyme solution was prepared anaerobically and added to the culture tubes anaerobically at a final concentration of 1 mg/ml.

6.3 Results

6.3.1 Isolation experiment 1 (selection of optimum media and blending time)

Isolation experiment 1 (ISO1) was conducted to select the best medium and blending time to isolate previously uncultured rumen methanogens. The change in the apparent MPN count of the initial number of methanogens over the 30 day incubation period after inoculation is shown in Figure 6.1. BY⁺ medium gave higher MPN counts of methanogens (Treatment 2 and 4) than when using the RM02 medium (Treatment 1 and 3). Also, in BY⁺ medium the MPN counts were affected little by blending time. The shorter blending time (Treatment 2) resulted in slightly higher MPN counts using BY⁺ medium. In contrast, the MPN counts using the RM02 medium with both blending time were lower than the BY⁺ medium and the longer blending time (Treatment 3) gave higher MPN counts than the shorter blending time (Treatment 1).

6.3.2 Isolation experiment 2 (selection of optimum media for RCC isolation)

Isolation experiment 2 (ISO2) was conducted to check the suitability of both media for isolation of RCC. The number of methane positive tubes with BY⁺ medium was always higher than the tubes with RM02 medium at similar dilutions (Table 6.5). Some of the methane positive tubes from high dilutions were selected and DNA was extracted from these tubes. Using this DNA, the numbers of 16S rRNA genes of total Archaea and RCC in culture tubes was estimated by qPCR (Table 6.6). In some of the tubes, the total RCC 16S rRNA genes nearly equalled the total archaeal 16S rRNA genes, suggesting that RCC were the dominant or only Archaea in these tubes (Table 6.6). None of the methane-positive tubes with RM02 medium tested with qPCR had RCC 16S rRNA genes. However, not all of the methane positive tubes were checked for RCC 16S rRNA genes. As such, it is not possible to definitely say that RM02 medium was not suitable for RCC isolation. However, BY⁺ medium did support the growth of RCC, and it was decided to use the BY⁺ medium for RCC isolation.

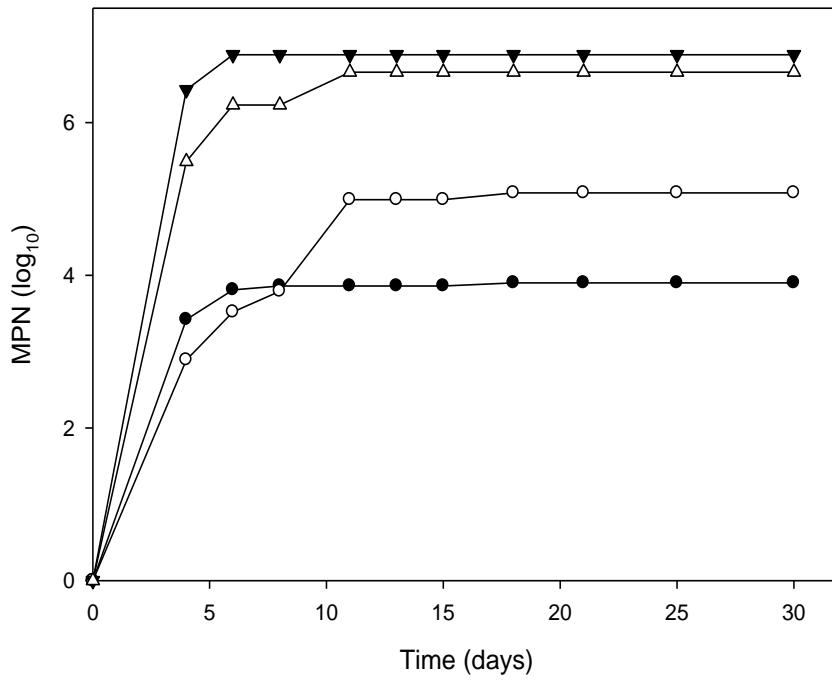


Figure 6.1. Change in the most probable number (MPN) estimation of methanogens with incubation time for combinations of two media and blending time in isolation experiment 1 (ISO1). (○) RM02 medium and long blending time, (●) RM02 medium and short blending time, (△) BY⁺ medium with long blending time, (▲) BY⁺ medium with short blending time.

Table 6.5. The number of methane-positive tubes obtained in ISO2 in each dilution with different media after 21 days of incubation

Series name	Total number of tubes inoculated per each medium	Number of methanogens inoculated/tube	Methane-positive tubes after 21 days	
			BY ⁺ medium	RM02 medium
A	10	500	10	5
B	10	50	8	2
C	10	5	3	0
D	10	0.5	1	0

6.3.3 Isolation experiment 3

This experiment was performed to attempt to isolate previously uncultured rumen methanogens, including members from the RCC group, by adding CHCl_3 to some tubes. However, none of the culture tubes to which CHCl_3 had been added showed methane production. Among the culture tubes without CHCl_3 , several methane-positive tubes were obtained. Direct sequencing of archaeal PCR products obtained using the DNA extracted from these tubes showed that these tubes contained RCC, *Methanosphaera* spp. and *Mbb. gottschalkii* (Table 6.7). Unfortunately, with repeated sub-culturing and the treatments used to eliminate bacteria (Section 6.2.10), most of these cultures lost viability. Only one culture, ISO3-F5, remained viable after all sub-culturing attempts and treatments to eliminate bacteria. The bacteria in this culture were successfully removed by several heat treatments (50°C for 30 minutes after the inoculation in each sub-culturing). The purity of this culture was confirmed by several methods, and initial characterization of this isolate was performed and described in Chapter 7.

Table 6.6. The number of 16S rRNA genes of total archaea and RCC estimated by qPCR from the DNA from methane-positive tubes obtained in isolation experiment 2 (ISO2)

Medium	Name of culture	Number of total Archaea	Number of total RCC
RM02	A9	$9.22 \times 10^5 \pm 8.5 \times 10^4$	0
RM02	B4	$6.02 \times 10^5 \pm 5.04 \times 10^4$	0
RM02	B2	$8.78 \times 10^5 \pm 4.78 \times 10^4$	0
BY	D9	$2.12 \times 10^5 \pm 8.63 \times 10^3$	$3.12 \times 10^5 \pm 3.55 \times 10^4$
BY	C9	$7.10 \times 10^6 \pm 6.47 \times 10^4$	$7.12 \times 10^6 \pm 7.05 \times 10^5$
BY	C7	$1.98 \times 10^6 \pm 3.42 \times 10^5$	$1.37 \times 10^7 \pm 2.15 \times 10^6$
BY	C4	$4.17 \times 10^5 \pm 2.90 \times 10^4$	0
BY	B3	$3.20 \times 10^5 \pm 1.63 \times 10^4$	$4.12 \times 10^5 \pm 4.93 \times 10^4$
BY	A1	$6.60 \times 10^5 \pm 7.12 \times 10^4$	$5.98 \times 10^5 \pm 9.90 \times 10^4$

Table 6.7. The different groups of methanogens identified in the methane-positive tubes from isolation experiment 3 (ISO3)

Methanogen group	Number of tubes
<i>Methanobrevibacter gottschalkii</i>	6
<i>Methanosphaera</i> spp.	3
RCC	24

6.3.4 Isolation experiment 4

A fourth isolation experiment was set up, using BY⁺ medium containing 10 μM coenzyme M. The different methanogen groups that were identified in the methane positive tubes in isolation experiment 4 are shown in Table 6.8. After several sub-culturing and bacterial elimination attempts, no pure cultures were obtained from this experiment. However, four cultures that contained only one type of methanogen were obtained. Efforts to eliminate the contaminating bacteria from these tubes were not fully successful. However, the number of contaminating bacterial species was reduced and this was confirmed by morphological analysis by phase contrast and epifluorescence

microscopy, and by analysis of libraries of bacterial 16S rRNA genes. The four cultures obtained from this experiment were ISO4-G1, ISO4-G11, ISO4-G16 and ISO4-H5. Attempts to purify these cultures are continuing.

Table 6.8. The different groups of methanogens identified in the methane-positive tubes from isolation experiment 4

Methanogen group	Number of tubes
<i>Methanobrevibacter gottschalkii</i>	7
<i>Methanobrevibacter ruminantium</i>	3
<i>Methanosphaera</i> spp.	5
RCC	16

6.3.5 Archaeal clone library analysis

Small archaeal clone libraries (≈ 20 clones) were constructed using the DNA extracted from cultures ISO3-F5, ISO4-G1, ISO4-G11, ISO4-G16 and ISO4-H5. All of these cultures possess only one type of archaeal sequence and the sequence information is given in Appendix 1. All sequences in any one culture were identical. The methanogen group identified in these cultures are given in Table 6.9.

6.3.6 Bacterial clone library analysis

Only 3 cultures (ISO4-G1, ISO4-G11 and ISO4-H5) produced bands with bacterial 16S rRNA gene primers. Libraries of bacterial 16S rRNA genes showed that 2 of these cultures (ISO4-G11 and ISO4-H5) contained only one type of bacteria, while the third culture (ISO4-G1) contained two bacterial contaminants. The number of clones per clone library was small (≈ 20 clones), therefore, there may be more bacteria in these cultures. However, the microscopic analysis of live cells of these cultures also confirmed the results of the clone library analysis. Surprisingly, one of the contaminating bacteria was found in all three cultures. This observation suggests that there may be some interactions between this culture and the RCC. More studies are needed to check this hypothesis. The different bacterial clones identified in the DNA of ISO4-G1, ISO4-G11 and ISO4-H5 are given in Table 6.10.

Table 6.9. Methanogens identified in the cultures

Culture	Number of clones	Closest relatives identified in GenBank (accession number), sequence identity	Methanogen group
ISO3-F5	20	<i>Methanosphaera stadtmanae</i> (CP000102), 96%	<i>Methanosphaera</i> spp.
ISO4-G1	16	<i>Aciduliprofundum boonei</i> (CP001941), 80%	RCC
ISO4-G11	18	<i>Aciduliprofundum boonei</i> (CP001941), 78%	RCC
ISO4-G16	18	<i>Methanobrevibacter thaueri</i> (U55236), 97%	<i>Mbb. gottschalkii</i> clade
ISO4-H5	17	<i>Aciduliprofundum boonei</i> (CP001941), 78%	RCC

Table 6.10. Bacteria identified in the cultures

Culture	Total number of clones	Closest relatives identified in GenBank (accession number), sequence identity	number of clones
ISO4-G1	16	<i>Succinivibrio dextrinosolvens</i> (NR026476), 99%	12
		Uncultured bacteria (EU843167), 98%	4
ISO4-G11	18	<i>Succinivibrio dextrinosolvens</i> (NR026476), 99%	18
ISO4-H5	15	<i>Succinivibrio dextrinosolvens</i> (NR026476), 99%	15

6.3.7 Microscopy

Phase contrast and fluorescent photomicrographs of cultures ISO4-G16 and ISO3-F5 are shown in Figures 6.2 and 6.3. The cells of ISO3-F5 were spherical in shape, and mostly occurred in pairs or as clumped packets. All of the cells showed the same morphology and these cells fluoresced when observed with UV, indicative of the presence of F₄₂₀ in the cells. No non-fluorescing cells were observed, which indicated this culture was free from bacteria.

The cells of culture ISO4-G16 were coccobacillus in shape. However, occasionally a few spherical shaped cells were also observed. All the coccobacillus-shaped cells fluoresced when observed with UV, but the spherical-shaped cells did not fluoresce, which suggested that culture ISO4-G16 was contaminated with bacteria or non-fluorescing archaea. Interestingly, the DNA extracted from this culture did not produce bands with bacterial primers.

The other three cultures, ISO4-G1, ISO4-G11 and ISO4-H5, which were obtained from isolation experiment 4, still contained bacterial cells after several sub-culturing steps and a number of attempts to eliminate the bacteria. However, after a number of sub-culturing steps, dilution and heat treatments, the number of bacterial contaminants was reduced to 1-2 cell types. The use of antibiotics (streptomycin, ampicillin and vancomycin) was not successful, as they were inhibitory to the RCC cultures as well. These cultures contained cocci and curved rods (Figure 6.4). *Succinivibrio dextrinosolvens*, which was identified in the bacterial clone library, may be the curved rods and the cocci may be the RCC cells. *Succinivibrio dextrinosolvens* cells are reported to be curved rods (Hippe *et al.*, 1999). Since both these cells did not autofluoresce, the actual morphology of RCC cell cannot be confirmed. Methane production was observed in these tubes, but no cells fluoresced like known methanogens. This suggests that RCC cells may not possess cofactor F₄₂₀ like all known methanogens or that they have much lower levels of F₄₂₀. More studies are needed to confirm this observation.

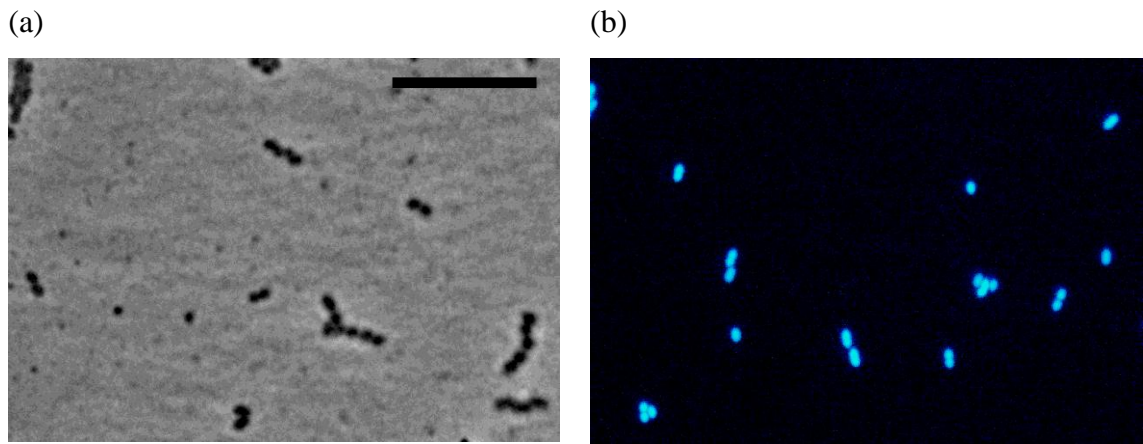


Figure 6.2. Photomicrographs of culture ISO4-G16. (a) Phase contrast photomicrograph. (b) Epifluorescence photomicrograph of cells displaying autofluorescence under UV illumination. Scale bar, 10 μm for both panels.

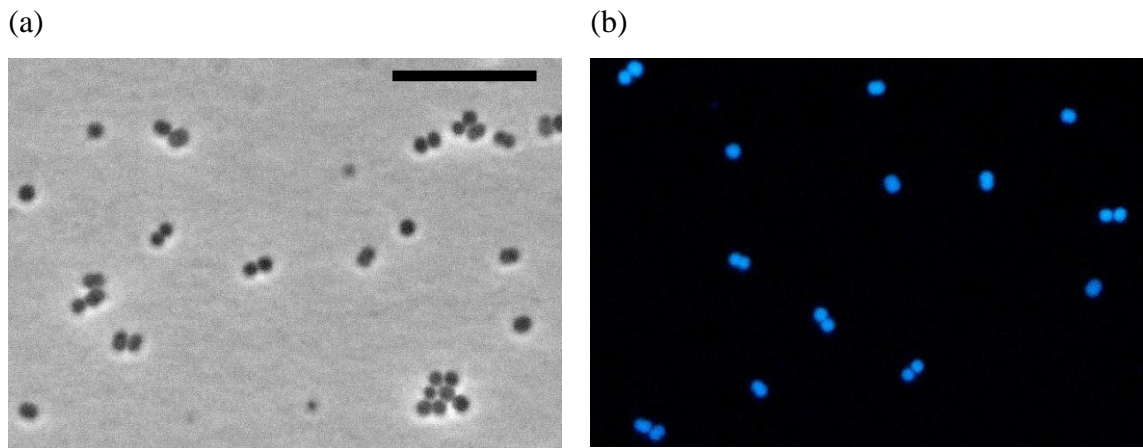


Figure 6.3. Photomicrographs of culture ISO3-F5. (a) Phase contrast photomicrograph. (b) Epifluorescence photomicrograph of cells displaying autofluorescence under UV illumination. Scale bar, 10 μm for both panels.

6.3.8 Fluorescence *in situ* hybridization (FISH)

Phase contrast microscopy of culture ISO4-G11 showed that this consisted of two cell types: cocci and curved rods (Figure 6.4a). To determine the phylogenetic affiliations of the two cell forms, fluorescence *in situ* hybridisation (FISH) was performed using probe EUB338, which targets most members of the domain *Bacteria* (Daims *et al.*, 1999). The curved rods hybridised with this probe (Figure 6.4b), indicating that these were bacteria. Dual staining was performed with the probes ARC915, which targets most members of the domain Archaea (Loy *et al.*, 2007), and the newly designed probe RCC771 (Section 2.20) designed to target members of the RCC group. The same cocci were stained with both probes (Figures 6.4c and 6.4d), indicating that these cells were the RCC in this culture.

6.3.9 Phylogenetic position

Phylogenetic trees were constructed using 16S rRNA gene sequences and deduced amino acid sequences of *mcrA* genes from the isolated cultures, along with reference sequences downloaded from GenBank (Figures 6.5 and 6.6). The sequence of the pure culture ISO3-F5 clustered within the radiation of cultured *Methanosphaera* species. Several clones obtained from rumen samples clustered with ISO3-F5. The sequence of ISO4-G16 clustered within the *Methanobrevibacter gottschalkii* clade. The cultured relatives of this sequence were *Methanobrevibacter thaueri* strain CW and *Methanobrevibacter millerae* ZA-10, but both showed only 97% similarity to ISO4-G16. This culture also showed 97% similarity with the *Methanobrevibacter* sp. SM9, which was isolated from a grazing sheep. ISO4-G16 is possibly a new species from the *Methanobrevibacter gottschalkii* clade.

The sequences of ISO4-G1, G11 and H5 clustered within the RCC group in the 16S rRNA gene phylogenetic tree. In the *mcrA* gene phylogenetic tree, these three cultures grouped within an uncultured archaeal group which probably represents the RCC group (Chapter 5). These three cultures were fell into separate sub-clusters, each with high bootstrap support, suggesting that these sequences may be belonged to three different species.

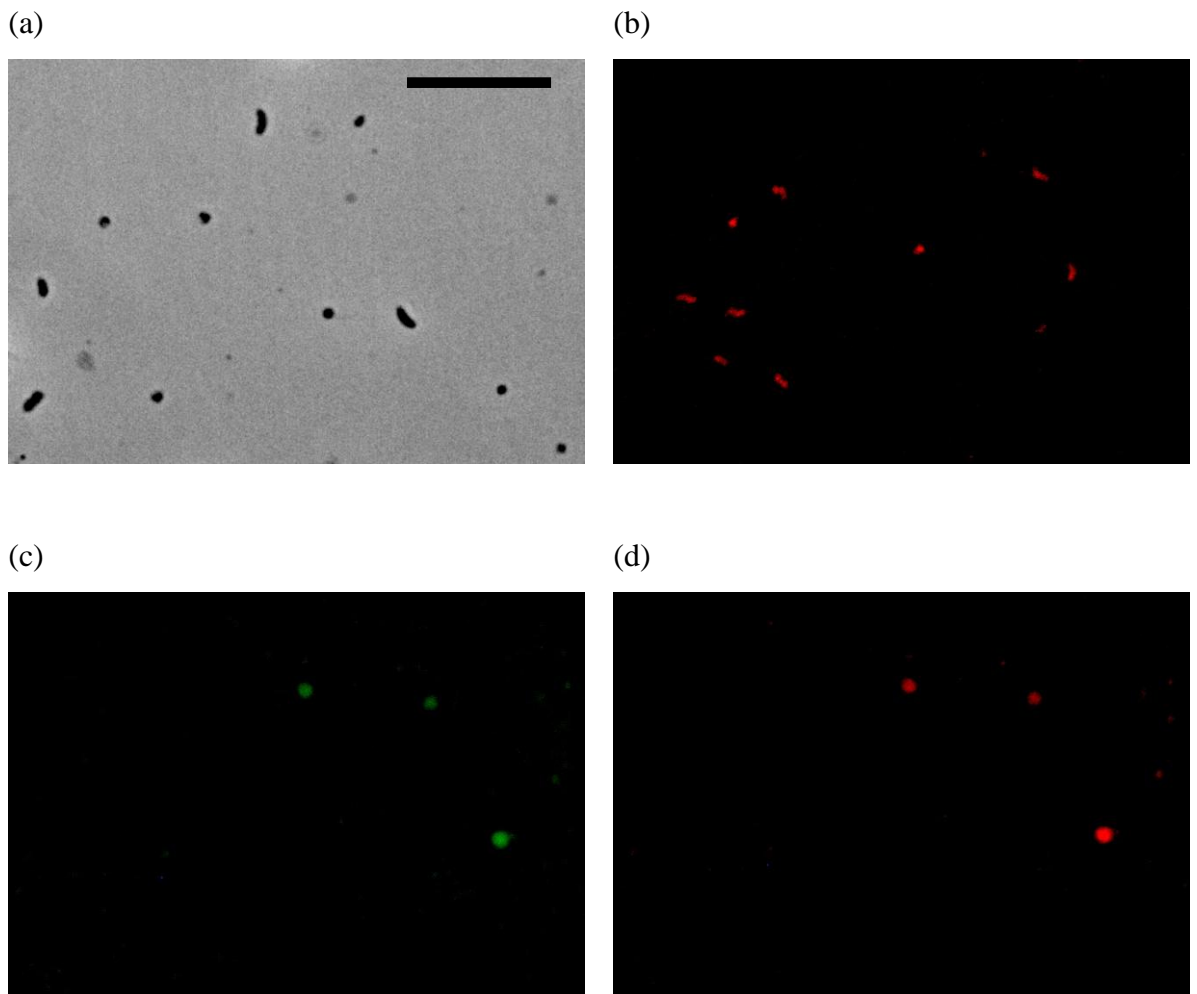


Figure 6.4. Photomicrographs of culture ISO4-G11. (a) Phase contrast photomicrograph showing two cell types, cocci and curved rods, in the culture. (b) Epifluorescence photomicrograph of the culture after *in situ* hybridisation with Cy3-labelled EUB338 probe, showing curved rods labelled with the probe. (c, d) Epifluorescence photomicrographs of identical microscope fields, showing the culture after *in situ* hybridisation with two probes: (c) FITC-labelled ARC915 probe, and (d) Cy3-labelled RCC771 probe, indicating that the same cocci hybridise with both probes. Scale bar, 10 μm for all panels.

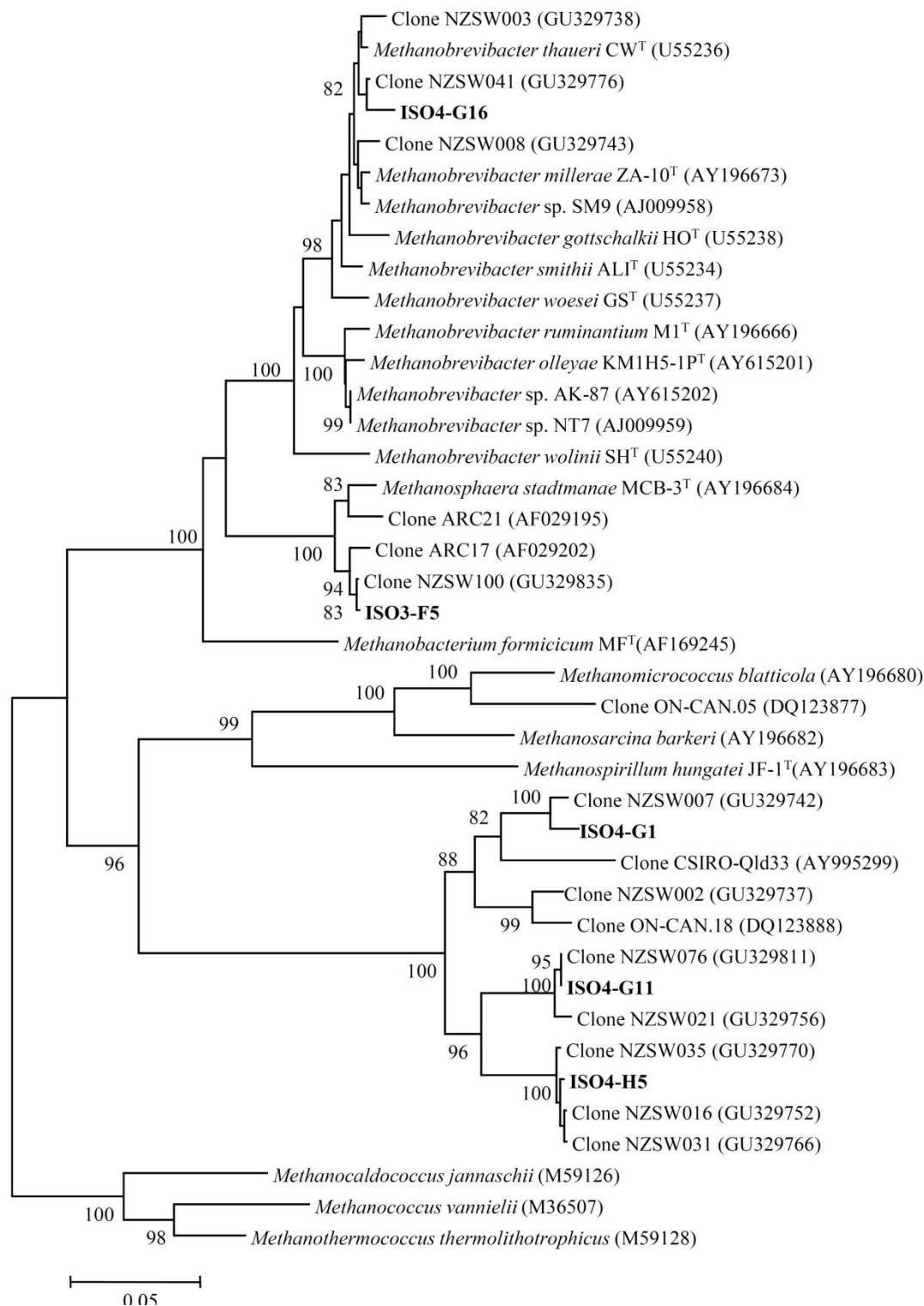


Figure 6.5. Phylogenetic relationships of isolated cultures and reference 16S rRNA gene sequences. In parentheses are the GenBank accession numbers for reference sequences. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates nucleotide substitutions per nucleotide position.

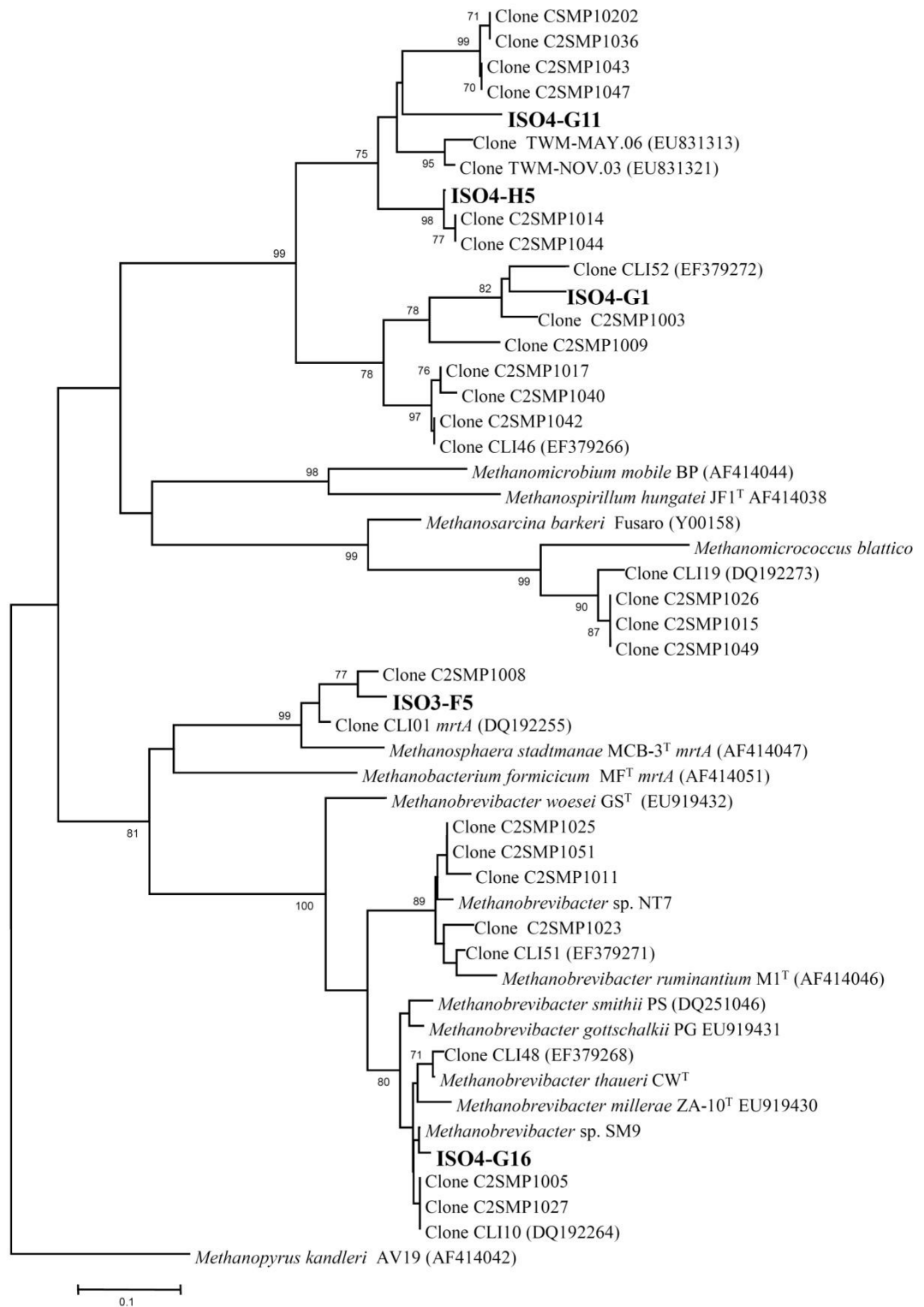


Figure 6.6. Phylogenetic relationships of isolated cultures and reference *mcrA* amino acid sequences. In parentheses are the GenBank accession numbers for reference sequences. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates amino acid substitutions per amino acid position.

6.4 Discussion

Molecular ecological approaches have made tremendous progress in identifying the microbial diversity in the rumen. For a better understanding of the physiology and ecological significance of members of the microbial community in the rumen, cultivation of pure cultures is still considered to be important. Availability of pure cultures of different microbial groups in the rumen will allow easier assessment of their physiologies, provide access to their genomes and their functional significance in rumen, and clearly be useful in improving our understanding of rumen microbiology.

A simple cultivation strategy was followed in attempts to isolate uncultured methanogens from the rumen. In a similar approach, Kenters *et al.*, (2010) isolated a significant number of previously-uncultured bacteria from the rumen. Methanogens as a group have a very limited substrate range that includes acetate, hydrogen, methanol, formate and methylamine (Garcia *et al.*, 2000). Therefore, a mixture of substrates (acetate, formate, methanol and hydrogen) was used for these isolation experiments. Initial experiments showed that BY⁺ medium was suitable for isolation of methanogens, including members from RCC. Antibiotics were not used in these experiments, as the effect of these on members of RCC was not known. It was known that some members of RCC group were less affected by low concentrations of CHCl₃ (Chapter 5). However, use of CHCl₃ in one of the experiment (ISO3) did not allow isolation of members of RCC. The concentration of CHCl₃ chosen (50 µM) may have been higher than their tolerance level.

Five cultures, each apparently containing only one type of methanogen, were obtained in these experiments (Table 6.11). One of the cultures, ISO3-F5, belonged to the *Methanosphaera* spp. and was confirmed as a pure culture (Table 6.11). The initial characterization of this isolate is reported in Chapter 7. This isolate appears to be the first report of cultivation of a *Methanosphaera* species from the rumen. Since *Methanosphaera* spp. are one of the dominant methanogen group in New Zealand ruminants (Chapter 4), isolation of this rumen strain will be valuable in methane mitigation strategies. Its genome is currently being sequenced (Attwood *et al.*, 2010).

Table 6.11. Purity checking of cultures

Cultures	Methanogen present	BY + glucose	Phase contrast microscopy	Fluorescent (F ₄₂₀) microscopy	Gram stain	Archaeal PCR	Bacterial PCR	RCC PCR
ISO3-F5	<i>Methanosphaera</i> sp.	No growth	Uniform cocci	All the cocci cells were fluoresced	Positive	Band	No bands	No bands
ISO4-G16	<i>Methanobrevibacter</i> sp.	No growth	Coccobacilli and very few cocci	All coccobacilli fluoresced	Positive	Band	No bands	No bands
ISO4-G1	RCC	Growth, curved rods	Cocci and curved rods	No fluorescent cells	Not determined	Band	Band	Band
ISO4-G11	RCC	Growth, curved rods	Cocci and curved rods	No fluorescent cells	Not determined	Band	Band	Band
ISO4-H5	RCC	Growth, curved rods	Cocci and curved rods	No fluorescent cells	Not determined	Band	Band	Band

Isolate ISO4-G16 clustered within the *Methanobrevibacter gottschalkii* clade. However, this culture was contaminated with another, as yet unidentified, organism (Table 6.11). Efforts are continuing to remove this contaminant. The 16S rRNA gene sequence shows that this isolate may be a new species within the *Methanobrevibacter gottschalkii* clade, but detailed study and characterization are needed to confirm this. Its 16S rRNA gene sequence showed 97% identity to other isolates. This is at the threshold of identity, 97%, often used to distinguish microbial species (Stackebrandt & Goebel, 1994). Detailed phenotypic characterization and comparison with related species will be required to determine its status as a new species.

Three possible RCC cultures, ISO4-G1, ISO4-G11 and ISO4-H5, were mixed cultures with bacteria (Table 6.11). Efforts to remove these bacteria by heat treatment and antibiotics addition were not successful. However, some of the bacterial cells were eliminated by these different treatments. Phase contrast microscopic examination confirmed only one or two bacterial contaminants in these cultures. Archaeal and bacterial clone library analysis of the RCC enrichment cultures confirmed the presence of RCC in these cultures along with at least one or two bacterial contaminants. The common bacteria present in these cultures were identified as *Succinivibrio dextrinosolvens*. All the cells were non-fluorescent and the cultures produced methane. Comparative sequence analysis of the archaeal 16S rRNA and *mcrA* genes showed that these three isolates belong to three different groups, possibly three different species. Efforts to purify these isolates are continuing. This may require use of a solid medium support, such as anaerobic roll-tubes or plates.

Culture ISO4-G11 contained two cell types, cocci and curved rods. The clone library analysis suggested that there were both bacteria and RCC (archaea) in this culture. FISH indicated that the curved rods were members of the domain *Bacteria*. The general morphology of these cells is consistent with detection of *Succinivibrio* sp. in the clone library of 16S rRNA genes from bacteria. Also using FISH, the cocci hybridised with probes that targeted most members of the domain Archaea and members of the RCC group. The probe targeting archaea has been widely used and is well validated (Loy *et al.*, 2007), but the RCC probe has not yet been validated, due to the absence of verified control cultures. However, the fact that the same cocci hybridised with both

probes strongly supports the conclusion that these cells are the members of RCC that were detected in this culture.

Although a pure culture of RCC was not obtained in these cultivation experiments, some of their important characteristics were identified. Importantly, they produce methane and should therefore to be included in the target group for vaccine and small molecule inhibitor development. They contain a *mcrA* gene, so that they can be considered as methanogens. However, no fluorescence cells were observed in this culture suggesting that they lack or have very little F₄₂₀ cofactor which is present in all known methanogens (Lin & White, 1986).

Cofactor F₄₂₀ is a derivative of 7, 8-didemethyl-8-hydroxyl-5-deazariboflavin (Ashton *et al.*, 1979; Eirich *et al.*, 1978). This cofactor has been identified in many euryarchaea (including all methanogens), one crenarchaeon (*Sulfolobus solfataricus*) and several bacterial genera of phylum *Actinobacteria*, including *Mycobacterium* (Graham, 2010). In methanogens, F₄₂₀ serves as a reductant carrier in the methane generation (Ellefson & Wolfe, 1980; 1981). It mediates two-electron hydride transfer reactions among hydrogenase, methylenetetrahydromethanopterin dehydrogenase and heterodisulfide reductase enzymes. All methanogens identified to date have contained this cofactor. Lack of F₄₂₀ in RCC suggests that they may produce methane using a different pathway than the normal methanogenesis pathway of known methanogens. Genome analysis of members of this group will provide more details of this organism. Further investigation on this group of archaea is warranted, since they do appear to be methanogens and may be biochemically different to known methanogens. Rumen methane mitigation strategies, whether inhibitors or vaccines, should target this group.

Chapter 7

Characterization of *Methanosphaera* isolate ISO3-F5

7.1 Introduction

Methanogens belonging to *Methanosphaera* spp. are one of the dominant genera residing in New Zealand ruminants (Chapter 4). Studies performed all over the world with different ruminant species fed different diets have also shown this species to be common inhabitants of the rumen (Table 1.2). A recent study of the relationship between feed efficiency and rumen methanogen diversity found a high prevalence of *Methanosphaera* spp. in inefficient animals, which produce more methane compared to efficient animals (Zhou *et al.*, 2009). Culture-independent studies performed using rumen samples have also suggested that there is more than one species of *Methanosphaera* in the rumen (Whitford *et al.*, 2001). To date, however, no members of this genus have been isolated from the rumen.

Methanosphaera spp. belong to the order *Methanobacteriales* and most of the members of this order produce methane using CO₂ as the electron acceptor and H₂ as the electron donor (Liu & Whitman, 2008). However, the genus *Methanosphaera* is unusual because they use H₂ to reduce CH₃OH to methane and, when using this pathway, CO₂ is not involved in CH₄ production (Miller & Wolin, 1985). In addition, studies with cultured *Methanosphaera* species have shown that acetate is absolutely required for their growth (Biavati *et al.*, 1988; Miller & Wolin, 1983). This is not necessarily the case for other rumen methanogens studied thus far. Currently, two species from this genus have been isolated and characterized, and these share similar characteristics. The first species described was *Methanosphaera stadtmanae*, which was isolated from human faeces and was the first human archaeon whose genome was sequenced (Fricke *et al.*, 2006). The second species, *Methanosphaera cuniculi*, was isolated from rabbit rectum, but has not been studied in detail. Although the two isolated *Methanosphaera* spp. show similarities in many characteristics, rumen strains may not possess the same characteristics.

Research aiming to develop methane mitigation techniques for farmed ruminants requires a clear understanding of methanogen diversity and their role in the rumen. For these reasons there is a need to isolate and investigate *Methanosphaera* species from the rumen, as they probably use a different pathway of methane production compared to other common rumen methanogens. A rumen isolate of *Methanosphaera* spp. would provide more information of their role in methane production in rumen. One isolate, ISO3-F5, was obtained in pure culture (Chapter 6). An initial characterization of isolate ISO3-F5 was performed and is presented in this chapter.

7.2 Materials and Methods

7.2.1 Source of inoculum

ISO3-F5 was isolated from a rumen sample collected from a ruminally-fistulated wether sheep (S4) grazing on perennial rye grass (*Lolium perenne*) and white clover (*Trifolium repens*) mix. This sheep was kept at the AgResearch Grasslands campus, Palmerston North, New Zealand, and had unlimited access to water at all times. The sample was collected during the summer 2009, 2 h after the animal was taken from the pasture.

7.2.2 Enrichment and isolation

Sample collection and sample processing methods for isolation of ISO3-F5 were described in Chapter 2 (Section 2.21) and Chapter 6 (Section 6.2.4.3). All preparations and transfers were carried out under an O₂-free CO₂ atmosphere using anaerobic procedures and the culture techniques. Enrichment and initial isolation procedures were carried out with BY⁺ medium (Section 2.22.2). Once ISO3-F5 was isolated, purification (elimination of bacteria) and experiments for characterization were performed in RM02 medium (Section 2.22.3). Initially this isolate was grown with a gas mixture of 80% H₂ and 20% CO₂ with the following substrates in the media: sodium acetate (20 mM), sodium formate (60 mM) and methanol (20 mM). The isolation procedures and all the experiments were conducted using Hungate tubes. All the inoculated tubes were incubated at 39°C under shaking condition (200 rpm) in the dark. Initially, growth of ISO3-F5 in mixed culture (with bacteria) was determined by gas chromatography

(Section 2.28.1). Once ISO3-F5 was purified, all experiments for characterization were performed in RM02 media and methanogen growth was determined by measuring the culture density at 600 nm by inserting the culture tubes directly into an Ultrospec 1100 Pro UV/Vis spectrophotometer (Section 2.28.2). All the characterization experiments were conducted with four replicates.

7.2.3 Culture purity

The purity of ISO3-F5 (absence of bacteria and other methanogens) was checked by the following methods.

7.2.3.1 Microscopy

Live cells were examined under phase contrast and epifluorescence microscopy for cell morphology and F₄₂₀ fluorescence using a DM2500 microscope (Leica Microsystems, Wetzlar, Germany). Live cultures of ISO3-F5 were mounted on agarose-coated slides (Pfenning & Wagener, 1986) and observed under 100× oil phase contrast and epifluorescence objectives. Since all the known methanogens contain F₄₂₀ fluorescence, occurrence of cells without fluorescence suggests the presence of bacteria or non-methanogenic archaea.

7.2.3.2 Inoculation with mixed sugar, peptone and yeast extract solution (RFgenV)

ISO3-F5 was inoculated into RM02 medium with 0.5 ml of RFgenV solution (Section 2.23.2) which contains glucose, cellobiose, xylose, arabinose, lactate, casamino acid bacto-peptone and yeast extract. All of these components are common bacterial substrates. No methanogenic substrates were added, including H₂/CO₂. These tubes were incubated at 39°C in the dark under shaking condition (200 rpm) for one week and checked for turbidity each day.

7.2.3.3 PCR with bacterial primers

DNA was extracted from a culture of ISO3-F5 by the phenol-chloroform method (Section 2.3.2b) and PCR was performed with the universal bacterial primers 27f and

1492r (Table 2.2). Failure to recover bacterial 16S rRNA gene amplification was an indicator of culture purity.

7.2.3.4 Archaeal 16S rRNA gene-based clone analysis

A small archaeal clone library (20 clones) was constructed with the universal archaeal primer pair 8f and 1510r (Table 2.2) using DNA extracted from ISO3-F5. Recovery of a single sequence type helped demonstrate culture purity.

7.2.4 Gram stain

The Gram reaction of ISO3-F5 was determined by Hucker's modification of the Gram stain method (Section 2.24) and observed using a DM2500 microscope (Leica Microsystems, Wetzlar, Germany) under 100× oil phase contrast objective.

7.2.5 Electron microscopy

Electron microscopic examination (Philips CM10 Transmission Electron Microscope with SIS Morada high-resolution digital imaging) of ISO3-F5 was carried out at the Manawatu Microscopy and Imaging Centre (MMIC), Massey University, Palmerston North, New Zealand. Both negative staining and thin section electron microscopy were performed with this isolate. Using the electron microscope cell images, the detailed morphology of cells (e.g. average diameter of cells, cell wall thickness, cytoplasm structures, etc.) was studied. Preparation of cells and the procedure for the negative staining and TEM are described in Chapter 2 (Section 2.26).

7.2.6 Growth factor requirements

An experiment was conducted to check the requirement for rumen fluid (RF), yeast extract (YE), coenzyme M (CoM), vitamins (Vit) and volatile fatty acids (VFA) on the growth of ISO3-F5. RM02 medium was used for this experiment and acetate (20 mM), methanol (20 mM) and H₂/CO₂ (80:20) were used as substrates. The requirements for growth factors in the additions were determined by the single or multiple omissions of the components. The experiment continued for 3 serial transfers to remove the carry over effect of the initial inocula. Growth was measured every day by measuring culture

density at 600 nm by inserting the tubes directly into an Ultrospec 1100 pro UV/Vis spectrophotometer.

The RF, YE, CoM, Vit and VFA solutions were prepared anaerobically and filter sterilized into N₂-filled sterile serum vials sealed with a butyl rubber stopper and an aluminium cap (Bellco) through 0.22 µm pore size Millex GP sterile filters (Millipore Corp., Bedford, MA, USA) using sterile syringes and needles. Solutions were added to the media to obtain final concentrations of 5% (v/v) RF, 1 g/l (w/v) YE and 10 µM 2-mercaptoethanesulfonic acid (CoM). The preparation of vitamin mixture and VFA solution was described in Chapter 2 (Section 2.23.3 and 2.23.5), and 0.1 ml of these solutions was added per 10 ml of medium.

7.2.7 Substrate requirements

ISO3-F5 was tested for its ability to utilize the following substrates with and without H₂/CO₂: sodium acetate (20 mM), sodium formate (20 mM), methanol (20 mM), ethanol (20 mM), isopropanol (20 mM), and methylamine (20 mM). RM02 medium was used for this experiment, and YE (1 g/l w/v) and vitamin mixture (0.1 ml/10 ml medium) were added to the RM02 base solution along with the substrates. Rumen fluid was not added in this experiment. The effects of these additions were determined by single or multiple omissions of the components and the experiment was continued for 3 serial transfers to remove the carry over effect of the initial inoculums. Growth was measured every day by measuring culture density at 600 nm.

Substrate solutions were prepared anaerobically in serum vials sealed with a butyl rubber stopper and an aluminium cap (Bellco) and autoclaved for 20 min at 121°C. These solutions were stored at room temperature in the dark and were added to the media on the day of inoculation.

7.2.8 Optimum pH for growth

The pH optimum for the growth of ISO3-F5 was determined using 2 different media adjusted to different pH values; RM02 medium and RM02 medium amended with a stock solution of 500 mM MOPS (3-[N-morpholino] propanesulfonic acid) designated as RM02-MOPS hereafter. MOPS is sufficiently heat-stable to be

autoclaved. For the RM02 medium, the pH was adjusted by adding HCl (5 M) and NaOH (5 M) solutions. For the RM02-MOPS medium, 500 mM MOPS solution was prepared to different pH values with NaOH (5 M) and added to RM02 media at 1 ml/10 ml medium. Methanol (20 mM) and acetate (20 mM) were used as the substrates and rumen fluid was not used in this experiment. The pH measurement of the pH-adjusted media was taken at 37°C to minimise temperature effects. The pH of the media was also measured at the end of the experiment. Growth was measured every day by measuring culture density at 600 nm.

7.2.9 Optimum temperature

ISO3-F5 was grown at different temperatures either in a water bath or in heat blocks to determine the range and optimum temperature for their growth. RM02 medium with YE (1 g/l) and vitamin mixture (0.1 ml/10 ml media) was used with methanol, acetate and H₂/CO₂ as the substrates. Growth was measured every day by measuring culture density at 600 nm.

7.2.10 Cell lysis

The resistance of ISO3-F5 cells to sodium dodecyl sulphate (SDS) (0.5% and 2% w/v), lysozyme (1 mg/ml and 10 mg/ml) and proteinase K (1 mg/ml and 10 mg/ml) were determined by adding these solutions to actively growing cultures and examining these cells after 2 h with a Leica DM2500 microscope under 100× oil phase contrast objective.

7.2.11 G+C content of genomic DNA

Genomic DNA was extracted from ISO3-F5 using the phenol-chloroform method (Section 2.3.2b). The quality of DNA was checked by NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the G+C content of the genomic DNA of ISO3-F5 was determined by a high-performance liquid chromatography (HPLC) method (Section 2.27).

7.2.12 Phylogenetic analysis of ISO3-F5

PCR was performed with archaeal 16S rRNA gene specific primers 8f and 1510r and *mcrA* gene primers ML1 and ML2 (Tables 2.2 and 2.3) using DNA extracted from ISO3-F5. These PCR products (16S rRNA and *mcrA* genes) were cloned into a plasmid vector (pCR 2.1; Invitrogen) and used to transform competent *Escherichia coli* TOP-10 cells using a TOPO-TA cloning system (Invitrogen). Positive clones were randomly selected and sequenced. Sequencing was done by Macrogen Inc. (Seoul, Republic of Korea).

The phylogenetic position of the ISO3-F5 was determined by comparative analysis of 16S rRNA and *mcrA* gene sequences using selected reference sequences. The two previously isolated *Methanosphaera* species (*Methanosphaera stadtmanae* and *Methanosphaera cuniculi*) were included in the tree. The 16S rRNA and *mcrA* gene sequences of *Methanosphaera stadtmanae* strain MCB-3 were downloaded from GenBank (<http://ncbi.nlm.nih.gov>). However, there were no gene sequence data for the 16S rRNA or *mcrA* gene sequences of *Methanosphaera cuniculi*. A culture of *Methanosphaera cuniculi* strain IR7, which had been purchased from the DSMZ was available from the AgResearch culture collection. DNA was extracted from this culture and 16S rRNA and *mcrA* gene information was obtained by PCR with 16S rRNA (86f and 1340r) and *mcrA* gene primers (ML1 and ML2) and then cloning and sequencing. These sequences were included in the phylogenetic analysis of ISO3-F5.

The phylogenetic tree of 16S rRNA gene sequences was constructed using the neighbor-joining tree inference method with the Jukes-Cantor substitution model, assuming uniform rates among sites and using the complete deletion of gaps option implemented in Version 4 of the MEGA software package. Deduced amino acid sequences were used to construct the *mcrA* gene phylogenetic tree. Amino acid sequences of *mcrA* gene clones and reference sequences were aligned with ClustalW and phylogenetic trees were constructed using the neighbour-joining tree inference method with the Jones-Taylor-Thornton matrix amino acid substitution model using the complete deletion of gaps option (Section 2.14).

7.2.13 Long term preservation of ISO3-F5

Cultures of ISO3-F5 were preserved with dimethyl sulfoxide (DMSO) solution and stored in -80°C (Section 2.29.1). Some of the cultures were also preserved with glycerol solution and stored in -80°C (Section 2.29.2). The viability of these preserved cultures was checked after one month at -80°C.

7.2.14 Gene sequences (16S rRNA and *mcrA* genes)

The 16S rRNA and *mcrA* gene sequences of ISO3-F5 and *Methanosphaera cuniculi* strain IR7 are shown in Appendix 1.

7.3 Results

7.3.1 Culture purity

All of the cells that were observed under phase contrast microscopy fluoresced when excited with UV, indicative of the presence of F₄₂₀ in the cells. This confirmed that there were no bacterial cells, which are non-fluorescent, in this culture. In addition, the morphology of all of the cells was similar: spherical-shaped organisms. This culture did not grow with a mixture of substrates that would support growth of many rumen heterotrophs (RFgenV). Inoculated tubes were kept for a week to confirm there were no slow growing bacteria. Furthermore, the PCR performed with bacterial 16S rRNA gene primers did not produce any products, while DNA from *Escherichia coli*, used as a positive control, produced a product of the expected size (1465 bp). All the above observations confirmed that this culture was free from bacteria.

In a clone library constructed using archaeal 16S rRNA gene specific primers, all the clones had the same sequence. This suggested that there was only one type of methanogen present in this culture. A Blast search with this sequence revealed that ISO3-F5 was related to *Methanosphaera stadtmanae* strain MCB-3 (96% sequence identity).

7.3.2 Morphology

Phase contrast and fluorescent photomicrographs of ISO3-F5 are shown in Figure 6.3. Single cells were spherical in shape and cells mostly occurred in pairs or as clumped packets. The pairs were often dividing cells. There was no sign of spores. Motile cells were never observed by phase-contrast microscopy of living cultures, and whole cells observed by electron microscopy (EM) by negative staining did not possess flagella (Figure 7.1). The cells were 0.6 - 1.2 µm in diameter (mean 0.82 µm and standard deviation = 0.196 µm [$n = 30$]). No surface structures were visible by EM examination of negatively stained whole cells. The cells stained Gram positive.

Electron microscopy of thin sections revealed that the cells divided by formation of a cross wall (Figure 7.2). The cleavage furrows were clearly visible in dividing cells. The cell envelope appeared to consist of a thick single layer (12-22 nm; mean 17.4 nm

and standard deviation = 3.8 nm [$n = 35$]) and its surface was smooth. All the cells were surrounded by a clear layer. This layer is most probably a mucous layer.

The cytoplasm appeared to contain two different types of structures that are yet to be identified (csi and csii in Figure 7.2). The electron dense inclusion csii were usually, but not always, located near the cell wall (Figures 7.2). In contrast, the cell structure csi was found in different places within the cell (Figures 7.2). The cell structure csi was larger than the csii and it was irregular in shape, sometimes reaching about 50 nm in diameters. The enlargement of these two cell structures are shown in Figure 7.3.

7.3.3 RF, YE, CoM, Vit and VFA requirements

An experiment conducted to check the requirements for RF, YE, CoM, vitamins and VFA for growth of ISO3-F5 revealed that this isolate absolutely requires YE for growth (Table 7.1). Different combinations of the additives were added and the growth of ISO3-F5 was followed by measuring increase in OD at 600 nm each day. The culture tube with these additives but without an inoculum was used as a blank tube to correct the OD measurement due to these additives.

Although rumen fluid was not required for the growth of ISO3-F5, inclusion of RF slightly increased the growth yield (Figure 7.4). Addition of a VFA mixture increased the growth rate and yield of ISO3-F5 (Figure 7.5). In addition, this experiment showed that the growth was not affected by the inclusion of CoM and vitamins (Figures 7.6 and 7.7). Since ISO3-F5 absolutely needs YE for its growth, the requirement of vitamins for growth could not be determined in this experiment. Addition of YE could supply many vitamins, such as B-group vitamins, for growth.

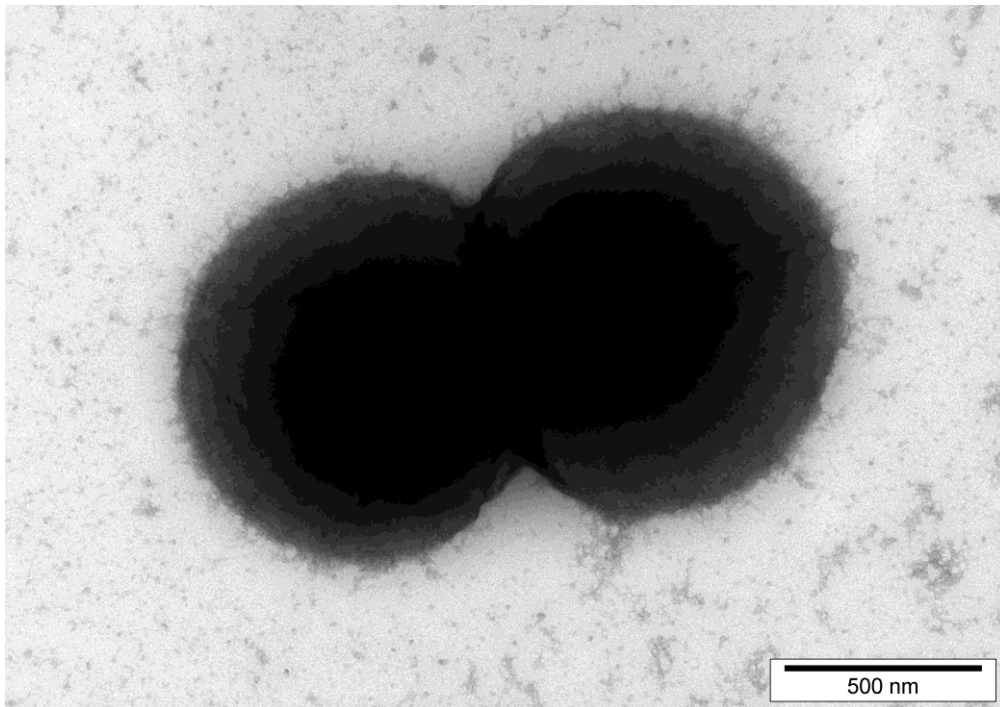
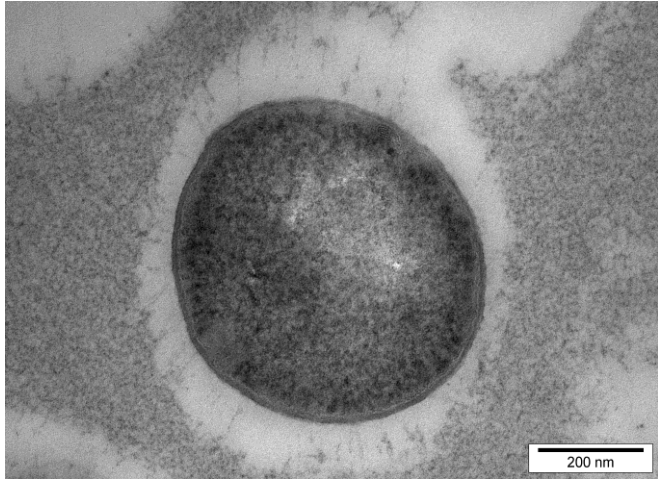


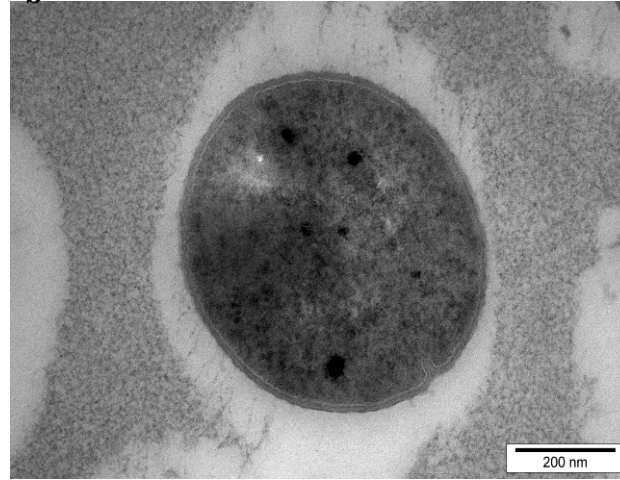
Figure 7.1. Electron photomicrograph of negatively-stained dividing cells of ISO3-F5.

Scale bar, 500 nm.

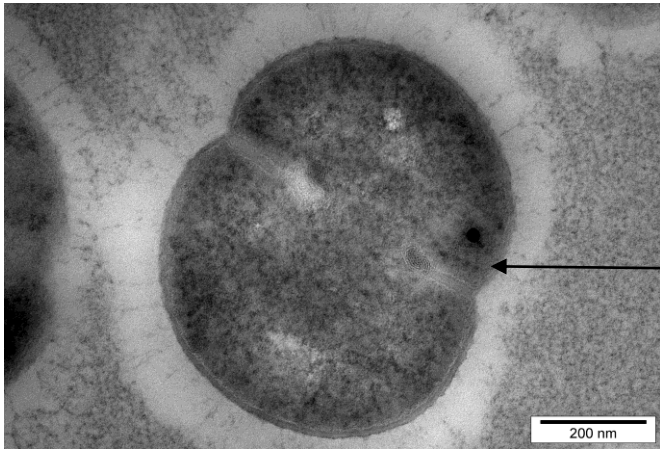
a



b

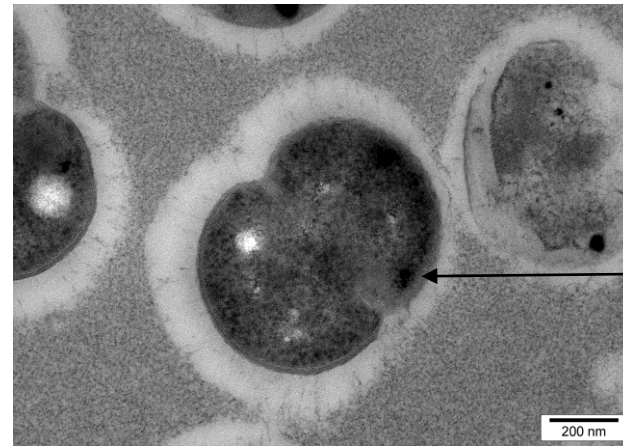


c



csi

d



csii

Figure 7.2 a-d. Electron microscopy of ISO3-F5. a. Thin section through a single cell. b. At the early stage of cell division when the cleavage furrows has formed and cross-wall formation is just beginning. c. Dividing cell with the cleavage furrow clearly visible, with the cross-wall forming and containing a cytoplasmic structure labelled csi. d. Dividing cell containing ctytoplasmic structure csii. Scale bar, 200 nm in all panels.

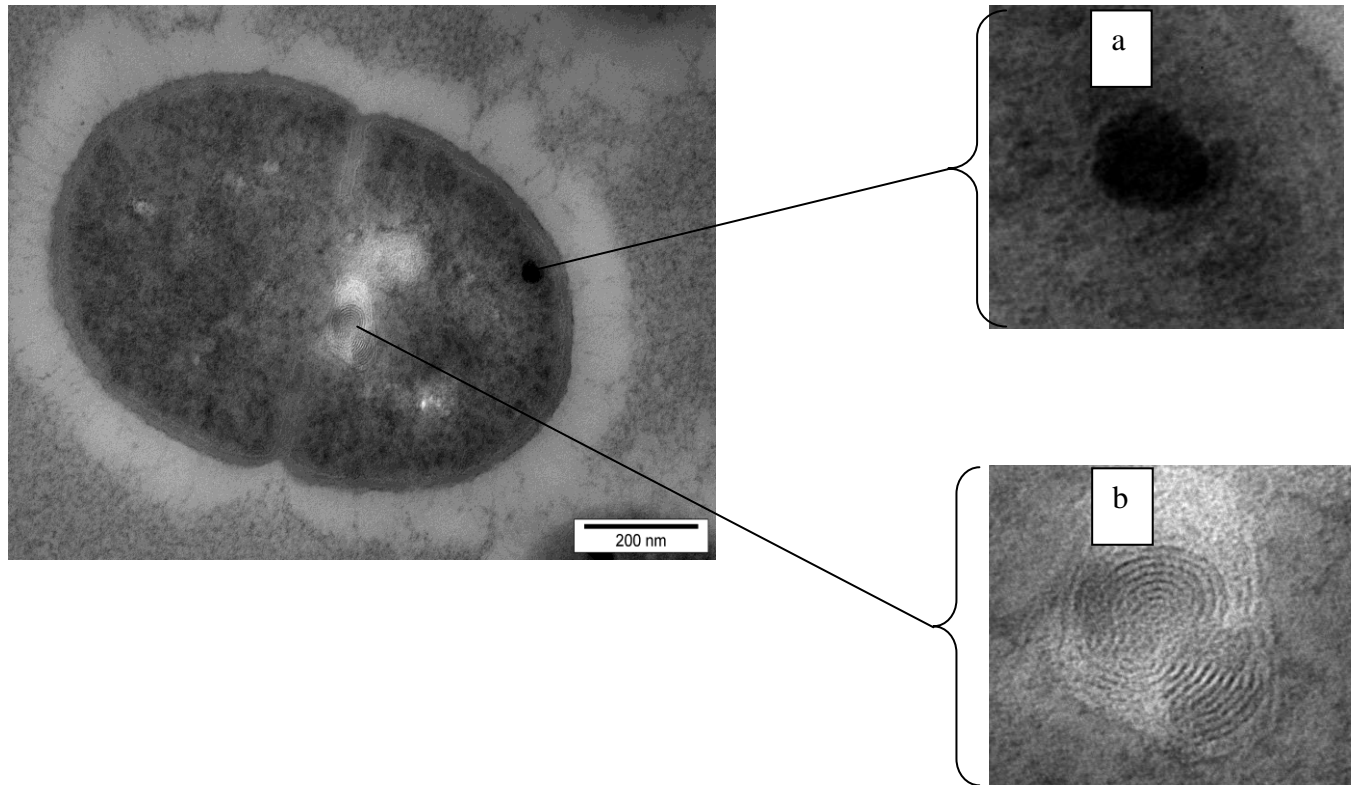


Figure 7.3. Enlargement of cytoplasmic structures csi and csii in a dividing cell of ISO3-F5. a. Cytoplasmic structure csii.
b. Cytoplasmic structure csi.

Table 7.1. The combinations of growth factors tested for their effects on growth of ISO3-F5. The growth factors tested were rumen fluid (RF), yeast extract (YE), coenzyme M (CoM), vitamin mixture (Vit) and volatile fatty acid mixture (VFA). Requirement of these growth factors were determined with 3 serial transfers to eliminate the carry over effect of the initial inoculums. Substrates added (Subs) were same in all the combinations: sodium acetate (20 mM), methanol (20 mM) and H₂/CO₂.

Additions	Growth		
	Transfer 1	Transfer 2	Transfer 3
RF + CoM + VFA + YE + Vit + Subs	+++	+++	+++
CoM + VFA + YE + Vit + Subs	+++	+++	+++
VFA + YE + Vit + Subs	+++	+++	+++
YE + Vit + Subs	++	++	++
Vit + Subs	+	-	-
CoM + VFA + Vit + Subs	+	-	-
RF + YE + Subs	+++	+++	+++
RF + VFA + Vit + Subs	+	-	-
RF + Vit + Subs	+	-	-
CoM + YE + Vit + Subs	++	++	++
YE + Subs	++	++	++

+++ very good growth, ++ good growth, + little growth and – no growth

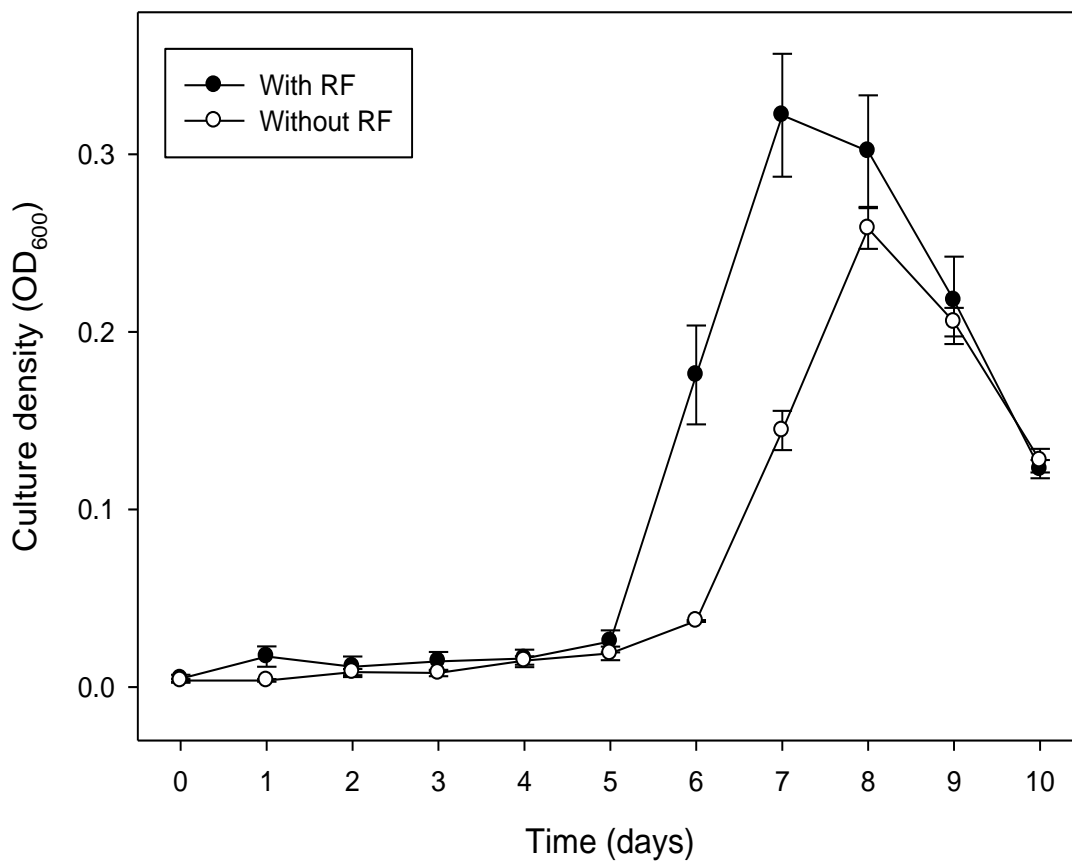


Figure 7.4. Growth curve of ISO3-F5 with and without 5% (v/v) rumen fluid in the 3rd serial transfer. RM02 medium contained yeast extract (1 g/l w/v) and substrate (20 mM sodium acetate, 20 mM methanol and H₂/CO₂) was used in this experiment. Each point is the mean of 4 replicates, and the vertical bars represent the standard error.

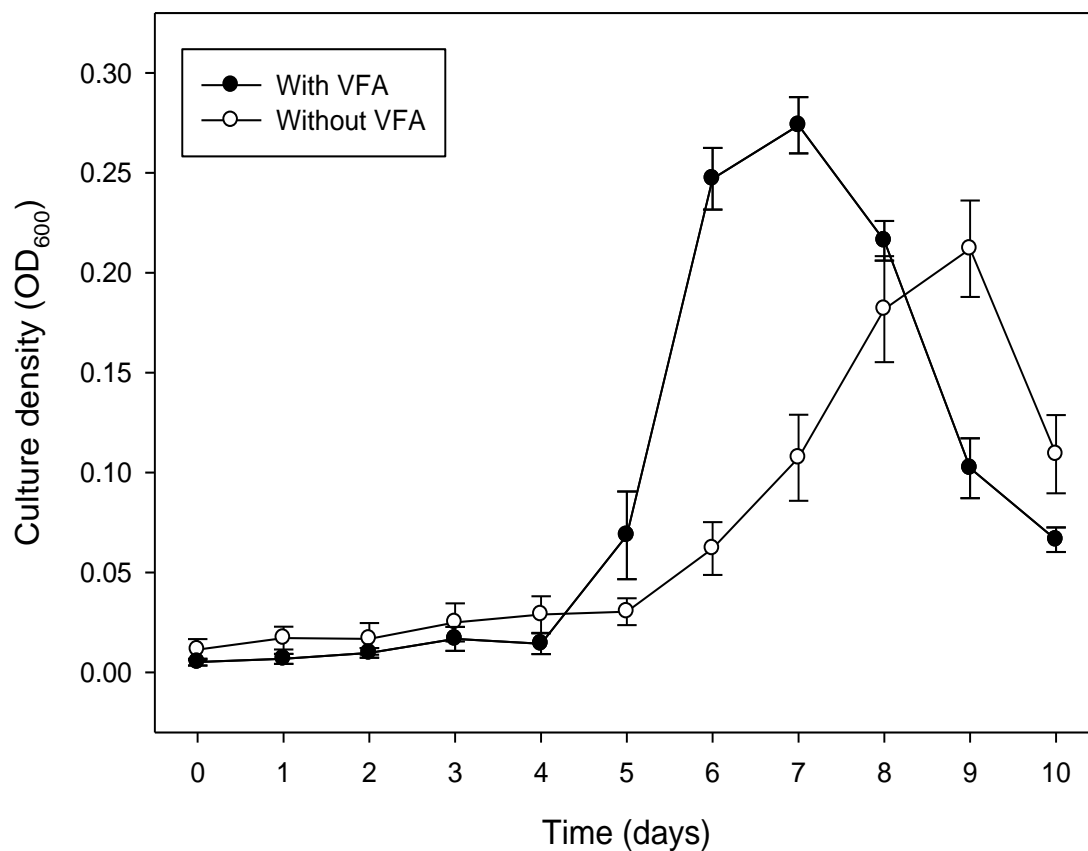


Figure 7.5. Growth curve of ISO3-F5 with and without volatile fatty acid mixture in the 3rd serial transfer. RM02 medium contained yeast extract (1 g/l w/v), vitamins and substrate (20 mM sodium acetate, 20 mM methanol and H₂/CO₂) was used in this experiment. Each point is the mean of 4 replicates, and the vertical bars represent the standard error.

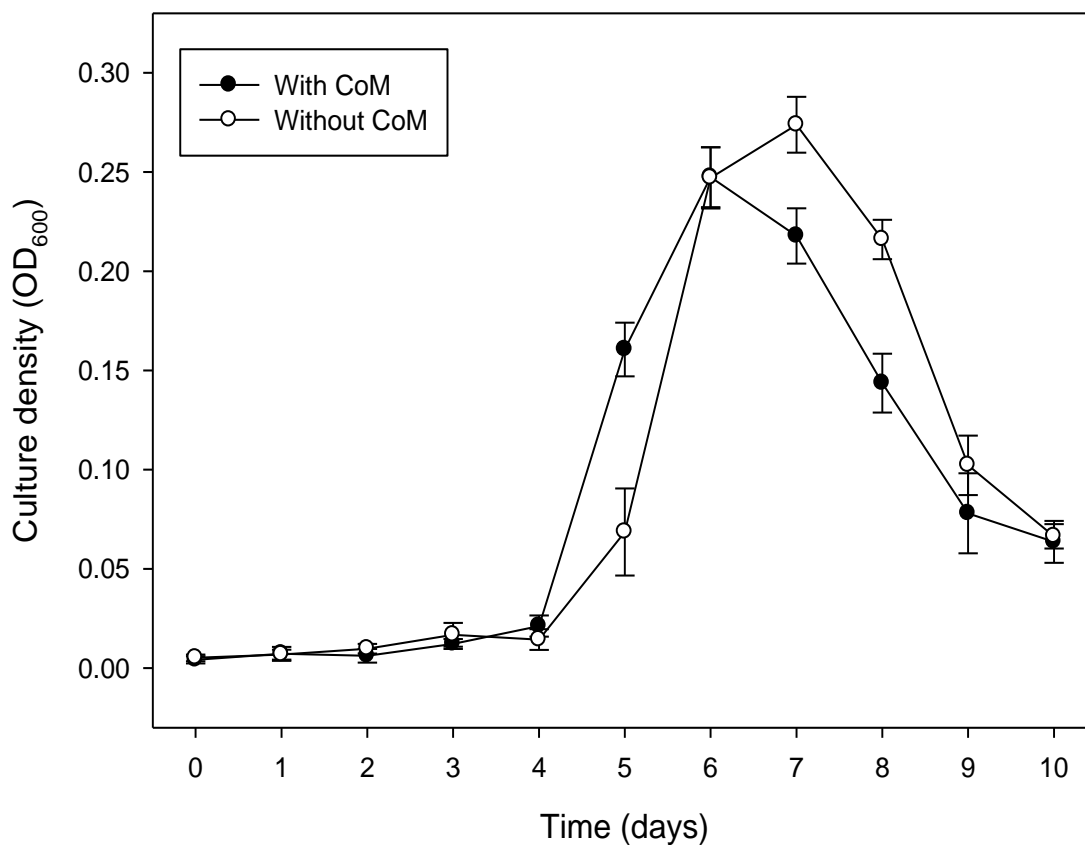


Figure 7.6. Growth curve of ISO3-F5 with and without coenzyme M (CoM) in the 3rd serial transfer. RM02 medium contained yeast extract (1 g/l w/v), vitamins (0.1 ml/10 ml medium), volatile fatty acids (0.1 ml/10 ml medium) and substrate (20 mM sodium acetate, 20 mM methanol and H₂/CO₂) was used in this experiment. Each point is the mean of 4 replicates, and the vertical bars represent the standard error.

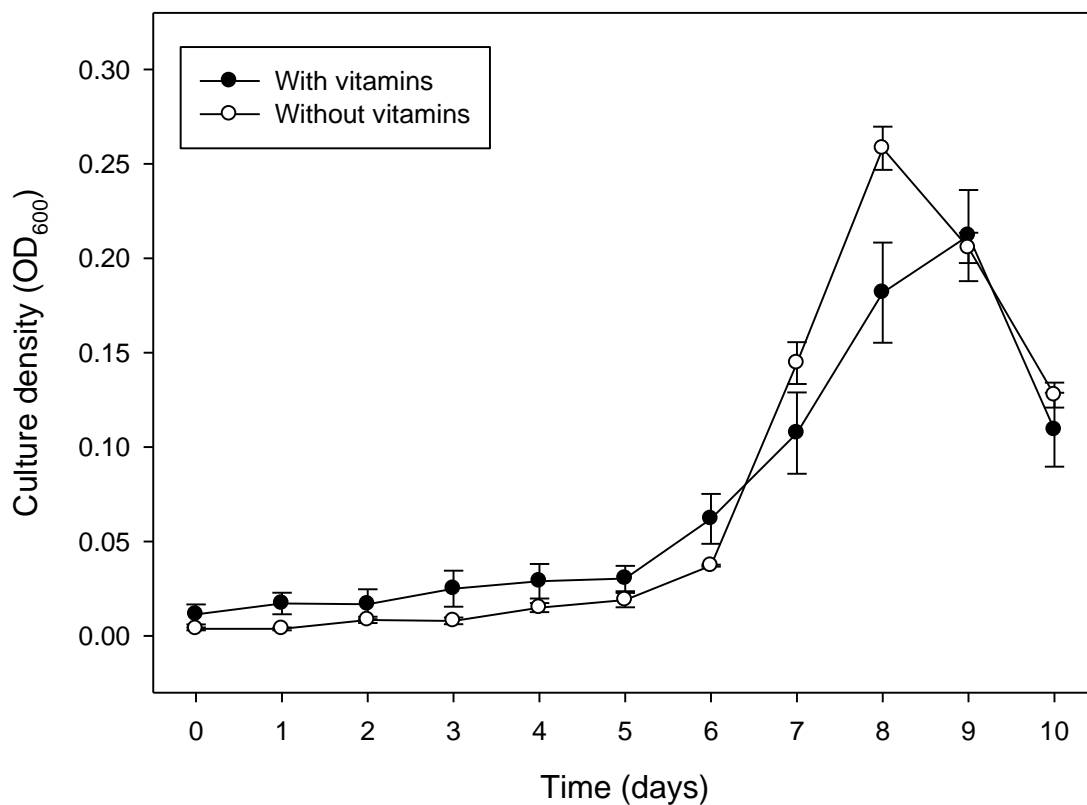


Figure 7.7. Growth curve of ISO3-F5 with and without vitamins in the 3rd serial transfer. RM02 medium contained yeast extract (1 g/l w/v) and substrate (20 mM sodium acetate, 20 mM methanol and H₂/CO₂) was used in this experiment. Each point is the mean of 4 replicates, and the vertical bars represent the standard error.

7.3.4 Substrate requirements

ISO3-F5 failed to grow without H₂/CO₂ (Table 7.2). Growth only occurred in the combination of substrates, methanol, acetate and H₂/CO₂ (Figure 7.8). No growth occurred with Na formate (20 mM), ethanol (20 mM), isopropanol (20 mM), and methylamine (20 mM) alone or in different combinations. Thus, ISO3-F5 absolutely requires H₂/CO₂, acetate and methanol for its growth. The small amount of growth in the absence of acetate and methanol in the initial transfer was probably due to the presence of small amounts of these substrates in the inoculums. Three serial transfers used in this experiment removed the carry-over effect of the inoculums.

7.3.5 Optimum pH

The optimum pH for growth of ISO3-F5 at 39°C was 6.7-6.8 (Figure 7.9). However, only the pH range of 6.3 to 6.9 was tested in this experiment, and so the range is not known.

7.3.6 Optimum temperature

No growth was observed at 30°C and 46°C. The temperature optimum for the growth of ISO3-F5 was 39°C to 42°C (Figure 7.10).

7.3.7 Cell lysis

Cells were resistant to lysis by sodium dodecyl sulphate (SDS) (0.5% and 2% w/v), lysozyme (0.1 mg/ml and 1 mg/ml) and proteinase K (0.1 mg/ml and 1 mg/ml).

7.3.8 G+C content

The mol% G + C in the genomic DNA of ISO3-F5 was 31.6 ± 0.28.

Table 7.2. Combinations of substrates tested for growth of ISO3-F5 and the results obtained. Growth with these substrates was determined for 3 serial transfers to eliminate the carry over effect of the initial inocula. RM02 medium with yeast extract (1 g/l) and vitamins was used in this experiment. The concentrations of the substrates were always 20 mM, except H₂/CO₂ (80:20), which was added at 200 kPa.

Additions	Growth		
	Transfer 1	Transfer 2	Transfer 3
H ₂ /CO ₂	+	-	-
H ₂ /CO ₂ + Na formate	+	-	-
H ₂ /CO ₂ + Na acetate	+	-	-
H ₂ /CO ₂ + Methanol	+	-	-
H ₂ /CO ₂ + Ethanol	+	-	-
H ₂ /CO ₂ + Isopropanol	+	-	-
H ₂ /CO ₂ + Methylamine	+	-	-
H ₂ /CO ₂ + Na formate + Na acetate	+	-	-
H ₂ /CO ₂ + Na formate + Methanol	+	-	-
H ₂ /CO ₂ + Na acetate + Methanol	+++	+++	+++
H ₂ /CO ₂ + Na acetate + Ethanol	+	-	-
H ₂ /CO ₂ + Na acetate + Isopropanol	+	-	-
H ₂ /CO ₂ + Na acetate + Methylamine	+	-	-
H ₂ /CO ₂ + Na formate + Na acetate + Methanol	+++	+++	+++

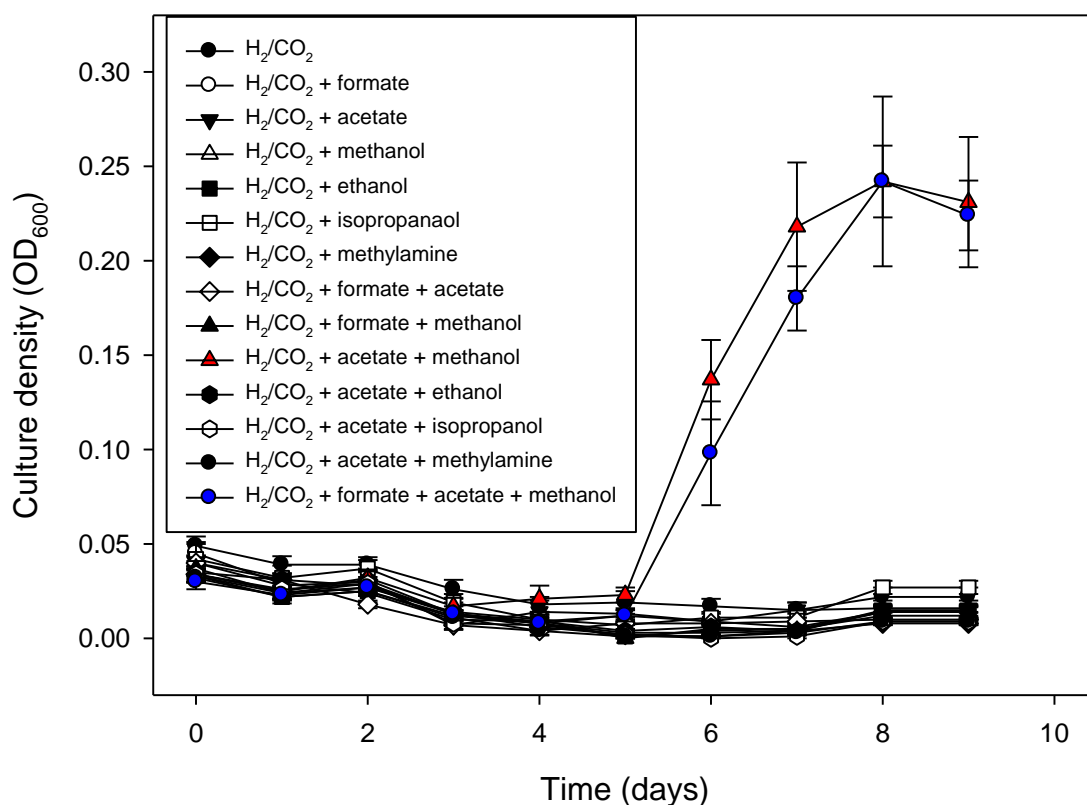


Figure 7.8. Growth curve of ISO3-F5 with different substrates. RM02 medium containing yeast extract (1 g/l w/v) and vitamin mixture was used in this experiment. The concentration of substrates was always 20 mM, except H₂/CO₂ (80:20), which was added at 200 kPa. Each point is the mean of 4 replicates, and the vertical bars represent the standard error.

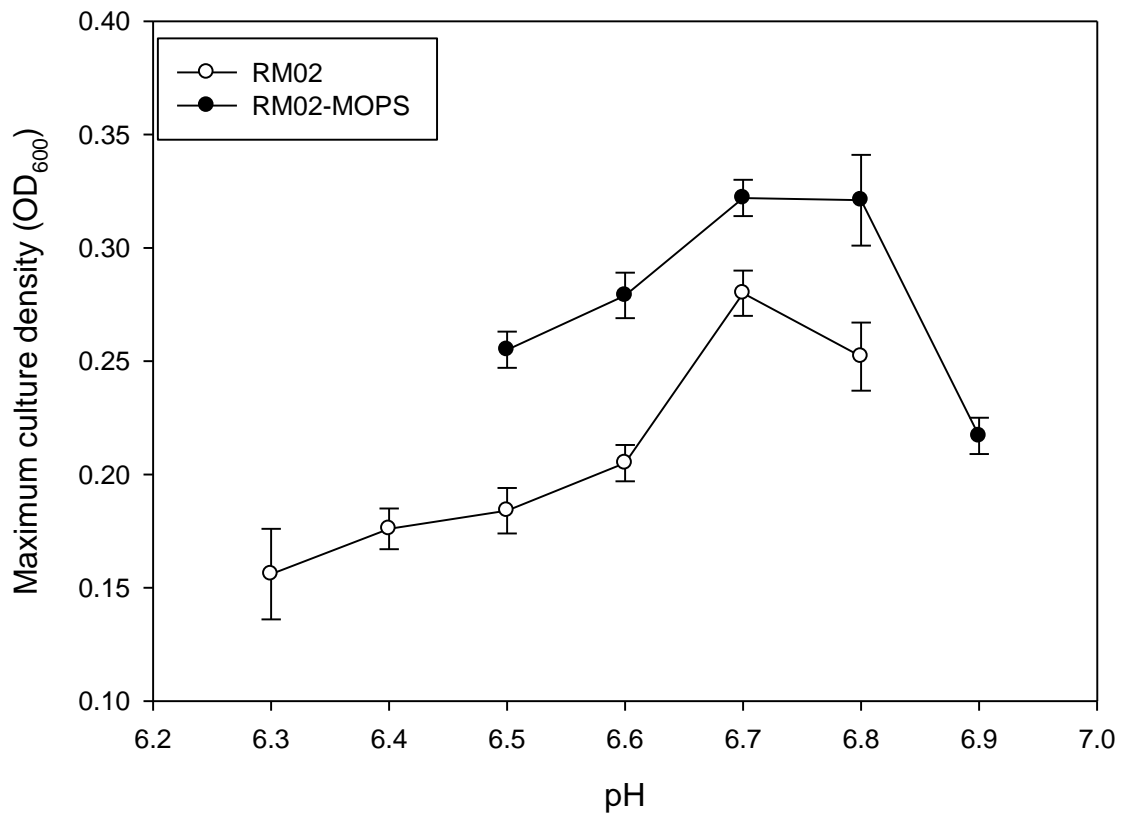


Figure 7.9. Effect of pH on maximum culture density. Experiments were performed using two different media. Each point is the mean of 4 replicates and the vertical bars represent the standard error.

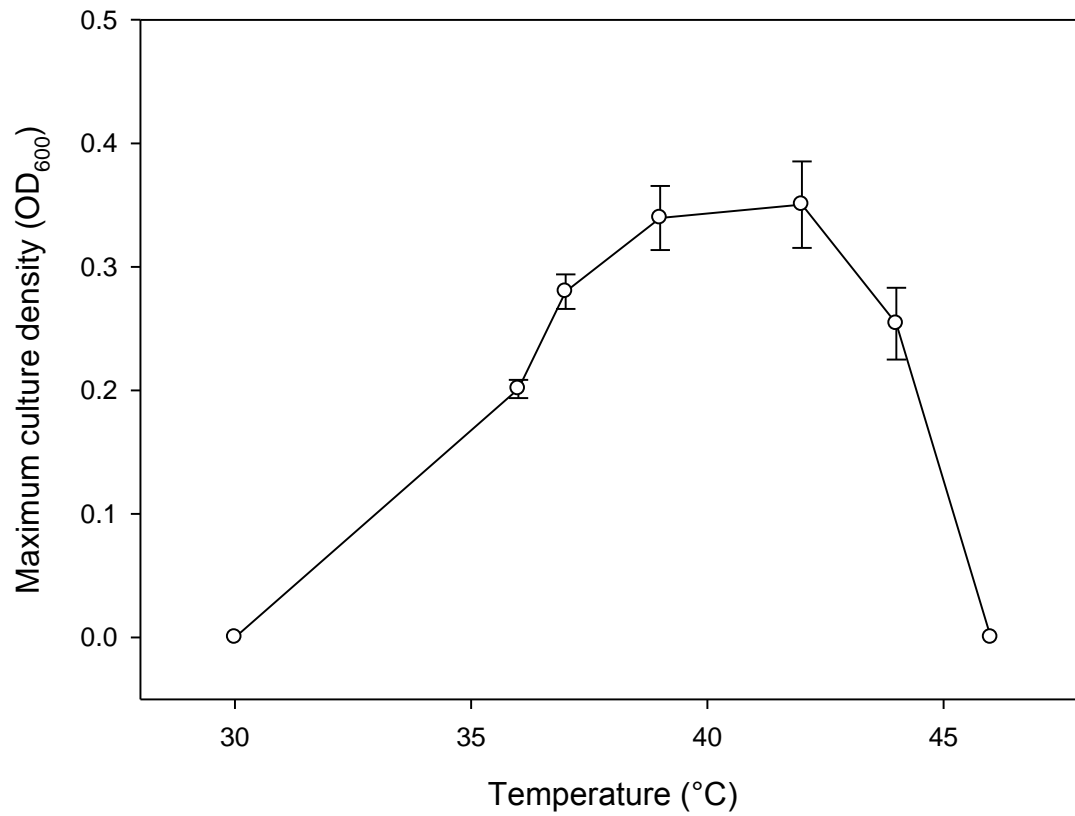


Figure 7.10. Effect of incubation temperature on maximum culture density. Each point is the mean of 4 replicates and the vertical bars represent the standard error.

7.3.9 Phylogenetic position

Phylogenetic trees were constructed using 16S rRNA gene and *mcrA* gene sequences of ISO3-F5 along with reference sequences downloaded from GenBank (Figure 7.11 and 7.12). Since no gene sequence information was available for *Methanosphaera cuniculi* strain IR7, these genes were sequenced and included in the phylogenetic trees.

The 16S rRNA gene sequence of ISO3-F5 clustered within the radiation of cultured *Methanosphaera* species (Figure 7.11). The sequence of ISO3-F5 was 96% identical to that of *Methanosphaera stadtmanae* and 95% identical to that of *Methanosphaera cuniculi*. The *Methanosphaera stadtmanae* and *Methanosphaera cuniculi* sequences were 95% identical to each other. In addition, several clones obtained from rumen samples clustered with ISO3-F5.

The *mcrA* gene sequence of ISO3-F5 clustered with *mrtA* gene sequences of *Methanosphaera stadtmanae* and the related clones. The phylogenetic analysis of these *mrtA* gene sequences showed that ISO3-F5 clustered away from *Methanosphaera stadtmanae* and *Methanosphaera cuniculi*. The *mcrA* sequence of ISO3-F5 was 88% identical to that of *mrtA* gene sequence of *Methanosphaera stadtmanae* and 86% identical to that of *Methanosphaera cuniculi*. The *Methanosphaera stadtmanae* and *Methanosphaera cuniculi* sequences were 90% identical to each other. Some clones obtained from the rumen closely clustered with ISO3-F5 (Figure 7.12).

The analysis of both 16S rRNA and *mcrA* gene sequences of ISO3-F5 suggested that they may be from a new species of *Methanosphaera* genus. However, more studies are still warranted to confirm this.

7.3.10 Long term preservation of ISO3-F5

Cultures preserved with DMSO or glycerol and stored at -80°C freezer were successfully revived after one month of storage. Thus, both these methods seem suitable for preservation of viable cultures of ISO3-F5.

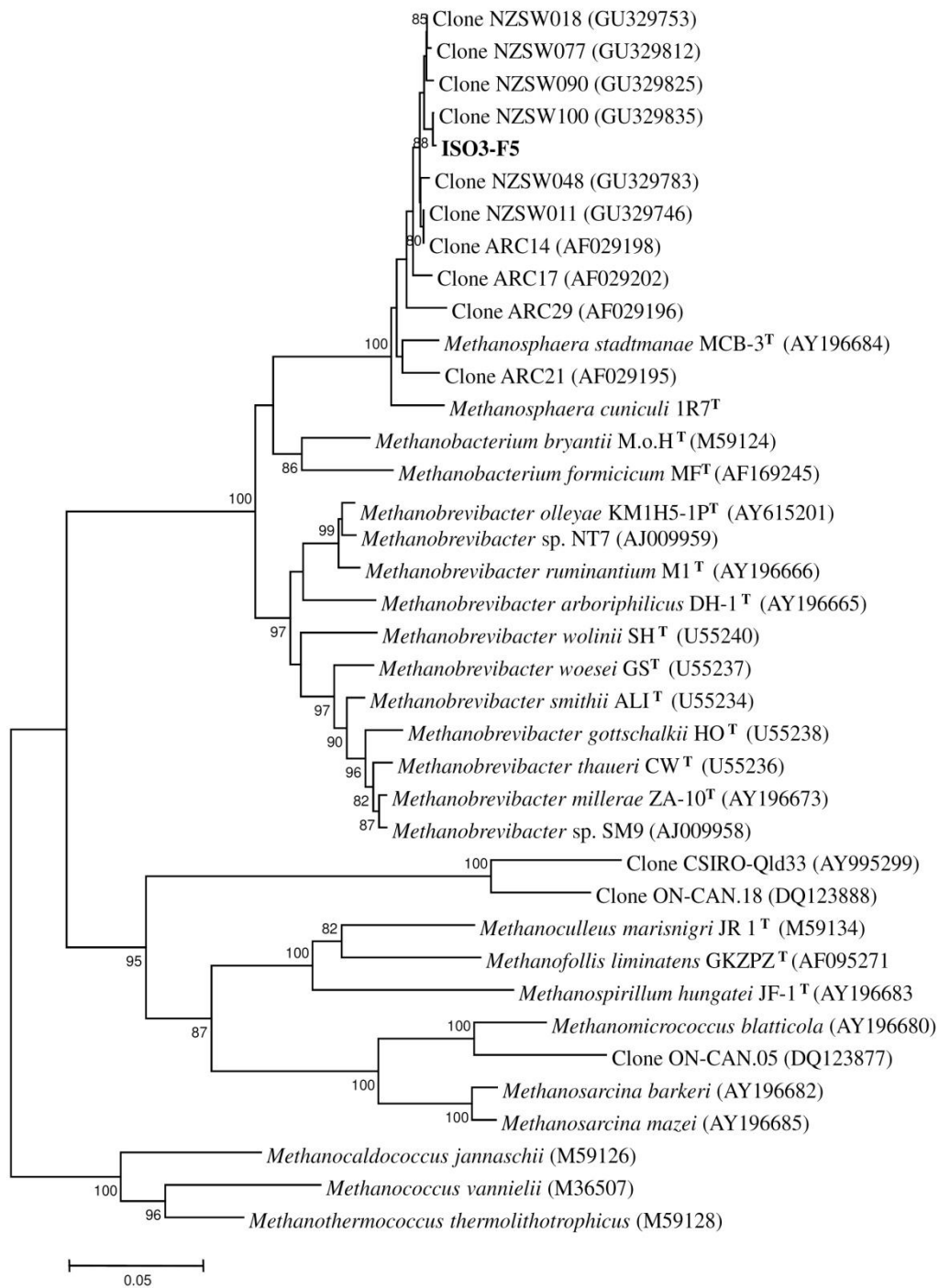


Figure 7.12. Phylogenetic relationships of isolate ISO3-F5 and reference 16S rRNA gene sequences. In the parentheses are the GenBank accession numbers for reference sequences. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates nucleotide substitutions per nucleotide position. The 16S rRNA genes of *Methanocaldococcus jannaschii* (GenBank accession M59126), *Methanococcus vannielii* (M36507) and *Methanothermococcus thermolithotrophicus* (M59128) were used as out-group sequences.

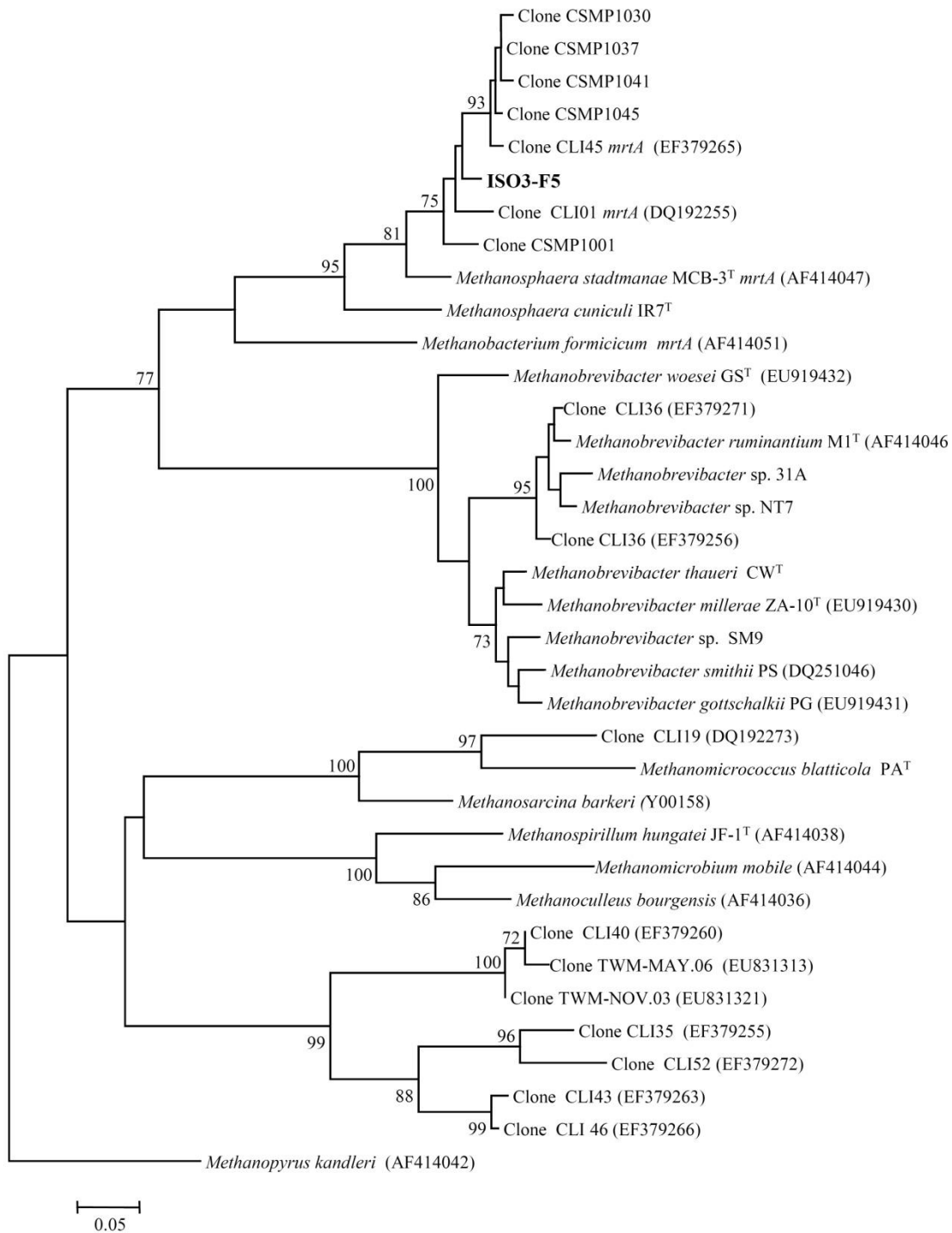


Figure 7.13 Phylogenetic relationships of isolate ISO3-F5 and reference *mcrA* gene sequences. In the parentheses are the GenBank accession numbers for reference sequences. Bootstrap values (>70%) are shown at selected nodes, and are percentages based on 1000 resamplings. The scale bar indicates nucleotide substitutions per nucleotide position. The tree was rooted with the sequence from *Methanopyrus kandleri*.

7.4 Discussion

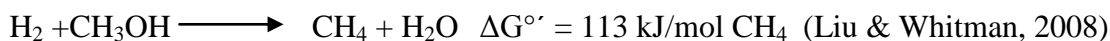
Methanogens of the genus *Methanosphaera* are common inhabitants in the rumen (Hook *et al.*, 2009; Pei *et al.*, 2008; Skillman *et al.*, 2006; Whitford *et al.*, 2001; Wright *et al.*, 2004b; Zhou *et al.*, 2009). In the study conducted in New Zealand ruminants (cows, sheep and red deer), *Methanosphaera* spp. were found to be one of the important methanogens that reside in these animals (Chapter 4). Furthermore, *Methanosphaera* spp. have been found in the intestinal tracts of other animals such as the foregut of the Tamar Wallaby (*Macropus eugenii*) (Evans *et al.*, 2009) and in the crop of the folivorous South American bird, the hoatzin (*Opisthocomus hoazin*) (Wright *et al.*, 2009). In the human gut, members of *Methanosphaera* spp. are one of the two methanogen species identified to date (Dridi *et al.*, 2010). In addition to being found in the intestinal tracts of animals, they have also been reported in a thermophilic municipal biogas plants which runs at elevated temperatures of 55°C-60°C (Weiss *et al.*, 2008). A study on bioaerosols in swine confinement buildings found that *Methanosphaera* spp. dominated (94.7%) the archaeal bioaerosols in the building (Nehme *et al.*, 2009).

Methanosphaera spp. belong to the family *Methanobacteriaceae* within the order *Methanobacteriales*. Significantly, the substrate requirements of *Methanosphaera* spp. have been found to be different from other members of this family. Studies performed with cultured *Methanosphaera* species found that they absolutely require H₂, CO₂, methanol and acetate for their growth (Biavati *et al.*, 1988; Miller & Wolin, 1985). In addition, among the members of family *Methanobacteriaceae*, only *Methanosphaera* species have a coccal morphology. *Methanosphaera* species contain pseudomurein in their cell walls (Biavati *et al.*, 1988; Konig, 1986), which is a characteristic of members of the family *Methanobacteriaceae*. The cell walls of members of other families of methanogens do not contain pseudomurein. Therefore, the genus *Methanosphaera* is included within the family *Methanobacteriaceae*. However, the amino acid composition of *Methanosphaera stadtmanae* and *Methanosphaera cuniculi* pseudomurein is distinguished from that of other members of the family by the presence of serine (Biavati *et al.*, 1988; Miller & Wolin, 1985).

The isolation and characterization of *Methanosphaera* isolate ISO3-F5 in this study is the first report of a cultured rumen strain of *Methanosphaera* species. The

morphology and substrate requirements of this isolate were similar to the previously isolated *Methanosphaera* species, *Methanosphaera stadtmanae* and *Methanosphaera cuniculi*. Phylogenetic analysis of 16S rRNA and *mcrA* genes suggested that this isolate may be a new species within the genus *Methanosphaera*. However, more studies are needed to confirm this. Whole genome sequence analysis of *Methanosphaera stadtmanae* revealed that it does not possess a typical *mcrA* gene. Instead, they have the *mrtA* isoform (Fricke *et al.*, 2006). The sequences obtained from the PCR amplification of DNA of ISO3-F5 with *mcrA* specific primers clustered with *mrtA* sequence of *Methanosphaera stadtmanae* and *Methanosphaera cuniculi*. Therefore, it can be assumed that all the members of genus *Methanosphaera* possess only the *mrtA* gene.

According to the studies conducted with isolated *Methanosphaera* species, these organisms use H₂ to reduce CH₃OH to CH₄ and H₂O. Therefore, they are obligately methylotrophic and hydrogenotrophic organisms (Biavati *et al.*, 1988; Fricke *et al.*, 2006; Miller & Wolin, 1985). The rumen isolate ISO3-F5 also possesses similar substrate requirements. Thus, it may also produce methane according to following equation.



Acetate and CO₂ are used as carbon sources for the growth of *Methanosphaera* species and CO₂ is not used for methane production. The only other known methanogen having the similar growth requirements is *Methanomicrococcus blatticola* isolated from the hindgut of the cockroach (Sprenger *et al.*, 2000). Like *Methanosphaera* species, they are also methylotrophic and hydrogenotrophic organisms. However, the metabolism of *Methanosphaera* spp. is restricted to methanol, while *Methanomicrococcus blatticola* can use both methanol and methylamine. While it appears that there is an obligate requirement for H₂, it is not clear if there is also a requirement for CO₂, which may be required for biosynthesis. This has not been determined.

The limited energy metabolism of *Methanosphaera* species is thought to be the reason for their relative scarcity in the human gut compared to *Methanobrevibacter smithii*. However, Dridi *et al.* (2010) argued that limitations in the experimental

protocols, such as sample size and DNA extraction methods used, may be the reason for this observation in many experiments. In the research conducted in 700 human fecal specimens with a modified DNA extraction method, they found that one third of these samples (29.4%) were positive for *Methanosphaera* spp. and that they highly varied in numbers among these specimens. The reason for this variation is not known yet. More research is needed to determine whether feeding habits have any influence on this observation. The methanol used by *Methanosphaera stadtmanae* in the human intestinal tract is a product of pectin degradation by *Bacteroides* spp. and other anaerobic bacteria (Dongowski *et al.*, 2000; Jensen & Canale-Parola, 1986). New Zealand ruminants are mainly fed with pastures compared to most of the other developed countries, which provides more pectin to be degraded to methanol. The high occurrence of *Methanosphaera* species in New Zealand ruminants (Chapter 4) may be due to this.

The isolate ISO3-F5 absolutely needs YE for growth. Interestingly, similar to isolate ISO3-F5, *Methanomicrococcus blatticola* is strictly dependent on the presence of yeast extract (Sprenger *et al.*, 2005). *Methanosphaera stadtmanae* is dependent on thiamine for growth and does not require yeast extract (Miller & Wolin, 1985). Rumen fluid was not required for growth of ISO3-F5, but it was stimulatory. Similarly, *Methanosphaera stadtmanae* also did not require rumen fluid for growth although, being a human isolate, that may not be surprising. The requirement of rumen fluid was not checked for the growth of *Methanosphaera cuniculi* as it was isolated from rabbit rectum. It was found that a volatile fatty acid mixture was stimulatory for the growth of ISO3-F5. *Methanosphaera stadtmanae* absolutely requires isoleucine, and some amino acids such as leucine were stimulatory. Stimulation of the growth of ISO3-F5 by amino acids was not checked in this study, but growth was possible in media containing no added amino acids.

Methanomicrococcus blatticola, which shows similar substrate requirements to those of *Methanosphaera* species, belongs to the order *Methanosarcinales*, and the other members of this order, the *Methanosarcina* species, can also utilize the methanol. However, they do not need H₂ to reduce the CH₃OH to CH₄. From the genome analysis of *Methanosphaera stadtmanae*, Fricke *et al.*, (2006) suggested that some of genes (e.g. mtaBC) may have been acquired by *Methanosphaera* spp. from members of the order

Methanosarcinales by lateral gene transfer. The similarity of some characteristics, especially with *Methanomicrococcus blatticola*, may be due to lateral gene transfer.

A comparison of the characteristics of ISO3-F5 with those of isolated *Methanosphaera* species is given in Table 7.3. Many of the characteristics (e.g. morphology, substrates required for growth, motility, and cell lysis) were common for all three isolates, which suggests that these are unifying characteristics of the genus *Methanosphaera*. Differences have been observed between *Methanosphaera stadtmanae* and *Methanosphaera cuniculi* (Biavati *et al.*, 1988; Miller & Wolin, 1985). For example, *Methanosphaera stadtmanae* immunologically cross-reacted with *Methanobacterium thermoautotrophicum*. In contrast, *Methanosphaera cuniculi* did not cross react with that species. Furthermore, there were differences in protein profiles between these two species. The G+C contents of the genomic DNA of *Methanosphaera stadtmanae* is 28% and of *Methanosphaera cuniculi* is 23%. In this study the G+ C content of genomic DNA of ISO3-F5 was found to be $31.6 \pm 0.28\%$. Whether these differences are related in differences in gene complements remains to be determined.

Genome sequence analysis of *Methanosphaera stadtmanae* revealed that it is adapted to the human large intestine habitat (Fricke *et al.*, 2006). This suggests that the rumen strain of *Methanosphaera* species may be adapted to the unique environment of the rumen and that may be a different species of *Methanosphaera*. Genome sequence analysis of ISO3-F5 will provide more details on this aspect and may reveal rumen-specific adaptations of this species in comparison to the human isolate. Sequencing of genome of ISO3-F5 is under way (Attwood *et al.*, 2010) and this will provide more information about this species. Also, the genome sequence of ISO3-F5 will be used to identify common targets for vaccine and small-molecule inhibitor development to target rumen methanogens.

7.5 Summary

Cells are spherical in shape, 0.6-1.2 μm in diameter, usually occurring in pairs or packed clumps. Gram positive organisms. Cells have a single thick cell wall (12-22 nm) and the cells divided by the formation of a cross wall. Flagella are absent. Optimum temperature is 36 to 42°C and optimum pH is 6.7 to 6.8. Energy for growth is obtained

by using methanol and H₂. Acetate and CO₂ are probably carbon sources. Methane is not produced from acetate, formate, ethanol, methylamine, isopropanol with or without H₂/CO₂. Yeast extract is absolutely required for growth. Coenzyme M is not required. Volatile fatty acids and rumen fluid enhance growth. Cells are resistant to lysis by SDS, lysozyme and proteinase K. The G+C content of genomic DNA is 31.6 ± 0.28 mol%. Isolated from the rumen of a sheep grazing on pasture.

7.6 Future work

Due to time constraints, a full characterization of ISO3-F5 was not completed. Further work is needed to confirm whether ISO3-F5 belongs to a new species or if it is a strain of an already isolated species. These include tests of antibiotic sensitivity, investigation of the amino acid composition of pseudomurein, analysis of protein and membrane lipid profile, and comparison of genomic DNA homology.

Table 7.3. Comparison of characteristics of ISO3-F5 with *Methanosphaera stadtmanae* and *Methanosphaera cuniculi*

Characteristics	Isolate		
	<i>Methanosphaera stadtmanae</i> MCB-3 ^T	<i>Methanosphaera cuniculi</i> IR7 ^T	ISO3-F5
Source of inoculum	Human faeces	Rectum of rabbit	Rumen of grazing sheep
Shape	Cocci. Usually occurs in pairs or tetrad or clusters	Cocci. Usually occurs in pairs or tetrads	Cocci. Usually occurs in pairs or clumped packets
Diameter	1.0-1.2 µm	0.6-1.2 µm	0.6-1.2 µm
Cell wall	Cell wall consisted of a single electron dense layer 8-20 nm thick	n.r.*	Cell wall consisted of a single electron dense layer. 12-22 nm thick
Gram stain	Gram positive	Gram positive	Gram positive
Optimum pH	6.5-6.9	6.8	6.7-6.8
Optimum temperature	n.r.*	35 - 40°C	36 - 42°C
G+C content	28 mol%	23 mol%	31.6 mol%
YE requirement	No	n.r.*	Yes
Substrates required**	Acetate, methanol, H ₂	Acetate, methanol, H ₂	Acetate, methanol, H ₂
Reference	Miller & Wolin 1983, 1985	Biavati <i>et al.</i> , 1988	This study

* n.r., not reported

** it is not known if CO₂ is required

Chapter 8

Summary and General discussion

Agriculture is New Zealand's largest industry and New Zealand's largest export earner. In 2009, animal products contributed 93% of New Zealand's total agricultural exports earnings, and 45% of total export earnings (Ministry of Agriculture and Forestry, 2010). These are mainly from pastoral agricultural production of meat, dairy products, and wool from ruminants. In addition to their economic importance, farmed ruminants also contribute significantly to New Zealand's greenhouse gas emission profile. Greenhouse gas emissions from the agricultural sector dominate New Zealand's national GHG emissions inventory, contributing 46.6% in 2008. Of that, 65.1% (i.e. 30.3% of the total) is methane (CH₄) emitted by ruminant farm animals (Ministry for the Environment, 2009). As a party to the United Nations Framework Convention on Climate Change, New Zealand has an obligation to reduce GHG emissions. However, it is generally accepted that reducing the number of ruminants being farmed is not the best option. This is because the worldwide demand for meat and milk is expected double by 2050 (FAO, 2008) and because New Zealand's economy relies on ruminant animal products. Therefore, research is being conducted to identify technologies to mitigate ruminant methane emissions without affecting ruminant production (Buddle *et al.*, 2011).

Ruminant production in New Zealand relies mainly on a pasture-based grazing system. Most of the techniques proposed to mitigate methane production from ruminants are more suitable for housed animal production (Jordan *et al.*, 2006; Lovett *et al.*, 2003). A practical and cost-effective mitigation technique is needed to reduce methane emissions from extensively-grazed ruminants in New Zealand. One attractive option is the manipulation of the rumen microbes such as methanogens (Wedlock *et al.*, 2010; Williams *et al.*, 2009; Wright *et al.*, 2004), acetogens (Lopez *et al.*, 1999; Nollet *et al.*, 1998) and protozoa (Hegarty, 1999; Morgavi *et al.*, 2008; Williams *et al.*, 2008). However, directly targeting rumen methanogens to reduce methane emissions is the most promising microbial manipulation method. The recent sequencing and analysis of the genome sequence of dominant rumen methanogen *Methanobrevibacter ruminantium* (Leahy *et al.*, 2010) has provided new opportunities to reduce methane

emissions from ruminants. The genome sequences of rumen methanogens help to identify the genes encoding key conserved enzymes that could be targets for small molecule inhibitors and genes encoding cell surface proteins that may be suitable as vaccine targets.

The research objectives of this thesis were formulated to contribute towards successful development of methane mitigation techniques for use in New Zealand ruminants. Significantly, as much of world ruminant animal production is also based on forage diets, this research also has potential implications for enhancing development of methane emission reduction techniques worldwide.

The first step for the development of successful methane mitigation strategy is to understand the methanogen diversity in New Zealand ruminants, which allows identification of the target methanogens for genome sequencing prior to vaccine or inhibitor development. Prior to this work, only a few studies had been performed to identify methanogen diversity in New Zealand ruminants (Nicholson *et al.*, 2007; Skillman *et al.*, 2006). Therefore, the first objective of this thesis was to study the methanogen community structure of farmed ruminants (cows, sheep and red deer) in New Zealand. The major feeding system in New Zealand is grazing pastures. In addition, many farmers feed their animals with silage during periods of feed shortage (e.g. the winter period). Therefore, rumen samples were collected from cows, sheep and red deer fed pasture during winter and summer and also from the animals fed silage during winter. For comparison purposes, rumen samples were also collected from a flock of sheep fed a concentrate-based diet, which is more common diet in North American and European countries than in New Zealand. Some of the sheep used to collect samples were from different flocks and also from different locations within New Zealand.

Molecular techniques were chosen to study the methanogen diversity in rumens of New Zealand ruminants. Many primers for amplification of marker genes from bacteria and archaea have been published for these molecular techniques. However, there were not many studies performed to check the validity of these primers to detect all rumen methanogens. Since it was not possible to test all of these primers for suitability to use in rumen samples, a few published primers were selected and a brief study was performed with these primers on a selected rumen sample. Based on this brief study,

primers were selected to use in this thesis for DGGE, real-time PCR and clone library construction. It was found that different primers gave very different methanogen community compositions, and the final selection was based the closeness of the results to the consensus obtained using all primers and data from literature reporting methanogen diversity in the rumen.

To compare the methanogen diversity in rumen samples, a validated DGGE method was used. DGGE analysis of archaeal PCR products obtained from different rumen samples showed only few differences among the archaeal populations. The dominant bands were mostly common to all the samples analyzed. The species representing the common dominant bands were from the *Methanobrevibacter ruminantium* clade, the *Methanobrevibacter gottschalkii* clade, and *Methanosphaera* spp. These three groups were identified as the dominant groups in all the samples analyzed. However, there have been some limitations reported for the DGGE technique (Kowalchuk *et al.*, 1997; Muyzer *et al.*, 1993; Muyzer & Smalla, 1998; Nubel *et al.*, 1996). The results from the DGGE experiments were validated using two other molecular techniques; clone library construction and real-time PCR.

Two different primer pairs (915af/1386r and 109f/915r) were chosen for the clone library construction in an effort to overcome potential primer bias. One of the primer pairs (915af/1386r) was the same one used for the DGGE. The methanogen groups identified by these two clone libraries were largely similar and the three groups which were identified as the common dominant methanogens by the DGGE technique were also the dominant groups in the clone libraries. However, the RCC group was also identified as the dominant group in this rumen sample in these two clone libraries. Interestingly, the RCC group was not, however, represented by a dominant band in DGGE analysis. The sequences of the RCC group in the clone libraries showed high variation among themselves and they clustered into different subgroups in the phylogenetic analysis. Therefore, there may be multiple different low intensity bands which represent the RCC group in archaeal DGGE gels.

To investigate the diversity of the RCC group in the rumen samples, DGGE was performed with the PCR products obtained using RCC-specific primers (762f/1099r). The high variation of sequences within the RCC group was confirmed in this analysis. Bands were observed on all positions throughout the gel and the number of bands was

also higher than observed in the archaeal DGGE. Variation in banding patterns among individual animals was also high compared to total archaeal DGGE. Most interestingly, some species-specific bands were observed in the cows, sheep and red deer fed with pasture during summer. However, only one comparison with one type of feed was performed to check RCC diversity in this study, and thus more studies are needed on the RCC group. For further confirmation of the presence and relative abundance of the RCC group in these rumen samples, a real-time PCR was developed and performed on the rumen samples collected from sheep fed different diets using archaeal-specific, RCC-specific and bacterial-specific primers. The results of the real-time PCR confirmed the presence of RCC group at a dominant level (average 26.5%) in the selected rumen samples.

Of the dominant methanogen groups identified in New Zealand ruminants, representatives of *Methanobrevibacter ruminantium* and *Methanobrevibacter gottschalkii* clades have been isolated from the rumen (Rea *et al.*, 2007; Smith & Hungate, 1958). One of these isolates, *Methanobrevibacter ruminantium*, has been studied in detail, including genome sequencing (Leahy *et al.*, 2010). Less is known about rumen *Methanosphaera* species and members of the RCC group. Thus, my next objectives were to study and isolate members of RCC and *Methanosphaera* species, which could then be used to enhance development of an anti-methanogen vaccines or small molecule inhibitors.

Members belonging to the RCC group have been found in animal and human intestinal guts (Evans *et al.*, 2009; Mihajlovski *et al.*, 2008; Sundset *et al.*, 2009a; 2009b; Wright *et al.*, 2006; 2007), a swine waste storage pit (Whitehead & Cotta, 1999), biogas reactor systems (Klocke *et al.*, 2008) and leachate from landfill (Huang *et al.*, 2003). However, the role of this group in these environments is not yet clear. Previous researchers have amplified the RCC sequences using archaeal primers. Even though all of the archaea identified thus far in the rumen are methanogens, there was no proof yet to conclusively demonstrate that RCC are methanogens. There have been some studies that have suggested that RCC may possess the *mcrA* gene (Denman *et al.*, 2007; Evans *et al.*, 2009), but supporting experimental evidence has not been described yet. In the past, studies have shown that a low concentration of chloroform (CHCl₃) selectively inhibits the methanogens without affecting other microbial activities (Acht nich *et al.*,

1995; Chidthaisong & Conrad, 2000; Chin & Conrad, 1995; De Graaf *et al.*, 1996). Therefore, samples collected from CHCl_3 -treated and control cows were used to study the physiology of the RCC group. Upon the CHCl_3 treatment, archaea that are methanogens should be susceptible to CHCl_3 and be eliminated from the rumen, while non-methanogenic archaea may be resistant. If RCC are methanogens, they should have been affected by CHCl_3 treatment.

Measurement of CH_4 production before and after CHCl_3 treatment clearly showed that methanogenesis had been affected after CHCl_3 . This was expected, as a low concentration of CHCl_3 is known to inhibit cobamide-dependent methyl-transfer reactions (Wood *et al.*, 1968; Kenealy & Zeikus, 1981) that normally lead to the formation of methane. CHCl_3 also inhibits the methyl coenzyme M reductase, an enzyme diagnostic of methanogens that catalyzes the formation of CH_4 from methyl-coenzyme M (Gunsalus & Wolfe, 1978). However, a small amount of residual methane production was still observed immediately after the CHCl_3 treatment. Therefore, there may have been some methanogens still present in these animals after the CHCl_3 treatment. Real-time PCR analysis showed that there were some archaea still present in these animals. Real-time PCR quantification with RCC-specific primers suggested that the archaea that remained in the treated cows may have been from the RCC group. The RCC group that remained in the rumen may have been responsible for the residual methane production. However, the number of RCC in the treated rumen decreased, suggesting they are sensitive to CHCl_3 , or dependent on CHCl_3 -sensitive organisms.

Archaeal DGGE analysis of rumen samples from CHCl_3 -treated and control cows before and after treatment clearly showed that CHCl_3 treatment affected the methanogens. All the dominant bands disappeared after the CHCl_3 treatment in CHCl_3 -treated cows. DGGE analysis was also performed on the same sample using PCR products obtained with RCC-specific primers. Interestingly, some of these bands disappeared after CHCl_3 treatment, while others were not affected by the CHCl_3 treatment. The control cows showed no difference before and after the CHCl_3 treatment. This showed that some sub-groups of RCC were affected by CHCl_3 , but some sub-groups were more resistant. Thus, the RCC group may be methanogens and some of them may not be affected by the CHCl_3 at the concentration used in the study. These

resistant RCC may have been responsible for the residual CH₄ production after CHCl₃ treatment. This suggests that the RCC group is not homogeneous.

To determine whether the RCC group possesses an *mcrA* gene, clone libraries were constructed using archaeal-specific primers and primers targeting *mcrA*. Only methanogens possess the *mcrA* gene, with the exception of a small subgroup of methane-oxidizing archaea (Hallam *et al.*, 2003). Clone libraries (16S rRNA and *mcrA* genes) constructed using rumen samples collected from CHCl₃-treated and control cow clearly showed that RCC possess a *mcrA* gene. Taken all together, these data suggest that at least some members of RCC are methanogens, and that probably all are. The high diversity among sequences within RCC group suggests that there may be different genera or species in this group. Isolating members from this group is very important, as this group needs to be included when developing methane reduction strategies.

A set of cultivation experiments was set up to isolate previously uncultured rumen methanogens. After several attempts, a pure culture of rumen *Methanosphaera* species was isolated from a grazing sheep. In addition, three enrichment cultures of RCC and an enrichment culture of a member of the *Methanobrevibacter gottschalkii* clade were obtained. There has not yet been any report of the isolation of a pure culture of *Methanosphaera* from the rumen thus far. Initial characterization of this *Methanosphaera* isolate suggests that it may belong to a separate species. Genome sequencing of this isolate is currently under way (Attwood *et al.*, 2010). The isolation of this rumen strain of *Methanosphaera* has provided the opportunity to learn more about this group, particularly in relation to the dominance of this species in New Zealand grazing ruminants. Also, genome sequencing of this isolate will help to identify common targets for methane reduction strategies, both vaccine and small molecule inhibitors.

Three enrichment cultures of RCC were obtained. Two of these enrichments contained only one common bacterial species. Efforts are still under way to purify these isolates. The enrichment cultures also provided some of the important characteristics of this group. Significantly, it has been shown that they do possess a *mcrA* gene. Most importantly, they produce methane. Thus, this group has to be targeted in methane reduction studies. Interestingly, one obvious characteristic of these isolates is that they do not fluoresce like normal methanogens. This suggests that their biochemistry may be

different to that of other methanogens, which could have implications for methane mitigation research. Additional studies are needed on this group once the pure cultures are obtained.

All the findings of this study will be useful for enhancing development of strategies for reducing methane emissions from New Zealand ruminants. Investigation of methanogen diversity in farmed ruminants fed different diets helped to identify dominant methanogen groups in these animals. Identifying some of the physiological characteristics of RCC group offers new possibilities to study this group further. Purification of RCC isolates from enrichment cultures will provide more information of this group. The pure culture of a rumen *Methanosphaera* species is now available to study its physiology, ecology and genome, to better understand this group in the rumen.

Massively parallel next generation sequencing is a recent development that allows the study of microbial diversity in an environment more accurately (Hugenholtz & Tyson, 2008; Nyren, 2007). When DGGE analysis was performed to study the methanogen diversity in NZ ruminants in this study, pyrosequencing was new and quite expensive. It was also thought at the time that the limited diversity of methanogens could be distinguished using DGGE. The DGGE technique can only pick the very obvious differences among samples, while massively parallel next generation sequencing technique can pick up very small differences. It is not expected that the general findings of this study would be changed by application of newer techniques. The dominant archaea would be the same, and the general conclusion that the same major groups dominate is expected to hold. However, more subtle difference of ecological interest may be uncovered.

In this study, only the dominant methanogen groups were focused on. However, the minor groups of rumen methanogens cannot be neglected, as once the dominant methanogens eliminated from the rumen these minor groups may fill the niches created by the dominant groups. In the future, minor groups of rumen methanogens such as *Methanomicrococcus* spp., *Methanobacterium* spp., *Methanomicrobium* spp., *Methanoculleus* spp. and *Methanosarcina* spp. have to be further studied. However, it seems that at least the dominant rumen methanogens in New Zealand ruminants are the same as those found in the other parts of the world. The findings of this thesis will be useful in the effort to reduce ruminant methane emissions worldwide.

References

- Achenbach L & Woese C (1995) 16S and 23S rRNA-like primers. In *Archaea: a laboratory manual* (Robb FT, Sowers KR, DasSarma S, Place AR, Schreier HJ & Fleischmann EM, eds), pp. 521-523. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY.
- Achtnich C, Bak F & Conrad R (1995) Competition for electron donors among nitrate reducers, ferric iron reducers, sulfate reducers and methanogens in anoxic paddy soil. *Biol Fert Soils* **18**: 65-72.
- Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403-410.
- Amann RI, Binder BJ, Olsen RJ, Chisholm SW, Devereux R & Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919-1925.
- Amann RI & Ludwig W (2000) Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. *FEMS Microbiol Rev* **24**: 555-565.
- Amann RI, Ludwig W and Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143-169.
- Animut G, Puchala R, Goetsch AL, Patra AK, Sahlu T, Varel VH & Wells J (2008) Methane emission by goats consuming different sources of condensed tannins. *Anim Feed Sci Technol* **144**: 228-241.
- Arahal DR, Dewhirst FE, Paster BE, Volcani BE & Ventosa A (1996) Phylogenetic analyses of some extremely halophilic archaea isolated from Dead Sea water, determined on the basis of their 16S rRNA sequences. *Appl Environ Microbiol* **62**: 3779-3786.
- Asanuma N & Hino T (2000) Activity and properties of fumarate reductase in ruminal bacteria. *J Gen Appl Microbiol* **46**: 119-125.

- Asanuma N, Iwamoto M & Hino T (1999) Effect of the addition of fumarate on methane production by ruminal microorganisms *in vitro*. *J Dairy Sci* **82**: 780-787.
- Ashton WT, Brown RD, Jacobson FS & Walsh CT (1979). Synthesis of 7, 8-didemethyl-8-hydroxy-5-deazariboflavin and confirmation of its identity with the deazaisoalloxazine chromophore of *Methanobacterium* redox coenzyme F₄₂₀. *J Am Chem Soc* **101**: 4419-4420.
- Attwood GT, Altermann E, Kelly WJ, Leahy SC, Zhang L & Morrison M (in press) Exploring rumen methanogen genomes to identify targets for methane mitigation strategies. *Anim Feed Sci Technol*, in press.
- Baker SK (1999) Rumen methanogens, and inhibition of methanogenesis. *Aust J Agric Res* **50**: 1293-1298.
- Balch WE & Wolfe RS (1976) New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Appl Environ Microbiol* **32**: 781-791.
- Banning N, Brock F, Fry JC, Parkes RJ, Hornibrook ER & Weightman AJ (2005) Investigation of the methanogen population structure and activity in a brackish lake sediment. *Environ Microbiol* **7**:947-960.
- Bauchop T (1967) Inhibition of rumen methanogenesis by methane analogues. *J Bacteriol* **94**:171-175.
- Bauchop T & Mountfort DO (1981) Cellulose Fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. *Appl Environ Microbiol* **42**: 1103-1110.
- Beauchemin KA, Kreuzer M, O'Mara F & McAllister TA (2008) Nutritional management for enteric methane abatement: a review. *Aust J Exp Agric* **48**: 21-27.

- Beijer WH (1952) Methane fermentation in the rumen of cattle. *Nature* **170**: 576-577.
- Bergmann I, Mundt K, Sontag M, Baumstark I, Nettmann E & Klocke M (2010) Influence of DNA isolation on Q-PCR-based quantification of methanogenic Archaea in biogas fermenters. *Syst Appl Microbiol* **33**: 78-84.
- Biavati B, Vasta M & Ferry JG (1988) Isolation and characterization of "*Methanosphaera cuniculi*" sp. nov. *Appl Environ Microbiol* **54**: 768-771.
- Bird SH, Hegarty RS & Woodgate R (2008) Persistence of defaunation effects on digestion and methane production in ewes. *Aust J Exp Agric* **48**: 152-155.
- Bodas R, López S, Fernández M, Garcia-González R, Rodriguez AB, Wallace RJ & González JS (2008). *In vitro* screening of the potential of numerous plant species as antimethanogenic feed additives for ruminants. *Anim Feed Sci Technol* **145**: 245-258.
- Bollmann A, Lewis K & Epstein SS (2007) Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates. *Appl Environ Microbiol* **73**: 6386-6390.
- Božić AK, Anderson RC, Carstens GE, Ricke SC, Callaway TR, Yokoyama MT, Wang JK & Nisbet DJ (2009) Effects of the methane-inhibitors nitrate, nitroethane, lauric acid, Lauricidin[®] and the Hawaiian marine algae *Chaetoceros* on ruminal fermentation *in vitro*. *Bioresource Technol* **100**: 4017-4025.
- Brochier-Armanet C, Boussau B, Gribaldo S & Forterre P (2008) Mesophilic *Crenarchaeota* : proposal for a third archaeal phylum, the *Thaumarchaeota*. *Nat Rev Microbiol* **6**: 245-252.
- Brosius J, Palmer ML, Kennedy PJ & Noller HF (1978) Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* **75**: 4801-4805.

- Brulc JM, Antonopoulos DA, Miller MEB, Wilson MK, Yannarell AC, Dinsdale EA, Edwards RE, Frank ED, Emerson JB, Wacklin P, Coutinho PM, Henrissat B, Nelson KE & White BA (2009) Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. *Proc Natl Acad Sci, USA*. **106**: 1948-1953.
- Bryant MP, Wolin EA, Wolin MJ & Wolfe RS (1967) *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. *Arch Microbiol* **59**:20–31
- Buddle BM, Denis M, Attwood GT, Altermann E, Janssen PH, Ronimus RS, Pinares-Patiño CS, Muetzel S & Wedlock DN (2011) Strategies to reduce methane emissions from farmed ruminants grazing on pasture. *Vet J* **188**: 11-17.
- Busquet M, Calsamiglia S, Ferret A, Carro MD & Kamel C (2005) Effect of garlic oil and four of its compounds on rumen microbial fermentation. *J Dairy Sci* **88**: 4393-4404.
- Callaway TR, Carneiro De Melo AMS & Russell JB (1997). The effect of nisin and monensin on ruminal fermentations *in vitro*. *Curr Microbiol* **35**: 90-96.
- Casamayor EO, Massana R, Benlloch S, Øvreås L., Diez B, Goddard V, Gasol JM, Joint I, Rodríguez-Valera F & Pedrós-Alió C (2002) Changes in archaeal, bacterial and eukaryal assemblages along a salinity gradient by comparison of genetic fingerprinting methods in a multi-pond solar saltern. *Environ Microbiol* **4**: 338-348.
- Chaban B, Ng SYM & Jarrell KF (2006) Archaeal habitats - From the extreme to the ordinary. *Can J Microbiol* **52**: 73-116.
- Chagan I, Tokura M, Jouany JP & Ushida K (1999) Detection of methanogenic archaea associated with rumen ciliate protozoa. *J Gen Appl Microbiol* **45**: 305-308.
- Chagunda MGG, Römer DAM & Roberts DJ (2009) Effect of genotype and feeding regime on enteric methane, non-milk nitrogen and performance of dairy cows during the winter feeding period. *Livest Sci* **122**: 323-332.

- Chaucheyras F, Fonty G, Bertin G & Gouet P (1995) *In vitro* H₂ utilization by a ruminal acetogenic bacterium cultivated alone or in association with an archaea methanogen is stimulated by a probiotic strain of *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **61**: 3466-3467.
- Chaudhary PP & Sirohi SK (2009) Dominance of *Methanomicrobium* phylotype in methanogen population present in Murrah buffaloes (*Bubalus bubalis*). *Lett Appl Microbiol* **49**: 274-277.
- Cheng YF, Mao SY, Liu JX & Zhu WY (2009) Molecular diversity analysis of rumen methanogenic archaea from goat in Eastern China by DGGE methods using different primer pairs. *Lett Appl Microbiol* **48**: 585-592.
- Chidthaisong A & Conrad R (2000) Specificity of chloroform, 2-bromoethanesulfonate and fluoroacetate to inhibit methanogenesis and other anaerobic processes in anoxic rice field soil. *Soil Biol Biochem* **32**: 977-988.
- Chin KJ & Conrad R (1995) Intermediary metabolism in methanogenic paddy soil and the influence of temperature. *FEMS Microbiol Ecol* **18**:85-102.
- Clark H, Pinares-Patiño CS & de Klein C (2005) Methane and nitrous oxide emissions from grazed grasslands. In *Grassland: A global resource* (McGilloway DA, eds), pp. 279-293. Wageningen Academic Publishers, Netherlands.
- Clemens J & Ahlgrimm (2001) Greenhouse gases from animal husbandry: mitigation options. *Nutr cycl Agroecosys* **60**: 287-300.
- Conrad R, Erkel C & Liesack W (2006) Rice Cluster I methanogens, an important group of Archaea producing greenhouse gas in soil. *Curr Opin Biotechnol* **17**: 262-267.
- Connon SA & Giovannoni SJ (2002) High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. *Appl Environ Microbiol* **68**: 3878-3885.

- Cord-Ruwisch R, Sietz HJ & Conrad R (1988) The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. *Arch Microbiol* **149**: 350-357.
- Czerkawski JW (1969) Methane production in ruminants and its significance. *World Rev Nutr Diet* **11**: 240-282.
- Daims H, Bruhl A, Amann R, Schleifer KH & Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**: 434-444.
- Darland G, Brock TD, Samsonoff W & Conti SF (1970) A thermophilic acidophilic mycoplasma isolated from a coal refuse pile. *Science* **170**: 1416-1418.
- DeGraaf W, Wellsbury P, Parkes RJ & Cappenberg TE (1996) Comparison of acetate turnover in methanogenic and sulfate reducing sediments by radio-labelling and stable isotope labelling and by use of specific inhibitors: evidence for isotopic exchange. *Appl Environ Microbiol* **62**: 772-777.
- Denman SE & McSweeney (2005) Quantitative (real-time) PCR. In *Methods in gut microbial ecology for ruminants* (Makkar HPS & McSweeney CS, eds), pp. 105-115. Kluwer Academic Publisher, Netherlands.
- Denman SE, Tomkins NW & McSweeney CS (2007) Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiol Ecol* **62**: 313-322.
- Demeyer DI, Fiedler D & DeGraeve KG (1996) Attempted induction of reductive acetogenesis into the rumen fermentation *in vitro*. *Reprod Nutr Dev* **36**: 233-240.
- Dongowski GA, Lorenz A & Anger H (2000) Degradation of pectins with different degrees of etherification by *Bacteroides thetaiotaomicron* isolated from human gut flora. *Appl Environ Microbiol* **66**:1321-1327

- Doré J, Morvan B, Rieu-Lesme F, Goderel I, Gouet P & Pochart P (1995) Most probable number enumeration of H₂-utilizing acetogenic bacteria from the digestive tract of animals and man. *FEMS Microbiol Lett* **130**: 7-12.
- Dridi B, Henry M, Khechine, AE, Raoult D & Drancourt M (2010) High prevalence of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* detected in the human gut using an improved DNA detection protocol. *PLoS ONE* **4**(9).
- Eckard RJ, Grainger C & Klein CAM (2010) Options for the abatement of methane and nitrous oxide from ruminant production: A review. *Livest Sci* **130**: 47-56.
- Edwards JE, McEwan NR, Travis AJ & Wallace (2004) 16S rDNA library-based analysis of ruminal bacterial diversity. *Anton van Lee* **86**:263-281.
- Edwards T & McBride BC (1975) New method for the isolation and identification of methanogenic bacteria. *Appl Microbiol* **29**: 540-545.
- Eirich LD (1978) Proposed structure for coenzyme F₄₂₀ from *Methanobacterium*. *Biochemistry*. **17**: 4583-4593.
- Ellefson WL & Wolfe RS (1980) Role of component C of the methylreductase system of *Methanobacterium*. *J Biol Chem* **255**: 8388-8389.
- Ellefson WL & Wolfe RS (1981) Component C of the methylreductase system of *Methanobacterium*. *J Biol Chem* **256**: 4259-4262.
- Embley TM, Finlay BJ, Thomas RH & Dyal PL (1992) The use of rRNA sequences and fluorescent probes to investigate the phylogenetic positions of the anaerobic ciliate *Metopus palaeformis* and its archaeobacterial endosymbiont. *J Gen Microbiol* **138**: 1479-1487.
- Etokebe GE & Spurkland A (2000) Method for avoiding PCR-inhibiting contaminants when eluting DNA from polyacrylamide gels. *Biotechniques* **29**: 694-696.
- Evans PN, Hinds LA, Sly LI, McSweeney CS, Morrison M & Wright AD (2009) Community composition and density of methanogens in the foregut of the Tammar wallaby (*Macropus eugenii*). *Appl Environ Microbiol* **75**: 2598-2602.

- Food and Agriculture organization of the United Nations (FAO) (2008) The state of food insecurity in the world. Available: <http://www.fao.org/docrep/011/i0291e/i0291e00.htm>. Accessed 2010 Sept 30.
- Fievez V, Dohme F, Danneels M, Raes K & Demeyer D (2003) Fish oils as potent rumen methane inhibitors and associated effects on rumen fermentation *in vitro* and *in vivo*. *Anim Feed Sci Technol* **104**: 41-58.
- Finlay BJ, Esteban G, Clarke KJ, Williams AG, Embley TM & Hirt RP (1994) Some rumen ciliates have endosymbiotic methanogens. *FEMS Microbiol Lett* **117**: 157-161.
- Firkins JL & Yu Z (2006) Characterization and quantification of the microbial populations of the rumen. In *Ruminant physiology. Digestion, metabolism, and impact of nutrition on gene expression, immunology and stress* (Sejrsen K, Hvelplund T & Nielsen MO, eds), pp.19-54. Wageningen Academic Publishers, Wageningen, Netherlands.
- Fonty G, Gouet P, Jouany JP & Senaud J (1987) Establishment of the microflora and anaerobic fungi in the rumen of lambs. *J Gen Microbiol* **133**: 1835-1843.
- Fonty G, Joblin K, Chavarot M, Roux R, Naylor G & Michallon F (2007) Establishment and development of ruminal hydrogenotrophs in methanogen-free lambs. *Appl Environ Microbiol* **73**: 6391-6403.
- Freudenberger DO, Toyakawa K, Barry TN, Ball AJ & Suttie JM (1994) Seasonality in digestion and rumen metabolism in red deer (*Cervus elaphus*) fed on a forage diet. *Br J Nutr* **71**: 489-499.
- Fricke WF, Seedorf H, Henne A, Kruer M, Liesegang H, Hedderich R, Gottschalk G & Thauer RK (2006) The genome sequence of *Methanosphaera stadtmanae* reveals why this human intestinal archaeon is restricted to methanol and H₂ for methane formation and ATP synthesis. *J Bacteriol* **188**: 642-658.
- Friedrich MW (2005) Methyl-coenzyme M reductase genes: unique functional markers for methanogenic and anaerobic methane-oxidizing Archaea. *Method Enzymol* **397**: 428-442.

- Garcia JL, Patel BKC & Ollivier B (2000) Taxonomic, phylogenetic, and ecological diversity of methanogenic Archaea. *Anaerobe* **6**: 205-226.
- García-Martínez J & Rodríguez-Valera F (2000) Microdiversity of uncultured marine prokaryotes: the SAR11 cluster and the marine Archaea of group I. *Mol Ecol* **9**: 935-948.
- Genthner BR, Davis CL & Bryant MP (1981) Features of rumen and sewage sludge strains of *Eubacterium limosum*, a methanol- and H₂-CO₂-utilizing species. *Appl Environ Microbiol* **42**: 12-19.
- Gephart P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR & Phillips GB (1981) Manual of methods for general bacteriology, ASM Press, Washington, USA.
- Gill HS, SHU Q & Leng RA (2000) Immunization with *Streptococcus bovis* protects against lactic acidosis in sheep. *Vaccine* **18**: 2541-2548.
- Giovannoni SJ, Rappé MS, Vergin KL & Adair NL (1996) 16S rRNA genes reveal stratified open ocean bacterioplankton populations related to the green non-sulfur bacteria. *Proc Natl Acad Sci USA* **93**: 7979-7984.
- Graham DE (2010) A new role for coenzyme F₄₂₀ in aflatoxin reduction by soil mycobacteria. *Mol Microbiol* **78**: 533-536.
- Grosskopf R, Janssen PH & Liesack W (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl Environ Microbiol* **64**: 960-969.
- Gunsalus RP & Wolfe RS (1978) ATP activation and properties of the methyl coenzyme M reductase system in *Methanobacterium thermoautotrophicum*. *J Bacteriol* **135**:851-857.

- Guo YQ, Liu JX, Lu Y, Zhu WY, Denman SE & McSweeney CS (2008) Effect of tea saponin on methanogenesis, microbial community structure and expression of *mcrA* gene, in cultures of rumen micro-organisms. *Lett Appl Microbiol* **47**: 421-426.
- Hales BA, Edwards C, Ritchie DA, Hall G, Pickup RW & Saunders JR (1996) Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Appl Environ Microbiol* **62**: 668-675.
- Hallam SJ, Girguis PR, Preston CM, Richardson PM & DeLong EF (2003) Identification of methyl coenzyme M reductase A (*mcrA*) genes associated with methane-oxidizing archaea. *Appl Environ Microbiol* **69**: 5483-5491.
- Hallam SJ, Putnam N, Preston CM, Detter JC, Rokhsar D, Richardson PM & DeLong EF (2004) Reverse methanogenesis: testing the hypothesis with environmental genomics. *Science* **305**: 1457-1462.
- Hamilton SW, DePeters EJ, McGarvey JA, Lathrop J & Mitloehner FM (2010) Greenhouse gas, animal performance, and bacterial population structure responses to dietary monensin fed to dairy cows. *J Environ Quality* **39**: 106-114.
- Hammer O, Harper DAT & Ryan PD (2001) PAST: Paleontological statistics software package for education and data analysis. *Palaeontol Electron* **4**: 9.
- Hedderich R & Whitman W (2006) Physiology and biochemistry of the methane-producing Archaea, In *The prokaryotes* (Dworkin M, eds), pp. 1050-1079. Springer, New York.
- Hegarty RS (1999) Reducing rumen methane emissions through elimination of rumen protozoa. *Aust J Agric Research* **50**: 1321-1327.
- Hegarty RS, Bird SH, Vanselow BA & Woodgate R (2008) Effects of the absence protozoa from birth or from weaning on the growth and methane production of lambs. *Br J Nutr* **100**: 1220-1227.
- Herd RM & Arthur PF (2009) Physiological basis for residual feed intake. *J Anim Sci* **87**: 64-71.

- Herd RM & Bishop SC (2000) Genetic variation in residual feed intake and its association with other production traits in British Hereford cattle. *Livest Prod Sci* **63**: 111-119.
- Hermann M, Noll KM & Wolfe RS (1986) Improved agar bottle plate for isolation of methanogens or other anaerobes in a defined gas atmosphere. *Appl Environ Microbiol* **51**: 1124-1126.
- Hillman K, Lloyd D & Williams AG (1988) Interactions between the methanogen *Methanosarcina barkeri* and rumen holotrich ciliate protozoa. *Lett in Appl Microbiol* **7**: 49-53.
- Hippe H, Hagelstein A, Kramer I, Swiderski J & Stackebrandt E (1999) Phylogenetic analysis of *Formivibrio citricus*, *Propionivibrio dicarboxylicus*, *Anaerobiospirillum thomasii*, *Succinimonas amylolytica* and *Succinivibrio dextrinosolvans* and proposal of *Succinivibrionaceae* fam. nov. *Int J Syst Bacteriol* **49**: 779-782.
- Hook SE, Northwood KS, Wright ADG & McBride BW (2009) Long-term monensin supplementation does not significantly affect the quantity or diversity of methanogens in the rumen of the lactating dairy cow. *Appl Environ Microbiol* **75**: 374-380.
- Hu B & Chen SL (2007) Pretreatment of methanogenic granules for immobilized hydrogen fermentation. *Int J Hydrogen energy* **32**: 3266-3273.
- Hu B, Zhou X, Forney L & Chen S (2009) Changes in Microbial community composition following treatment of methanogenic granules with chloroform. *Environ Prog Sustain Energ* **28**:
- Huang LN, Chen YQ, Zhou H, Luo S & Qu LH (2003) Characterization of methanogenic archaea in the leachate of a closed municipal solid waste landfill. *FEMS Microbiol Ecol* **46**: 171-177.

- Huber T, Faulkner G & Hugenholtz P (2004). Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20**: 2317–2319.
- Hugenholtz P & Tyson GW (2008) Microbiology: metagenomics. *Nature* **455**: 481-483.
- Hungate RE (1950) The anaerobic mesophilic cellulolytic bacteria. *Bacteriol Rev* **14**: 1-49.
- Hungate RE (1967) Hydrogen as an intermediate in the rumen fermentation. *Arch Microbiol* **59**: 158-164.
- Hungate RE, Smith W, Bauchop T, Yu I & Rabinowitz JC (1970) Formate as an intermediate in the bovine rumen fermentation. *J Bacteriol* **102**: 389-397.
- Hurley MA & Roscoe ME (1983) Automated statistical analysis of microbial enumeration by dilution series. *J Appl Bacteriol* **55**:159-164.
- Ihara K, Watanabe S & Tamura T (1997) *Haloarcula argentinensis* sp. nov. and *Haloarcula mukohataei* sp. nov., two new extremely halophilic archaea collected in Argentina. *Int J Syst Bacteriol* **47**: 73–77.
- IPCC (Intergovernmental Panel on Climate Change) (2007) The physical science basis, contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change. Available: <http://www.ipcc.ch/publications>. Accessed 2010 March 30.
- Irbis C & Ushida K (2004) Detection of methanogens and proteobacteria from a single cell of rumen ciliate protozoa. *J Gen Appl Microbiol* **50**: 203-212.
- Jäger D, Sharma CM, Thomsen J, Ehlers C, Vogel J & Schmitz RA (2009) Deep sequencing analysis of the *Methanosarcina mazei* Gö1 transcriptome in response to nitrogen availability. *Proc Natl Acad Sci U S A* **106**: 21878-21882.
- Janse I, Bok J & Zwart G (2004) A simple remedy against artifactual double bands in denaturing gradient gel electrophoresis. *J Microbiol Meth* **57**: 279-281.

- Janssen PH & Kirs M (2008) Structure of the archaeal community of the rumen. *Appl Environ Microbiol* **74**: 3619-3625.
- Jarvis GN, Strompl C, Burgess DM, Skillman LC, Moore ER & Joblin KN (2000) Isolation and identification of ruminal methanogens from grazing cattle. *Curr Microbiol* **40**: 327-332.
- Jensen NS & Canale-Parola E (1986) *Bacteroides pectinophilus* sp. nov. and *Bacteroides galacturonicus* sp. nov.: two pectinolytic bacteria from the human intestinal tract. *Appl Environ Microbiol* **52**: 880-887.
- Joblin KN (1999) Ruminal acetogens and their potential to lower ruminant methane emissions. *Aust J Agric Research* **50**: 1307-1313.
- Joblin KN (2005) Methanogenic archaea. In *Methods in Gut Microbial Ecology for Ruminants* (Makkar HPS & Mcsweeney C, (eds), pp. 47-53. Kluwer Academic Publisher, The Netherlands.
- Jones DT, Taylor WR & Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. *Comp Appl Biosci* **8**:275-282.
- Jordan E, Kenny D, Hawkins M, Malone R, Lovett DK & O'Mara FP (2006). Effect of refined soy oil or whole soybeans on intake, methane output, and performance of younger bulls. *J Anim Sci* **84**: 2418-2425.
- Jukes TH & Cantor CR (1969) Evolution of protein molecules. In *Mammalian protein metabolism* (Munro HN, eds), pp. 21-123. Academic Press, New York, NY, USA.
- Juottonen H, Galand PE & Yrjala K (2006) Detection of methanogenic Archaea in peat: comparison of PCR primers targeting the *mcrA* gene. *Res Microbiol* **157**:914-921.
- Kaeberlein T, Lewis K & Epstein SS (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science* **296**: 1127-1129.
- Kalmakoff ML, Bartlett F & Teather RM (1996) Are ruminal bacteria armed with bacteriocins. *J Dairy Sci* **79**: 2297-2306.

- Kemnitz D, Kolb S & Conrad R (2005) Phenotypic characterization of Rice Cluster III archaea without prior isolation by applying quantitative polymerase chain reaction to an enrichment culture. *Environ Microbiol* **7**: 553-565.
- Kenealy W & Zeikus JG (1981) Influence of corrinoid antagonists on methanogen metabolism. *J Bacteriol* **146**: 133-140.
- Kenters N, Henderson G, Jeyanathan J, Kittelmann, S & Janssen PH (in press) Isolated of previously-uncultured rumen bacteria by dilution to extinction using a new liquid culture medium. *J Microbiol Meth* (in press, MIMET-D-10-00482).
- Kim BS, Kim BK, Lee JH, Kim M, Lim YW & Chun J (2008) Rapid phylogenetic dissection of prokaryotic community structure in tidal flat using pyrosequencing. *J Microbiol* **46**, 357-363.
- Klieve AV, O'Leary MN, McMillen L & Ouwkerk D (2007) *Ruminococcus bromii*, identification and isolation as a dominant community member in the rumen of cattle fed a barley diet. *J Appl Microbiol* **103**: 2065-2073.
- Klocke M, Nettmann E, Bergmann I, Mundt K, Souidi K, Mumme J & Linke B (2008) Characterization of the methanogenic archaea within two-phase biogas reactor systems operated with plant biomass. *Syst Appl Microbiol* **31**: 190-205.
- Knight T, Ronimus RS, Dey D, Tootill C, Naylor G, Evans P, Molano G, Smith A, Tavendale M, Pinares-Patiño CS and Clark H (2011) Chloroform mitigates methane emissions and strongly affects methanogen populations without altering rumen function in cattle. *Anim Feed Sci Technol*, in press.
- Kocherginskaya SA, Aminov RI & White BA (2001) Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing and statistical ecology approaches. *Anaerobe* **7**: 119-134.
- Koike S, Yoshitani S, Kobayashi Y & Tanaka K (2003) Phylogenetic analysis of fiber-associated rumen bacterial community and PCR detection of uncultured bacteria. *FEMS Microbiol Lett* **229**: 23-30.

- Kolganova TV, Kuznetsov BB & Tourova TP (2002) Designing and testing oligonucleotide primers for amplification and sequencing of archaeal 16S rRNA genes. *Mikrobiologiya* **71**: 283–286.
- Konig H (1986) Chemical composition of cell envelopes of methanogenic bacteria isolated from human and animal feces. *Syst Appl Microbiol* **8**: 159-162
- Kowalchuk GA, Stephen JR, DeBoer W, Orosser JI, Embley TM & Woldendorp JW (1997) Analysis of ammonia-oxidising bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Appl Environ Microbiol* **63**: 1489-1497.
- Krause DO & Russell JB (1996) How many ruminal bacteria are there? *J Dairy Sci* **79**: 1467-1475.
- Krause DO, Smith WJ & McSweeney CS (2001) Extraction of microbial DNA from rumen contents containing plant tannins. *Biotechniques* **31**: 294-298.
- Krause DO, Smith WJM, Ryan FME, Mackie RI & McSweeney CS (1999) Use of 16S rRNA based techniques to investigate the ecological succession of microbial populations in the immature lamb rumen: tracking of a specific strain of inoculated *Ruminococcus* and interactions with other microbial populations *in vivo*. *Microbial Ecol* **38**: 365-376.
- Krober M, Bekel T, Diaz NN, Goesmann A, Jaenicke S, Krause L, Miller D, Runte KJ, Viehöver P, Puhler A & Schluter A (2009) Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing. *J Biotechnol* **142**: 38-49.
- Kruger M, Meyerdierks A, Glockner FO, Amann R, Widdel F, Kube M, Reinhardt R, Kahnt J, Bocher R, Thauer RK & Shima S (2003) A conspicuous nickel protein in microbial mats that oxidize methane anaerobically. *Nature* **426**: 878-881.

- Krumholz LR, Forsberg CW & Veira DM (1983) Association of methanogenic bacteria with rumen protozoa. *Can J Microbiol* **29**: 676-680.
- Lana RP, Russell JB & Van Amburgh ME (1998) The role of pH in regulating ruminal methane and ammonia production. *J Anim Sci* **76**: 2190-2196.
- Lane DJ (1991) 16S/23S rRNA sequencing. In *Nucleic acid techniques in bacterial systematics* (Stackebrandt E & Goodfellow M, eds), pp. 115-175. John Wiley and Sons, New York.
- Le Van TD, Robinson JA, Ralph J, Greening RC, Smolenski WJ, Leedle JA & Schaefer DM (1998) Assessment of reductive acetogenesis with indigenous ruminal bacterium populations and *Acetitomaculum ruminis*. *Appl Environ Microbiol* **64**: 3429-3436.
- Leahy SC, Kelly WJ, Altermann E, Ronimus RS, Yeoman CJ, Pacheco DM, Li D, Kong Z, McTavish S, Sang C, Lambie SC, Janssen PH, Dey D & Attwood GT (2010) The genome sequence of the rumen methanogen *Methanobrevibacter ruminantium* reveals new possibilities for controlling ruminant methane emissions. *PLoS ONE* **5**, e8926 (17 pages).
- Leedle JA & Greening RC (1988) Postprandial changes in methanogenic and acidogenic bacteria in the rumens of steers fed high- or low-forage diets once daily. *Appl Environ Microbiol* **54**: 502-506.
- Leslie M, Aspin M & Clark H (2008) Greenhouse gas emissions from New Zealand agriculture: issues, perspectives and industry response. *Aust J Exp Agric* **48**: 1-5.
- Lila ZA, Mohammed N, Kanda S, Kurihara M & Itabashi H (2005) Sarsaponin effects on ruminal fermentation and microbes, methane production, digestibility and blood metabolites in steers. *Asian-Australasian J Ani Sci* **18**: 1746-1751.
- Lin C, Raskin L & Stahl DA (1997) Microbial community structure in gastrointestinal tracts of domestic animals: Comparative analyses using rRNA-targeted oligonucleotide probes. *FEMS Microbiol Ecol* **22**: 281-294.

- Lin C & Miller TL (1998) Phylogenetic analysis of *Methanobrevibacter* isolated from feces of humans and other animals. *Arch Microbiol* **169**: 397-403.
- Liu W & Saint DA (2002) Validation of a quantitative method for real time PCR kinetics. *Biochem Bioph Res Co* **294**: 347-353.
- Liu Y & Whitman WB (2008) Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Ann NY Acad Sci.* **1125**: 171-189.
- Lopez S, Valdes C, Newbold CJ & Wallace RJ (1999) Influence of sodium fumarate addition on rumen fermentation *in vitro*. *Br J Nutr* **81**: 59-64.
- Lovley DR, Greening RC & Ferry JG (1984) Rapidly growing rumen methanogenic organism that synthesizes coenzyme M and has a high affinity for formate. *Appl Environ Microbiol* **48**: 81-87.
- Loy A, Maixner F, Wagner M, Horn M (2007) probeBase - an online resource for rRNA-targeted oligonucleotide probes: new features 2007. *Nucleic Acids Res* **35**: D800-D804.
- Ludwig W & Schleifer KH (1994) Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbiol Rev* **15**: 155-173.
- Lueders T & Friedrich M (2003) Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small subunit rRNA and *mcrA* genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. *Appl Environ Microbiol* **69**: 320-326.
- Lueders T, Chin KJ, Conrad R & Friedrich M (2001) Molecular analyses of methyl-coenzyme M reductase alpha-subunit (*mcrA*) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. *Environ Microbiol* **3**:194-204.
- Lueders T, Manefield M & Friedrich MW (2004) Enhanced sensitivity of DNA- and rRNA based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* **6**:73-78.

- Luton PE, Wayne JM, Sharp RJ & Riley PW (2002) The *mcrA* gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. *Microbiology* **148**: 3521-3530.
- Marcin A & Sudekum KH (2009) Nutritive defaunation of the rumen in steers with subsequent refaunation using a cryopreserved monoculture of *Entodinium caudatum*. *J Anim Physiol An N* **93**: 44-51.
- Martin C, Morgavi DP & Doreau (2010) Methane mitigation in ruminants: from microbe to the farm scale. *Animal* **4**: 351-365.
- Marvin-Sikkema FD, Richardson AJ, Stewart CS, Gottschal JC & Prins RA (1990) Influence of hydrogen-consuming bacteria on cellulose degradation by anaerobic fungi. *Appl Environ Microbiol* **56**: 3793-3797.
- Marvin-Sikkema FD, Rees E, Kraak MN, Gottschal JC & Prins RA (1993) Influence of metronidazole, CO, CO₂, and methanogens on the fermentative metabolism of the anaerobic fungus *Neocallimastix* sp. strain L2. *Appl Environ Microbiol* **59**: 2678-2683.
- McAllister TA & Newbold CJ (2008) Redirecting rumen fermentation to reduce methanogenesis. *Aust J Exp Agric* **48**: 7-17.
- McInerney MJ, Mackie RI & Bryant MP (1981) Syntrophic association of a butyrate-degrading bacterium and *Methanosarcina* enriched from bovine rumen fluid. *Appl Environ Microbiol* **41**: 826-828.
- McSweeney CS, Denman SE, Wright ADG & Yu Z (2007) Application of recent DNA/RNA-based techniques in rumen ecology. *Asian-Australasian J Ani Sc* **20**: 283-294.
- Mihajlovski A, Alric M & Brugere JF (2008) A putative new order of methanogenic archaea inhabiting the human gut, as revealed by molecular analyses of the *mcrA* gene. *Research Microbiol* **159**: 516-521.

- Miller TL & Lin C (2002) Description of *Methanobrevibacter gottschalkii* sp. nov., *Methanobrevibacter thaueri* sp. nov., *Methanobrevibacter woesei* sp. nov. and *Methanobrevibacter wolinii* sp. nov. *Int J Syst Evol Micr* **52**: 819-822.
- Miller TL & Wolin MJ (1974) A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. *Appl Microbiol* **27**: 985-987.
- Miller TL & Wolin MJ (1985) *Methanosphaera stadtmaniae* gen. nov., sp. nov.: a species that forms methane by reducing methanol with hydrogen. *Arch Microbiol* **141**: 116-122.
- Miller TL & Wolin MJ (1983) Oxidation of hydrogen and reduction of methanol to methane is the sole energy source for a methanogen isolated from human feces. *J Bacteriol* **153**: 1051-1055.
- Miller TL, Wolin MJ & Kusel EA (1986a) Isolation and characterization of methanogens from animal feces. *Syst Appl Microbiol* **8**: 234-238.
- Miller TL, Wolin MJ, Zhao HX & Bryant MP (1986b) Characteristics of methanogens isolated from bovine rumen. *Appl Environ Microbiol* **51**, 201-202.
- Ministry of Agriculture and forestry (2010) Agriculture and forestry in New Zealand- An overview. Available: <http://www.maf.govt.nz/mafnet/publications>. Accessed 2010 Nov 10.
- Ministry for the Environment. New Zealand's greenhouse gas inventory 1990-2007 (2009). Available:<http://www.mfe.govt.nz/publications>. Accessed 2010 Sept 30.
- Morgavi DP, Jouany JP & Martin C (2008) Changes in methane emission and rumen fermentation parameters induced by refaunation in sheep. *Aust J Exp Agric* **48**: 69-72.
- Morvan B, Dore J, Rieu-Lesme F, Foucat L, Fonty G & Gouet P (1994) Establishment of hydrogen-utilizing bacteria in the rumen of the newborn lamb. *FEMS Microbiol Lett* **117**: 249-256.

- Morvan B, Bonnemoy F, Fonty G & Gouet P (1996) Quantitative determination of H₂-utilizing acetogenic and sulfate-reducing bacteria and methanogenic archaea from digestive tract of different mammals. *Curr Microbiol* **32**: 129-133.
- Moss AR, Jouany JP & Newbold J (2000) Methane production by ruminants: its contribution to global warming. *Ann Zootech* **49**: 231-253.
- Mountfort DO, Asher RA & Bauchop T (1982) Fermentation of cellulose to methane and carbon dioxide by a rumen anaerobic fungus in a triculture with *Methanobrevibacter* sp. strain RA1 and *Methanosarcina barkeri*. *Appl Environ Microbiol* **44**: 128-134.
- Mukhopadhyay B, Purwantini E, De Macario EC & Daniels L (1991) Characterization of a *Methanosarcina* strain isolated from goat feces and that grows on hydrogen-carbon dioxide only after adaptation. *Curr Microbiol* **23**: 165-174.
- Muyzer G, de Waal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**: 695-700.
- Muyzer G & Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Anton van Lee* **73**: 127-141.
- Muyzer G, Teske A, Wirsen CO & Jannasch HW (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164**: 165-172.
- Myers RM (1985) Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel-electrophoresis. *Nucl Acids Res.* **13**: 3131-3145.
- Nehme B, Gilbert Y, Letourneau V, Forster RJ, Veillette M, Villemur R & Duchaine C (2009) Culture-independent characterization of archaeal biodiversity in swine confinement building bioaerosols. *Appl Environ Microbiol* **75**: 5445-5450.

- Nakamura K, Terada T, Sekiguchi Y, Shinzato N, Meng XY, Enoki M & Kamagata Y (2006) Application of pseudomurein endoisopeptidase to fluorescence *in situ* hybridization of methanogens within the family *Methanobacteriaceae*. *Appl Environ Microbiol* **72**: 6907-6913.
- Nicholson MJ, Evans PN & Joblin KN (2007) Analysis of methanogen diversity in the rumen using temporal temperature gradient gel electrophoresis: Identification of uncultured methanogens. *Microb Ecol* **54**: 141-150.
- Nkrumah JD, Okine EK, Mathison, JW, Schmid K, Li C, Basarab JA, Price MA, Wang Z & Moore SS (2006) Relationships of feedlot feed efficiency, performance, and feeding behaviour with metabolic rate, methane production, and energy partitioning in beef cattle. *J Anim Sci* **84**: 145-153.
- Nollet L, Demeyer D & Verstraete W (1997a) Effect of 2-bromoethanesulfonic acid and *Peptostreptococcus productus* ATCC 35244 addition on stimulation of reductive acetogenesis in the ruminal ecosystem by selective inhibition of methanogenesis. *Appl Environ Microbiol* **63**: 194-200.
- Nollet L, Vande Velde I & Verstraete W (1997b) Effect of the addition of *Peptostreptococcus productus* ATCC35244 on the gastro-intestinal microbiota and its activity, as simulated in an *in vitro* simulator of the human gastro-intestinal tract. *Appl Microbiol Biotechnol* **48**: 99-104.
- Nollet L, Mbanzamihiho L, Demeyer D & Verstraete W (1998) Effect of the addition of *Peptostreptococcus productus* ATCC 35244 on reductive acetogenesis in the ruminal ecosystem after inhibition of methanogenesis by cell-free supernatant of *Lactobacillus plantarum* 80. *Anim Feed Sci Technol* **71**: 49-66.
- Nubel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, Ludwig W & Backhaus H (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J Bacteriol* **178**: 5636-5643.

- O'Farrell KA & Janssen PH (1999) Detection of verrucomicrobia in a pasture soil by PCR-mediated amplification of 16S rRNA genes. *Appl Environ Microbiol* **65**:4280-4284.
- Ohene-Adjei S, Teather RM, Ivan M & Forster RJ (2007) Postinoculation protozoan establishment and association patterns of methanogenic archaea in the ovine rumen. *Appl Environ Microbiol* **73**: 4609-4618.
- Ohene-Adjei S, Chaves AV, McAllister TA, Benchaar C, Teather RM & Forster RJ (2008) Evidence of increased diversity of methanogenic archaea with plant extract supplementation. *Microb Ecol* **56**: 234-242.
- Oppermann RA, Nelson WO & Brown RE (1957) *In vitro* studies on methanogenic rumen bacteria. *J. Dairy Sci* **40**: 779-788.
- Orpin CG (1975) Studies on the rumen flagellate *Neocallimastix frontalis*. *J Gen Microbiol* **91**: 249-262.
- Osborne CA, Galic M, Sangwan P & Janssen PH (2005) PCR-generated artefact from 16S rRNA gene-specific primers. *FEMS Microbiol Lett* **248**: 183-187.
- Ouwerkerk D, Turner AF & Klieve AV (2008) Diversity of methanogens in ruminants in Queensland. *Aust J Exp Agric* **48**: 722-725.
- Patterson JA & Hespell RB (1979) Trimethylamine and methylamine as growth substrates for rumen bacteria and *Methanosarcina barkeri*. *Current Microbiol* **3**: 79-83.
- Paynter MJ & Hungate RE (1968) Characterization of *Methanobacterium mobilis*, sp. n., isolated from the bovine rumen. *J Bacteriol* **95**: 1943-1951.
- Pei C, Mao S & Zhu W (2008) Molecular diversity of rumen Archaea from Jinnan cattle [in Chinese]. *Wei Sheng Wu Xue Bao* **48**: 8-14.
- Pei CX, Mao SY, Cheng YF & Zhu WY (2010) Diversity, abundance and novel 16S rRNA gene sequences of methanogens in rumen liquid, solid and epithelium fractions of Jinnan cattle. *Animal* **4**: 1-10.

- Peng J, Lu Z, Rui J & Lu Y (2008) Dynamics of the methanogenic archaeal community during plant residue decomposition in an anoxic rice field soil. *Appl Environ Microbiol* **74**: 2894-2901.
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucl Acids RES* **29**: e45.
- Pfennig N & Wagener S (1986) An improved method of preparing wet mounts for photomicrographs of microorganisms. *J. Microbiol Meth* **4**:303-306.
- Pinares-Patiño CS, Machmuller A, Molano G, Smith A, Vlaming JB & Clark H (2008) The SF₆ tracer technique for measurement of methane emissions from cattle-effect of tracer permeation rate. *Can J Anim Sci* **88**: 309-320.
- Pinares-Patiño CS, Ulyatt MJ, Holmes CW, Barry TN & Lassey KR (2003) Persistence of the between-sheep variation in methane emission. *J Agric Sci* **140**: 227-233.
- Polz MF & Cavanaugh CM (1998) Bias in template-to-product ratios in multitemplate PCR. *Appl Environ Microbiol* **64**: 3724-3730.
- Purwantini E and Daniels L (1996) Purification of a novel coenzyme F₄₂₀-dependent glucose-6-phosphate dehydrogenase from mycobacterium smegmatis. *J bacteriol* **178**: 2861-2866.
- Ramírez-Restrepo CA, Barry TN, Marriner A, López-Villalobos N, McWilliam EL, Lassey KR & Clark H (2010) Effects of grazing willow fodder blocks upon methane production and blood composition in young sheep. *Anim Feed Sci Technol* **155**: 33-43.
- Rea S, Bowman JP, Popovski S, Pimm C & Wright ADG (2007) *Methanobrevibacter millerae* sp. nov. and *Methanobrevibacter olleyae* sp. nov., methanogens from the ovine and bovine rumen that can utilize formate for growth. *Int J Syst Evol Micr* **57**: 450-456.

- Regensbogenova M, McEwan NR, Javorsky P, Kisidayova S, Michalowski T, Newbold CJ, Hackstein JH & Pristas P (2004) A re-appraisal of the diversity of the methanogens associated with the rumen ciliates. *FEMS Microbiol Lett* **238**: 307-313.
- Reysenbach AL & Pace NR (1995) Reliable amplification of hyperthermophilic archaeal 16S rRNA genes by the polymerase chain reaction (PCR). In *Archaea: a laboratory manual* (Robb FT, Sowers KR, DasSarma S, Place AR, Schreier HJ & Fleischmann EM, eds), pp. 101-106. Cold Spring Harbor Press, Cold Spring Harbor, NY, USA.
- Rouviere PE & Wolfe RS (1988) Novel biochemistry of methanogenesis. *J Biol Chem* **263**: 7913-7916.
- Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, van den Hoff MJB & Moorman AFM (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucl Acids Research* **37**:e45.
- Sait L, Galic M, Strugnell RA & Janssen PH (2003) Secretory antibodies do not affect the composition of the bacterial microbiota in the terminal ileum of 10-week-old mice. *Appl Environ Microbiol* **69**: 2100-2109.
- Saitou N & Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406-425.
- Sakai S, Imachi H, Sekiguchi Y, Ohashi A, Harada H & Kamagata Y (2007) Isolation of key methanogens for global methane emission from rice paddy fields: a novel isolate affiliated with the clone cluster rice cluster I. *Appl Environ Microbiol* **73**: 4326-4331.
- Sauer FD, Fellner V, Kinsman R, Kramer JK, Jackson HA, Lee AJ & Chen S (1998) Methane output and lactation response in Holstein cattle with monensin or unsaturated fat added to the diet. *J Anim Sci* **76**: 906-914.

- Sauvant D & Giger-Reverdin S (2007) Empirical modelling meta-analysis of digestive interactions and CH₄ production in ruminants. In *Energy and protein metabolism and nutrition* (Ortigue-Marty I, Miraux N & Brand-Williams, eds), pp. 561. EAAP publication No.124, Wageningen Academic Publishers, Wageningen, The Netherlands.
- Scanlan PD, Shanahan F & Marchesi JR (2008) Human methanogen diversity and incidence in healthy and diseased colonic groups using *mcrA* gene analysis. *BMC Microbiol* **8**: 79.
- Schluter A, Bekel T, Diaz NN, Dondrup M, Eichenlaub R, Gartemann KH, Krahn I, Krause L, Kromeke H, Kruse O, Mussnug JH, Neuweger H, Niehaus K, Puhler A, Runte KJ, Szczepanowski R, Tauch A, Tilker A, Viehover P & Goesmann A (2008) The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. *J Biotechnol* **136**: 77-90.
- Seegerer A, Langworthy TA & Stetter KO (1988) *Thermoplasma acidophilum* and *Thermoplasma volcanium* sp. nov. from solfatara fields. *Syst Appl Microbiol* **10**: 161-171.
- Sekiguchi Y, Kamagata Y, Nakamura K, Ohashi A & Harada H (1999) Fluorescence *in situ* hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. *Appl Environ Microbiol* **65**: 1280-1288.
- Shannon CE & Weaver W (1963) *The Mathematical Theory of Communication*. Univ. of Illinois Press, Urbana, IL, USA.
- Sharp R, Ziemer CJ, Stern MD & Stahl DA (1998) Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. *FEMS Microbiol Ecol* **26**: 71-78.
- Shi PJ, Meng K, Zhou ZG, Wang YR, Diao QY & Yao B (2008) The host species affects the microbial community in the goat rumen. *Lett Appl Microbiol* **46**: 132-135.

- Shibata M & Terada F (2010) Factors affecting methane production and mitigation in ruminants. *J Anim Sci J* **81**: 2-10.
- Shin EC, Choi BR, Lim WJ, Hong SY, An CL, Cho KM, Kim YK, An JM, Kang JM, Lee SS, Kim H & Yun HD (2004) Phylogenetic analysis of archaea in three fractions of cow rumen based on the 16S rDNA sequence. *Anaerobe* **10**: 313-319.
- Shu Q, Bir SH, Gill HS, Duan E, Xu Y, Hiliard MA & Rowe JB (2001) Antibody response in sheep following immunization with *Streptococcus bovis* in different adjuvants. *Vet Res Commun* **25**: 43-54.
- Simpson EH (1949) Measurement of diversity. *Nature* **163**: 688.
- Skillman LC, Evans PN, Naylor GE, Morvan B, Jarvis GN & Joblin KN (2004) 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. *Anaerobe* **10**: 277-285.
- Skillman LC, Evans PN, Strompl C & Joblin KN (2006) 16S rDNA directed PCR primers and detection of methanogens in the bovine rumen. *Lett Appl Microbiol* **42**: 222-228.
- Smith PH & Hungate RE (1958) Isolation and characterization of *Methanobacterium ruminantium* n. sp. *J Bacteriol* **75**: 713-718.
- Soliva CR, Meile L, Hindrichsen IK, Kreuzer M & Machmüller A (2004) Myristic acid supports the immediate inhibitory effect of lauric acid on ruminal methanogens and methane release. *Anaerobe* **10**: 269-276.
- Sprenger WW, Hackstein JHP & Keltjens JT (2005) The energy metabolism of *Methanomicrococcus blatticola*: Physiological and biochemical aspects. *Anton van Leeuwen* **87**: 289-299.
- Sprenger WW, van Belzen MC, Rosenberg J, Hackstein JH & Keltjens JT (2000) *Methanomicrococcus blatticola* gen. nov., sp. nov., a methanol- and methylamine-reducing methanogen from the hindgut of the cockroach *Periplaneta americana*. *Int J Syst Evol Micr* **50**: 1989-1999.

- Springer E, Sachs MS, Woese CR & Boone, DR (1995) Partial gene sequences for the A subunit of methyl-coenzyme M reductase (mcrI) as a phylogenetic tool for the family *Methanosarcinaceae*. *Int J Syst Bacteriol* **45**:554-559.
- Stackebrandt E & Goebel BM (1994) Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**: 846-849.
- Stahl DA, & Amann RI (1991) Development and application of nucleic acid probes. In *Nucleic acid techniques in bacterial systematics* (Stackebrandt E & Goodfellow M, eds), pp. 205-248. John Wiley & Sons, New York.
- Steinfeld H, Gerber P, Wassenaar T, Castel V, Rosales M & Haan C (2006) Livestock's long shadow: environmental issues and options. Rome: Food and Agriculture Organization of the United Nations. Available: <http://www.fao.org/docrep/010/a0701e/a0701e00.HTM>. Accessed 2010 Sep 28.
- Stewart CS & Richardson AJ (1989) Enhanced resistance of anaerobic rumen fungi to the ionophores monensin and lasalocid in the presence of methanogenic bacteria. *J Appl Bacteriol* **66**: 85-93.
- Stewart CS, Flint HJ & Bryant MP (1997) The rumen bacteria. In *The Rumen Microbial Ecosystem* (Hobson PN & Stewart CS eds), pp.10-72. Chapman and Hall, London.
- Stubner S (2004) Quantification of Gram-negative sulphate-reducing bacteria in rice field soil by 16S rRNA gene-targeted real-time PCR. *J Microbiol Meth* **57**: 219-230.
- Stumm CK, Gijzen HJ & Vogels GD (1982) Association of methanogenic bacteria with ovine rumen ciliates. *Br J Nutr* **47**: 95-99.
- Sundset MA, Edwards JE, Cheng YF, Senosiain RS, Fraile MN, Northwood KS, Praesteng KE, Glad T, Mathiesen SD & Wright AD (2009a) Molecular diversity of the rumen microbiome of Norwegian reindeer on natural summer pasture. *Microb Ecol* **57**:335-348.

- Sundset MA, Edwards JE, Cheng YF, Senosiain RS, Fraile MN, Northwood KS, Praesteng KE, Glad T, Mathiesen SD & Wright AD (2009b) Rumen microbial diversity in Svalbard reindeer, with particular emphasis on methanogenic archaea. *FEMS Microbiol Ecol* **70**: 553-562.
- Sundset MA, Præsteng KE, Cann IKO, Mathiesen SD and Mackie RI (2007) Novel rumen bacterial diversity in two geographically separated sub-species of reindeer. *Microbial Ecology* **54**:424-438
- Swainson NM, Hoskin SO, Clark H, Pinares-Patiño CS & Brookes I (2008) Comparative methane production and yields from adult cattle, red deer and sheep. *Aust J Exp Agric* **48**: lxxix-lxxx.
- Tajima K, Aminov RI, Nagamine T, Matsui H, Nakamura M & Benno Y (2001a) Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Appl Environ Microbiol* **67**: 2766-2774.
- Tajima K, Nagamine T, Matsui H, Nakamura M & Aminov RI (2001b) Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. *FEMS Microbiol Lett* **200**: 67-72.
- Tajima K, Nonaka I, Higuchi K, Takusari N, Kurihara M, Takenaka A, Mitsumori M, Kajikawa H & Aminov RI (2007) Influence of high temperature and humidity on rumen bacterial diversity in Holstein heifers. *Anaerobe* **13**: 57-64.
- Tatsuoka N, Mohammed N, Mitsumori M, Hara K, Kurihara M & Itabashi H (2004) Phylogenetic analysis of methyl coenzyme-M reductase detected from the bovine rumen. *Lett Appl Microbiol* **39**: 257-260.
- Tamura K, Dudley J, Nei M & Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**: 1596-1599.
- Teunissen MJ, Kets EP, Op den Camp HJ, Huis in 't Veld JH & Vogels GD (1992) Effect of coculture of anaerobic fungi isolated from ruminants and non-ruminants with methanogenic bacteria on cellulolytic and xylanolytic enzyme activities. *Arch Microbiol* **157**: 176-182.

- Tichopad A, Didier A & Pfaffl MW (2004) Inhibition of real-time RT-PCR quantification due to tissue-specific contaminants. *Mol Cell Probe* **18**:45-50.
- Tichopad A, Dilger M, Schwarz G & Pfaffl MW (2003) Standardized determination of real-time PCR efficiency from a single reaction set-up. *Nucl Acids Res* **31**: e122.
- Tokura M, Ushida K, Miyazaki K & Kojima Y (1997) Methanogens associated with rumen ciliates. *FEMS Microbiol Ecol* **22**: 137-143.
- Tokura M, Chagan I, Ushida K & Kojima Y (1999) Phylogenetic study of methanogens associated with rumen ciliates. *Curr Microbiol* **39**: 123-128.
- Tokura M, Ohkuma M & Kudo T (2000) Molecular phylogeny of methanogens associated with flagellated protists in the gut and with the gut epithelium of termites. *FEMS Microbiol Ecol* **33**: 233-240.
- Tschech A & Pfennig N (1984) Growth yield increase linked to caffeine reduction in *Acetobacterium woodii*. *Arch Microbiol* **137**:163-167.
- United-Nations (1997) Kyoto protocol to the United Nations Framework Convention on Climate Change. UNEP.IUC/99/10. Chatlelaine, Switzerland, United Nations Environment Programme's Information Unit for Conventions, for the Climate change secretariat. Available: <http://unfccc.int/resource/docs/convkp/kpeng.pdf>. Accessed 2009 Oct 10.
- Ushida K & Jouany JP (1996) Methane production associated with rumen-ciliated protozoa and its effect on protozoan activity. *Lett Appl Microbiol* **23**: 129-132.
- Vogels GD, Hoppe WF & Stumm CK (1980) Association of methanogenic bacteria with rumen ciliates. *Appl Environ Microbiol* **40**: 608-612.
- von Wintzingerode F, Gobel UB & Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* **21**: 213-229.
- Watanabe T, Asakawa S, Nakamura A, Nagaoka K & Kimura M (2004) DGGE method for analyzing 16S rDNA of methanogenic archaeal community in paddy field soil. *FEMS Microbiol Lett* **232**: 153-163.

- Wedlock DN, Pedersen G, Denis M, Dey D, Janssen PH & Buddle BM (2010) Development of a vaccine to mitigate greenhouse gas emissions in agriculture: Vaccination of sheep with methanogen fractions induces antibodies that block methane production *in vitro*. *NZ Vet J* **58**: 29-36.
- Weimer PJ (1998). Manipulating ruminal fermentation: a microbial ecological perspective. *J Anim Sci* **76**: 3114-3122.
- Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S Ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**: 697-703.
- Weiss A, Jerome V, Freitag R & Mayer HK (2008) Diversity of the resident microbiota in a thermophilic municipal biogas plant. *Appl Microbiol Biotechnol* **81**: 163-173.
- Whitehead TR & Cotta MA (1999) Phylogenetic diversity of methanogenic archaea in swine waste storage pits. *FEMS Microbiol Lett* **179**: 223-226.
- Whitford MF, Teather RM & Forster RJ (2001) Phylogenetic analysis of methanogens from the bovine rumen. *BMC Microbiol* **1**: 5.
- Whitman WB, Bowen TL & Boone DR (1991) The methanogenic bacteria. In *The Prokaryotes* (Balows A, Truper HG, Dworkin M, Harder W & Schleifer KJ, eds), pp. 719-767. Springer Verlag, New York.
- Widdel F, Kohring GW & Mayer F (1983) Studies on dissimilatory sulfate reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov., and *Desulfonema magnum* sp. nov. *Arch Microbiol* **134**: 286-294.
- Williams AG & Coleman GS (1997) The Rumen Protozoa. In *The Rumen Microbial Ecosystem* (Hobson PN & Stewart CS, eds), pp. 73-139. Chapman and Hall, London.

- Williams YJ, Rea SM, Popovski S, Pimm CL, Williams AJ, Toovey AF, Skillman LC & Wright AD (2008) Responses of sheep to a vaccination of entodinal or mixed rumen protozoal antigens to reduce rumen protozoal numbers. *Br J Nutr* **99**: 100-109.
- Williams YJ, Popovski S, Rea SM, Skillman LC, Toovey AF, Northwood KS & Wright AD (2009) A vaccine against rumen methanogens can alter the composition of archaeal populations. *Appl Environ Microbiol* **75**:1860-1866.
- Wintzingerode F, Gobel UB & Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* **21**: 213-229.
- Woese CR (1987) Bacterial evolution. *Microbiol Rev* **51**: 221-271.
- Woese CR, Kandler O & Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc Natl Acad Sci USA* **87**: 4576-4579.
- Wolin MJ, Miller TL & Stewart CS (1997) Microbe-microbe interactions. In *The Rumen Microbial Ecosystem* (Hobson PN & Stewart CS, eds), pp. 10-72. Chapman & Hall, London.
- Wood JM, Kennedy FS & Wolfe RS (1968) The reaction of multihalogenated hydrocarbons with free and bound reduced Vitamin B₁₂. *Biochemistry* **7**: 1707-1713.
- Wood J, Scott KP, Avgustin G, Newbold CJ & Flint HJ (1998) Estimation of the relative abundance of different *Bacteroides* and *Prevotella* ribotypes in gut samples by restriction enzyme profiling of PCR-amplified 16S rRNA gene sequences. *Appl Environ Microbiol* **64**: 3683-3689.
- Wright AD, Auckland CH & Lynn DH (2007) Molecular diversity of methanogens in feedlot cattle from Ontario and Prince Edward Island, Canada. *Appl Environ Microbiol* **73**: 4206-4210.

- Wright AD, Kennedy P, O'Neill CJ, Toovey AF, Popovski S, Rea SM, Pimm CL & Klein L (2004a) Reducing methane emissions in sheep by immunization against rumen methanogens. *Vaccine* **22**: 3976-3985.
- Wright AD, Ma X & Obispo NE (2008b) *Methanobrevibacter* phylotypes are the dominant methanogens in sheep from Venezuela. *Microb Ecol* **56**: 390-394.
- Wright AD, Northwood KS & Obispo NE (2009) Rumen-like methanogens identified from the crop of the folivorous South American bird, the hoatzin (*Opisthocomus hoazin*). *ISME J* **3**: 1120-1126.
- Wright AD & Pimm C (2003) Improved strategy for presumptive identification of methanogens using 16S riboprinting. *J Microbiol Methods* **55**: 337-349.
- Wright AD, Toovey AF & Pimm CL (2006) Molecular identification of methanogenic archaea from sheep in Queensland, Australia reveal more uncultured novel archaea. *Anaerobe* **12**: 134-139.
- Wright AD, Williams AJ, Winder B, Christophersen CT, Rodgers SL & Smith KD (2004b) Molecular diversity of rumen methanogens from sheep in Western Australia. *Appl Environ Microbiol* **70**: 1263-1270.
- Wuebbles DJ & Hayhoe K (2002) Atmospheric methane and global change. *Earth-Sci Rev* **57**: 177-210.
- Yanagita K, Kamagata Y, Kawaharasaki M, Suzuki T, Nakamura Y & Minato H (2000) Phylogenetic analysis of methanogens in sheep rumen ecosystem and detection of *Methanomicrobium mobile* by fluorescence *in situ* hybridization. *Biosci Biotechnol Biochem* **64**:1737-1742.
- Yu Z, Garcia-Gonzalez R, Schanbacher FL & Morrison M (2008) Evaluations of different hypervariable regions of archaeal 16S rRNA genes in profiling of methanogens by Archaea-specific PCR and denaturing gradient gel electrophoresis. *Appl Environ Microbiol* **74**: 889-893.
- Zengler K, Toledo G, Rappe M, Elkins J, Mathur EJ, Short JM & Keller M (2002) Cultivating the uncultured. *Proc Natl Acad Sci U S A* **99**: 15681-15686.

- Zhang H, Banaszak JE, Parameswaran P, Alder J, Krajmalnik-Brown R & Rittmann BE (2009) Focused-Pulsed sludge pre-treatment increases the bacterial diversity and relative abundance of acetoclastic methanogens in a full-scale anaerobic digester. *Water Research* **43**: 4517-4526.
- Zhou M, Hernandez-Sanabria E & Guan LL (2009) Assessment of microbial ecology of ruminal methanogens in cattle with different feed efficiency. *Appl Environ Microbiol* **75**: 6524-6533.
- Zhou M, Hernandez-Sanabria E & Guan LL (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 Microbiol* **76**: 3776-3786
- Zinder SH (1993) Physiological ecology of methanogens. In *Methanogenesis: ecology, physiology, biochemistry, and genetics* (Ferry JG, eds), pp. 128-206. Chapman and Hall, New York, USA.

Appendix 1

The details of gene sequences of clones and pure cultures of the thesis

The 16S rRNA and *mcrA* gene sequences obtained in this study are saved on the enclosed and the key for the sequences is given below.

Folders	Sub folders	No of sequences	Name of the sequences	Details	
Chapter 3	16SC2P1	47	C2SP1001- C2SP1048	Partial 16S rRNA gene clone sequences of primer pair P1	
	16SC2P2	59	C2SP2001- C2SP2059	Partial 16S rRNA gene clone sequences of primer pair P2	
	16SC2P3	61	C2SP3001- C2SP3061	Partial 16S rRNA gene clone sequences of primer pair P3	
	16SC2P4	47	C2SP4001- C2SP4047	Partial 16S rRNA gene clone sequences of primer pair P4	
	16SC2P5	46	C2SP5001- C2SP5046	Partial 16S rRNA gene clone sequences of primer pair P5	
	16SC2P6	49	C2SP6001- C2SP6049	Partial 16S rRNA gene clone sequences of primer pair P6	
	MC2M-P1	51	C2SMP1001- C2SMP1051	Partial <i>mcrA</i> gene clone sequences of primer pair M-P1	
	MC2M-P2	49	C2SMP2001- C2SMP2049	Partial <i>mcrA</i> gene clone sequences of primer pair M-P2	
	MC2M-P3	64	C2SMP3001- C2SMP3064	Partial <i>mcrA</i> gene clone sequences of primer pair M-P3	
	MC2M-P4	14	C2SMP4001- C2SMP4014	Partial <i>mcrA</i> gene clone sequences of primer pair M-P4	
	MPureculture		5	<i>Methanobrevibacter</i> sp. NT7	Partial <i>mcrA</i> gene sequence of <i>Methanobrevibacter</i> sp. NT7
				<i>Methanobrevibacter</i> sp. SM9	Partial <i>mcrA</i> gene sequence of <i>Methanobrevibacter</i> sp. SM9
				<i>Methanobrevibacter</i> sp. 31A	Partial <i>mcrA</i> gene sequence of <i>Methanobrevibacter</i> sp. 31A
			<i>Methanobrevibacter thaueri</i>	Partial <i>mcrA</i> gene sequence of <i>Mbb thaueri</i> (DSM 11995)	
		<i>Methanomicrococcus blatticola</i>	Partial <i>mcrA</i> gene sequence of <i>Methanomicrococcus blatticola</i> (DSM 13328)		

Chapter 4	All the sequences of this chapter were deposited in the GenBank and the details are given in the Chapter 4			
Chapter 5	16SC1-T	24	C1SP1T001-C1SP1T024	Partial 16S rRNA gene sequences from CHCl ₃ -treated cow 1
	16SC2-C	47	C2SP1001-C2SP1047	Partial 16S rRNA gene sequences from control cow 2
	16SC3-T	41	C3SP1T001-C3SP1T041	Partial 16S rRNA gene sequences from CHCl ₃ -treated cow 3
	16SC4-C	21	C4SP1001-C4SP1021	Partial 16S rRNA gene sequences from control cow 4
	16SC6-C	24	C6SP1001-C6SP1024	Partial 16S rRNA gene sequences from control cow 6
	16SC7-T	22	C7SP1T001-C7SP1T022	Partial 16S rRNA gene sequences from CHCl ₃ -treated cow 7
	MCRC2-C	51	C2SMP1001-C2SMP1051	Partial <i>mcrA</i> gene sequences from control cow 2
	MCRC3-T	48	C3SMP1T001-C3SMP1T048	Partial <i>mcrA</i> gene sequences from CHCl ₃ -treated cow 3
Chapter 6	16S-ISO3-F5	1	16S-ISO3-F5	Partial 16S rRNA gene sequence of ISO3-F5
	16S-ISO4-G1	1	16S-ISO4-G1	Partial 16S rRNA gene sequence of ISO4-G1
	16S-ISO4-G11	1	16S-ISO4-G11	Partial 16S rRNA gene sequence of ISO4-G11
	16S-ISO4-G16	1	16S-ISO4-G16	Partial 16S rRNA gene sequence of ISO4-G16
	16S-ISO4-H5	1	16S-ISO4-H5	Partial 16S rRNA gene sequence of ISO4-H5
	M-ISO3-F5	1	MCR-ISO3-F5	Partial <i>mcrA</i> gene sequence of ISO3-F5
	M-ISO4-G1	1	MCR-ISO4-G1	Partial <i>mcrA</i> gene sequence of ISO4-G1
	M-ISO4-G11	1	MCR-ISO4-G11	Partial <i>mcrA</i> gene sequence of ISO4-G11
	M-ISO4-G16	1	16S-MCR-G16	Partial <i>mcrA</i> gene sequence of ISO4-G11
	M-ISO4-H5	1	16S-MCR-H5	Partial <i>mcrA</i> gene sequence of ISO4-H5

	Bac-ISO4-G1	2	Bac-ISO4-G1-1- Bac-ISO4-G1-2	Partial 16S rRNA gene sequences of bacterial contaminants
	Bac-ISO4-G11	1	Bac-ISO4-G11	Partial 16S rRNA gene sequence of bacterial contaminant
	Bac-ISO4-H5	1	Bac-ISO4-H5	Partial 16S rRNA gene sequences of bacterial contaminants
Chapter 7	16S-ISO3-F5	1	16S-ISO3-F5	Partial 16S rRNA gene sequence of ISO3-F5
	M-ISO3-F5	1	MCR-ISO3-F5	Partial <i>mcrA</i> gene sequence of ISO3-F5
	16S- <i>Msp.cuniculi</i>	1	<i>Methanosphaera cuniculi</i>	Partial 16S rRNA gene sequence of <i>Methanosphaera cuniculi</i>
	mcrA- <i>Msp.cuniculi</i>	1	<i>Methanosphaera cuniculi</i>	Partial <i>mcrA</i> gene sequence of <i>Methanosphaera cuniculi</i>
