Identification of rumen methanogens, characterization of substrate requirements and measurement of hydrogen thresholds

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Abstract

In New Zealand, exported farmed commodities derived from ruminants make up about one-third of the nation's economy. However, farming ruminants creates a significant environmental impact by emitting methane which is a by-product of the microbial fermentation occurring in the rumen. Accumulated methane in the atmosphere is considered to be an important contributing factor to global warming and climate change. Methanogenic archaea, collectively called methanogens, inhabiting the rumen are responsible for the production of ruminal methane. These organisms are capable of anaerobically reducing CO_2 to CH_4, using H_2, formate, methanol, a range of methyl-compounds, or acetate as electron-donors. Currently, all known methanogens that have been isolated from a diverse range of habitats are classified into 28 genera and 113 species based on the study of pure cultures and analysis of small subunit rRNA gene sequence data. Less than 10% of these species were isolated from the rumen and these reflect only a small portion of the true rumen methanogen diversity that has been determined by cultivation-independent methods. This project has been derived from the necessity to characterise genome sequences of a greater diversity of rumen methanogens than is currently covered in public culture collections. 14 methanogen strains were isolated as pure cultures and identified based on 16S rRNA and *mcrA* gene sequences in order to create a comprehensive phylogenetic tree comparing the genetic distances between the newly identified strains and the few named species. Strains 229/11, AbM4, M1, SM9, G16, D5, BRM9, YCM1, ISO3-F5, and A4 were then selected to be characterised for their substrate requirements for growth, by systematically omitting single or multiple components from the growth medium. Finally, the threshold levels of hydrogen, below which the methanogens fail to use it as a substrate, were measured for these strains by gas chromatography. Overall, the H_2 thresholds of rumen methanogens fell within the range between 0.5 and 5.8 Pa. *Methanobrevibacter*, the most predominant group of methanogens

occurring in the rumen, had relatively higher H_2 thresholds compared to the genus *Methanosphaera*, a group of methanogens frequently isolated from New Zealand ruminants, and the genus *Methanobacterium.*

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Chapter 1

Literature review

1.1 Introduction

This literature review aims to summarize the current state of knowledge regarding (1) the phylogenetic diversity of rumen methanogens based on comparative studies of 16S rRNA and *mcr*A gene sequences; (2) currently available culture-dependent and culture-independent techniques for surveying rumen methanogens; (3) the process of methanogenesis and substrate utilization by methanogens; (4) the competition for H_2 among microorganisms occupying the rumen; and (5), methane mitigation strategies that have been developed and attempted in real-life applications.

Greenhouse gases often elicit a negative response from the general public, as the term is frequently used by the media to illuminate the causes of climate change. Without the natural greenhouse effect, the life we know would not have been possible, as the average Earth surface temperature would likely to have remained below the freezing point of water (IPCC, 2007). The sun radiates its energy at very short wavelengths, a large part of it in the form of the ultraviolet spectrum (IPCC, 2007). Approximately two-thirds of the solar energy that reaches the earth is absorbed by the atmosphere, while the remaining energy is reflected back into the space (IPCC, 2007). The Earth radiates the same amount of energy back into the space as the incoming energy, but at much longer wavelengths, predominantly in the infrared part of the spectrum (IPCC, 2007). This emitted energy, however, becomes trapped by the atmosphere, which acts similarly to the walls in a greenhouse, and reradiates the energy back into the Earth (IPCC, 2007). An quilibrium is reached, which determines the mean global temperature. If the properties of the atmosphere change, a different equilibrium is reached, and so the mean temperature will be different.

Changes in the composition of the Earth's atmosphere can be caused by a wide range so-called greenhouse gases produced by human activities including burning of fossil fuels and changing land uses (e.g. clearing of forests for logging, ranching and agriculture). Many greenhouse gases occur naturally, such as water vapour, carbon dioxide, methane, nitrous oxide and ozone (IPCC, 2007). Water vapour is the most important greenhouse gas, closely followed by carbon dioxide. Others such as hydrofluorocarbons (HFCs), perfluorocarbons (PFCs) and sulphur hexafluoride (SF_6), are the by-products of human industrial processes (IPCC, 2007). Accumulation of these heat-trapping gases in the atmosphere results in an increase in the average global temperature, leading to alteration of weather patterns which may hasten species extinction, melting of ice in Arctic and Antarctic areas, and desertification (IPCC, 2007).

Faced with apparent signs of climate change, the UN launched the Framework Convention on Climate Change (UNFCCC), with a goal of fostering conferences in which international decisions on effective reduction of greenhouse gas emissions at a global level would be made. In 1997, 174 countries, including New Zealand, signed an agreement called the "Kyoto Protocol' which enforced strict limitations on the level of allowed greenhouse gas emissions (United Nations, 1997).

Although on a global scale, New Zealand's contribution to total greenhouse gas emissions is small, only 0.2% of total estimated anthropogenic emissions, New Zealand's commitments under the Kyoto Protocol requires that the nation be responsible for emissions excesses of 1990 levels in the first commitment period (2008-2012) (IPCC, 2007). Exported dairy products, meat, wool, and leather products are the New Zealand's biggest source of earnings, and make up 42% of the nation's commodity exports (Statistics New Zealand, 2011). These products are derived almost totally from farmed ruminants. The heavy economic dependence of New Zealand on its farmed ruminants makes the nation particularly

vulnerable to agriculture-responsible methane emissions. Enteric emissions of methane from farmed ruminants raised in New Zealand are responsible for 48% of the country's total greenhouse gas emissions (IPCC, 2007). Annually, an estimate of 80 million tonnes of methane gas is produced by ruminant livestock bred in New Zealand, which accounts for 36% of the total national atmospheric methane emissions, making ruminant farming the most significant source of anthropogenic methane emissions by far (Ministry for the Environment, 2012). Methane is one of the most important greenhouse gases. Its heat-trapping potential is twenty five-fold larger than CO_2 (Forster *et al*., 2007).

This unique situation of agricultural methane emissions being the single largest source of the nation's total greenhouse gas emissions propelled the livestock industry (dairy, sheep, beef and deer) in partnership with the NZ government to form the Pastoral Greenhouse Gas Research Consortium (PGgRc) in 2002, as an industry investor whose goal is to develop greenhouse gas mitigation solutions that can be implemented within the agricultural industry of New Zealand (Attwood *et al*., 2008; Leslie *et al*., 2008).

Methane is a metabolic by-product of methanogenic microbes that inhabit the rumen. The rumen is essentially a compartmentalised bioreactor that harbours bacteria, archaea, protozoa, fungi, and phage (Frey *et al*., 2009). These organisms carry out the degradation of ingested plant materials into fermentation products (e.g. H_2, acetate, propionate, and butyrate), some of which are absorbed across the rumen epithelium where they are used as the energy for ruminants (Janssen, 2010). Under the anaerobic conditions of the rumen, the build-up hydrogen gas from the partial oxidation of fermentation products can be detrimental to proper functioning of the rumen (Janssen, 2010). Methanogens essentially keep the fermentation processes in the rumen running efficiency by removing H_2 during the reduction of CO_2 to methane (Janssen, 2010). The process of methanogenesis has been studied extensively. The current research efforts are focused on elucidating a complete phylogenetic diversity of

methanogen species inhabiting the rumen. The most challenging aspect of developing effective strategies for methane mitigation is devising a way to reduce methane production by the majority of the methanogen species that inhabit the rumen. Thus, it is important to gain a comprehensive understanding of the diversity of methanogens, the complexity of rumen microbial interactions, the role of rumen methanogens, and the strategies which they adopt for survival in the rumen environment. Until now, methane mitigation strategies involving vaccination, the use of feed additives and small molecule inhibitors, controlled diets, and defaunation efforts have been attempted, but without notable success. A genome-based approach might provide more insight into various aspects of rumen methanogens. Unfortunately, *Methanobrevibacter ruminantium* is the only currently available rumen methanogen species whose genome has been fully sequenced (Leahy *et al*., 2010). However, the number of complete genome sequences of rumen methanogens is expected to increase, which opens up the possibility of using genome-derived information to find solutions for reducing the methane production from rumen methanogens (Attwood *et al*., 2011).

1.2 Rumen methanogens and methanogenesis

The rumen forms the larger part of the reticulorumen, and is effectively a chamber that retains ingested feedstuffs (Russell & Rychlik, 2001). Large particles of digest become propelled up into the oesophagus and mouth during contractions of the reticulum (Russell & Rychlik, 2001). The partially digested food is chewed in the mouth in a process known as rumination, and then is swallowed back down the oesophagus to be settled down in the rumen once more (Russell & Rychlik, 2001). Reticular contractions mix the small particles with liquid, and push them through reticulo-omasal/orifice, which leads to the next chamber in the ruminant digestive tract, the omasum. Ruminants thus provide suitable habitats and a continuous supply of fresh nutrients to fibrolytic rumen microorganisms that convert plant cell wall polysaccharides into fermentation products such as proteins, vitamins, volatile fatty

acids (VFAs) such as acetic, propionic and butyric acid, and short-chain organic acids that become absorbed across the rumen epithelium (Mitsumori & Sun, 2008). Ruminant animals digest fibrous plant materials by harbouring bacteria, fungi and protozoa that produce fibre-degrading enzymes (Janssen, 2010). Microbial proteins constitute as much as 90% of amino acids reaching the small intestine. A variety of ruminal bacteria-produced end products including formate and H_2 become subjected to secondary fermentation by other microbial species (Mitsumori & Sun, 2008). The rumen is a strictly anaerobic habitat in which substrates are only partially oxidized. Thus, removal of hydrogen gas derived from reducing equivalents (e.g. NADH) is a critical feature of rumen fermentation, since the build-up of hydrogen within the rumen is thermodynamically unfavourable for plant fibre fermentation (Morgavi *et al*., 2010). Rumen methanogens are at the bottom of the trophic chain and are capable of using H_2, formate and methanol to produce methane via a pathway coupled to ATP synthesis (Morgavi *et al*., 2010). Efficient H_2 removal by methanogens has a profound effect on functioning of the rumen fermentation system as the build-up of H_2 inhibits the re-oxidation of coenzymes involved in redox reactions within bacterial cells, creating a less favourable environment for VFA formation (Janssen, 2010). Although only a small proportion of the rumen microbial ecosystem is occupied by the methanogens (Janssen, 2008), these organisms contribute a large part of rumen function and therefore are potentially significant for the animal's nutrition.

Methanogens are archaea, and fall within the kingdom Euryarchaeota. They are obligate anaerobes that produce methane as a major metabolic end product. In a new-born ruminant, the size of the rumen is smaller than that of the abomasum in accordance with their milk-constituent diet (Skillman *et al*., 2004). As their diet changes to solid feeds, the rumen develops rapidly, and become quickly colonized by obligate anaerobes, facultative anaerobes and aerobic bacteria (Skillman *et al*., 2004). A fully-developed rumen quickly becomes taken

over by a large population of obligate anaerobes (Skillman *et al*., 2004). Methanogens establish themselves in the rumen soon after birth, and increase in number in an exponential manner in the first few weeks after birth (Skillman *et al*., 2004).

1.3 Substrate utilization and methane production

Methanogens produce methane by using three major substrates; CO_2, methyl group-containing compounds, or acetate. However, the slow rate of methane formation from acetate and the high turnover rate in the rumen prevent the aceticlastic pathway from being significantly used to produce methane in the rumen (Attwood & McSweeney, 2008). The hydrogenotrophic methanogens that use CO_2 or acetate as their carbon source and H_2 as the main electron donor play a dominant role during methanogenesis in the rumen (Fig. 1.1). During the oxidation of sugars via the Embden-Meyerhof-Parnas pathway in bacteria, fungi and protozoa, electron carrying cofactors such as NADH must be reoxidized to NAD^+ to allow fermentation to continue (McAllister & Newbold, 2008). Under the anaerobic conditions of the rumen, where it is impossible to use oxygen as an acceptor of electron transfers to regenerate NAD^+, the reduction of CO_2 to CH_4 is the major sink that allows recycling of reduced cofactors (Morgavi *et al*., 2010). In hydrogenotrophic methanogenesis, CO_2 is initially carried by methanofuran (MFR) and is reduced to formate (Liu & Whitman, 2008). The electrons involved in this first step are donated by ferredoxin (Fd) reduced with H_2. The formyl group is transferred to tetrahydromethanopterin (H_4MPT), forming formyl-H_4MPT in a reaction catalysed by formyl methanofuran:tetrahydromethanopterin formyltransferase (Liu & Whitman, 2008). The formyl group is then successively reduced to methenyl-H_4MPT and then to methylene-H_4MPT in reactions catalysed by 5,10-methenyl tetrahydromethanopterin cyclohydrolase, and methylene-tetrahydromethanopterin:coenzyme F_{420} oxidoreductase, respectively (Liu & Whitman, 2008). In the next step, a reaction catalysed by methyl-H_4MPT:HS-CoM methyltransferase (Mtr) transfers the methyl group to

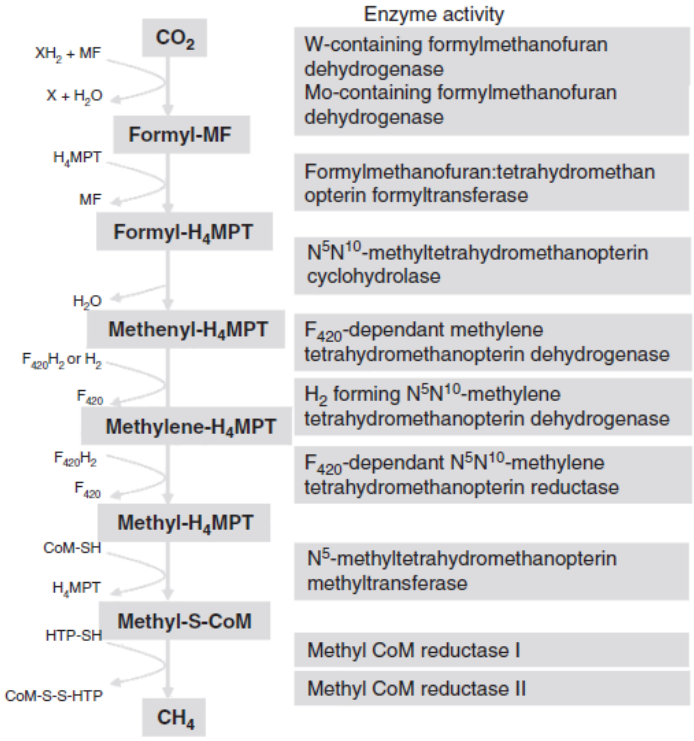

Figure 1.1 Methanogenesis pathway from H_2 +CO_2. The seven-step enzymatic pathway for the formation of methane in hydrogenotrophic methanogens is shown (Attwood & McSweeney, 2008).

H_4MPT, forming methyl-H_4MPT. In the last step of methanogenesis, methyl-CoM is reduced to methane by the action of methyl coenzyme M reductase (Mcr) (Liu & Whitman, 2008). Coenzyme B-coenzyme M (CoB-S-S-CoM) heterodisulfide forms and becomes subsequently reduced to generate the CoB-SH and CoM-SH thiols (Liu & Whitman, 2008). The methyl transfer from H_4MPT to CoM and the reduction of heterodisulfide are both exergonic reactions that are highly favourable for ATP synthesis. The exact mechanism of ATP formation remains controversial (Liu & Whitman, 2008).

Methanogens isolated from a wide range of extreme habitats have been described, and among these, hydrogenotrophs use H_2 to reduce CO_2 to generate methane (Garcia *et al.*, 2000). Hydrogenotrophic methanogens are capable of simultaneously using formate to form methane (Garcia *et al.*, 2000). Methane production from formate makes up approximately 15-

20% of the total methane production in the rumen (Hungate *et al*., 1970). During formatotrophic methanogenesis, four molecules of formate are oxidized to form CO_2 by formate dehydrogenase (FdH). The members of the order *Methanosarcinales* and *Methanosphaera* spp. from the order *Methanobacteriales* have been classified into methylotrophs that use methyl-containing compounds such as methanol, methylamines or dimethylsulfide to produce methane. During methanogenesis, a methyl group from methyl-containing compounds enter the pathway in the form of methyl-CoM, which is subsequently reduced to methane. Acetate is a preferred substrate for *Methanosarcina* and *Methanosaeta* that produce methane via aceticlastic pathway (Liu & Whitman, 2008). Acetate is a preferred substrate for methanogens inhabiting fresh water ecosystems; approximately 60~80% of methane produced from the fresh water environments is generated from acetate (Castro *et al*., 2004). During acetoclastic methanogenesis, acetate splits to form carboxyl compounds that become oxidized to CO_2. Methyl groups from CO_2 then enters the hydrogenotrophic pathway to form methane. Contribution of methyl groups and acetate as substrates for methanogenesis in the rymen is likely to be minimal as the methanogens that depend on these conversions for producing methane have a very slow growth rate *in vitro*, suggesting the short retention time exerted by normal rumen conditions would prevent them from thriving (Janssen & Kirs, 2008).

1.4 Techniques for culturing methanogens

Cultivation-based studies of the majority of microorganisms isolated from the natural habitats are typically unsuccessful due to the difficulties of growing these organisms under laboratory conditions. Methanogens are strict anaerobes and are extremely difficult to cultivate, as a sufficient exclusion of oxygen is essential for their growth. Furthermore, methanogens require a long incubation period and the growth of some methanogens is heavily dependent on a partnership with the syntrophic organisms (Bryant *et al*., 1967). The

first pure cultures of *Methanobacterium formicicum* and *Methanobrevibacter ruminantium*, both isolated from the bovine rumen, were obtained by using anaerobic cultivation techniques developed by Hungate (1950). The technique consists of preparing and inoculating media in a continuous presence of the hydrogen/CO_2 mixture. The tubes are sealed with butyl rubber stoppers to maintain anaerobic conditions. Manipulating Petri dishes is difficult under strict anaerobic conditions; instead, anaerobic culture tubes are often used because they allow inoculation of methanogens onto an agar surface which was spread evenly over the inner surface of the Hungate tubes (roll tubes), and sometimes, that of the serum bottles (Miller and Wolin, 1974). Later, simple modifications of the Hungate technique were developed to maintain and grow cells under strict anaerobic conditions. Uffen *et al*. (1970) used a prescription bottle onto which the agar was allowed to solidify on its flat side of the bottle, while a stream of O_2-free, sterile gas was passed into the bottle. However, the use of roll tubes or agar bottles is disadvantageous when picking isolated colonies, as well as when proceeding with standard genetic techniques such as replica plating. To overcome these drawbacks, a system that makes use of an anaerobic glove "box" or "chamber", which permits cultivating methanogens on Petri dishes has been developed (Edwards and McBride, 1975). The "box" itself contains an oxygen-free atmosphere, thus minimizing the risk of potential exposure of the microorganisms to lethal oxygen levels. The anaerobic chamber provides the convenience of using conventional methodology that does not involve complicated and time-consuming anaerobic techniques to isolate single colonies of methanogens. The anaerobic chamber techniques were later modified to grow methanogens on Petri dishes stacked inside a pressurized anaerobic cylinder which enables the cultures to be taken out from the chamber and then incubated at a desirable temperature (Hermann *et al*., 1986).

Even after the anaerobic requirements are fulfilled, cultivating methanogens has proven to be difficult, probably because of the inability to emulate the nutrients and substrates, the physical conditions, and perhaps even biotic interactions in their environment.

Sakai *et al*. (2008) demonstrated the effectiveness of a co-culture method for isolating methanogens from a variety of environmental samples, including soils from rice fields, and sediments from lakes and marine waters. To obtain detectable growth, a high H_2 partial pressure is generally applied during the cultivation and isolation of the organisms, but this is not a true reflection of their natural habitats, as such high levels of hydrogen rarely occur in nature. While high H_2 levels may favour the growth of fast-growing methanogens that thrive under high H_2 concentrations, the selective advantage might come at the expense of other methanogens that do not grow as fast even when hydrogen concentrations are high. Methanogens were enriched from various samples in the presence of a pure culture of *Syntrophobacter fumaroxidans* strain MP08. *S. fumaroxidans* is a syntrophic bacterium that oxidises propionate, butyrate or ethanol, to produce H_2. The taxonomic positions of the methanogens recovered from these low-hydrogen co-cultures were different to the conventional methanogen taxonomic profiles obtained under high H_2 partial pressure, from which only a very limited range of phylotypes are recovered. The 16S rRNA gene analysis of the co-cultures revealed the presence of methanogens species such as *Methanolinea tarda*, and *Candidatus Methanoregula boonei* of the group E1/E2, an archaeal lineage which has only a few cultivated representatives so far. Novel methanogens belonging to the group E1/E2 and/or the order *Methanocellales* were also found in large numbers and one of the novel phylotypes was successfully isolated. The co-culture method doubtless demonstrated its effectiveness by circumventing the difficulties of growing methanogens that avoid cultivation in traditional ways. However, considering the extended incubation period required

to grow these methanogens, it is likely that they would be easily outcompeted by the fast-growing strains in the rumen environment.

In most cases, the medium which was used to isolate and cultivate the methanogenic archaea is a design by Hungate (1950). The medium includes bovine rumen fluid as an essential culture ingredient, along with other substrates, such as $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, $CaCl_2$ and $MgSO_4$, that are commonly found in the animal rumen. The medium also contains a reducing agent and an oxidation-reduction indicator (e.g. resazurin). Cysteine hydrochloride is commonly used as a reducing agent, while the exclusion of oxygen is indicated by the reaction of resazurin under reduced conditions to become colourless.

1.5 Identification of rumen methanogens

1.5.1 16S rRNA gene sequence analysis

Culture-based techniques are not very adequate tools for studying methanogens because (1) methanogens are slow to grow; (2) only a minor fraction of any microbial ecosystems is represented by methanogens; (3) some methanogens are more readily cultivated than others, thus giving an impression of more frequent appearances in the environment than the reality; and (4) phenotypic discrimination of methanogens are not practical as they have a very limited physiological diversity. The advent of molecular techniques, mainly the Polymerase Chain Reaction (PCR), has enabled a qualitative, and a semi-quantitative description of the community structure of natural microbial ecosystems based on the rRNA gene sequencing method, without having to obtain pure cultures of the organisms. Of course, data from cultured strains are heavily used in the databases used to compare new data from environmental samples.

Methanogens were one of the first microbial groups to have their phylogenetic framework constructed based on 16S rRNA gene sequencing. A typical 16S rRNA molecule is approximately 1540 nucleotides in length and contains enough information for more reliable analysis than 5S rRNA molecules (Amann *et al*., 1995 & Theron and Cloete, 2000). The highly conserved nature of 16S rRNA in a wide range of archaea and bacteria has been exploited for designing rRNA-specific universal primers, allowing the use of standard PCR/cloning based method to selectively amplify 16S rRNA gene fragment from mixed microbial samples to assess their natural abundances in the environments (Amann *et al*., 1995). Large and constantly-updated electronic databases of 16S rRNA gene sequences are available to provide identification of bacteria, archaea and fungi to the species levels. Boone (1987) suggested a 16S rRNA gene sequence similarity of 98% or less can be considered as the parameter for species differentiation, while Stackebrandt & Goebel (1994) suggested a 97% boundary based on correlations with DNA-DNA hydridization differences between accepted species. Dighe *et al*. (2004) also suggested that a 16S rRNA gene sequence similarity of less than 98% is equivalent to the DNA-DNA hybridization difference of >30% between members of the genus *Methanobrevibacter*, and therefore can be used as an indicator to separate sequences into species.

16S rRNA genes (and 16SrRNA itself) can be analysed in environmental samples to determine the structure of a microbial community. Highly sensitive molecular approaches such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (TRFLP) and automated ribosomal intergenic spacer analysis (ARISA) are capable of separating a target DNA of a minor species constituting less than 1% of the microbial population of a mixed culture (Zhou *et al*., 2010, Nicholson *et al*., 2006). Libraries of amplified 16S rRNA genes can be prepared and sequenced, and this has been expanded with the emergence of next-

generation DNA sequencing (Mardis, 2008). The massively parallel DNA sequencing instruments that sequence >100,000 DNA sequences in libraries constructed from amplified genomic DNA are now available for detecting extremely rare variants of DNA sequences in microbial populations (Mardis, 2008). Another benefit of next-generating DNA sequencing is that the conventional intermediate cloning-step prior to DNA sequencing can be omitted, thus considerably reducing the amount of time and labour required to achieve the same outcome (Mardis, 2008). Also, multiple samples can be easily handled using bar-coding technologies to identify fsequences from different samples. However, 16S rRNA may provide a biased assessment of the relative abundance of SSU rRNA genes occurring in nature in that (1) some SSU rRNA genes are more preferentially amplified than others by using degenerate universal primers; (2) the number of rRNA loci per genome varies between different groups of organisms (Suzuki & Giovannoni, 1996)

1.5.2 *mcr*A gene sequence analysis

The utility of 16S rRNA as a marker gene substantially deteriorates when discrimination extends down to a sub-species level. The higher levels of sequence variation commonly found in protein-coding genes may better serve as an indicator for differentiation at intra-species levels or between very closely related species. Methyl coenzyme M reductase (MCR) catalyzes the reduction of a methyl group bound to coenzyme M, leading to the eventual release of methane. The vital role played by MCR during methanogensis is reflected in the highly conserved nature of the protein in all methanogen species. Two different types of MCR exist: MCR-I is encoded by *mcrBDCGA* operon, and is universally present in all methanogens, whereas MCR-II, encoded by *mrtBDGA*, can only be found in members of the *Methanococcales* and the *Methanobacteriales* (Friedrich, 2005). The genes encoding the α-subunits of MCR, known as *mcr*A and *mrt*A, show low sequence variations since even minor sequential changes within the catalytic region may lead to a functional loss of the protein

(Hallam *et al*., 2003 & Friedrich, 2005). Based on the conserved sequence of *mcr*A gene, degenerate primers were designed to investigate the potential use of *mcr*A gene as a diagnostic indicator of methanogenesis (Friedrich, 2005). Comparison of *mcr*A and 16S rRNA phylogeny revealed that overall, the tree topologies of both marker genes very closely resemble each other, indicated by a similar branching order (Lueders *et al*., 2001, Luton *et al*., 2002, Tatsuoka *et al*., 2004 & Friedrich, 2005). The degenerate *mcr*A primers, however, also amplify the isoenzyme *mrt*A gene, which has a serious consequence when analysing the clone libraries constructed from environmental samples-the organism becomes over-represented in the clone libraries as both copies of *mcr*A and *mrt*A are sequenced (Tatsuoka *et al*., 2004).

1.6 Cultured rumen methanogens

So far, only 10 named species of methanogens have been successfully isolated from the ruminants using culture-based techniques. Beijer (1952) reported isolation of the first pure culture of a rumen methanogen, a *Methanosarcina* from the fistula of a goat, followed shortly after by isolation of *Methanobacterium formicicum* (Oppermann *et al*., 1957) from bovine rumen fluid. However, it was not until *Methanobrevibacter ruminantium* was isolated by Hungate and Smith (1958) that a formal characterization of a rumen methanogen species was carried out. The only rumen methanogen species known to possess features required for motility, thus named *Methanobacterium mobilis* (later renamed *Methanomicrobium mobile),* was isolated from the rumen of Holstein heifers and characterized by Paynter and Hungate (1968). Many years after Beijer's discovery, the first fully characterized pure culture of *Methanosarcina barkeri* was obtained from goat faeces (Mukhopadhyay *et al*., 1991). *Methanosarcina barkeri* and *Methanomicrobium mobile* were also isolated from a grazing Friesian cow in the study conducted by Jarvis *et al*. (2000). Phylogenetic analysis of rumen methanogens based on 16S rRNA gene sequences was initiated by Miller and Lin in 1998.

All of the five methanogen strains isolated from the faecal samples collected from horse, pig, sheep and cow were grouped within the genus *Methanobrevibacter* (Miller and Lin, 1998). Genomic DNA reassociation studies and comparative 16S rRNA sequence analysis of these animal isolates support the conclusion that these isolates represent novel species of the genus *Methanobrevibacter*. Full descriptions of *Methanobrevibacter gottschalkii* strains HO^T and PG^T from horse and pig, *Methanobrevibacter thaueri* strain CW^T from cow, and *Methanobrevibacter wolinii* strain SH^T from sheep were reported in 2002 (Miller and Lin). Recently, Rea *et al*. (2007) proposed creation of two novel species of methanogens that utilize formate and CO_2; *Methanobrevibacter olleyae* strain $KM1H5-1P^T$ and *Methanobrevibacter millerae* strain $ZA-10^T$ were isolated from ovine and bovine rumen, respectively, and were fully characterized. The isolation of *Methanobacterium bryantii*, *Methanoculleus olentangyi* and *Methanobrevibacter smithii* were reported by Joblin (2005), although no formal phylogenetic analysis or physiological characterizations of these species were carried out.

1.7 Methanogens isolated based on culture-independent method

Culture-independent molecular techniques are valuable tools for surveying the structure and microbial diversity of naturally occurring ecosystems without experiencing the difficulties of obtaining pure microbial cultures (Frey *et al*., 2009). Comparative analysis of 16S rRNA gene sequence is the most widely used molecular method for establishing the phylogenetic relationships between microbial DNA isolated from various environments.

The study of microbial diversity of total rumen archaea has been carried out by various research groups (Table 1.1). The species composition of methanogen populations within the rumen varied significantly depending on the species and geological locations of ruminant animals, the types of feed and the choice of PCR primers. However, the global data

sets suggest a large portion of the rumen methanogen populations is dominated by three major groups; the genus *Methanobrevibacter* (61.6%), the genus *Methanomicrobium* (14.9%) and a group of uncultured rumen archaea, rumen cluster C (RCC) (Janssen & Kirs, 2008).

Cultivation-independent studies of rumen archaea suggest that *Methanobrevibacter* spp. outnumber all the other methanogen genera inhabiting the animal rumen (Janssen & Kirs, 2008). Yanagita *et al*. (2000) demonstrated that the members of the genus *Methanobrevibacter* are the most prevalently present methanogenic species within the rumen of Japanese sheep by using 16S-targeting fluorescent *in situ* hybridization method. Universal PCR primers were used in the studies conducted by Wright and co-workers in 2004 and 2008, to amplify the archaeal 16S rRNA genes from the rumen content collected from Australian and Venezuelan sheep. In both occasions, 16S rRNA gene sequences that are closely related to the genus *Methanobrevibacter* were most frequently isolated. Pei *et al*. (2010) reported the predominant presence of *Methanobrevibacter* spp. in Chinese cattle, suggesting the distribution of *Methanobrevibacter* spp. in the animal rumen is not limited by the geological location of the ruminants. *Methanobrevibacter* spp. are also the major methanogen species occurring in other types of ruminant animals including merino, steers, reindeer, as well as in non-ruminant animals such as wallabies according to the recent studies carried out by Ouwerkerk *et al.* (2008), Sundset *et al.* (2009), Evans *et al.* (2009) and Zhou *et al.* (2010). In several of these studies, 16S rRNA sequences similar to the members of the genus *Methanosphaera* and *Methanomicrococcus* were also detected at low levels. Whitford *et al*. (2001) reported that approximately 60% of rumen 16S rRNA sequences had 98.5~98.8% similarity to the 16S rRNA gene sequence of *Methanobrevibacter ruminantium*. The author also reported 37% of the sequences has sequence similarities ranging from 97.2~97.7% with the 16S rRNA sequence of *Methanobrevibacter ruminantium*. The explanation given by Whitford *et al*. (2001) was that the latter cluster of 16S rRNA gene sequences belong to a

distinct species of the genus *Methanobrevibacter*, because the levels of sequence similarity do not fall within the recommended parameter of identification to be considered as *Methanobrevibacter ruminantium*, yet these organisms share 99.4~100% sequence similarities with each other. Studies performed by Skillman *et al*. (2006) also demonstrated the predominance of *Methanobrevibacter* spp. including *Methanobrevibacter ruminantium, Methanobrevibacter smithii* and *Methanobrevibacter thaueri* within the rumen methanogen populations in a Jersey cow. The study carried out by Skillman *et al*. (2006) revealed that among the isolated methanogen species, *Methanobrevibacter thaueri* occurs the most frequently in the rumen of a dairy calf fed on pasture, closely followed by *Methanobrevibacter ruminantium*. Zhou *et al*. (2009) investigated the methanogen species composition within the rumen of Canadian steers that were fed either with high efficiency- or low efficiency-diet. Among the two clone libraries constructed for each feed group, clones containing 16S rRNA sequences of *Methanobrevibacter ruminantium* strain NT7 accounted for 89.2% and 73.0% of the total number of clones in each library.

Tajima *et al*. (2001), Regensbogenova *et al*. (2004), Shin *et al*. (2004) and recently, Chaudhary and Sirohi (2008), reported to have observed a predominant presence of the genus *Methanomicrobium* in the rumen microbial community. According to Shin (2004), the majority of the sequences in their clone library belonged to the family *Methanomicrobiaceae*, with *Methanomicrobium mobile* representing the single largest source of DNA. When the 45 clones obtained from the rumen fluid samples were analyzed, 15 clones showed sequence similarity values high enough to be grouped into *Methanomicrobium mobile*. Of the 39 clones obtained from the epithelium samples, 37 clones were placed in the same cluster with *Methanomicrobium mobile*. The majority of the clones obtained from the rumen solid sample were phylogenetically classified as *Methanobacteriaceae* and *Methanomicrobiaceae. mobile*. Among the 20 clones obtained from the rumen solid samples, 9 were identified as

Table 1.1 Methanogen diversity in rumen samples, based on analysis of 16S rRNA gene clone libraries. In each clone library, the dominant group of methanogens is indicated with bold font.

Reference	DNA extraction method	Primers	Methanogen species	Diet	Source
Yamagita *et al*., 2000	FastRNA kit-Blue(BIO101)/bead-beating method	Arc1000f Arc1500r	***Methanobrevibacter* spp.** *Methanomicrobium mobile*	Timothy hay, alfafa hay, commercial formula feed	Corriedale sheep Japan
Tajima *et al*., 2001	Phenol-chloroform/bead-beating method	D30 D33 0025eF 1492R	**RCC** *Methanomicrobium mobile* *Methanobrevibacter* spp. Uncultured	Alfafa-timothy hat and concentrate in a 4:1 ratio	Holstein cow Japan
Whitford *et al*., 2001	Phenol-ethanol precipitation/glass bead-beating method	1Af 1100Ar	***Methanobrevibacter ruminantium*** *Methanosphaera* spp. Uncultured	A mixed ration containing 9% hay, 26% alfafa haylage, 30% corn silage, and 35% concentrate (13% barley, 50.8% corn, 28.1% roasted soybeans, plus vitamin/minerals)	Holstein cow Canada
Regensbogenova *et al*., 2004	Heat-disruption method	ArcF7 ArcR1326	***Methanomicrobium mobile*** *Methanomicrococcus* spp.	Not mentioned	Cow Netherlands
Shin *et al*., 2004	Genomic DNA extraction kit (iNtRON BIOTECHNOLOGY, Korea)	21f 958r	***Methanomicrobium mobile*** *Methanobacterium formicicum*	A mixed ration of rice hull and concentrated feed (Daehan food, Korea) in a 4:1 ratio	Hanwoo cow Korea
Wright *et al*., 2004	Cetyltrimethylammonium method	Met86F Met1340R	***Methanobrevibacter* spp.** *Methanosphaea* spp.	Normal grazing condition	Merino Australia
Skillman *et al*., 2006	Phenol-ethanol precipitation/glass bead-beating method	Archf364 Archr1386	***Methanobrevibacter ruminantium*** *Methanobrevibacter gottschalkii* *Methanosphaera* spp.	Rye grass/clover pasture	Jersey cow New Zealand

		21f 958r	***Methanosphaera*** *spp.* Crenarchaeota		
Wright *et al*., 2006	Cetyltrimethylammonium method	Met86F Met1340R	**RCC** *Methanobrevibacter* spp. *Methanomicrobium* spp. *Methanobacterium* spp.	Rhodes grass hay	Sheep Australia
Wright *et al*., 2007	Qiagen DNeasy Plant Kit	Met86F Met1340R	**RCC** *Methanobrevibacter* spp. *Methanomicrococcus* spp. *Methanosphaera* spp.	Corn-based diet	Hereford-Cross cattle Canada
			RCC *Methanobrevibacter* spp.	Potato products	
Chaudhary and Sirohi, 2008	Bacterial genomic DNA isolation kit	Met86F Met1340R	***Methanomicrobium mobile***	A standard diet (40% concentrate/60% roughage)	Murrah buffaloes India
Ouwerkerk *et al*., 2008	Phenol-ethanol precipitation/glass bead-beating method	Arch46f Arch1017r	***Methanobrevibacter*** **spp.** *Methanosphaera spp.*	Cattles fed rye grass hay, spear grass hay, Pangola grass hay, a barley-based feedlot ration, or a leucaena--grass pasture mix Merino wethers fed lucerne pellets or fresh cut kikuyu grass	Cattle/merino Australia
Wright *et al*., 2008	Qiagen DNeasy Plant Kit	Met86F Met1340R	***Methanobrevibacter gottschalkii***	Alfalfa pellets and Bermuda hay	West African hair sheep Venezuela

Evans *et al*., 2009	Cetyltrimethylammonium method	Met86F Met1340R	***Methanobrevibacter gottschalkii*** RCC *Methanosphaera* spp	Not mentioned	Tammar wallaby Australia
Sundset *et al*., 2009	Glass milk extraction method	Met86F Met1340R	***Methanobrevibacter* spp.** *Methanomicrococcus* spp. RCC	Grazing on late summer pastures composed of woody plants, graminoids, mosses and lichens	Reindeer Norway
Zhou *et al*., 2009	Phenol-chloroform/physical disruption with Zirconium beads	Met86F Met915R	***Methanobrevibacter ruminantium*** *Methanobrevibacter* spp. *Methanosphaera* spp.	Oats, hay, feedlot supplement	Steer Canada
Pei *et al*., 2010	Phenol-chloroform/bead-beating method	Met86F Met1340R	***Methanobrevibacter* spp.** *Methanobacterium* spp. *Methanosphaera* spp. Methanomicrobium *mobile* RCC	Corn meal, cottonseed meal, whole corn stalk, wheat stalk	Jinnan cattle China
Zhou *et al*., 2010	Phenol-chloroform/physical disruption with Zirconium beads	Met86F Met915R ARC344F ARC519R	***Methanobrevibacter* spp.** *Methanosphaera* spp.	Oats, hay, feedlot supplement	Steer Canada

Methanomicrobium. In a similar experiment carried out by Tajima *et al*. (2001), two libraries of clones obtained from the bovine rumen samples were amplified by using two different sets of primers. 21% of the clones in the first library showed a high level of sequence similarity with the 16S rRNA gene of *Methanomicrobium mobile*, while the rest of the clones did not show any apparent sequence affiliations with any cultured methanogens. 56% of the clones in the second library were identified as *Methanomicrobium mobile*. A FISH-based study of the sheep rumen carried out by Jarvis (2001) also reported that *Methanomicrobium mobile* was present within the range of 10^6~ 10^8 cells per ml of rumen content. From these studies, the predominance of genus *Methanomicrobium* in the rumen is apparent, while *Methanobrevibacter* spp. is either not detected or present at insignificant levels. A phylogenetic analysis of 16S rRNA clones obtained from cilate-associated bovine/ovine rumen methanogens was carried out by Regensbogenova and the co-workers (2004). Of the 20 SSU clones sequenced, 10 were identified as *Methanomicrobium mobile*, while the rest of the clones were grouped with either the members of the genus *Methanomicrococcus* or uncultured rumen archaeon. Chaudhary and Sirohi (2009) conducted comparative 16S rRNA gene sequence analysis of the clones obtained from the rumen content collected from Indian buffaloes. Of the total of 108 clones examined, 94.42% of the clones were identified as *Methanomicrobium mobile*. These data sets are obviously in disagreement with the numerous studies that reported the dominance of *Methanobrevibacter* spp. in animal rumen. The discrepancies may have resulted from the different methodologies used, the different animals used, and the choice of the diets. Diet is one of the controlling factors which affect the composition of the rumen microbial community. Wright *et al*. (2007) examined three groups of sheep fed on three different diets consisting of pasture, oaten hay or Lucerne hay. The species composition of the rumen archaeal community varied markedly between the three groups, reflecting the shift in the rumen microbial composition due to the changing diet.

Similarly, Jeyamalar *et al*. (2011) also showed that diet has an influence on rumen archaeal community structure in sheep, cattle, and deer. The choice of PCR primers may also have influenced the results (Janssen and Kirs, 2008). It should not be overlooked that the observed predominance of *Methanobrevibacter* spp. and *Methanomicrobium* spp. in the rumen is possibly the result of PCR bias, rather than the true reflection of the rumen microbial diversity.

The protozoa-methanogen symbiosis is also an important factor which may influence the rumen methanogen composition. The intimate association between protozoa and methanogens is frequently found in anaerobic systems. Such a relationship can be mutually beneficial to both species in that protozoa provide substrates for methanogens while methanogens continuously remove H_2 that can inhibit the protozoan metabolism. Sharp *et al*. (1998) reported a shift in the rumen methanogen composition in the absence of protozoa that normally co-exist with methanogens in the rumen. Although the abundance of different groups of protozoan-associated methanogens varies in different studies, the majority of protozoan-associated rumen methanogens was found to belong to genera *Methanobrevibacter*, *Methanomicrobium* and the RCC clade. Sharp (1998) used dual flow continuous culture fermenters to constantly remove protozoa over an extended period of time. At the period when protozoan loss is the greatest, disturbance in the rumen archaeal composition was observed. The relative abundance of *Methanomicrobiales* increased from 9.6% to 26.3%, whereas the relative abundance of *Methanobacteriaceae* exhibited a decline from 84.2% to 54.9% over the 240 h period of operation.

The majority of the data sets substantiate the idea that methanogens belonging to a group of methanogens that have not been cultures thus far exists in the rumen. This group has been designated “RCC” by Janssen and Kirs (2008). The members of RCC have only few distantly related cultured isolates, and are only distantly related to aerobic thermoacidophilic

archaea, most notably *Thermoplasma acidophilum* and *Picrophilus oshimae*. According to the published data, sequences in the RCC clade make up a significant fraction of the total 16S rRNA gene pool of the rumen archaea, and tend to show relatively large sequence variations (>3%) between members of different subgroups (Janssen & Kirs 2008). The RCC clade formed the largest constituent of the clone libraries constructed in the studies of Tajima *et al*. (2001) and Wright *et al*. (2006 & 2007). Wright and his co-workers constructed clone libraries of 16S rRNA sequences obtained from the rumens of Australian and Canadian sheep. Approximately 80% and 50% of the total number of clones in each library were identified as RCC, respectively. In a similar study conducted by Tajima *et al*. (2001), RCC formed the dominant group of methanogens in the rumens of Japanese cows.

1.8 Methods for controlling rumen methanogens

A number of mitigation strategies have been attempted, but so far without substantial success. Difficulties arise due of the need for selectively targeting all methanogens inhabiting the rumen to prevent the unaffected species from replacing the niche emptied of affected species. It is also important that other rumen microorganisms continue to carry out their normal digestive functions without being targeted for inhibition to maintain the balance in the rumen ecosystem.

1.8.1 Inhibitory compounds

A number of compounds have been tested for their potential use as feed additives to abate methane emissions from the rumen. Recent research shows certain plant extracts may have ability to directly inhibit methanogenesis by disturbing the symbiotic relationship between methanogens and rumen protozoa (McAllister & Newbold, 2008). Saponins are another group of plant secondary compounds that are known to repress methanogen-associated protozoal activity in the rumen (Patra & Saxena, 2009). Studies by Hass *et al*.

(2003), Agarwal *et al*. (2006) and Hu *et al*. (2005) demonstrated the effects of saponin components extracted from various plant sources in reducing the methane release from supplemented animals.

Although there is reluctance to include chemically synthesized additives in animal feeds, halogenated analogues are in fact very potent inhibitors of methane production in the rumen. Dong *et al*. (1999) reported a significant reduction in methane production following addition of bromoethanesulfonic acid (BES). This corresponds with the recent study carried out by Tomkin *et al*. (2009) in which BES was shown to reduce methane emissions from 3.9%~0.6% of the total energy intake in steers. A halogenated methane analogue, bromochlomethane (BCM), reacts with reduced vitamin B_{12}, subsequently blocking the essential methyl group transfer step during methanogenesis. Goel *et al*. (2009) studied the anti-methanogenic activity of BCM in batch cultures and continuous fermentation, and observed significant methane reduction values (89-94%) from both, demonstrating the potential use of BCM as a methane inhibitor. However, it is unlikely such halogenated analogues will be used in a wide scale mitigation scheme, as methane inhibition by these chemicals only had transient effects. The rumen methanogen populations may rapidly shift to the species that are insensitive to the chemical analogues (McAllister & Newbold, 2008). Taking advantage of the unique presence of 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) in archaea, the inhibitors of HMG-CoA, mevastatin and lovastatin, were successfully tested for their inhibitory effects on methane production in *Methanobrevibacter* strains (Miller and Wolin, 2001) *in vitro*.

1.8.2 Immunization

An attractive methane mitigation approach is to develop vaccines that may establish a salivary immune response against methanogens in ruminants. Whole-cell extract vaccines

that target 20% and 52% of the total methanogen species/strains inhabiting the ovine rumen were formulated by Wright *et al*. (2004) and Williams *et al*. (2009), respectively. In both studies, a significant increase in IgG antibody titres in plasma, saliva and rumen fluid samples was observed in vaccine-treated sheep, but not in control-vaccinated sheep. These results suggest that the raised antibodies are delivered to the rumen via saliva, and a robust immune response is maintained in the hostile rumen environment through a continuous production of active antibodies. Williams and his co-workers (2009) reported that the 5 methanogen strains *(Methanobrevibacter* strains AK-87 and 1Y, *Methanobrevibacter millerae* ZA- 10^T , *Methanomicrobium mobile* BP^T, *Methanosphaera stadtmanae* MCB-3^T) targeted by their anti-methanogen vaccine made up 67% of the total 16S rRNA sequences retrieved from the control sheep but only 47% of the clones obtained from the vaccine-treated sheep. Targeting a greater representation of methanogen strain/species was expected to enhance the vaccine's effectiveness by eliminating a larger proportion of the methanogen population and leading to a reduction of methane emissions from the rumen. However, no significant changes in the methane emissions levels were observed in both studies. This was affirmed by Wedlock et al. (2010), who surmised that the whole-cell vaccines containing immunodominant antigens present on methanogens may not necessarily generate immune responses that prevent the growth of methanogens or the production of methane. Wedlock *et al*. (2010) prepared antigenic fractions from whole-cells, cytoplasm, cell wall-derived proteins and the cell walls of *Methanobrevibacter ruminantium* strain M1 and tested their effects on the *in vitro* growth and methane production of *Methanobrevibacter ruminantium* M1. Wedlock and the co-authors reported that treating the *in vitro* cultures of *Methanobrevibacter ruminantium* M1 with antisera raised against fractions from whole cells, cytoplasm and the cell wall-derived proteins led to a reduction in both the cell density and the amount of methane accumulated. An interesting observation was that antisera directed against cell-wall fractions had no effect

in reducing methane emissions, suggesting the ability of antisera to mitigate the methane production is limited to certain fractions containing antigens that both induce immune responses and are essential for growth. The research effort to develop broad-spectrum vaccines that include the less conspicuous species of methanogens is continuing, as these minor species could quickly adapt to occupy the empty niches in the absence of more competitive methanogen species.

1.8.3 Alternative hydrogen sinks

In the rumen, methanogens assume the role of terminal reducers of carbons by using the hydrogen by-products generated from fungal, bacterial and protozoal energy metabolism. This process, called "interspecies hydrogen transfer" (IHT), is important in maintaining the microbial fermentations and plant fibre degradation that occurs in the rumen by oxidizing and reusing reduced cofactors such as NADH. An alternative pathway to methanogenesis in the rumen is reductive acetogenesis in which H_2 and CO_2 are used to yield acetate as the final product instead of methane. Reductive acetogenesis is carried out by homoacetogens that are part of the normal ruminant flora. Homocetogens can divert H_2 away from methanogens, and direct it to the acetogenesis metabolic pathway, so that less H_2 is available for methane formation. Methanogens act as a preferred hydrogen sink because reduction of CO_2 to acetate is thermodynamically less favorable than reduction of CO_2 to methane (McAllister & Newbold, 2008). Furthermore, the hydrogen threshold levels of methanogens are considerably lower than acetogens, placing methanogens in an advantageous position during the competition for hydrogen (Joblin, 1999). Taken together, these observations open up the possibility that once the methanogen populations are eliminated, the vacant niches may be occupied by acetogens. Faichney *et al*. (1999) observed 51-67% less methane emissions from lambs that were reared in isolation since birth. Examination of fermentation balance revealed that only 33-43% of reducing equivalents were recovered from the rumen than what was

expected, suggesting the presence of an alternative hydrogen sink, in this case, acetogenesis. Inhibiting methanogen growth will increase the hydrogen partial pressure until the hydrogen threshold levels required for acetogenesis are reached. Rumen acetogens can then assume the role of scavenging metabolic by-product hydrogen in place of methanogens, thus providing an effective way to control H_2 build-up in the rumen.

Propionate is another metabolic intermediate that can act as an alternative sink for hydrogen in that the propionate producers in the rumen compete with methanogens for H_2 (Attwood *et al*., 2008). Propionate producers stimulated by addition of precursors of the propionate biosynthesis pathway, such as furmarate and malate, were shown to effectively compete with the rumen methanogens, and eventually resulted in 38% reduction in methane production (Attwood *et al*., 2008).

1.9 Competition for H_2 in anoxic environments

Hydrogen (H_2) is an important intermediate in the microbial oxidation and degradation of organic matter in anaerobic environment. Despite its important role as an energy source, hydrogen exhibits a fast turnover, and occurs at a very low concentration in anaerobic environments (Conrad, 1998). The main consumers of hydrogen are sulfate reducers, methanogens, homoacetogens, and organisms that respire NO_3^-, Fe (III) or Mn (IV). A keen competition for hydrogen exists between these organisms. Microorganisms that exhibit higher affinities for hydrogen outcompete others by more effectively utilizing traces of H_2, thus yielding higher growth (Cord-Ruwisch *et al*., 1988). Studies show that an organism's ability to scavenge hydrogen is thermodynamically controlled by the redox potential of the terminal electron acceptor. Organisms that use NO_3^-, Fe (III) or Mn (IV) as terminal electron acceptors usually outcompete sulfate reducers, which in turn outcompete methanogens and homoacetogens (Valentine *et al*., 2000). Each terminal electron accepting process

(denitrification, iron and manganese reduction, sulfate reduction, methanogenesis and acetogenesis) is associated with a unique hydrogen concentration, called the hydrogen threshold. A hydrogen threshold can be defined as (1) the minimal partial pressure below which H_2 uptake is no longer possible (Kotsyurbenko *et al*., 2001) or (2) the minimum concentration of hydrogen at which thermodynamically favourable reactions associated with negative Gibbs free energy can occur (Karadagki & Rittmann, 2007). According to the threshold model, a successful organism places itself in an advantageous position for H_2-scavanging by maintaining the H_2 partial pressure below the threshold H_2 levels used by the competitors (Cord-Ruwisch *et al*., 1988). Experimental studies on competition for H_2 among aquatic sediment dwellers show that the sulfate reducers pull hydrogen away from methanogens by lowering the H_2 partial pressure below the threshold level that is necessary to allow hydrogen oxidation by methanogens (Lovley *et al*., 1982).

In aquatic sediments, degradation of organic matter is often coupled to the reduction of inorganic terminal electron acceptors including nitrate, Fe (III), Mn (VI), carbon dioxide and most notably, sulfate. The large contribution made by sulfate reducers in oxidation of organic matter explains the low level of H_2 distribution in the sediments, as well as the dependence of methanogens on non-competitive precursors such as trimethylamine during the production of methane (Conrad, 1999 & Thauer *et al*., 2008).

In the rumen, inorganic terminal electron acceptors other than CO_2 are generally not available, thus methanogens and homoacetogenic bacteria are solely responsible for the consumption of hydrogen (Conrad, 1999). Ruminal hydrogen is constantly generated from the fermentation of cellulose and hemicelluloses by cellulolytic bacteria, rumen protozoa and various species of anaerobic fungi. However, under normal conditions, the concentration of hydrogen in the rumen is maintained at low levels (<0.2% of the total gas phase), suggesting that the hydrogen generated from fermentation is quickly used by the action of

hydrogenotrophic methanogens (Morvan *et al*., 1996). Very little H_2 consumption occurring in the rumen can be attributed to homoacetogenesis because the hydrogen thresholds of methanogenic H_2 consumption are usually lower than those of homoacetogens (Kotsyurbenko *et al*., 2001).

1.9.1 Determination of H_2 threshold

Development of mathematical models that describe the kinetics of hydrogen thresholds, substrate consumption, biomass growth and end-product generation is an important aspect of understanding microbial interactions and their competitions for hydrogen under anaerobic conditions. Table 1.2 summarizes the experimental results from previous studies that investigated H_2 threshold of methanogens isolated from various anaerobic environments. Earlier studies use kinetic models to explain the complete inhibition of methanogenesis upon addition of sulfate in co-culture experiments (Lovley *et al*., 1982; Achtnich *et al*., 1995 and 1995; Robinson & Tiedje, 1984; Kristjansson *et al*., 1982). The idea is that the specific kinetic parameters (μ_{max}, V_{max}, K_s and K_M) for a given microorganism can be used to predict the outcome of competition between H_2 utilizing organisms. Robinson and Tiedje (1984) and Kristjansson *et al*. (1982) determined H_2-uptake kinetic parameter values (K_m and V_{max}) for methanogens belonging to various genera and several strains of sulfidogens, which they then incorporated into a two-term Michaelis-Menten equation. However, evaluation of substrate depletion or the outcome of bacterial competition based on a Michaelis-Menten kinetic model does not accurately reflect the natural phenomenon because it fails to acknowledge the effect of long-term competition which results in a numerical predominance of one organism over the other. Lovley (1985) also noted the Michaelis-Menten model is only suitable for describing H_2 consumption in a habitat where the hydrogen concentration is more than 100-fold greater than that in aquatic sediments and where a higher steady-state H_2 concentration does not persist long due to a constant consumption by hydrogen utilizing microorganisms

(Conrad, 1999). Indeed, when Robinson and Tiedje (1984) fitted the sigmoidal H_2 depletion data into an integrated Monod equation, the K_s value obtained for *Methanospirillum* JF-1 was more correct than the K_s value determined by using the Michaelis-Menten equation, indicating the Monod equation more correctly describes the successful nature of some microorganisms during competition for hydrogen in the presence of growth.

Measuring hydrogen thresholds for mixed/pure cultures of microorganisms isolated from various places revealed that there is a defined thermodynamic trend in which H_2 thresholds progressively decrease in the order of nitrate reduction→ iron reduction→ sulfate reduction→ methanogenesis→homoacetogenesis. This trend remains the same for the Gibbs free energies (ΔG°) of these terminal electron accepting reactions, suggesting H_2 thresholds can be calculated using the Gibbs free energy equation (Karadagli and Rittmann, 2007). Theoretically, H_2 threshold should occur when thermodynamic equilibrium is achieved between the product and the reactant, that is when ΔG=0 (Conrad, 1999). In reality however, a small negative free energy was obtained at the point where H_2 utilization stopped, which the researchers associated with the critical minimal energy necessary for the survival of microorganism (Karadagli & Rittmann, 2007). Conrad (1999) attributed this critical value as a consequence of the energetic threshold determined by the cellular energy generating system, which is approximately 1/3 ATP or -23 KJ mol^{-1} of the energy generating reaction. Indeed, the H_2 threshold values measured in methanogenic environments corresponded closely to the calculated critical minimal energy value, indicating methanogenesis is under a thermodynamic control of the threshold of H_2 utilization.

1.9.2 Hydrogen thresholds of rumen methanogens

Although the rumen is one of the most important sites of methanogenic activity occurring in nature, little attention has been devoted to the study of the competition for

hydrogen by teh different methanogens inhabiting the rumen. Considering the very limited range of substrates utilized by the rumen methanogens, it is a mystery that such a diverse methanogen population exists in the rumen. Whether the H_2 use kinetics (including thresholds) affect the inter-species competition for survival among the rumen methanogens remain unknown. However, the amount of H_2 that these organisms are exposed to may differ greatly depending on various conditions such as the feeding frequency, the types of feeds, the feed passage rate, syntrophic associations with rumen ciliates, physical attachment of methanogens to the rumen epithelium walls, and the types of the host animals. Therefore, in the constantly changing rumen environment, it is likely that different methanogen species have evolved separate H_2 utilization strategies which guarantee the best chance of survival by making the best use of available H_2. So far, the lack of experimental data makes it difficult to validate any of above suggestions. Further studies are needed in order to determine if the relative abundance of methanogens occupying the rumen is influenced by the unique H_2 utilization threshold that is associated with each species.

1.10 Conclusion

Understanding the diversity of methanogens inhabiting the rumen has a large impact on developing mitigation strategies which aim to minimize the methane emissions from the rumen. The molecular-based techniques that analyse the gene sequences obtained from the environmental samples are increasingly replacing the use of conventional culture based methods used to study rumen methanogen diversity. Due to the inconsistency of the methodology used to collect the rumen samples, the geographical location of the host animals, the types of the feeds, and most notably, the PCR primers used to amplify 16S rRNA and *mcr*A gene sequences from the environmental samples, the prevalent methanogen species varied considerably in different studies. The global data-set indicates that the members of the genera *Methanobrevibacter*, *Methanomicrobium*, and *Methanosphaera* and of the RCC

cluster constitute the most prevalent methanogens in the rumen. A number of mitigation strategies have been attempted, including the use of inhibitory chemical compounds, vaccination approaches, the change of diets, and the use of alternative hydrogen sinks, but so far without substantial success. Difficulties arise due of the need for selectively targeting all methanogens inhabiting the rumen to avoid the unaffected species from replacing the niche emptied of affected species. It is also important that other rumen microorganisms continue to carry out their normal digestive functions without being targeted for inhibition to maintain the balance in the rumen ecosystem.

Considering that the methanogens utilize relatively limited substrate ranges, the fact that there is such a large number of methanogen species inhabiting the rumen comes as a surprise. In the rumen, where the effect of H_2 accumulation must be relieved in order to maximize the fermentation efficiency, the ability of methanogens to scavenge H_2 is considered essential for proper rumen functioning. During the inter-species competition for survival, the methanogen species with higher H_2 utilization efficiency would likely to outcompete the species possessing less competitive system for H_2 utilization. The varying H_2 utilization thresholds of different methanogen species could indicate that there are different strategies of H_2 use, thus shaping the methanogen diversity in the rumen. So far, the little attention which has been devoted to studying the H_2 use (including thresholds) of methanogens has been focused on the methanogens isolated from sediments of ponds, the ocean, and rice paddies, and not on methanogens isolated from the rumen.

Table 1.2 H_2 thresholds of methanogens isolated from various anaerobic environments have been determined in a number of studies. The H_2 threshold values that were recorded in ppm or nM have been converted into Pascal to enable a direct comparison between related studies.

Reference	Source	organism	H2 threshold concentrations in literatures	H2 threshold concentrations in Pascal
Lovley, 1985	Aquatic sediments	*Methanobacterium formicicum* JF-1	6.5 ± 0.6 Pa	6.5 ± 0.6 Pa
		Methanobacterium bryantii M.o.H	6.9 ± 1.5 Pa	6.9 ±1.5 Pa
		Methanospirillum hungatei JF-1	9.5 + 1.3 Pa	9.5 ± 1.3 Pa
Cord-Ruwisch *et al*., 1988	Pure culture collection from the German Collection of Microorganisms (DSM)	*Methanospirillum hungatei* (DSM864)	30 ppm	3.5 Pa
		Methanobrevibacter smithii (DSM861)	100 ppm	11.6 Pa
		Methanobrevibacter arboriphilus(DSM744)	90 ppm	10.5 Pa
		Methanobacterium formicicum (DSM1535)	28 ppm	3.3 Pa
		Methanococcus vannielii (DSM1224)	75 ppm	8.7 Pa
Lee *et al*., 1988	Aquatic sediments	Co-culture of acetate-oxidising bacterium and *Methanothermobacter thermoautotrophicus* ΔH	12-14 Pa	12-14 Pa
		Co-culture of acetate-oxidising bacterium and *Methanothermobacter thermoautotrophicus* THF	12-14 Pa	12-14 Pa
Kotsyurbenko *et al*., 2001	Pond sediments	Methanogenic strain MSB	0.8-1 Pa	0.8-1 Pa
		Methanogenic strain MSP	3-4 Pa	3-4 Pa

Conrad and Wetter, 1990		*Methanobacterium bryantii* strain Bab 1.	16 nM	0.04 Pa
		Methanobacterium thermoautotrophicum	71 nM	0.19 Pa
Chong *et al*., 2002	Oregon Collection of Methanogens (OCM), USA	*Methanogenium frigidum* Ace-2	0.57 Pa	0.57 Pa
Karadagli and Rittmann, 2007	Culture collection from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ 863)	*Methanobacterium bryantii* M.o.H	0.4 nM	0.001 Pa

1.11 Research aims

In the light of our limited understanding of methanogen community within the rumen, my research represents the initial characterisation of methanogen species in the rumen and their physiology.

My research had two aims:

Aim 1. To investigate diversity of culturable methanogens isolated from the rumen.

Aim 2. To determine basic metabolic traits of isolates representing different clades.

Aim 1 had 2 objectives:

a. Obtain pure cultures of methanogen isolates in the AgResearch collection and carry out phylogenetic analysis using 16S rRNA and *mcr*A genes.

b. Describe the morphology of cells of the chosen strains as observed under fluorescent and phase-contrast microscopes and demonstrate purity of the cultures.

Aim 2 had two objectives:

a. Characterize the minimum substrate requirements of the methanogen isolates chosen based on their distinctive features and genetic novelty.

b. Measure the hydrogen consumption thresholds for chosen methanogen isolates.

Chapter 2

Materials and methods

2.1 Regenerating frozen cultures

The methanogen culture collection at AgResearch contains a large number of isolates which have been obtained from various ruminant animals raised both in and out of New Zealand. The cultures were stored frozen at -83℃ for up to 20 years. For those cultures that went through a lengthy period of preservation, some details such as the source of the isolates; the types of medium used to grow these organisms; the age of the cultures; the growth requirements; and the purity status of the cultures had not adequately been recorded. The frozen cultures were taken out in time for inoculation and thawed on ice. 0.5 ml of the inoculums cultures was transferred into 9.5 ml of freshly prepared BY medium that contains fluid extracted from the contents of cow rumen and a mixture of substrates. The methods used for the preparation of media and substrate solutions and the culture techniques were those of Hungate (1950), as modified by Balch *et al*. (1976). The medium was dispersed into 16 x 125 mm Hungate tubes with open top screw caps and septum stoppers (Bellco Glass Inc., NJ, USA). All media were prepared under O_2-free CO_2, then autoclaved. Prior to inoculation, filter-sterilized stock solutions of vitamins (1%), methanol (1M), sodium formate (3M) and sodium acetate (2M) solutions were added under O_2-free conditions. After inoculation, each culture was pressurized with high-pressure H_2:CO_2 (80:20). The inoculated cultures were incubated at 39℃ under a continual 195 rpm rotation and in the dark. Growth of cultures was determined by gas chromatography to identify released methane and by spectrophotometry.

2.2 Media

2.2.1 BY medium

The components of BY medium (salt solution A (170 ml/L), salt solution B (170 ml/L), centrifuged rumen fluid (300 ml/L), distilled water (360 ml/L), $NaHCO_3$ (5 g/L), resazurin (8 drops/L) and yeast extract (1 g/L)) were thoroughly mixed before being boiled under O_2-free 100% CO_2. The medium was then place on ice while being continuously bubbled with 100% CO_2. Once the medium cooled down, L-cysteine-HCl (500 mg/L) was added. 9.5 ml of medium was then dispersed into CO_2-washed 16 x 125 mm Hungate tubes with open top screw caps and septum stoppers (Bellco Glass Inc., NJ, USA). The sealed tubes were sterilized by autoclaving for 20 min at 121℃. The medium was kept it dark overnight before use.

2.2.2 Salt solution A

Salt solution A was prepared by mixing NaCl (6 g/l [w/v]), KH_2PO_4 (3 g/l [w/v]), $(NH_4)_2SO_4$ (1.5 g/l [w/v]), $CaCl_2{\cdot}2H_2O$ (0.79 g/l [2/v]), and $MgSO_4{\cdot}7H_2O$ (1.2 g/l [w/v]) in distilled water.

2.2.3 Salt solution B

Salt solution B was prepared by dissolving $K_2HPO_4{\cdot}3H_2O$ (7.86 g/l [w/v]) in distilled water.

2.2.4 Centrifuged rumen fluid

The rumen fluid was collected from fistulated cows that had been fed pasture hay and rye-grass clove pasture. The rumen contents were filtered though cotton cheesecloth. The filtrate was then centrifuged at 8,000 rpm for 20 min before being stored frozen at -20℃. The one-time centrifuged rumen fluid was taken out for thawing in time for use. Remaining fine particle materials were removed by centrifugation at 8,000 rpm for 20 min.

2.2.5 RM02 medium

1 litre of RM02 medium was prepared by mixing KH_2PO_4 (1.4 g/L), $(NH_4)_2SO_4$ (0.6 g/L), KCl (1.5 g/L), trace element solution (SL10)* (1 ml/L), selenite/tungstate solution* (1 ml/L) and resazurin solution (4 drops/L) in 950 ml of distilled water. The medium was then place on ice while being continuously bubbled with 100% CO_2. Once the medium cooled down, $NaHCO_3$ (4.2 g/L) and L-cysteine-HCl (500 mg/L) were added. 9.5 ml of medium was then dispersed into CO_2-washed Hungate tubes with open top screw caps and septum stoppers. The sealed tubes were sterilized by autoclaving for 20 min at 121℃. The medium was kept in the dark overnight before use. Some types of media contain dyes and other light sensitive ingredients. Excessive exposure to light can produce the formation of toxic peroxides that can inhibit growth. At the time of inoculation, 0.5 ml of the rumen fluid, vitamin and yeast extract mixture (section 2.3.1) needed to be added into 9.5 ml of RM02 to complete the medium.

* Refer to Tschech and Pfennig, 1984

2.3 Media additives

2.3.1 Rumen fluid additive (NoSubRFV)

Spun-down rumen fluid (2.2.1) was bubbled with 100% N_2 for 20 min before being dispersed into N_2-washed serum bottles sealed with black butyl rubber stoppers and aluminium caps (Bellco Glass Inc., NJ, USA). The sealed bottles were autoclaved at 121℃ for 20 min. The cooled-down autoclave rumen fluid was then poured into a beaker in which it was thoroughly mixed with $MgCl_2 \cdot 6H_2O$ (1.63 g per 100 ml) and $CaCl_2 \cdot 2H_2O$ (1.18g per 100 ml) by stirring for 30 min. The heavy precipitate formed in this mixture was removed by centrifuging at 8,000 rpm for 20 min. The clear supernatant was then mixed with yeast extract (2 g per 100 ml) before being subject to bubbling under N_2 gas for 20 min. While under N_2 gas, the mixture was filter-sterilized though a 0.22 µm pore size sterile filter into

N_2-washed serum bottles sealed with black butyl rubber stoppers and aluminium caps. At this stage, this basal rumen fluid is termed “Clarified rumen fluid”. Finally, 2% vitamin 10 concentrate was added by using a syringe and needle.

2.3.2 Vitamin 10 concentrate

Vitamin 10 concentrate solution was prepared by dissolving 10 mg of folic acid, 30 mg of riboflavin, 30 mg of D,L-6,8-thioctic acid, 50 mg of cyanocobalamin, 100 mg of thiamine chloride hydrochloride, 150 mg of pyridoxine hydrochloride, 50 mg of hemicalcium D-(+)-pantothenate, 100 mg of nicotinic acid, 10 mg of D-(+)-biotin, and 40 mg of 4-aminobenzonate in 1 L of distilled water. The solution was bubbled with nitrogen for 20 min before being filter-sterilized into N_2-washed, sealed serum bottles through a 0.22 µm pore size sterile filter.

2.3.3 Substrate solutions

All substrates were added to RM02 and BY media at the time of inoculation. Sodium formate (50 mM) and sodium acetate (20 mM) dissolved in 100 ml of distilled water were bubbled with CO_2 for 20 min and then transferred into CO_2-washed serum bottles. The sealed serum bottles were autoclaved at 121℃ for 20 min. Methanol solution was prepared by carrying out a 10-fold dilution of absolute methanol with distilled water. Diluted methanol was bubbled with CO_2 for 20 min before being filter-sterilized into CO_2-washed, sealed and sterile serum bottles through a 0.22 µm pore size sterile filter. For preparation of coenzyme M solution (5 mM), sodium-2-mercaptoethanesulfonate was dissolved in distilled water, which was then bubbled with CO_2 for 20 min before being filter-sterilized into CO_2-washed, sealed, and sterile serum bottles through a 0.22 µm pore size sterile filter. All solutions were kept in the dark except when in use.

2.3.4 RFgenV

A sugar-mix solution containing D-glucose, D-cellobiose, D-xylose, L-arabinose, sodium-L-lactate, casamino acid, Bacto-peptone and yeast extract was prepared by mixing the components in clarified rumen fluid. The solution was bubbled with N_2 for 20 min before being filter-sterilized into N_2-washed, sealed, and sterile serum bottles through a 0.22 µm pore size sterile filter. The completed solution was named RFgenV.

2.4 Purification

Methanogen cultures showing signs of bacterial contamination were subjected to a 3-stage purification process involving treatments of cultures with heat and antibiotics, and a serial dilution of cultures. Not all strains survived the purification process. In addition, the methods employed did not show 100% effectiveness at eliminating bacterial contaminations.

Prior to inoculation, cultures were heat-treated at 55℃ for 30 min by completely submerging the tubes in a water bath. After the growth, cultures were subject to a second round of heat treatment at 55℃ for an hour, and then were re-inoculated into a fresh BY medium. The cultures were then subject to the antibiotic treatment. The antibiotic mix was prepared by mixing ampicillin (10 µg/ml), streptomycin (10 µg/ml) and vancomycin (86.7 µg/ml) with distilled water under O_2-free CO_2. The solution was filter-sterilized into CO_2-washed, sealed, and sterile serum bottles through a 0.22 µm pore size sterile filter. 0.1 ml of the mix was added into 10 ml BY medium during the inoculation. Finally, in order to isolate a single cell of methanogens, cultures treated with antibiotics were serially diluted by 10-fold until a 10^7-fold dilution was achieved. In order to investigate the persistence of bacterial growth, the cultures were inoculated into BY media along with 0.5 ml of RFgenV solution containing a sugar mixture that enhances the bacterial growth. None of the methanogenic substrates was added, e.g. sodium formate, sodium acetate, methanol, Coenzyme M, and

H_2/CO_2 .Cultures were incubated at 39℃ under shaking condition at 195 rpm in the dark. The entire process of purification was repeated until pure cultures of methanogen were obtained.

2.5 Microscopy

Rumen methanogens naturally fluoresce under UV light due to the presence of coenzyme F_{420}. The cell morphology of methanogens was observed by fluoresecent microscopy in combination with phase contrast microscopy (DM2500 microscope, Leica Microsystems, Wetzlar, Germany). Prior to imaging, cells were fixed onto the slide surface coated with 1% agarose (Pfenning & Wagener, 1986).

2.6 DNA extraction and PCR amplification

DNA was extracted from the pelleted bacterial cells by using phenol-chloroform method. Cells were physically disrupted by beating with Zirconium beads. DNA was separated from RNA and proteins by centrifugation of a mix of the aqueous sample and solutions containing water-saturated phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/isoamylalcohol (24:1). The RNA- and protein-free DNA formed at the end of the separation step was ethanol-precipitated, washed with 70% ethanol, dried, and DNA resuspended in EB buffer. The DNA was used as a template for PCR amplification. The near-full length (≈ 1500 bp) 16S rRNA gene sequence was obtained by PCR amplification with universal primers 8F (5`-AGAGTTTGATCCTGGCTCAG-3`) and 1510R (5`-GGTTACCTTGTTACGACTT-3`). The PCR contained 5 µl of 1.25 mM $MgCl_2$ PCR reaction buffer, 4 µl of 2 mM dNTP mix, 0.5 µM of each primer, 5 U of Taq Polymerase, 25 ng of template DNA and DNA-free water. Full length 16S rRNA genes were amplified in a Px2 thermal cycler (Thermo Electron Corp., Milford, MA, USA) using the following cycle

parameters: an initial denaturation for 3 min at 95℃; 35 cycles each of denaturation for 30 sec at 95℃, annealing for 30 sec at 56℃, and primer extension for 1 min at 72℃; and a final extension of 10 min at 72℃. The *mcr*A genes were amplified using *mcr*A gene-specific primers, MLF (5`-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3`) and MLR (5`-TTCATTGCRTAGTTWGGRTAGTT-3`) to generate PCR product whose size ranges between 464 and 491 bp. The PCR contained 5 µl of 1.5 mM $MgCl_2$ PCR reaction buffer, 4 µl of 2 mM dNTP mix, 1 µM of each primer, 5 U of Taq Polymerase, 25 ng of template DNA and DNA-free water. The *mcr*A genes were amplified in a Px2 thermal cycler (Thermo Electron Corp., Milford, MA, USA) using the following cycle parameters: an initial denaturation for 3 min at 94℃; 35 cycles each of denaturation for 30 sec at 94℃, annealing for 25 sec at 52℃, and primer extension for 1 min at 72℃; and a final extension of 7 min at 72℃. Prior to sequencing, the PCR products were purified using ProMega Wizard SV Gel and PCR Clean-Up System.

2.7 Cloning and sequencing

PCR products were directly cloned into the pCR® 2.1 vector (Invitrogen) and recombinant colonies were randomly picked and screened for inserts using colony PCR. Approximately 1,800-bp gene sequence containing the16S rRNA gene sequence was obtained by PCR amplification with primers GEM2987FF (5`-CCCAGTCACGACGTTAAACG-3`) and TOP168R (5`-ATGTTGTGTGGAATTGTGAGCGG-3`). The 10 µL PCR reaction contained 1 µl of 15 mM $MgCl_2$ PCR reaction buffer, 0.2 µl of 25 mM $MgCl_2$, 2 µl of 2 mM dNTP mix, 0.1 µl of each primer (10 pmol/µl), 0.1 µl of 5 U of Taq Polymerase, a loopful of recombinant colony and DNA-free water. DNA inserts were amplified in a Px2 thermal cycler (Thermo Electron Corp., Milford, MA, USA) using the following cycle parameters: an initial denaturation for 1 min at 94℃; 35 cycles each of denaturation for 15 sec at 94℃, annealing for 30 sec at 56℃,

and primer extension for 1 min at 72℃; and a final extension of 10 min at 72℃. The products of colony PCR were directly sequenced at the Allan Wilson Center, Massey University with M13 Forward (5`- GTAAAACGACGGCCAG-3`) and M13 Reverse (5`- CAGGAAACAGCTATGAC -3`) primers. All reference sequences were obtained from the GenBank database.

2.8 Phylogenetic analysis

Similarity searches against database entries were performed using online BLAST searches. For analysis, the 16S rRNA and *mcr*A gene sequences were aligned with ClustalW and compared using MEGA 4.0 software (Tamura *et al*., 2007). A distance matrix tree was constructed using the neighbor-joining methods and bootstrap re-sampled 1000 times. *Methanococcus vannielii* SB, *Methanococcus jannaschii* JAL-1 and *Methanococcus thermolithotrophicus* SN-1 were used as outgroups.

2.9 Substrate requirements

Selected strains were tested for their ability to utilize formate (50 mM), methanol (30 mM) and acetate (20 mM) with or without H_2/CO_2. In order to minimize the interference during optical density reading, clear-colored RM02 medium was used instead of the rumen fluid-infused BY medium to grow the isolates. 9.5 ml of RM02 medium was dispensed into Balch tubes (Bellco Glass Inc., NJ, USA) that were later sealed with butyl rubber stoppers (20 mm in diameter, Bellco Glass Inc., NJ, USA) and aluminium caps (Bellco Glass Inc., NJ, USA) before being autoclaved at 121℃ for 20 min. Prior to adding the substrates, 0.5 ml of NoSubRFV and 60 µl of coenzyme M (5 mM) were universally added into the medium. The effect of substrate addition on methanogen growth was determined by systemically omitting single or multiple components from the addition. A total of eleven different substrate combinations were tested, as summarized in Table 2.1. The experiment continued for 3 serial

transfers to dilute out any residual substrates carried over from the initial inocula. Growth was measured every day by measuring culture optical density at 600 nm by inserting the tubes directly into an Ultrospec 1100 pro UV/Vis spectrophotometer until a stationary phase had been reached.

Table 2.1 A summary of substrate combinations. Selected strains were tested for their ability to utilize formate (50 mM), methanol (30 mM) and acetate (20 mM) with or without the addition of H_2/CO_2. The effect of substrate addition on methanogen growth was determined by systemically omitting single or multiple components from the addition. Circles (+) indicate the added component. Omitted substrates are indicated by bars (-).

	H_2/CO_2 (80:20)	Formate	Methanol	Acetate
1	+	-	-	-
2	-	+	-	-
3	-	-	+	-
4	-	-	-	+
5	+	+	-	-
6	+	-	+	-
7	+	-	-	+
8	+	+	+	-
9	+	+	-	+
10	+	-	+	+
11	+	+	+	+

2.10 Hydrogen threshold analysis

2.10.1 Preparation of cultures

Selected strains were tested for their hydrogen threshold levels below which the methanogens fail to use it as a substrate. Cultures were grown in a clear RM02 medium prepared in 16 x 125 mm Hungate tubes (Bellco Glass Inc., NJ, USA). For each strain, only the minimum substrate requirement was satisfied, so that the added substrates are assumed to have been completely used up when the growth ceased. After inoculation, each culture was pressurized with high-pressure H_2:CO_2 (80:20). The cultures were left to grow for 5~6 days

with no further addition of H_2/CO_2. Growth of cultures was determined by gas chromatography to identify released methane.

2.10.2 Preparation of gas-free Hungate tubes

An empty Hungate tube was submerged completely under saturated sodium sulphate solution until all air bubbles were expelled from the tube. The tube was then held upside down so that only its opening remains submerged in the solution. The solution in the tube was replaced by 100% CO_2 by placing the gassing needle though the opening of the tube. While still submerged under the solution, the tube opening was closed with open top screw caps and butyl rubber stoppers that were specially designed to minimize the gas leakage. Using CO_2-washed syringes and needles, 1 ml of sodium sulphate solution was dispensed into each tube. All tubes were stored upside down until the time of use.

2.10.3 Threshold measurement

The threshold level to which H_2 was consumed was monitored through three cycles of H_2 injection and consumption for each culture. Prepared cultures were injected with 1,000 ppm pure hydrogen gas. In order to obtain desired initial hydrogen concentration, pure hydrogen gas was diluted by 1000-fold inside the gas-free Hungate tubes. Using gas-tight syringes, 600 µl of pure hydrogen gas was taken out, and injected into a 16 ml Hungate tube containing 1 ml of sodium sulphate solution. The tube was shaken well before taking out 400 µl of gas, which was then injected into the tubes containing 9.5 ml of culture. Twice the amount of pure hydrogen gas (2,000 ppm) than the first was added into the cultures at second and third injections by carrying out a 500-fold dilution. At second and third injections, 800 µl of diluted gas was removed from the dilution tube to be injected into the cultures. The hydrogen concentration of each tube was measured immediately after the injection by gas chromatography equipped with a reduction gas detector (SRI Instrument, U.S.A). SRI RGD

consists of a mercuric oxide reaction tube and a mercury lamp in a heated UV detector cell. The eluted gas in the heated reaction tube reacts with the mercuric oxide to form mercury vapour. As the vapour flows through the detector cell, the UV light emitted from the mercury lamp inside the cell becomes absorbed by the gaseous mercury. The changes in UV light detection are transmitted to the data system which converts them into an absorbance output. The only time that the cultures were exposed to room temperature was during sampling. From each tube, duplicate samples of the headspace gas (0.5 ml) were taken out and injected directly into SRI RGD using gas-tight syringes.

2.10.4 Controls

For each strain, 5 control tubes were prepared to test for the viability of the cells. These control tubes contained 9.5 ml of RM02 medium into which 0.5 ml of inoculums is added along with the minimal growth substrates. After inoculation, each culture was pressurized with high-pressure H_2:CO_2 (80:20). The growth of cultures was determined by gas chromatography to identify released methane. A set of control tubes into which no biomass was added was also prepared. The H_2 concentration remained constant up to 40 days during the time which the cultures went through three cycles of H_2 injection and consumption.

2.10.5 Calibration

The amount of hydrogen in the number of moles was calculated by plotting a calibration curve using reference hydrogen in N_2 (1:200) (BOC). Two ranges of calibration curves (0.07 nM – 1.5 nM and 0.9 nM – 22.0 nM) were prepared to measure the hydrogen concentration. To achieve the H_2 concentrations within the higher range, the reference H_2 in N_2 (1:200) was directly injected into SRI RGD in various volumes using gas-tight syringes. The volumes of the reference gas injected into SRI RGD and their relevant numbers of moles of H_2 are summarized in Table 2.2. The reference hydrogen in nitrogen (1:200) was diluted

by up to 300-fold in gas-free Hungate tubes containing sodium sulphate solution to achieve the hydrogen concentrations within the lower range (Table 2.3). 1 ml of diluted gas was taken out from the tube and injected into SRI RGD using gas-tight syringes.

Table 2.2 The amount of H_2 in gas samples. The volumes of the reference gas injected into SRI RGD for calibration and the relevant amount of H_2 calculated in the numbers of moles of H_2.

Volume (ml) of H_2 in N_2 injected	Amount (nM) of H_2 in the injected gas
1	22.0
0.8	17.6
0.6	13.2
0.5	11.0
0.4	8.8
0.3	6.6
0.2	4.4
0.1	2.2
0.08	1.8
0.06	1.3
0.04	0.88

Table 2.3 The amount of H_2 contained in the diluted gas. The reference hydrogen in nitrogen (1:200) was diluted up to 300-fold in gas-free Hungate tubes containing sodium sulphate solution to achieve the hydrogen concentrations within the lower range during the calibration. The amount of H_2 (nM) contained in 1 ml of diluted gas was calculated.

Dilution fold	Amount of H_2 (nM) in 1 ml
15	1.50
25	0.88
30	0.73
50	0.44
100	0.22
150	0.15
200	0.11
250	0.09
300	0.07

2.10.6 Calculation

At 0℃, 1 mole of H_2 occupies 22.414 L. Since the molar gas volumes differ at varying temperatures, the volume occupied by a specified number of particles (moles) of an ideal gas at 39℃ can be calculated using Charles' law. The gas-in-gas concentration of hydrogen at 39℃ is 0.039041mol/L based on the calculation using the ideal gas law. It is assumed that the absolute partial pressure of the gas is 1 atm. Based on the formulae derived from the

calibration curves, the number of moles of H_2 contained in the volume of the gas-tight syringe (0.5 ml) was calculated. These values were converted into mol/L, which in turn were converted into atmosphere (atm) by dividing with the gas-in-gas concentration of hydrogen at 39℃ (0.039041mol/L).

The solubility of H_2 in pure water was calculated from the Ostwald coefficient (*L*) from the tabulated data of Wilhelm *et al*. (1977). *L* is the ratio of the volume of gas dissolved into the medium and the volume of the absorbing liquid under 1 atm. The *L* value at 39℃ calculated by interpolation is 0.01887. Based on the ideal gas law, corrected for temperature using Charles' law, the concentration of dissolved H_2 at 39℃ under STP is 0.737 mmol/L. A ratio value (52.9729) was obtained between the gas-in-gas concentration of hydrogen at 39℃ (0.039041 mol/L) and the concentration of dissolved H_2 at 39℃ under STP (0.737 mmol/L). This value was used to calculate the number of moles of dissolved H_2 contained within the volume of the syringe (0.5 ml). These values were converted into mol/L prior to the conversion into atm by dividing with the concentration of dissolved H_2 at 39℃ under STP (0.737 mmol/L).

The total H_2 partial pressure (atm) in the culture tubes was recorded in the unit of Pascal (Pa). 101,300 Pa is equivalent to 1 atm.

Chapter 3

Identification and purification of rumen methanogens

3.1 Introduction

As part of New Zealand's Pastoral Greenhouse Gas Research Consortium (PGgRc), AgResearch has been accumulating a collection of cultures of rumen methanogens. During the process, quite a few cultures of methanogens were stored without being identified. In this chapter, work is described that aimed at purifying culture of some of these rumen methanogen isolates and identifying them based on 16S rRNA and *mcr*A gene sequences. A phylogenetic tree comparing the genetic distances between the newly identified isolates and the few named species was constructed. A number of isolates was chosen for further characterizations based on their phylogenetic positions or unique phenotypic characteristics. The cultures were inspected for purity by comparing the fluorescent and the phase-contrast microscopic images of the cells. As a significant number of isolates were shown to retain contamination by rumen bacteria, the cultures were alternatively treated with heat and antibiotics to remove the presence of undesirable contaminants. The cultures containing more than two types of methanogens were serially diluted to isolate a single cell of methanogens. The data obtained here may be used to guide ensuing research, aimed at characterizing novel methanogen species and sequencing the genomes of a greater diversity of rumen methanogens than is currently available in public culture collections.

3.2 Materials and methods

Frozen cultures were regenerated (section 2.1) and purified (section 2.4), and purity was confirmed by microscopy (section 2.5). DNA was extracted, and 16S rRNA and *mcr*A

genes were amplified by PCR using gene-targeted oligonucleotide orimers (section 2.6). Amplified genes were cloned and sequenced (section 2.7) and the primary sequences were used to construct phylogenetic trees to identify the isolates (section 2.8).

3.3 Results

A total of 32 isolates that were stored frozen at -85℃ were selected to go through the revival process. 14 isolates were successfully revived, as summarized in Table 3.1. The growth of these cultures was determined using gas chromatography that detects the presence of methane.

By courtesy of Debjit Dey, Gemma Henderson, Faith Cox, and Jeyanathan Jeyamalar of AgResearch, live cultures of isolates AbM4, M1, SM9, G16, D5, BRM9, YCM1, ISO3-F5, and MCB-3 were obtained. A brief description of these cultures is summarized in Table 3.2.

3.3.1 Phylogenetic analysis of 16S rRNA genes

16S rRNA gene sequences were obtained from the isolates 229/4, 229/5, 229/11, 229/14, 229/15, AbM1, AbM23, AbM25, Alpaca, CM2, CM3, Wallaby, AL10, YLM1, AbM4, M1, SM9, G16, D5, BRM9, YCM1 and ISO3-F5. The identities of these isolates were uncovered by comparing their 16S rRNA gene sequences using BLAST similarity searches against database entries (Table 3.3). More than 90% of the 16S rRNA gene sequence was recovered for most isolates, using the complete 16S rRNA from *Methanobrevibacter ruminantium* strain M1 (1,366 base pairs) as a reference.

Isolates 229/4, 229/5, 229/11, 229/14 and 229/15 shared a high degree of 16S rRNA gene sequence similarity (99%) with each other, suggesting that these isolates in fact, belong to a single species of methanogen. The closest recognized relatives of the 229 these five isolates were *Methanobrevibacter olleyae* strain KM1H5-1P (98.8%) and

Methanobrevibacter sp. FM1 (99.0%). Boone (1987) proposed that a 16S rRNA gene sequence similarity of 98% or less can be used for species differentiation. Dighe *et al*. (2004) showed that a 16S rRNA gene sequence identity of less than 98% is equivalent to the genome-wide identity of less than 30% within the genus *Methanobrevibacter*, and therefore can be used as an indicator to separate sequences into species. Using this sequence similarity for species differentiation, these five isolates and *Methanobrevibacter* sp. strain FM1 are classified into *Methanobrevibacter olleyae.*

YLM1 was previously thought to belong to the genus *Methanobacterium*. However, the analysis of the16S rRNA gene revealed that YLM1 is identical to the five isolates that were identified as *Methanobrevibacter olleyae*.

Isolates AbM1 and AbM23 had very high 16S rRNA gene sequence similarity (99.6%) to each other, suggesting that these of the same species repeatedly isolated from the abomasum of the sheep. The closest relative of AbM1 and AbM23 was *Methanobrevibacter* strain AbM4 that first appeared as unpublished work cited by Jarvis *et al*. (1999).

Isolates AL10, CM2, CM3 and ABM25 were 99.7% identical to each other. The closest recognized relative of these four isolates was *Methanosarcina barkeri* CM1 (99.5%).

The Alpaca isolate grouped with a number of unidentified members of the genus *Methanobrevibacter*, including *Methanobrevibacter* sp. Z4, sp. AK-87, sp. NT7, and sp. Z6. The sequence similarity between all of these strains and the Alpaca isolate was over 99.4%, suggesting that they belong to the same species of methanogen.

16S rRNA sequences of *Methanobrevibacter smithii* and an unknown species of the genus *Methanosphaera* closely related to *Methanosphaera cuniculi* (96.0%) were recovered from the mixed culture named Wallaby. The two methanogenisolates that were recovered

Table 3.1 Methanogen cultures revived from the frozen state. Due to the prolonged preservation period, some details of the cultures had not been recorded adequately. Only the viable cultures were subjected to the purification process which categorically eliminated the bacterial contaminants. A number of cultures were shown to harbour more than one methanogen species; these cultures were termed "mixed" in order to differentiate them from the cultures harbouring bacterial contaminants.

Culture name	Source of animals	Isolated by	Viability	Bacterial contamination	Mixed culture	Reference
C5/1	Cow	Graham Naylor	No	N/A	N/A	
229/4	Lamb rumen	Graham Naylor	Yes	Yes	No	
229/5	Lamb rumen	Graham Naylor	Yes	Yes	No	
229/11	Lamb rumen	Graham Naylor	Yes	Yes	No	
229/14	Lamb rumen	Graham Naylor	Yes	Yes	No	
229/15	Lamb rumen	Graham Naylor	Yes	Yes	No	
AbM1	Sheep abomasum	Keith Joblin	Yes	Yes	No	
AbM2	Sheep abomasum	Keith Joblin	No	N/A	N/A	
AbM4	Sheep abomasum	Keith Joblin	Yes	Yes	No	Jarvis *et al*., 2004 (Unpublished)
AbM11	Sheep abomasum	Keith Joblin	No	N/A	N/A	
AbM12	Sheep abomasum	Keith Joblin	No	N/A	N/A	
AbM13	Sheep abomasum	Keith Joblin	No	N/A	N/A	
AbM14	Sheep abomasum	Keith Joblin	No	N/A	N/A	
AbM21	Sheep abomasum	Keith Joblin	No	N/A	N/A	
AbM22	Sheep abomasum	Keith Joblin	No	N/A	N/A	
AbM23	Sheep abomasum	Keith Joblin	Yes	Yes	No	
AbM24	Sheep abomasum	Keith Joblin	No	N/A	N/A	
AbM25	Sheep abomasum	Keith Joblin	Yes	N/A	N/A	
AbM41	Sheep abomasum	Keith Joblin	No	N/A	N/A	
AbM42	Sheep abomasum	Keith Joblin	No	N/A	N/A	
AbM43	Sheep abomasum	Keith Joblin	No	N/A	N/A	
AbM44	Sheep abomasum	Keith Joblin	No	N/A	N/A	
AbM45	Sheep abomasum	Keith Joblin	No	N/A	N/A	
Alpaca	Alpaca rumen	Graham Naylor	Yes	Yes	No	
AS9/11/19	Unknown	Lucy Skillman	Yes	Yes	Unknown	
CM1	Cow	Keith Joblin	Yes	N/A	N/A	
CM2	Cow	Keith Joblin	Yes	N/A	N/A	
CM3	Cow	Keith Joblin	Yes	N/A	N/A	
DM2	Deer	Diana Pacheco	No	N/A	N/A	
DM22	Deer	Diana Pacheco	No	N/A	N/A	
DM6	Deer	Diana Pacheco	No	N/A	N/A	
Wallaby	Wallaby hindgut	Graham Naylor	Yes	Yes	Yes	
YLM1	Lamb rumen	Graham Naylor	Yes	Yes	No	
AL10	Alpaca	Gemma Henderson	Yes	N/A	N/A	Isolated in parallel with rumen fungus

Table 3.2 Methanogen strains obtained as live cultures. The isolates listed below were obtained as live cultures by courtesy of researchers at AgResearch.

Strain name	Source of animals	Isolated by	Viability	Bacterial contamination	Mixed culture	Reference
G16	Sheep	Jeyanathan Jeyamalar	Yes	Yes	No	
H6	Sheep	Gemma Henderson	Yes	Yes	Yes	
BRM9	Cow rumen		Yes	Yes	No	Jarvis *et al*., 2000
YCM1	Calf rumen	Paul Evans	Yes	Yes	No	
ISO3-F5	Sheep rumen	Jeyanathan Jeyamalar	Yes	No	No	Jeyamalar, 2010
MCB-3	Human intestine	Miller and Wolin	Yes	No	No	Miller and Wolin, 1985
SM9	Sheep rumen	Diana Pacheco	Yes	No	No	Skillman *et al*., 2006
M1	Cow rumen	Smith and Hungate	Yes	No	No	Smith and Hungate, 1958

from a single Wallaby isolate were differentiated by giving them new strains names; *Methanobrevibacter smithii* isolate was designated as R4C, while the close relative of *Methanosphaera cuniculi* was named A4.

Several attempts were made to obtain the 16S rRNA gene sequence of isolate AS9/11/19, but without success. The culture of AS9/11/19 was contaminated with an unusually high degree of bacterial diversity which may have interfered with the sequencing efforts.

The 16S rRNA sequence from strain H6 grouped the organism with *Methanobrevibacter millerae* strain ZA-10, although with relatively low sequence similarity (96.2%) between the two species.16S rRNA sequences from strain G16 showed 98% sequence similarity with *Methanobrevibacter thaueri* strain CW.

As expected, the BRM9, YCM1, and SM9 were identified as *Methanobacterium formicicum*, *Methanobacterium bryantii*, and *Methanobrevibacter* sp. SM9, respectively.

ISO3-F5 was previously identified as a novel species belonging to the genus *Methanosphaera* (Jeyanathan, 2010). MCB-3 strain is a human isolate of *Methanosphaera stadtmanae*, and the revived culture was confirmed as this by 16S rRNA gene sequencing.

The 16S rRNA gene sequences were used to construct a phylogenetic tree, along with the reference sequences downloaded from GenBank (Fig. 3.1). Strains H6, SM9, G16, R4C, AbM1, AbM4 and AbM23 clustered within the *Methanobrevibacter gottschalkii* clade. Isolates Alpaca, M1, 229/4, 229/5, 229/11, 229/14, 229/15 and YLM1 clustered within the *Methanobrevibacter ruminantium* clade. YCM1 and BRM1 grouped with *Methanobacterium* spp. Isolates A4 and ISO3-F5 grouped with *Methanosphaera stadtmanae*. A parallel tree was

obtained using *mcr*A gene as the genetic marker (Fig. 3.2), in which the strains clustered with the expected reference sequences. Overall, the 16S rRNA and *mcr*A tree topologies resembled each other, validating the use of *mcr*A gene as a genetic marker for rumen methanogen species differentiation.

3.3.2 Isolating a single species of methanogen from mixed cultures

All strains except those that belong to the genus *Methanosarcina* (AbM25, AL10, CM2 and CM3) were observed under fluorescent and phase-contrast microscope to inspect the purity of the cultures. Non-fluorescent cells were observed from the phase-contrast microscopic images of all the cell cultures examined. Furthermore, the cultures showed a heavy growth when a mixture of substrates enhancing bacterial growth (section 2.3.4) was added into the medium. These observations indicated that the strains were contaminated with heterotrophic bacteria. A measure of purification was taken to obtain pure cultures of these strains.

The 16S rRNA gene sequence analysis revealed that the Wallaby culture was a mixed culture of *Methanobrevibacter smithii* and a member of the genus *Methanosphaera*. Based on the 16S rRNA gene analysis, strain H6 showed no indication of harbouring methanogen species other than a close relative of *Methanobrevibacter millerae*. However, the microscopic observation of H6 revealed that the culture also harboured large, spherical cells that are characteristic of the genus *Methanosphaera*. Mixed cultures were serially diluted until pure cultures consisting of a single methanogen species were obtained. From the Wallaby strain, pure cultures of *Methanobrevibacter smithii* and a member of the genus *Methanosphaera*

Table 3.3 Identification of methanogen isolates based on 16S rRNA gene sequence similarity

Isolate name	Closest relatives	Sequence identity (%)	Length (bp)	Purified from
229/4	*Methanobrevibacter olleyae* KM1H5-1P *Methanobrevibacter* sp. FM1	99.0 99.0	1,415	
229/5	*Methanobrevibacter olleyae* KM1H5-1P *Methanobrevibacter* sp. FM1	99.0 99.0	1,415	
229/11	*Methanobrevibacter olleyae* KM1H5-1P *Methanobrevibacter* sp. FM1	99.0 99.0	1,415	
229/14	*Methanobrevibacter olleyae* KM1H5-1P *Methanobrevibacter* sp. FM1	99.0 99.0	1,415	
229/15	*Methanobrevibacter olleyae* KM1H5-1P *Methanobrevibacter* sp. FM1	99.0 99.0	1,415	
AbM1	*Methanobrevibacter* sp. AbM4	99.0	1,414	
AbM4	*Methanobrevibacter* sp. AbM4	100.0	1,415	
AbM23	*Methanobrevibacter* sp. AbM4	99.0	1,415	
AbM25	*Methanosarcina barkeri* CM1	99.0	1,411	
SM9	*Methanobrevibacter* sp. SM9	99.0	1,443	
AL10	*Methanosarcina barkeri* CM1	99.0	1,415	
CM1	*Methanosarcina barkeri* CM1	99.0	1,415	
CM2	*Methanosarcina barkeri* CM1	99.0	1,415	
R4C	*Methanobrevibacter smithii* ALI *Methanobrevibacter smithii* b181	99.0 99.0	1,415	Isolate Wallaby
Alpaca	*Methanobrevibacter* sp. NT7 *Methanobrevibacter* sp. AK-87 *Methanobrevibacter* sp. Z6 *Methanobrevibacter* sp. Z4	99.0 99.0 99.0 99.0	1,415	
G16	*Methanobrevibacter thaueri* CW	98.0	1,415	
M1	*Methanobrevibacter ruminantium* M1	100.0	1,415	
D5	*Methanobrevibacter millerae* ZA-10	97.0	1,415	Isolate H6
A4	*Methanosphaera cuniculi* DSM 4103T	96.0	1,415	Isolate Wallaby
ISO3-F5	*Methanosphaera stadtmanae* ISO3-F5	97.0	826	
MCB-3	*Methanosphaera stadtmanae* MCB-3	100.0	1,415	
BRM9	*Methanobacterium formicicum* MFT	100.0	1,415	
YCM1	*Methanobacterium bryantii* M.o.H	97.0	1,415	
YLM1	*Methanobrevibacter olleyae* strain KM1H5-1P *Methanobrevibacter* sp. FM1	99.0 99.0	1,415	

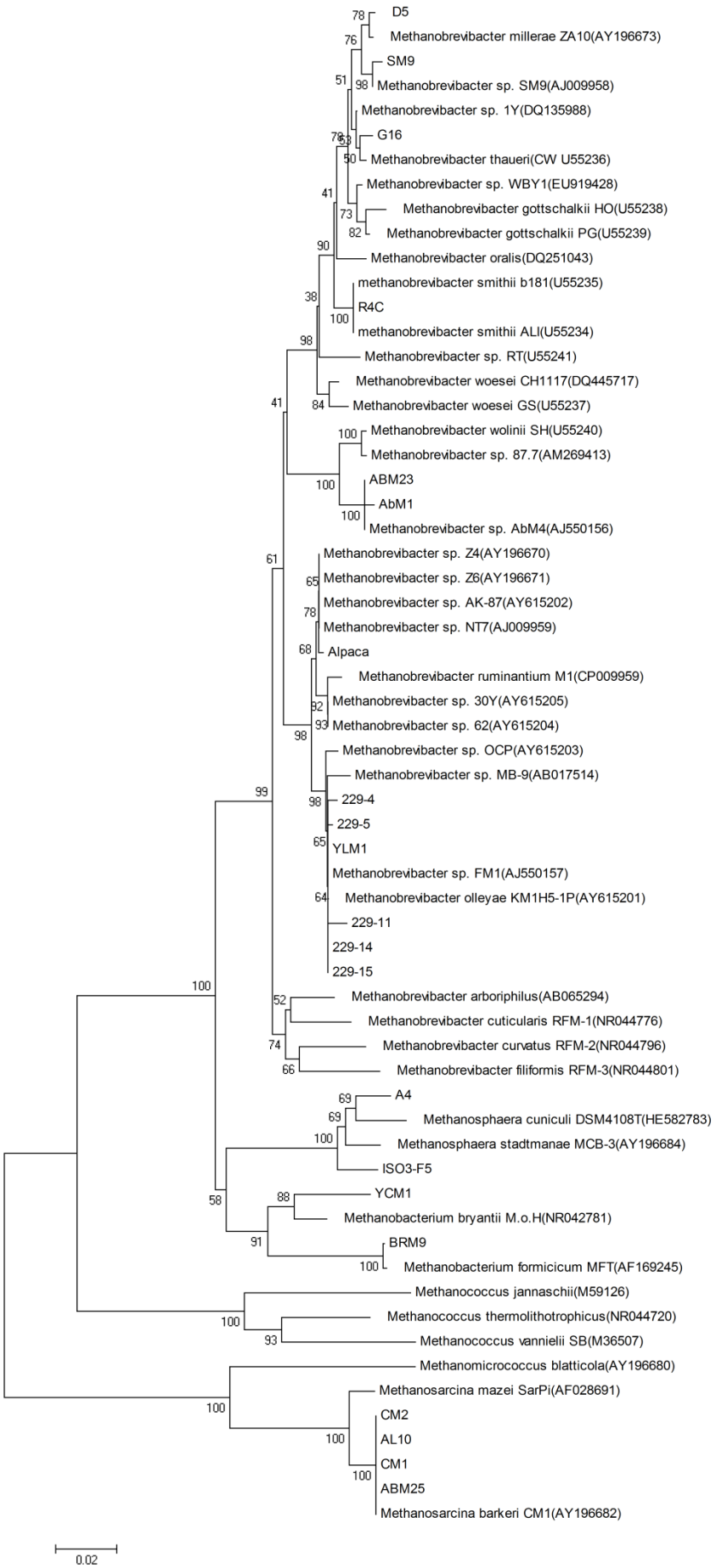
D5
Methanobrevibacter millerae ZA10(AY196673)
SM9
Methanobrevibacter sp. SM9(AJ009958)
Methanobrevibacter sp. 1Y(DQ135988)
G16
Methanobrevibacter thaueri(CW U55236)
Methanobrevibacter sp. WBY1(EU919428)
Methanobrevibacter gottschalkii HO(U55238)
Methanobrevibacter gottschalkii PG(U55239)
Methanobrevibacter oralis(DQ251043)
methanobrevibacter smithii b181(U55235)
R4C
methanobrevibacter smithii ALI(U55234)
Methanobrevibacter sp. RT(U55241)
Methanobrevibacter woesei CH1117(DQ445717)
Methanobrevibacter woesei GS(U55237)
Methanobrevibacter wolinii SH(U55240)
Methanobrevibacter sp. 87.7(AM269413)
ABM23
AbM1
Methanobrevibacter sp. AbM4(AJ550156)
Methanobrevibacter sp. Z4(AY196670)
Methanobrevibacter sp. Z6(AY196671)
Methanobrevibacter sp. AK-87(AY615202)
Methanobrevibacter sp. NT7(AJ009959)
Alpaca
Methanobrevibacter ruminantium M1(CP009959)
Methanobrevibacter sp. 30Y(AY615205)
Methanobrevibacter sp. 62(AY615204)
Methanobrevibacter sp. OCP(AY615203)
Methanobrevibacter sp. MB-9(AB017514)
229-4
229-5
YLM1
Methanobrevibacter sp. FM1(AJ550157)
Methanobrevibacter olleyae KM1H5-1P(AY615201)
229-11
229-14
229-15
Methanobrevibacter arboriphilus(AB065294)
Methanobrevibacter cuticularis RFM-1(NR044776)
Methanobrevibacter curvatus RFM-2(NR044796)
Methanobrevibacter filiformis RFM-3(NR044801)
A4
Methanosphaera cuniculi DSM4108T(HE582783)
Methanosphaera stadtmanae MCB-3(AY196684)
ISO3-F5
YCM1
Methanobacterium bryantii M.o.H(NR042781)
BRM9
Methanobacterium formicicum MFT(AF169245)
Methanococcus jannaschii(M59126)
Methanococcus thermolithotrophicus(NR044720)
Methanococcus vannielii SB(M36507)
Methanomicrococcus blatticola(AY196680)
Methanosarcina mazei SarPi(AF028691)
CM2
AL10
CM1
ABM25
Methanosarcina barkeri CM1(AY196682)
0.02

Figure 3.1 A phylogenetic tree of methanogen isolates constructed based on 16S rRNA gene sequence similarity. The GeneBank accessions numbers for reference sequences are in the paranthesis. For analysis, the 16S rRNA gene sequences were aligned with ClustalW and compared using MEGA 4.0 software. A distance matrix tree was constructed using the neighbor-joining methods and bootstrap re-sampled 1000 times.The scale bar indicates 0.02 nucleotide substitutions per nucleotide position. *Methanococcus vannielii* SB, *Methanococcus jannaschii* and *Methanococcus thermolithotrophicus* were used as outgroups.

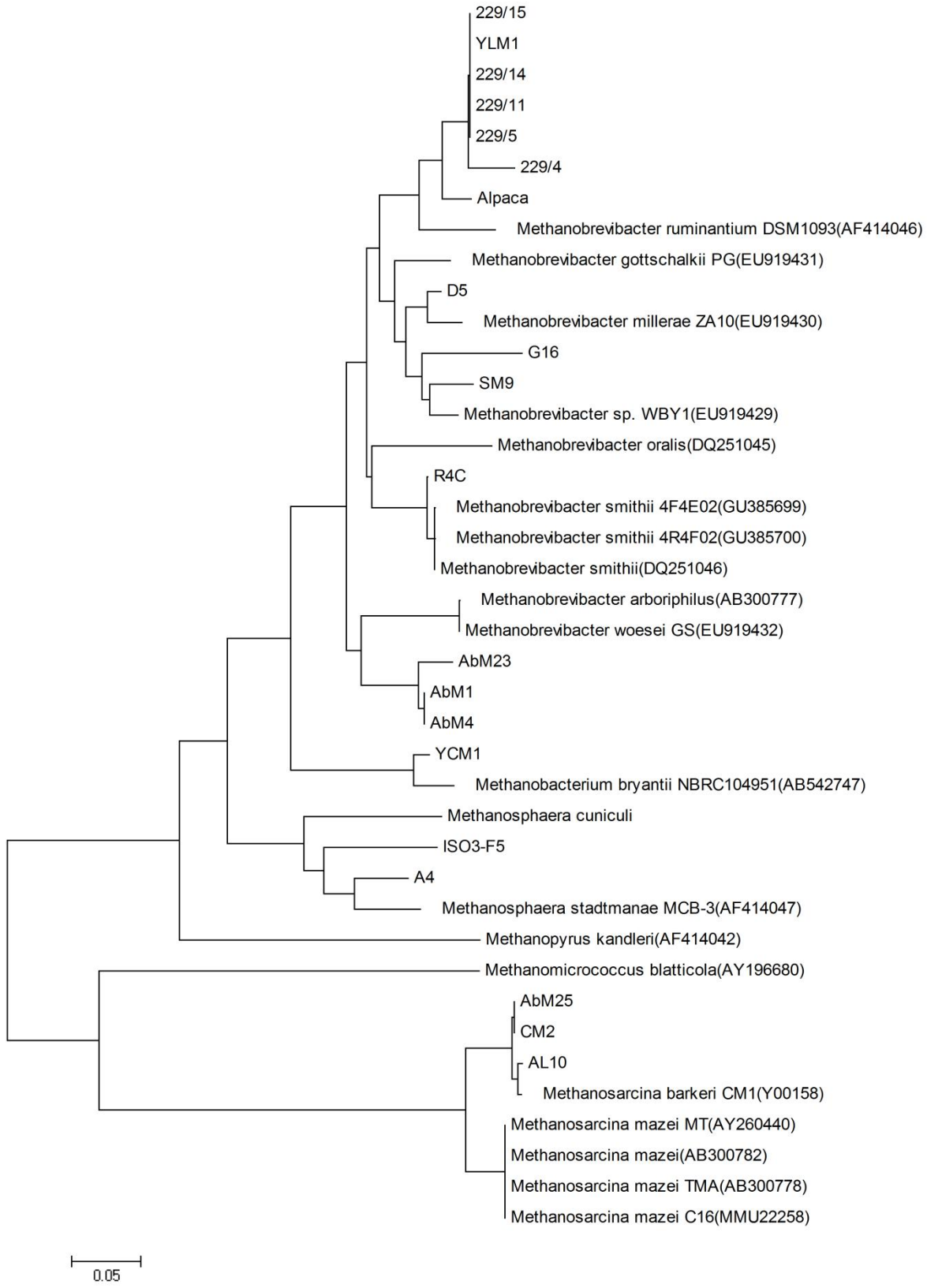
229/15
YLM1
229/14
229/11
229/5
229/4
Alpaca
Methanobrevibacter ruminantium DSM1093(AF414046)
Methanobrevibacter gottschalkii PG(EU919431)
D5
Methanobrevibacter millerae ZA10(EU919430)
G16
SM9
Methanobrevibacter sp. WBY1(EU919429)
Methanobrevibacter oralis(DQ251045)
R4C
Methanobrevibacter smithii 4F4E02(GU385699)
Methanobrevibacter smithii 4R4F02(GU385700)
Methanobrevibacter smithii(DQ251046)
Methanobrevibacter arboriphilus(AB300777)
Methanobrevibacter woesei GS(EU919432)
AbM23
AbM1
AbM4
YCM1
Methanobacterium bryantii NBRC104951(AB542747)
Methanosphaera cuniculi
ISO3-F5
A4
Methanosphaera stadtmanae MCB-3(AF414047)
Methanopyrus kandleri(AF414042)
Methanomicrococcus blatticola(AY196680)
AbM25
CM2
AL10
Methanosarcina barkeri CM1(Y00158)
Methanosarcina mazei MT(AY260440)
Methanosarcina mazei(AB300782)
Methanosarcina mazei TMA(AB300778)
Methanosarcina mazei C16(MMU22258)
0.05

Figure 3.2 A phylogenetic tree of methanogen isolates constructed based on *mcr*A rRNA gene sequence similarity. The GeneBank accessions numbers for reference sequences are in the paranthesis. For analysis, *mcr*A gene sequences were aligned with ClustalW and compared using MEGA 4.0 software. A distance matrix tree was constructed using the neighbor-joining methods and bootstrap re-sampled 1000 times.The scale bar indicates 0.05 nucleotide substitutions per nucleotide position. *Methanopyrus kandleri* and *Methanomicrococcus blatticola* were used as outgroups.

were obtained and were designated R4C and A4, respectively. Numerous attempts were made to isolate the cells of the genus *Methanosphaera* from the mixed culture of H6, but without success. On the other hand, the close relative of *Methanobrevibacter millerae* was readily isolated due to the relatively large number of the cells present in the culture. The pure culture of the genus *Methanobrevibacter* isolated from H6 was renamed D5. 16S rRNA gene Sequences from the purified cultures were identical to those originally generated from the mixed culture, confirming the identities of isolates R4C, A4, and D5.

3.3.3 Elimination of bacterial contamination

The archaeal cell wall of methanogens is resistant to the action of conventional anti-bacterial drugs. Moreover, many archaea are extremophiles inhabiting harsh environments including hot springs and salt lakes, and are better adapted to enduring high temperatures than bacteria. By exploiting these characteristics of archaea, a 3-stage purification process involving treatments with heat and antibiotics and a serial dilution of cultures was designed to eliminate the rumen bacteria from the methanogen cultures (see section 2.4). Jeyamalar *et al.* (2010) previously demonstrated the effectiveness of these methods at eliminating bacterial contaminants from methanogen cultures. Nearly all strains survived the purification process, and a majority of methanogen cultures became free of bacterial contamination at the end of the purification procedures. The purity of cultures was thoroughly ensured by inoculating into BY media along with 0.5 ml of RFgenV solution containing a sugar mixture that enhances the bacterial growth (section 2.3.4). None of the cultures showed the signs of growth when examined with microscopy and spectrophotometry, suggesting the presence of bacterial contaminants was eliminated.

Strain AS9/11/19 did not survive through the purification process, and was not studied further. Among the homologous strains, isolates 229/11 and AbM4 were chosen to

represent their respective groups. After taking various purification measures, purity of cultures was confirmed through fluorescent and phase-contrast microscopic imaging of cells All the cells observed under phase-contrast microscopy fluoresced when excited with UV light, indicating the cultures were free of bacterial contaminants (Fig. 3.3).

3.3.4 Morphology

The cell morphologies of the methanogen strains are shown in fig 3.3 and are summarized in Table 3.4. Isolates 229/4, 229/5, 229/11, 229/14, 229/15 and YLM1 were short, round cocci (2.0~3.0 µm) occurring singly or in pairs. AbM1, AbM4 and AbM23 were elongated rods (2.5~6.0 µm) with transparent spaces at both ends of each cell. In these irregular clumps, individual cells were hardly discernable during microscopic observation. G16 cells were coccobacilli less than 2 µm in length when occurring singly, but become elongated (2.0~4.0 µm) when in chains. The Alpaca isolate grew as coccobacilli that were 1.3~2.7 µm in length. A4 cells were large almost completely spherical cocci occurring in pairs or in grape-shaped clumps (2.7~3.3 µm). R4C cells were short rods (2.0~3.3 µm). D5 cells were rods (2.7~4.0 µm) occurring in chains. AS9/11/19 grew rods that occur in a scarcer number compared to other strains of methanogens studied in this thesis. The phenotypic details of strains MCB-3, ISO3-F5, SM9, BRM9 and YCM1 were in agreement with the formal descriptions of these strains found in the literature YCM1 and BRM9 are bent, long rods that grew up to 15 µm in length. Some of the cells were in long chains while few filamentous cells were occasionally observed. MCB-3 and ISO3-F5 are large, spherical cocci (2.7~3.3 µm) that occured in pairs or in grape-shaped clumps. None of the above isolates showed any signs of motility when observed under the phase contrast microscope. The motility in methanogens has been associated with the presence of flagella. However, little is known about their structure, attachment to the cell envelop, and energetics of motility

(Dworkin *et al*., 2006). Detailed genome analysis is usually needed in order to determine the presence of genes encoding structural components of flagella or pili.

3.4 Discussion

The culture collection at AgResearch covered a broad diversity of rumen methanogens representing various genera. Due to the large number of cultures that had been accumulating for decades, some were stored without being formally identified. This project was initiated from the necessity to inspect the viability of these cultures and to assign phylogenetic identities to the strains. In the parallel phylogenetic trees constructed based on 16S rRNA and *mcr*A gene sequence similarities, the majority of strains grouped within the genus *Methanobrevibacter*. The 16S rRNA gene sequence similarity of the strain D5 to its closest relative (*Methanobrevibacter millerae*) was slightly less than the 98% cut-off threshold for species differentiation, suggesting the potential phylogenetic novelty of this strain.

So far, only 3 species of the genus *Methanosphaera* have been isolated in pure cultures. *Methanosphaera stadtmanae,* isolated from human faeces, was the first species of the genus *Methanosphaera* that had the full-genome sequenced (Fricke *et al*., 2006). Genome analysis of *Methanosphaera stadtmanae* revealed that the organism has specially adapted to colonize the human gut (Fricke *et al*., 2006). *Methanosphaera cuniculi* was isolated from rectal samples of rabbits (Biavati *et al*., 1988). Strain ISO3-F5 has been very recently isolated from sheep and is the first true rumen isolate of the genus *Methanosphaera* (Jeyamalar, 2010). Genome sequencing of ISO3-F5 is currently under way to reveal its adaptive strategies to enable the survival of the strain in the rumen environment.

In this study, a pure culture of strain A4 was obtained from a culture derived from the foregut of a Tammar Wallaby (*Macropus eugenii*). According to the phylogenetic analysis, isolate A4 is considered a novel species of the genus *Methanosphaera* closely related to

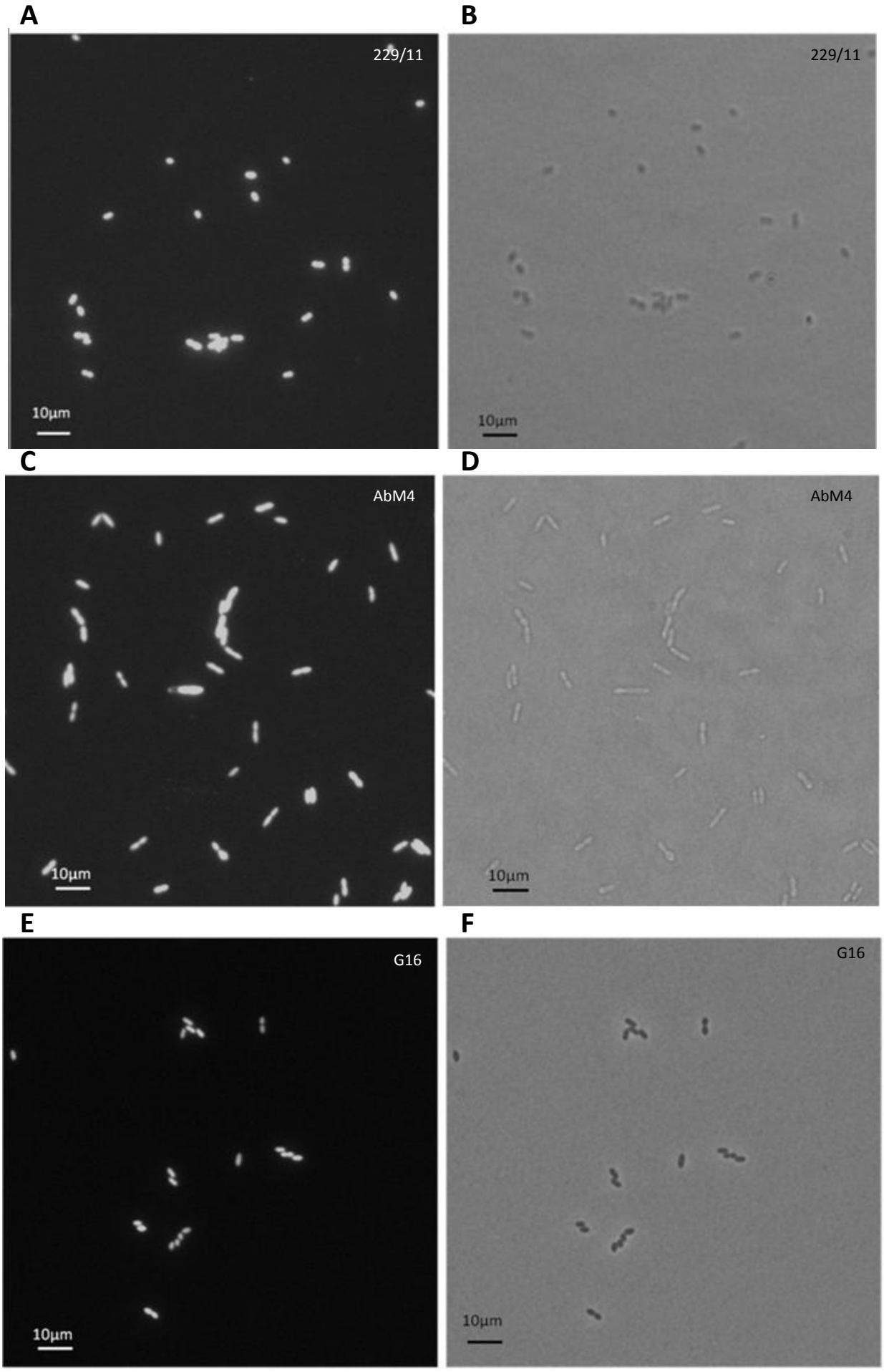

Figure 3.3 Fluorescence and phase-contrast microscopic images of methanogen isolates. Fluorescent (A,C,E) and phase-contrast (B,D,F) microscopic images of isolates 229/11, AbM4 and G16.

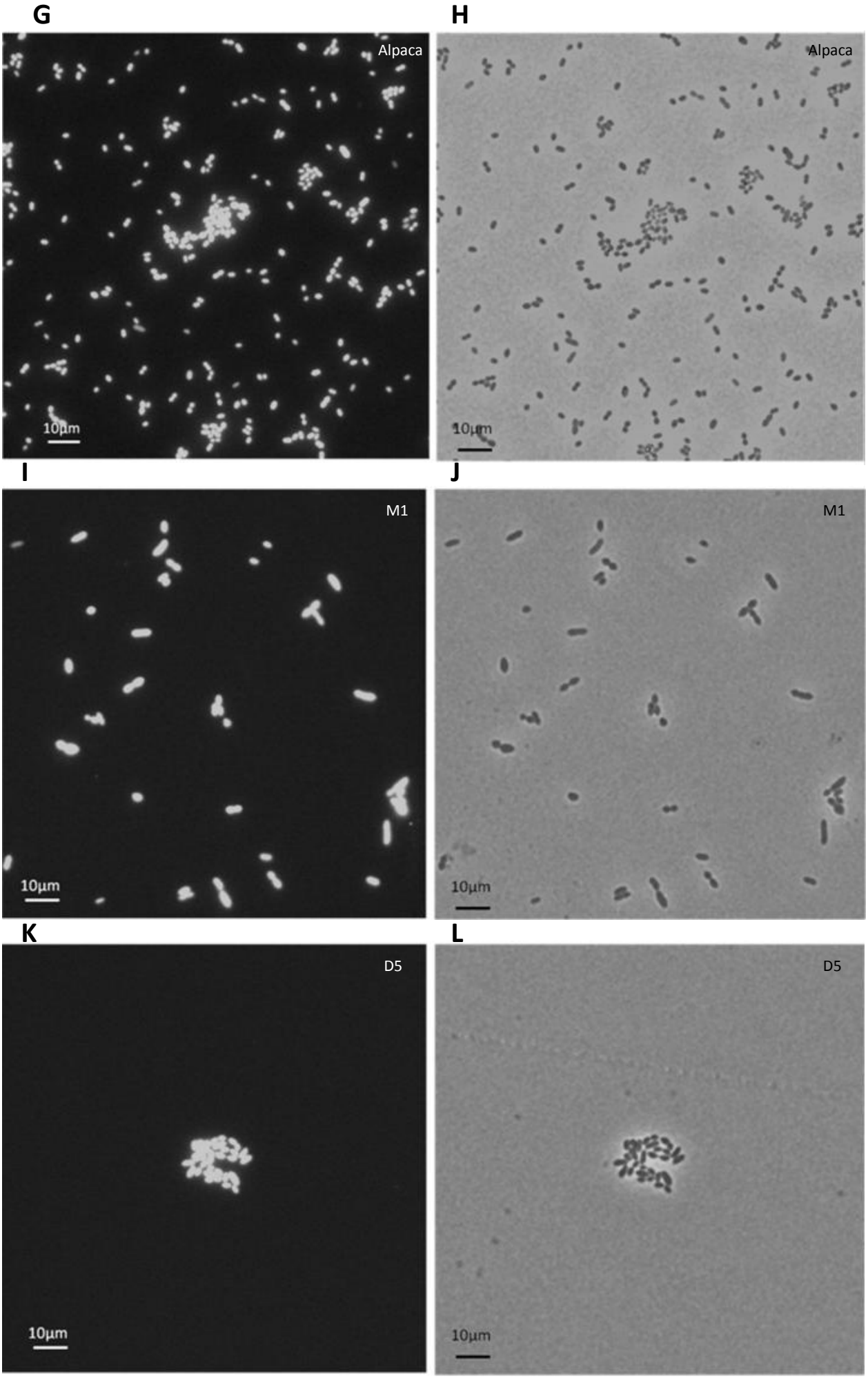

Figure 3.3 continued. Fluorescent (G,I,K) and phase-contrast (H,J,L) microscopic images of isolates Alpaca, M1 and D5.

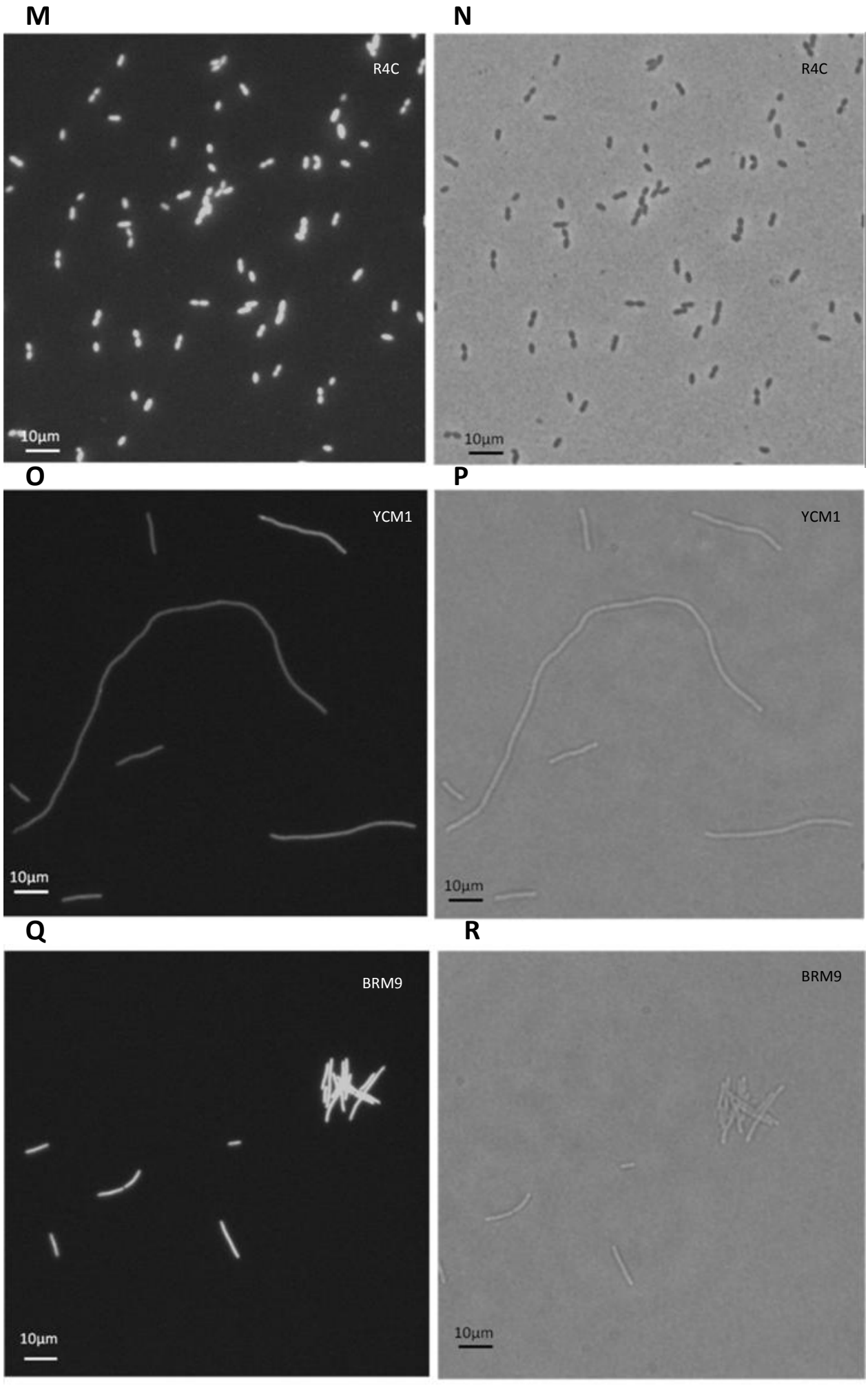

Figure 3.3 continued. Fluorescent (M,O,Q) and phase-contrast (N,P,R) microscopic images of isolates R4C, YCM1 and BRM9.

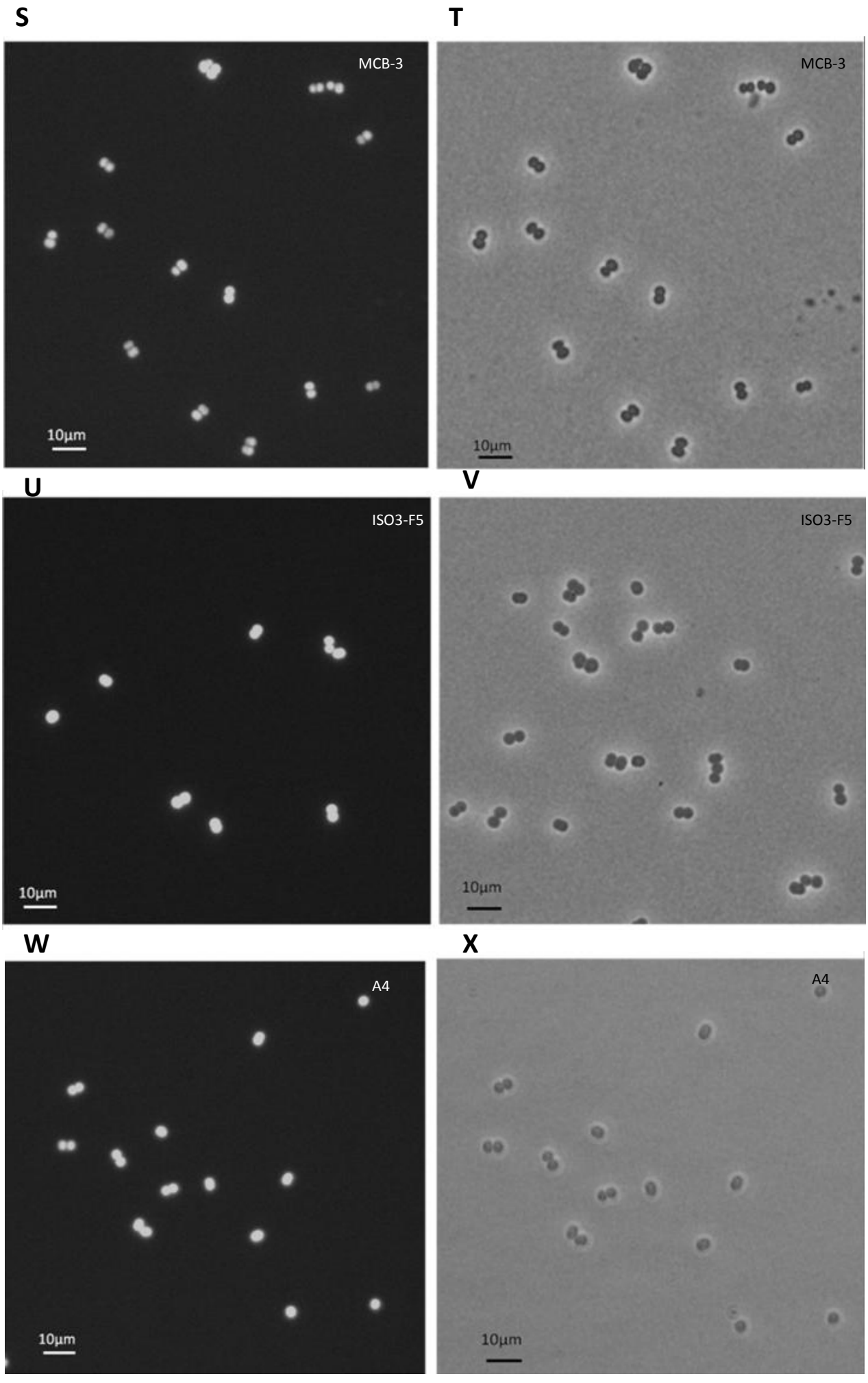

Figure 3.3 continued. Fluorescent (S,U,W) and phase-contrast (T,V,X) microscopic images of isolates MCB-3, ISO3-F5 and A4.

Table 3.4 Morphological descriptions of methanogen isolates used in this study.

Isolate	Cell shape	Cell length (µm)	Cell width (µm)	Specific characteristics
229/4	rod	2.0-3.0	1.0-1.3	Sometimes occur in chains
229/5	rod	2.0-3.0	1.0-1.3	
229/11	rod	2.0-3.0	1.0-1.3	
229/14	rod	2.0-3.0	1.0-1.3	
229/15	rod	2.0-3.0	1.0-1.3	
AbM4	rod	2.7-6.0	1.3-2.0	Transparent spaces at cell ends
AbM4	rod	2.7-6.0	1.3-2.0	
AbM23	rod	2.7-6.0	1.3-2.0	
AbM25	pseudosarcina	Irregular	irregular	
AL10	pseudosarcina	Irregular	irregular	Irregular cell clumps
CM2	pseudosarcina	Irregular	irregular	
CM3	pseudosarcina	Irregular	irregular	
R4C	rod	2.0-3.3	1.7-2.0	Isolated from Wallaby
Alpaca	coccobacillus	1.3-2.7	1.3-2.0	
SM9	coccobacillus	2.0-3.3	1.5-2.5	
AS9/11/19	rod	0.7-0.9	0.5-0.8	Occur in chains
G16	coccobacillus	2.0-3.3	2.0-2.7	
D5	rod	2.7-4.0	2.0-2.7	Derived from H6
A4	coccus	2.7-3.7	2.7-3.7	Isolated from wallaby
MCB-3	coccus	3.3-4.0	3.3-4.0	Human isolate
ISO3-F5	coccus	2.7-3.3	2.7-3.3	
BRM9	Ccooked long rod	> 4.0	0.7-1.3	Filamentous
YCM1	long rod	> 4.0	0.7-1.3	Filamentous
YLM1	rod	2.0-3.0	1.0-1.3	Sometimes occur in chains

Methanosphaera cuniculi. Wallabies, along with kangaroos, belong to the marsupial family Macropodidae. Native to Australia, marsupial animals evolved in geographically separated regions from the ruminant animals. Strictly speaking, macropod marsupials do not possess the rumen, but their foregut carries out similar functions to the rumen by harbouring a complex microbiome in which bacteria, archaea, fungi and protozoa interact to breakdown plant materials. For an unknown reason, the methane production from marsupial animals is relatively low compared to the ruminants (Dellow *et al*., 1988, Kempton *et al*., 1976, Engelhardt *et al*., 1978). It is still too early to describe the contribution that the members of the genus *Methanosphaera* have on the overall production of ruminant methane, as there are so few isolates available for study. Nevertheless, methanogens of the genus *Methanosphaera* are one of the predominant inhabitants of the rumen of New Zealand livestock (Jeyamalar *et al*., 20011). A comparative genome analysis of *Methanosphaera* spp. isolated from the rumen and the foregut of marsupials may reveal the genomic variations that cause the production of varying amounts of methane from these species. Genomic variations between these species may suggest (1) that the contribution of the genus *Methanosphaera* towards the overall production of ruminant methane may be relatively small; (2) methanogens inhabiting the foregut of marsupials are too small in numbers to produce a significant amount of methane; and (3) the marsupial foregut microbial ecosystem is not optimized for methanogenesis.

With the phylogenetic identities of the methanogens at hand, the next step will be to characterize the phenotypic properties of the strains of interest. A majority of the methanogen strains used in this study still has not been formally described. The complete description of a microbial species is carried out through the characterizations of the substrate and growth factor requirements, the optimal pH and temperature ranges, G+C contents of genomic DNA

taxonomic and morphological properties, and tolerance against chemicals inducing cell lysis such as sodium dodecyl sulphate (SDS), lysozymes, proteases and NaCl solutions. It was not the aim of this work to complete a full characteristization; rather, growth characteristics that would allow further experimentation were determined.

Chapter 4

The minimum substrate requirements for rumen methanogens

4.1 Introduction

Majority of the methanogen strains that were phylogenetically defined in Chapter 3 have still not been formally described. To begin describing these strains, the minimum substrate requirements for growth were determined. Nine pure strains derived from initial isolates, each representing a differen methanogen species, were tested for growth using different combinations of substrates including formate, acetate, and methanol with or without the addition of H_2/CO_2.

It was planned to measure the minimum H_2 threshold concentrations below which methanogens fail to use H_2. It was necessary to establish the minimal growth conditions for individual methanogen strains prior to the measurement of the threshold levels for hydrogen H_2. Furthermore, the results obtained from this study may be used as a part of characterization studies to support the findings from the on-going analysis of the methanogen genomes or for any description of new strains or species.

4.2 Materials and methods

Selected isolates were tested for their ability to utilize formate (50 mM), methanol (30 mM) and acetate (20 mM) with or without H_2/CO_2 using the methods described in section 2.9.

4.3 Results

The minimum substrate requirements of ten methanogen isolates belonging to the genus *Methanobrevibacter*, *Methanobacterium*, and *Methanosphaera* were determined. Two or more isolates were chosen to represent each genus. The effect of different combinations of

H_2/CO_2, formate, methanol, and acetate on the growth of the selected strains was investigated by measuring the daily increase in optical density of cultures was measured to determine the time course of growth and maximal cell density. The results are summarized in Tables 4.1-4.10.

4.3.1 The substrate requirements of *Methanobrevibacter olleyae* 229/11

Methanobrevibacter olleyae 229/11 did not grow on formate, methanol or acetate alone, and absolutely required H_2/CO_2 to grow (Fig. 4.1). Although there was no indication of formate alone being utilized as a growth substrate, consistently high OD values were obtained when H_2/CO_2, formate and acetate were present together in the medium.

4.3.2 The substrate requirements of *Methanobrevibacter* spp. AbM4

Methanobrevibacter spp. AbM4 was not able to grow without the addition of H_2/CO_2 (Fig. 4.2). The strain grew relatively well using H_2/CO_2 as the sole source of energy. The growth pattern of AbM4 was not significantly altered by the inclusion of additives other than acetate, which appeared to stimulate growth.

4.3.3 The substrate requirements of *Methanobrevibacter smithii* R4C

The optical density of cultures of *Methanobrevibacter smithii* R4C reached a peak within 1-2 days of the growth, immediately followed by a sharp decline (Fig. 4.3). Regardless of the substrates added, the maximum OD was below 0.1, indicating that under the normal growth condition used, the growth requirements of *Methanobrevibacter smithii* strain R4C may not have been met. H_2/CO_2 sufficed as the minimum substrate

Table 4.1 Effect of different substrate combinations on the growth of *Methanobrevibacter olleyae* 229/11.

The substrates tested were H_2/CO_2, formate (50 mM), methanol (30 mM) and acetate (20 mM). Requirements of these substrates were determined with 3 serial transfers to eliminate the carry-over effect on the initial inocula. Coenzyme M (5 nM) and rumen fluid (NoSubRFV) were added universally into all cultures.

Additions	**Growth**		
	Transfer 1	**Transfer 2**	**Transfer 3**
H_2/CO_2	+++	+++	+++
Formate	-	-	-
Methanol	-	-	-
Acetate	-	-	-
H_2/CO_2 + Formate	++	++	+
H_2/CO_2 + Methanol	++	++	++
H_2/CO_2 + Acetate	++	++	++
H_2/CO_2 + Formate + Methanol	++	++	++
H_2/CO_2 + Formate + Acetate	++	++	++
H_2/CO_2 + Methanol + Acetate	++	++	++
H_2/CO_2 + Formate + Methanol + Acetate	++	++	++

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

Table 4.2 Effect of different substrate combinations on the growth of *Methanobrevibacter* sp. AbM4.

The substrates tested were H_2/CO_2, formate (50 mM), methanol (30 mM) and acetate (20 mM). Requirements of these substrates were determined with 3 serial transfers to eliminate the carry-over effect on the initial inocula. Coenzyme M (5 nM) and rumen fluid (NoSubRFV) were added universally into all cultures.

Additions	**Growth**		
	Transfer 1	**Transfer 2**	**Transfer 3**
H_2/CO_2	++	++	++
Formate	-	-	-
Methanol	-	-	-
Acetate	-	-	-
H_2/CO_2 + Formate	++	++	++
H_2/CO_2 + Methanol	++	++	++
H_2/CO_2 + Acetate	++	++	+++
H_2/CO_2 + Formate + Methanol	++	++	++
H_2/CO_2 + Formate + Acetate	+++	+++	++
H_2/CO_2 + Methanol + Acetate	++	+++	++
H_2/CO_2 + Formate + Methanol + Acetate	++	++	++

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

Table 4.3 Effect of different substrate combinations on the growth of *Methanobrevibacter smithii* R4C.

The substrates tested were H_2/CO_2, formate (50 mM), methanol (30 mM) and acetate (20 mM). Requirements of these substrates were determined with 3 serial transfers to eliminate the carry-over effect on the initial inocula. Coenzyme M (5 nM) and rumen fluid (NoSubRFV) were added universally into all cultures.

Additions	Growth		
	Transfer 1	Transfer 2	Transfer 3
H_2/CO_2	+	+	+
Formate	-	-	-
Methanol	-	-	-
Acetate	-	-	-
H_2/CO_2 + Formate	+	+	+
H_2/CO_2 + Methanol	+	+	+
H_2/CO_2 + Acetate	+	+	+
H_2/CO_2 + Formate + Methanol	+	+	+
H_2/CO_2 + Formate + Acetate	+	+	+
H_2/CO_2 + Methanol + Acetate	+	+	+
H_2/CO_2 + Formate + Methanol + Acetate	+	+	+

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

Table 4.4 Effect of different substrate combinations on the growth of *Methanobrevibacter ruminantium* M1.

The substrates tested were H_2/CO_2, formate (50 mM), methanol (30 mM) and acetate (20 mM). Requirements of these substrates were determined with 3 serial transfers to eliminate the carry-over effect on the initial inocula. Coenzyme M (5nM) and rumen fluid (NoSubRFV) were added universally into all cultures.

Additions	Growth		
	Transfer 1	Transfer 2	Transfer 3
H_2/CO_2	-	-	-
Formate	-	-	-
Methanol	-	-	-
Acetate	-	-	-
H_2/CO_2 + Formate	+++	++	++
H_2/CO_2 + Methanol	+++	++	+
H_2/CO_2 + Acetate	+++	+	+
H_2/CO_2 + Formate + Methanol	+++	++	+
H_2/CO_2 + Formate + Acetate	+++	+	+
H_2/CO_2 + Methanol + Acetate	+++	++	+
H_2/CO_2 + Formate + Methanol + Acetate	+++	++	++

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

Table 4.5 Effect of different substrate combinations on the growth of *Methanobrevibacter* sp. D5.

The substrates tested were H_2/CO_2, formate (50 mM), methanol (30 mM) and acetate (20 mM). Requirements of these substrates were determined with 3 serial transfers to eliminate the carry-over effect on the initial inocula. Coenzyme M (5 nM) and rumen fluid (NoSubRFV) were added universally into all cultures.

Additions	**Growth**		
	Transfer 1	**Transfer 2**	**Transfer 3**
H_2/CO_2	+	++	++
Formate	-	-	-
Methanol	-	-	-
Acetate	-	-	-
H_2/CO_2 + Formate	+	++	++
H_2/CO_2 + Methanol	+	++	++
H_2/CO_2 + Acetate	+	++	++
H_2/CO_2 + Formate + Methanol	++	++	++
H_2/CO_2 + Formate + Acetate	++	++	++
H_2/CO_2 + Methanol + Acetate	+	++	++
H_2/CO_2 + Formate + Methanol + Acetate	+	++	++

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

Table 4.6 Effect of different substrate combinations on the growth of *Methanobacterium formicicum* BRM9.

The substrates tested were H_2/CO_2, formate (50 mM), methanol (30 mM) and acetate (20 mM). Requirements of these substrates were determined with 3 serial transfers to eliminate the carry-over effect on the initial inocula. Coenzyme M (5 nM) and rumen fluid (NoSubRFV) were added universally into all cultures.

Additions	**Growth**		
	Transfer 1	**Transfer 2**	**Transfer 3**
H_2/CO_2	++	+	++
Formate	-	-	-
Methanol	-	-	-
Acetate	-	-	-
H_2/CO_2 + Formate	++	++	+++
H_2/CO_2 + Methanol	++	+	+
H_2/CO_2 + Acetate	+	++	++
H_2/CO_2 + Formate + Methanol	++	++	++
H_2/CO_2 + Formate + Acetate	++	++	++
H_2/CO_2 + Methanol + Acetate	++	++	++
H_2/CO_2 + Formate + Methanol + Acetate	++	+++	+++

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

Table 4.7 Effect of different substrate combinations on the growth of *Methanobacterium bryantii* YCM1.

The substrates tested were H_2/CO_2, formate (50 mM), methanol (30 mM) and acetate (20 mM). Requirements of these substrates were determined with 3 serial transfers to eliminate the carry-over effect on the initial inocula. Coenzyme M (5 nM) and rumen fluid (NoSubRFV) were added universally into all cultures.

Additions	**Growth**		
	Transfer 1	**Transfer 2**	**Transfer 3**
H_2/CO_2	+	+	+
Formate	-	-	-
Methanol	-	-	-
Acetate	-	-	-
H_2/CO_2 + Formate	++	+	+
H_2/CO_2 + Methanol	++	+	+
H_2/CO_2 + Acetate	++	++	++
H_2/CO_2 + Formate + Methanol	+	+	++
H_2/CO_2 + Formate + Acetate	++	+	+
H_2/CO_2 + Methanol + Acetate	++	+	+
H_2/CO_2 + Formate + Methanol + Acetate	++	+	+

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

Table 4.8 Effect of different substrate combinations on the growth of *Methanosphaera stadtmanae* MCB-3.

The substrates tested were H_2/CO_2, formate (50 mM), methanol (30 mM) and acetate (20 mM). Requirements of these substrates were determined with 3 serial transfers to eliminate the carry-over effect on the initial inocula. Coenzyme M (5 nM) and rumen fluid (NoSubRFV) were added universally into all cultures.

Additions	**Growth**		
	Transfer 1	**Transfer 2**	**Transfer 3**
H_2/CO_2	-	-	-
Formate	-	-	-
Methanol	-	-	-
Acetate	-	-	-
H_2/CO_2 + Formate	-	-	-
H_2/CO_2 + Methanol	+++	+++	+++
H_2/CO_2 + Acetate	-	-	-
H_2/CO_2 + Formate + Methanol	+++	+++	+++
H_2/CO_2 + Formate + Acetate	-	-	-
H_2/CO_2 + Methanol + Acetate	+++	+++	+++
H_2/CO_2 + Formate + Methanol + Acetate	+++	+++	+++

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

Table 4.9 Effect of different substrate combinations ects on the growth of *Methanosphaera* sp. ISO3-F5.

The substrates tested were H_2/CO_2, formate (50 mM), methanol (30 mM) and acetate (20 mM). Requirements of these substrates were determined with 3 serial transfers to eliminate the carry-over effect on the initial inocula. Coenzyme M (5 nM) and rumen fluid (NoSubRFV) were added universally into all culture.

Additions	**Growth**		
	Transfer 1	**Transfer 2**	**Transfer 3**
H_2/CO_2	-	-	-
Formate	-	-	-
Methanol	-	-	-
Acetate	-	-	-
H_2/CO_2 + Formate	-	-	-
H_2/CO_2 + Methanol	+++	+	-
H_2/CO_2 + Acetate	-	-	-
H_2/CO_2 + Formate + Methanol	-	-	-
H_2/CO_2 + Formate + Acetate	-	-	-
H_2/CO_2 + Methanol + Acetate	+	+++	+++
H_2/CO_2 + Formate + Methanol + Acetate	+++	+	++

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

Table 4.10 Effect of different substrate combinations on the growth of *Methanosphaera* sp. A4.

The substrates tested were H_2/CO_2, formate (50 mM), methanol (30 mM) and acetate (20 mM). Requirements of these substrates were determined with 3 serial transfers to eliminate the carry-over effect on the initial inocula. Coenzyme M (5 nM) and rumen fluid (NoSubRFV) were added universally into all culture.

Additions	**Growth**		
	Transfer 1	**Transfer 2**	**Transfer 3**
H_2/CO_2	-	-	-
Formate	-	-	-
Methanol	-	-	-
Acetate	-	-	-
H_2/CO_2 + Formate	-	-	-
H_2/CO_2 + Methanol	+++	+++	-
H_2/CO_2 + Acetate	-	-	-
H_2/CO_2 + Formate + Methanol	+++	-	-
H_2/CO_2 + Formate + Acetate	-	-	-
H_2/CO_2 + Methanol + Acetate	+++	+++	+++
H_2/CO_2 + Formate + Methanol + Acetate	+++	+++	+++

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

requirements of R4C. It was difficult to tell if the strain utilized formate, acetate or methanol due to the premature termination of the growth.

4.3.4 The substrate requirements of *Methanobrevibacter ruminantium* M1

Methanobrevibacter ruminantium strain M1 did not grow on H_2/CO_2, and required addition of any one, or combination of formate, methanol or acetate for growth (Fig.4.4). In the final transfer, the growth of M1 increased almost by 2-fold at the inclusion of all four substrates. In the second and the final transfers, the growth of M1 was initiated only after experiencing a lag phase which lasted for 3~4 days when H_2/CO_2 was present in the medium together with any one of methanol or acetate. An extensive incubation period was required before the growth of M1 on H_2/CO_2 plus formate was initiated in the final transfer. While cultivating methanogens, it was observed the cultures that were maintained for a prolonged period even after the stationary phase has been reached are susceptible to cell lysis. The increasingly dissipating cellular mass in the medium as a result of the cell lysis is reflected by a gradual decline of OD readings from the peak growth until the growth curve reaches a similar level to the initial starting point. When these aged cultures are used as the inocula, the poor cell qualities may either prevent the growth or delay the initiation of the growth. In the second transfer, the M1 cultures growing on H_2/CO_2 plus formate reached the peak OD barely two days after the time of inoculation. Because these cultures had to be maintained without additional injections of H_2/CO_2 until the next transfer, the carried-over cells from the second transfer may have been in poor health by the time the final inoculation was carried out. As a consequence, a longer-than-usual lag phase may have resulted in the final transfer before the growth of M1 cultures on H_2/CO_2 plus formate was triggered.

4.3.5 The substrate requirements of *Methanobrevibacter* sp. D5

H_2/CO_2 was absolutely required to support the growth of *Methanobrevibacter* sp. D5. In the second transfer, a slightly higher growth was observed when formate and acetate were included in the substrate combination (Fig. 4.5). In the final transfer, the growth of D5 was stimulated when acetate was present alone or together with other additives. However, there was no direct evidence of formate or acetate being utilized by D5 for growth when provided alone with H_2/CO_2.

4.3.6 The substrate requirements of *Methanobacterium formicicum* BRM9

Methanobacterium formicicum BRM9 could grow solely on H_2/CO_2, although the growth was substantially enhanced with the inclusion of formate (Fig. 4.6). The strain absolutely required H_2/CO_2 for growth, and did not grow on CO_2 plus formate in the absence of H_2/CO_2.

4.3.7 The substrate requirements of *Methanobacterium bryantii* YCM1

Methanobacterium bryantii YCM1 could grow well solely on H_2/CO_2 (Fig. 4.7). No signs of formate or methanol utilization were observed, as the strain failed to grow on either substrate without the presence of H_2/CO_2. In the second and the third transfers, the addition of acetate substantially stimulated the growth compared to the growth on H_2/CO_2 alone.

4.3.8 The substrate requirements of *Methanosphaera stadtmanae* MCB-3

Methanosphaera stadtmanae MCB-3 was able to grow by utilizing H_2/CO_2 and methanol as the growth substrates (Fig. 4.8). Interestingly, there was no indication of acetate being stimulatory for this strain. The optical density of MCB-3 measured at its peak growth was approximately 5-fold higher than that of M1, which was also demonstrated in the relatively high turbidity of the cultures.

4.3.9 The substrate requirements of *Methanosphaera* sp. ISO3-F5

A majority of the substrate combinations from the first transfer was eliminated as *Methanosphaera* sp. ISO3-F5 did not grow unless H_2/CO_2, methanol and acetate were all included in the combination (Fig. 4.9). The strain was able to grow without acetate until the second transfer, probably due to the carry-over of residual acetate remaining in the medium from the first transfer. However, it was apparent that growth of ISO3-F5 is required by acetate as the strain survived through all three transfers only when acetate was present in the medium. The cultures of ISO3-F5 was characterized by a very high turbidity, which was mirrored in the subsequent OD measurement in which almost 2-fold higher optical density was obtained for ISO3-F5 compared to M1.

4.3.10 The substrate requirements of *Methanosphaera* sp. A4

From the first transfer, only five substrate combinations containing H_2/CO_2 and methanol were able to support the growth of *Methanosphaera* sp. A4 (Fig.4.10). A4 was able to grow without acetate until the second transfer. At the end of the third transfer however, it became apparent that the isolate requires acetate in that A4 survived through the three transfers only when all of H_2, CO_2, methanol and acetate were present in the medium. The cultures of A4 became very turbid after 2-4 days of inoculation, and the heavy growth was reflected on the relatively high optical density measured during the stationary phase.

4.4 Discussion

The experimental results revealed that a majority of strains was able to grow using H_2/CO_2 as the sole source of energy. As expected from published work (Biavati *et al*., 1988 Fricke *et al*., 2006 Jeyamalar, 2010), the members of the genus *Methanosphaera* gave no signs of growth when any one of H_2/CO_2, acetate or methanol was absent from the substrate

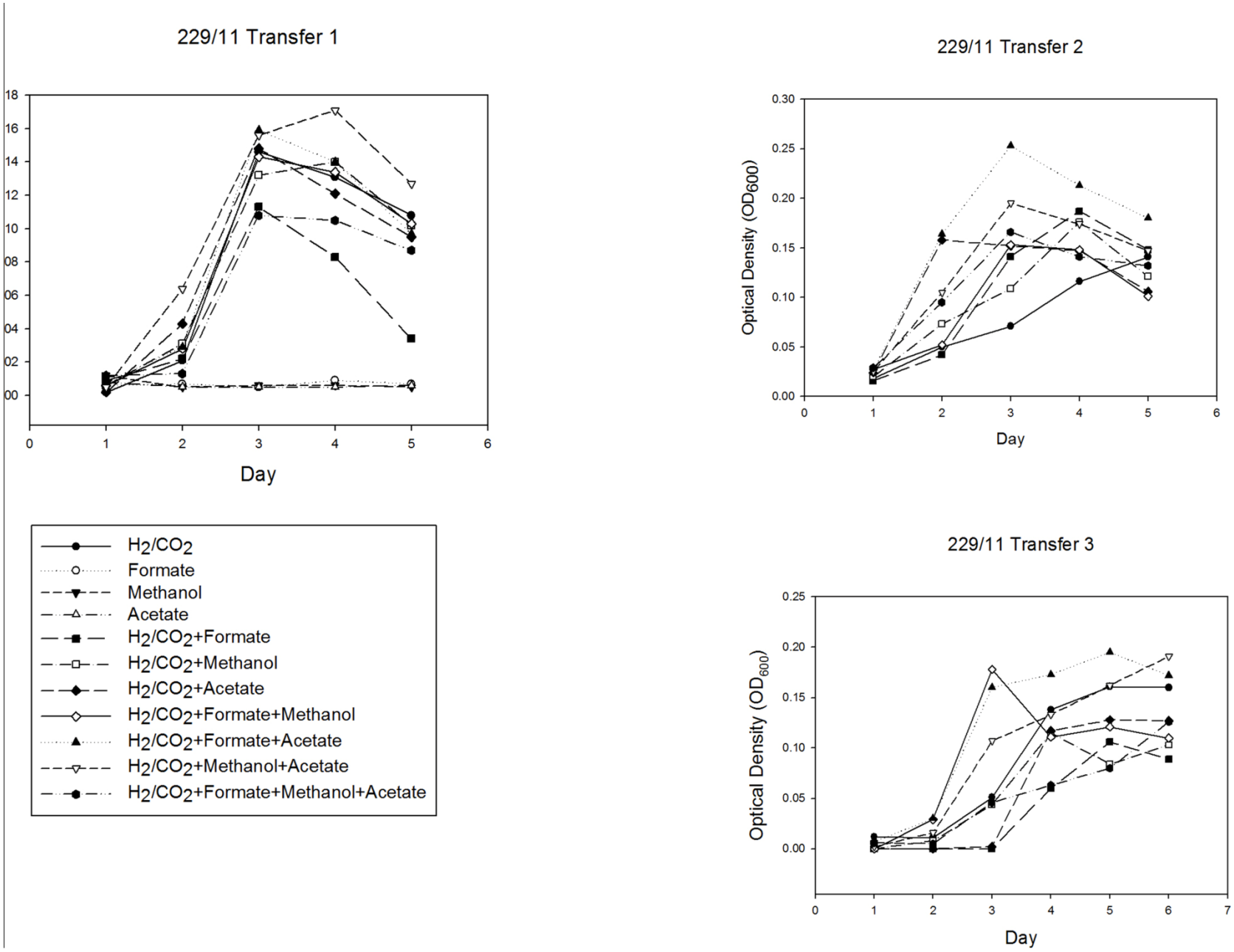

Figure 4.1 Growth curve of *Methanobrevibacter olleyae* 229/11 growing on combinations of formate (50 mM), methanol (30 mM) and acetate (20 mM) with or without H_2/CO_2 (80:20) at 200 kPa. Growth of these substrates was determined for 3 serial transfers to eliminate the carry-over from the initial inocula. Each point is a mean of 3 replicates.

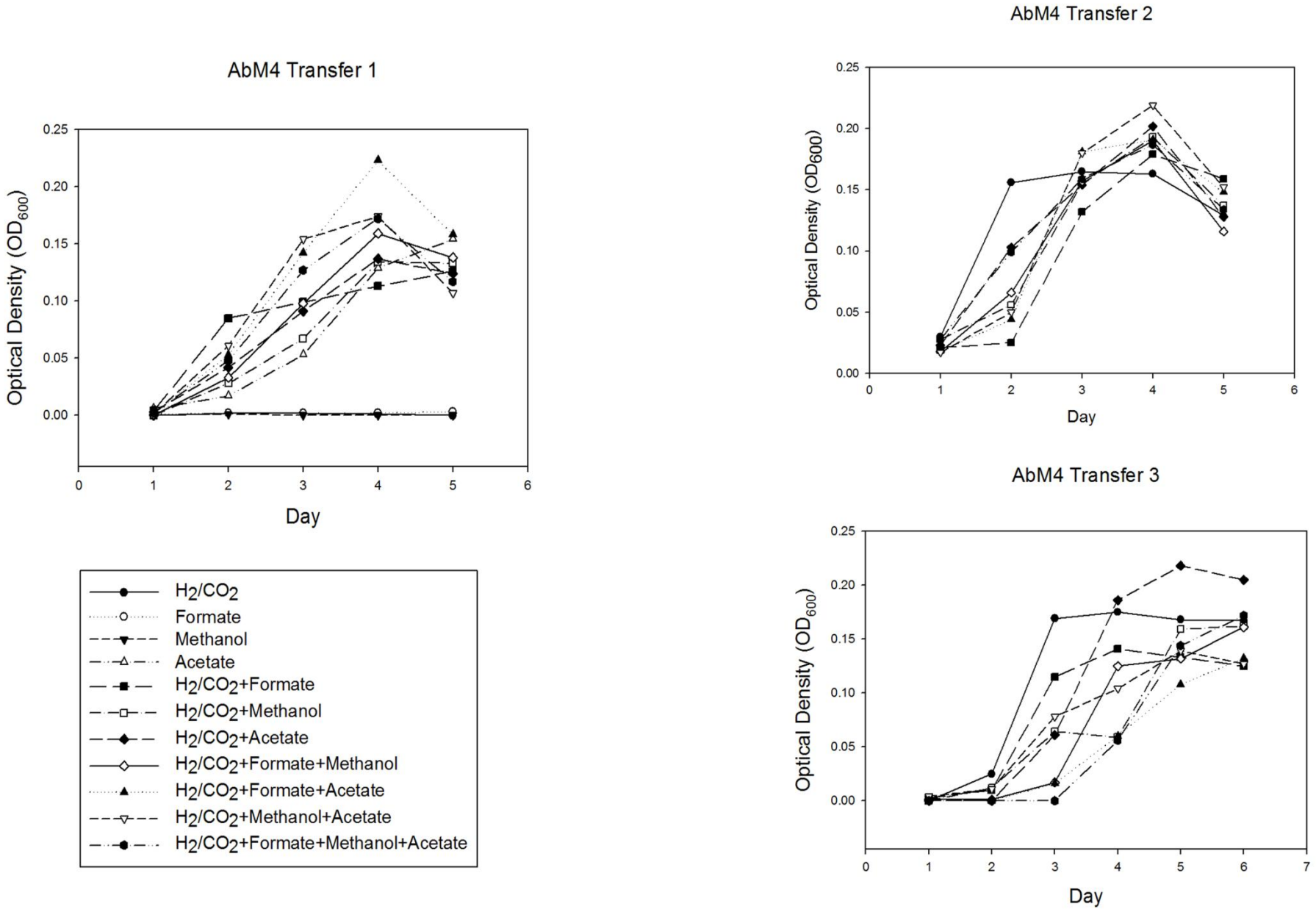

Figure 4.2 Growth curve of *Methanobrevibacter* sp. AbM4 growing on combinations of formate (50 mM), methanol (30 mM) and acetate (20 mM) with or without H_2/CO_2 (80:20) at 200 kPa. Growth of these substrates was determined for 3 serial transfers to eliminate the carry-over from the initial inocula. Each point is a mean of 3 replicates.

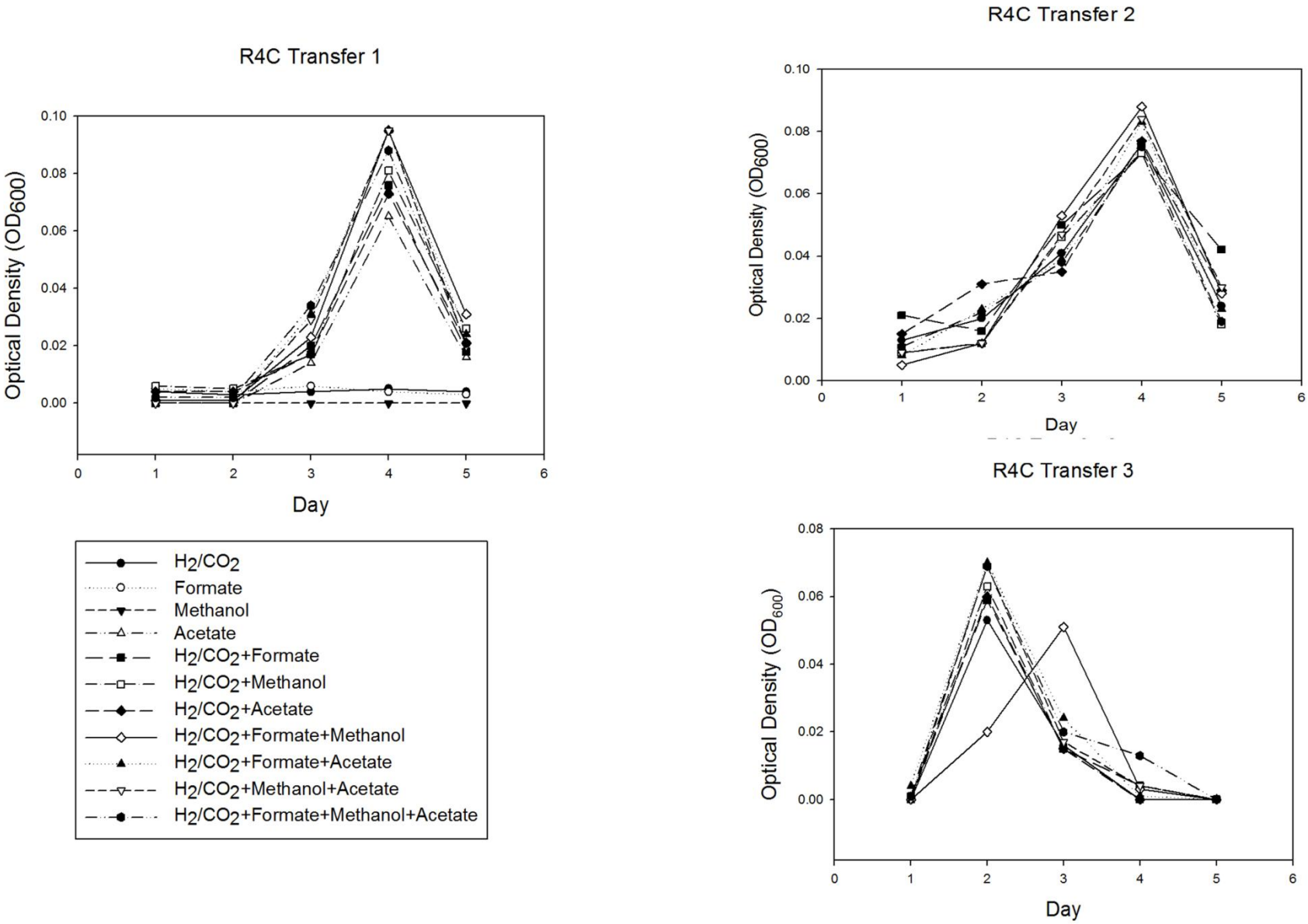

Figure 4.3 Growth curve of *Methanobrevibacter smithii* R4C growing on combinations of formate (50 mM), methanol (30 mM) and acetate (20 mM) with or without H_2/CO_2 (80:20) at 200 kPa. Growth of these substrates was determined for 3 serial transfers to eliminate the carry-over from the initial inocula. Each point is a mean of 3 replicates.

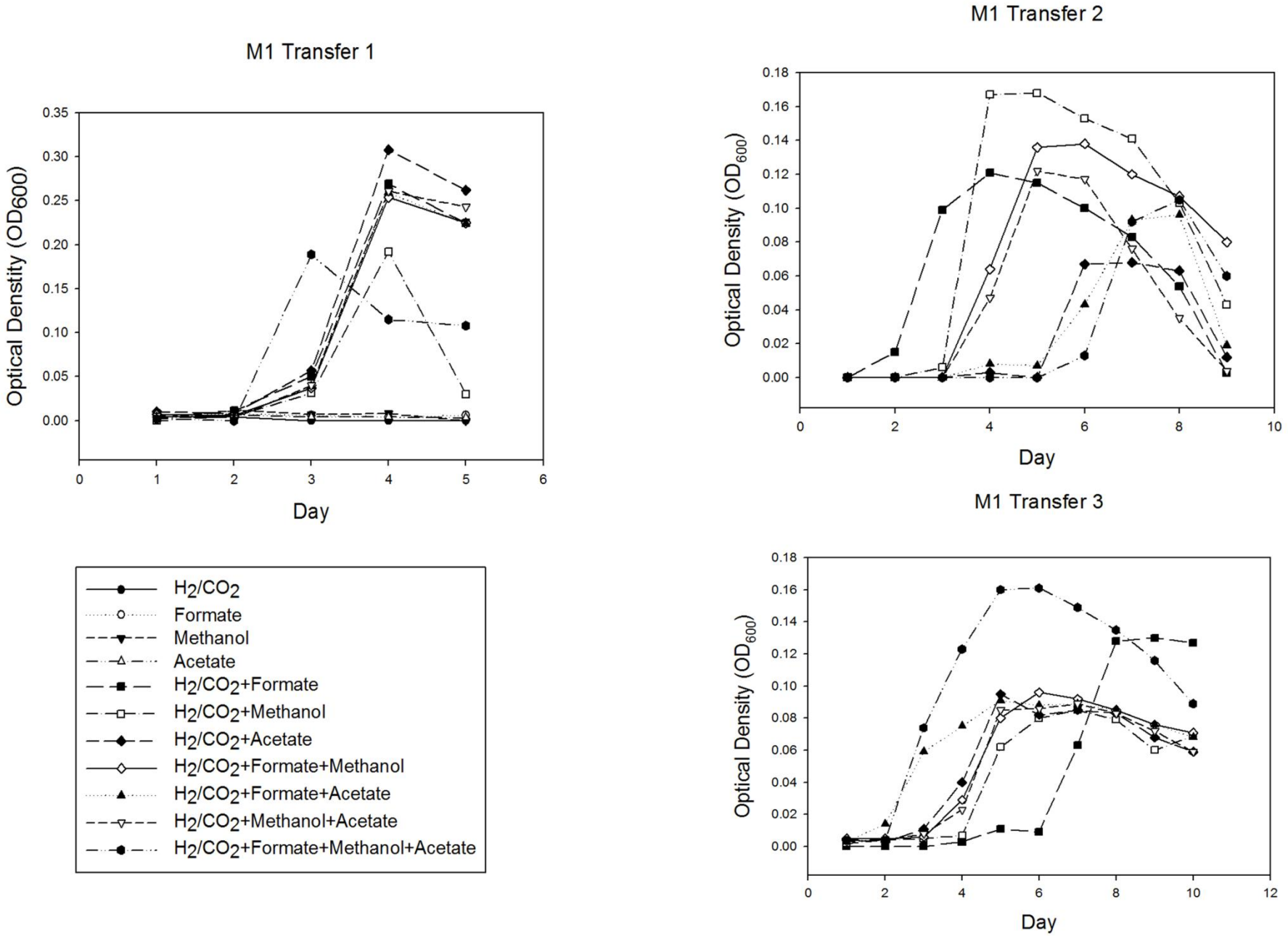

Figure 4.4 Growth curve of *Methanobrevibacter ruminantium* M1 growing on combinations of formate (50 mM), methanol (30 mM) and acetate (20 mM) with or without H_2/CO_2 (80:20) at 200 kPa. Growth of these substrates was determined for 3 serial transfers to eliminate the carry-over from the initial inocula. Each point is a mean of 3 replicates.

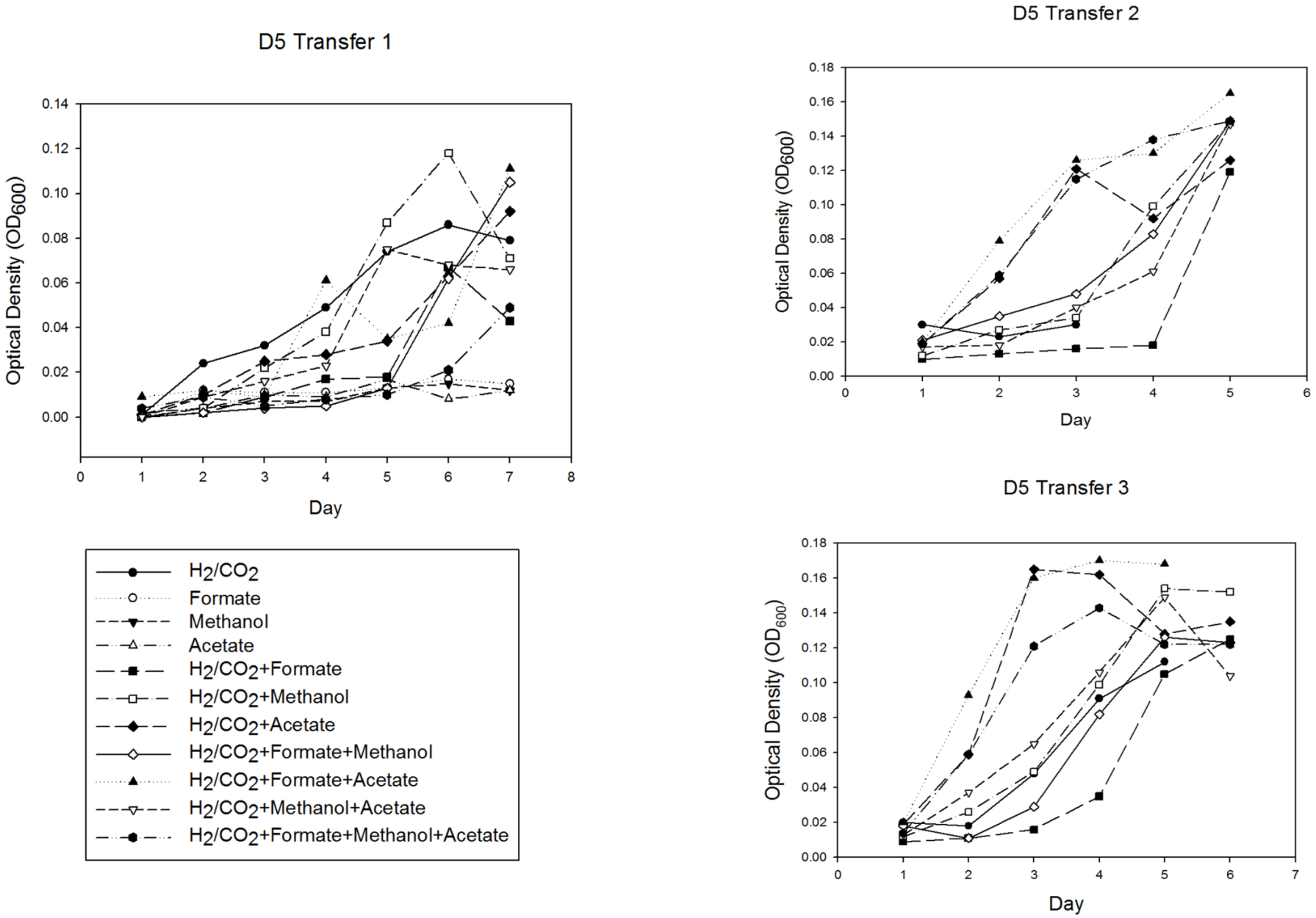

Figure 4.5 Growth curve of *Methanobrevibacter* sp. D5 growing on combinations of formate (50 mM), methanol (30 mM) and acetate (20 mM) with or without H_2/CO_2 (80:20) at 200 kPa. Growth of these substrates was determined for 3 serial transfers to eliminate the carry-over from the initial inocula. Each point is a mean of 3 replicates.

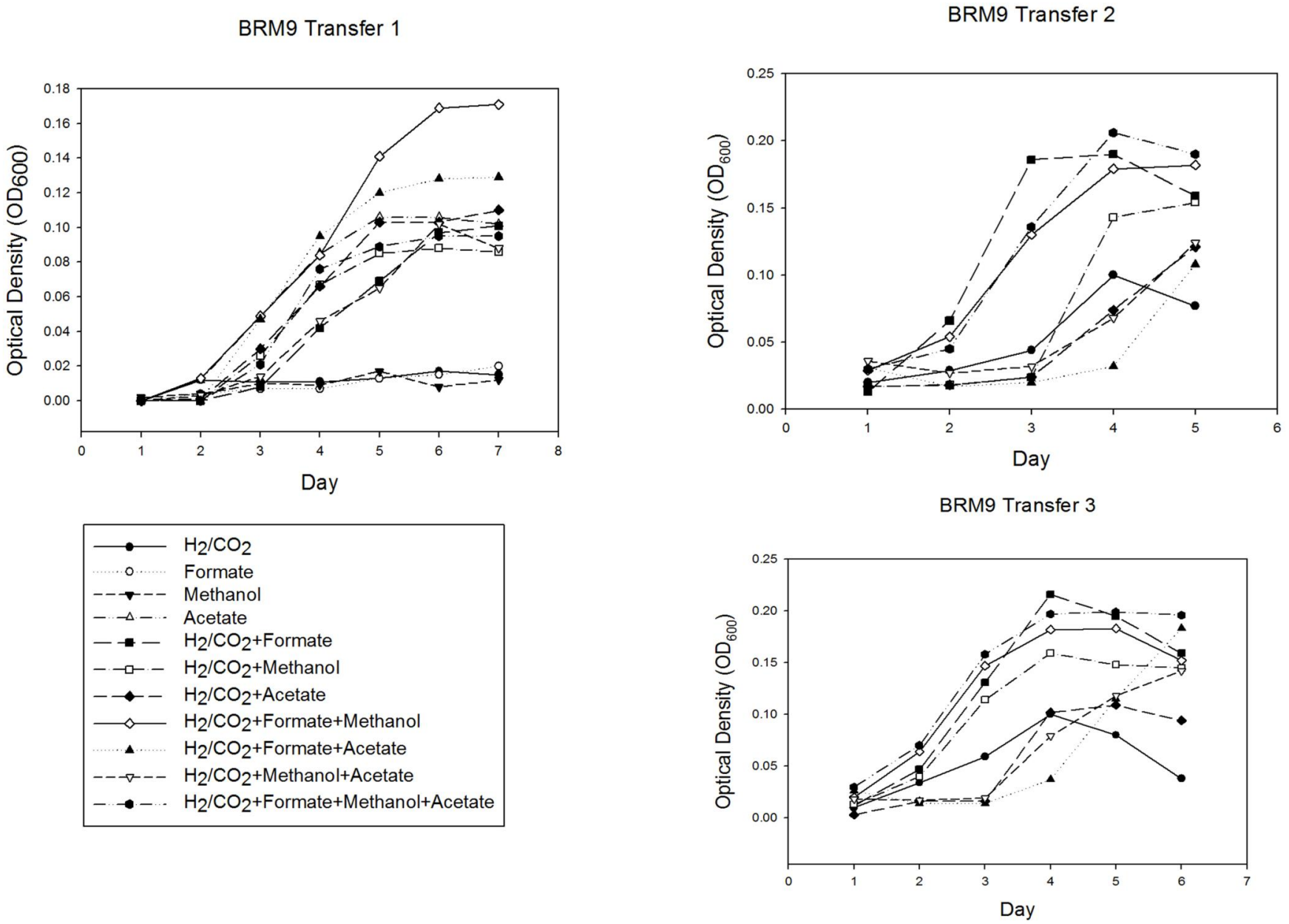

Figure 4.6 Growth curve of *Methanobacterium formicicum* BRM9 growing on combinations of formate (50 mM), methanol (30 mM) and acetate (20 mM) with or without H_2/CO_2 (80:20) at 200 kPa. Growth of these substrates was determined for 3 serial transfers to eliminate the carry-over from the initial inocula. Each point is a mean of 3 replicates.

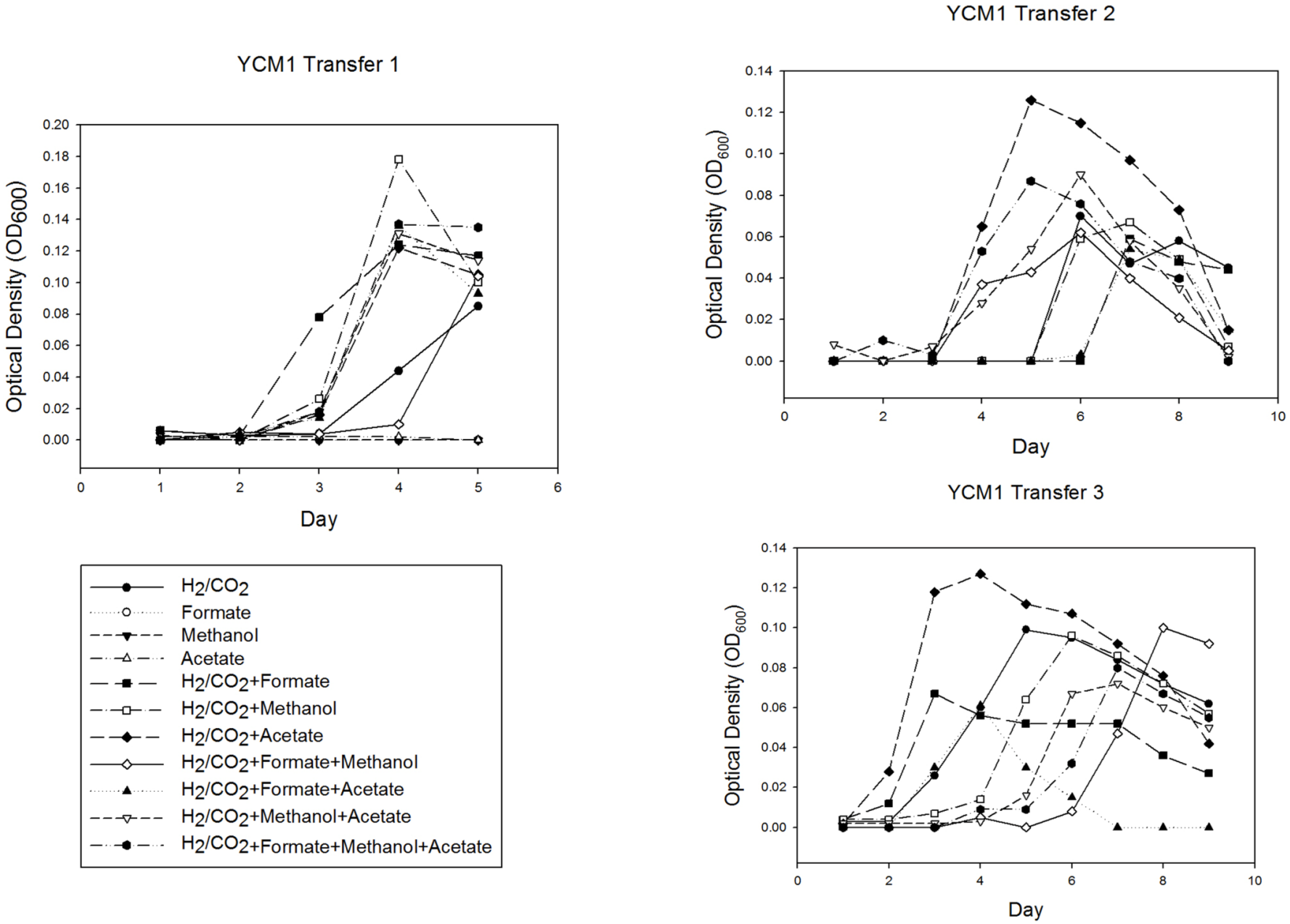

Figure 4.7 Growth curve of *Methanobacterium bryantii* YCM1 growing on combinations of formate (50 mM), methanol (30 mM) and acetate (20 mM) with or without H_2/CO_2 (80:20) at 200 kPa. Growth of these substrates was determined for 3 serial transfers to eliminate the carry-over from the initial inocula. Each point is a mean of 3 replicates.

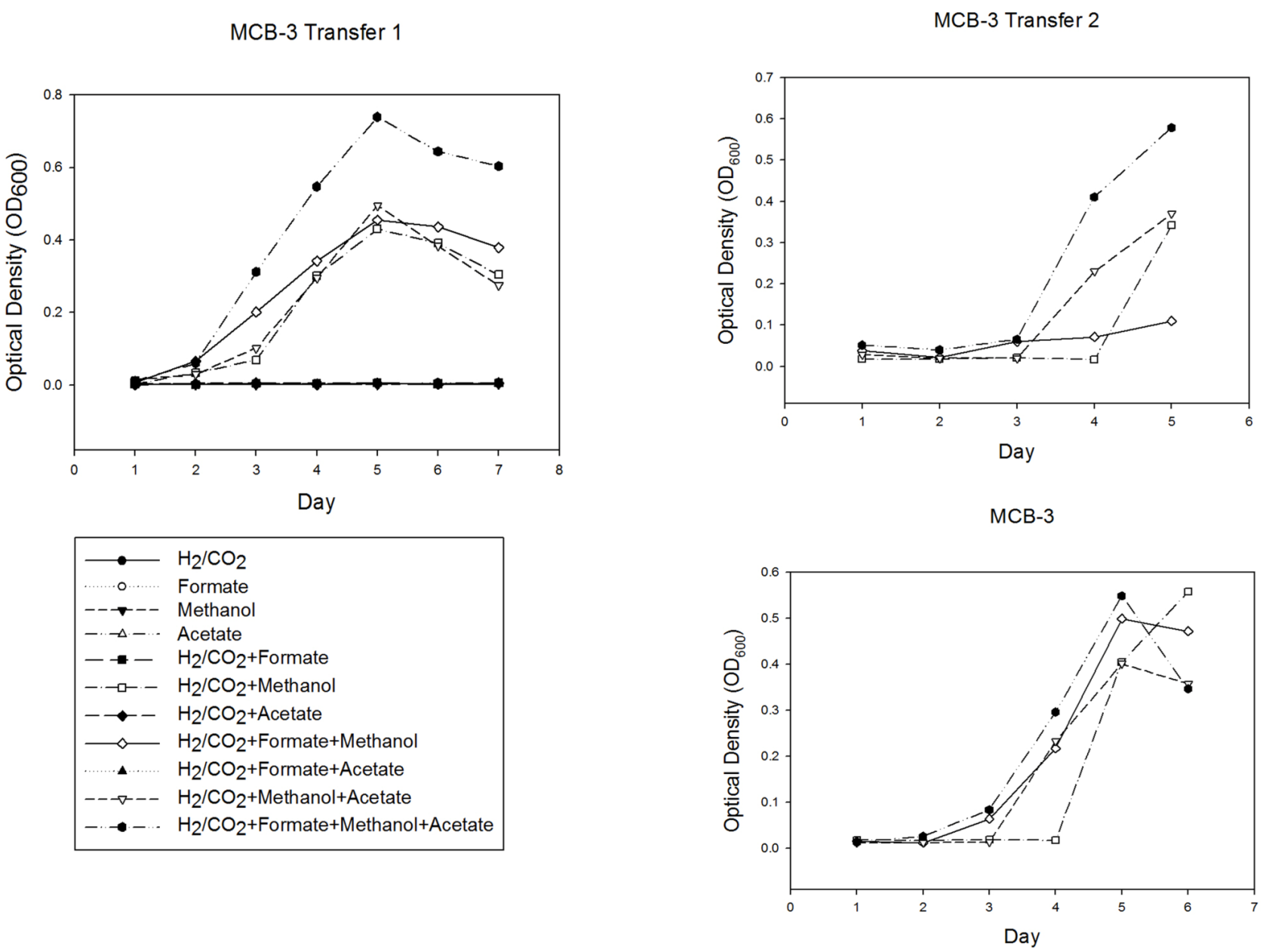

Figure 4.8 Growth curve of *Methanosphaera stadtmanae* MCB-3 growing on combinations of formate (50 mM), methanol (30 mM) and acetate (20 mM) with or without H_2/CO_2 (80:20) at 200 kPa. Growth of these substrates was determined for 3 serial transfers to eliminate the carry-over from the initial inocula. Each point is a mean of 3 replicates.

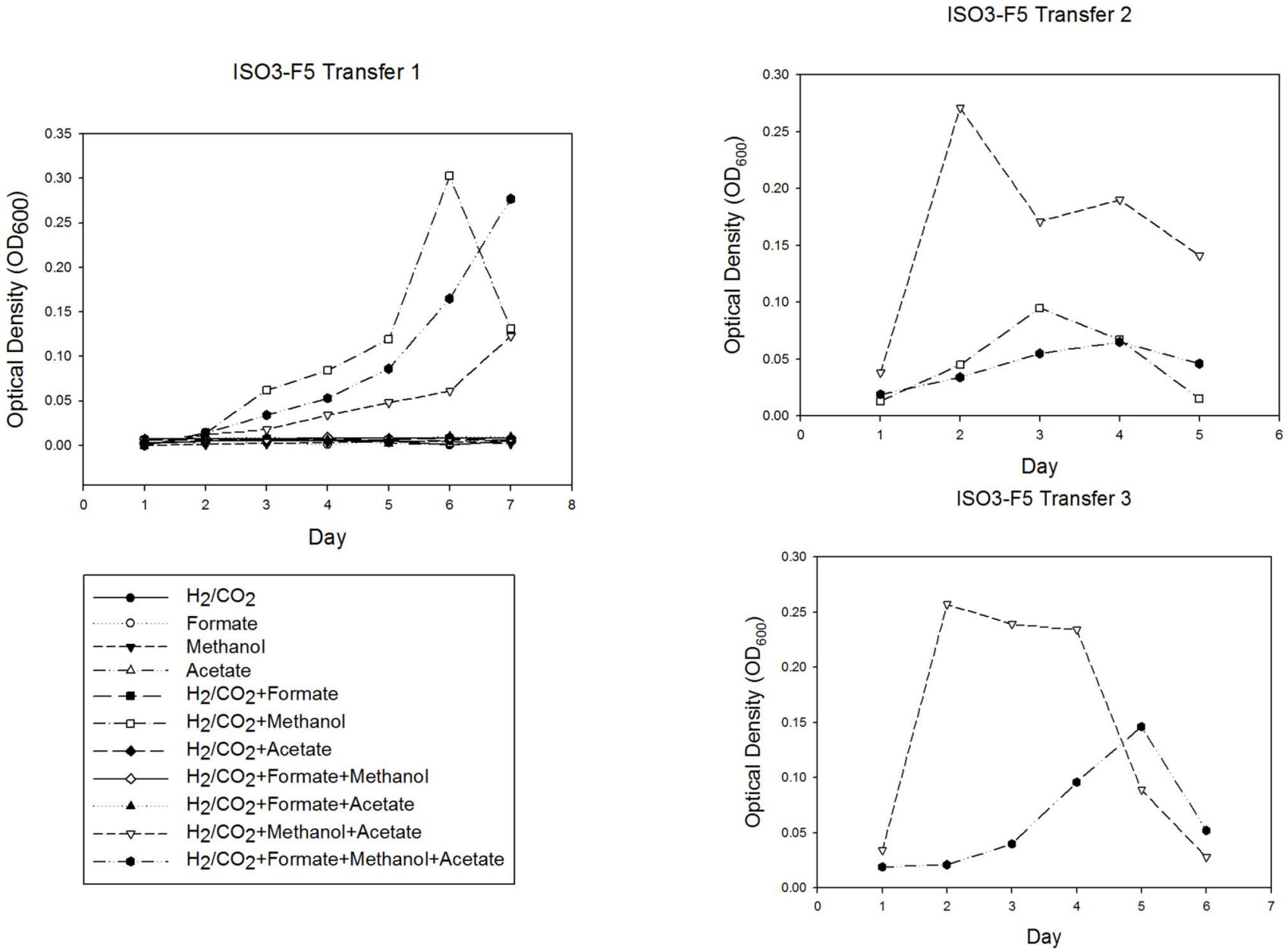

Figure 4.9 Growth curve of *Methanosphaera* sp. ISO3-F5 growing on combinations of formate (50 mM), methanol (30 mM) and acetate (20 mM) with or without H_2/CO_2 (80:20) at 200 kPa. Growth of these substrates was determined for 3 serial transfers to eliminate the carry-over from the initial inocula. Each point is a mean of 3 replicates.

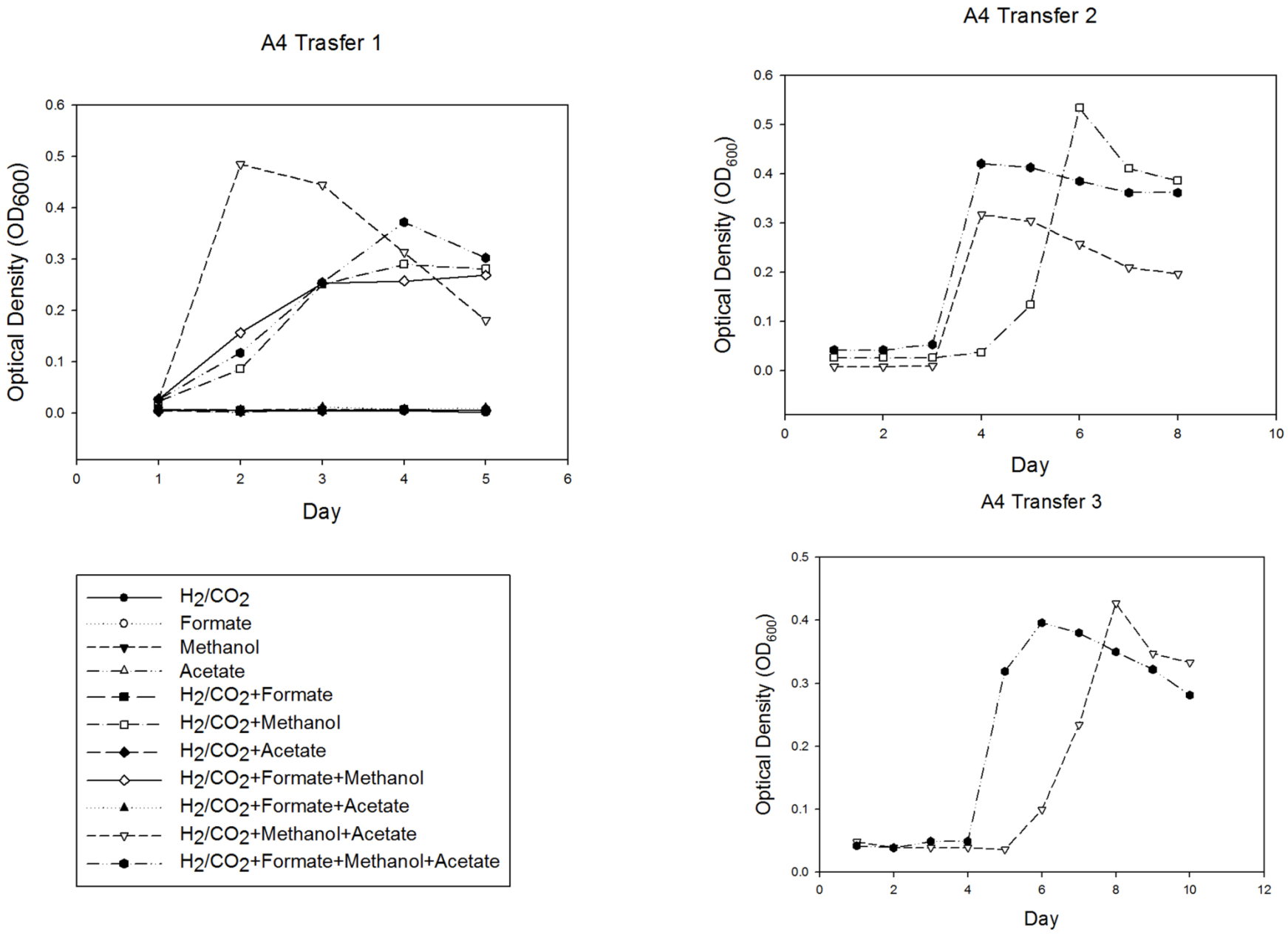

Figure 4.10 Growth curve of *Methanosphaera* sp. A4 growing on combinations of formate (50 mM), methanol (30 mM) and acetate (20 mM) with or without H_2/CO_2 (80:20) at 200 kPa. Growth of these substrates was determined for 3 serial transfers to eliminate the carry-over from the initial inocula. Each point is a mean of 3 replicates.

composition. No strain was able to grow without H_2/CO_2, demonstrating the essential involvement of hydrogen in the process of methanogensis.

The genus *Methanobrevibacter* is characterized by the ability to utilize H_2/CO_2, and in some cases CO_2 plus formate as the energy and carbon source. The few rumen strains of the genus *Methanobrevibacter* that were shown to be capable of growing on CO_2 plus formate include *Methanobrevibacter ruminantium* M1, *Methanobrevibacter olleyae* KM1H5-1P Methanobrevibacter thaueri CW, *Methanobrevibacter wolinii* SH and *Methanobrevibacter millerae* ZA-10.

Methanobrevibacter olleyae strain KM1H5-1P was isolated from sheep rumen contents and was characterized in a study reported by Rea *et al*. (2007). In that study, KM1H5-1P was tested for its ability to use various substrates including formate, acetate, methanol, ethanol, 2-propanol, 2-butanol, methylamine and trimethylamine under the presence of either CO_2/N_2 (20:80) or H_2/CO_2 (80:20), and was found to grow and produce methane using H_2/CO_2 or CO_2 plus formate. Interestingly, Rea *et al*. (2007) reported that the two reference strains used (*Methanobrevibacter* sp. OCP and *Methanobrevibacter* sp. AK-87) were not able to grow on CO_2 plus formate, and absolutely required H_2/CO_2 for growth. According to the 16S rRNA and *mcr*A phylogenetic trees obtained from the previous chapter, *Methanobrevibacter* sp. AK-87 belongs to a sister group to M*ethanobrevibacter ruminantium* M1, and is distantly related to *Methanobrevibacter olleyae*. On the other hand, *Methanobrevibacter* spp. OCP, a bovine rumen isolate, grouped with *Methanobrevibacter olleyae* KM1H5-1P and the five 229 strains that share a 98% 16S rRNA gene sequence similarity with *Methanobrevibacter olleyae*. Thus, of the three strains of *Methanobrevibacter olleyae* tested for formate utilization, only strain KM1H5-1P was able to grow on CO_2 plus formate, while strains OCP and 229/11 required H_2/CO_2.

Methanobrevibacter millerae ZA-10 was a bovine rumen isolate characterized by Rea *et al*. (2007). From the previous chapter, D5 was identified as a close relative of ZA-10, possibly a separate strain of *Methanobrevibacter millerae*. Despite the phylogenetic proximity between two organisms, only ZA-10 demonstrated the ability to utilize formate plus CO_2.

The substrate utilization by *Methanobrevibacter* sp. AbM4 has been studied for the first time in this thesis. Isolated from sheep abomasum, AbM4 forms its own cluster within the *Methanobrevibacter gottschalkii* clade. Being a typical *Methanobrevibacter*, the strain was able to grow solely on H_2/CO_2, and gave no clear signs of formate, methanol or acetate utilization.

The ability to utilize formate is not limited to the members of the genus *Methanobrevibacter*, as it is also found in some members of the genus *Methanobacterium*. *Methanobacterium formicicum* strain BRM9 was one of the first methanogen species that has been isolated from grazing cattle (Jarvis *et al*., 2000). The author reported that BRM9 grew on H_2/CO_2 or formate. However, when BRM9 was tested for formate utilization in this thesis, the strain failed to grow on formate without the presence of H_2/CO_2. Under H_2/CO_2, formate appeared to stimulate the growth of BRM9, as reflected by the high OD readings that were consistently obtained throughout the three transfers. The substrate utilization by *Methanobacterium bryantii* strain YCM1 was tested for the first time in this thesis. YCM1 was able to grow and produce methane solely on H_2/CO_2. There were no apparent signs of formate utilization by this strain, but the addition of acetate slightly enhanced the growth. The formatotrophic lifestyle is also observed in non-rumen isolates of the genus *Methanobacterium* that share a high degree of phylogenetic and morphological similarities

with the rumen strains such as BRM9 and YCM1. Unlike the rumen methanogens, non-rumen methanogens do not require rumen fluid and yeast extract for growth. Benstead *et al*. (1991) reported to have observed methane production using formate from the methanogens isolated from aquatic sediments including *Methanobacterium formicicum* MF and *Methanobacterium bryantii* M.o. H.G but not *Methanobacterium* strain FR-2 and *Methanobacterium bryantii* M.o.H. On the other hand, Joulian *et al* (1998) has reported that only the hydrogenotrophic lifestyle was found in *Methanobacterium bryantii* isolated from rice soils.

These observations suggest that strains belonging to a single methanogen species may not necessarily share the same capacity for substrate utilization. It appears the ability to use formate plus CO_2 is found only in a limited number of strains, and these strains frequently occur in the same species with the strains that grow on H_2 but not on formate. Whether there is a survival advantage for the strains capable of using both pathways to generate methane remains unknown.

Methanobrevibacter ruminantium was the first methanogen species isolated from the bovine rumen (Smith and Hungate, 1958). *Methanobrevibacter ruminantium* M1 is the only species of rumen methanogens whose full genome sequence has been made available to the public. M1 is known to grow with H_2 plus CO_2 and formate. The enzymes and cofactors involved in this pathway have been studied in detail. Microarray analysis of gene expression by M1 that was co-cultured with *Butyrivibrio proteoclasticus* B316 revealed a significant up-regulation in the expression of the formate utilization genes (*fdh*AB), suggesting formate is an important substrate for growth and the production of methane. Consistent with these findings, M1 was incapable of growing in the absence of H_2, confirming the soundness of the

methodology used in this study. Interestingly, even when formate was absent, M1 was able to grow with H_2/CO_2 as long as methanol or acetate was present (Rea *et al*., 2007). The full genome analysis of M1 revealed that the strain lacks the methanophenazine-reducing hydrogenase (VhoACG), methanophenazine-dependent heterodisulphide reductase (HdrDE) and the homologues of *mta* genes that are involved in methanol utilization by the members of the order Methanosarcinales (Leahy *et al*., 2010). Acetate was found to be essential for cell carbon biosynthesis after activation to acetyl CoA (acs, acsA), followed by reductive carboxylation to pyruvate (porABCDEF). Leahy *et al*. (2010) noted that in the presence of a limiting amount of H_2/CO_2, methanol and acetate stimulated the growth of M1. In this thesis, M1 was capable of growing on methanol plus H_2/CO_2 or acetate plus H_2/CO_2, but the growth started 3~6 days after the time of inoculation. Because the cultures were pressurized with H_2/CO_2 only at the beginning of each transfer, the level of H_2 is expected to gradually deplete as H_2 is slowly used by the methanogen. These results suggest that under low levels of H_2/CO_2, M1 is capable of growing on methanol or acetate, confirming the results obtained by Leahy *et al*. (2010).

Methanobrevibacter smithii is a numerically dominant group of methanogens that makes up 10% of all anaerobes in the colon of healthy adults that harbour a methanogenic flora (Lin and Miller, 1998, Samuel *et al*., 2007). The genome analysis of *Methanobrevibacter smithii* strain PS revealed that the genes involved in utilization of CO_2, H_2 and formate were highly enriched (Samuel *et al*., 2007). In addition, genes encoding enzymes that facilitate the use of acetate, ethanol and methanol in other methanogens were also discovered in the genome of *Methanobrevibacter smithii* PS, although the conversion of these substrates into methane by this organism has not yet been experimentally proved (Samuel *et al*., 2007). R4C, which has been isolated from the foregut contents of the Tamar

Wallaby, is one of the few non-human isolate of *Methanobrevibacter smithii* that have been made available in pure cultures. Very recently, *Methanobrevibacter smithii* strain GMS-01 was isolated from the goat rumen and its identity confirmed based on the *mcr*A gene sequence similarity (Gupta *et al*., 2010). Kumar *et al*. (2011) also isolated strains BRM-1 and BRM-3 from the rumen of Indian buffalos, and these share a 100% 16S rRNA gene sequence similarity to *Methanobrevibacter smithii*. The characterization of the buffalo isolates revealed that these strains use H_2 plus CO_2, formate and acetate as substrate, but failed to grow on ethanol and methanol. Consistent with the results obtained by Kumar *et al*. (2011), R4C was unable to grow without the presence of H_2/CO_2. Furthermore R4C did not utilize the CO_2 plus formate pathway to produce methane. The inverted v-shaped growth curves and low final optical densities of R4C suggest that under the growth condition used here, the optimal growth requirements for the strain are not met. As a result, it was difficult to determine the effects of formate, methanol and acetate on the growth of R4C. Further optimization process will be needed to carry out a complete characterization of substrate requirements for R4C.

The ability to produce methane using CO_2 as the electron acceptor and H_2 as the electron donor is a characteristic of the members of the order *Methanobacteriales.* *Methanosphaera* sp. is unique because they use H_2 to reduce methanol to methane without involving CO_2 in the pathway. Furthermore, studies of *Methanosphaera* spp. revealed that acetate is absolutely required to support their growth. So far, three species of the genus *Methanosphaera* have been isolated and characterized. *Methanosphaera stadtmanae* is a common inhabitant of the human gut, and was the first species of the genus

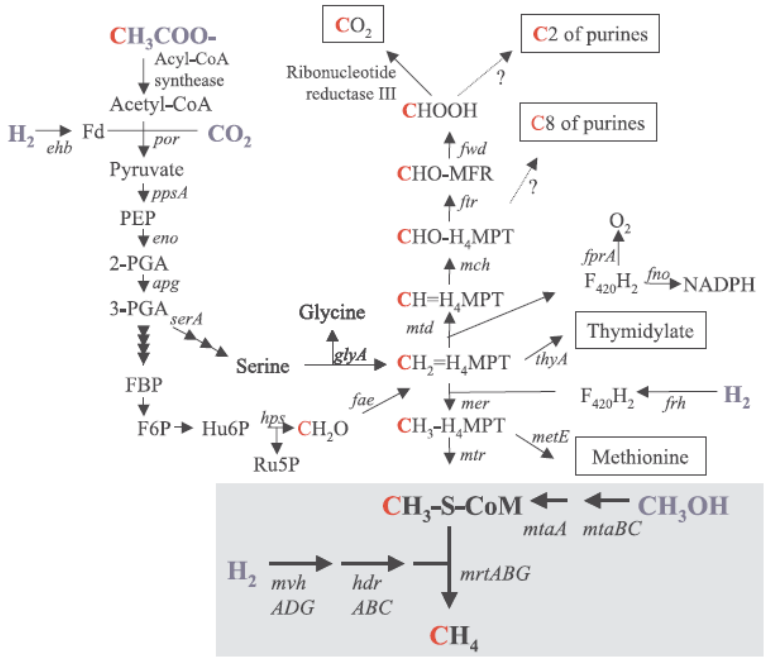

Figure 4.11 Metabolic pathways involved in methanol reduction to methane with H_2 and in acetate used by *Methanosphaera stadtmanae* (Fricke *et al*., 2006).

Methanosphaera to have its genome studied in detail (Fricke *et al*., 2006). The genome of *Methanosphaera stadtmanae* was highly enriched with genes encoding enzymes involved in methanol reduction with H_2 to methane coupled to ATP synthesis (Fricke *et al*., 2006). Consistent with the proposed metabolic requirements of the genus *Methanosphaera* (Fig. 4.11), genes encoding methanol:coenzyme M methyltransferase (MtaABC), methyl-coenzyme M redeuctase (MrtABG), heterodisulfide reductase (HdrABC) and non-F_{420}-reducing hydrogenase (MvhADG) appeared to be highly expressed (Fricke *et al*., 2006). MtaABC genes encoding enzymes catalysing the methanol reduction by forming methyl-coenzyme M from methanol and coenzyme M are also found in the species of the genus *Methanosarcina*, the only other methanogens known to grow on methanol plus H_2. The four isoenzymes of *mta*A, *mta*B, *mta*C that were found in the genome of the *Methanosphaera stadtmanae* (*mta*B1C1, *mta*B2C2, *mta*B3C3 *mta*B4C4, *mta*A1, *mta*A2, *mta*A3 and *mta*A4) had less than 50% sequence similarity to *mta*BC isoenzymes of the genus *Methanosarcina*

(Fricke *et al*., 2006). The presence of *mta*BC isoenzymes in the genome of *Methanosphaera* differentiates the species from other cytochrome-free methanogens that are capable of growing solely on H_2/CO_2. So far, the genus *Methanosarcina* is the only group of methanogens known to possess cytochromes. Cytochromes are membrane-bound electron carriers involved in the oxidation of methyl groups to CO_2. Previous studies revealed that methanogens possessing cytochromes are capable of using the broadest range of substrates including hydrogen and carbon dioxide, acetate, methylamines and methanol, and show higher growth yields on hydrogen and CO_2 than those without cytochromes (Hook *et al*., 2010). Fricke *et al*. (2006) proposed that *Methanosphaera stadtmanae* acquired *mta*BC genes by lateral gene transfer from *Methanosarcina* species a long time ago, as reflected by the low sequence similarity to *mta*BC genes in *Methanosarcina* species. Furthermore, the authors interpreted the close relationship among *mta*BC isoenzymes in *Methanosphaera stadtmanae* as a result of three gene duplications, which occurred more recently than the lateral gene transfer from *Methanosarcina* species. Because *Methanosphaera* species are incapable of reducing CO_2 to methane or oxidizing methanol to CO_2, and rely on acetate to synthesize the methyl group of methionine, the Mtr complex involved in methanol oxidation to CO_2 and the methyl transfer from methyl-coenzyme M to H_4MPT does not appear to be vital to the survival of the organism (Fricke *et al*., 2006). Consistent with this finding, although the Mtr complex was found in the genome of *Methanosphaera stadtmanae*, enzyme assays revealed that the Mtr complex was present in the cell extract below the detection limit (van de Wijngaard *et al*., 1991).

Methanosphaera cuniculi was isolated from rabbit rectum, and has not been studied extensively (Biavati *et al*., 1988). ISO3-F5 was the first *Methanosphaera* sp. that was isolated from the rumen of sheep and was only partially characterized (Jeyamalar, 2010). Strain A4

characterised in this MSc thesis (chapters 3 and 4) was isolated from a mixed methanogen culture from Tamar Wallaby. 16S rRNA gene sequence analysis of the strain revealed that it was 96% similar to *Methanosphaera cuniculi*, suggesting that A4 is a potentially novel species of the genus *Methanosphaera*. Of the three species of the genus *Methanosphaera* tested for substrate utilization, A4 and ISO3-F5 required H_2/CO_2, methanol and acetate for growth. On the other hand, *Methanosphaera stadtmanae* strain MCB-3 was able to grow on H_2/CO_2 and methanol. MCB-3, a human isolate, does not require rumen fluid and yeast extract to support its growth. It is possible that a trace amount of acetate present in the rumen fluid might have enabled the growth of MCB-3 without an actual addition of acetate, suggesting that *Methanosphaera stadtmanae* (from human gut) may have developed a more efficient system for acetate utilization than species of *Methanosphaera* isolated from the rumen.

In this chapter, selected methanogen strains were tested for their ability to use formate, methanol and acetate as substrates in the presence or absence of H_2/CO_2. The results obtained in this chapter did not deviate much from what is known from published sources, suggesting the methodology used here is more thorough. Further characterization of the strains is expected to be carried out in the future, and the data obtained from this study may be used as preliminary information for designing the minimum substrate conditions for cultivating these strains. By doing so, any undesirable interference that may be caused by an excess of substrates in the medium will be minimized. Furthermore, the findings from this study may be linked to the functional genomics to elucidate the genetic foundations which enable the strains to utilize the specific substrate ranges.

Chapter 5

Measurement of H_2 consumption thresholds of rumen methanogens

5.1 Introduction

According to the threshold model, a successful organism places itself in an advantageous position for H_2-scavanging by maintaining the H_2 partial pressure below the threshold H_2 levels of its competitors (Cord-Ruwisch *et al*., 1988). The threshold is the concentration below which metabolism of the substrate ceases. However, this model was formulated based on observations from the relatively stable anaerobic environments of rice paddy soils, digesters, and marine sediments. In most cases, the emphasis has been the comparison of H_2 consumption by microorganisms (e.g.sulfate reducers) that have lower H_2 thresholds than methanogens (Conrad, 1999 & Thauer *et al*., 2008). The rumen constantly experiences a dynamic movement of partially digested feeds. Whether the threshold model will still be applicable in determining the relative competitiveness of different methanogens with generally similar physiologies inhabiting the dynamic rumen environment is currently unknown.

Although the rumen is one of the important sites of methanogenic activity occurring in nature, so far, relatively little attention has been devoted to studying the hydrogen thresholds of methanogens inhabiting the rumen. In this study, the H_2 threshold concentrations of rumen methanogens have been investigated for the first time. In future, the results of this study may be used to select model strains to determine (1) whether rumen methanogens behave according to the threshold model; and (2) whether varying H_2 thresholds associated with each methanogen species are partly responsible for creating the outcome of inter-species competition among methanogens.

5.2 Materials and methods

Ten methanogen strains belonging to three different genera were selected for H_2 threshold measurements (Fig. 5.1-5.10). Two or more species were selected to represent each genus in order to investigate the possibility of genus-level variations in the H_2 threshold concentrations. The strains used in this study were *Methanobrevibacter olleyae* 229/11, *Methanobrevibacter* spp. AbM4, *Methanobrevibacter ruminantium* M1, *Methanobrevibacter millerae* D5, *Methanobrevibacter* sp. SM9, *Methanobacterium formicicum* BRM9, *Methanobacterium bryantii* YCM1, *Methanosphaera stadtmanae* MCB-3, *Methanosphaera* spp. ISO3-F5, and *Methanosphaera* sp. A4.

5.3 Results

Freshly inoculated methanogen cultures were injected with a fixed amount of pure hydrogen gas, and subsequent changes in H_2 partial pressure in the headspace were monitored using gas chromatography. As the methanogens began to consume the added H_2, the H_2 partial pressure in the culture gradually decreased, until a threshold point was reached below which the strain fails to metabolise H_2 as a substrate. Once H_2 was consumed down to the threshold level, the H_2 partial pressure in the tube remained unchanged for several days until the next H_2 addition. The threshold level was monitored through three cycles of H_2 injection and consumption. At the end of each cycle, the H_2 partial pressure in the tube always reached the same threshold level as from the previous cycle (see section 2.10 detailed description of the methods).

Each methanogen strain was associated with a unique H_2 threshold concentration below which the strain failed to metabolise H_2. The average of the lowest H_2 partial pressure measured between H_2 injections was recorded as the H_2 threshold for each individual strain. Some replicate cultures did not remain viable throughout the duration of study, and were discarded before the three cycles of injection and consumption were complete. As a result, a

varying number of replicates was used to calculate the average H_2 threshold values for each strain. The variations of H_2 thresholds measured in multiple cultures were less than ~30%. Although this variance was relatively large, the mean H_2 thresholds steadily decreased with the time. Table 5.1 summarizes the H_2 threshold values and the identity of methanogen strains associated with the respective H_2 thresholds obtained from this study.

5.3.1 H_2 thresholds for *Methanobrevibacter* spp.

H_2 thresholds of the strains of the genus *Methanobrevibacter* were distinctly higher than those of other genera, falling within a range between 2.5 and 4.7 Pa (Table 5.1, Fig 5.1-5.5). *Methanobrevibacter olleyae* 229/11 had slightly lower H_2 threshold than other members of the genus. The ranges of H_2 thresholds measured for strains AbM4, M1, D5 and SM9 were remarkably similar. The cultures of SM9 were subject to a fourth H_2 injection and consumption cycle since the third addition of H_2 was carried out before the H_2 partial pressure inside the tube had reached the threshold.

5.3.2 H_2 thresholds for *Methanobacterium* spp.

The two strains of the genus *Methanobacterium*, BRM9 and YCM1, had H_2 thresholds of 2.1 ($\pm$1.27) Pa and 1.2 ($\pm$0.71) Pa, respectively (Table 5.1, Fig. 5.6-5.7). BRM9 had a slightly higher H_2 threshold than YCM1, but considering the experimental errors and uncertainties that inevitably occur when handling gas phase samples, the difference was not very substantial.

5.3.3 H_2 thresholds for *Methanosphaera* spp.

The two strains of the genus *Methanosphaera*, A4 and ISO3-F5, had the lowest H_2 thresholds among the strains used in this study, with the lower end of the threshold reaching well below 1.0 Pa (Table 5.1, Fig. 5.8-5.10). *Methanosphaera stadtmanae* MCB-3 (isolated

from human gut) had slightly higher H_2 thresholds than other members of the genus *Methanosphaera.* In fact, the H_2 threshold of MCB-3 occurred at 2.1 ($\pm$1.27) Pa, similar to levels observed with *Methanobrevibacter olleyae* 229/11 and *Methanobacterium formicicum* BRM9.

5.4 Discussion

So far, only a limited number of studies have been carried out to investigate the H_2 thresholds of methanogens. The results from these studies are summarized in Table 1.3. The majority of the methanogens whose H_2 thresholds have been studied were isolated from the sediments of ponds and the ocean, soils and rice paddies. In the published works of Lovley (1985), Lee *et al*. (1988), and Kotsyurbenko *et al*. (2001), the H_2 thresholds of *Methanobacterium formicicum* JF-1, *Methanobacterium bryantii* M.o.H, *Methanospirillum hungatei* JF-1, methanogenic strains MSB and MSP, and co-cultures of an acetate-oxidising bacterium with either *Methanothermobacter thermoautotrophicus* ΔH or *Methanothermobacter thermoautotrophicus* THF that were isolated from various aquatic sediments have been measured. Due to the inconsistency of methodology and equipments used in different studies, the obtained H_2 threshold values varied widely, falling within a range of 1.0 - 14.0 Pa. In a study conducted by Cord-Ruwisch *et al*. (1988), H_2 thresholds of *Methanospirillum hungatei*, *Methanobrevibacter smithii*, *Methanobrevibacter arboriphilus*, *Methanobacterium formicicum* and *Methanococcus vannielii* obtained from the German Collection of Microorganisms (DSMZ) were measured and found to be within the range of 3.0 - 12.0 Pa. Conrad and Wetter (1990) and Chong *et al*. (2002) investigated the H_2 thresholds of strains *Methanobacterium bryantii* strain Bab 1, *Methanobacterium thermoautotrophicum* and *Methanogenium frigidum* Ace-2 obtained from various culture collections, and reported that their thresholds are below 1.0 Pa. In this study, the H_2 thresholds of methanogens isolated from the rumen have been measured for the first time.

Table 5.1 The H_2 threshold values for ten methanogen strains. The average H_2 partial pressure measured during the static phase between H_2 injections were recorded as the H_2 thresholds for individual strains. Of the ten duplicate cultures, some did not remain viable throughout the duration of study, and were discarded before the three cycles of injection and consumption were complete.

Strain	Identity	H2 threshold (Pa)
229/11	*Methanobrevibacter olleyae*	2.5 (±0.71)
AbM4	*Methanobrevibacter* spp.	4.2 (±0.92)
M1	*Methnanobrevibacter ruminantium*	4.5 (±1.41)
SM9	*Methanobrevibacter* spp.	4.7 (±1.63)
D5	*Methanobrevibacter millerae*	4.3 (±1.06)
BRM9	*Methanobacterium formicicum*	2.1 (±1.27)
YCM1	*Methanobacterium bryantii*	1.2 (±0.71)
ISO3-F5	*Methanosphaera* spp.	1.1 (±0.85)
A4	*Methanosphaera* spp.	1.4 (±0.64)
MCB-3	*Methanosphaera stadtmanae* (human isolate)	2.1 (±1.27)

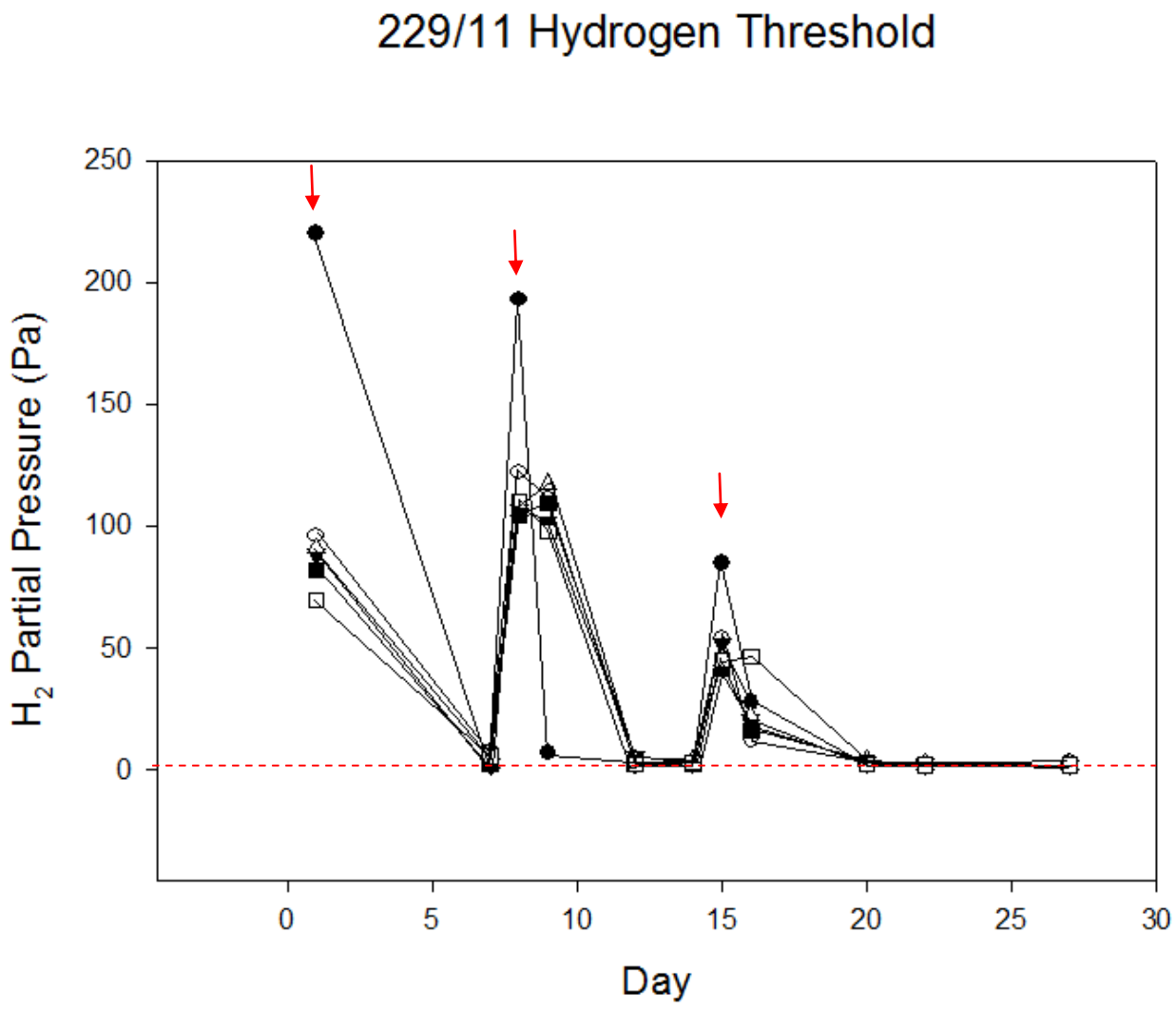

Figure 5.1 H_2 threshold of multiple cultures of *Methanobrevibacter olleyae* 229/11 growing on H_2/CO_2 (80:20). Arrows indicate the H_2 additions. The H_2 threshold is indicated by a dashed line. Symbols stand for five replicate cultures.

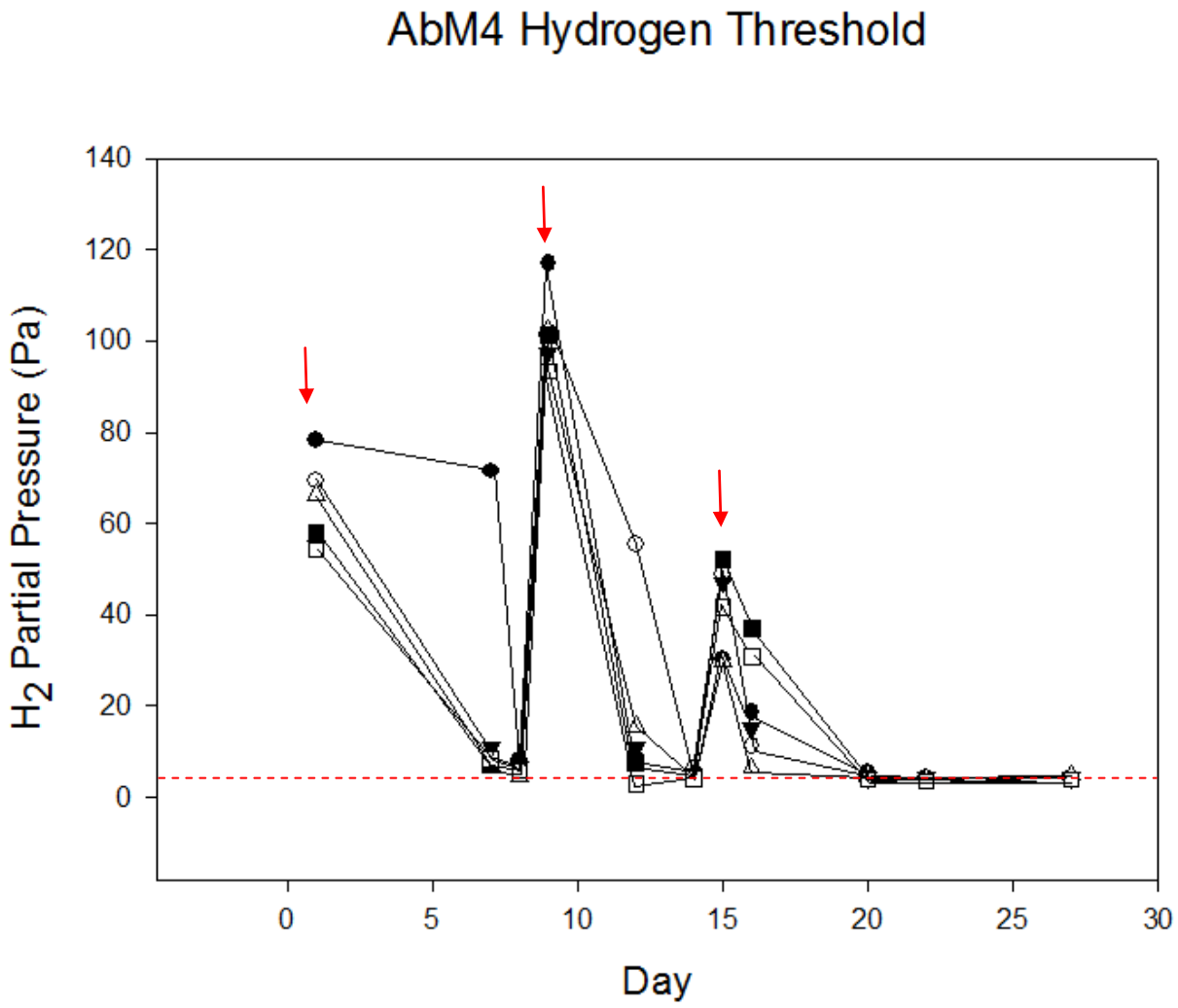

Figure 5.2 H_2 threshold of multiple cultures of *Methanobrevibacter* sp. AbM4 growing on H_2/CO_2 (80:20). Arrows indicate the H_2 additions. The H_2 threshold is indicated by a dashed line. Symbols stand for five replicate cultures.

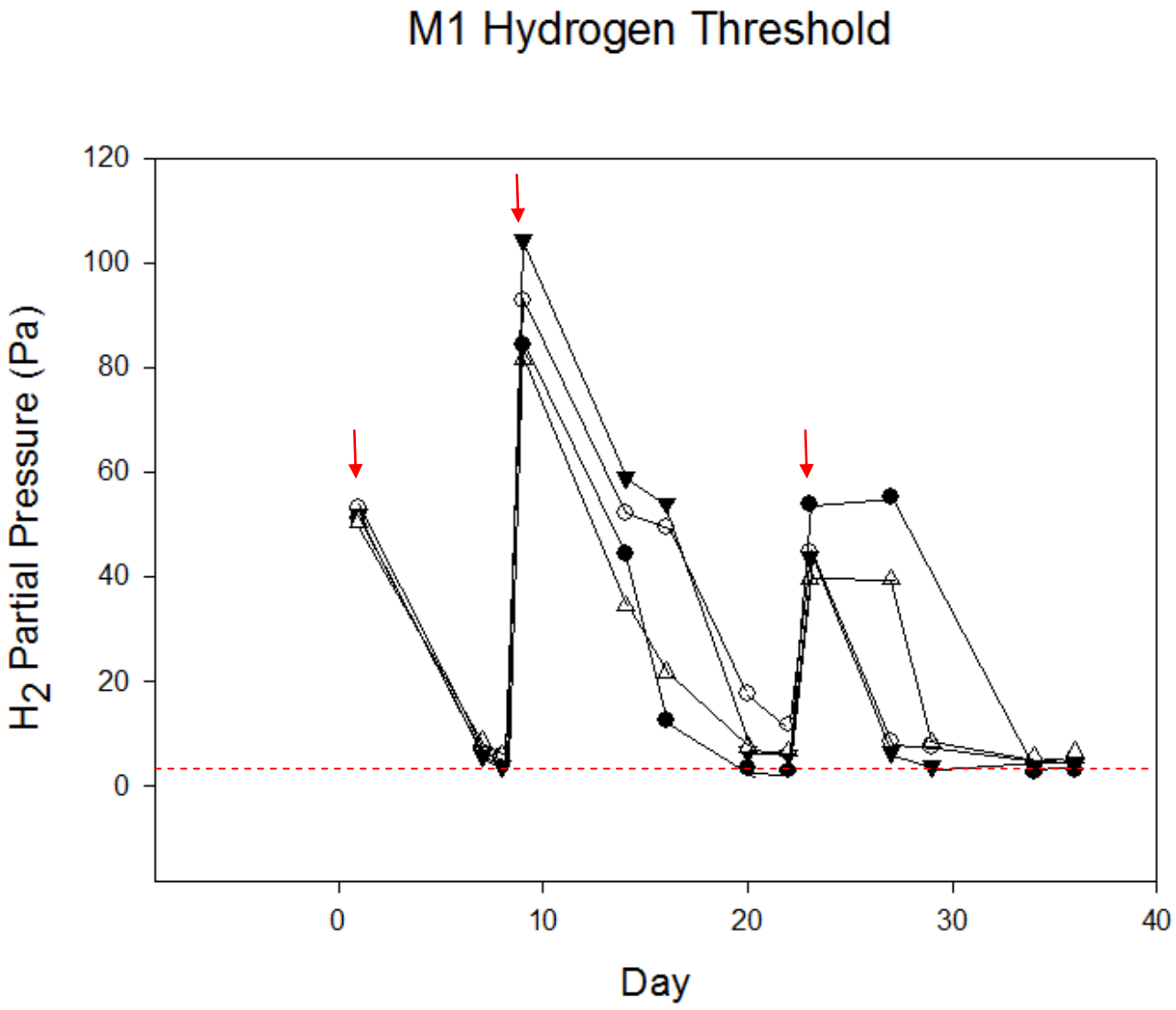

Figure 5.3 H_2 threshold of multiple cultures of *Methanobrevibacter ruminantium* M1 growing on H_2/CO_2 (80:20) plus formate. Arrows indicate the H_2 additions. The H_2 threshold is indicated by a dashed line. Symbols stand for five replicate cultures.

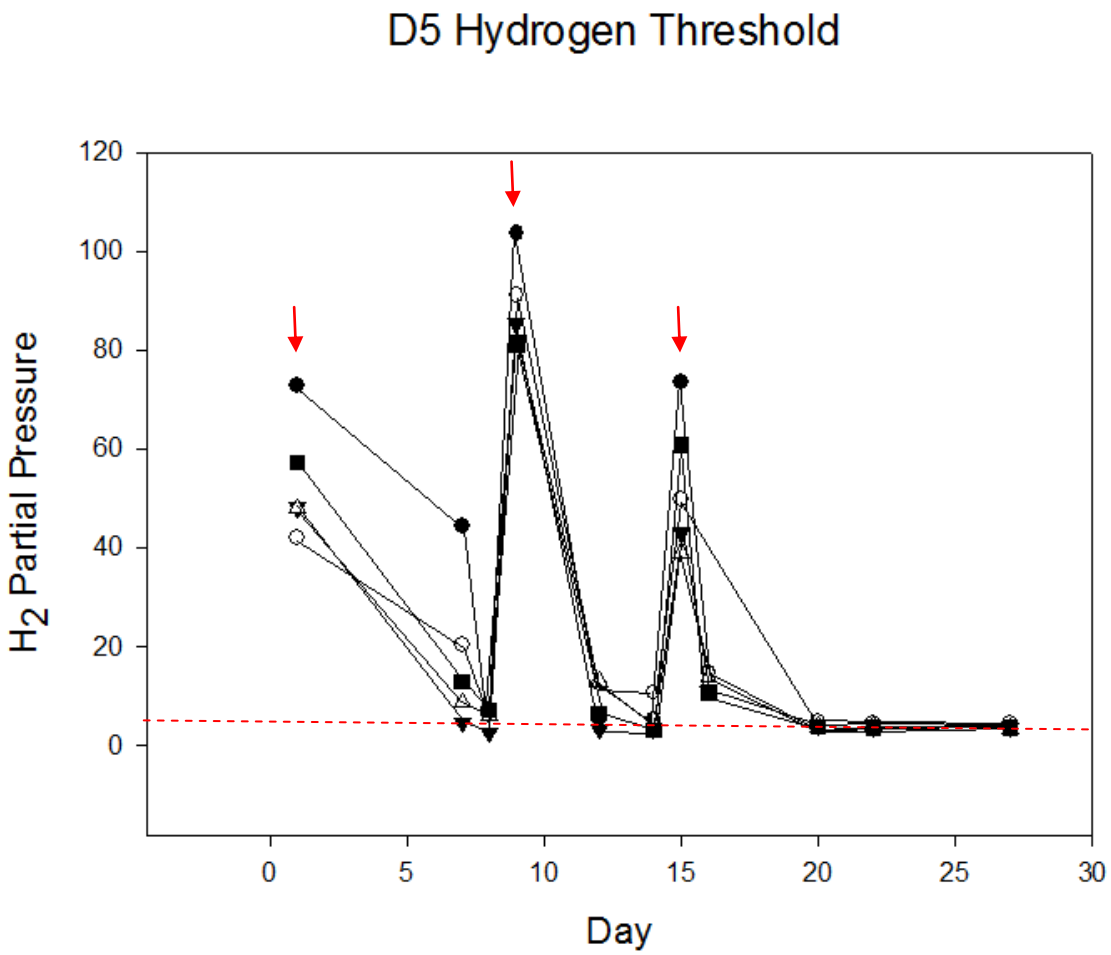

Figure 5.4 H_2 threshold of multiple cultures of *Methanobrevibacter millerae* D5 growing on H_2/CO_2 (80:20). Arrows indicate the H_2 additions. The H_2 threshold is indicated by a dashed line. Symbols stand for five replicate cultures.

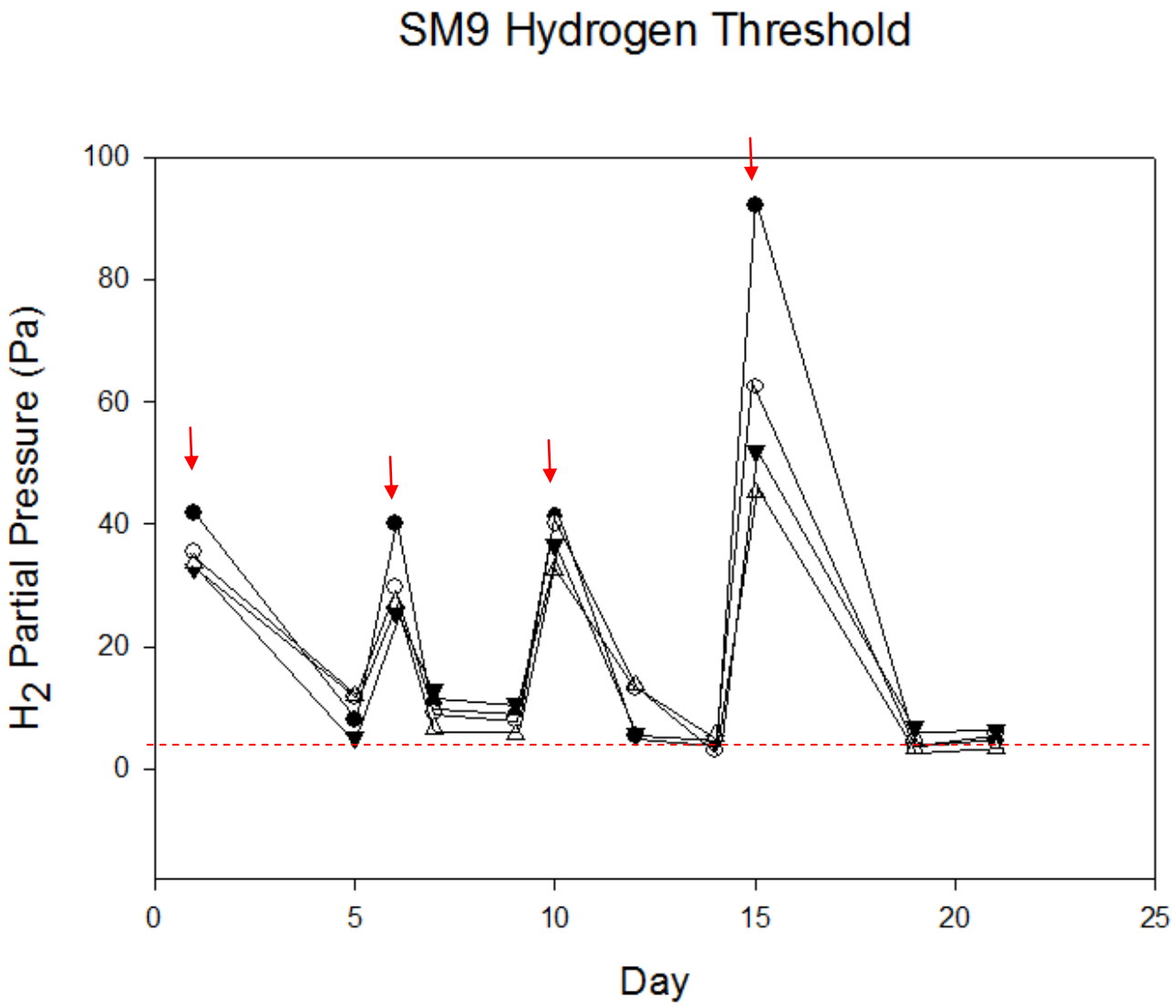

Figure 5.5 H_2 threshold of multiple cultures of *Methanobrevibacter* sp. SM9 growing on H2/CO2 (80:20) plus formate. Arrows indicate the H_2 additions. The H_2 threshold is indicated by a dashed line. Symbols stand for five replicate cultures.

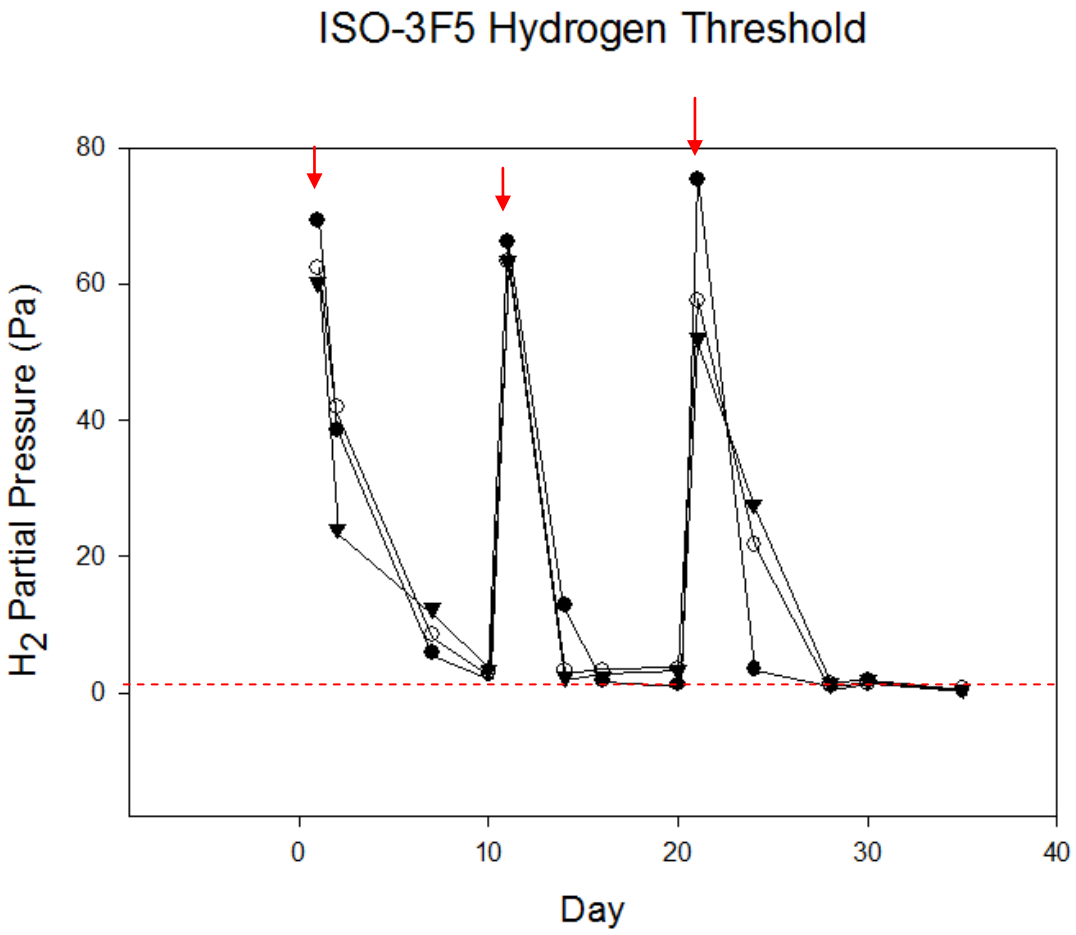

Figure 5.6 H_2 threshold of multiple cultures of *Methanosphaera* sp. ISO3-F5 growing on H_2/CO_2 (80:20) plus methanol and acetate. Arrows indicate the H_2 additions. The H_2 threshold is indicated by a dashed line. Symbols stand for five replicate cultures.

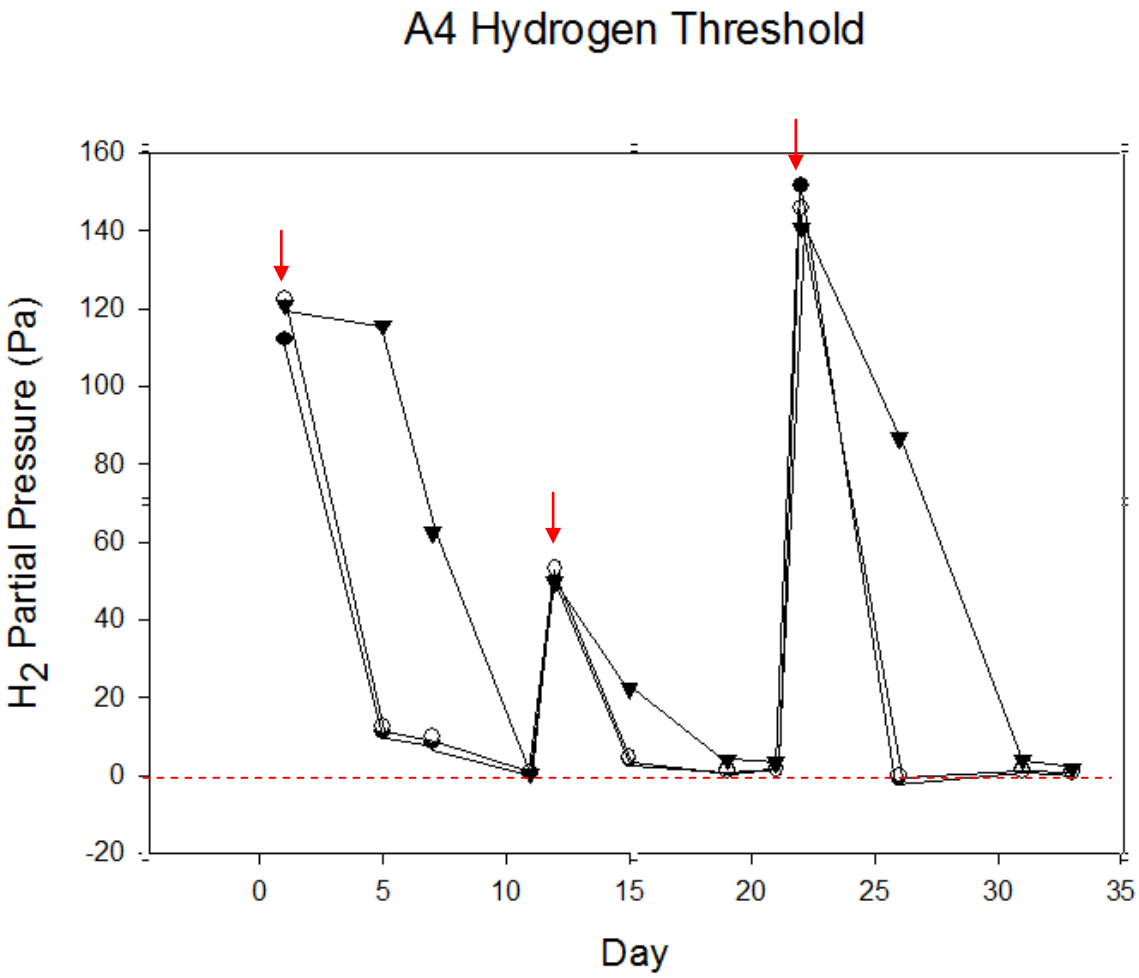

Figure 5.7 H_2 threshold of multiple cultures of *Methanosphaera* sp. A4 growing on H_2/CO_2 (80:20) plus methanol and acetate. Arrows indicate the H_2 additions. The H_2 threshold is indicated by a dashed line. Symbols stand for five replicate cultures.

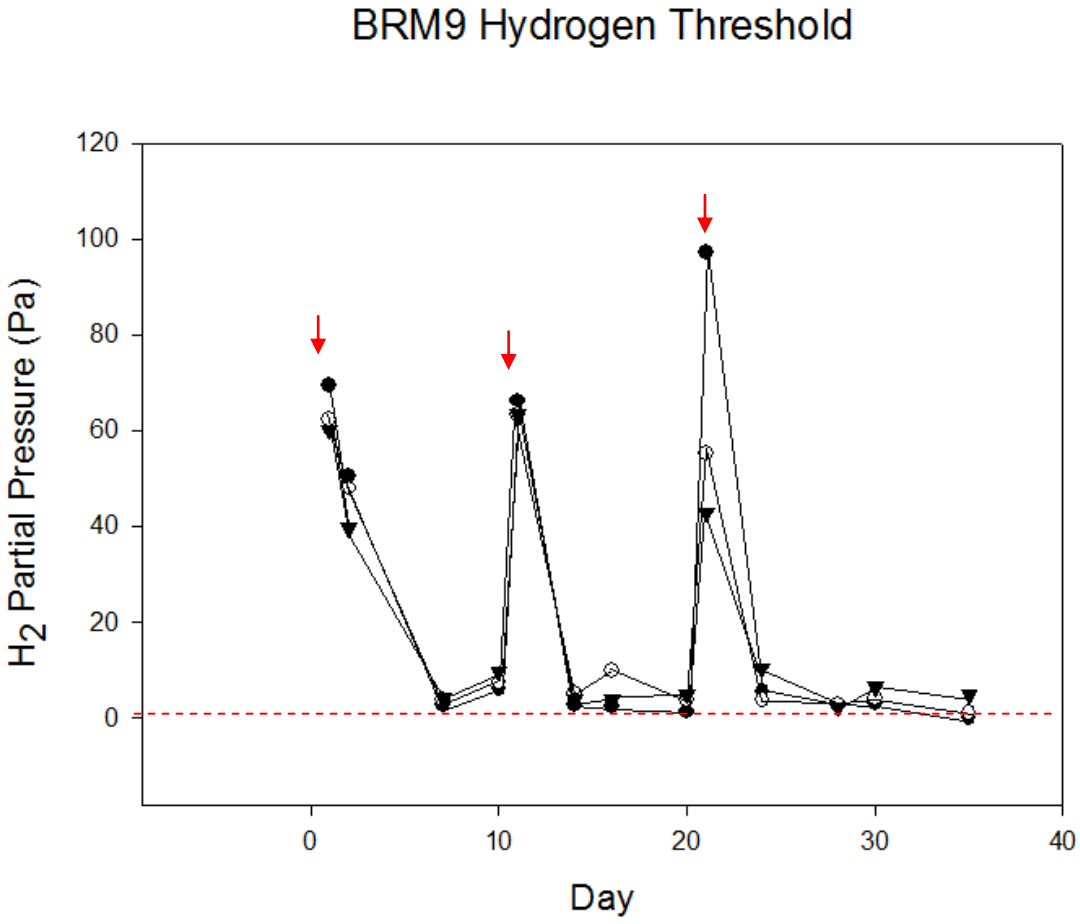

Figure 5.8 H_2 threshold of multiple cultures of *Methanobacterium formicicum* BRM9 growing on H_2/CO_2 (80:20). Arrows indicate the H_2 additions. The H_2 threshold is indicated by a dashed line. Symbols stand for five replicate cultures.

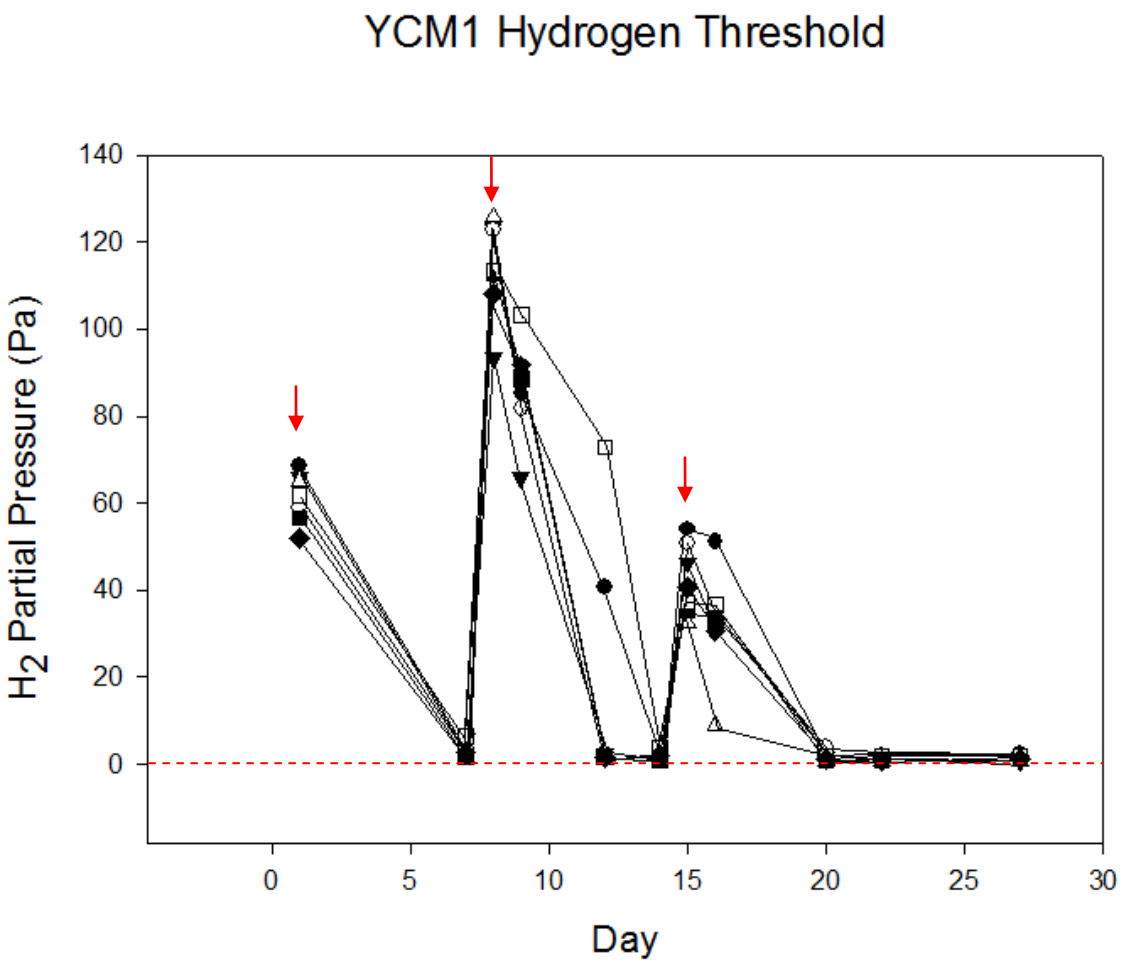

Figure 5.9 H_2 threshold of multiple cultures of *Methanobacterium bryantii* YCM1 growing on H_2/CO_2 (80:20). Arrows indicate the H_2 additions. The H_2 threshold is indicated by a dashed line. Symbols stand for five replicate cultures.

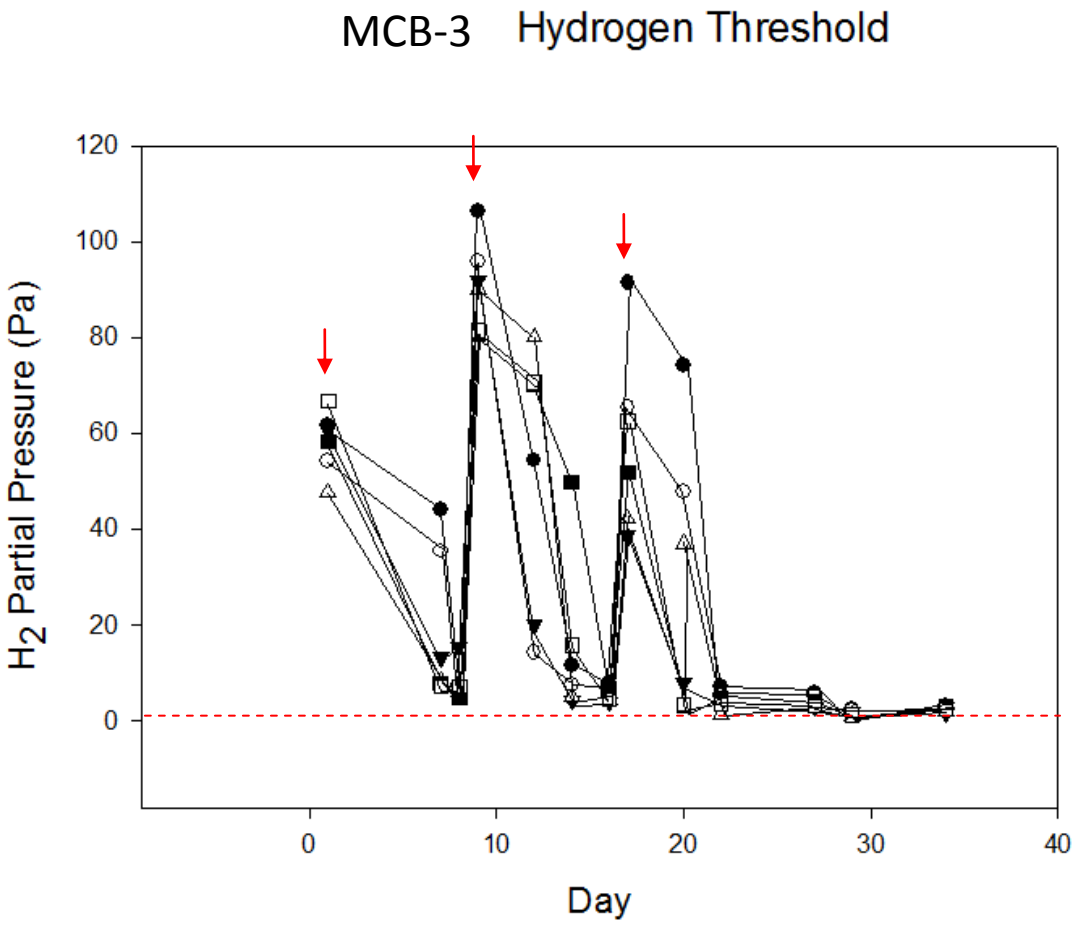

Figure 5.10 H_2 threshold of multiple cultures of *Methanosphaera stadtmanae* MCB-3 growing on H_2/CO_2 (80:20) plus methanol and acetate. Arrows indicate the H_2 additions. The H_2 threshold is indicated by a dashed line. Symbols stand for five replicate cultures.

Overall, the H_2 thresholds of rumen methanogens fell within the range between 0.5 and 5.8 Pa. Because no published data on H_2 threshold of the strains studied in this thesis are available, a direct comparison to published literature was not possible. Nonetheless, the values obtained are comparable with those for other methanogens, suggesting that the methodology used in this study was sound, and the results are reliable. The task of handling the gas phase samples is challenging in that they are sensitive to the subtle changes in temperature and humidity. However, various measures of caution were taken to ensure that experimental errors were minimized as much as possible. Prior to use, the reductive gas detector and the gas-tight syringes were carefully searched for the signs of gas leakage. The rubber stoppers and glass wool inserts at the injection port of the reductive gas detector and of the sampling port of the standard H_2/N_2 (1:200) gas cylinder were regularly replaced to prevent gas leakage due to perforation damage. A a set of control tubes to which no methanogen cells were added was prepared to ensure that the H_2 concentration remained constant for 40 days during the time which the cultures went through three cycles of H_2 injection and consumption. All experiments were conducted under temperature- and humidity-controlled conditions. Before the addition of pure H_2 gas, gassing lines connected to the external gas cylinders were flushed with pure H_2 for up to 20 min to ensure no residual gases apart from H_2 remained in the gassing system.

In general, the H_2 threshold values obtained from this study fell into two groups; the relatively higher H_2 threshold concentrations ranging between 2.0 and 5.8 Pa that were associated with strains of the genus *Methanobrevibacter*, and the relatively lower H_2 thresholds associated with members of the genus *Methanobacterium* and of the genus *Methanosphaera*, at 2.0 Pa or below. The study of microbial diversity of the total rumen

archaea has revealed that members of the genus *Methanobrevibacter* is the most dominant group of methanogens occurring in the rumen (Jeyamalar *et al*., 2011). Therefore, the results obtained from this study suggest that in the rumen, the most dominant group of methanogens have differences in their H_2 utilisation ability compared to the relatively more minor groups of methanogens.

One possible explanation is that there is an energetic trade-off which compensates for the competitive disadvantage of having high H_2 thresholds by enabling a fast rate of growth. According to the trade-off model, by diverting the cellular energy needed for maintaining low H_2 thresholds towards maximizing growth, members of the genus *Methanobrevibacter* are more competitivelysuccessfully in the rumen compared other genera. This was reflected in the experiments of Sakai *et al*. (2009), in which the methanogen species isolated under a very low H_2 pressure constituted relatively minor species of methanogens whose presence are only rarely detected in the rumen. The microorganisms inhabiting the rumen are exposed to the high-speed flow of digested feed material that constantly removes the microorganisms from the rumen. As a consequence, the ability to scavenge H_2 at very low levels is a poor alternative compared to the maximization of the growth rate, when the first priority is to establish a significant microbial population to resist a complete removal from the fast-changing rumen environment.

Methanobacterium spp. are rare members of the rumen microbial community, and are more frequently found in aquatic sediments. The methanogens inhabiting aquatic sediments are constantly outcompeted by sulfate reducers that pull down the H_2 partial pressure below the level of methanogen H_2 threshold, forcing methanogens to produce methane by resorting to less competitive substrates, such as acetate and trimethylamine (Lovley et al., 1982). In contrast, inorganic terminal electron acceptors other than CO_2 are generally not available in the rumen, thus methanogens and homoacetogenic bacteria are solely responsible for the

consumption of the ruminal hydrogen. Very little H_2 consumption occurring in the rumen can be attributed to the sulfate reducers. Homoacetogens, which utilise H_2 in the rumen, have much higher thresholds for H_2 consumption than methanogens (Joblin, 1999). Therefore, as only methanogens utilise H_2, at a low pressure, an inter-species competition for H_2 among methanogens dominates in the rumen. Assuming the H_2 threshold actually is an influential factor that determines the relative competitiveness of methanogens within the rumen, methanogens with lower H_2 thresholds should be more competitive than methanogens with relatively higher H_2 thresholds. According to the threshold theory, members of the genus *Methanobacterium* should outcompete members of the genus *Methanobrevibacter*, since the former has lower H_2 thresholds than the latter. However, in reality, cultivation-independent studies of rumen archaea suggest that the presence of *Methanobrevibacter* spp. outcompete the other methanogen genera inhabiting the animal rumen (Janssen and Kirs, 2008; Jeyamalar *et al*., 2011). The members of the *Methanobacterium* may have compromised the growth rate by concentrating the cellular energy towards maintaining low H_2 thresholds. Note that this is not reflected on the characterization study from previous chapter, because the cultures were grown at a higher H_2 concentration than the actual steady-state H_2 concentration within the rumen. In the rumen, where the microorganisms are constantly being washed out by the high-speed flow of rumen contents (Janssen, 2010), members of the genus *Methanobacterium* may not grow fast enough to maintain a sufficient number of cells to prevent the removal from the rumen. Therefore, it appears the rumen may not be an adequate environment for members of the genus *Methanobacterium*.

Methanosphaera spp. is some of the most dominant species of methanogens detected in ruminant animals raised in New Zealand (Jeyamalar *et al*., 2011). The members of the genus *Methanosphaera* are unusual among methanogens in that they reduce methanol to methane using H_2 without involving CO_2 in this pathway. The availability of an alternative

pathway to produce methane may have rendered the dependence of *Methanosphaera* on H_2 relatively less significant, thus enabling growth of the members of this genus in the rumen without having to compromise their ability to use H_2 at a low threshold. For an unknown reason, MCB-3, the only human strain of *Methanosphaera* used in this study, displayed slightly higher H_2 thresholds than other strains of the genus *Methanosphaera*. The detailed genome analysis of *Methanosphaera stadtmanae* (Fricke et al., 2006) revealed that the organism has evolved adaptive strategies that enable effective colonization of the human gut. In the human colon, selective retention of fibrous material does not occur, as the fibrous and fluid phases are diluted by inflow of nutrients at the same rate (Henderson and Demeyer, 1989). Because the rumen and the human gut are two broadly different microbial environments, it is likely that the methanogens inhabiting these ecosystems have developed separate adaptive strategies. Thus, the slight variations of the H_2 thresholds among the members of the genus *Methanosphaera* may be a consequence of evolving optimal strategies of survival in different environments.

Chapter 6

Summary and general discussion

Exported dairy products, meat, woll, and leather products are the New Zealand's biggest source of earnings, and make up 42% of the nation's commodity exports (Statistics New Zealand, 2011). These products are derived almost totally from farmed ruminants. The heavy economic dependence of New Zealand on its farming industry makes the nation particularly vulnerable to agriculture-responsible methane emissions. Faced with this challenge, the livestock industry (dairy, sheep, beef and deer) in partnership with the NZ government formed the Pastoral Greenhouse Gas Research Consortium (PGgRc) in 2002, as an industry investor whose goal is to develop greenhouse gas mitigation solutions that can be implemented within the agricultural industry of New Zealand (Attwood *et al*., 2008; Leslie *et al*., 2008).

As mentioned previously, a number of methane mitigation strategies, including the use of chemical inhibitors, vaccination approaches, and the use of alternative hydrogen sinks have been attempted, but so far without substantial success. Difficulties arise due to the lack of knowledge of the rumen methanogen diversity. The analysis of 16S rRNA gene sequences from the rumen samples has revealed that the rumen harbours a surprisingly large number of methanogen species (Janssen & Kirs, 2008; Jeyanathan et al., 2011). There are still large groups of rumen methanogens remain to be studied, and so far, no more than ten rumen methanogens species have been successfully cultivated under laboratory conditions.

AgResearch, one of the leading research institutions where methods to reduce the environmental impact of rumen methanogenesis are being actively investigated, has been accumulating a collection of cultures of rumen methanogens. This project was initiated for

the purpose of confirming the identities of methanogen isolates that have been included in the collection without having their identities determined. Phylogenetic trees constructed based on 16S rRNA and *mcr*A gene sequence similarities showed that the majority of strains grouped within the genus *Methanobrevibacter*. The phylogenetic positions of the isolates within both trees were remarkably similar. The validity of the mcrA gene as a marker gene was previously demonstrated in numerous studies (Lueders *et al*., 2001, Luton *et al*., 2002, Tatsuoka *et al*., 2004 & Friedrich, 2005), thus supporting the inter-changeable use of the 16S rRNA and *mcr*A genes as the genetic markers for species differentiation of methanogens in this study. The 16S rRNA gene sequence similarity of isolates D5 and A4 to their closest recognized relatives (*Methanobrevibacter millerae* and *Methanosphaera cuniculi*, respectively) was less than the suggested threshold for species differentiation, often placed at 3% sequence difference (eg., Stackebrandt & Goebel, 1994). In agreement with these results, phylogenetic novelty of these isolates has been proposed. Interestingly, *Methanosphaera cuniculi* and isolate A4 were both derived from non-ruminant digestive systems that carry out similar functions to the rumen. 16S rRNA gene sequence analysis revealed that *Methanosphaera* spp. were frequently observed in the rumen of New Zealand livestocks (Jeyamalar, 2010). However, these species were phylogenetically closer to *Methanosphaera stadtmanae* than *Methanosphaera cuniculi* (Jeyamalar, 2010). Thus, a possibility remains that the presence of *Methanosphaera cuniculi* and its close relatives is a unique feature of the methanogen species composition in non-ruminant animals. Dellow *et al*. (1988), Kempton *et al*. (1976), and Engelhardt *et al*. (1978) observed that, for unknown reasons, methane production from marsupial animals was relatively low compared to ruminants. At the present stage, because of the limited knowledge of the methanogen diversity and microbiome function in marsupial and ruminant animals, it is still early to suggest that there is a significant difference between the species composition of methanogens inhabiting these two

types of animals. However, the possibility remains that the low methane emissions observed from marsupial animals is partly due to a unique methanogen species composition that is not found in the ruminant counterpart.

The majority of the strains used in this study were able to grow solely on H_2/CO_2. Strains belonging to the same methanogen species did not necessarily share the same capacity for substrate use. The ability to use formate plus CO_2 was found only in a limited number of strains, and these strains frequently belonged to the same species with the strains that grow on H_2/CO_2 but not on formate. In contrast to the published data, MCB-3, the only human isolate used in this study, did not require the addition of acetate for growth. However, inclusion of rumen fluid in the growth medium may have provided sufficient acetate, enabling the growth of MCB-3 without an actual addition of acetate. Acetate concentrations in rumen fluid are usually in the region of 60 mM, and so addition of 5% (vol/vol) rumen fluid would have added about 3 mM acetate. This was not quantified. This may suggest *Methanosphaera stadtmanae* have developed a more efficient system for acetate utilization in the human gut compared to other species of *Methanosphaera* isolated from the rumen.

Members of the genus *Methanobrevibacter*, which is easily the most dominant group of methanogens in the rumen, were found to have a higher threshold for H_2 use than the relatively minor groups of methanogens. One possible explanation is that there is an energetic trade-off which compensates for the competitive disadvantage of having high H_2 thresholds by enabling fast growth. This could be tested by comparing growth rates under comparable conditions. The microorganisms inhabiting the rumen are exposed to the high-speed flow of digested feed that constantly removes the microorganisms from the rumen. As a consequence, the ability to scavenge H_2 at very low levels may be a poor alternative compared to the maximization of the growth rate, when the first priority is to establish a significant microbial population to resist a complete removal from the fast-changing rumen environment. The

availability of an alternative pathway to produce methane may have rendered the dependence of *Methanosphaera* on H_2 relatively less significant, thus enabling fast growth of members of this genus in the rumen without having to compromise its ability to utilize the low H_2 thresholds.

In this study, only three methanogen genera were focused on, only partially representing rumen methanogen diversity. Therefore, it is difficult to argue that the results from this study can be used to explain the effect of H_2 utilization thresholds on the relative dominance of some methanogen species over others. In the future, methanogens of greater diversity should be studied. As an increasing number of methanogen genomes are becoming available, comparative genome analysis may be used as a powerful tool to uncover the identity of the genes that confer competitive advantages to certain methanogen species.

The research effort to develop broad-spectrum vaccines that comprehensively cover the dominant, as well as low-abundance species of methanogens is ongoing. It was considered that the efficacy of the vaccine is largely determined by the spectrum of the vaccine targets, in that the minor species could quickly adapt to occupy the empty niches in the absence of more competitive methanogen species. At the moment, the lack of knowledge of a complete methanogen diversity in the rumen poses as a major challenge to accomplishing this task. The study of the threshold concentrations of H_2 consumption by rumen methanogens, and their effects on shaping the methanogen diversity in the rumen may play an important role in designing a narrow-spectrum vaccine that efficiently suppresses the methane production in the rumen. By specifically targeting the fast-growing species of methanogens that have high H_2 thresholds, the difficult task of developing a broad-spectrum vaccine which universally targets all methanogens may be significantly reduced. Once the fast-growing methanogenic strains are eliminated from the population, the growth rate of

those that use low H_2 threshold may be too low to maintain a sufficient cell number to support the production of a significant amount of methane.

In conclusion, rumen is a unique microbial ecosystem that harbours diverse populations of microorganisms whose consortium is vital for maintaining the host nutrition. Although methanogens occupy relatively small portion of the total rumen microbiome, phylogenetic analysis based on 16S rRNA and *mcr*A genes revealed the presence of unusually large species diversity among these organisms. However, the relatively minor species are rarely detected from the rumen, suggesting an inter-species competition for survival is keen among different species of methanogens. Characterization studies of rumen methanogen species that are available as pure cultures showed that species belonging to the genus *Methanosphaera* required methanol and acetate in addition to H_2/CO_2 for growth. With the exception of *Methanobrevibacter ruminantium* M1, members of the genus *Methanobrevibacter* and the genus *Methanobacterium* were able to grow solely on H_2/CO_2. In this study, the threshold concentrations for H_2 consumption were determined for methanogens belonging to three different genera. *Methanobrevibacter*, the most predominant group of methanogens occurring in the rumen, had relatively high H_2 thresholds. In contrast, the genus *Methanosphaera*, a group of methanogens frequently isolated from New Zealand ruminants, had relatively low H_2 thresholds. The notable difference between these two genera was the unique ability of the genus *Methanosphaera* to produce methane using methanol and acetate as substrates without involving CO_2 in this alternative pathway. From the results obtained, it can be assumed that the relative competitiveness of rumen methanogens is not determined by having low H_2 threshold concentrations. Instead, growth rate and the substrate utilization capacity may be more important. The members of the genus *Methanobacterium* have similar H_2 thresholds to *Methanosphaera*, but do not share the alternative pathway for methane production. *Methanobacterium* spp. are very rare in the rumen, in contrast to

members of the geenra *Methanosphaera* and *Methanobrevibacter*. It is possible that *Methanobacterium* spp. do not compete effectively because they have low H_2 thresholds (and thus are penalised by relatively slow growth), and do not compensate for this disadvantage by using alternative substrate utilization pathways that may render the dependence of these organisms on H_2 less significant. Overall, the currently existing threshold model does not adequately describe the rumen methanogen ecosystem, because the relative competitiveness of methanogen species appears to be determined by combinatory effects of several factors (e.g. H_2 threshold, substrate utilization capacity, growth rate, and the feed passage rate within the rumen).

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