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Reducing ruminal methanogenesis through alternative hydrogen and carbon sequestration pathways

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Abstract

Methane produced by ruminant livestock accounts for more than 10% of Australia's net total greenhouse gas emissions. Reducing these emissions will have an immediate benefit for the environment, will improve the 'clean and green' image of Australian beef and may also have a positive impact on the animals' energetic efficiency. Our current understanding of the microbial processes underpinning methane production is incomplete and the key to manipulating these emissions in the future will flow from fundamental improvements in our knowledge of metabolic schemes that provide alternatives to methanogenesis as sinks for hydrogen. This project has been able to define at the molecular level the key microbial populations involved in methanogenesis and the bacteria that could compete for hydrogen through the metabolic processes of reductive acetogenesis and fumarate reduction. This has enabled both the isolation of potent hydrogen utilising bacteria and the development of a suite of molecular tools to monitor the abundance and function of these populations in the rumen. Feeding strategies that reduce methanogenesis can now be evaluated accurately for their potential to promote alternative sinks for hydrogen that result in the capture of energy for use by the animal rather than being lost as methane.

Executive Summary

Methane produced by ruminant livestock accounted for 10.9% of Australia's net total greenhouse gas emissions in the year 2006 (Australian Greenhouse Office, 2008). Reducing these emissions will have an immediate benefit for the environment, will improve the 'clean and green' image of Australian beef and may also have a positive impact on the animals' energetic efficiency. Our current understanding of the microbial processes underpinning methane production is incomplete and the key to manipulating these emissions in the future will flow from fundamental improvements in our knowledge of methanogenesis and alternative hydrogenotrophic pathways.
Several strategies for utilising hydrogen in the rumen as alternatives to methanogenesis have been identified. Two approaches which could be promoted as alternative hydrogen sinks to methanogenesis are reductive acetogenesis as well as increased synthesis of propionate and butyrate through the provision of intermediates such as malate, fumarate and crotonate. If we are to employ these strategies successfully we need a more complete understanding of the physiology and ecology of the microorganisms underpinning these metabolic pathways.

The objectives of the project were to:

- Identify and characterize the microbiological and genetic attributes of the microbiomes possessing alternative routes of hydrogenotrophy and (or) methanotrophy in the rumen of grainfed and grassfed cattle.
- Develop novel strategies, which can be tested experimentally in cattle, for the practical implementation of project outcomes to inhibit rumen methanogens, reduce rumen methane emissions and improve animal energetic efficiency.

Batch fermentation systems designed to promote reductive acetogenesis were established with rumen inocula from either pasture or grain fed animals using ^{13}C-Sodium carbonate as the principal carbon and energy source, and in the presence or absence of bromoethanesulfonate (BES) to inhibit methanogenesis. Fermentation end-product analyses were performed to characterize and monitor short chain fatty acid changes and balances of hydrogen and carbon. Metagenomic DNA was extracted and isotope ratio mass spectrometry (IRMS) was used to confirm the uptake of ^{13}C from CO_2 into the DNA, followed by the separation of ^{13}C-labeled DNA via isopycnic gradient ultracentrifugation. The ^{13}C-labeled DNA was used as a template for the production of 16S rDNA phylogenetic and functional gene clone libraries, as well as metagenomic (fosmid) libraries and 454 short read sequencing. The main functional genes investigated were those of three key enzymes in the reductive acetogenesis pathway of homoacetogens: formyltetrahydrofolate synthetase (FTHFS), carbon monoxide dehydrogenase (CODH) and acetyl-CoA synthase (ACS). Putative FTHFS, CODH and ACS sequences were also generated from DNA of known homoacetogens from the rumen and other environments as reference genes. The majority of FTHFS sequences obtained from the batch fermentations grouped within the homoacetogenic cluster which is dominated by clostridial representatives. Based on this information it is now possible to affiliate FTHFS-encoded rumen-bacteria to either homoacetogenic or non-homoacetogenic clusters. Putative CODH and ACS sequences were also generated from DNA obtained from rumen enrichment cultures of homoacetogens, from the rumen of cattle. Clusters of sequences for the three key functional genes aligned close to *Blautia obeum* and other *Blautia* speciecs that are known to be homoacetogenic. There was a degree of similarity between the pasture fed and grain fed enrichments in relation to these key functional genes.

Based on this information, an extensive set of primers have been developed from the numerous phylogenetic and functional gene databases so that populations of homoacetogic microorganisms in the rumen can be monitored. By developing primer sets that can detect the ACS and CODH genes in conjunction with the FTHFS gene, we have been able to identify the bacteria that carry all these genes within the rumen microbial enrichments that were studied. The ACS and FTHFS primer sets have been further refined for realtime PCR analysis that will enable the monitoring of acetogenic populations in the rumen in response to the inhibition of methanogenesis.

While a large emphasis has been placed on understanding the microbiology and genetics underpinning reductive acetogenesis, other rumen bacteria also sequester H_2 in competition with methanogens. The reduction of fumarate through the coupling of H_2 to produce succinate and subsequent formation of propionate is thermodynamically very favourable within the rumen. However there is limited knowledge of the bacteria which are responsible for these steps and how they can be manipulated within the rumen. Fermentation systems were set up in such a manner as to enrich for these microorganisms and allow the capture of genetic material that could be functionally analysed. Analysis of fermentative end products was used to monitor shifts in microbial biochemical pathways.

The 16S rDNA libraries from fumarate enrichment cultures indicated that several Gram-negative bacteria including *Selenomonas ruminantium* and *Prevotella ruminicola* which are known to carry out fumarate reduction were present in these libraries. *Selenomonas ruminantium* uses extracellular H_2 to reduce fumarate to succinate and has the ability to carry out decarboxylation of succinate to propionate from soluble sugars including lactate. Therefore bacteria such as *Selenomonas ruminantium* must be considered as an important population within this system. The most prevalent population from the libraries was identified as bacteria that cluster with the low G+C Gram-positive Clostrida XIVa group. *Schwartzia succinivorans* which utilises succinate as its sole carbon source to produce propionate was also present in the fumarate enrichments. Promotion of this species in this system may lead to improved rates of conversion of succinate to propionate and whether this has a flow on effect to fumarate reduction needs to be examined. The studies showed that succinate was reduced mainly to fumarate but acetate was also formed which is undesirable because it results in the release of two pairs of reducing equivalents that counterbalances the uptake of reducing equivalents by fumarate. The contribution of the Gram-positive bacteria to this process was investigated by performing fermentations with the addition of the Gram-positive inhibitor monensin. The rate of fumarate reduction was significantly decreased as was its conversion to acetate. These data indicate that Gram-positive bacteria within this system may be responsible for the unfavorable conversion of fumarate to acetate. In light of these results experiments were performed to obtain pure cultures from the fumarate reducing cultures to define the bacteria responsible for reducing fumarate and those that can decarboxylate succinate to propionate. An *E. coli* isolate completely converted fumarate to succinate, while an *Enterococcus faecalis* isolate also produced significant levels of acetate. Neither of these isolates was capable of growing on succinate as a sole carbon source. Another isolate was identified as *Clostridium orbiscindens* which could not grow on fumarate, but could completely convert succinate to propionate. Several other bacteria isolated from the fumrarte/H_2 system are Gram-positive (*Enterococcus gallinarum, Lactobacillus catenaformis, Lactobacillus vitulinus, Streptococcus gallolyticus, Streptococcus* sp. and *Streptococcus infantarius*) and of these the *Streptococcus* and *Enterococcus* species are known to produce acetate from various substrates. Presumably monensin also inhibited the bacteria affiliated with the Gram-positive Clostrida XIVa cluster but a representative could not be isolated and thus it's function is still unknown.

In summary, this project has delivered new knowledge about the presence of acetogenic and hydrogenotrophic bacteria in the digestive tract of ruminants and enabled the development of a suite of molecular tools to evaluate the contribution of these organisms to hydrogen sequestration when methane is inhibited and hydrogen accumulates in the rumen. This information will provide insights into the ecology of organisms involved in alternative pathways to methanogenesis for carbon and hydrogen sequestration. The potential to enhance their role in competition with methanogens and thus reduce methane emissions (greenhouse gases) can now be explored based on this new knowledge and tools. Theoretically the feeding of organic acids such as fumarate and malate to ruminants should also result in less hydrogen being available for methanogenesis but in practise benefits have been variable. This project has demonstrated that the suppression of Gram-positive bacteria with rumen modifiers such as monensin may favour propionate rather than acetate production from fumarate and consequently less methane production.

Contents

1 Background

1.1 Purpose and description

Methane produced by ruminant livestock accounted for 10.9% of Australia's net total greenhouse gas emissions in the year 2006 (Australian Greenhouse Office, 2008). Reducing these emissions will have an immediate benefit for the environment, will improve the 'clean and green' image of Australian beef and may also have a positive impact on the animals' energetic efficiency. Our current understanding of the microbial processes underpinning methane production is incomplete and the key to manipulating these emissions in the future will flow from fundamental improvements in our knowledge of methanogenesis and alternative hydrogenotrophic pathways.
Ungerfeld and Kohn (2006) provided an excellent overview of the thermodynamics of ruminal fermentation and identified several strategies for utilising hydrogen in the rumen as an alternative to methanogenesis. Two options which have received most attention involved promoting reductive acetogenesis as well as increased synthesis of propionate and butyrate through the provision of intermediates such as malate, fumarate and crotonate. If we are to employ these strategies successfully we need a more complete understanding of the physiology and ecology of the microorganisms underpinning these metabolic pathways.

1.1.1 Reductive acetogenesis

An alternative pathway to methanogenesis (equation 1) in the rumen is reductive acetogenesis (equation 2) that converts H_2 and CO_2 into acetate as outlined in the following equations:

Methanogenesis:

$4\ H_2 + CO_2 \quad CH_4 + 2\ H_2O \quad \Delta G = -68.3\ kJ / mol \quad (1)$

Acetogenesis:

$4\ H_2 + 2\ CO_2 \quad CH_3COOH + 2\ H_2O \quad \Delta G = -10.2\ kJ / mol \quad (2)$

The potential for reductive acetogenesis in the rumen as an alternative hydrogen sink to methanogenesis has been the subject of considerable speculation (Boccazzi 1997; Chaucheyras et al. 1995; Joblin 1999; Morvan et al. 1994; Morvan et al. 1996a, 1996b; Nollet et al. 1997; Olsson et al. 2006; Russell & Wallace 1997). Hydrogen and carbon dioxide-utilizing acetogens have been isolated from the ruminants and it is likely that these bacteria are normal flora of the rumen and therefore acetogens may be able to occupy the niche of methanogens as terminal sinks for ruminal H_2 (Mackie and Bryant 1994; Joblin 1999). While this proposition seems feasible there are some reasons why methanogenesis predominates as the favoured pathway for disposal of H_2. Firstly, the conversion of H_2 and CO_2 into methane yields more energy and is thermodynamically more attractive than their metabolism to acetate (Thauer et al. 1977; Cord-Ruwisch et al. 1988). Secondly, methanogens tend to have lower utilisation thresholds for H_2 than acetogens, although some ruminal acetogens grow at thresholds below 1μM H_2 (Joblin 1999). Thirdly, ruminal acetogens do not appear to be obligate hydrogenotrophs and can also use other compounds such as simple sugars as energy yielding substrates (Genthner et al. 1981; Greening and Leedle 1989; Rieu-Lesme et al. 1998; Joblin 1999). Although reductive acetogenesis is dominated by methanogenesis in the rumen this does not appear to be the case in hind-gut fermentations and some non-ruminant forestomach fermenters (Demeyer and de Graeve 1991). Some of Australia's native marsupials appear to possess unique

patterns of carbon sequestration that result in minimal emissions of methane from kangaroos and wallabies (Kempton et al. 1976; Engelhardt et al. 1978). The ecological reasons for this difference in metabolism between fore- and hind-gut compartments are unknown and require examination.

Some studies demonstrate that promoting acetogenesis in the rumen while employing strategies to inhibit methanogens may be a feasible approach to reduce methane emissions (Nollett et al. 1997, 1998; Le Van et al. 1998). An experiment with newborn lambs reared in isolation from their dams produced 30-40% less methane than conventional animals and hydrogen appeared to be channelled into acetate production via reductive acetogenesis (Faichney et al. 1999). Our current knowledge of ruminal acetogens is primarily based on investigations which have relied on the isolation and identification of these organisms. However it is now accepted that only a small percentage of the microorganisms in the rumen can be cultured (Tajima et al. 1999; Whitford et al. 1998). In an attempt to identify uncultured rumen acetogens we have analysed 16S rDNA and acetogenic functional gene libraries of DNA from enrichments of rumen contents grown on acetogen enrichment media with and without a methanogen-inhibitor (Rieu-Lesme et al. 1996, 1998).

1.1.2 Succinate/Propionate precursors as sinks for hydrogen

Fumarate and malate have been used by several investigators to stimulate succinate/propionate producers in the rumen which compete with methanogens for hydrogen. Many of these organisms use the succinate-propionate (randomizing) pathway as a major route for propionate synthesis in the rumen (Baldwin et al. 1963). In this pathway, malate is dehydrated to fumarate, and the reduction of fumarate to succinate is coupled to ATP synthesis. Succinate is either an intermediate or an end-product in the pathway of different rumen bacteria. However, several researchers have shown in mixed ruminal cultures that fumarate (and malate) is converted to propionate and acetate in varying proportions (Ungerfield and Kohn 2006; Ungerfield et al. 2007). The relative amounts of propionate and acetate formed from fumarate will impact on the hydrogen pool available to methanogens. Stoichiometrically, propionate production from fumarate consumes one pair of reducing equivalent while acetate production from fumarate releases two pairs of reducing equivalents. Therefore the production of acetate from fumarate is counterproductive when the objective is to reduce hydrogen available for methane production. It is important therefore to identify the microorganisms involved in these pathways and determine the physiological and biochemical conditions which favour propionate rather than acetate production from fumarate.

This project will provide this understanding by identifying and characterising the microbiology and genetics underpinning the alternative hydrogenotrophic pathways in ruminants, using metagenomics. The knowledge and resources arising from this project can then be used as the basis of an integrated approach to develop mechanisms that successfully inhibit rumen methanogens and (or) methane emissions from ruminant livestock.

2 Project Objectives - Section

2.1 Project Objectives

Identify and characterize the microbiological and genetic attributes of the microbiomes possessing alternative routes of hydrogenotrophy and (or) methanotrophy in the rumen of grainfed and grassfed cattle.
Develop novel strategies, which can be tested experimentally in cattle, for the practical implementation of project outcomes to inhibit rumen methanogens, reduce rumen methane emissions and improve animal energetic efficiency.

3 Methodology - Section

3.1 Methodology

3.1.1 Animal feeding and rumen sampling

Brahman-cross cattle (4 animals, 347-377 kg liveweight) were housed at the Rendel Laboratory, Rockhampton in individual pens to allow recording of daily feed intakes. They were adapted for 14 days to a commercial feedlot diet (Betterblend) plus 20% roughage Rhodes/Angleton (*Chloris gayana/ Dichanthium annulatum*) grass *ad lib*. Rumen samples were collected and used as inocula for *in vitro* fermentation studies and termed “grain-enrichment”.

A grass diet comprising a mixture of green panic (*Panicum maximum var. trichoglume*), Buffel grass *(Cenchrus ciliaris)* and Rhodes grass (*Chloris gayana*) was fed to three mature Brahman-cross cattle. Rumen samples were collected and used as inocula for *in vitro* fermentation studies and termed “grass-enrichment”.

3.1.2 *In- vitro* fermentations

Rumen digesta collected from untreated cattle (controls) and animals maintained on methanogen-inhibitors (BES, lumazine and mevinolin) were used to inoculate duplicate serum bottles containing anaerobic media. For the control inocula, *in-vitro* fermentations were performed with (control + inhibitor) and without (control) the methane-inhibitor compounds. For the methanogen inhibited animals the fermentations contained their respective inhibitor: BES, 1 mM; lumazine, 1.37 mM and mevinolin, 50 μM. Each fermentation was performed in basal medium using either labelled (^{13}C) or unlabelled (^{12}C) $NaHCO_3$ as the main source of carbon. Serum bottles were initially pressurised with H_2 to 200 kPa and gas pressures monitored periodically. When culture pressures decreased to approximately 50 kPa they were re-pressurised with H_2 gas to 200 kPa. Cultures were incubated at 39^oC and fermentations were allowed to proceed for 21 days after which they were harvested. The entire sample was centrifuged and the cell pellet stored at -80^oC until further use. Headspace gas aliquots were collected into airtight gas syringes periodically (~3 day intervals) and analysed by GC-TCD (Perkin Elmer, USA) as described below, for the estimation of H_2, CH_4 and CO_2 levels. Aliquots of cultures were collected weekly for pH and VFA analysis.
A second fermentation system was established in similar basal media containing 30 mM fumaric acid, 30 kPa H_2 and inoculated with digesta as described above. After 72 h incubation at 39^oC a

transfer into new media was performed. The transfers were continued every 72 h. At transfer five the media contained 25mM fumaric acid (1-2-3-4 ^{13}C) as the main carbon source. Headspace gases and VFA concentrations were analysed as described below and the bacterial cell pellet was harvested after centrifugation for DNA analysis.

3.1.3 High molecular weight DNA extraction

High molecular weight DNA was extracted from microbial enrichment cell pellets after centrifugation to remove growth media. Cells were initially washed with 10 mM Tris-HC1 (pH 8.0) prior to resuspending in lysis buffer (6 mM Tris-HC1 (pH 8.0), 1 M NaC1, 100 mM EDTA). Lysis of cells was undertaken at 37^{o}C for 3 hr with the addition of 3 µl of lysozyme (1mg/ml), 1 µl of mutanolysin (20 unit/µl) and 5 µl of acrohompeptidase (200 units/µl). Upon completion SDS was added to produce a final concentration of 1% and then 20 µl of proteinase K (20 mg/ml) was added and incubated at 45^{o}C for 1.5 hrs. The NaCl concentration was raised to a final concentration of 0.7 M and a 1/10th volume of NaCl/CTAB buffer (0.7 M NaCl/ 10% CTAB) was added. The solution was incubated at 70^{o}C for 10 minutes and then an equal volume of phenol/chloroform was added. After mixing of the liquid phases the sample was centrifuged at 12,000 x g for 5 min and the aqueous phase was transferred to a new tube. Addition of a 0.8 vol of isopropanol was performed with gentle mixing until DNA was observed to spool. The genomic DNA was removed from the tube using a glass rod and washed in 70% ethanol before being resuspended in TE buffer.

3.1.4 Isopycnic gradient ultracentrifugation and gradient fractionation

Caesium chloride (CsCl) gradients were prepared by adding 1 g of solid CsCl to every gram of DNA solution, and ethidium bromide (10 mg/ml) was added to this and gently mixed (Manefield et al. 2002). CsCl solution in RNase/DNase-free deionized water (Gibco, Invitrogen corp.) was added to bring the final density to approximately 1.58 g/ml. Centrifugation was performed in an ultracentrifuge at 247,000 x g for 16-18 h at 20^{o}C. DNA bandings within the gradient were then visualised using a UV transilluminator.
Collection of the CsCl density fractions was achieved by displacement with sterile de-ionized water by piercing a 19G needle through the top of the centrifugation tube and the use of a peristaltic pump. Removal of ethidium bromide from each fraction was achieved by the addition of an equal volume of TE-saturated 1-butanol and dialysed. DNA was subsequently recovered from the dialysis and pelleted by centrifugation (Padmanabhan et al. 2003). The DNA was visualised for integrity on agarose gel and quantified by Nanodrop-ND1000.

3.1.5 Stable isotope probing and isotope ratio mass spectrometry

The percentage of ^{13}C-labelling of the DNA was determined by isotope ratio mass spectrometry as described by Manefield et al (2002). Samples were combusted to CO_2 and analysed for ^{12}C:^{13}C ratios by continuous flow isotope ratio mass spectrometry on a GV Isoprime mass spectrometer. Glucose was used as the primary standard and the carbon content was reported as ^{13}C atom% units obtained by the equation (equation 1);

$$^{13}C \text{ atom\%} = [R_{sample}/R_{sample}+1] \times 1000 \qquad (1)$$

where R_{sample} is the *m/z* 45/44 (^{13}C:^{12}C) determined by IRMS on analyte CO_2 gas released on combustion of the sample. ^{13}C atom% of DNA is calculated by the mass balance equation that converts bulk IRMS ^{13}C values to DNA ^{13}C atom% by subtracting the mass of ^{13}C values of the

glucose (G) from the mass of ^{13}C in the total sample (S), to give the mass of ^{13}C atom% of the DNA (equation 2).

$$\text{DNA } ^{13}C \text{ atom\%} = [(S_{13C\ atom\%} \times S_{mass}) - (G_{13C\ atom\%} \times G_{mass}) / (S_{mass} - G_{mass})] \quad (2)$$

3.1.6 Meta-genomic fosmid construction

HMW-DNA recovered using the method above was used for cloning into the Fosmid copy control system (pCC1FOS) and performed according to the manufacturer's instructions (Epicentre). Randomly sheared DNA (20µg) was loaded onto a sucrose gradient (50-20%; 10% increments) and centrifuged for 20 h at 25,000 x g. DNA was collected from the gradient in 250 µl fractions and resolved on a 0.8 % gel for the identification of the fraction containing DNA at approximately 40 kb. The DNA was cloned into the copy control fosmid vector after end repair. Ligated vector products were then packaged into Lambda phage particles before infection of *E. coli* cells. Recombinant cells were selected by growth on antibiotic plates. Insert size was judged by restriction digestion of the plasmid and then separation by pulse field gel electrophoresis (PFGE).

3.1.7 Meta-genomic 454 sequencing

A metagenomic assessment of the CO_2/H_2 enrichment DNA using 454 pyro-sequencing was undertaken. High molecular weight DNA extracted previously from enrichments was nebulized and adapter fragments were added. The DNA was then amplified on small DNA-capture beads in a water-in-oil emulsion. Each DNA-bound bead was placed into a ~44 µm well on a PicoTiterPlate for massively-parallel pyrosequencing by 454 GS-Flx (MACROGEN, Korea).

3.1.8 Gas sampling and analysis

Gas samples were collected at several time points in gas tight syringes and CO_2, H_2 and CH_4 were analysed by gas chromatography using a Shimadzu GC-17A gas chromatograph with a thermal conductivity detector (GC-TCD).

3.1.9 Organic acid sampling and analysis

For the detection of volatile fatty acids a 1ml sample of culture fluid was taken at various time points for organic and inorganic acid analysis. Samples were centrifuged (12,000 x g; 10 min). 400 µl of supernatant was set aside for inorganic acid analysis while to the remaining 600 µl was combined with 150 µl of meta-phosphoric/internal standard solution (20% meta-phosphoric acid/0.24% 4-methyl valeric acid). Organic acids analysis was performed using a Shimadzu GC-17A gas chromatograph on a packed glass column (2 m length; 0.6 mm OD, 0.2 mm ID) containing 10% FFAP/1% H_3PO_4 on Chromasorb WAW 100/120 mesh. The C2–C5 acids were separated over 16 min using nitrogen as a carrier at 12 ml/min)1. Peaks were detected by Flame Ionization (Playne, 1985). Organic acids were analysed by HPLC on a Dionex Ultimate 3000 LC (Dionex Corporation, CA) using a Dionex Acclaim OA Reversed-Phase column (4 x 150 mm). Cultures were centrifuged and supernatants filtered using a 0.22 µm Millex GP filter. A sample volume of 2.5 µl was injected and separation of compounds was performed using 50 mM NaH_2PO_4 pH 2.7 buffer, with an isocratic flow rate of 0.6 ml/min at 30°C column temperature. Organic acids were detected by UV absorbance

at 210 nm using a photodiode array. Analysis of data was achieved with the Chromeleon v 6.8 analysis software (Dionex Corporation, CA) using appropriate standards for the organic acids.

3.1.10 Phylogenetic analysis

Bacterial 16S rDNA clone libraries were constructed using the bacterial universal primers 27f-1492r. Archaeal 16S rDNA libraries were constructed using the universal primers 4fa-1492r or methanogenic specific libraries by targeting the methyl co-enzyme reductase gene (mcrA). The amplicons were cloned into pGemTeasy (Promega, Madison WI). Random clones were sequenced using Big dye Terminator sequencing reagents. The sequence data was quality checked and then imported into the ARB database environment. Sequences were aligned using fast-aligner and then manually edited where required. Phylogenetic trees were constructed using the nearest neighbour algorithm with Felsenstein correction.
The 16S rDNA database was used for the design of probe and primer sets to detect phylogenetic groups. Various design parameters were used in order to optimise target specificity. For the design of probe sets to detect functionally important genes (eg. CODH, ACS and mcrA) sequences for the functional genes of inertest were aligned into separate datasets using ARB (Ludwig et al. 2004) and possible target regions were selected.

A phylogenetic microarray comprising four replicates of 3000, 40 nt oligo probes was constructed and also used for phylogenetic analysis. The oligo probes were designed against the 16S rRNA gene predominately at the genus and species level and cover many of the known gut microbial populations. Probes were either designed directly against the 16S rDNA sequence or using the GoArray technique (Rimour *etal,* 2005). Probe hybridization temperatures were kept within similar limits (70 – 80^{O}C) to maintain optimized hybridization conditions for all probes. An optimised hybridization temperature of 42°C for 16 hrs was used for all treatments.
Microarray image acquisition was performed on a GenePix 4000B microarray scanner (Molecular Devices). Oligo spots with an intensity ratio of less than 10000 compared to the negative control spots were flagged and removed. The captured imaged was exported for further analysis with the GeneSpring GX software package (Agilent). Normalization of data was performed using the single dye global normalization to 50% of the array signal. Spots with a 3 fold differential were flagged and then used to generate conditional trees for clustering analysis.

3.1.11 PCR based analysis of functional genes involved in hydrogenotrophy

DNA libraries were created for several functional genes to enable the design of specific probes and realtime PCR primer sets for key genes involved in hydrogenotrophy of methanogens and acetogens.

Methanogens Libraries of the mcrA gene were established using DNA extracted from rumen samples of cattle fed a roughage + grain diet (Denman et al. 2007) using previously published primers and methods described by Luton and co-workers (2002). From the libraries, clones were chosen for sequencing based on their distinctive RFLP pattern. In addition several clones from the dominant RFLP patterns were sequenced to determine if any significant variation existed within these patterns. Sequences obtained in this study were aligned to mcrA gene sequences downloaded from GenBank including recently described rumen methanogenic archaeal sequences

(Tatsuoka et al. 2004). Gene sequences were translated within ARB and amino acid alignments were then generated to construct phylogentic trees.
Primers for the monitoring of general methanogenic populations were designed based on conserved amino acid sequences around the catalytic regions of the mcrA gene. Multiple alignments using deduced amino acid sequences indicated two conserved stretches approximately 40 amino acids apart that would correlate to a PCR product of around 140 bp. The reverse primer (5'-TTCGGTGGATCDCARAGRGC) was designed to target a conserved region within the catalytic site GFYGYDL while the forward primer (5'- GBARGTCGWAWCCGTAGAATCC) targets the conserved sequence FGGSQR. Validation of the primer set and PCR conditions for their use in a real time quantitative PCR assay have been published (Denman et al. 2007).

Acetogens These libraries were established using DNA recovered from hydrogenotrophic batch fermentations using degenerate primer sets to ensure maximal coverage of target sequences. The genes investigated were those of three key enzymes in the acetyl-CoA pathway (Figure 1): formyltetrahydrofolate synthetase (FTHFS), carbon monoxide dehydrogenase (CODH) and acetyl-CoA synthase (ACS).

FTHFS libraries were generated using previously published primers and the protocol outlined by Leaphart and Lovell (2001) but there is little information available for the CODH and ACS sequences that would enable the design of primers specific for these genes. CODH, ACS amino acid sequences were retrieved from public databases (Brenda and NCBI via BLAST analysis) and aligned. The only sequences from an authentic homoacetogen were from *Moorella thermoacetica*. All other sequences were from genomes of a variety of archaea and bacteria, none reported to be homoacetogenic. Newly developed degenerate PCR primers were designed across conserved amino acid regions for CODH and ACS. PCR primers for the CODH and ACS genes respectively were designed based on conserved regions of these genes in the authentic homoacetogen *Moorella thermoacetica*, sulfite-reducing bacterium *Desulfitobacterium hafniense* and a variety of putative bacterial CODH and ACS sequences from genome sequencing projects. After analysis of sequences retrieved with the ACS primers from authentic homoacetogens (*Moorella thermoacetica, Clostridium aceticum, Acetobacterium woodii, Eubacterium limosum, Blautia hydrogenotrophica, Blautia schinkii, Blautia producta, Acetitomaculum ruminis* and *Clostridium difficile*), less degenerate primers were designed within the ACS region. These primers amplified ACS more specifically and were also used in combination with very degenerate primers upstream and downstream of this region, to retrieve almost full-length ACS gene sequence. A combination of these degenerate and more specific ACS primers were used to generate ACS libraries from enrichment cultures.

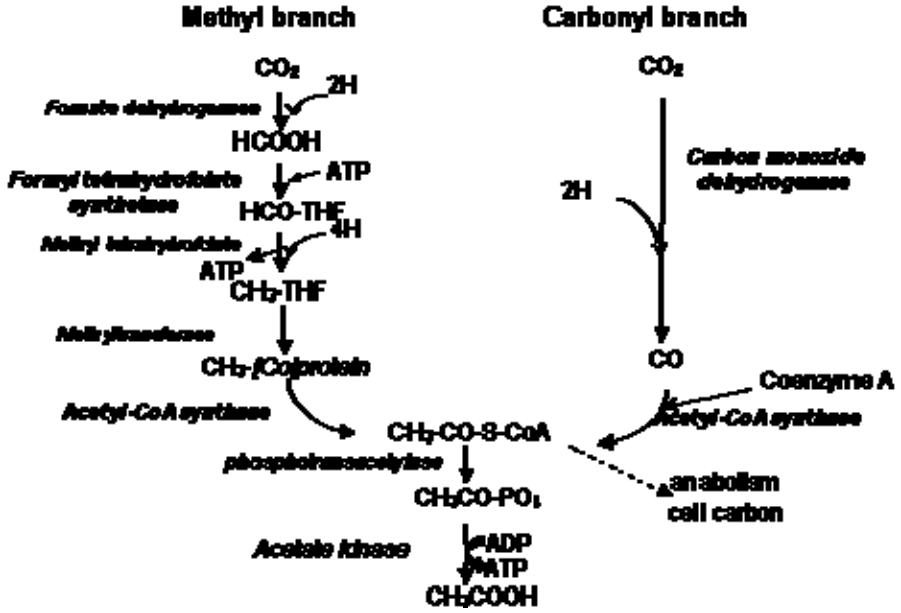


Figure 1. The reductive acetogenesis pathway. Adapted from Drake (1994).

3.1.12 Isolation of bacteria

The anaerobic techniques of Hungate (1969) as modified by Bryant (1972) were used to culture bacteria from the acetogenic and fumarate enrichments using a range of selective media for the major functional groups of microorganisms present in the gastrointestinal tract (Stewart et al. 1997; McSweeney et al. 2005). The basal medium used in the agar plates was the same as the respective enrichment medium. Colonies growing on acetogenic medium under a gas phase of H_2/CO_2 (1:3, 30 kPa) were picked into liquid broth medium and screened by PCR for the presence of genes involved in reductive acetogenesis. Bacteria from the fumarate enrichments were isolated from agar plates that contained either fumarate or succinate as the main source of carbon and picked into similar broth.

4 Results and Discussion

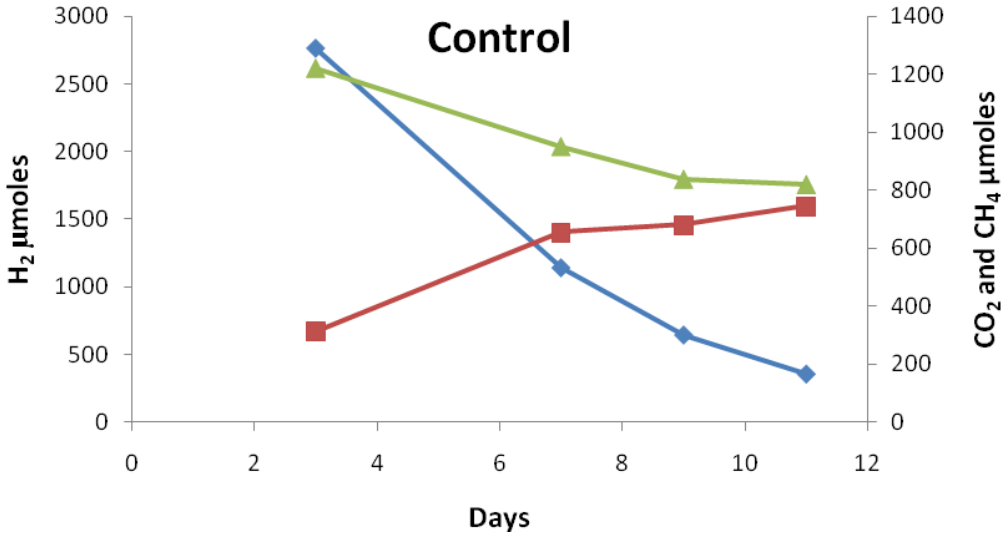
4.1 Results and Discussion

4.1.1 *In vitro* enrichments (Acetogenic)

The bacteria that are likely to be capable of hydrogen sequestration within the rumen are expected to be present as a minor population within the high bacterial diversity of the rumen. The ability to study these populations is hampered by the absence of tools that are available to detect these populations within diverse environments. Therefore, *in-vitro* fermentations were established in a

manner that would promote micro-organisms that were capable of utilizing H_2 and CO_2 as energy source. Furthermore, the use of stable isotope probing for the incorporation of ^{13}C from labelled CO_2 into the bacterial DNA actively utilizing CO_2 was used to further enrich this subpopulation from these enrichments. The *in-vitro* fermentation studies were performed several times for varying time periods ranging from 11 days to 36 days. The most accurate analysis with respect to gas pressure and composition data of these fermentations was achieved with the 11 day trial. Longer fermentations produced similar data with respect to microbial fingerprinting, but due to the need to re-pressurize these cultures with H_2 gas, accurate headspace gas data could not be guaranteed.

Fresh rumen digesta from four animals were used as inocula for *in-vitro* fermentations in the presence and absence of methane inhibitors from animals on a grain based diet. Hydrogen and CO_2 levels within the headspace declined gradually during the fermentation period, indicating actively consuming population(s) being present (Figure 2). Methane levels for the control fermentation (no inhibitor) increased to a maximum concentration of 745 µM over the 11 days, while the fermentation that was inhibited with BES showed no detectable levels of methane production for the 11 days



(Figure 2).

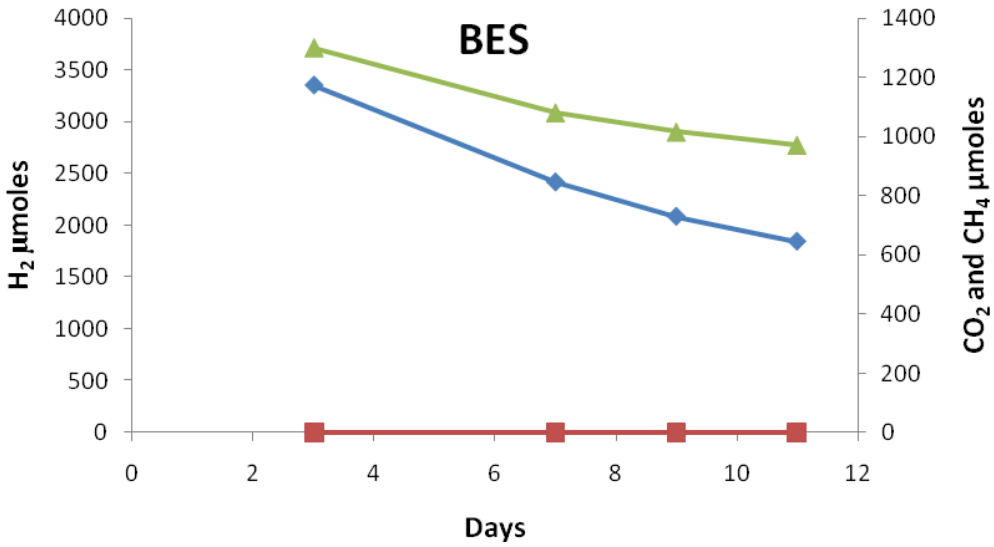


Figure 2. Concentration of gases (µmole) in the head space of fermentations control (A) and methane inhibited (BES) (B). Gas data is represented for CO_2 (▲), H_2 (♦) and CH_4 (■).

In addition to inhibiting production of methane, the BES fermentation produced significantly higher levels of acetate compared to the uninhibited fermentation (Table 1). These data indicate that reductive acetogenesis was occurring in the methane-inhibited fermentations and accounted for hydrogen and carbon dioxide utilisation. However the accumulation of acetate in the methanogenic fermentation may indicate that reductive acetogenesis was also occurring in the presence of methanogens but at a reduced level due to competition for substrate.

Table 1. VFA (mM) analysis of 12 day fermentations from grain and grass fed animals

	Acetic	Propionic	*i*-Butyric	*n* -Butyric	*i*-Valeric	*n*-Valeric
Control-grain	6.3	1.2	0.1	0.8	0.3	0.0
BES-grain	16.0	0.9	0.1	0.9	0.3	0.0
BES-grass	21.8	0.9	0.2	1.1	0.4	0.0

A second set of fermentations performed using inocula from animals being fed a grass diet and inhibited with BES showed no production of methane and acetate as the major fermentation end product (Table 1).
The data from the various fermentation systems clearly showed that these enrichments were being driven towards reductive acteogenesis and thus were an excellent source of material for investigating the bacterial populations responsible for hydrogenotrophy in the rumen.

4.1.2 Metagenomic analysis of acetogenic cultures.

4.1.2.1 Stable isotope probing

Cultures maintained on ^{13}C-labelled substrates accumulate ^{13}C in their genome, and due to their higher DNA mass will position lower when centrifuged in an isopycnic gradient compared with unlabelled DNA. Capture of this genetic material is then achieved by extraction of this heavier DNA

from the isopycnic gradient. DNA from the mixed rumen fermentations incorporating ^{13}C- labelled CO_2 did not produce two discrete bands, but rather produced a broader DNA band compared to unlabelled DNA. Several investigators have commented on the non-separation of the $^{12}C/^{13}C$ into distinct bands in environmental samples (Jeon et al. 2003; Padmanabhan et al. 2003). DNA was therefore extracted from the isopycnic gradient by targeting the bottom portion of the DNA band. The extent of labelling of the DNA in enrichment cultures was determined by IRMS as described by Manefield et al (2002). Labelled DNA showed enrichment of heavy labelled carbon as 8.9 atom% ^{13}C compared to 1.3 atom% ^{13}C for unlabelled cultures. Although no visible DNA separation was observed in the gradients there was enrichment of ^{13}C within the DNA that was extracted, denoting that this DNA originated from bacterial populations that were capable of consuming CO_2.

4.1.2.2 Phylogenetic classification (16S rDNA)

The construction of 16S rDNA libraries was performed to ascertain the diversity and identity of bacteria within these enriched cultures. 16S rDNA libraries from the control and BES CO_2/H_2 enrichments showed a wide variety of bacteria across many operational taxonomic units (OTU's). For BES treated fermentations, 53 sequences were placed into 19 operational taxanomic units (OTUs indicating a 97% sequence similarity) with the five predominant OTU's being most similar to *Escherichia coli* (99%), a Synergistes member *Dethiosulfovibrio* sp. (86%), *Desulfovibrio* sp. (97%), *Selenomonas ruminantium* (99%) and a *Clostridales* sp (95%). Several bacteria from these OTU's are known to produce aceteate as their main fermentation end product. The Clostridales OTU contained sequences that showed high similarity to an isolate from the kangaroo gut which was not able to ferment carbohydrates but produced acetate as its main end product (Ouwerkerk *et* al 2005) (Figure 3: OTU 991). Also within this OTU is *Balutia productus* a known homoacetogenic bacterium from the cattle rumen. The dominance of these organisms within the BES fermentation explains the higher levels of acetate detected from these fermentations (Table 1).

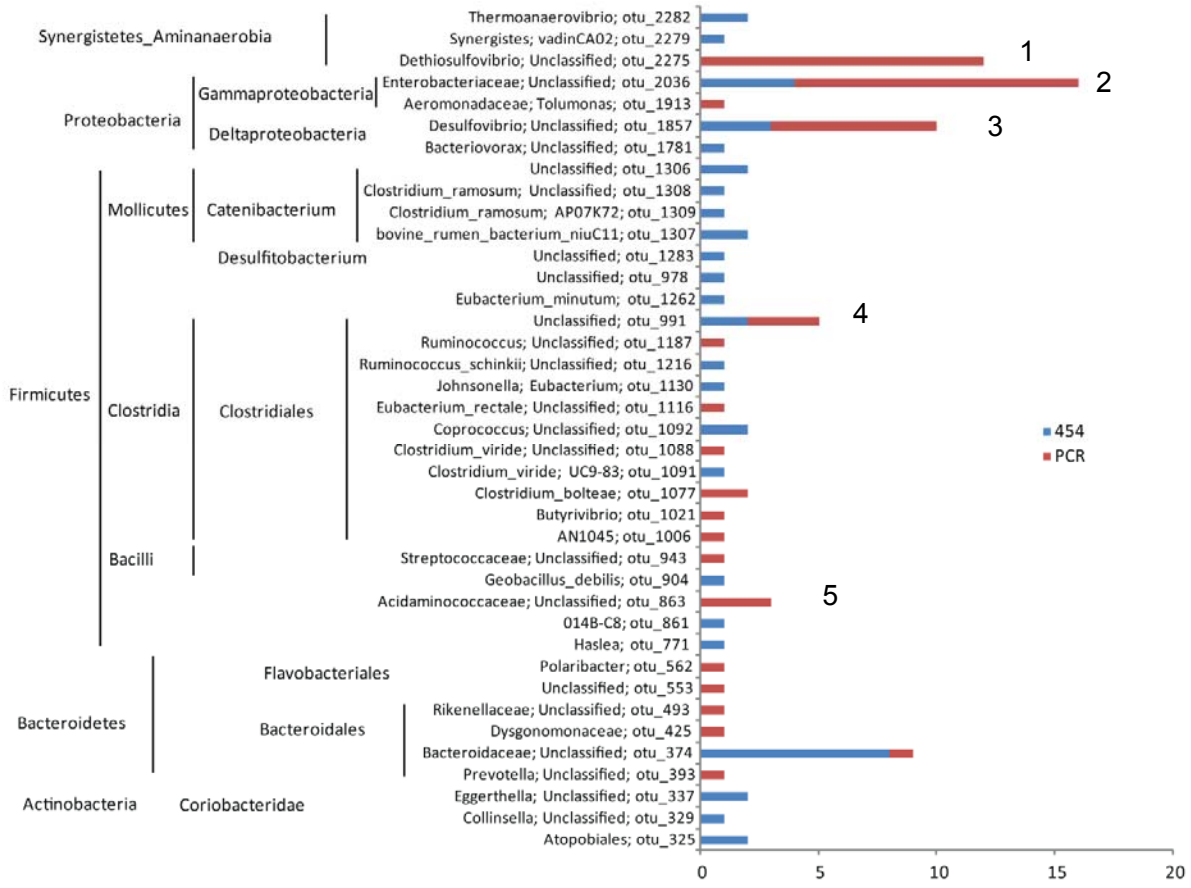


Figure 3. Placement of 16S rDNA sequences into defined OTU's via NAST alignment and Greengenes server using Hugenholtz taxonomy (www.greengenes.lbl.gov). OTU 1: *Dethiosulfovibrio* sp.; 2: *Escherichia coli*; 3: *Desulfovibrio* sp.; 4: *Clostridales* sp.; 5: *Selenomonas ruminantium*. Red bars represent the number of sequences classified from PCR generated libraries using universal primers 27f and 1492r; blue bars represent 16S rDNA sequence extracted from 454 metagenomic sequencing.

Rarefaction analysis of the OTUs shows that only a small fraction of the diversity of this enrichment was identified, thus indicating that those OTUs represented by multiple sequences are likely to be the predominant species. A greater number of OTU's was identified from the metagenomic DNA analysis using 454 pyrosequencing technology. Forty three 16S rDNA sequences were extracted from the first pass assembly data consisting of 69,651 contiguous sequences. These sequences could be assigned to 24 individual OTU's (Figure 3). The predominate OTU's identified by 454 sequencing correlated well with those that were identified using 16S rDNA PCR. A more extensive analysis of the 454 generated metagenomic data will be addressed later in the report.

4.1.2.3 PCR based analysis of functional genes:

Although 16S rDNA analysis is considered the most appropriate tool for identifying bacterial populations, it imparts very little information with regard to the function that these bacteria may

perform within these environments. Approaches targeting the functional genes contained within a metabolic pathway of interest will indentify those bacteria capable of performing this function. This is particular relevant to bacteria that are likely to be carrying out reductive acetogensis or fumarate reduction as these bacteria are found within diverse phyla. Tools for indentifying and tracking these populations were developed through the construction of functional gene libraries, mcrA gene for methanogenesis and the CODH, ACS and FTHFS genes for reductive acetogenesis.

4.1.2.4 PCR based analysis of functional genes: MCRA

Several groups have reported the monitoring of methanogen populations from environmental samples through targeting of the 16S ribosomal gene (Yu et al. 2005; Stewart et al. 2006). Whilst researchers have traditionally used the 16S rRNA gene for phylogenetic diversity, many researches are now addressing the diversity of the methanogenic archaea by studying sequence divergence within the mcrA gene (Lueders et al. 2001, Luton et al. 2002; Hallam et al. 2003). Methyl coenzyme-M reductase is ubiquitous to methanogens and is crucial to the terminal step of methanogenesis where it is involved in the reduction of the methyl group bound to coenzyme-M. Within this study we aimed to analyse the methanogenic diversity within the rumen of cattle using the mcrA gene as the phylogenetic marker and from this gathered information to design qPCR primers.
Amplicons generated from primers targeting the mcrA gene were used for phylogenetic analysis (Figure 4). Examination of clones showed that the majority were from the Methanobacteriales order, a similar result to that generated from 16S rDNA libraries. Minor groups of clones were placed within the orders Methanomicrobiales (*Methanomicrobium mobile*) and Methanosacinales (*Methanosarcina bakeri*). In addition to these another clone was most closely related to *Methanosphaera stadtmanae* mrtA gene with 90.4% identity. Several other clones clustered closest to an uncultured euryarchaeote clone OS55 (Luton et al. 2002).

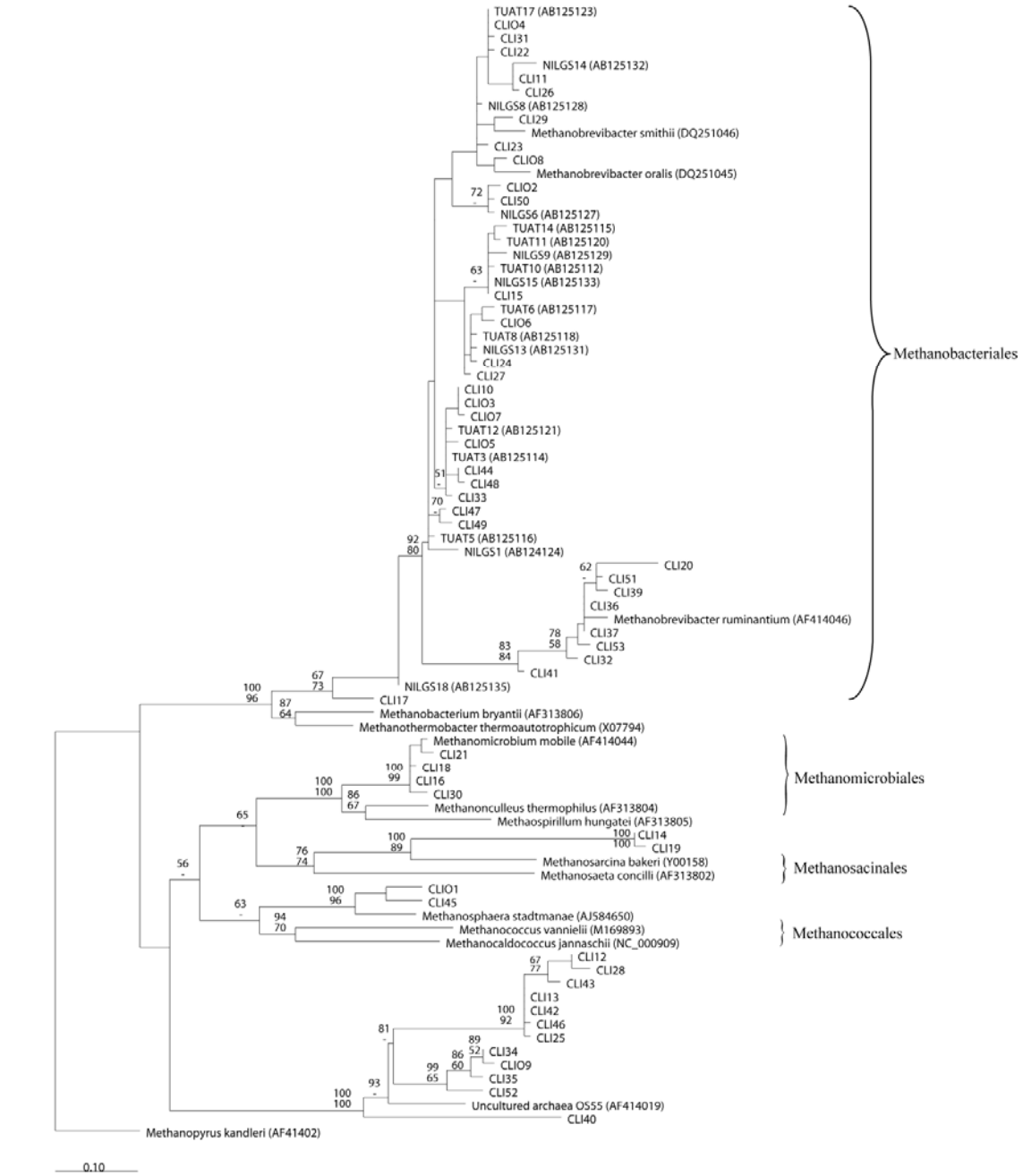


Figure 4. Phylogenetic placement of deduced amino acid sequences for the mcrA gene. GenBank accession numbers are presented in brackets for those sequences used. Clones retrieved from the mcrA library are designated as CLI.

PCR Primer design and validation Primers for the monitoring of general methanogenic populations (see material and methods) were designed based on conserved amino acid sequences around the catalytic regions of the mcrA gene. Conventional PCR using this primer set on total rumen microbial DNA resulted in a single amplicon corresponding to 140 bp. Sequencing of 24 clones derived from this amplicon all resulted in mcrA sequences. An identical product size was observed when templates derived from the CLI clones (rumen mcrA libraries) were used. Dissociation curve analysis for the general methanogenic archaeal primer set produced a dissociation curve with a single peak at 81^{o}C when *M. ruminantium* M1 DNA was used as template, while two peaks at 81 and 86^{o}C were observed, when using total rumen microbial DNA as template. CLI clones from the Methanobacteriaceae family all possessed a single dissociation curve between 80-82^{o}C, while all other CLI clones produced a single dissociation curve at 84-86^{o}C. The mrtA gene sequence clone CLI01, produced a single dissociation curve peak at 81^{o}C. These primers and assay conditions were further refined to produce a robust and accurate qPCR assay for the detection and quantification of methanogenic populations based on the detection of the mcrA gene.

4.1.2.5 PCR based analysis of functional genes: FTHFS

An published set of primers that is accepted for detection the FTHFS gene is already available (Leaphart and Lovell 2001). Placement of FTHFS sequences generated from PCR clone libraries within these trees was essential to the process of accurately identifying which genes were likely to be involved in the pathways of interest since some of these genes also reside in pathways/microorganisms not involved in hydrogenotrophy. FTHFS sequences were recovered from the grain enrichments were found predominantly in cluster A which align with FTHFS genes from known homoacetogenic microorganisms (Figure 5). This was further defined into two main groups of sequences which grouped closely to *Eubacterium limosum.* For grass enrichments there were two major groupings of genes within cluster A that aligned closely with *Sporomusa* sp., while another significantly represented group fell outside of cluster A and were most closely related to the non-homoacetogeneic bacteria *Proteus vulgaris.* Only a single representative from the grain enrichments fell within this region, suggesting a diet effect for these microorganisms. Metagenomic DNA sequencing data showed greater diversity of FTHFS genes than the PCR generated data, highlighting the limits of the PCR primer set to fully cover the entire diversity. However, the majority of 454 generated sequence data still fell within cluster A. Most notable was the detection of *Bacterodies* sp. like FTHFS sequences. Within cluster A the metagenomic data also identified a grouping of sequences more closely related to the known acetogenic bacteria *Blautia productus* and *Clostridium magnum*.
With respect to the FTHFS gene it is important to identify those genes that belong to cluster A, as these are from known homoacetogenic bacteria, while sequences in cluster B belong to the sulfate reducing bacteria (SRB). The SRB are known to catabolise acetate via the acetyl-CoA pathway functioning in reverse (Fuchs 1986; Schauder et al.1986). Thus any sequences found within this cluster are unlikely to be involved in acetogensis.

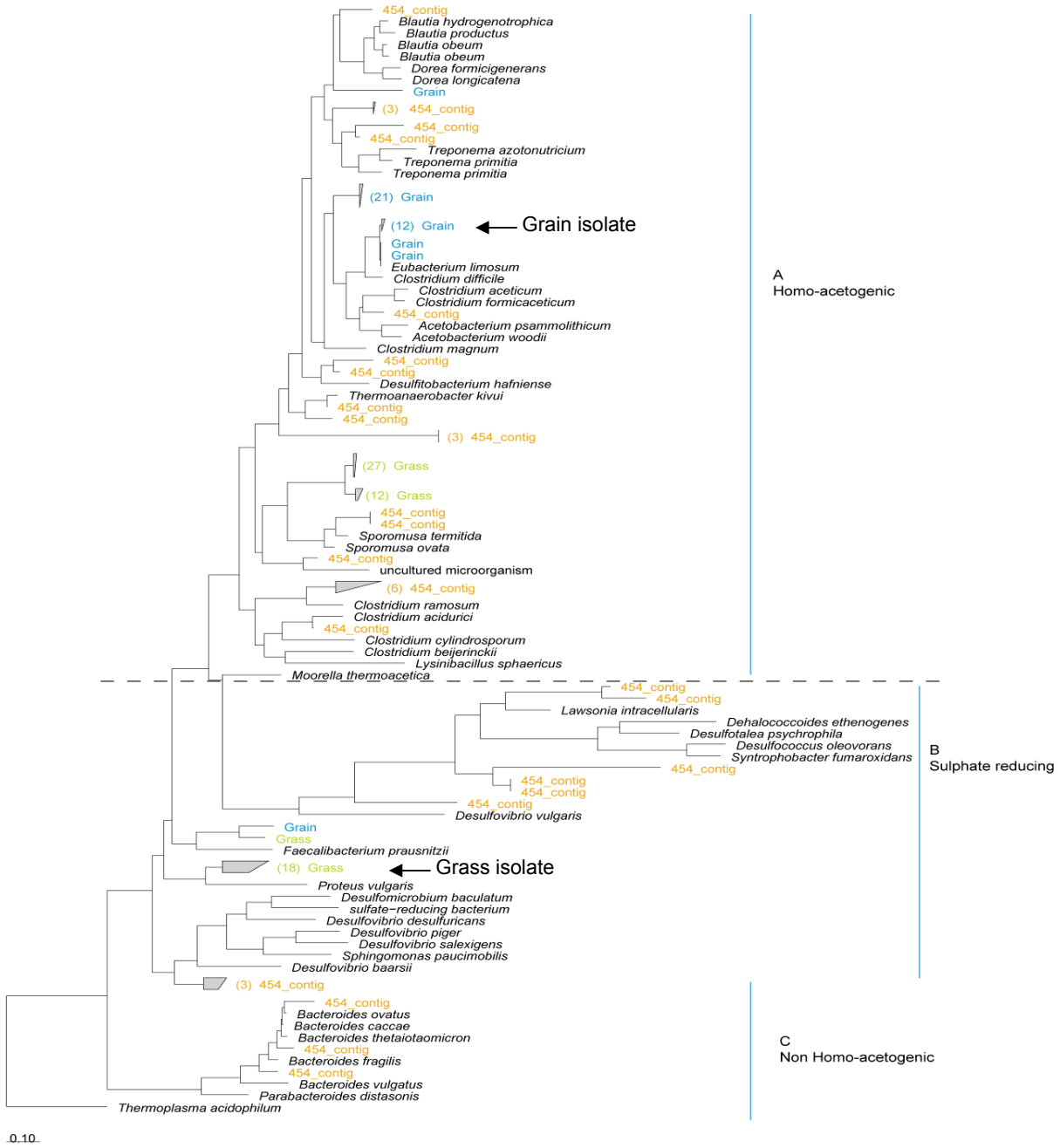


Figure 5. Phylogenetic tree illustrating the placement of FTHFS gene sequences from acetogenic enrichment cultures. A, B & C refer to FTHFS clusters associated with acetogenic and non-acetogenic bacteria (Lovell and Leaphart 2005). Grain and 454_contig labels indicates sequences derived from grain enrichments while Grass labels indicate sequences derived from grass enrichments. Two bacterial isolates obtained from these enrichments are indictaed by arrows.

4.1.2.6 PCR based analysis of functional genes: ACS/CODH

The bifunctional enzyme carbon monoxide dehydrogenase/acetyl-coenzyme A synthase comprises the CODH and ACS genes. The acetyl co-A synthase (ACS) gene is essential for reductive acetogenesis and is perhaps the best candidate gene for assigning function. However, very little sequence information is available for this gene particularly from known acetogenic bacteria. Initial sequence alignments were limited but did allow for the production of a first generation primer set which was successful in generating gene fragments from pure cultures of known homo-acetogenic bacteria. Addition of these newly generated sequences allowed for more refined alignments and the generation of a more specific primer set. Sequences from both grass and grain enrichments were seen to cluster together with known acetogenic clostridiales isolates (*Blautia hydrogenotrophicus, B. productus* and *B. schinkii*) (Figure 6). A smaller grouping consisting of three sequences from the grain enrichment was found to associate in a group containing known acetogenic isolates (*Clostridium aceticum, Eubacterium limosum* and *Acetobacter woodii*). Sequences generated from 454 sequencing for the grain enrichment showed similar clustering to the sequences obtain through PCR, suggesting that the new PCR primer set was capable of detecting the diversity of bacteria carrying the ACS gene within these enrichments.

Supporting these observations were the results for the carbon monoxide dehydrogenase gene (CODH) (Figure 7). Again limited sequence data from known acetogens restricted the development of a PCR primer set. CODH gene sequence data was obtained from known acetogenic bacterial isolates using this primer set, but no new regions could be found to refine this primer set. The primer set was considered sub-optimal for specificity as non-specific bands could be detected when applied to the metagenomic samples. PCR generated sequence data from both grass and grain enrichments produced a small number of gene sequences that grouped closely with *B. hydrogenotrophicus and B. obeum,* known homoacetogen. The 454 sequence data again supported the limited diversity of bacteria harbouring this gene. Expect for one short sequence read all 454 data generated CODH sequences clustered close to the PCR data. The single CODH sequence that grouped distantly with *Campylobacter* sp. will need to be further investigated since its short read length may affect the accuracy of this placement.

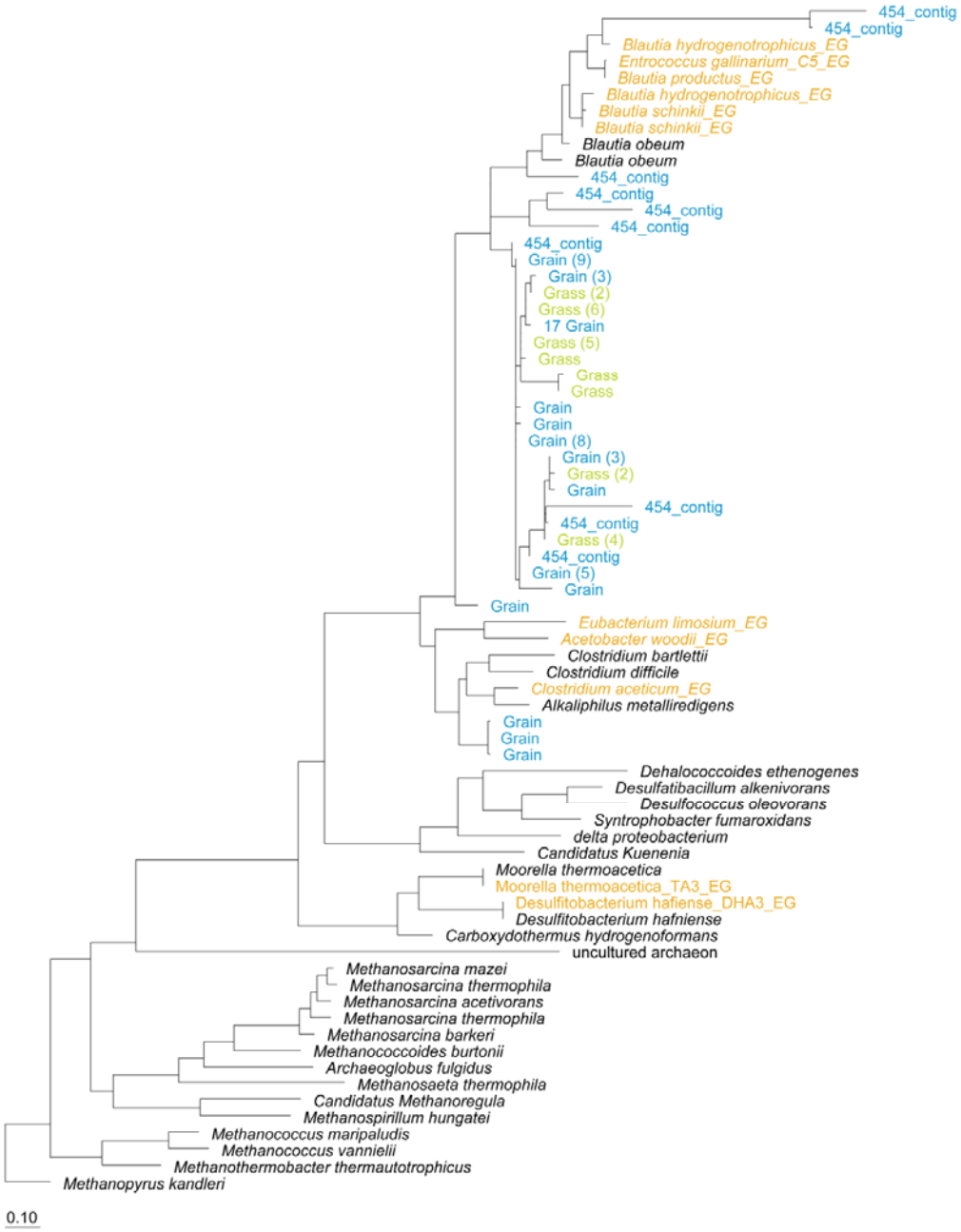


Figure 6. Phylogenetic tree illustrating the placement of ACS gene sequences. ‘Grain’ and ‘454_contig’ labels indicate sequences derived from grain enrichments, ‘Grass’ labels indicate sequences derived from grass enrichments. Numbers in brackets refer to the number of sequences represented by that clone. Bacterial isolate sequences in gold indicated with an asterix are novel from this study.

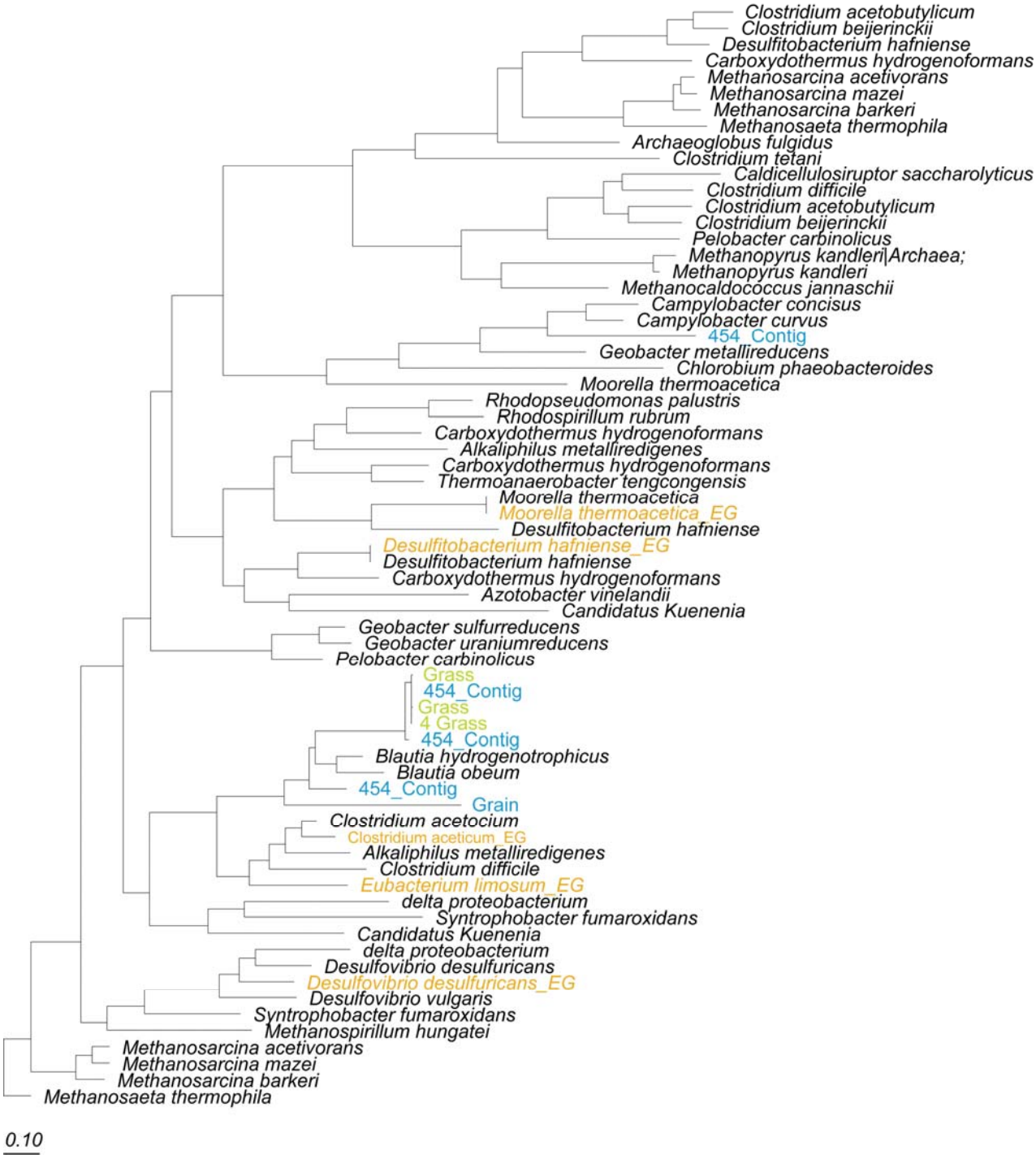


Figure 7. Phylogenetic tree illustrating the placement of CODH gene sequences. ‘Grain’ and ‘454_contig’ labels indicate sequences derived from grain enrichments, ‘Grass’ labels indicate sequences derived from grass enrichments. Bacterial isolate sequences in gold indicated with an asterix are novel from this study.

The most common molecular tool for the monitoring of acetogenic populations from environmental samples has been the use of the FTHFS primer set that was first described by Leaphart and Lovell (2001). Although this primer set has been readily taken up by most researchers it has its limitations; the primer set does not cover all diversity and is not capable of detecting some known acetogenic bacteria. The FTHFS gene is also involved in other pathways in addition to reductive acetogenesis, such as folate biosynthesis and therefore will detect bacteria that are not capable of reductive acetogenesis. Any analysis using the FTHFS gene requires its placement within an accurately generated tree, so that association within cluster A can be confirmed. By developing primer sets that can detect the ACS and CODH genes we have been able to ascertain that a limited number of bacteria carry these genes within the microbial enrichments that were studied compared to the diversity for the FTHFS gene. The ACS/CODH sequences clustered closely with the same bacteria that affiliate with the predominant clusters for the FTHFS cluster A data, indicating that these bacteria will carry all three of these essential functional genes.
The ACS primer set was further refined generating a primer set that is suitable for realtime PCR analysis and will help tracking and quantifying these bacteria within the cattle rumen.

4.1.2.7 454 metagenomic analysis of functional genes:

With the advent of 454 sequencing and associated advantage through deeper sequence coverage of samples (due to the massive amount of sequence generated), metagenomic sequencing analysis can now be performed successfully on complex environmental samples. A metagenomic assessment of the CO_2/H_2 enrichment DNA using 454 pyro-sequencing was undertaken. High molecular weight DNA extracted from grain based enrichments was nebulized and adapter fragments were added prior to sequencing. Approximately 544,000 sequences with an average read length of 256 bp were obtained. A first pass assembly was performed using the Newbler GS *de nova* assembly software. A total of 69651 contiguous sequences were generated ranging in size from 95 bp to 5 kb. A local BLAST database was constructed using these contiguous sequences and searches for functional genes were performed (Table 2, Figure 8).

Table 2. Frequency of acetogenic pathway genes in metagenomic sequence data

Functional gene	Reads	Contigs	Largest contig (bp)
NADP-dependent formate dehydrogenase	907	15	2395
Formyltetrahydrofolate synthase	260	36	1572
Methenyltetrahydrofolate cyclohydrolase	284	34	1707
Methenyltetrahydrofolate dehydrogenase	284	34	1707
Methenyltetrahydrofolate reductase	10	2	212
Methyltetrahydrofolate:corrinoid/iron-sulfur methyltransferase	295	34	2077
Acetyl-CoA synthase	48	12	422
Carbon monoxide dehydrogenase	143	4	2395
CODH chaperone	17	2	966
Corrinoid/iron-sulfur protein large subunit	113	19	1382
Corrinoid/iron-sulfur protein small subunit	11	2	227

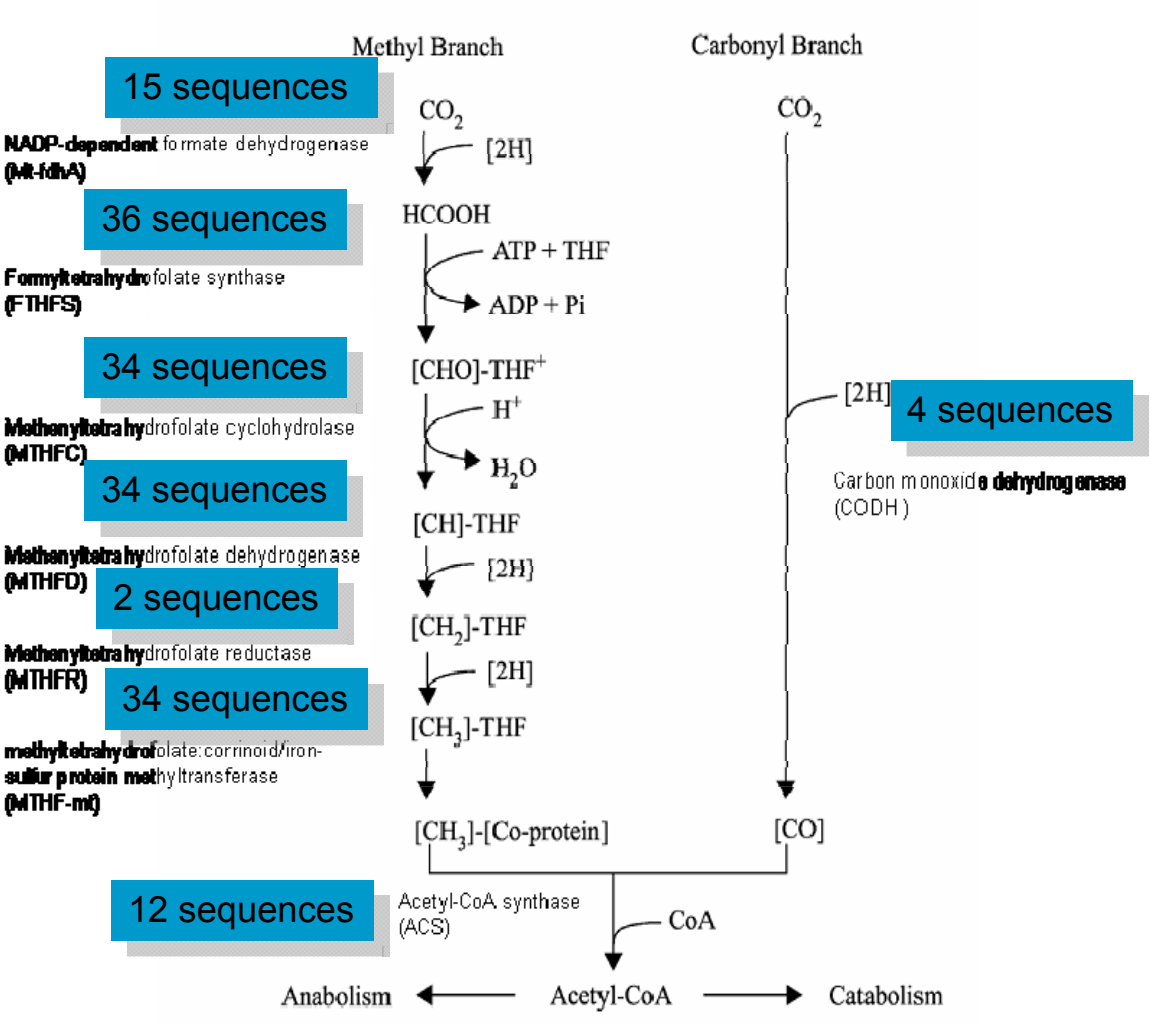


Figure 8. The reductive acetogensis pathway reconstructed from metagenomic data from grain enrichment fermentations. The number of sequences found for each enzyme is listed above the enzyme name.

The entire reductive acetogenic pathway could be reconstructed from the metagenomic data, including the corrinoid/iron-sulfur containing accessory proteins in the ACS/CODH complex and the CODH chaperone protein (cooC) involved in nickel insertion for CODH. Recently sequenced genome data for *Moorella thermoacetica* has identified that the genes involved in the carbonyl branch of the reductive pathway are co-located in a gene cluster and that this is true for most known homoacetogens (Pierce et al. 2008). Making these genes, the key marker genes for defining bacteria performing reductive acetogenesis.
Assignment of these genes into taxanomic grouping will aid in identifying which bacteria are responsible for this function. Provisional taxonomy association was performed using NCBI BLAST and MEGAN software (Altschul et al. 1990; Huson et al. 2007) (Figure 9). The only taxonomic grouping to contain all functional genes was that for clostridiales phyla. In addition, only genes from

the carbonyl branch (and ACS gene cluster) were found to associate with the clostridiales phyla and were absent from all other bacterial phyla (Figure 9).

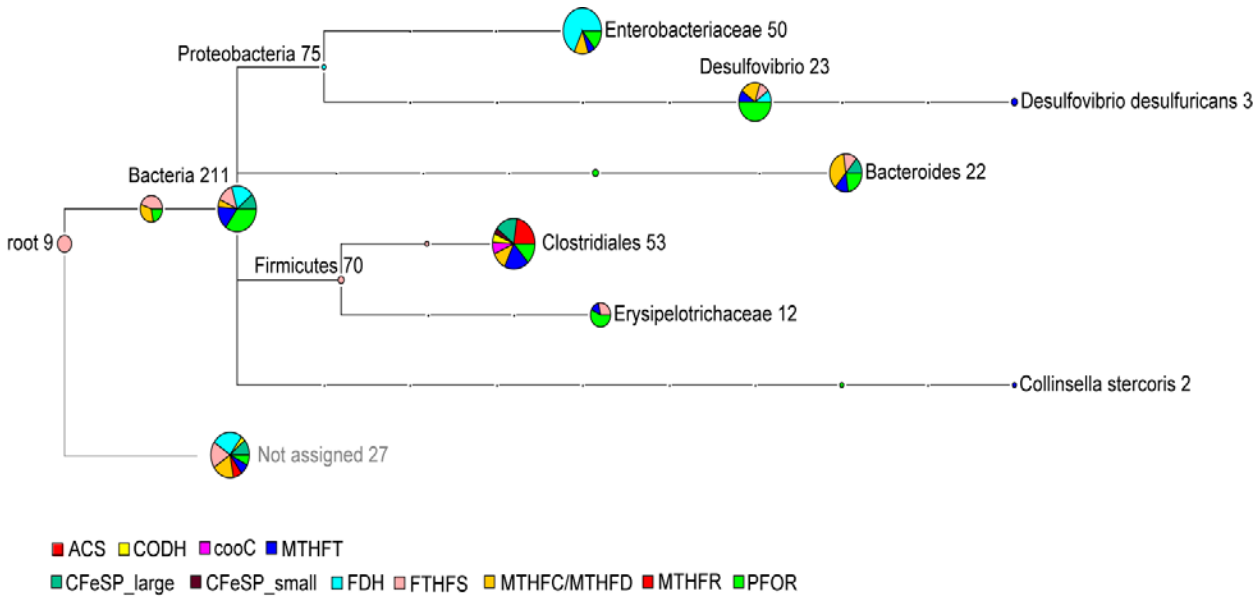


Figure 9. MEGAN assignment of functional gene reads to NCBI taxonomy data. Numbers beside taxonomy assignment represent the number of sequences. ACS: acetyl-coA-synthase; CODH: carbon monoxide dehydrogenase; cooC: accessory protein; MTHFT: methyltetrahydrofolate methyltransferase; CFeSP_large: corrinoid/iron-sulfur protein large subunit; CFeSP_small: corrinoid/iron-sulfur protein small subunit; FDH: formate dehydrogenase; FTHFS: formyl tetrahdrofolate synthase; MTFHC/MTHFD: methyltetrahydrofolate cyclohydrolase/dehydrogenase; MTHFR: methyltetrahydrofolate reductase; PFOR: pyruvate ferroxidoreductase.

4.1.3 Bacterial isolates from acetogenic enrichments.

Bacterial isolation experiments were performed in order to obtain pure cultures of reductive acetogenic bacteria from grass and grain enrichments. Bacteria were picked as single colonies from anaerobic agar plates and screened for the presence of the FTHFS gene. Several isolates from both enrichments were found to harbour the gene and further analysis was performed on these isolates. Sequencing of the FTHFS gene products placed the grain isolates within the homoacetogenic cluster for the FTHFS gene while the grass isolate fell outside of this cluster (Figure 5). The grain isolate grouped close to *Eubacterium limosum* and other FTHFS gene clones obtained from the grain enrichment. The grass isolate grouped closely to *Proteus vulgaris* and closely to a dominant grass PCR clone cluster (Figure 5). Only the isolate from the grain enrichment was found to carry the ACS gene, suggesting that it possesses the ability to perform reductive acetogenesis. This result further highlights that currently the most appropriate gene for identifying and quantifying acetogenic bacteria lies within the ACS gene cluster.

4.1.3 Fumarate reduction an alternative to acetogenesis.

4.1.3.1 *In vitro* fermentations

While a large emphasis has been placed on understanding the microbiology and genetics underpinning reductive acetogenesis, other bacteria can use alternative pathways to sequester H_2. Reducing fumarate through the coupling of H_2 to produce succinate and finally propionate by bacteria also exists within the rumen. There is little knowledge of which bacteria are responsible for these actions and how they can be manipulated within the rumen. The primary pathway for fumarate metabolism is hydrogenation to succinate and then decarboxylation of succinate to propionate. Fermentation systems were set up in such a manner as to enrich for these microorganisms and allow the capture of genetic material from these functionally active bacteria. Analysis of fermentative end products was used to monitor shifts in microbial biochemical pathways.

The 16S rDNA libraries from fumarate enrichment cultures indicate a less complex microbial enrichment compared to the CO_2/H_2 enrichments. *Selenomonas ruminantium* was present in these libraries at high numbers and this species is known to be a major population in the rumen that lacks the ability to degrade plant material, but will ferment soluble sugars to propionate. *Selenomonas ruminantium* uses extracellular H_2 to reduce fumarate to succinate and they have the ability to carry out decarboxylation of succinate to propionate from soluble sugars including lactate. The most prevelant population from the libraries was identified as bacteria that cluster with the low G+C Gram-positive Clostrida XIVa group. No strains from this cluster have currently been isolated, thus limiting our understanding of their function in this system. *Enterococcus faecalis* was also identified in these cultures and is a known acetogenic bacteria that has been isolated from the rumen of young lambs (Forano pers. comm.). Also identified in the 16S rDNA libraries was *Schwartzia succinivorans*, a bacteria that utilises succinate as its sole carbon source to produce propionate.

Fermentation end product results show a conversion rate to succinate that matches that of the reduction in fumarate, but it also reveals production of acetate (Figure 9). Acetate production from fumarate is undesirable because it counterbalances the uptake of reducing equivalents by fumarate in that it results in the release of two pairs of reducing equivalents. This novel observation highlights the lack of understanding that exists with respect to which microbial populations should be promoted within this strategy.

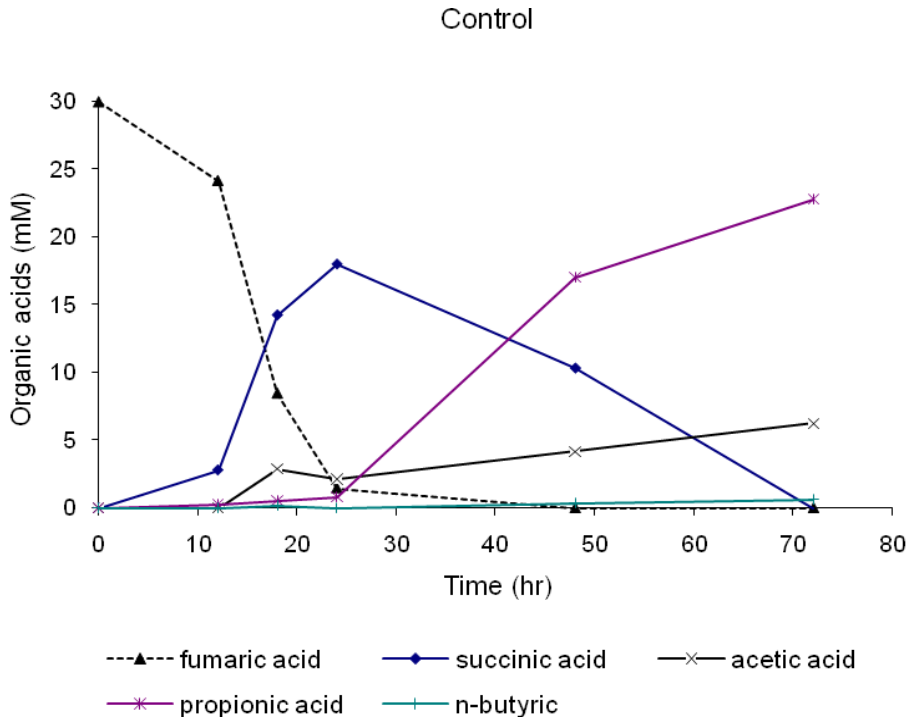


Figure 9. Organic acid concentrations from fumarate/H_2 fermentations performed for 72 hrs.

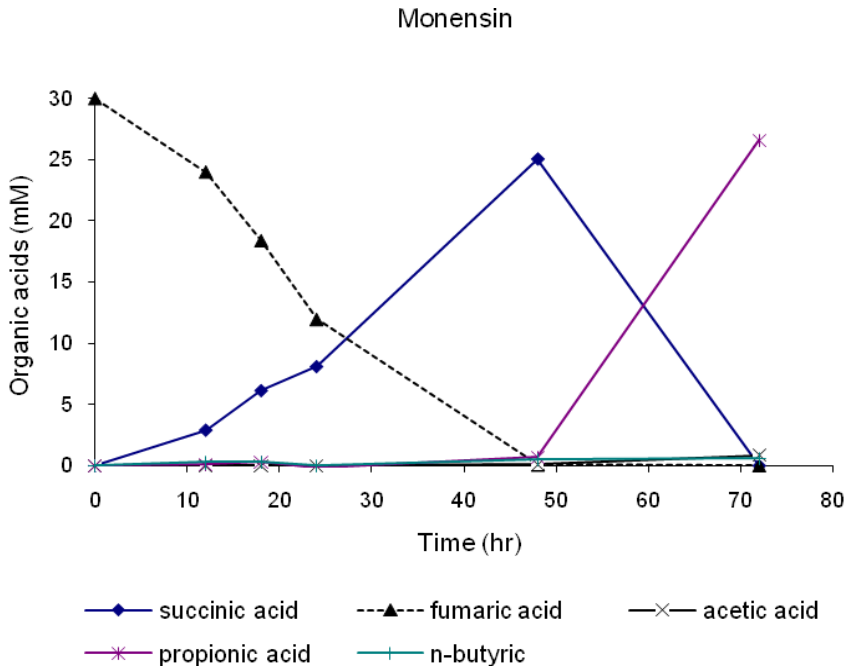


Figure 10. Organic acid concentrations from fumarate/H_2 fermentations with the addition of monensin performed for 72 hrs.

From the organisms that have been identified within this system several Gram-negative bacteria are known to carry out fumarate reduction. Both *Selenomonas ruminantium* and *Prevotella ruminicola* (to a lesser extent) have been reported to consume fumarate with the major end product being succinate (Asanuma et al. 1999).The contribution that the Gram-positive bacteria exhibit in this system was investigated by performing a fermentation with the addition of the Gram-positive inhibitor monensin. The rate of fumarate reduction was significantly decreased as was its conversion to acetate (Figure 10). Although the rates of conversion were altered the proportion of fumarate being reduced to succinate was also increased. These data indicate that Gram-positive bacteria within this system may be responsible for the unfavorable conversion of fumarate to acetate. Presumably monensin inhibited the bacteria affiliated with the Gram-positive Clostrida XIVa cluster, but as no isolate was obtained, it's function here is still unknown. Several Gram-positive bacteria isolated from the fumrarte/H_2 system are (*Enterococcus faecalis, Enterococcus gallinarum, Lactobacillus catenaformis, Lactobacillus vitulinus, Streptococcus gallolyticus, Streptococcus* sp. and *Streptococcus infantarius*) and of these the *Streptococcus* and *Enterococcus* species are known to produce acetate from various substrates.

4.1.3.2 Bacterial isolates from fumarate enrichments

In light of the results obtained from the fermentation trial, isolates were tested to define the bacteria responsible for reducing fumarate and those that can decarboxylate succinate to propionate. Two bacteria were investigated for their ability to reduce fumarate to succinate (Table 3), while another two were identified for their ability to convert succinate to propionate. The *E. coli* isolate completely converted fumarate to succinate, while the *Enterococcus faecalis* isolate also produced significant levels of acetate. Neither of these isolates was capable of growing on succinate as a sole carbon source (table 3). Two isolates where also investigated for their ability to decarboxylate succinate to propionate, both of these isolates were most similar to *Clostridium orbiscindens* (99%). Both isolates could not grow on fumarate, but did completely convert succinate to propionate (Table 3).

Table 3: VFA (mM) analysis of fermentations

	Fumaric	Succininc	Acetic	Propionic	i-Butyric	Butyric	i-Valeric	Valeric
Fumarate media								
*E.coli*_1	-30.25	30.33	-0.95	-0.29	-0.05	-0.19	-0.08	-0.04
*E.faecalis*_B5	-31.30	26.24	6.43	0.24	0.03	0.10	0.04	0.02
*C.orbiscindens*_S4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
*C.orbiscindens*_S5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Succinate media								
*E.coli*_1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
*E.faecalis*_B5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
*C.orbiscindens*_S4	0.00	-36.45	0.45	31.75	0.03	-0.63	0.01	0.01
*C.orbiscindens*_S5	0.00	-32.96	-1.77	30.45	-0.03	-0.70	-0.06	-0.04

Fumarate has been discussed in the literature as a way of encouraging alternative hydrogen sequestering (Asanuma *et al* 1999). The results of this experiment provide novel insights into the metabolism of fumarate in the rumen and the potential diversion of some hydrogen from methanogenic pathways. Most importantly it indicates some of the bacteria responsible for the

production of acetate and H_2 from fumarate that would diminish the expected reduction in methane. Tools to track these specific bacterial types enable the monitoring of these key bacterial populations and lead to a better understanding of fumarate conversion to acetate and H_2. We have developed and optimised a phylogenetic microarray for the monitoring of bacterial populations. The diversity coverage of the array exceeds that of the bacteria identified in the enrichment libraries to date, thus ensuring complete monitoring of the bacterial populations in these enrichments. Initial experiments with DNA extracted from these cultures provided data that both the *E. coli* and *Clostridia* species were the most dominant groups. This data correlated strongly with the 16S DNA clone libraries (data not shown). Monensin treatment of the fumarate enrichments was found to suppress predominantly Gram-positive bacteria, with *Enterococcus, Lactobacillus and Streptococcus* species probes showing the greatest change in intensity (Figure 11). This data along with the VFA fermentation patterns for the *Enterococcus* species indicates that this species requires further investigation within the rumen to determine whether it is contributing negatively to the hydrogen balance when supplemented with fumarate. Further work is being carried out on the *Streptococcus* species to elucidate if they are also contributing negatively to the hydrogen balance. Preliminary data suggests that these species are not producing high levels of acetate from fumarate consumption.

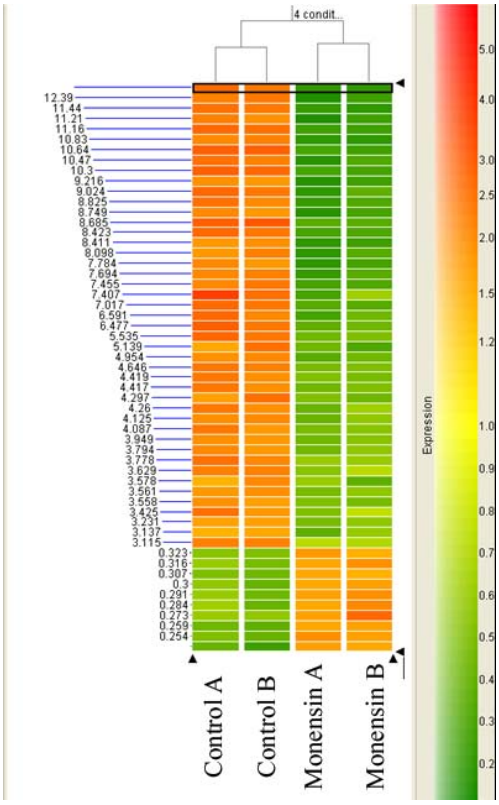


Figure 11. Genesprings clustering for 3 log differential display of replicate samples for control and monensin treated enrichments. Red colours indicated increased signal detection; green colours represent decreased signal detection.

5 Success in Achieving Objectives

5.1 Outcomes against objectives

5.1.1 Identify and characterize the microbiological and genetic attributes of the microbiomes possessing alternative routes of hydrogenotrophy and (or) methanotrophy in the rumen of grainfed and grassfed cattle Reductive acetogenesis

Reductive acetogenesis: Homoacetogens are present in the rumen and appear to cover a broad taxonomic group but they are poorly represented in culture collections and thus molecular tools are necessary to further investigate their ecology. However it is difficult to infer function to phylotypes when the acetogenic phenotype is presents in diverse taxonomic groups. Therefore in the current work we attempted to identify DNA sequences of genes present in homoacetogens that may be suitable as targets for the development of functional-group molecular tools for these microorganisms. The main functional genes in the reductive acetogenesis pathway of homoacetogens: formyltetrahydrofolate synthetase (FTHFS), carbon monoxide dehydrogenase (CODH) and acetyl-CoA synthase (ACS) were investigated. Putative FTHFS, CODH and ACS sequences were also generated from DNA of known homoacetogens from the rumen and other environments as reference genes. The majority of FTHFS sequences obtained from the batch fermentations grouped within the homoacetogenic cluster which is dominated by clostridial representatives. There was a degree of similarity between the pasture fed and grain fed enrichments particularly in relation to the ACS gene. Based on this information, an extensive set of primers have been developed from the numerous phylogenetic and functional gene databases so that populations of homoacetogic microorganisms in the rumen can be monitored. The ACS primer set has been further refined generating a primer set that is suitable for realtime PCR analysis that will enable the monitoring of acetogenic populations in the rumen in response to the inhibition of methanogenesis. Based on this information it is now possible to affiliate FTHFS-encoded rumen-bacteria to either homoacetogenic or non-homoacetogenic clusters.

Fumarate reduction: Fumarate has been used by several investigators to stimulate propionate producers in the rumen which compete with methanogens for hydrogen. We have shown in mixed ruminal cultures that fumarate is converted to propionate and acetate in varying proportions and thus the relative amounts of propionate and acetate formed from fumarate will impact on the hydrogen pool available to methanogens. Stoichiometrically, propionate production from fumarate consumes one pair of reducing equivalents while acetate production from fumarate releases two pairs of reducing equivalents. Therefore the production of acetate from fumarate is counterproductive when the objective is to reduce hydrogen available for methane production. It was important therefore to identify the microorganisms involved in these pathways and determine the conditions which favour propionate rather than acetate production from fumarate. We established enrichment cultures from the rumen which grew on an oligotrophic medium containing fumarate. In these experiments approximately 15-20% of the fumarate appeared to be converted to acetate while the remainder was converted to propionate. Based on 16s rDNA analysis the predominant populations in these cultures were *Selenomonas ruminantium* and *Prevotella ruminicola*, a group of bacteria affiliated with the Gram-positive Clostrida XIVa cluster, *Clostridium aminophilum* and *Lactobacillus* sp. We were able to isolate bacteria which were involved in the key steps in the pathway of fumarate fermentation to propionate. Bacteria which produced both succinate and acetate from fumarate were identified and it

was demonstrated that the Gram-positive inhibitor monensin could reduce the amount of acetate formed from fumarate.

5.1.2 Develop novel strategies, which can be tested experimentally in cattle, for the practical implementation of project outcomes to inhibit rumen methanogens, reduce rumen methane emissions and improve animal energetic efficiency.

The main outcome of this project has been the identification of the microorganisms involved in two hydrogen utilising pathways in the rumen that could compete with methanogens and theoretically reduce greenhouse gas emissions. This has enabled the development of molecular tools to monitor the responses in these populations particularly when methanogens are inhibited. In this regard a project will be funded under the MLA/DAFF Reducing Emissions from Livestock Research Program which will utilise the outcomes of this current project to determine the contribution of acetogenic and hydrogenotrophic bacteria in the digestive tract of ruminants to hydrogen sequestration when methane is inhibited and hydrogen accumulates in the rumen. The major objective of the new study is to determine the natural ability of the rumen to utilise hydrogen when methanogenesis is reduced and thus maintain normal rumen function. Specifically we aim to determine:
1. the degree of methane reduction and subsequent hydrogen accumulation that does not adversely affect rumen fermentation
2. the ability of the rumen to adapt and consume hydrogen when methanogenesis is reduced
3. the favourable changes in the microbial ecology involved in hydrogenotrophy in response to reduced methanogenesis and promote these under conditions of industry practice.

The path to commercialisation of technologies to limit methane production in ruminants will involve demonstration of the efficacy of antimethanogenic agents on methanogenesis in the rumen and the subsequent effects on rumen function.

6 Impact on Meat and Livestock Industry – now & in five years time

6.1 Impact on Meat and Livestock Industry

The immediate impact of this project on the meat and livestock industries will be the contribution it will make to the scientific tools and knowledge which will underpin projects recently announced in the $26.8 million MLA/DAFF Reducing Emissions from Livestock Research Program that will commence in 2009. Several of the projects in Methane Mitigation Program will employ the tools and information generated from the current project in their effort to devise practical strategies which reduce methanogenesis.

The Australian livestock sector which produces the greatest amount of enteric methane is the beef cattle industry. There are approximately 22 million beef cattle in Australia and about half of this population is found in northern Australia. Northern Australian production systems are typified by low rates of gain (< 1 kg/day), high turn-off ages (~ 3.5 years old), lack of grain for finishing, and an almost complete reliance on low quality forage based diets. It has been estimated that as little as a

5% increase in the efficiency of digestion could yield an economic benefit of at least A$100 million to the cattle industry. The excretion of methane from the rumen can represent a loss of 8-10% of the energy of the diet depending on the type of diet. A reduction in methanogenesis in the rumen can be associated with improvements in feed conversion efficiency without affecting intake. Therefore reducing methane production could benefit the ruminant energetically provided the efficiency of ruminal metabolism is not compromised.

This research from the current project is likely to have impact on both the intensive and extensive sectors of the ruminant livestock industries particularly if there are productivity benefits associated with a reduction in methane emissions. Manipulation of ruminal metabolism will be more amenable under intensive production conditions but it is also possible that pathways for hydrogen utilization are influenced by host-microbe interactions which are linked to genetic characteristics of the host animal such as feed conversion efficiency. These heritable traits could be selected for in breeding programs focussed on cattle under rangeland conditions such as tropical northern Australia.
In addition, this proposal will provide the scientific and industrial communities with a wide range of knowledge (structure of the hydrogenotrophic and methanotrophic ruminal population, general ecology of the rumen), that will help in the future to design new ecological molecules or probiotics to limit methane production and improve microbial fermentation by ruminants.

7 Conclusions and Recommendations

7.1 Conclusions

- Metagenomic analysis of hydrogenotrophic fermentations from the rumen has led to the discovery of novel microorganisms and gene sequences involved in methanogenesis, the reductive acetogenesis pathway, and fumarate reduction.
- This new information has enabled the development of a suite of molecular tools to monitor and quantify methanogenic and hydrogenotrophic populations in the rumen in response to dietary manipulations and methanogen inhibitors.
- Homoacetogenic bacteria have been isolated which could be used in a probiotic strategy to promote reductive acetogenesis
- Analysis and manipulations of *in-vitro* rumen fermentations promoting fumarate reduction to propionate showed that the inhibition of Gram-positive bacteria in these fermentations leads to a reduction in the unfavourable production of acetate from fumarate which would not result in H_2 utilisation.
- Feeding cattle diets enriched in fumarate and monensin may be a practical strategy to consume H_2 and reduce methane production

7.2 Recommendations

- The molecular tools and information generated from this project should now be applied in controlled animal studies to determine the natural ability of the rumen to utilise hydrogen when methanogenesis is reduced and thus maintain normal rumen function. This will provide a target for the level of methane reduction which is feasible and desirable in practical strategies that will lead to significant reductions in greenhouse gas emissions without adversely affecting productivity.

- Feeding of organic acids such as malate or fumarate should be considered as a strategy to reduce methane production provided it is done in conjunction with a rumen modifier which prevents the formation of acetate from these acids. This strategy will be dependant upon the availability of succinate precursors which are inexpensive and readily available. Condensed distillers by-products may provide a source of these organic acids for feeding to ruminants.

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9 Appendices

9.1 Publications

Refereed journal publications

Denman, S.E., Tomkins, N. and McSweeney, C.S. (2007). Quantitation and diversity analysis of ruminal methanogenic populations in response to the anti-methanogenic compound bromochloromethane. FEMS Microbial Ecology 62, 313-322.

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