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Methanotrophs from natural ecosystems as biocontrol agents for ruminant methane emissions

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1 Abstract

In ruminant cattle, the anaerobic fermentation of ingested plant biomass results in the production of methane (CH_4). This CH_4 is subsequently eructated to the environment, where it acts as a potent greenhouse gas and is one of the leading sources of anthropogenic CH_4 in Australia. Methane oxidising microorganisms are an important environmental sink for CH_4; however the possibility that methanotrophs are native to the rumen has received little attention. This project aimed to characterise methanotrophs from a range of environments, and to subsequently determine the metabolic activity of these microorganisms under *in vitro* rumen-like conditions. This study is the first to characterise rumen methanotrophs using molecular methodology. Using a combination of denaturing gradient gel electrophoresis and phylogenetic analysis, it was found that simple communities of Proteobacterial methanotrophs can be native residents of the rumen microbial community in grain-fed *Bos indicus* steers. A putative methanotrophic Gamma-proteobacterial *Methylobacter* species was also enriched from grain-fed whole rumen contents using novel techniques. However, the activity of these organisms *in situ* remains to be fully understood. Furthermore, the possibility that a grain-based dietary affect influences the diversity and activity of methanotrophs *in situ* is intriguing. Future work to address these questions is necessary to evaluate the potential for methanotrophs to act as biocontrol agents for ruminant CH_4 emissions.

2 Executive Summary

Methane (CH_4) is a potent greenhouse gas with a global warming potential approximately twenty-five times greater than carbon dioxide (CO_2) over a one hundred year time span. Agriculture represents a dominant source of global anthropogenic CH_4 emissions with enteric fermentation and eructation of CH_4 by ruminant livestock contributing between 20 – 25% of annual global anthropogenic CH_4 emissions. On a national scale, cattle and sheep produce 53% of Australia's annual anthropogenic CH_4 emissions. In many anaerobic environments, such as the rumen, methanogenic archaea act as the terminal step of biodegradation, by converting metabolic waste end products CO_2 and hydrogen gas (H_2) to CH_4. This CH_4 is subsequently eructated to the environment, where it acts as an environmental pollutant.

Methane oxidising microorganisms, or methanotrophs, play a vital role in the environment by recycling carbon and by regulating the concentration of CH_4 in the atmosphere. Because of this, methanotrophs represent a novel approach to the biocontrol of ruminant CH_4 emissions. The biodiversity and activity of methanotrophs in a variety of environments has been well documented in the past, mostly in regards to soils and fresh water/marine environments. The possibility that methanotrophs are actively oxidising CH_4 in the rumen has received very little attention from the international scientific community. Two examples of the isolation of methanotrophs from cattle do exist (Stocks & McCleskey, 1964, Whittenbury*, et al.*, 1970). However, no work was conducted to establish whether these isolates were in fact active, native residents of the rumen microbial community, or were simply transient microorganisms ingested with feed material. Furthermore, as isolation procedures typically understate the biodiversity of microbial communities *in situ*, these studies may not accurately reflect methanotroph presence in the rumen. It is unknown whether these isolates are metabolically active in the rumen. The concept that CH_4 eructated from cattle is actually a result of active methanogenesis vs. methanotrophy *in situ* is highly intriguing.

This project sought to characterise methanotrophs from a range of environments, and to subsequently determine the metabolic activity of methanotrophs under *in vitro* rumen-like conditions. In particular, it sought to determine if active methanotrophic communities are native to the rumen environment.

Specifically, the aims of this project were:

1) To characterise the biodiversity of methanotrophic organisms from a range of Australian environments, including landfill soils, piggery effluent, wastewater, and the gastrointestinal tract of herbivore hosts using a range of molecular techniques.
2) To assess the capacity for methanotrophs to oxidise CH_4 emissions in an *in vitro* rumen-like fermentation environment using gas chromatography (GC) analysis.
3) To confirm methane oxidation under *in vitro* rumen-like conditions using a combination of techniques to assess the activity, abundance, distribution and morphology of methanotrophs, for example Quantitative PCR, Stable Isotope Probing (SIP) and Fluorescent *In Situ* Hybridisation (FISH).

PCR screening for methanotroph 16S rRNA and functional genes indicated that Proteobacterial methanotrophs were present in a number of environments, including

landfill soils, piggery effluent, wastewater, and on the rumen epithelium and in the whole rumen contents of grain-fed *Bos indicus* cattle. Denaturing gradient gel electrophoresis of partial 16S rRNA genes suggested that simple communities of methanotrophs were present in these environments. Of particular interest, phylogenetic analysis of partial 16S rRNA genes identified a Gamma-proteobacterial *Methylobacter* species and an Alpha-proteobacterial *Methylocystis* species present on the rumen epithelium and in the rumen contents of four grain-fed *Bos indicus* cattle. Phylogenetic analysis of functional genes supported a dominance of *Methylocystis* species. Quantification of *Methylocystis* cells using a real time PCR based on functional genes suggested that, in a total of four animals, an average number of 4.38×10^4 *Methylocystis* cells mL^{-1} were present in whole rumen contents. While low, this cell number is high enough to strongly suggest that *Methylocystis* species are native, and not simply transient organisms ingested with feed material. No methanotrophs were detected using these molecular assays in grass-fed cattle (a total of 12 animals), or in the kangaroo foregut (a total of 16 animals).

Using a modified Nitrate Mineral Salts medium containing 33% sterile rumen fluid (RF-NMS) and 40 μM mL^{-1} CH_4 supplemented to the gas headspace, a *Methylobacter* species was aerobically enriched from grain-fed cattle rumen contents. Currently, an *in vitro* rumen-like fermentation vessel is being designed to accurately mimic the potential for active CH_4 oxidation by methanotrophs to occur *in situ*. This activity will be established using a combination of SIP, GC and FISH methodologies.

This work indicates that under specific conditions, simple communities of methanotrophs are present as native residents of the rumen microbial community. This work also represents the first molecular characterisation of ruminant methanotrophs. The enrichment methods used confirm the earlier work that suggested that methanotrophs are present in the rumen. However, the activity of these organisms *in situ* remains to be fully understood. Furthermore, the possibility that a grain-based diet influences the diversity and activity of methanotrophs *in situ* is intriguing. A better understanding of the microbial ecology of methanotrophs in the rumen holds great potential in lowering CH_4 emissions from Australian livestock. Future work to address these questions is necessary to evaluate the potential for methanotrophs to act as bio-remediation agents for ruminant CH_4 emissions.

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4 Background

Methane (CH_4) is a potent greenhouse gas with a global warming potential approximately twenty-five times greater than carbon dioxide (CO_2) over a one hundred year time span (Forster*, et al.*, 2007). Agriculture represents a dominant source of global anthropogenic CH_4 emissions (Smith*, et al.*, 2008) with enteric fermentation and eructation of CH_4 by ruminant livestock contributing between 20 – 25% of annual global anthropogenic CH_4 emissions (Thorpe, 2009). On a national scale, ruminant livestock produce 54.7 Mt CO_2 equivalent emissions, or roughly 53% of Australia's annual anthropogenic CH_4 emissions (NGGIC, 2009). Ruminant livestock consist of domesticated mammals of the family *Bovidae* (for example cattle, buffalo, sheep and goats) with cattle acting as the foremost global producers of CH_4 emissions (Johnson & Ward, 1996).

Methanogenic archaea are the terminal step of biodegradation in many anaerobic environments, such as the rumen (Hungate, 1966, Holland*, et al.*, 1987, Madigan & Martinko, 2006). The anaerobic fermentation of ingested plant biomass results in the production of waste by-products CO_2 and hydrogen gas (H_2) (Ellis*, et al.*, 2008). Methanogens are the dominant H_2 sink in the rumen, where they reduce CO_2 with H_2 to produce CH_4 (Nelson*, et al.*, 2003, Nicholson*, et al.*, 2007, Wright*, et al.*, 2008). CH_4 is subsequently eructated into the environment.

Aerobic CH_4 oxidising bacteria (MOB) play a fundamental role in the carbon cycle. MOB are found in many different terrestrial and marine environments, and are metabolically active at the oxic/anoxic interface where diffusion of dioxygen (O_2) and CH_4 meet (Horz*, et al.*, 2001, Eller*, et al.*, 2005, Urmann*, et al.*, 2009). MOB use O_2 as an electron acceptor for CH_4, and can utilise CH_4 as a sole carbon and energy source (Whittenbury*, et al.*, 1970). The oxidation of CH_4 is carried out by the following enzymes: a membrane-bound, copper dependent particulate methane monooxygenase (pMMO) (Semrau*, et al.*, 1995, Lieberman & Rosenzweig, 2004) and a cytoplasmic, copper independent soluble methane monooxygenase (sMMO) (Murrell*, et al.*, 2000).

MOB are separated into several major groups based on 16S rRNA gene phylogeny, phospholipid fatty acid analysis, morphological characteristics and biochemical pathways of carbon assimilation (Hanson & Hanson, 1996, Semrau*, et al.*, 2010). The Methylococcales are Gamma-proteobacteria, and the following genera have been demonstrated to be methanotrophs: *Methylobacter*, *Methylomicrobium*, *Methylomonas*, *Methylocaldum*, *Methylosphaera*, *Methylothermus*, *Methylosarcina*, *Methylohalobius*, *Methylosoma, Clonothrix* and *Crenothrix*. These organisms are obligate methanotrophs, with the sole exception being *Crenothrix polyspora* which can utilise acetate and glucose (Stoecker*, et al.*, 2006). For the purposes of this study, Gamma-proteobacterial MOB shall be referred to as Type I MOB. Specific Alpha-proteobacteria have also been shown to oxidise CH_4. The following genera from the Methylocystaceae and Beijerinckiaceae are known methanotrophs: *Methylocystis*, *Methylosinus, Methylocella* and *Methylocapsa*. Isolates from the *Methylocystis*, *Methylocella* and *Methylocapsa* genera have been identified as facultative methanotrophs capable of growth on multi-carbon substrates, such as ethanol, acetate, pyruvate, succinate and malate (Theisen*, et al.*, 2005, Dunfield*, et al.*, 2010, Im*, et al.*, 2011). Alpha-proteobacterial MOB shall be referred to as Type II MOB. Finally, a novel genus of thermoacidophilic methanotrophs from the Phylum Verrucomicrobia, *Methyloacidiphilum*, have also been identified (Dunfield*, et al.*, 2007, Pol*, et al.*, 2007, Islam*, et al.*, 2008, Op den Camp*, et al.*, 2009). With the singular exception of the Type II genus *Methylocella*, pMMO is expressed by all

characterised MOB (Semrau*, et al.*, 2010). Phylogenetic comparisons of the *pmoA* gene, which encodes a 27-kDa subunit of pMMO, suggest that pMMO in Proteobacteria and Verrucomicrobia have diverged from a common ancestor, as opposed to being shared via horizontal gene transfer (Op den Camp*, et al.*, 2009). Conversely, the distribution of sMMO is sporadic, and has only been identified from isolates of the following genera: *Methylococcus*, *Methylomonas*, *Methylomicrobium*, *Methylocystis* and *Methylosinus* (Murrell*, et al.*, 2000).

Anaerobic CH_4 oxidising bacteria of the NC10 division have recently been identified as a CH_4 sink in freshwater and wastewater ecosystems (Ettwig*, et al.*, 2009, Hu*, et al.*, 2009, Zhu*, et al.*, 2010). Extensive phenotypic and genotypic work with enrichments dominated by *Candidatus* Methylomirabilis oxyfera demonstrate that this organism is capable of coupling denitrification of nitrite to the oxidation of CH_4 in the absence of O_2 (Ettwig*, et al.*, 2010). Interestingly, this process is facilitated by a novel pMMO enzyme specific to *Ca*. M. oxyfera (Ettwig*, et al.*, 2010).

Anaerobic methanotrophic archaea (ANME) are associated with CH_4 oxidation in marine sediments, primarily at the sulphate/CH_4 transition zone (Valentine, 2002, Stams & Plugge, 2009). It is thought that ANME facilitate the reversal of methanogenesis, using a novel methyl coenzyme M reductase (MCR), which is necessary for the anaerobic oxidation of CH_4 (Meyerdierks*, et al.*, 2010, Scheller*, et al.*, 2010, Lloyd*, et al.*, 2011). ANME often form symbiotic relationships with sulphate reducing bacteria, however it is not certain whether this interaction is essential for the anaerobic oxidation of CH_4. Due to the difficulty of studying this group under *in vitro* conditions (with estimated cell doubling times of more than three months (Orphan*, et al.*, 2009)) there are still many questions in regards to the metabolism and ecology of ANME.

Although the rumen is generally considered to be anoxic, O_2 concentrations of up to 0.3 μM L^{-1} and 6.7 μM L^{-1} have been measured in the cattle rumen fluid and gas, respectively (Scott*, et al.*, 1983). The rumen epithelium is also highly oxygenated, with blood flow to the rumen epithelial tissue rising to 3.1 mL min^{-1} $gram^{-1}$ post-feeding (Dobson, 1984). MOB have been enriched from the mouth of cattle (Whittenbury*, et al.*, 1970) and the rumen of fistulated cattle previously (Stocks & McCleskey, 1964). However, it is unclear whether these isolates represented native populations, or if they were simply transient organisms ingested with feed material. Previous attempts to screen the rumen for methanotrophs using molecular methods have suggested that methanotrophs are not present (Mitsumori*, et al.*, 2002). *In vivo* studies using MOB feed supplements to sheep have generated intriguing results, with decreases in CH_4 emissions of up to 1.73 L day^{-1}, showing that MOB can indeed be active under O_2 limiting conditions in the rumen (Moss, 2008).

Due to these previous studies, and considering that conditions vary greatly between environments where methanotrophs have been characterised (i.e. soils, fresh and marine sediments) and the rumen in regards to temperature, pH, O_2 availability and gastrointestinal flow, this study focused on confirming the presence of natural communities of methanotrophs in the rumen environment. Greater knowledge of any natural CH_4 sinks in the rumen would not only improve our understanding of rumen microbiology, but ruminant methanotrophs are more likely to be metabolically active *in situ*, and therefore more likely to act as potential CH_4 biocontrol agents. This study outlines the first molecular characterisation of native ruminant methanotrophs, in addition to the enrichment of a putative methanotrophic Gamma-proteobacterial *Methylobacter* species.

5 Project Objectives

The purpose of the project was to evaluate the potential for methanotrophs to act as bio-remediation agents in lowering ruminant CH_4 emissions. This was to be done by characterising methanotrophs from the environment, and then determining their activity under *in vitro* rumen-like conditions. During the course of this study, it became apparent that it was not currently known if active methanotrophs exist naturally within the rumen. This project focused on confirming the presence of ruminant methanotrophs, characterising the biodiversity of these microorganisms, and determining their activity under *in vitro* rumen-like conditions.

The major milestones for this project were:

1) By 1st July 2010 complete a literature review on methanotrophs and their effect on methane reduction and rumen microbial population dynamics including a review of currently active research on both aerobic and anaerobic methane oxidation in natural ecosystems.
2) By 1st June 2012 isolate and characterise methanotrophs from a range of natural ecosystems that can be demonstrated to have potential to reduce methanogenesis in rumen-like environments.

6 Methods

6.1 Bacterial strains and culture conditions

The bacterial strains used in this study are outlined in Appendix 1. Positive controls for MOB were obtained from the American Type Culture Collection (ATCC). MOB were grown in nitrate mineral salts (NMS) medium (ATCC medium: 1306). NMS was amended to include copper (II) sulfate pentahydrate ($CuSO_4.5\ H_2O$, Alfa Aesar, Thomastown, Australia) to a final concentration of 10 µM. MOB were grown aerobically in 20 mL Hungate tubes fastened with butyl-rubber stoppers, with a medium: gas headspace ratio of 1:1. A final concentration of 60 µM mL^{-1} CH_4 (ultra high purity >99%, BOC Gas, Australia) was injected in to the gas headspace. Cultures were grown under shaking conditions (200 rpm) in an NB-205 Orbital Shaking Incubator (Progen Scientific, London, UK). The Type I MOB *Methylomonas methanica* ATCC 51626 was grown at 30ºC for 2 – 4 weeks. The Type II MOB *Methylocystis rosea* ATCC BAA-1196 was grown at 26ºC for 7 – 10 days. An enrichment containing *Ca.* M. oxyfera was obtained for use as a positive control for NC10 anaerobic methanotrophs from the Advanced Wastewater Manaegment Centre, University of Queensland, St Lucia (Hu*, et al.*, 2011). For cloning purposes, chemically competent TOP10 *Escherichia coli* (Invitrogen, CA, USA) transformants were grown in LB supplemented with 50 µg mL^{-1} ampicillin where necessary.

6.2 Environmental sampling and genomic DNA extraction

A wide range of environments were chosen for sampling. Soil samples from Nudgee landfill in Brisbane, Australia, were collected. Fifteen grams of soil, roughly 0 – 10 cm and 20 – 30 cm in depth, was extracted using a coring device and soil was resuspended in sterile phosphate buffered saline pH 7.4 (PBS; 0.14 M NaCl, 2.7 mM KCl, 1.4 mM $Na_2HPO_4.7H_2O$, 1.8 mM KH_2PO_4) and samples taken for genomic DNA extraction. For culturing purposes, whole soil slurry was frozen at -80ºC in 10% methanol, anoxic 50% rumen fluid/glycerol and 50% glycerol to preserve bacterial cells. Water samples from Grantham Piggery effluent pond and the Elanora Wastewater Management Centre were also taken. Two hundred millilitres of water was centrifuged at 14, 000 g for 30 minutes; the supernatant was discarded and pelleted cells resuspended in sterile PBS. As mentioned above, samples were taken for genomic extraction and environmental samples stored at -80ºC in 10% methanol, anoxic 50% rumen fluid/glycerol and 50% glycerol. Samples were taken from the gastrointestinal tract of cattle. Whole rumen contents and faecal grab samples were taken from Victorian DPI *Bos taurus* dairy cattle fed a diet of concentrates and tannins. These samples were frozen and stored at – 20°C. A collection of four adult Greyman steers (*Bos indicus* cross) fed a mixed diet of grain and grass, were sampled at Highchester Abattoir, Gleneagle, Australia. Tissue swabs were taken from the nasal passages, rumen epithelium and intestinal epithelium using Amies medium Transwabs (MW&E, Corsham, England). Transwabs were stored at - 20°C. Liquid fractions of whole rumen contents were also taken and stored at - 20°C. Four adult Brahman steers (*Bos indicus*) fed a diet of barley-based concentrate at the Centre for Advanced Animal Science (CAAS) at UQ, Gatton, were slaughtered at a commercial abattoir and rumen contents were sampled, filtered through a nylon stocking, and rumen fluid was stored at – 20°C. In addition, whole foregut samples from Eastern Grey kangaroos, Red kangaroos and Wallaroos, part of a previous study (Klieve, 2009) were sampled. These samples were also kept at – 20°C.

Prior to genomic extraction, environmental samples were resuspended in 1 mL PBS and pelleted by centrifugation at 17, 000 x g for 10 minutes. DNA was extracted using a modified version of the QIAamp Stool Kit (Qiagen, Valencia, CA, USA) that incorporates an initial 10 minute bead beating step using 0.5 g of 0.1 mm sterile glass beads (Morgan*, et al.*, 2009).

6.3 Type I and II MOB 16S rRNA gene primer design

16S rRNA gene primer design was performed in the DNA sequence alignment tool ARB (Ludwig*, et al.*, 2004) using the Greengenes database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) (DeSantis*, et al.*, 2006). Nine Type I MOB strains from the Order *Methylococcales* were aligned against the *Escherichia coli* 16S rRNA gene as a reference. The following strains were aligned: *Methylocaldum szegediense* strain OR2 (Genbank Accession number U89300), *Methylosphaera hansonii* strain AM6 (U67929), *Methylococcus capsulatus* (X72771), *Methylomonas methanica* strain S1 (NR_041815), *Methylomonas rubra* (M95662), *Methylobacter luteus* strain ACM 3304 (X72772), *Methylomicrobium album* strain BG8 (NR_029244), *Methylosarcina fibrata* strain AML-C10 (AF177296) and *Methylobacter alcaliphilum* strain 20Z (EF495157). Six Type II MOB strains from the Families *Methylocystaceae* and *Beijerinckiaceae* were also aligned against the *E. coli* 16S rRNA reference gene. These were: *Methylocystis hirsute* strain CSC1 (NR_043754), *Methylocystis parvus* strain 57 (AJ458508), *Methylosinus trichosporium* strain OB3b (NR_044947), *Methylosinus sporium* strain SE2 (AJ458478), *Methylocella palustris* strain ch3 (AJ563926) and *Methylocella tundrae* strain ch1 (AJ563929). Once aligned, conserved regions of the 16S rRNA gene were used to design degenerate primers. *In silico* analysis to confirm primer specificity was carried out using the Ribosomal Database Project version 10 (RDP v 10) (http://rdp.cme.msu.edu/) (Cole*, et al.*, 2009). The primers designed for *Methylococcales* MOB, TImob117 and TImob1144, target the 16S rRNA gene variable regions 1 – 7 and produce a product of approximately 1.03 kb in size. The primers designed for *Methylocystaceae/Beijerinckaceae* MOB, TIImob445 and TIImob1416, target the 16S rRNA gene variable regions 3 – 8 and produce a product approximately 970 bp in size (Appendix 2).

6.4 Polymerase chain reaction

All primer oligonucleotides used in this study are outlined in Appendix 2. PCRs were run on a Bio-rad S1000 Thermocycler (Bio-rad, Gladesville, Australia). Fifty micro-litre reactions were performed using premixed MyTaq Red DNA Polymerase according to the manufacturer's instructions (Bioline, Alexandria, Australia). Final concentrations of primers were 0.2 µM. Final concentration of DNA template was 10 – 20 ng. A nested PCR approach was adopted to target the 16S rRNA gene of Type I and II MOB separately. Initially, the primers TImob117/TImob1144 and TIImob445/TIImob1416 were used as follows: 1 cycle of 95°C denaturation for 3 mins, 35 cycles of 95°C denaturation for 30 sec, 57°C primer annealing for 1 min, 72°C extension for 30 sec, 1 cycle of 72°C extension for 3 mins. This PCR product

was used as a template for a second round of PCR using 341F_GC/Type I R for Type I MOB, and 518F_GC/Type II R for Type II MOB (Chen*, et al.*, 2007). The second stage of PCR was carried out as above. Screening for *pmoA* was performed as described previously (Costello & Lidstrom, 1999). Two bases of the forward *pmoA* primer, A189, were altered to reduce non-specific amplification (refer to Appendix 2). Additionally, screening for *mmoX*, a functional genetic marker for sMMO, was carried out as previously reported (Iwamoto*, et al.*, 2000). The 16S rRNA gene of anaerobic methanotrophic NC10 bacteria were screened as described previously (Ettwig*, et al.*, 2009). Screening for the specific *pmoA* of *Ca*. M oxyfera was performed as described previously (Luesken*, et al.*, 2011). One percent agarose gels (Bio-rad, Gladesville, Australia) were supplemented with 3 x Gelred (Biotium, Hayward, CA, USA) and run in Tris-acetate-EDTA buffer (TAE; 0.04 M Tris base, 0.07 M acetic acid, 1 mM EDTA) to visualise DNA.

6.5 Denaturing gradient gel electrophoresis

Prior to denaturing gradient gel electrophoresis (DGGE), PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and standardised to 50 ng μL^{-1} using a Nanodrop 8000 (Thermo Scientific, Rockford, IL, USA). DGGE was performed as described previously (Muyzer*, et al.*, 1993). Fifty nanograms of DNA per sample were run on 8% (w/v) polyacrylamide gels with a denaturing gradient of 20% - 55%, where 100% corresponded to 7 M urea and 40% formamide solution (Bio-rad, Gladesville, Australia and Roche, Castle Hill, Australia respectively). DGGE was performed on a Bio-rad DCode Universal Detection Mutation System (Bio-rad, Hercules, CA, USA). Gels were run at 100 V for 18 hours at 60°C in 1 x TAE. Gels were subsequently silver stained for DNA visualisation (Kocherginskaya, 2005).

6.6 Clone library construction, sequencing and phylogenetic analysis

The TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA) was used to transform purified PCR products into chemically competent TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DGGE was performed on Type I and II MOB 16S rRNA gene clones to screen for clones with variable inserts. Cloned inserts were compared to the original bands present in environmental samples based on having the same mobility evident using DGGE. Restriction fragment length polymorphism (RFLP) was used to select *pmoA* clones with variable inserts, using a combination of *Msp*I and *Hae*III, as described previously (Costello & Lidstrom, 1999). A total of three clones per band/pattern were sequenced to confirm the identity of each band/pattern present in DGGE and RFLP for quality control purposes (Klieve*, et al.*, 2007). Selected clones were grown overnight at 37°C in 5 mL LB broth supplemented with 50 μg mL^{-1} ampicillin and plasmid DNA was extracted using a QIAquick miniprep kit (Qiagen, Valencia, CA, USA). Purified DNA was prepared for sequencing by the Australian Genome Research Facility (AGRF, Brisbane, Australia) as per their instructions. Operational taxonomic units (OTUs) were viewed using the DNA analysis tool Sequencher version 4.10.1 (Gene Codes, MI, USA). Reference sequences were obtained from online databases (Greengenes, RDP v 10, National Centre for Biotechnology Information, NCBI)

(http://www.ncbi.nlm.nih.gov/) and aligned in Sequencher v 4.10.1. Prior to phylogenetic analysis, an optimal nucleotide substitution model was calculated using the program jModelTest (Guindon & Gascuel, 2003, Posada, 2008). Maximum likelihood analysis was carried out using Phylogenetic Analysis Using Parsimony (PAUP) version 4.0 (Sinauer Associates Inc, Sunderland, MA, USA). One thousand bootstrap replications were conducted to ensure the tree topology was constructed accurately.

6.7 Quantitative PCR for *Methylocystis* species

Primers and dual-labelled fluorescent probes were designed specific to the *pmoA* OTUs sequenced in this study, using OligoArchitect (Sigma-Aldrich, Sydney, Australia; sigma.com/designmyprobe). *In silico* analysis to confirm primer and probe specificity was carried out using the NCBI Basic Local Alignment Search Tool (BLAST) (Altschul*, et al.*, 1990). Appendix 2 outlines the *pmoA* primers and probes used for *Methylocystis* sp. *pmoA* qPCR in this study. Quantitative PCRs were run on a Rotor-Gene RG-6000 (Qiagen, Valencia, CA, USA). Twenty-five micro-litre reactions were performed using premixed Real MasterMix Probe – ROX in triplicate (Eppendorf, Hamburg, Germany). Primer and probe concentrations used were 0.2 µM and 0.1 µM, respectively. The pCR4 TOPO plasmid containing the *pmoA* insert was used as a standard for the qPCR. Genomic DNA extracted from cattle faeces was used as an environmental negative control in addition to a no-template control. A two-step qPCR protocol was used as follows: 1 cycle of 95°C denaturation for 1 min, followed by 40 cycles of 95°C denaturation for 10 sec and 60°C annealing/extension for 30 sec. Copies mL^{-1} were calculated based on comparisons of cycle thresholds with standard curves, and translated to cells mL^{-1}, as most known MOB have two *pmoA* copies $cell^{-1}$ (Gilbert*, et al.*, 2000). Cloning and sequencing of qPCR *pmoA* amplicons was carried out to ensure specificity of primers/probes. Analysis of Variance (ANOVA) of cells mL^{-1} was carried out in Genstat 11^{th} edition (VSN International, Hempstead, UK).

6.8 Enrichment methods

Isolation procedures were carried out as described previously (Whittenbury*, et al.*, 1970). Briefly, 0.5 g or mL of environmental sample was used to inoculate 10 mL sterile aerobic NMS in 20 mL gas-tight Hungate tubes, with a final concentration of 60 µM mL^{-1} CH_4 supplemented to the gas headspace. Environmental samples were incubated at 28°C and 39°C, shaking, for two weeks. Enrichments were degassed every 72 hours, with CH_4 re-supplemented to the gas headspace as above. After 2 weeks, enrichments were serially diluted to fresh NMS up to 10^{-3} and the above procedures were repeated. After a further two weeks, enrichments were streaked to NMS agar (1.5%) and a universal bacterial 16S rRNA gene PCR was performed on single colonies to confirm the identity of isolated microorganisms as previously described (Weisburg*, et al.*, 1991).

A novel medium was designed in this study for the enrichment of rumen methanotrophs. This was done with an NMS based medium supplemented with 33% sterile rumen fluid (RF-NMS) at pH 6.8, under aerobic conditions. Enrichments were performed in 250 mL gas-tight serum bottles. Ten percent whole rumen contents was used to inoculate sterile RF-NMS, with a final concentration of 40 µM mL^{-1} CH_4

injected into the gas headspace of serum bottles. Enrichments were kept at 39°C, shaking, for eight weeks in a Ratek Orbital Mixer Incubator (Ratek, Victoria, Australia). Enrichments were degassed weekly, with CH_4 resupplemented to the gas headspace as above. Weekly, gDNA was extracted from 5 mL enrichments as above, and was screened for *pmoA* using aforementioned PCR procedures. Putative *pmoA* OTUs were cloned, sequenced and analyzed as above. Methanotrophs were cryopreserved at – 80°C with 10% methanol.

7 Results

7.1 Screening for methanotroph molecular markers

Table 1 outlines the results of PCR screening for MOB and *Ca*. M. oxyfera in this study. A combination of 16S rRNA and functional gene based PCRs were chosen to screen for both aerobic and anaerobic methanotrophs. MOB were detected in the Nudgee Landfill soil, Grantham Piggery effluent and Elanora wastewater. Of interest, MOB were also detected on the rumen epithelium and in the rumen contents of cattle sampled from the Highchester Abattoir, in addition to the rumen contents of CAAS cattle. MOB appeared to be either absent or below detectable limits in Victorian DPI and Tullimba cattle rumen contents, and kangaroo foregut contents. Anaerobic methanotrophic *Ca*. M. oxyfera 16S rRNA and functional genes were not detected by PCR in the environments sampled in this study.

Table 1: Detection of methanotroph molecular markers.

	Type I MOB 16S	Type II MOB 16S	*pmoA*	*mmoX*	*Ca*. M. oxyfera 16S	*Ca*. M. oxyfera *pmoA*
Nudgee Landfill soil	+	+	+	+	-	-
Grantham Piggery effluent	+	+	+	-	-	-
Elanora Wastewater Treatment Center	+	+	+	+	-	-
Vic DPI cattle rumen contents	-	-	-	-	-	-
Vic DPI cattle faeces	-	-	-	-	-	-
Highchester Abattoir cattle nasal mucosa	-	-	-	-	-	-
Highchester Abattoir cattle rumen epithelium	+	+	+	-	-	-
Highchester Abattoir cattle rumen contents	+	+	+	-	-	-
Highchester Abattoir cattle intestinal epithelium	-	-	-	-	-	-
CAAS cattle rumen contents	+	+	+	-	-	-
Tullimba cattle rumen contents	-	-	-	-	-	-
Kangaroo foregut contents	-	-	-	-	-	-

7.2 Molecular analysis of environmental samples

Initially, the landfill soil, wastewater and piggery effluent samples were used to optimise molecular methods for methanotroph screening. Although *in silico* analysis suggested that the Type I and II MOB primers designed in this study were specific to MOB, it was necessary to confirm this *in situ*. DGGE and phylogenetic analysis of the Nudgee landfill soil samples indicated that the Type I and II MOB primers were specific to MOB in well defined environments. Figure 1 shows DGGE images comparing the diversity of Type I and II MOB from environmental samples, based on partial 16S rRNA genes. Type I MOB communities appeared to display greater biodiversity than Type II MOB in all samples. Sequencing was performed on OTUs present in the landfill soil samples from site b at 10 cm depth (lane 6) and 30 cm depth (lane 8). All DNA bands sequenced clustered with known Type I and II MOB (Figure 2 and Figure 3).

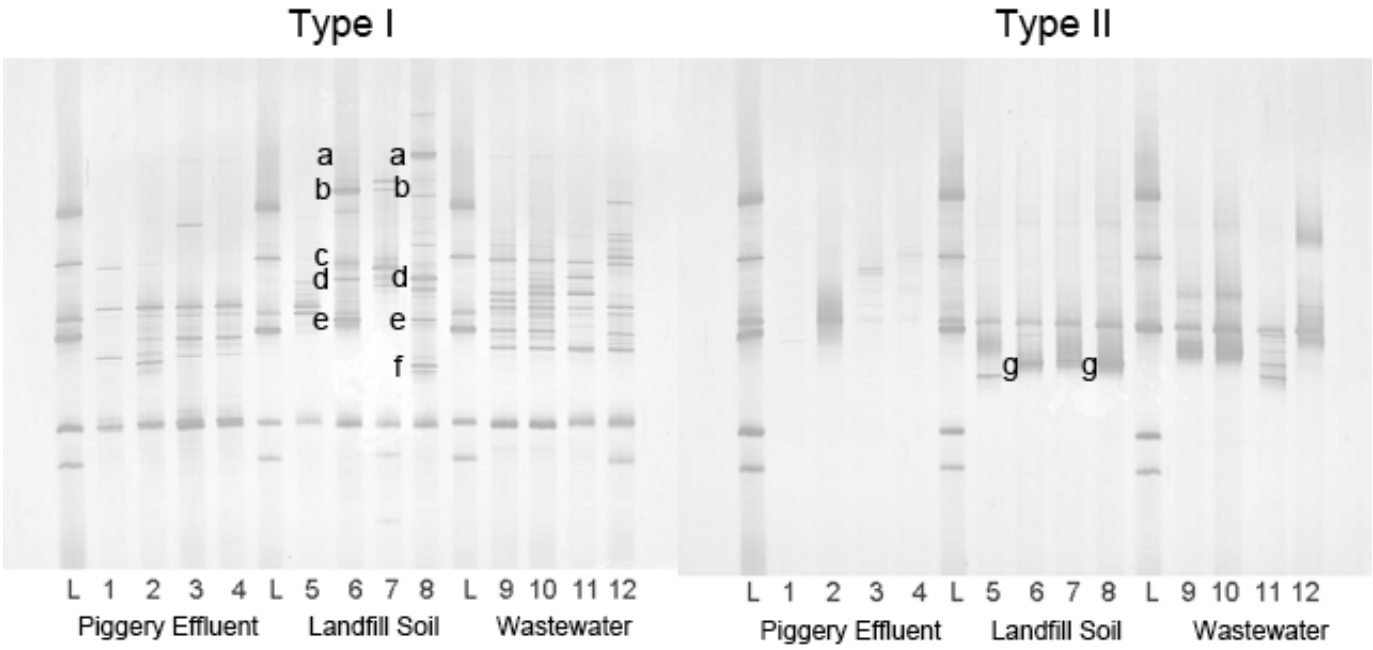

Figure 1: Denaturing gradient gel electrophoresis of piggery effluent, landfill soil and wastewater samples, targeting the variable region 3 of the 16S rRNA gene of Type I and II MOB. L corresponds to a DNA ladder marker. Samples 1 and 2 are planktonic samples from piggery effluent; 3 and 4 are sediment samples from piggery effluent ponds. Samples 5 and 7 are landfill soil from site a, at depths 10 cm and 30 cm, respectively. Samples 6 and 8 are landfill soil from site b at depths 10 cm and 30 cm, respectively. Samples 9 and 10 are aerobically treated wastewater; samples 11 and 12 are anaerobically treated wastewater. Uncultured Type I MOB of the Gamma-proteobacteria (band a) and Methylococcaceae (band b) and *Methylobacter* (band d) were identified. Cultured representatives of the *Methylosoma* (band c) and *Methylocaldum* were also present (bands e and f). Type II MOB of the *Methylosinus* genus were also present (band g).

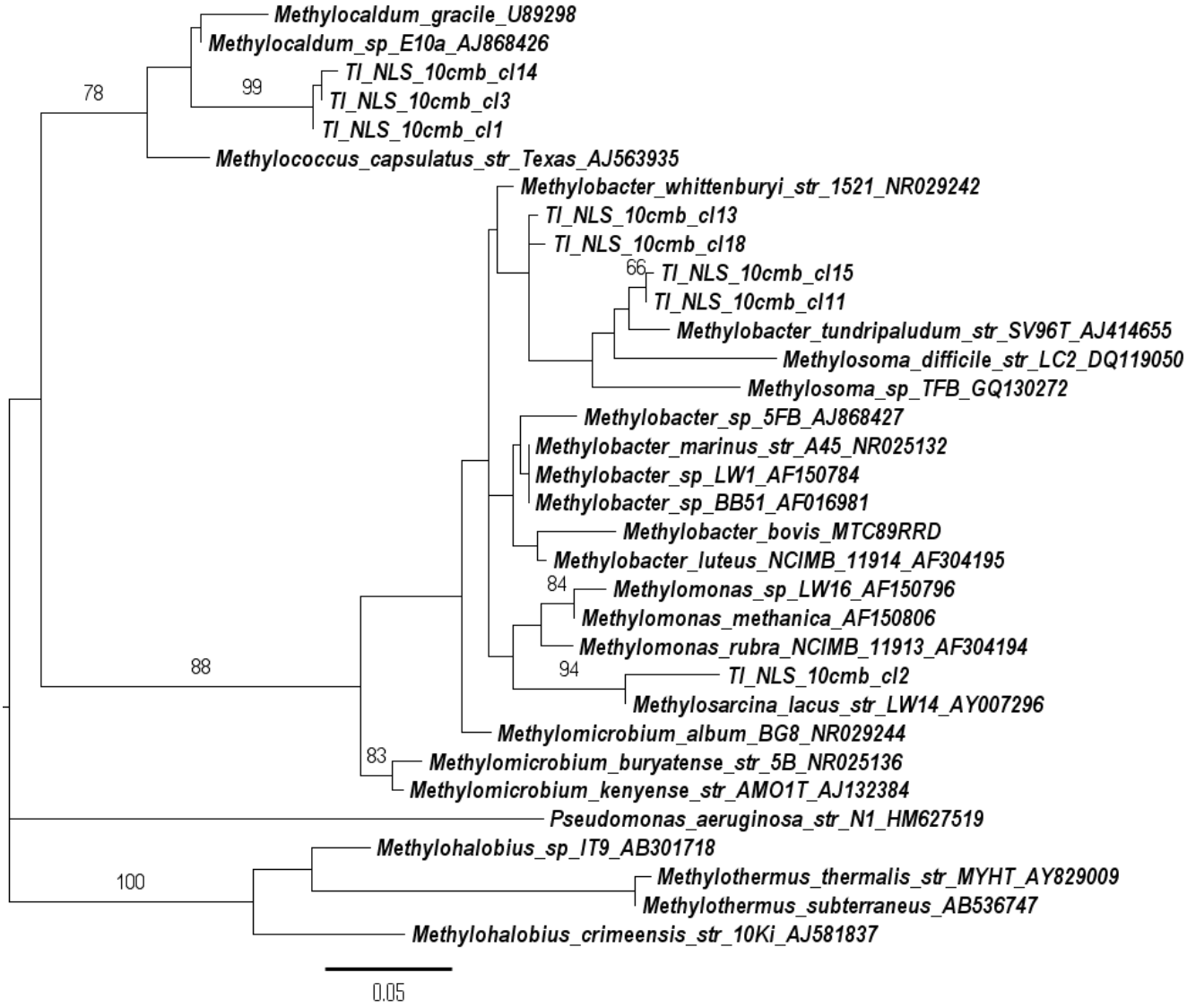

Figure 2: Maximum likelihood analysis of Type I MOB partial 16S rRNA genes present in Nudgee landfill soil site b depths 10 cm and 30 cm. OTUs cluster tightly with members of the *Methylococcus, Methylocaldum*, *Methylobacter* and *Methylosarcina* genera. *Pseudomonas aeruginosa* strain N1 was used as the outgroup. Bootstrap values above 60% are shown and are based on 1,000 replications. The scale bar represents 5% sequence divergence.

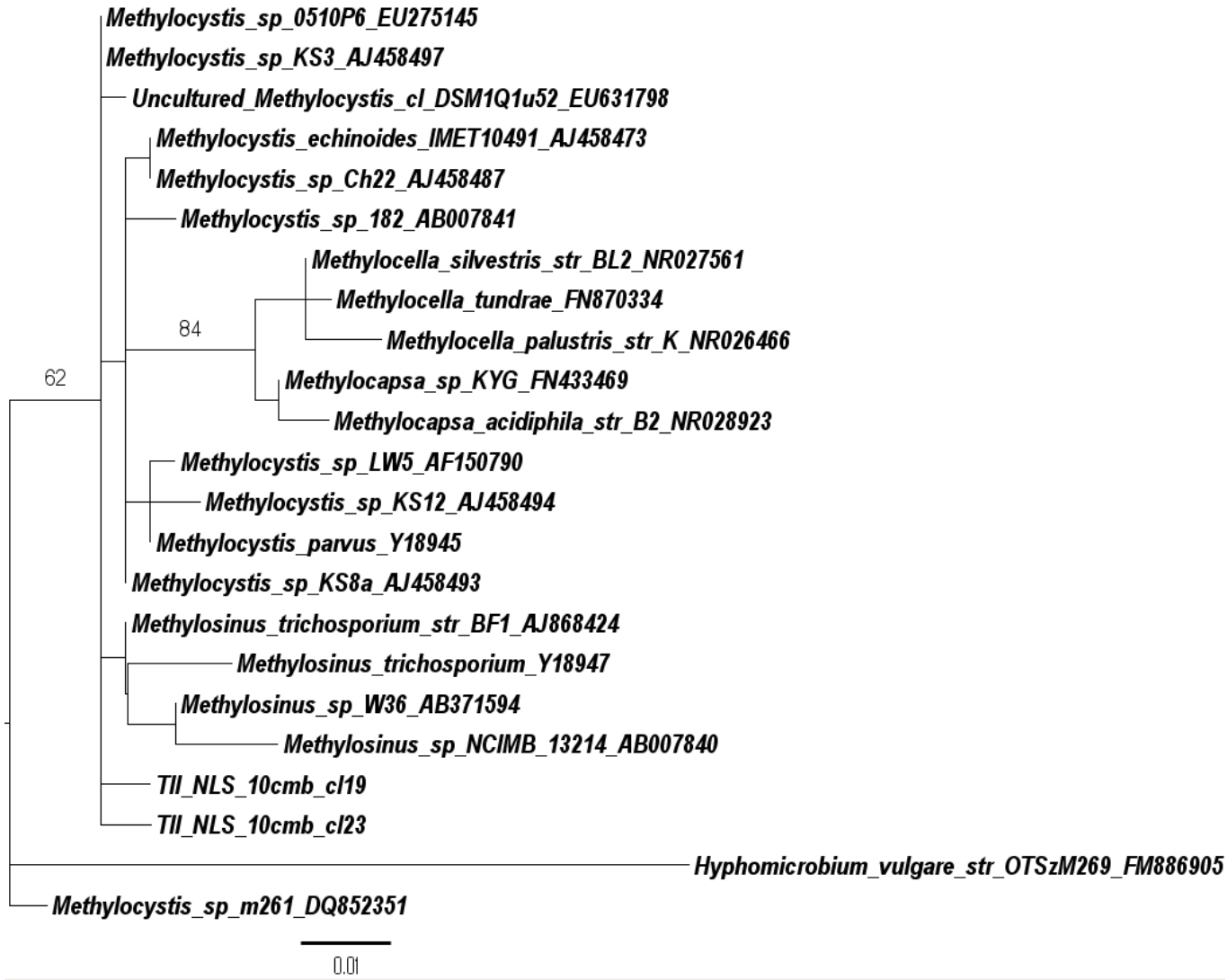

Figure 3: Maximum likelihood analysis of Type II MOB partial 16S rRNA genes present in Nudgee landfill soil site b depths 10 cm and 30 cm. OTUs cluster tightly with members of the *Methylosinus* genus. *Hyphomicrobium vulgare* strain OTSzM269 was used as the outgroup. Bootstrap values above 60% are shown and are based on 1,000 replications. The scale bar represents 1% sequence divergence.

7.3 Molecular analysis of Highchester Abattoir cattle

After optimising methanotroph screening methods on environmental samples, screening began on animal samples. DGGE and phylogenetic analysis was used to characterise the biodiversity of MOB communities present in the rumen of Highchester Abattoir cattle. Figure 4 is a DGGE image comparing Type I and II MOB partial 16S rRNA genes between the rumen epithelium and contents of four animals. A Type I *Methylobacter* species was detected on the rumen epithelium of Animal 3 (DNA sequence identity of 94% over 360 bp to *Methylobacter* sp. LW1; bands a and b) and in the rumen contents of Animal 4 (DNA sequence identity of 98% over 360 bp to *Methylobacter* sp. BB5.1; bands c and d). In contrast, a Type II *Methylocystis* species was detected on the rumen epithelium and in the rumen contents of all four animals screened (DNA sequence identity of 99% over 377 bp to *Methylocystis* sp. KS12; bands e, f, g and h). Some non-specific amplification of non-methanotrophic

16S rRNA genes was apparent. For example, the primer pair TIImob 445/1416 amplified the 16S rRNA gene of an uncharacterised microorganism distantly related to *Roseburia intestinalis* strain XB6B4 (DNA sequence identity of 91% over 377 bp) in Animals 1 and 2.

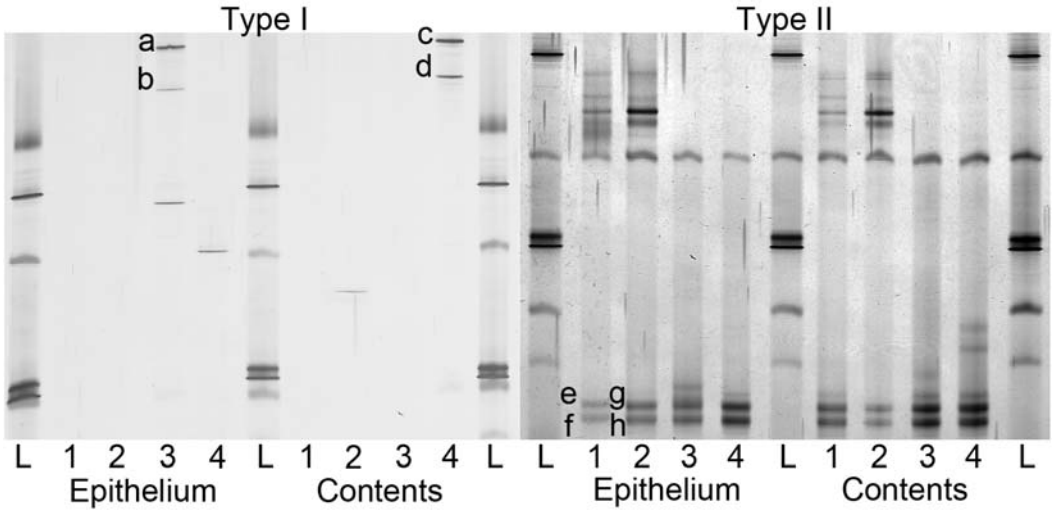

Figure 4: Denaturing gradient gel electrophoresis of Highchester Abattoir rumen epithelium and contents samples from Animals 1 - 4, targeting the 16S rRNA gene of Type I and II MOB. Type I MOB of the *Methylobacter* genus were identified on the rumen epithelium of Animal 3 (bands a and b) and in the rumen contents of Animal 4 (bands c and d). Type II MOB of the *Methylocystis* genus were present on the rumen epithelium and in the contents of all four animals screened (bands e, f, g and h). L corresponds to a DNA ladder marker.

Phylogenetic analysis of methanotroph OTUs supported the identification of *Methylobacter* and *Methylocystis* species in the rumen of these cattle. Figure 5, Figure 6 and Figure 7 show the results of maximum likelihood analysis of Type I and II MOB partial 16S rRNA and *pmoA* genes, respectively. The *Methylobacter* 16S rRNA gene OTUs identified on the epithelium of Animal 3 and in the contents of Animal 4 appear to represent different species, whereas the *Methylocystis* 16S rRNA gene OTUs clustered tightly irrespective of animal. Analysis of *pmoA* OTUs supported the presence of *Methylocystis* species. No *Methylobacter* species *pmoA* was detected *in situ*.

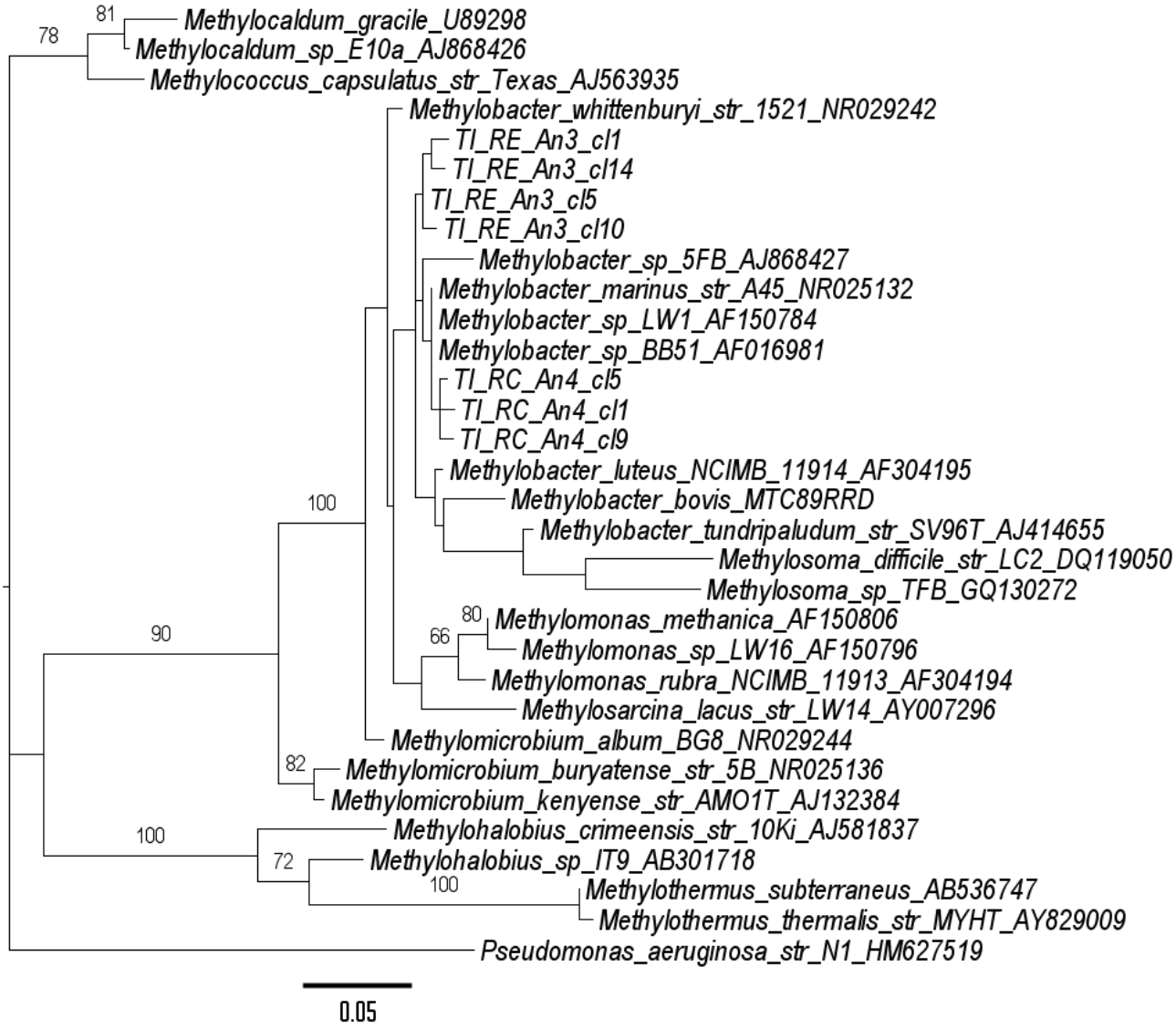

Figure 5: Maximum likelihood analysis of Type I MOB partial 16S rRNA genes present in Highchester Abattoir animals. Rumen epithelium (RE) and contents (RC) Animal 3 and 4 OTUs clustered with *Methylobacter* species. *Pseudomonas aeruginosa* strain N1 was used as the outgroup. Bootstrap values above 60% are shown and are based on 1,000 replications. The scale bar represents 5% sequence divergence.

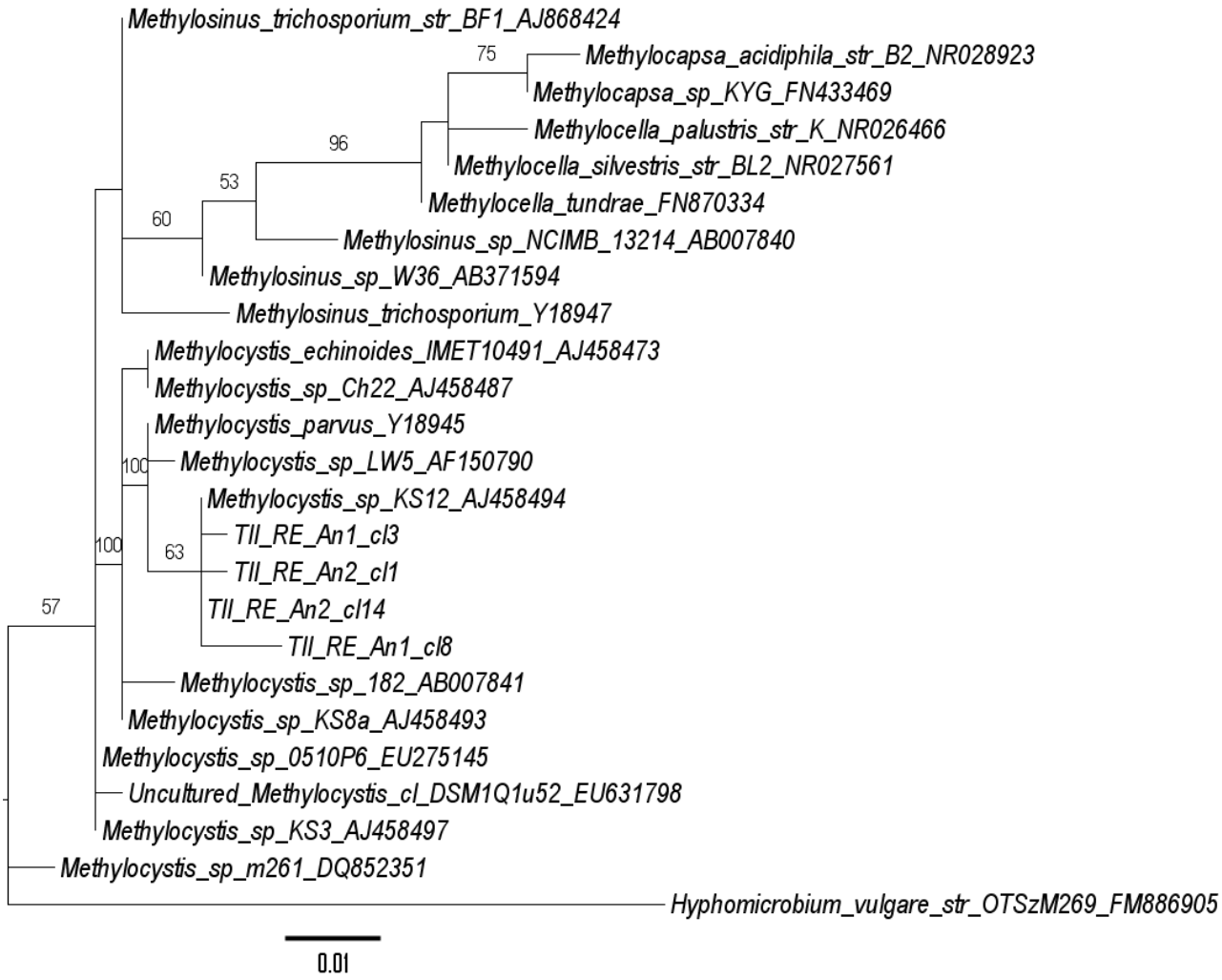

Figure 6: Maximum likelihood analysis of Type II MOB partial 16S rRNA genes present in Highchester Abattoir animals. Rumen epithelium (RE) Animal 1 and 2 OTUs clustered with *Methylocystis* species. *Hyphomicrobium vulgare* strain OTSzM269 was used as the outgroup. Bootstrap values above 50% are shown and are based on 1 000 replications. The scale bar represents 1% sequence divergence.

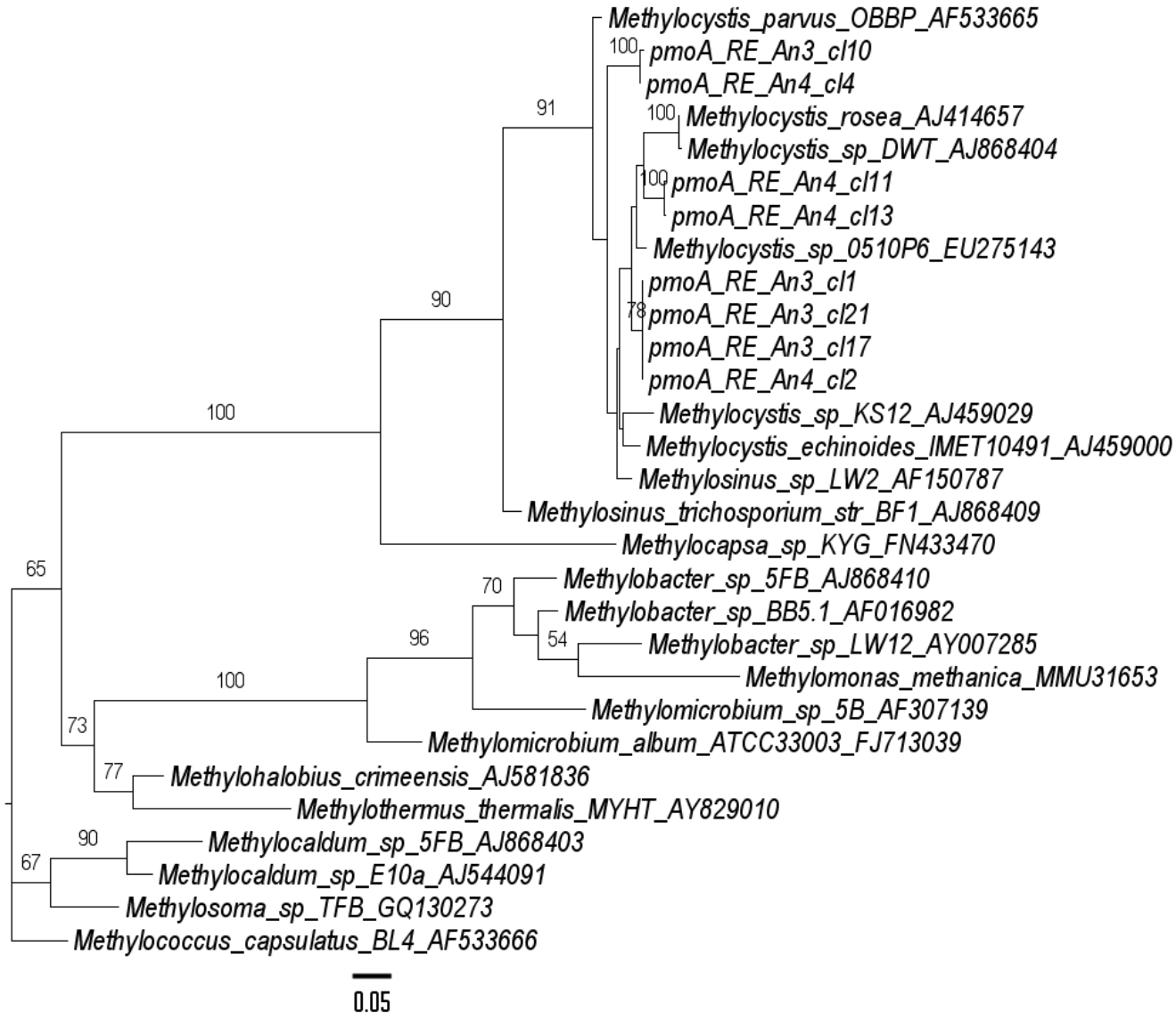

Figure 7: Maximum likelihood analysis of *pmoA* present in Highchester Abattoir animals. Rumen epithelium (RE) Animal 3 and 4 OTUs clustered with *Methylocystis* species. No *Methylobacter* species *pmoA* was identified *in situ*. *Methylococcus capsulatus* BL4 was used as the outgroup. Bootstrap values above 50% are shown and are based on 1 000 replications. The scale bar represents 5% sequence divergence.

Quantitative PCR was used to enumerate *Methylocystis* populations *in situ* (Figure 8). Average *Methylocystis* cells present in whole rumen contents ranged from 7.67 x 10^3 to 6.92 x 10^4 cells mL^{-1}, based on three individual replicates performed on whole rumen contents samples from each animal. The average *Methylocystis* cells mL^{-1} of whole rumen contents across the four animals was 4.38 x 10^4 cells mL^{-1}. Vic DPI cattle faecal samples were used as a negative control; average cell numbers in negative controls were 4.44 x 10^0 cells mL^{-1}. There were significantly more *Methylocystis* cells mL^{-1} in the rumen contents than the negative control (ANOVA P = 0.019). There were no significant differences between animals (ANOVA P = 0.821). Cloning and sequencing performed on *Methylocystis pmoA* qPCR amplicons confirmed the sequences as *Methylocystis pmoA*. All qPCR assays were performed

in triplicate. Because no *Methylobacter pmoA* was identified, *Methylobacter* cells were not quantified.

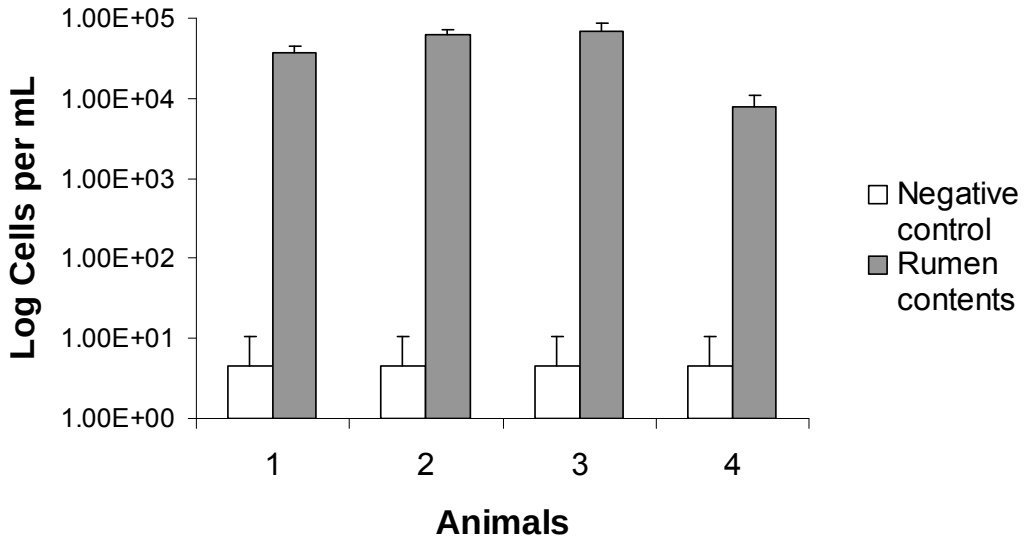

Figure 8: Quantitative PCR targeting *Methylocystis* species cells mL^{-1} present in whole rumen contents of Highchester Abattoir animals. Assays were performed in triplicate. Cattle faeces from the Vic DPI cattle was used as a negative control.

7.4 Methanotroph enrichments using NMS

No characterised methanotrophs were isolated using previously established isolation methods (Whittenbury*, et al.*, 1970). Table 2 outlines the microorganisms isolated in this study, the environmental samples they were isolated from, and the main form of metabolism attributed to them in the literature. Of interest was the isolation of a putative methanotrophic *Klebsiella* species from landfill soil and piggery effluent, a methylotrophic *Methylobacterium* species from landfill soil, and an autotrophic, micro-aerobic *Pelomonas* species from Vic DPI rumen contents. The majority of isolated microorganisms have been characterised as typical respirers/fermenters of complex multi-carbon substrates.

Table 2: Table of microorganisms isolated from environmental samples using NMS.

Isolate (% similarity over 1.5 kb)	Environmental Sample	Metabolism
Methylobacterium species (99% similarity)	Landfill soil	Methylotroph
Aquabacterium species (98 – 99% similarity)	Landfill soil, Piggery effluent, Vic DPI cattle rumen contents	Respiration of multi-carbon substrates
Klebsiella species (98 – 100% similarity)	Landfill soil, Piggery effluent	Putative facultative methanotroph
Enterobacteriaceae (97% similarity)	Piggery effluent	Respiration/fermentation of multi-carbon substrates
Acinetobacter species (97 – 100% similarity)	Landfill soil, Piggery effluent, Kangaroo foregut contents	Respiration of multi-carbon substrates
Lysobacter species (99 – 100% similarity)	Landfill soil, Piggery effluent, Kangaroo foregut contents	Respiration of multi-carbon substrates
Pseudoxanthomonas species (98 – 100% similarity)	Landfill soil	Respiration of multi-carbon substrates
Stenotrophomonas species (99% similarity)	Piggery effluent	Respiration of multi-carbon substrates
Bacillus species (99 – 100% similarity)	Piggery effluent	Respiration of multi-carbon substrates
Paenibacillus species (97 – 99% similarity)	Landfill soil, Kangaroo foregut contents	Respiration/fermentation of multi-carbon substrates
Pelomonas species (97% similarity)	Vic DPI rumen contents	Autotrophic metabolism under microaerobic conditions

7.5 Highchester Abattoir methanotroph enrichments using RF-NMS

Using a novel modification of the NMS media, a *Methylobacter* species was enriched from Highchester Abattoir cattle whole rumen contents. Enrichments were checked weekly for the detectable presence of methanotrophs using PCR methods targeting *pmoA*. Methanotrophs were detected after four weeks of *in vitro* enrichment in RF-NMS with 40 μM mL^{-1} CH_4 supplemented to the gas headspace. At eight weeks, *pmoA* remained detectable in RF-NMS enrichments. Subcultures were set up by inoculating sterile RF-NMS with four week old enrichments. After a further four weeks of enrichment, *pmoA* was detectable in subcultures. Figure 9 shows phylogenetic analysis of *pmoA* OTUs enriched in RF-NMS from Highchester Abattoir cattle. In agreement with the prior molecular screening, a *Methylobacter* species was enriched. However, no *Methylocystis* was enriched using the methods developed in this study.

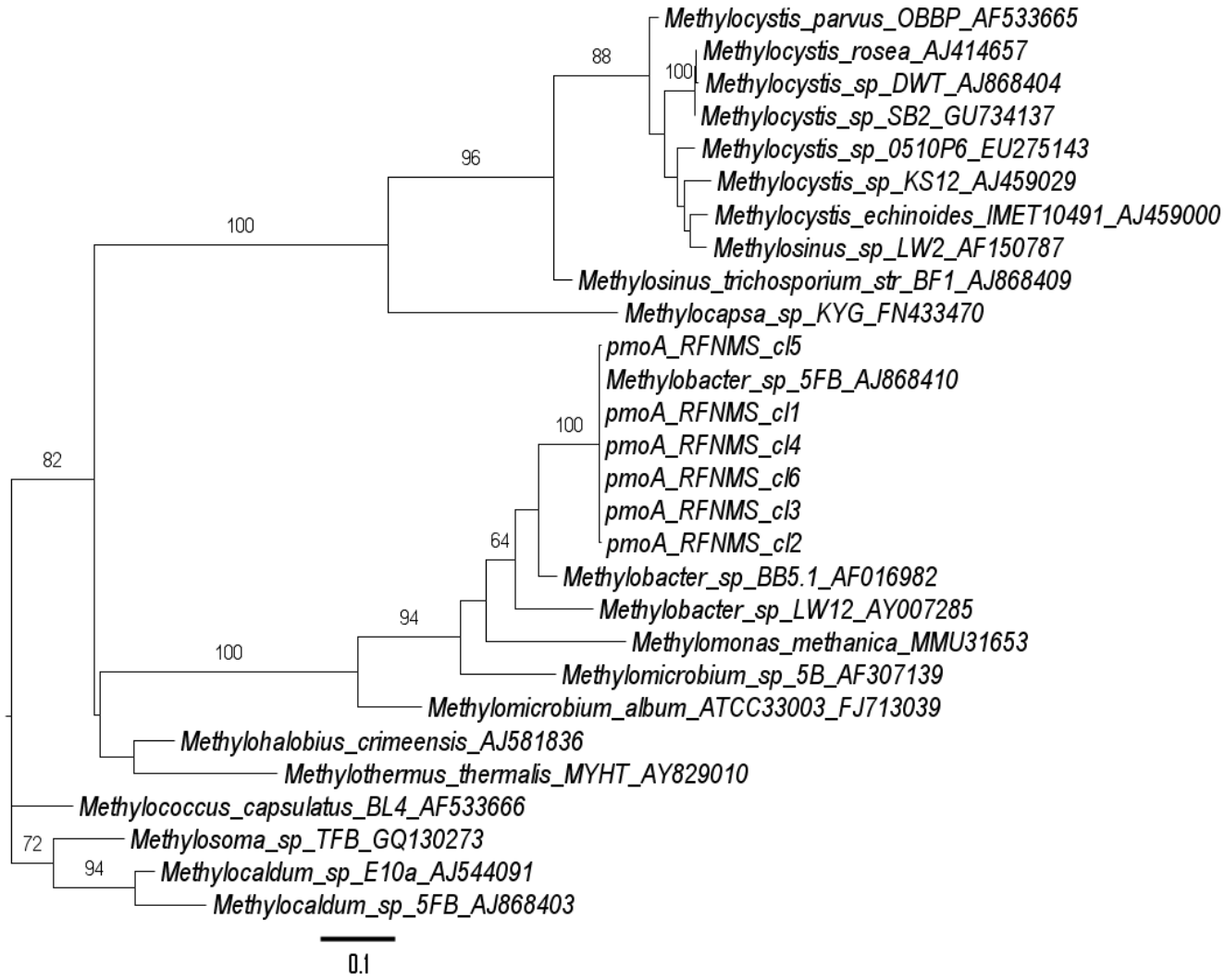

Figure 9: Maximum likelihood analysis of *pmoA* enriched from Highchester Abattoir animal rumen contents. A *Methylobacter* species was enriched from whole rumen contents. *Methylococcus capsulatus* BL4 was used as the outgroup. Bootstrap values above 50% are shown and are based on 1,000 replications. The scale bar represents 10% sequence divergence.

8 Discussion / Conclusion

The purpose of this project was to evaluate the potential for methanotrophs to act as bio-remediation agents in lowering ruminant CH_4 emissions. Specifically, by the 1st of June 2012, to have characterised and isolated methanotrophs that can be demonstrated to oxidise CH_4 under *in vitro* rumen-like conditions. An issue that became apparent while reviewing the scientific literature regarding CH_4 oxidation and the rumen environment was that it is unknown whether methanotrophs are present in the rumen. To date, the only methanotrophs isolated from cattle are aerobic MOB, but it was never determined if these isolates were native to the rumen, or transient organisms ingested with feed material (Stocks & McCleskey, 1964, Whittenbury*, et al.*, 1970). Oxygen can enter the rumen through feed/water and via diffusion across the rumen epithelium (Czerkawski, 1969). Facultative aerobes play an essential role in scavenging this O_2 (Ellis*, et al.*, 1989). It is tempting to speculate that MOB may play a role in O_2 scavenging along the rumen epithelium (Mitsumori*, et al.*, 2002). In addition to O_2, alternate electron acceptors that could be used in CH_4 oxidation are present in the rumen, such as nitrite and sulphate. Given the presence of electron acceptors and CH_4 *in situ*, a niche for CH_4 oxidation seems entirely plausible. If methanotrophs are indeed native to the rumen, then these isolates would be well adapted to the rumen environment, and would represent the most likely source for potential ruminant CH_4 biocontrol agents. This study outlines the first molecular characterisation of native communities of MOB in the rumen, in addition to novel culturing techniques designed to enrich ruminant methanotrophs.

Novel primers targeting partial 16S rRNA genes of Type I and II MOB were designed in this study, as *in silico* analysis indicated that previously designed primers would bind and amplify the 16S rRNA gene of non-methanotrophic bacteria common to the rumen environment (Chen*, et al.*, 2007). It was necessary to optimise TImob117/1144 and TIImob445/1416 using well characterised environments previously established to host diverse communities of MOB. This was done using landfill soil, piggery effluent and wastewater samples (Table 1). DGGE and sequence analysis of partial 16S rRNA gene OTUs from landfill soil confirmed that the primers designed in this study were sensitive (binding to a range of genera, including *Methylococcus*, *Methylocaldum*, *Methylobacter*, *Methylosarcina* and *Methylosinus*) and specific to MOB in well characterised environments (Figure 1).

A variety of micro-environments that could potentially support methanotrophs were sampled from Highchester Abattoir cattle. These included the nasal mucosa, rumen epithelium, rumen contents and intestinal epithelium. Interestingly, MOB were detected on the rumen epithelium and in the rumen contents of Highchester Abattoir and CAAS cattle but not detected in Vic DPI cattle rumen contents and faeces, Tullimba cattle rumen contents, or in kangaroo foregut contents. No anaerobic *Ca*. M. oxyfera methanotrophs were detected in this study. Ultimately due to time constraints and a lack of established methods to study the diversity of ANME separately from methanogenic archaea, it was decided that investigating the biodiversity and activity of ANME within Australian environments was best approached as an independent study.

A number of factors can affect the rumen microbial community, including host genetics, diet, season and geographical location (Kim*, et al.*, 2011). It has been shown that grain-based diets can have a positive impact upon Proteobacteria on the rumen epithelial community (Chen*, et al.*, 2011). Pyrosequencing studies to monitor rumen microbial diversity based on partial 16S rRNA genes also indicate that Proteobacteria in the rumen contents are enriched on grain-based diets (unpublished

data from the Klieve lab). As MOB predominantly belong to the Gamma and Alpha-proteobacteria, it is tempting to speculate that a dietary effect may influence the enrichment of MOB in the rumen. MOB molecular markers for the 16S rRNA and *pmoA* genes were only detected from the Highchester Abattoir and CAAS cattle groups. Both of these groups consisted of *Bos indicus* cattle on a grain-based diet. MOB were not detected in the rumen or faeces of Vic DPI *Bos taurus* grass-fed dairy cows nor in the rumen of the Tullimba bull group, *Bos taurus* cattle also on a grain-based diet. Future work to assess a potential dietary effect of grain on the enrichment of Proteobacteria in the rumen, in particular the time it takes for Proteobacterial methanotrophs to be enriched, should be considered.

A Type II Alpha-proteobacterial *Methylocystis* species was identified on the rumen epithelium and in the whole rumen contents of Highchester Abattoir cattle Animals 1 – 4 (Figure 4; bands e, f, g and h). Type I Gamma-proteobacterial *Methylobacter* species were found to be variable in this sample set, with species identified on the rumen epithelium of Animal 3 (bands a and b) and in the rumen contents of Animal 4 (bands c and d). These were found to be different species, based on phylogenetic analysis of partial 16S rRNA genes (Figure 5). In agreement with the 16S rRNA gene phylogeny, the *pmoA* gene sequence which clustered with *Methylocystis* was present in all Highchester Abattoir animals (Figure 7). The presence of aerobic methanotrophs, particularly associated with the rumen epithelium, suggests a possible niche for CH_4 oxidation *in situ*.

The overall bacterial community in the rumen contents is typically considered to be up to 10^{11} cells mL^{-1} (Hobson, 1988). At approximately 4.38 x 10^4 cells mL^{-1} in the rumen contents, the *Methylocystis* population is quite low in Highchester Abattoir cattle (Figure 8). Whilst low, it is high enough to be a native member of the rumen community rather than a transient organism ingested with feed. The average volume of the bovine rumen is 80 L, and the flow rate of liquid digesta is approximately 0.156 volumes $hour^{-1}$ (Hungate, 1966). This means that the cattle in this study would need to be ingesting 5.47 x 10^8 *Methylocystis* cells $hour^{-1}$ to retain the abovementioned population, which is unlikely to occur through feed/water. It is also worth noting that inhibitors are often present in DNA extracted from rumen samples, which can interfere with qPCR (Ouwerkerk*, et al.*, 2002). Due to this inhibition, *in situ* cell numbers may be underestimated by up to 10-fold.

Culture dependent methods were used to enrich and isolate methanotrophs from environmental samples. Established methods to culture MOB involve the use of the selective medium NMS (Whittenbury*, et al.*, 1970). This medium is devoid of carbon sources; the only carbon available to microorganisms is supplied as CH_4 supplemented into the gas headspace of air-tight culturing vessels. Thus, through subculturing of isolates, this method selects purely for microorganisms that can grow using CH_4 as a sole carbon source. A disadvantage to this method is that not all microorganisms are capable of synthesising all cellular requirements; syntrophic interactions with other microorganisms or alternate carbon sources may be essential for the activity and growth of methanotrophs *in situ*. No characterised methanotrophs were isolated from any environments using NMS in this study. *Klebsiella* were isolated from two environments, the landfill soil and piggery effluent, and this genus has been shown to be capable of acquiring and expressing a CH_4 oxidising phenotype (Zhao*, et al.*, 2009). However, this has only been noted in specific strains, and it is worth noting that the isolates in this study were negative for the *pmoA* molecular marker. Methylotrophic *Methylobacterium* were also isolated via NMS methods. Methylotrophs oxidise methanol, a substrate produced by methanotrophs during active growth – therefore, as methanol is absent from NMS, the presence of methylotrophs suggests the presence and activity of methanotrophs. Regardless, the

isolation of a range of non-methanotrophic microorganisms (*Enterobacteriaceae*, *Pseudoxanthomonas*, *Aquabacterium*, *Lysobacter*, *Acinetobacter*) indicates that despite the use of carbon-free selective media, many microorganisms are capable of long-term survival in minimal media. In future, it may be necessary to increase the duration of enrichments so that slow growing methanotrophs can dominate the culture.

Using a novel medium, RF-NMS, a *Methylobacter* species was enriched from the whole rumen contents of Highchester Abattoir cattle (Figure 9). RF-NMS consists of 33% sterile rumen fluid, which appears to be essential for the growth of this ruminant *Methylobacter* species. The enrichment of *Methylobacter* from Highchester Abattoir rumen contents is in accordance with molecular methods, which identified *Methylobacter* in two of the four animals. *Methylocystis* species were not enriched, possibly due to reasons mentioned above, such as a lack of essential elements supplied in RF-NMS.

Due to the presence of aerobic methanotrophs on the rumen epithelium, it is possible that CH_4 oxidation may be occurring at the CH_4/O_2 interface along the rumen epithelium *in situ*. In order to test this, a novel fermentation apparatus that allows for a CH_4/O_2 transition zone across a surface is currently being developed. Figure 10 shows a preliminary design for such a fermenter. While the fermenter itself will run under anoxic conditions (verified by a dissolved O_2 (DO) probe inserted into the enrichment) a peristaltic pump will deliver a known concentration of DO in sterile salts solution to a nylon membrane surface suspended in the anaerobic enrichment. This will create a slight O_2 gradient across the nylon membrane designed to mimic the diffusion of O_2 across the rumen epithelium. CH_4 produced by anaerobic methanogenic archaea will diffuse across the nylon membrane, forming a CH_4/O_2 transition zone. After enrichment procedures, the nylon membrane can be removed and gDNA from the microbial biofilm present screened for the presence of methanotrophs. A combination of SIP and GC will be used to study the activity of putative methanotrophs, in addition to the activity of the *Methylobacter* enrichment. Currently, this fermenter is under construction.

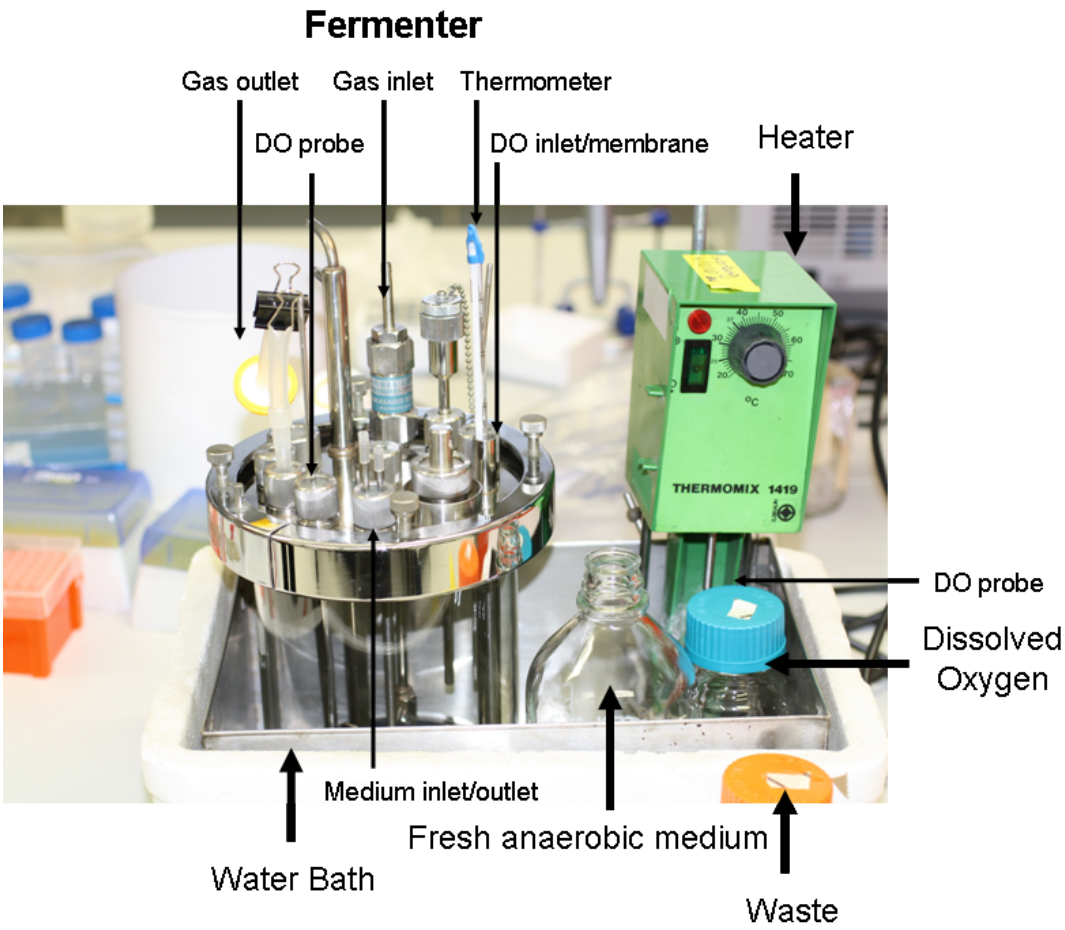

Figure 10: Preliminary design of fermenter for *in vitro* rumen-like CH_4 oxidation assays.

In conclusion, the combination of culture independent and dependent techniques used in this study suggest that simple communities of Proteobacterial methanotrophs are associated with the rumen epithelium and whole contents of *Bos indicus* cattle under specific dietary conditions. At this stage, the activity of methanotrophs under *in vitro* rumen-like conditions and the impact of these organisms on total rumen CH_4 production is unknown. Considering the low cell numbers of MOB detected *in situ*, it is unlikely to be significant. However, the presence of MOB, particularly associated with the rumen epithelium, suggests a role for CH_4 oxidation in the rumen. Currently, an experimental procedure to demonstrate CH_4 oxidation under *in vitro* rumen-like conditions, specifically associated with a CH_4/O_2 transition zone, is under way.

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11 Appendix 1

Table 3: Bacterial strains used in this study.

Bacterial strain	Description	Reference
Methylomonas methanica ATCC 51626	Positive control for Type I 16S rRNA, *pmoA* and *mmoX* gene PCR assays	(Whittenbury, *et al.*, 1970)
Methylocystis rosea ATCC BAA-1196	Positive control for Type II 16S rRNA gene and *pmoA* gene PCR assays	(Wartiainen, *et al.*, 2006)
Candidatus Methylomirabilis oxyfera enrichment	Positive control for *Ca.*M. oxyfera *pmoA* gene PCR assay.	Donated by Prof Zhiguo Yuan, Advanced Wastewater Management Centre, University of Queensland.

12 Appendix 2

Table 4: Primer oligonucleotides used in this study to target MOB molecular markers.

Primer name	Sequence (5' – 3')	Target	Reference
TImob117	GTAAYGCRTAGGAATCTGCC	16S rRNA gene of Type I MOB	This study
TImob1144	CGGCAGTCTCCYTAGAGTTC	16S rRNA gene of Type I MOB	This study
TIImob445	GGGAMGATAATGACGGTACCWGG	16S rRNA gene of Type II MOB	This study
TIImob1416	GCCTTCGGGTARARCCAACTCC	16S rRNA gene of Type II MOB	This study
341F_GC[a]	CCTACGGGAGGCAGCAG	Universal bacterial 16S rRNA gene	(Muyzer*, et al.*, 1993)
Type I R	CCACTGGTGTTCCTTCMGAT	16S rRNA gene of Type I MOB	(Chen*, et al.*, 2007)
518F_GC[a]	CCAGCAGCCGCGGTAAT	Universal bacterial 16S rRNA gene	(Muyzer*, et al.*, 1993)
Type II R	GTCAARAGCTGGTAAGGTTC	16S rRNA gene of Type II MOB	(Chen*, et al.*, 2007)
A189[b]	GGKGACTGGGACTTCTSG	*pmoA* gene	(Holmes*, et al.*, 1995)
mb661	CCGGMGCAACGTCYTTACC	*pmoA* gene	(Costello & Lidstrom, 1999)
mmoXI	CGGTCCGCTGTGGAAGGGCATGAAGCGCGT	*mmoX* gene	(Iwamoto*, et al.*, 2000)
mmoX r1403	TGGCACTCGTAGCGCTCCGGCTC	*mmoX* gene	(Iwamoto*, et al.*, 2000)
mmoX f901 GC	TSAARACSTGGAACCGCTGGGT	*mmoX* gene	(Iwamoto*, et al.*, 2000)
Mcystis pmoA f	GTCTGCTCTGATCGTTCC	*Methylocystis pmoA* gene	This study
Mcystis pmoA r	CCAGTTGTTCGGGTAGAA	*Methylocystis pmoA* gene	This study
Mcystis pmoA probe[c]	AACCGACAACCGCCGTGATC	*Methylocystis pmoA* gene	This study
27F	AGAGTTTGATCMTGGCTCAG	Universal bacterial 16S rRNA gene	(Weisburg*, et al.*, 1991)
1525R	AAGGAGGTGWTCCARCC	Universal bacterial 16S rRNA gene	(Weisburg*, et al.*, 1991)

[a]A GC clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGCCAGCAGCCGCGG) was attached to the 5' end of the primer.
[b]This primer was slightly altered from the original study: the third base was replaced with a K (G/T), and the seventeenth base was replaced with an S (C/G).
[c]The fluorescent reporter dye 6-carboxyfluorescein (6-FAM) was attached to the 5' end of this probe, and the non-fluorescent quencher Black Hole Quencher 1 (BHQ1) at the 3' end (Sigma-Aldrich, Sydney, Australia).

Table 5: Primer oligonucleotides used in this study to target *Ca*. M. oxyfera molecular markers.

Primer name	Sequence (5' – 3')	Target	Reference
qP2F	GGGGAACTGCCAGCGTCAAG	16S rRNA gene of NC10 bacteria	(Ettwig*, et al.*, 2009)
qP2R	CTCAGCGACTTCGAGTACAG	16S rRNA gene of NC10 bacteria	(Ettwig*, et al.*, 2009)
A189b	GGNGACTGGGACTTYTGG	*Ca*. M. oxyfera *pmoA* gene	(Luesken*, et al.*, 2011)
cmo682	TCGTTCTTYGCCGGRTTT	*Ca*. M. oxyfera *pmoA* gene	(Luesken*, et al.*, 2011)
cmo182	TCACGTTGACGCCGATCC	*Ca*. M. oxyfera *pmoA* gene	(Luesken*, et al.*, 2011)
cmo568	GATGGGGATGGAGTATGTGC	*Ca*. M. oxyfera *pmoA* gene	(Luesken*, et al.*, 2011)

13 Conferences, meetings and publications

Attended a workshop on stable isotope probing techniques hosted by the Manefield lab, University of New South Wales (UNSW), June 2011.

Finn, D., D. Ouwerkerk and A. Klieve. 2011. Isolation of a novel bacterial carbon dioxide sink in the rumen of cattle. *4th Congress of European Microbiologists (FEMS 2011),* Geneva, SWITZERLAND (Proceedings datastick – pdf number 1899).

Invited speaker for the Australian Society for Microbiology (ASM) Queensland state branch annual seminar series, November 2011.

Finn, D.R., D. Ouwerkerk and A.V. Klieve. 2012. Molecular characterisation of Proteobacterial methanotroph communities present in the rumen of Australian cattle. *FEMS Microbiology Letters.* (Submitted).

Finn, D.R., D. Ouwerkerk and A.V. Klieve. 2012. Investigating the biodiversity of Proteobacterial methanotroph communities in the rumen of Australian cattle. *Australian Society for Microbiology – ASM 2012.* (submitted).