Methanotrophy in Movile Cave

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Abbreviations

ANMS	Ammonium nitrate mineral salts
bp	Base pairs
CBB	Calvin Benson-Bassham
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
FISH	Fluorescent *in situ* hybridisation
GC	Gas Chromatography
HTS	High-throughput sequencing
kDa	kilo dalton
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LB	Lysogeny broth
mRNA	Messenger RNA
NMS	Nitrate mineral salts
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
pMMO	Particulate methane monooxygenase
RuMP	Ribulose monophosphate
rRNA	Ribosomal RNA
RNA	Ribonucleic acid
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulfate
SIP	Stable Isotope Probing
sMMO	Soluble methane monooxygenase
sp.	Species
TCA	Tricarboxylic acid
TE	Tris EDTA
tris	Tris (hydroxymethyl) methylamine
UCL	University College London
USC	Upland soil cluster

UV	Ultra-violet
µ	Micro

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Declaration

I declare that the work presented in this thesis was conducted by me under the direct supervision of Professor J. Colin Murrell, with the exception of those instances where the contribution of others has been specifically acknowledged. None of the work presented has been previously submitted for any other degree.

Jason Stephenson

Abstract

Movile Cave is an isolated cave ecosystem that receives no input of photosynthetically fixed carbon. Instead, carbon is primarily fixed through light-independent bacterial processes such as chemolithoautotrophy and methanotrophy. Distinctive microbial floating mats appear at the surface of groundwater flooding the cave, at the redox interface between the oxygenated air above (7-10%) and the anaerobic water below. Methane, of geological origin, bubbles up into the cave and is present in the cave atmosphere (0.5-1%).

The *in situ* methanotroph community of Movile Cave microbial floating mat was determined by examination of metagenomic sequencing and *pmoA* gene microarray data sets. The metagenonomic sequencing approach indicated a *Methylococcus capsulatus* -like organism to be the most abundant methanotroph in Movile Cave. *pmoA* microarray analysis indicated a high abundance of *Methylocystis pmoA* gene sequences with *Methylococcus capsulatus*-like *pmoA gene* sequences being relatively abundant.

The methane oxidising bacterium *Methylomonas* strain LWB was isolated from a sample of lake water from Movile Cave. Phylogenetic analysis of the genes encoding 16S rRNA and the soluble and particulate methane monooxygenase functional gene markers *pmoA* and *mmoX,* respectively, confirmed that strain LWB belongs to the genus *Methylomonas*. *Methylomonas* LWB has a second putative copy of the particulate methane monooxygenase pXM which displays an unusual gene orientation. The *Methylomonas* LWB genome contains all genes encoding the typical Type I methanotroph ribulose monophosphate pathway for formaldehyde assimilation and all genes required for a complete TCA cycle.

Active methane oxidisers in Movile Cave were identified by DNA Stable-isotope probing. Organisms belonging to the genera *Methylomonas, Methylocystis, Methylococcus* and *Methylobacter*- were identified from ^{13}C-enriched DNA. Cross-feeding of the ^{13}C label into non-methanotrophic organisms identified from the ^{13}C-enriched DNA indicated that methanotrophs provide a carbon source for other microorganisms in Movile Cave.

Chapter 1

Introduction

1.1 Cave and karst systems

Caves and karst are natural and abundant land morphologies that are found across the globe. There can be cracks and crevices to entire networks of cathedral sized hollows within the Earth's crust. Some caves will be devoid of life, impenetrable to living organisms and have no interaction with the external environment. Others may be teaming with life due to biochemical exchange with external environments and the surface ecosystem. Whilst some of these environments would be considered benign, others are some of the most extreme and inhospitable places on Earth, highlighting them as areas of focus for research. Speleology is the study of cave and karst formations, covering geology, shape and structure, speleogenesis - the process of cave formation, organisms and also the recreational activity of cave exploration.

Karst by definition are land features predominantly found in limestone that are eroded through processes, such as condensation corrosion leading to characteristic formations including sinkholes, fissures and often include underground streams. Karst structures predominantly form due to the descent of meteoric surface water carrying with it enough energy, in the form of water, air and gravity, to carve out passages and support life (Palmer, 1991). Much effort has been directed at understanding hypogenic speleogenesis, the formation of these karst structures below the surface of the earth that form due to the ascension of subsurface water (Forti et al., 2002). One such case investigating the speleogenesis of Buso della Rana-Pisatela, a karst system in the Venetian Alps, indicated that the oxidation of pyrite (FeS_2) leads to acidification of the ground water. The acidic ground water then dissolves the

limestone (a by-product of which is the formation of gypsum crystals, which can be found throughout the cave system), and this is deposited elsewhere as the water flows (Tisato et al., 2012). It is thought that the dissolution of limestone and deposition process that forms these karst structures expand the dimensions of the hollow by only a few millimetres per 100 years, where the flow of water is low to moderate (Dreybrodt et al., 2002).

Karst structures can lead to the production of vast cave systems such as Frasassi, Monte Cucco and Acquasanta Terme caves, found at the central Umbria and Marche regions in Italy (Galdenzi & Menichetti, 1995). The Frasassi cave system is estimated to have over 25km of solutional passages. The waters running through Frasassi are rich in H_2S, and there is a regular supply of O_2 coming into the cave from the atmosphere or carried by seepage water (Galdenzi et al., 2008). As a result, there is the presence of sulfuric acid in the cave water which drives the speleogensis. Galdenzi et al., (2008) noted that there was an increase of CO_2 in the atmosphere where the corrosion was taking place and explained the process through the following geochemical reactions:

1. $H_2S + 2O_2 \rightarrow H_2SO_4$
2. $H_2SO_4 + 2H_2O \rightarrow 2H_3O^+ + SO_4^{2-}$
3. $H_3O^+ + CaCO_3 \rightarrow Ca^{2+} + HCO_3^- + H_2O$
4. $HCO_3^- + H_3O^+ \rightarrow H_2CO_3 + H_2O$
5. $H_2CO_3 \rightarrow H_2O + CO_2(g)$

An experiment in the Frasassi cave system was carried out, in which, limestone blocks were placed around the cave. After 5 years the change in limestone mass was observed. It was noted that the dissolution of the limestone reached rates of around 20 mg cm^{-1} $year^{-1}$, both when the blocks were placed in areas containing H_2S vapour and also when they were directly within the acidic water (Galdenzi et al., 1997; Galdenzi, 2012).

1.1.2 Cave Microbiology

With the discovery of more and more caves, scientists have become interested in discovering how microorganisms survive and interact with these unusual environments. Most cave microbiology research has focused on microbial sulfur metabolism. The Italian Frasassi and Acquasanta Terme caves mentioned previously (Galdenzi and Menichetti, 1995) have been subject to much scrutiny of the microbial populations living within. White microbial floating mats have been discovered on the waters of Acquasanta cave. In addition, an investigation by Jones et al., (2010) found they had low biodiversity consisting mainly of lithotrophic *Gamma*- and *Epsilonproteobacteria*, which is a consistent finding with other caves systems with fast flowing waters that have a high dissolved sulfur to oxygen ratio. In the study by Macalady et al., (2008), it was also shown that filamentous sulfur oxidising bacteria thrive where a high dissolved sulfur to oxygen ratio was present, and also that *Thiothrix* species were more dominant when the sulfur to oxygen ratio was relatively lower. Snottites, highly acidic viscous biofilms found to dangle from the cave walls in Frasassi, are thriving with microbial life dominated by the bacterial species *Acidithiobacillus* along with the Thermoplasmata archaeal group (Macalady et al.,

2007). A later metagenomics study of cave Snottites by Jones et al., (2014) indicated rare species belonging to *Ferroplasma*, G-plasma and some *Acidimicrobium* species to potentially oxidise sulfide in the Snottites along with the more abundant *Acidithiobacillus* previously identified. Other than Movile Cave, no other cave has been found to have high concentrations of methane, Methanotrophy in cave environments is so far unique to Movile Cave (Hutchens et al., 2004).

1.2 Movile Cave

1.2.1 Formation of Movile Cave

In 1986, a power company was digging large shafts in the south eastern region of Dobrogea, Romania to see if the region would be a suitable area in which to build a geothermal power plant. The region of Dobrogea is well known by the locals to be very geologically active. There are several sulfidic springs in the local vicinity as well as milky white sulfidic lakes and methane seeps. One shaft in particular was excavated about 2 km from the Black Sea in a disused field. The shaft was situated on a small hill (Movile in Romanian), that was a part of a series of hills surrounding a sinkhole depression feature in the landscape (**Figure 1.1**). The shaft of about 25 m was found to have tapped into a cave system that ran below the series of hills. After this discovery, the power company were unable to carry on with their investigations, and a group of speleologists investigated and described the extensive cave structure beneath (personal communication, Dr Alexandra-Maria Hillebrand-Voiculescu, Emil Racoviţă, Institute for Speleology, Romania).

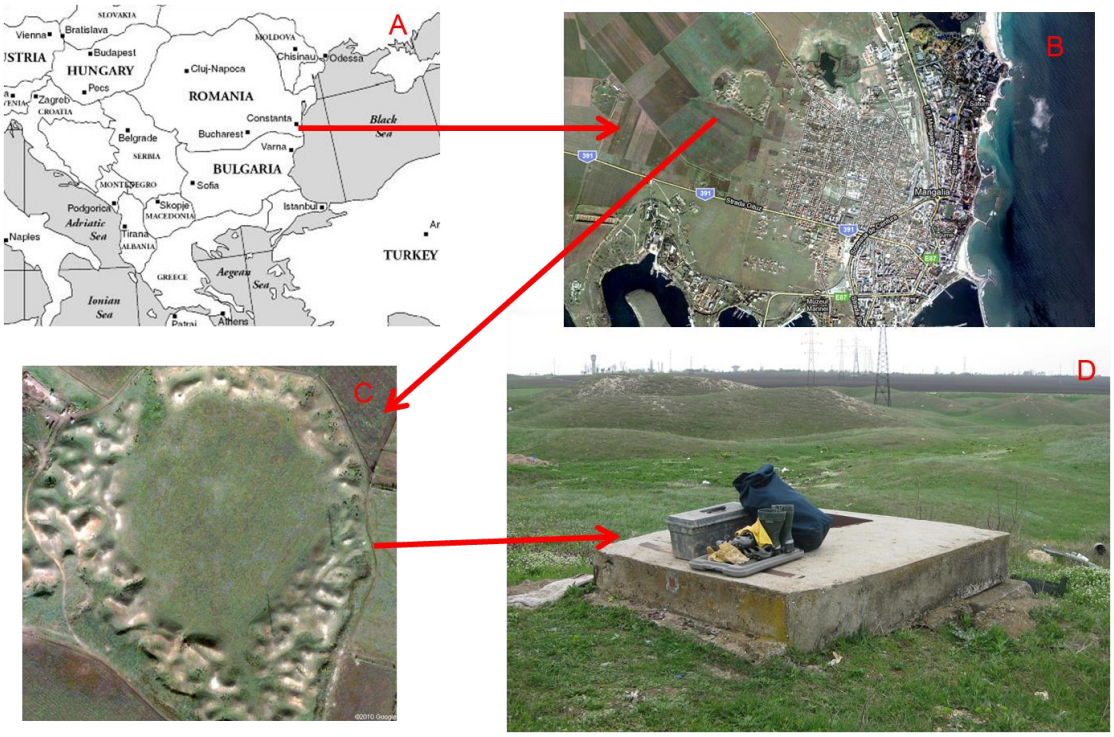


Figure 1.1: Location of Movile Cave. The images B and C were obtained from Google maps (https://maps.google.co.uk/). The cave is situated in the south eastern region of Romania (Dobrogea) just off of the coast of the Black Sea. The series of hills around the sinkhole is west of the coastal town on Mangalia, about 2 km from the beach. On top of one of the small hills to the eastern side of the sinkhole lies the entrance to Movile Cave.

Movile Cave is a hypogenic karst system situated around 25 m below the Earth's surface. It consists of an entrance shaft, a descending passageway, a room with a lake, flowing ground water and two air bell structures (**Figure 1.2**). At the surface, the limestone is covered with clay and loess, which prevent meteoric waters from entering the cave system and also prevent gaseous exchange between the cave and the surface (Sarbu and Kane, 1995). The upper cave tunnels covering a distance of around 200 m are dry, highlighting the fact that meteoric water does not penetrate the

cave. The lower region of the cave is flooded with thermal ground water. The temperature of the cave water is 21 °C, while the wall and air temperature is approximately 19-20 °C, which is much higher than the average temperature above ground of 8 °C. The water is rich in hydrogen sulfide at a concentration of 8-12 mg/l (Sarbu and Kane, 1995). The flow rate of the water is estimated to be 5 L/s, and there is a dissolved methane concentration of 0.2 mM (Sarbu and Lascu, 1997). The air found in the air bells has an increased concentration of CO_2 (2.5-3.5%) and high methane concentrations of about 0.5-1%. Conversely, there is a reduced concentration of O_2 (7-10%) (Sarbu et al., 1996). Measurements of carbon isotope ratios in the cave indicate that most of the CO_2 in the cave atmosphere is produced from the isotopically light geological methane that enters the cave (Sarbu et al., 1996). There is a redox interface at the surface of the water in Movile Cave due to the presence of reduced compounds in the water and oxygen in the atmosphere. Microorganisms thrive at this redox interface and organic floating mats form at the water-air interface (Sarbu et al., 1994). These floating mats are made up of bacteria, fungi and protozoa feeding on primary substrates and taking advantage of the redox potential (Sarbu et al., 1994).

The pH inside the cave is maintained at 7.3, due to the buffering ability of the limestone walls, through condensation corrosion producing bicarbonate (**Figure 1.3**) (Sarbu and Lascu, 1997). The condensation corrosion process, along with sulfuric acid corrosion, are the two main driving forces behind Movile Cave speliogenesis (Sarbu and Kane, 1995; Sarbu and Lascu, 1997). The condensation corrosion process occurs due to the warm water releasing water vapour into the cave atmosphere. The water combined with the CO_2 to produce carbonic acid, which in turn, reacts with the

limestone walls. The limestone dissolves, the solution moves and then the limestone is deposited elsewhere as the water evaporates again (**Figure 1.3**). The sulfuric acid corrosion is contained within the regions flooded by water. Oxidation of the sulfide in the cave water occurs due to the presence of oxygen in the cave atmosphere. This produces sulfuric acid which reacts with the limestone again causing deposition much like the process occurring in the Frasassi Cave (Forti et al., 2002). The sulfuric acid corrosion generates CO_2 which will also feed into the condensation corrosion process.

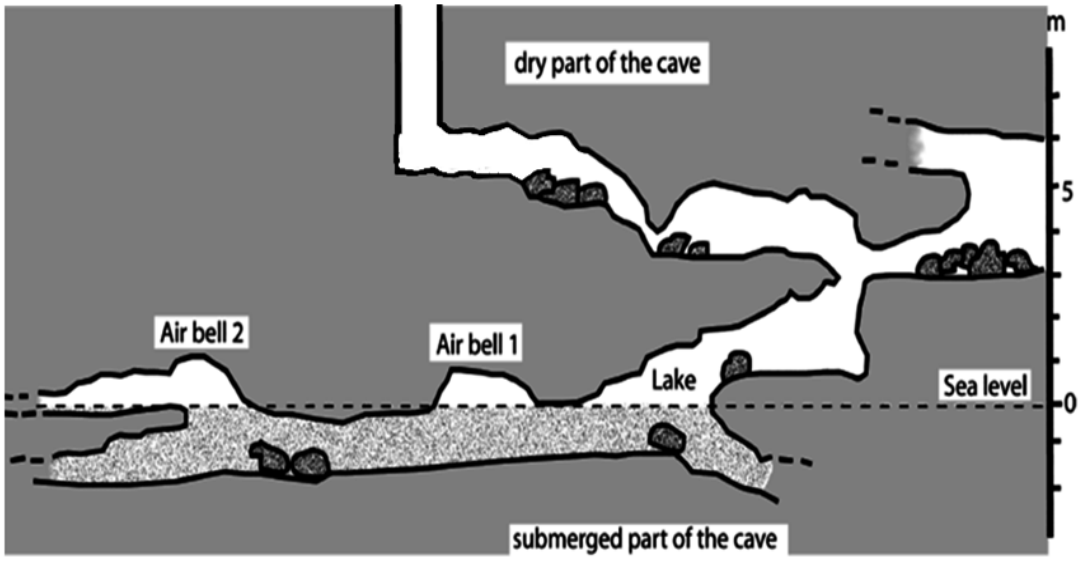


Figure 1.2: Schematic representation of Movile Cave adapted from Muschiol and Traunspurger, (2007). The 20 m descent into Movile Cave leads to limestone clay covered passages. These passages end at a small lake roughly 6 m^2. Beyond the wall of the lake are two air bell structures, air bell 1 and air bell 2, that are formed where the water level reaches into two dome structures within the limestone.

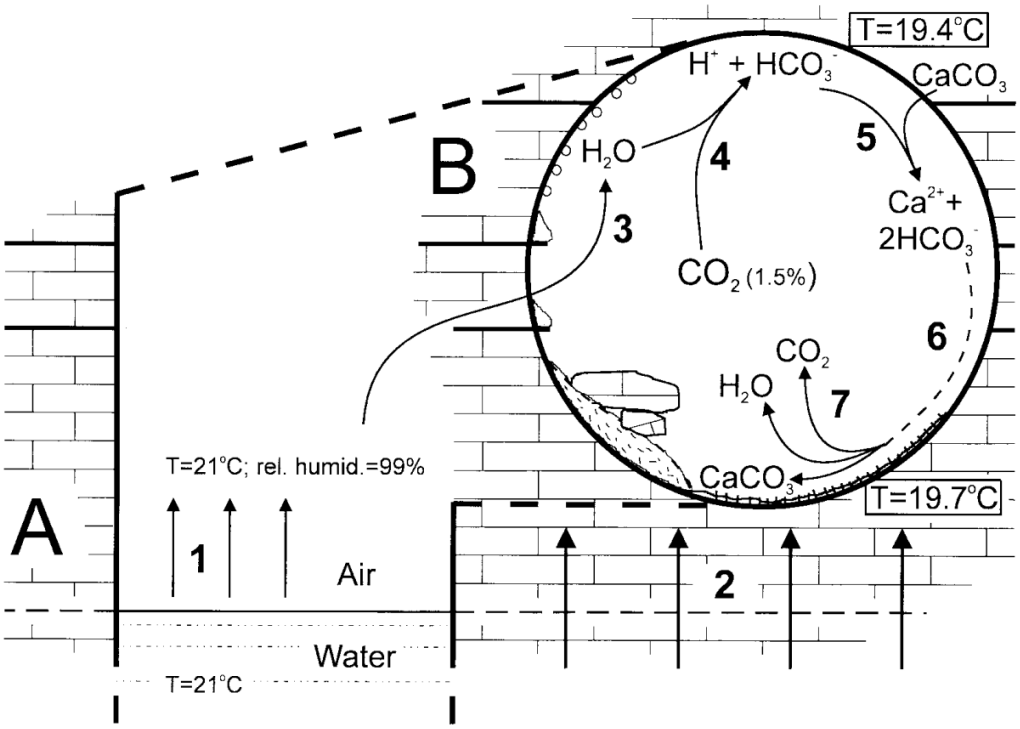


Figure 1.3: A representation of the condensation corrosion process occurring inside Movile Cave taken from Sarbu and Lascu, (1997). The production of the bicarbonate acts as a buffer, keeping the pH of the cave at 7.3. The constant deposition of the limestone ($CaCO_3$) is responsible for part of the Movile Cave speleogenesis, the process of which is boosted by biogenic CO_2 produced by the cave fauna.

1.2.2 Movile Cave, an isolated ecosystem

There are several sources of evidence that suggest Movile Cave is an isolated ecosystem and does not benefit from carbon fixed by photosynthetic organisms. After the 1986 Chernobyl accident, the artificial nuclides ^{137}Cs and ^{90}Sr could be found in the surface soils and water bodies in the area located near Movile Cave. They were also found within other caves and also in the Black Sea (Sarbu and Kane, 1995). In the study by Sarbu and Kane, (1995) it is stated that the artificial nuclides ^{137}Cs and ^{90}Sr were completely absent in Movile Cave. Much of the area around Movile Cave is farmland, however, despite this there was no evidence of pesticides or faecal streptococci having entered Movile Cave (Sarbu et al., 1994). Stable isotope ratio analysis was used by Sarbu et al., (1996) in order to determine the source of carbon and nitrogen in organic matter obtained from Movile Cave. It was shown through this process that Movile Cave is a self-sustaining eco-system. Organisms from Movile Cave were isotopically lighter than those from the surface. The organisms at the surface showed typical isotope ratios dependant on photosynthetic production, whereas the organisms from Movile Cave displayed ratios more indicative of chemoautotrophic production (Sarbu et al., 1996).

1.2.3 Movile Cave Biology

There are 48 species of invertebrates that are adapted for aquatic and terrestrial life in Movile Cave, 33 of which are endemic to this environment (Rohwerder et al., 2003, Muschiol and Traunspurger, 2007; Sarbu et al., 1994; Sarbu and Kane, 1995; Sarbu et al., 1996). Troglomorphy is the adaptation to living in constant darkness and includes the loss of eyes, pigment and the development of long sensitive antennae and appendages for feeling instead of seeing the surroundings. The majority of the invertebrates in the cave have evolved through a process of troglomorphy during the time the cave has been isolated, estimated to be around 5 million years (Sarbu and Kane, 1995) (**Figure 1.4)**. Carnivores in the cave include leeches, spiders, pseudoscorpions and centipedes. The carnivores likely feed on the abundant bacterivors including the bacterivorous nematodes. Bacterivors play a vital role in the Movile Cave food web, as they provide the transition route for biomass that is produced by the microbial community into the larger organisms that inhabit the cave (Muschiol and Traunspurger, 2007).

Figure 1.4: Some of the invertebrate inhabitants of Movile Cave. Images provided by Emil Racoviţă, Institute for Speleology, Romania. Left to right: millipede, water-scorpion and spider.

The microbial mats that form at the redox interface on the surface of the water contain a dense network of fungal hyphae including *Plasmopara*, *Glicocadium*, *Penicillium* and *Trichoderma* (Sarbu et al., 1994). Bacteria live amongst the network of fungi and include sulfur oxidisers and methylotrophs (Hutchens et al., 2004; Vlasceanu et al., 2000; Chen et al., 2009). Lazar et al., (2005) used different selective growth media to analyse the bacteria in Movile Cave and found them to be much more diverse than was previously thought. The methods used by Lazar et al., (2005) were by no means quantitative, but they did show a large diversity of bacteria including chemoheterotrophs, sulfate reducers, sulfur oxidisers, methylotrophs, nitrifiers and N_2 fixers.

1.2.4 Microbiology of Movile Cave

The Movile Cave ecosystem is believed to be built on a foundation of primary producing bacteria, including organisms that gain energy from reduced sulfur compounds, methane and ammonia (Chen et al., 2009; Hutchens et al., 2004). Since the discovery of Movile Cave in 1986, there have been several studies conducted to investigate the microbiology present. The earlier studies tended to focus on the bacteria that participated in the cycling of sulfur (Sarbu et al., 1994; Rohwerder et al., 2003; Vlasceanu et al., 1997). Notable sulfur oxidisers identified from the microbial mats in Movile include *Beggiatoa*, *Thiobacillus*, *Thiosphaera* and *Thiomicrospira* (Sarbu et al., 1994; Sarbu et al., 1995). Rohwerder et al., 2003 identified that there were also facultatively anaerobic sulfur oxidisers in Movile Cave that used nitrate as a terminal electron acceptor, and found they were relatively abundant. It is likely that these facultatively anaerobic sulphur oxidiser groups of microbes also contribute

significantly to the primary production in the cave. DNA Stable-Isotope Probing (DNA-SIP) performed by Chen et al., (2009) identified species including *Thiovirga*, *Thiothrix*, *Thioploca* and *Sulfuricurvum* (anaerobic sulfur oxidiser), with *Thiobacillus* being the most active in assimilation of the ^{13}C bicarbonate ions. Further sulfur oxidisers identified from Movile Cave by Porter et al., (2009) include *Halothiobacillus* and *Thiomonas*. Porter et al., (2009) were able to estimate that the chemolithoautotrophic productivity of Movile Cave would be around 281 g $C/m^2/yr$.

Sulfate reducing bacteria have been identified in Movile Cave, but it was suggested by Rohwerder et al., (2003) that they are not primary producers. It is thought that the sulfate reducing bacteria utilise carbon sources that are bio-available made by the primary producers. Both Chen et al., (2009) and Porter et al., (2009) identified the presence of *Desulfobulceae* from 16S rRNA gene clone libraries. It was noted by Engel, (2007) that the sulfate reducers in Movile Cave fall mostly into the Deltaproteobacteria, despite sulfate reduction being a phylogenetically diverse phenotype.

There is relatively little known of the microbiology of nitrogen cycling in Movile Cave. Sarbu et al., (1996) carried out nitrogen stable isotope ratio measurements and found that ammonia in the cave water was relatively high in ^{15}N, while in the floating microbial mats it was relatively light. It had been reported (Sarbu et al., 1996) that a similar fractionation pattern of nitrogen isotopes to that observed in Movile Cave was observed when microbes growing on ammonia were not substrate limited. It was also noted (Sarbu et al., 1996) that the process of nitrification can deplete ^{15}N in a

biological sample. Either or both of these phenomena could be the cause of the nitrogen stable isotope signature in the floating microbial mat. In the study by Chen et al., (2009) ^{13}C-bicarbonate DNA-SIP experiment indicated that both ammonia and nitrite oxidisers were active in assimilating the ^{13}C label. This study provided evidence that the nitrification process would contribute to the nitrogen isotope ratios seen by Sarbu et al., (1996). Denitrification and or assimilatory nitrate reduction may be taking place in Movile Cave since nitrate was not observed in the cave waters, indicating rapid turnover of any nitrate available (Sarbu, 2000). Some of the bacteria found in Movile Cave are known to be able to fix nitrogen gas (N_2), including *Beggiatoa* and *Methylocystis* (Sarbu and Kane, 1995; Hutchens et al., 2004).

Isotope ratio measurements of carbon from within Movile Cave suggested that biological methane oxidation may be a contributing factor to the results obtained (Sarbu et al., 1996). Active methane oxidising bacteria were identified in Movile Cave by DNA-SIP experiments (Hutchens et al., 2004); a sample of floating microbial mat was incubated with $^{13}CH_4$ in order to identify those bacteria that could incorporate the ^{13}C-label into their DNA. It was found that species of the genera *Methylomonas*, *Methylococcus* and *Methylosinus*/*Methylocystis* were among the most active methane utilisers (Hutchens et al., 2004). Furthermore, through functional gene analysis several strains of the *Methylomonas*, *Methylococcus* and *Methylosinus*/*Methylocystis* that contained the genes encoding the active sites of methane monooxygenase enzymes (*pmoA* and *mmoX*) were detected. This study indicated that there was relatively high diversity among the methane oxidising bacteria in Movile Cave and that the phenotype was not dominated by a single organism. CO_2 produced as a result of methane oxidation will contribute to the pool

of CO_2 in the environment thus feeding the autotrophic bacteria present. Methanotrophs may also release methyl compounds, such as methanol, into the ecosystem, providing a carbon and energy source for non-methanotrophic methylotrophs (e.g. methanol utilising bacteria).

Non-methanotrophic methylotrophs have also been shown to be both present and active in Movile Cave. The first evidence of methylotrophy in Movile cave came from Rohwerder et al., (2003) with cultivation of methylotrophs from the floating mats growing on methanol. Methylotrophs were shown to be active in Movile Cave through the DNA-SIP study peformed by Hutchens et al., (2004). In this study, clone libraries of the functional gene marker *mxaF* encoding the enzyme for the methanol dehydrogenase enzyme were identified from ^{13}C-enriched DNA, including sequences from the non-methanotrophic methylotrophs *Methylophilus* and *Hyphomicrobium*. Hutchens et al., (2004) speculated that *Methylophilus* and *Hyphomicrobium* utilised $^{13}CH_3OH$, which may have been excreted by the methanotrophs. Chen et al., (2009) identified more examples of non-methanotrophic methylotrophs from a 16S rRNA gene study, noting that the obligate methylated-amine utiliser *Methylotenera mobilis* was relatively abundant and also other methylotrophs including *Methylophilu*s and *Methylovorus* species were present. The detection of *Methylotenera* and *Methylophilus* species in Movile Cave was also noted by Porter et al., (2009).

1.3 Methane oxidising bacteria

1.3.1 Characteristics of methane oxidising bacteria

Bacterial oxidation of methane was first identified in the late 1900's (Kaserer, 1905, 1906 and Söhngen, 1906, 1910). Methanotrophy, the ability to grow on methane is a sub-phenotype of methylotrophy, the ability of organisms to grow on C_1 compounds, i.e. compounds which contain no carbon-carbon bonds. Isolation and characterisation of methanotrophic bacteria have been key to the understanding of these organisms. Since the isolation of the first methane oxidising bacterium *Pseudomonas* (*Bacillus*) *methanica* (Söhngen, 1906), there have been many methane oxidising bacteria isolated covering (currently) 19 formally described genra (*Methylomonas, Methylobacter, Methylococcus, Methylomicrobium, Methylosphaera, Methylocaldum, Methylosarcina, Methylothermus, Methylohalobius, Methylogaea, Methylosoma, Methylomarinarum, Methylovulum, Methylosinus, Methylocystis, Methylocella, Methylocapsa, Methyloferula and Methylacidiphilum*). The majority of methanotrophs identified belong to the *Alphaproteobacteria* and *Gammaproteobacteria* phyla. The validly published genus *Methylacidiphilum* belongs to the *Verrucomicrobia* phylum.

The classic aerobic methanotrophs can be grouped based on their carbon assimilation pathway into Type I and Type II methanotrophs (Trotsenko and Murrell, 2008). The methanotrophs that assimilate carbon via the ribulose monophosphate cycle (RuMP) (Lawrence and Quayle, 1970) are deemed to be Type I methanotrophs. The

methanotrophs that assimilate carbon through the serine cycle (Shishkina et al., 1976) are deemed to be Type II methanotrophs. The difference in methanotroph type is also defined by the 16S rRNA gene taxonomy of the organisms. Type I methanotrophs fall into the *Gammaproteobacteria*, while the Type II methanotrophs are *Alphaproteobacteria* (**Figure 1.5**). Interestingly the Type I and Type II methanotrophs differ physically in their intracytoplasmic membrane structure (**Figure 1.6**). Type I methanotrophs tend to have stacks of vesicular disc-shaped intracytoplasmic membranes throughout the cell, whereas Type II methanotrophs have the intracytoplasmic membranes arranged around the periphery of the cell (reviewed in Trotsenko and Murrell, 2008).

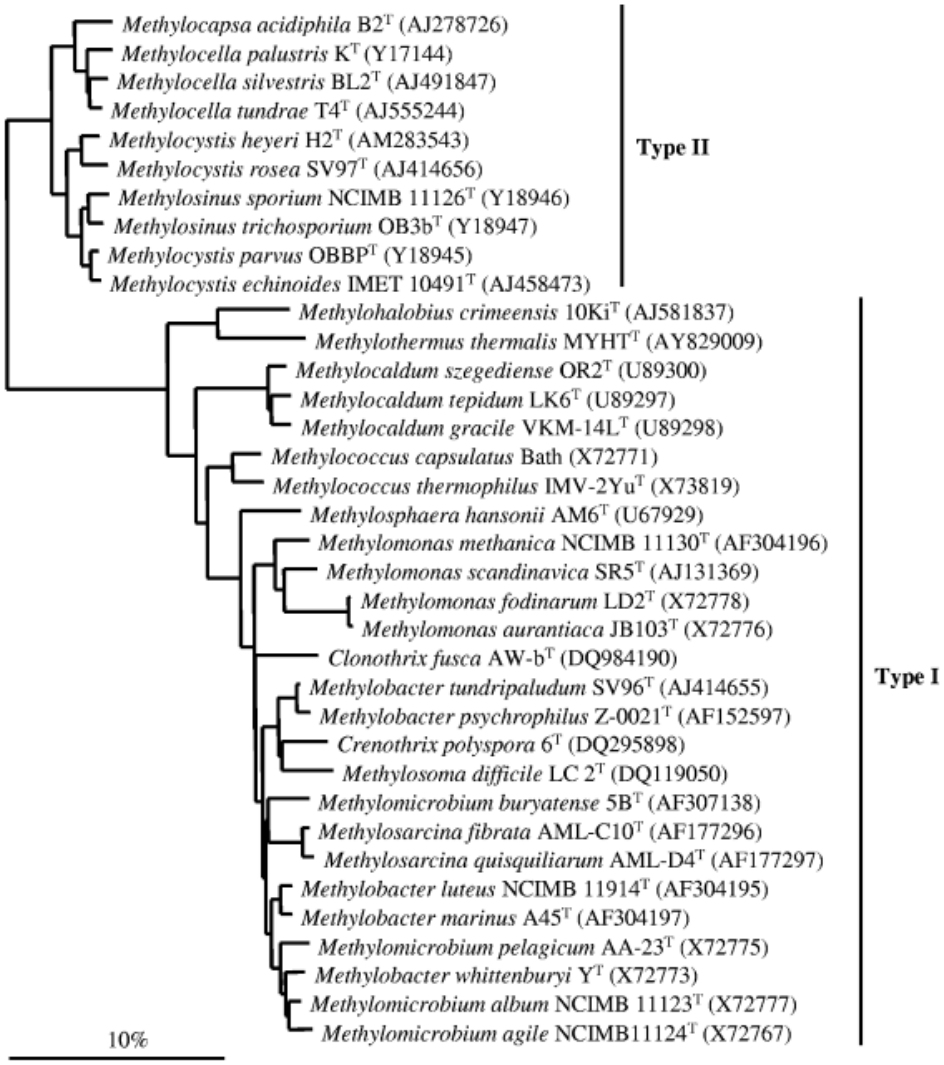


Figure 1.5: 16S rRNA gene phylogenetic tree of proteobacterial methanotrophs. There is a clear distinction phylogenetically between the Type I (*Gammaproteobacteria*) and Type II (*Alphaproteobacteria*) methanotrophs. The phylogenetic tree was constructed using the Neighbour joining method covering 1,245 nucleotide positions. (Image taken from McDonald et al., 2008).

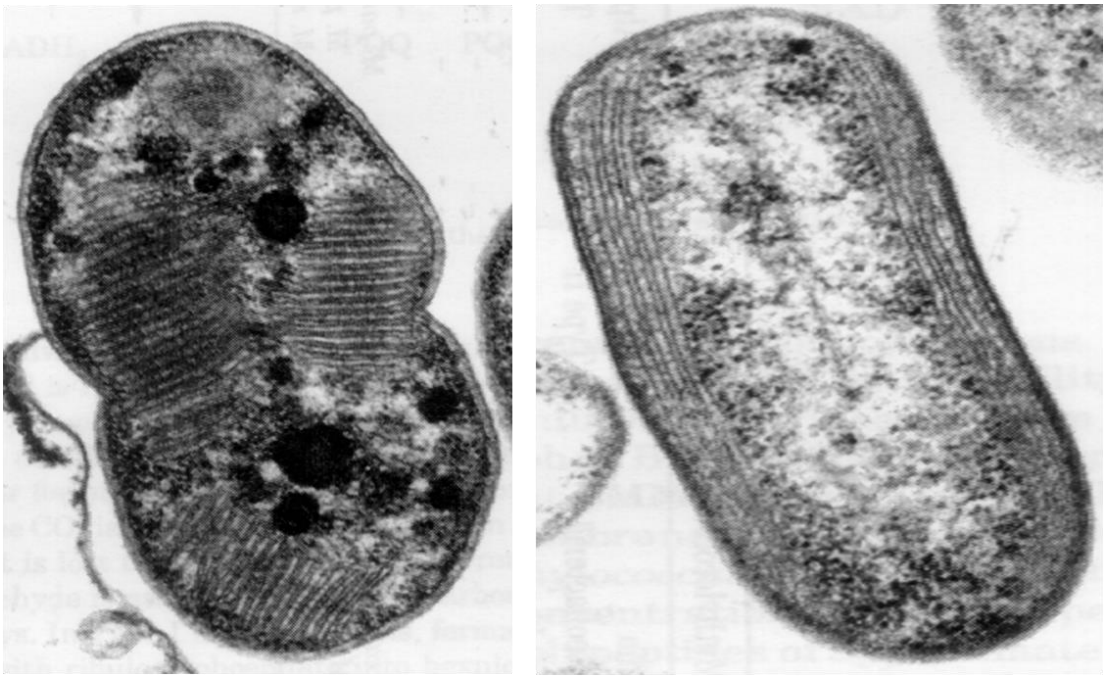


Figure 1.6: Electron micrographs showing the difference in intracytoplasmic membrane structure. Left is *Methylomonas methanica*, a Type I methanotroph, displaying stacked vesicular disc shaped membranes throughout the cell, indicative of Type I methanotrophs. Right is *Methylocystis parvus*, a Type II methanotroph, displaying intracytoplasmic membranes arranged around the periphery of the cell, indicative of Type II methanotrophs. Image from Green, (1992).

The first *Verrucomicrobial* methanotrophs *Methylacidiphilum fumarolicum* (Pol et al., 2007) and *Methylacidiphilum infernorum* (Dunfield et al., 2007) were published back to back in Nature due to the substantial importance of the finding of methane oxidation in a new phylum. *Methylacidiphilum fumarolicum* was isolated from a sample of mud at the Solfatara volcano in Italy, and the *Methylacidiphilum infernorum* was isolated from a geothermal field on the North island of New Zealand (Pol et al., 2007; Dunfield et al., 2007). These *Methylacidiphilum* species are extremophiles able to oxidise methane at pH values as low as 1.0, and grow at temperatures in excess of $50^{O}C$. A further *Verrucomicrobial* isolate, *Methylacidiphylum kamchatkense*, was isolated from an acidic hot spring in Russia (Islam et al., 2008). *Methylacidiphilum infornorum* and *fumarolium* both lack the genes required for the ribulose monophosphate and serine cycles, the two formaldehyde assimilation pathways. It was found that *Methylacidiphilum* contained the genes required to produce a fully functional ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme and a full set of genes for the Calvin-Benson-Bassham (CBB) cycle for the fixation of CO_2 by Khadem et al., (2011). Khadem et al., (2011) demonstrated that *Methylacidiphilum* was able to grow autotrophicaly with CO_2 as carbon source, whilst using methane as an energy source only.

1.3.2 Ecology of methane oxidising bacteria

Methane oxidising bacteria are ubiquitous in the environment. They have been found in landfill cover soil, coal mines, forest soil, peat bogs, rice paddies, wetlands and in fresh and ocean water sediment (Hanson and Hanson, 1996) (Dedysh et al., 1998).

They have also been found in more extreme environments such as volcanic mud, geothermal soils, hot springs, alkaline lakes and arctic wetland soil (Op den Camp et al., 2009; Wartiainen et al., 2006; Lin et al., 2004). Most known species of methanotroph tend to be mesophilic in nature but those isolated from more extreme environments tend to be more thermophilic, psychrophilic, acidophilic, alkalophilic or halophilic. Methanotroph distribution is dependent on several environmental factors including substrate availability, moisture, temperature and pH (Amaral and Knowles, 1995). Thermophilic methanotrophs include *Methylococcus capsulatus* Bath isolated from the thermal waters of a Roman bath house (Whittenbury et al., 1970), and *Methylothermus thermalis* isolated from a hot spring in Japan (Tsubota et al., 2005). *Methylomonas*, *Methylobacter*, *Methylosinus* and *Methylocapsa* species were all identified from alkali soda lake sediments reaching pH of 9.5 (Lin et al., 2004). *Methylohalobius crimeensis* was isolated from a hypersaline lake in the Crimean Peninsula of the Ukraine and is halophilic. Psychrophilic methanotrophs can grow at temperatures as low as 5 °C, and include *Methylomonas scandinavica* isolated from a sample of deep igneous rock in Sweden (Kalyuzhnaya et al., 1999) and *Methylobacter tundripaludum* isolated from arctic wetland soil (Wartiainen et al., 2006). The extreme acidophilic *Verrucomicrobial* methanotrophs of the genus *Methylacidiphilum*, that can grow as low as pH 1.0, were isolated from a geothermal field in New Zealand and volcanic mud pool in Italy (Pol et al., 2007; Dunfield et al., 2007).

1.3.3 Bacterial oxidation of methane

1.3.3.1 Overview of bacterial methane oxidation

The aerobic bacterial methane oxidation pathway involves converting methane through to the central intermediate, formaldehyde, which can then be assimilated or further oxidised to CO_2 (Hanson and Hanson, 1996; Trotsenko and Murrell, 2008). Methane can be oxidised anaerobically by some bacteria (Knittel and Boetius, 2009), but this process is not considered further here as this study is focused on aerobic oxidation of methane. Methane is initially oxidised to methanol by the enzyme methane monooxygenase, of which there are two types, a particulate methane monooxygenase (pMMO) and a soluble methane monooxygenase (sMMO) (Hanson and Hanson, 1996; Trotsenko and Murrell, 2008) (**Figure 1.7**). Methanol is further oxidised to formaldehyde via the enzyme methanol dehydrogenase. It was suggested by Myronova et al., (2006) that methanol dehydrogenase could be the electron donor for pMMO with direct transfer between the docked enzymes. Formaldehyde can be assimilated into biomass at this oxidation level via the RuMP cycle or the serine cycle. Alternatively, the formaldehyde may be further oxidised to formate via the enzyme formaldehyde dehydrogenase. Formate is then oxidised into CO_2 by the enzyme formate dehydrogenase.

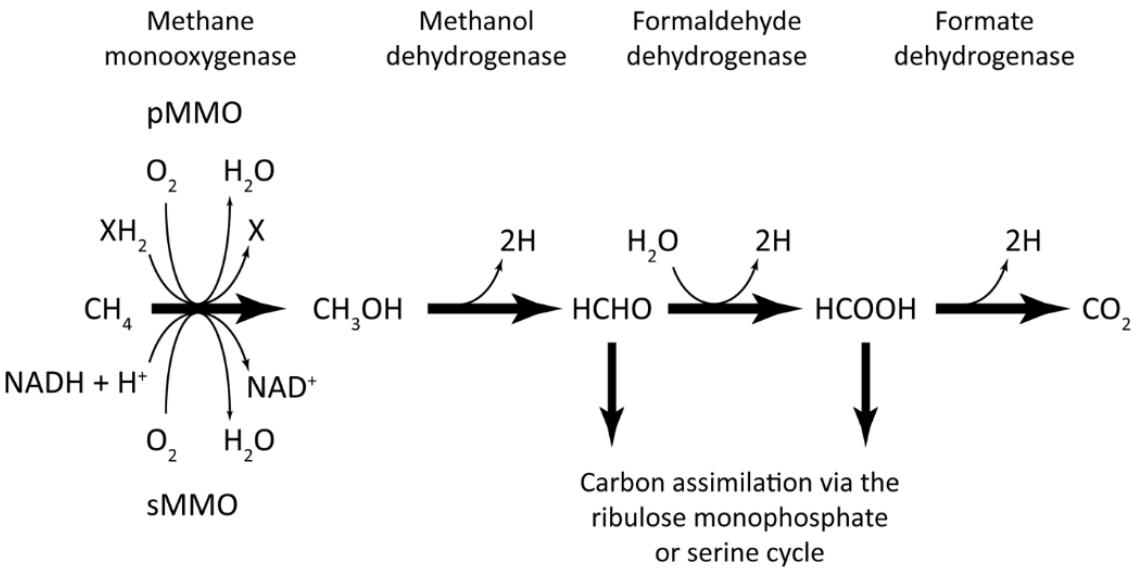


Figure 1.7: Methane oxidation pathway. pMMO requires an electron donor represented by X while sMMO receives electrons via NADH, Crombie, (2011).

1.3.3.2 Particulate methane monooxygenase

Particulate methane monooxygenase is known to be present in all known methane oxidising bacteria, with the exception of *Methylocella* and *Methyloferula* (Theisen et al., 2005; Vorobev et al., 2011). This enzyme is membrane bound and, as such, has been relatively little studied due to the difficulties in isolating the protein in its active form compared to the sMMO, which is much easier to purify from whole cells. Smith and Dalton, (1989) made attempts to isolate pMMO and were successful in isolating the protein from *Methylococcus capsulatus* Bath. Improved solubilisation protocols enabled the whole pMMO complex to be purified. The complex consisted of two

components, a putative reductase composed of 8 and 63 kDa proteins and a hydroxylase component comprised of 3 subunits; α (47 kDa), β (24 kDa) and γ (22 kDa) (Basu et al., 2003; Zahn and DiSpirito, 1996). The genes encoding the pMMO can all be found as a single operon and always in the order *pmo*CAB and are controlled by a σ^{70} promoter directly upstream of the gene cluster (Gilbert et al., 2000) (**Figure 1.8**).

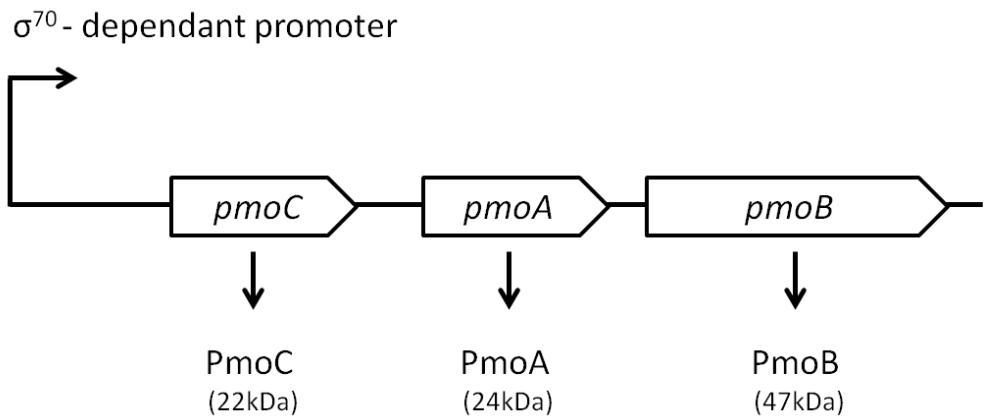


Figure 1.8: Arrangement of the pMMO gene cluster consisting of the genes *pmoC, pmoA* and *pmoB.* (Adapted from Gilbert et al., 2007).

The particulate methane monooxygenase is a copper containing enzyme and has been shown to have from 2 to 15 copper ions bound per αβγ complex (Yu et al., 2003; Lieberman et al., 2003; Basu et al., 2003; Zahn and DiSpirito, 1996). In 2005, Lieberman and Rosenzweig, (2005) determined the crystal structure of the particulate methane monooxygenase of *Methylococcus capsulatus* Bath to a resolution of 2.8 Å. The enzyme was determined to be a trimeric structure consisting of a $\alpha_3\beta_3\gamma_3$ polypeptide arrangement. Further crystal structures have been determined for the

pMMO of *Methylosinus trichosporium* OB3B and *Methylocystis strain* M (Hakamien et al., 2008; Smith et al., 2011).

1.3.3.3 Soluble methane monooxygenase

Soluble methane monooxygenase is a much more stable enzyme than the particulate, membrane bound enzyme. Whereas pMMO has been found in almost all methanotrophs, sMMO has only been described present in the genera *Methylococcus*, *Methylocystis*, *Methylosinus*, *Methylomicrobium*, *Methylomonas*, *Methylovulum* and is the only MMO present in *Methylocella* and *Methyloferula* (Theisen et al., 2005; Vorobev et al., 2011). The sMMO is part of the soluble diiron centre monooxygenase family (Leahy et al., 2003). The diiron centre at the active site of the sMMO has roles as both an oxygen carrier and also has a catalytic function (Trotsenko and Murrell, 2008). As the sMMO is a relatively stable enzyme and is readily purified from cell free extract, it has been the focus of many more investigations compared to pMMO. sMMO was first purified from *Methylococcus capsulatus* Bath and characterised by Dalton, (1980). The sMMO enzyme is made up of three components; the active site containing hydroxylase subunit ($\alpha_2\beta_2\gamma_2$), a NADH-dependent reductase for electron transfer to the active site and a coupling protein. The hydroxylase component containing the active site is made up of 3 subunits; MmoX (α) 60 kDa, MmoY (β) 45 kDa and MmoZ (γ) 19 kDa. The gene arrangements of the sMMO operons differ from one organism to the next, but all contain the genes for the 3 components of the enzyme (**Figure 1.9**).

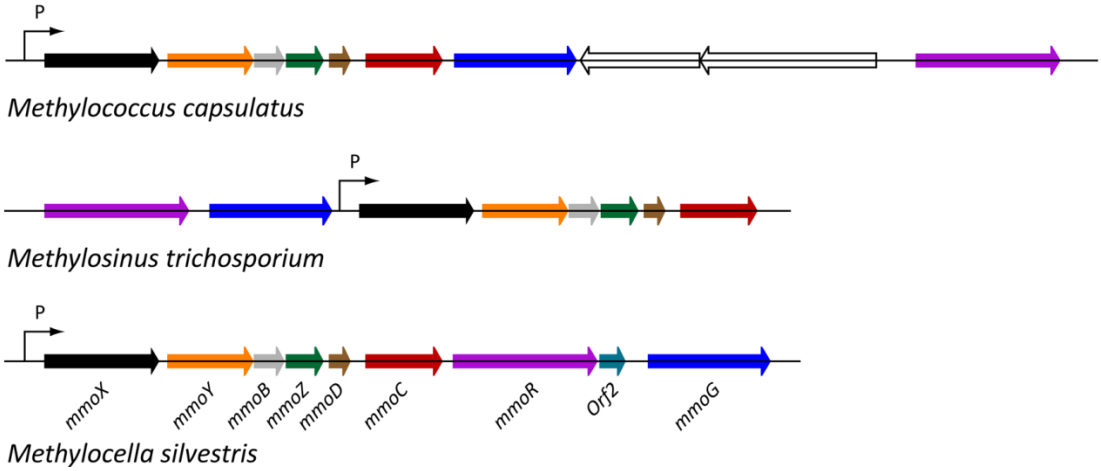


Figure 1.9: Arrangement of the genes encoding the soluble methane monooxygenase from *Methylococcus capsulatus* Bath, *Methylosinus trichosporium* OB3B and *Methylocella silvestris* BL2. Homologous genes are represented in the same colours. All operons are controlled by a σ^{54} promoter. Data taken from Csaki et al., (2003); Stafford et al., (2003) and Theisen et al., (2005). *mmoXYBZDC* encode the sMMO, *mmoR* is a transcriptional regulator and *mmoG* encodes a homologue of the chaperone GroEL (Crombie, 2011).

1.3.3.4 The copper switch

Methanotrophs that contain both the soluble and particulate methane monooxygenase genes control the expression of these genes depending on the availability of copper ions to the cell. It is the available copper-to-biomass ratio that determines whether the soluble or particulate form of the enzyme is expressed (Murrell et al., 2000). When the copper-to-biomass ratio is high, the pMMO will be expressed. Conversely, when the copper to biomass drops below a certain threshold, the sMMO will be expressed.

The pMMO was seen to be transcribed during growth under low copper-to-biomass ratios by Ali (2006), which suggests that the pMMO is constitutively transcribed under the low copper conditions. It was suggested that there is also a level of post-translational regulation. pMMO is known to have copper ions at its active site (Lieberman and Rosenzweig, 2005). Without copper ions in the pMMO active site its function is significantly reduced. The sMMO is controlled by a σ^{54} promoter which is only activated under low copper conditions. A similar repression of the sMMO is noted in *Methylocella silvestris* where sMMO transcription is repressed by the presence of other available substrates such as acetate as opposed to copper (Theisen et al., 2005).

1.3.3.5 Formaldehyde assimilation

Proteobacterial methanotrophs have two main cycles through which formaldehyde is assimilated, the Ribulose monophosphate pathway (RuMP) and the serine cycle. The *Verrucomicrobial* methanotrophs have been shown to use the Calvin-Benson-Bassham cycle for CO_2 fixation and some *Proteobacterial* methanotrophs have also been shown to have RuBisCO (Taylor et al., 1981; Khadem et al., 2011). The RuMP pathway is operational in the Type I *Gammaproteobacterial* methanotrophs (**Figure 1.10).** Three molecules of formaldehyde are used in the production of phosphoglycerate, which leads into central biosynthesis and metabolism. The serine cycle is operational in the *Alphaproteobacterial* Type II methanotrophs. Formaldehyde is fed into the serine cycle bound to tetrahydrofolate (H_4F), or via formate (Vorholt et al., 1999; Crowther et al., 2008) (**Figure 1.11**).

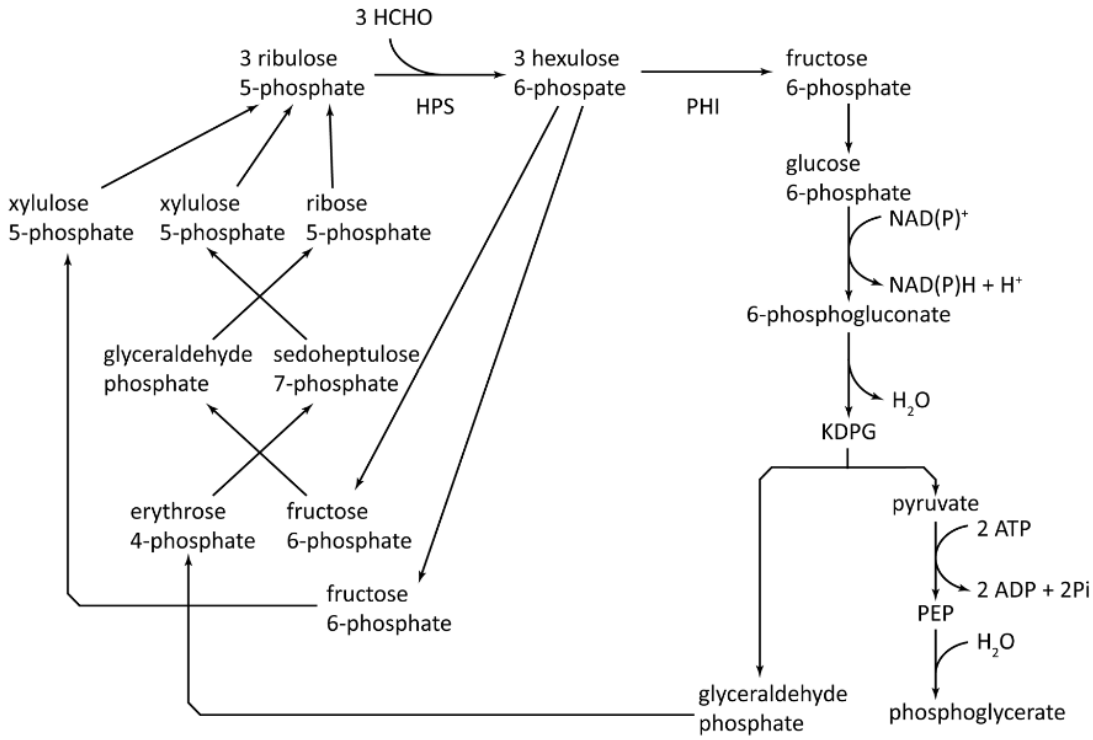


Figure 1.10: The ribulose monophosphate pathway that occurs in *Gammaproteobacterial* methanotrophs. Adapted from Anthony, (1982).

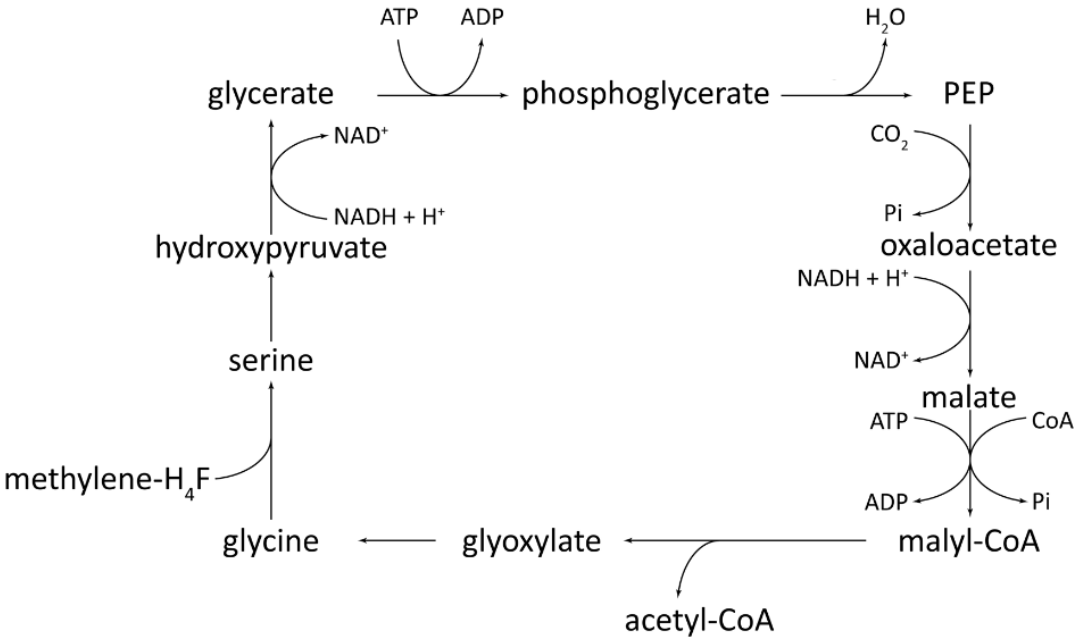


Figure 1.11: The serine cycle that occurs in *Alphaproteobacterial* methanotrophs showing the production of acetyl-CoA from methylene-H_4F (Formaldehyde bound to tetrahydrofolate). Anthony, 2011.

The RuMP and Serine cycles are elaborated on in more detail in Chapters 3 and 4.

1.4 The study of methanotrophic bacteria in the environment

In the attempt to study microorganisms and how they function and interact with their environment, many creative techniques have been developed and reiterated to accomplish this. The methods used rely on cultivation dependent, the isolation and analysis of an organisms physiology and cultivation independent techniques; these involve the retrieval and analysis of bio-markers including functional genes, proteins and lipids obtained from target communities. In most cases the use of one tool for analysing a microbial community will not provide enough robust evidence to draw

accurate conclusions due to limitations of the individual techniques and so many of the techniques are performed in a polyphasic approach to overcome this. Many of the cultivation independent techniques rely on the retrieval and sequencing of nucleic acids. Over the past few decades the emergence of metagenomics facilitated by ever improving high throughput sequencing (HTS) has emphasised how little of the microbial community in many environments was know when microbiology relied on cultivation dependant study (Sleator et al., 2008; Singh et al., 2009; Wilson and Piel, 2013).

1.4.1 Cultivation dependent studies of methanotrophs

Many of the earlier studies on methane oxidising bacteria relied on the enrichment and isolation of bacteria, since obtaining genetic (DNA and RNA sequence) information was not as common, easy or cheap as it is now. One of the largest notable efforts into the isolation of methanotrophic bacteria was performed by Roger Whittenbury and colleagues particularly noted in the paper (Whittenbury et al., 1970). Whittenbury describes in detail the process of enrichment and isolation using two defined media, NMS and ANMS, both of which are still routinely used for the isolation of methanotrophs today. Numerous samples were obtained of mud and water from ponds, rivers, streams and ditches from the UK, France, Germany, Russia, North America, South America, East and North Africa and Egypt (Whittenbury et al., 1970). From this study many strains of the genera *Methylosinus*, *Methylocystis*, *Methylomonas*, *Methylobacter* and *Methylococcus* were isolated. Notably two of the isolates *Methylococcus capsulatus* and *Methylosinus trichosporium* OB3B have been extensively studied by several research groups since their isolation.

More recently, cultivation of methane oxidising bacteria from more extreme environments have delivered methanotrophic isolates with properties that reflect their origin, including thermophilic, psychrophilic, acidophilic, alkylophilic and halophilic isolates (Tsubota et al., 2005; Lin et al., 2004; Kalyuzhnaya et al., 1999; Wartiainen et al., 2006; Pol et al., 2007; Dunfield et al., 2007). There are several advantages to isolating microorganisms; the physical limitations of the organisms can be tested by measuring growth rate and yield with different substrates and by changing the environment (pH, temperature, salinity). Enzymes can be purified and functions physically tested, including testing substrate specificity and range, co-factor requirements and optimal pH. All of these parameters will give an indication of how these isolates might function in different environments (Dalton, 1980; Grosse et al., 1999).

Cultivation also makes genome sequencing more successful as good quality high yield DNA, all from the same organism, can be purified and sequenced. It is possible to sequence genomes from single cells from the environment by applying processes such as multiple displacement amplification (Arakaki et al., 2010), but this is likely to give more erroneous sequence compared to isolation of DNA from a batch culture of a pure isolate (Binga et al., 2008). With a genome sequence of a pure isolate, one is able to identify potential metabolic versatility that may not be apparent or tested for during the isolation and characterisation process. The sequences of the genes and the arrangement of genes for particular operons can be used to determine phylogeny and possible evolution of some metabolic units. The genomes of several methanotrophs have been published (**Table 1.1**). Dam et al., 2012 sequenced the genome of *Methylocystis* sp. SC2, and then carried out a comprehensive comparison of this

genome with those of other methanotrophs. From this they were able to identify all of the common genes that were shared among the organisms. For instance, there were 1,853 homologues across the genomes of three *Methylocystaceae* methanotrophs, including *Methylocystis* sp. SC2 (Dam et al., 2012).

Table 1.1: List of published methanotroph genomes that are complete or at the final draft stage.

Organism	Accession Genbank	Stage	Reference
Methylocapsa acidiphila	CP001280 ABLP01000000	*Complete*	Chen et al., 2010
Methylocystis sp. ATCC 49242	AEVM00000000	*Final draft*	Stein et al., 2011
Methylocystis sp. SC2	HE956757	*Complete*	Dam et al., 2012
Methylocystis parvus OBBP	AJTV00000000	*Final draft*	Del Cerro et al., 2012
Methylosinus trichosporium OB3b	ADVE00000000	*Final draft*	Stein et al., 2010
Methylococcus capsulatus BATH	AE017282.2	*Complete*	Ward et al., 2004
Methylomonas methanica MC09	CP002738	*Complete*	Boden et al., 2011
Methylobacter tundripaludum SV96	AEGW00000000	*Final draft*	Svenning et al., 2011
Methylomicrobium alcaliphilum 20Z	FO082060	*Complete*	Vuilleumier et al., 2012
Methylomicrobium buryatense 5G	AOTL00000000	*Final draft*	Khmelenina et al., 2013
Methylomicrobium album BG8	CM001475	*Final draft*	Kits et al., 2013
Methylacidiphilum infernorum V4	CP000975.1	*Final draft*	Hou et al., 2008

1.4.2 Cultivation-independent studies of methanotrophs

Cultivation-independent techniques are vital for the study of organisms that are as yet uncultivated or unlikely to be cultivated with currently used methods (Hugenholtz et al., 1998). The basis of many cultivation-independent techniques is the PCR and subsequent DNA sequencing of PCR gene products and determining phylogeny from sequence variation of particular genes. The presence or absence of key functional genes can also be determined.

1.4.2.1 Phylogenetic probes

Functional gene analysis is reliant on the design of oligonucleotide probes that are specific enough to target genes of only a particular function, but have a broad enough coverage to be able to identify unknown gene sequences of enzymes that offer the same function. Functional gene probes for the enzymes involved in the methane oxidation pathway have been vital in understanding methanotroph ecology in the environment (Holmes et al., 1995; McDonald et al., 1995). Several sets of functional gene primers have emerged over the years as primer sequences have been refined to include coverage of novel sequences (Costello and Lidstrom, 1999; Bourne et al., 2001; Hutchens et al., 2004). Functional gene probes for methane monooxygenase focus on the *pmoA* gene of the pMMO and the *mmoX* gene of the sMMO. These sets of probes have been used extensively for the analysis of methanotrophs, and there are now thousands of sequences for each gene in public databases (reviewed in McDonald et al., 2008).

Phylogenetic probes are designed based on known sequences of the target gene of interest. Regions of sequence homology are identified along the target gene, and primers are designed to amplify the internal region of DNA sequence. This brings an inherent limitation when trying to identify novel homologous sequences, for example, from an environmental sample, as the unknown sequences of interest may differ in sequence at the region of primer design despite encoding a similar functioning enzyme to the target gene of interest and therefore be missed in the study. This can in some way be dealt with by designing a degree of degeneracy into the primers, but a balance needs to be made so the primers are still amplifying the original genes of interest along with any novel environmental sequences.

1.4.2.2 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was originally intended for use as a mutation detection system. This is a powerful technique that is able to distinguish Single Nucleotide Polymorphisms (SNP), based on the different bond strengths between Adenine & Guanine and Thymine & Cytosine. The DGGE technique was adapted for use in microbial ecology by Muyzer et al., (1993) and has formed the basis of many microbial ecology studies (Schäfer and Muyzer, 2001). PCR products generated from DNA isolated from the environmental samples should be able to pick up all sequences with the same function, but the sequences may vary by only a single nucleotide between two organisms. PCR products used for DGGE require the use of a "GC Clamp" at one end of the PCR product which forms a stable connection between the two strands of DNA. As the PCR products move through the DGGE gel, they will meet increasing denaturants with increasing temperature or chemical denaturant can be used. Upon

reaching a position in the gel individual PCR products will reach a sequence dependent threshold of denaturant. Here the two strands of DNA will split, but will still be connected by the GC clamp. The electrophoretic pull on the PCR product through the gel is retarded due to this split. PCR products of different sequence will reach different positions along the gel when they reach their individual denaturant thresholds, and so produce a sequence-dependent fingerprint. Sequences that are more abundant will create brighter bands as they will all be retarded at the same position. The DGGE technique has been used most extensively with environmental 16S rRNA gene PCR products for profiling bacterial communities in the environment. (Chen et al., 2007; Eller and Frenzel, 2001; Henckel et al., 2000; Henckel et al., 1999).

Functional gene DGGE has been used to specifically target methanotrophs in the environment with *pmoA* gene PCR products (Lin et al., 2005; Knife et al., 2003; Horz et al., 2001; Henckel et al., 1999) and *mmoX* gene PCR products (Iwamoto et al., 2001). McDonald et al., 2008 found that using DGGE with functional genes would sometimes result in multiple bands representing the same DNA sequence.

As with other fingerprinting techniques, there are limitations to the use of DGGE. DGGE relies firstly on DNA being extracted from a community, which can be variable depending on the method used (Starke et al., 2014). It is generally the opinion that within a given study, consistent use of the same method of DNA extraction for all samples is acceptable. This applies to all methods of DNA fingerprint analysis. DGGE is based on a PCR amplification of the target DNA that

will potentially introduce primer bias on the sequences amplified (Ishii and Fukui, 2001). There is the potential to miss dominant novel sequences if the primers used have not been designed to recognise and amplify them. DGGE being designed as a SNP mutation detection system means that any error introduced to sequences during the PCR amplification process will potentially result in the production of a second band on a DGGE, that would otherwise be in the same position of the original sequence making that individual band brighter. Retrieval of sequences from bright dominant DGGE bands can be difficult, especially in the situation where several bright bands may occur next to each other or very close to other bands. There is again another round of PCR amplification after the excision of bright DGGE bands for sequencing which can potentially introduce errors. In the event of a co-migration where two or more sequences, occupy the same position on a DGGE gel, erroneoussequences will likely be retrieved due to the sequencing of multiple target sequences at once (Gafan and Spratt, 2005). However, this can however be overcome by producing a small clone library of the originally extracted sequences and subsequent sequencing of the target sequence from the clones.

1.4.2.3 Metagenomics

Metagenomics is becoming much more popular and accessible due to the reduction in the cost of high throughput sequencing. The ever improving sequencing platforms provide more robust data sets, as sequences obtained are longer and coverage (number of sequences) of sequence gets higher. Metagenomes provide a snapshot view of potential metabolic processes that could be active within a given sample.

DNA is extracted from all organisms in an environmental sample and the metagenome is the sum of genetic information available from all of the organisms in that sample, providing sufficient coverage is achieved. Once the sequences are collected, they then need to be annotated in order to understand what processes and organisms may be present (Tyson et al., 2004). Annotation is carried out by comparing all of the metagenome sequences to sequences in reference databases. Once sequences have been annotated, the metagenomic datasets can be screened for specific genes of interest and compared between metagenomes to get a gross overview of genetic difference between different environmental communities. With the increase in popularity of metagenomic sequencing, robust analysis platforms are required. Websites such as MG-RAST provide public access to tools for the preparation, annotation and analysis of metagenomic data sets (Meyer et al., 2008). Metagenomics has been used for the analysis of microbial communities from myriad environments including a termite gut, Yellowstone hot spring, indoor urban environment, Alaskan oil facility and the oceanic dead zone (Warnecke et al., 2007; Schoenfeld et al., 2008; Tringe et al., 2008; Duncan et al., 2009; Walsh et al., 2009).

1.4.2.4 Microbial diagnostic microarrays

Microbial diagnostic microarrays are used as high throughput tools for the analysis of genes present in a sample. The first microarrays were designed to study expression of genes from genomes. The technology was then adapted for use with specific functional genes as a screening method for those genes from environmental samples. For example, functional gene microarrays were designed to test for the functional genes *nirS*, *nirK*, *amoA* and *pmoA* by Wu et al., (2001). Bodrossy et al., (2003)

designed a microbial diagnostic microarray that was designed to specifically target methane oxidising bacteria. This first microarray of 59 oligonucleotide probes covered all known sequences of *pmoA* and *amoA* genes, thus targeting methane and ammonia oxidising bacteria respectively. The *pmoA* microarray has undergone improvement with the addition of more probes targeting the *pmoA*- and *amoA*-like sequences; and in this study, a microarray of 198 probes was used. The *pmoA* microarray has been used to successfully determine the diversity of methane oxidising bacteria from a range of environments, including including saline soil, pond water and landfillcover soil (Saidi-Mehrabad et al., 2013; Bisset et al., 2011; Kumaresan et al., 2011).

The microbial diagnostic microarray, as with other fingerprinting techniques, relies on the PCR amplification of the target gene sequences of interest. This means the community observed will be dependent on the specificity of the primers being used. The primers will be designed based on current known target gene sequences, which will result in them potential not picking up novel and possibly abundant sequences. The approach used by Bodrossy et al., (2003) uses two different primer sets targeting the *pmoA* gene to somewhat compensate for these biases. Where microarrays are concerned ,there are also probe hybridisation binding efficiencies to be taken into account which could skew results (Bodrossey et al., 2003; Koltai and Weingarten-Baror, 2008).

1.4.2.5 DNA-Stable Isotope Probing (DNA-SIP)

There are studies that cover a range of stable isotope labelling techniques, probing a number of organic molecules including RNA, mRNA, protein and phospholipid fatty acids (PLFA) (Manfield et al., 2002; Dumont et al., 2011; Jehmlich et al., 2008 and Boschker et al., 1998). This study only focuses on DNA-Stable Isotope Probing (Radajewski et al., 2000). Stable Isotope Probing is a tool which is used to link microbial identity with function. A stable isotope labelled substrate such as $^{13}CH_4$ is incubated with an environmental sample. Organisms that are able to metabolise the labelled substrate will incorporate the stable isotope (^{13}C) into their cellular material, for instance DNA, during synthesis and replication. Once DNA is extracted from all of the bacteria in the environmental sample, the heavy, ^{13}C labelled DNA can be separated from the lighter (^{12}C labelled) DNA by density gradient ultracentrifugation (Radajewski et al., 2000; Neufeld et al., 2007) (**Figure 1.12**). Ideally, only the DNA from organisms able to utilise the ^{13}C labelled substrate will be in the heavy DNA, which means that targeted analysis can be carried out, focusing on the active community. The heavy and light DNA are separated by a process of fractionation. The individual fractions will contain DNA from the different buoyant densities achieved in the ultracentrifugation process. Once the DNA has been fractionated, it is ready for downstream processing, such as 16S rRNA and functional gene analysis, DGGE profiling and high throughput sequencing.

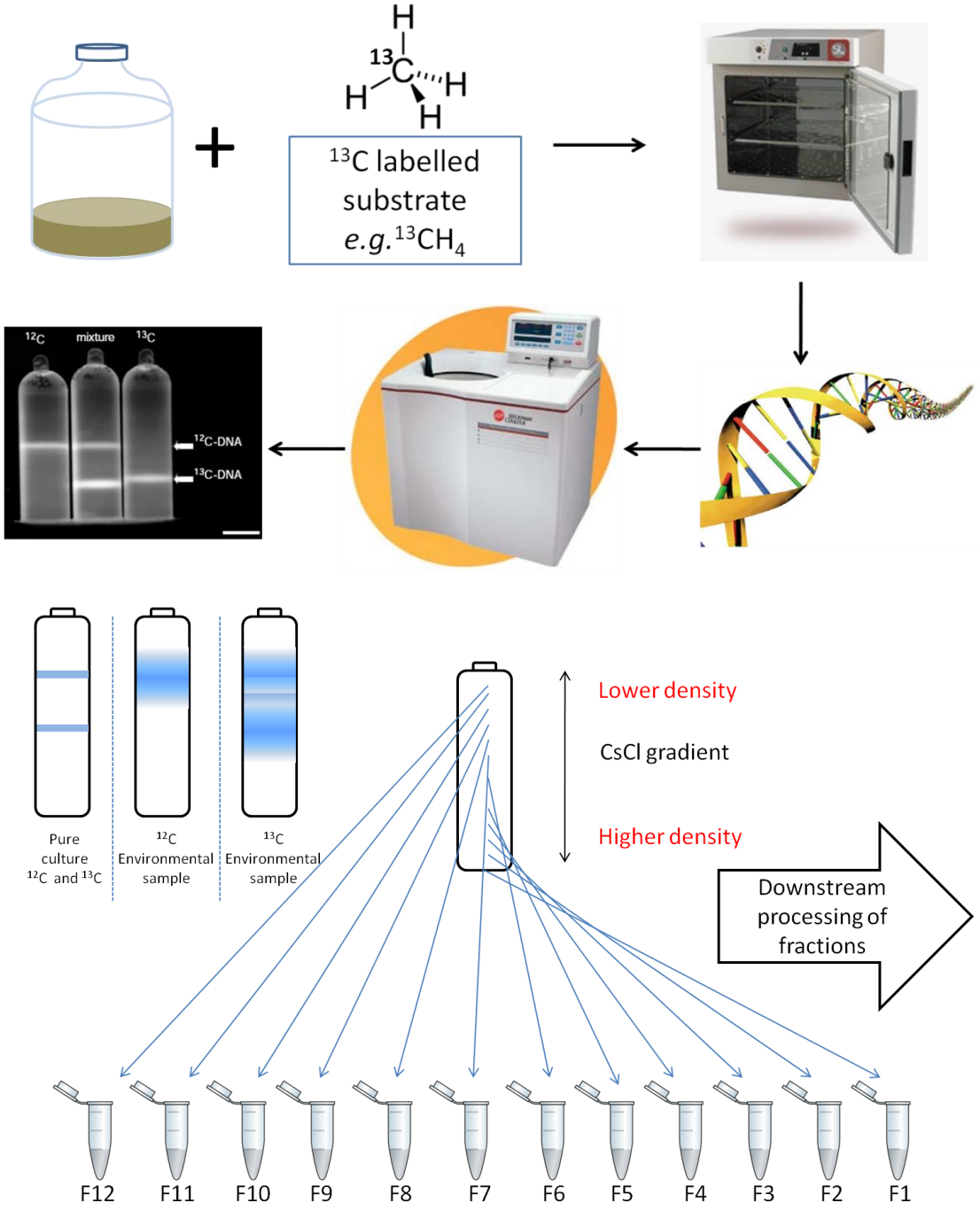


Figure 1.12: DNA-Stable Isotope Probing. An environmental sample is incubated with a ^{13}C labelled substrate. After a period of incubation, DNA is extracted and subject to density gradient ultracentrifugation. Heavy and light DNA are separated by a process of fractionation by collecting contents of the centrifuge tube as it drains from the bottom into separate collection tubes. The fractionated DNA can then be analysed.

DNA-SIP has been used to analyse organisms that can utilise a wide variety of labelled substrates including $^{13}CH_4$, $^{13}CH_3OH$, $^{13}CH_3Cl$, $^{13}CO_2$,^{13}C-acetate and $^{15}N_2$ (Hutchens et al., 2004; Neufeld et al., 2008; Borodina et al., 2005; Lu et al., 2006; Schwarz et al., 2007; Buckley et al., 2007). Using different labelled substrates and different stable-isotopes, one is able to probe different organisms within communities carrying out different functions.

With DNA-SIP requiring sufficient incorporation of an isotope label, the labelled substrate is often added in excess and much higher than what might be considered environmentally relevant. This likely alters the community of organisms observed and would otherwise be apparent in the natural environment. The heavy-isotope substrate could be metabolised differently to what would be ideal for the experimental set up. For example, when adding $^{13}CH_4$ to a microcosm, some of the methane may be fully oxidised to $^{13}CO_2$ rather than being fixed into biomass. This has a two-fold negative effect in that isotope incorporation into the target organism DNA may be reduced, leading to incomplete separation of heavy and light DNA during ultracentrifugation; secondly, that the liberated isotope label may be metabolised and incorporated into the DNA of non-target organisms.

1.4.2.6 Raman spectroscopy

Raman spectroscopy is another technique which takes advantage of organisms incorporating stable isotopes into their cellular material. It is a non-destructive tool for monitoring cellular constituents at the single cell level by analysing scattered light (Huang et al., 2007). Monochromatic light is shone onto a sample. Photons of light interact with molecules in the sample and are reflected and scattered in different

wavelengths. The wavelength of the scattered light is dependent on the bond frequency of the molecule it interacts with, which can also differ from molecule to molecule. For example, a C=O bond will reflect light of different wave lengths dependent on the other bonds that the elements are bound to, as this shifts the electron density of the molecule. If the ^{12}C=O bond becomes a ^{13}C=O bond, then the reflected light wavelength will shift predictably to another wavelength. This shift in wavelength caused by an alternative isotope is called the "Red shift" as the wave length becomes smaller. Raman spectroscopy was combined with FISH to identify single cells using fluorescence to link function with identity of organisms that had metabolised a ^{13}C labelled substrate (Huang et al., 2007).

Similarly to DNA-SIP, Raman spectroscopy relies on a certain amount of isotope label to be incorporated into the microbial biomass in order to distinguish a "Red shift" in key peak positions. The ^{13}C isotope label could of methane could again be fully oxidised to $^{13}CO_2$ and be incorporated by non-target organisms. If combining the technique with FISH to identify the organisms that have incorporated a detectable amount of label, a limitation occurs in the design of the probe. Similarly to PCR primer design, FISH probe design is often based on currently known sequence data. If cells are identified to have incorporated the isotope label, unless a FISH probe designed to target the isotope labelled cell is used, the cells' identity will remain unknown.

1.5 Project hypotheses

- There is a large diversity of Methane oxidising bacteria in Movile Cave
- Methane oxidising bacteria are active in Movile Cave
- Methane oxidising bacteria of Movile Cave are genetically similar to other known methane oxidising bacteria
- Methane oxidising bacteria in Movile Cave are primary producers providing a carbon source for organisms at subsequent trophic levels

Chapter 2

Materials and Methods

2.1 Sampling from Movile Cave

Samples of microbial floating mat and water from Movile Cave were collected by Alexandra-Maria Hillebrand Voiciulescu. The microbial floating mat was obtained by scuba-diving through to airbell 2. Samples of the microbial floating mat were obtained by resting a 1 L Nalgene bottle at the surface of the water and collection of the mat into the bottle. The microbial floating mat consisted of a ~1 mm thick large sheet of tissue paper coloured beige with orange and brown patches. The mat lost its flat shape and tore upon collection into the Nalgene bottle. Water samples were collected by dunking a 1 L Nalgene bottle approximately 5-10 cm below the surface of the water until filled. Samples used for bacterial isolation were transported from the cave to the University of Warwick at 4 °C. Sample used for DNA extraction for metagenomic and microarray analysis was frozen within 2 hours of being taken from the cave and remained frozen until processing. DNA-SIP experiments were set up inside the cave moments after sampling by Professor Colin Murrell and Dr Rich Boden.

2.2 Bacterial strains and cultivation

2.2.1 *Escherichia coli* JM109

Escherichia coli JM109 competent cells were cultivated for use when cloning PCR products. JM109 cells were obtained commercially from Promega. *E.coli* was cultivated in Lysogeny Broth (LB) and incubated at 37 °C on an orbital shaker rotating at 150 rpm. Where solid media was required, 1.5% (w/v) Bacto agar (DB Diagnostic) was added to the LB media and autoclaved. When required, the media was supplemented with an appropriate concentration of Ampicillin.

2.2.2 *Methylomonas* LWB

Methylomonas sp. Strain LWB was isolated from a lake water sample from Movile Cave. LWB has had its genome sequenced, and the strain was used for the carbon cross-feeding experiment. LWB was isolated on a modified Ammonium Nitrate Mineral Salts media (mANMS) (Tsubota et al., 2005), and subsequently maintained on Nitrate Mineral Salts (NMS) (Whittenbury et al.,1970) media at 30 °C. Where solid media was required, 1.5% (w/v) Bacteriological agar, Agar No.1 (Oxoid) was added to the mANMS or NMS liquid media. *Methylomonas* was always cultivated in the presence of methane.

2.2.3 *Methylobacterium* strain

Methylobacterium Strain. Movile was isolated by Daniela Wischer from a sample of microbial floating mat from Movile Cave. The *Methylobacterium* strain was used for the carbon cross-feeding experiment. *Methylobacterium* was isolated on Dilute Basal Salts (DBS) media and subsequently maintained on NMS media at 30 °C. Where solid media was required, 1.5% (w/v) Bacteriological agar, Agar No.1 (Oxoid) was added to the mANMS or NMS liquid media. *Methylobacterium* was always cultivated in the presence of methanol unless otherwise stated.

2.2.4 *Methylococcus capsulatus* Bath

Methylococcus capsulatus Strain Bath was obtained from the University of Warwick culture collection. DNA extracted from *Methylococcus* was used as a positive control for most PCRs. *Methylococcus* was maintained on NMS media in the presence of methane at 45 °C.

2.3 Media and solutions

All chemicals used for media preparation were obtained from Sigma Aldrich (UK).

2.3.1 DBS

Per litre:

$(NH_4)_2SO_4$	0.5 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$CaCl_2 \cdot 2H_2O$	0.05 g
K_2HPO_4	0.11 g
KH_2PO_4	0.085 g

Add 1ml/L thiothrix trace elements solution after autoclaving.

2.3.2 Thiothrix trace elements solution

Per litre:

Na-EDTA	50 g
NaOH	11 g
$ZnSO_4 \cdot 7H_2O$	5 g
$CaCl_2 \cdot 2H_2O$	7.34 g
$MnCl_2 \cdot 6H_2O$	2.5 g
$CoCl_2 \cdot 6H_2O$	0.5 g
$(NH_4)_2MoO_4$	0.5 g
$FeSO_4 \cdot 7H_2O$	5 g
$CuSO_4 \cdot 5H_2O$	0.5 g

The solution is filter sterilised before use

2.3.3 NMS

Solution I (10X)

Per litre:

$MgSO_4 \cdot 7H_2O$	10 g
$CaCl_2 \cdot 2H_2O$	2 g
KNO_3	10 g

Solution II (1000X)

Per litre

Fe-EDTA	3.8 g

Solution III (1000X)

Per litre

$NaMoO_4 \cdot 6H_2O$	0.26 g

Solution IV (1000X)

Per litre

$CuSO_4 \cdot 5H_2O$	0.2 g
$FeSO_4 \cdot 7H_2O$	0.5 g
$ZnSO_4 \cdot 7H_2O$	0.4 g
H_3BO_3	0.015 g
$CoCl_2 \cdot 6H_2O$	0.05 g
Na-EDTA	0.25 g
$MnCl_2 \cdot 4H_2O$	0.02 g
$NiCl_2 \cdot 6H_2O$	0.01 g

Solution V (100X)

Per litre

KH_2PO_4	26 g
$Na_2HPO_4 \cdot 12H_2O$	71.6 g

NMS is made by autoclaving a 1X solution of I-IV together and 100X solution V separately. Autoclaved solution V (10 ml) is added to 990 ml of autoclaved 1X solution I-IV.

2.3.4 mANMS

Solution I

Per litre

KNO_3	0.25 g
NH_4Cl	0.25 g
$MgSO_4 \cdot 7H_2O$	0.4 g
$CaCl_2$	0.1 g
Fe-EDTA	0.19 mg
$NaMoO_4 \cdot 6H_2O$	0.13 mg

Solution II

Per litre

KH_2PO_4	13 g
$NaHPO_4$	35.8 g

Solution I and II are autoclaved separately. Once cool, 10 ml of solution II is added to 989 ml of solution I. 1 ml of thiothrix trace elements solution was also added to the medium.

2.3.5 LB

Per litre

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

pH is adjusted to 7.4 before autoclaving.

2.3.6 Tris-EDTA (TE) buffer

Tris-HCL	10 mM
Na_2HPO_4	1 mM

The solution is prepared from 1 M Tris-HCl (pH8) and 0.5 M Na_2EDTA (pH8)

2.3.7 SET buffer

EDTA	40 mM
Tris-HCl, pH9	50 mM
sucrose	0.75 mM

2.3.8 Lysozyme solution

Sterile water	990 µl
lysozyme	9 mg
1 M Tris-HCl, pH 8	9 µl

2.3.9 SDS solution

SDS	10 %

2.3.10 Proteinase K solution

sterile water	950 µl
proteinase K	20 mg
1 M Tris-HCl, pH 8	50 µl

2.3.11 Ammonium acetate solution

NH_4acetate	7.5 M

2.4 DNA purification

DNA was extracted by enzymatic lysis and the presence of SDS (Neufeld et al., 2007a), or in some cases using the FastDNA SPIN Kit for soil from MP Biomedicals (UK).

2.4.1 DNA Extraction

DNA was extracted from all biological samples using the following protocol unless otherwise stated. The DNA extraction method was modified from the Neufeld et al., (2007) protocol by using twice as much SDS to maximise DNA recovery.

Biomass was collected at the bottom of a 15 ml falcon tube and re-suspended in 1.6 ml of SET buffer. To this, 180 µl of 9 mg/ml lysozyme was added and the sample incubated at 37 °C for 30 minutes. Next, 400 µl of 10 % SDS and 55 µl of 20 mg/ml proteinase K was added. The sample was then incubated at 55 °C for 2 hours. Following incubation another 1 ml of SET buffer was added to the sample and mixed

by inversion. The sample was then transferred to a Maxtract™ High Density phase lock tube (Qiagen, UK). 3 ml of Phenol:Chloroform:Isoamylalcohol (25:24:1) was added to the phase lock tube and the sample was inverted until it appeared milky white. The tube was then centrifuged at 6500 *g* for 10 minutes. The addition of 3 ml of Phenol:Chloroform:Isoamylalcohol (25:24:1) and centrifugation at 6500 *g* for 10 minutes was then repeated. Next, the addition of 3 ml of Chloroform:Isoamylalcohol to remove any residual phenol was carried out along with another centrifugation step at 6500 *g* for 10 minutes. The top liquid layer above the white phase lock matrix was then transferred to a new 15 ml falcon tube. DNA was precipitated from the sample with the addition of 5 µl of Glycogen (Roche), 1 ml of 7.5 M ammonium-acetate and 6 ml of ethanol. The sample was inverted to mix and kept at -20 °C overnight while the DNA precipitated. DNA was retrieved from the sample by centrifugation at 16,000 *g* for 20 minutes, then aspiration of the precipitation buffer. The DNA was re-suspended in water or TE buffer. DNA quality was assessed by running a sample on a 0.8% agarose gel stained with Ethidium bromide and visualised by UVtransluminesence in a GeneGenious imaging system (Syngene) and visualised using the Gene-snap software. DNA was quantified on a Nanodrop-1000.

2.5 Polymerase Chain Reaction (PCR)

PCR was carried out with the following mix of reagents per 50 µl reaction as an example of a typical set up. Variations were used when required for particular primer sets according to published protocols.

Water	36.1µl
BSA (3.4 %)	1 µl
PCR buffer (10x)	5 µl
dNTPs 25 mM	0.4 µl
Forward primer 10 mM	2 µl
Reverse primer 10 mM	2 µl
$MgCl_2$ 1.5 mM	3 µl
Taq DNA polymerase (5U/µl) (Fermentas UK)	0.5 µl

PCR reactions were performed using a Tetrad thermocylcer (Bio-Rad). Analysis of DNA fragments was carried out by electrophoresis through a 1% agarose gel. Agarose gels were analysed by UVtransluminesence in a GeneGenious imaging system (Syngene) and visualised using the Gene-snap software.

2.5.1 List of primers used

Table 2.1

Primer	Sequence	Target	Reference
27F	AGAGTTTGATCMTGGCTCAG	16S rRNA gene	Lane, 1991
907R	CCGTCAATTCMTTTGAGTTT	16S rRNA gene	Lane, 1991
341F	CCTACGGGAGGCAGCAG	16SrRNA gene	Muyzer et al., 1993
341F_ GC*	CGCCCGCCGCGCCCCGCGCCCGTCC CGCCGCCCCCGCCCGCCTACGGGAG GCAGCAG	16S rRNA gene	Muyzer et al., 1993
1492r	TACGGYTACCTTGTTACGACTT	16S rRNA gene	Lane, 1991
189F	GGNGACTGGGACTTCTGG	*pmoA*	Holmes et al., 1995
661R	CCGGMGCAACGTCYTTACC	*pmoA*	Costello and Lidstrom, 1999
682R	GAASGCNGAGAAGAASGC	*pmoA*	Holmes et al., 1995
206F	ATCGCBAARGAATAYGCSCG	*mmoX*	Hutchens et al., 2004
886R	ACCCANGGCTCGACYTTGAA	*mmoX*	Hutchens et al., 2004
M13F	GTAAAACGACGGCCAG	pGEM-T vector	Invitrogen
M13R	CAGGAAACAGCTATGAC	pGEM-T vector	Invitrogen

*The GC clamp is added to the 5' end of the primer for use in DGGE.

2.6 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed according to the protocol first described by Muyzer et al., (1993). PCR products amplified using a primer set with a GC clamp were analysed using the DCode™ universal mutation detection system (BioRad, USA). The DGGE gels were 8 % (w/v) polyacrylamide with a 30 % - 70 % linear denaturant gradient, where 100 % was equivalent to 7.0 M urea and 40 % UltraPure™ formamide (Invitrogen). Gels were run in 1 x TAE buffer for 16 hr, 80 V at 60 °C. DGGE gels were stained with 5 µL of 10 000 x SYBR® Gold in 200 ml of 1 x TAE buffer for 60 min. Gels were then imaged by UVtransluminesence in a GeneGenious imaging system (Syngene) and visualised using the Gene-snap software.

Dominant bands of interest were excised from the DGGE gel using a sterile razor blade while being viewed on a 365 nm long wavelength UV transilluminator. Excised bands were transferred to a 250 µL eppendorf tube containing 20 µL of sterile nuclease free water and incubated over night at 4 °C. A sample of the 20 µL water was then used as a PCR template for re-amplification of the target sequence. PCR products were purified using the NucleoSpin® Extract II Nucleic Acid and Protein Purification kit (Macherey-Nagel, Germany). Purified PCR products were then sequenced at the University of Warwick Molecular Biology Facility.

2.7 Cloning

Cloning was carried out using the pGEM®-T Easy Vector system for the cloning of purified PCR products (according to the manufactures instructions).

2.8 DNA sequencing

PCR amplicon sequencing was carried out at the University of Warwick molecular biology service. Between 10 and 80 ng of PCR product was combined with 5.5 ρmol primer and sequenced by Sanger sequencing. High-throughput metagenomic sequencing was carried out at the UCL genomics facility. Genome sequencing of *Methylomonas* LWB was carried out at TGAC, Norwich research park. The DNA sample was sequenced using an Illumina MiSeq benchtop sequencer. Data sets were produced for both 150 bp and 250 bp paired end reads. A combined assembly of both data sets were used to produce a genome scaffold. The assembled data were then uploaded to the RAST website for annotation and analysis (http://rast.nmpdr.org).

2.9 Phylogenetic analysis

Phylogeny of sequences was determined by alignment by ClustalW with BLAST hits obtained from the GenBank database using Mega5 (Tamura et al., 2011). Phylogenetic trees were constructed using the Neighbour-joining method with bootstrap values calculated with 1000 replicates in all cases.

2.10 Metagenomic Analysis

Metagenomic data was uploaded to the MG-RAST website (http://metagenomics.anl.gov/) for analysis (Meyer et al., 2008). The analysis pipeline first involved de-replication of sequences to remove artificial duplicate reads that can occur due to artefacts during the sequencing process. Sequences containing five or more consecutive ambiguous base pairs were removed. A step of gene calling was then implemented which predicts coding DNA sequence from sequences of 75 base pairs or longer using the inbuilt tool “FragGeneScan”. The amino acid

sequences were then determined and clustered, using the uclust component of QIIME, on a basis of 90% sequence similarity, maintaining relative abundances for function abundance comparison. Protein identifications were determined for representatives of each cluster using the sBLAT component of the BLAT algorithm. Sequence similarities were identified by comparison to the M5nr non redundant protein database which contains identities from a range of integrated databases including, but not limited to, GenBank, IMG, KEGG, EBI and NCBI. Abundance profiles were generated for all functions which can then be scanned for comparison and analysis. The MG-RAST website was used to search the Movile Cave metagenome for genes of interest by keyword search.

2.11 Genome Analysis

A DNA sample from *Methylomonas* LWB was sequenced using an Illumina MiSeq benchtop sequencer. Data sets were produced for both 150 bp and 250 bp paired end reads. A combined assembly of both data sets were used to produce a genome scaffold. The assembled data were then uploaded to the RAST website for annotation and analysis (http://rast.nmpdr.org). Further analysis was also carried out by uploading the assembled genome to the IMG genome analysis website (http://img.jgi.doe.gov/). A Microsoft Excel spreadsheet with all annotations was used to identify and manipulate genes of interest for phylogenetic analysis. Kegg recruitment plots were created based on the gene annotations of the *Methylomonas* LWB genome from the Seed viewer comparison tools. These were then used to determine complete metabolic pathways encoded by the *Methylomonas* LWB genome.

2.12 DNA-Stable Isotope Probing (DNA-SIP)

DNA-SIP was performed following the protocol described by Neufeld et al., (2007). Microcosms with a mixture of Movile Cave floating microbial mat and water were incubated in 120 ml serum vials with approximately 20 ml of sample per vial. The microcosms were incubated with either $^{12}CH_4$ or $^{13}CH_4$ in the headspace at 2.0 % (v/v). Methane consumption was monitored by gas chromatography. Samples of 5 ml of the mat and liquid were taken from the microcosm at different time points and then resealed and re-spiked with methane. DNA was extracted from the samples as described above and subject to CsCl density gradient ultracentrifugation. Fractionation of gradients was carried out by allowing the CsCl to drop from the centrifugation tube into collection tubes. Gradient formation was confirmed by measurement of the density of every fraction of CsCl using a digital refractometer (Reichert AR2000, Reichert Analytical instruments, USA). DNA was precipitated from all fractions as described by Neufeld et al., (2007). DNA from every fraction was then frozen at -20 °C for analysis.

2.13 Gas chromatography

Gas chromatography was used for measuring CH_4 concentration from bacterial cell cultures and microcosms. An Agilent 6890 instrument was used with a Porapak Q column with N_2 carrier gas flowing at 20 ml min^{-1}.The temperature set up was; injector 150 °C, column 125 °C, detector 200 °C. An injection volume of 100 µl was used for all measurements.

2.14 *pmoA* microarray

The *pmoA* microarray was carried out by Dr D. Kumaresan at the CSIRO institute, Hobart, Tasmania. The microarray consisted of 198 probes covering all known methanotroph sequences. The analysis was performed as stated by Stralis-Pavse et al., (2004).

Chapter 3

Analysis of Movile Cave Microbial Community

3.1 Metagenome of Movile Cave microbial floating mat

Metagenomics is a tool being used by the microbial ecology community to gain an insight into microorganisms and their genetic potential in an ecosystem. One of the key advantages of using the metagenomics approach is that it eliminates PCR primer bias (Wooley et al., 2010). A limitation to the technique is that it can only display genetic potential and it does not give evidence for the function of each individual bacterium (Takami et al., 2012). However, despite this limitation, metagenomic datasets can be important for formulating hypotheses about microbial diversity and function in a given environment. If one knows the genes that are present, one can make assumptions about possible functions occurring in the environment which can then be tested. Large noteworthy metagenomics studies include the Sorcerer II Global Ocean Sampling Expedition (http://collections.plos.org/plosbiology/gos-2007, a collection of papers from the Venter institute) and The Human Microbiome Project (http://commonfund.nih.gov/Hmp/, a collection of works funded by the NIH). In this study, a metagenomic data set from the DNA extracted from microbial mats floating in the Movile Cave water was produced and analysed in order to create hypotheses for further research in this project.

The purpose of this metagenome was to identify the methane oxidising bacterial community from a non-enriched sample of microbial floating mat that had been frozen shortly after leaving the cave. This would be the best opportunity to get a snapshot of the methanotroph community as it exists in the cave environment.

Method

A sample of microbial mat and water was retrieved from air bell 2 of Movile Cave and was frozen at -20 °C two hours after being harvested. The sample remained frozen at -20 °C for three weeks until processing. DNA was extracted from the microbial mat following the protocol described in section 2.4.1. (Neufeld et al., 2007/sterivex method). An aliquot of the extracted DNA was run on an agarose gel (0.7 % agarose) to determine quality, while quantity was determined using a Nano-drop 1000 Spectrophotometer.

The DNA extracted (20 µg) was subsequently sent to the UCL Genomics sequencing facility for analysis. 500 ng of the DNA was sheared using a Corvis S2 focused-ultrasonicator. The sequence library was prepped using the Roche Titanium Rapid Library kit (http://454.com/downloads/my454/documentation/gs-flx-plus/Rapid-Library-Preparation-Method-Manual_XLPlus_May2011.pdf). Quality of the library was assessed on the Bioanalyser and then sequenced using Titanium chemistry on the two large regions of a PTP on a Roche 454 GS FLX. Image and signal processing were carried out using GS software v2.5.3. Sequence data were analysed using the MG-RAST online metagenomics analysis server and pipeline (Meyer et al., 2008). The analysis pipeline first involved de-replication of sequences to remove artificial duplicate reads that can occur due to artefacts during the sequencing process. Sequences containing five or more consecutive ambiguous base pairs were removed. A step of gene calling was then implemented, which predicts coding DNA sequence from sequences of 75 base pairs or longer using the inbuilt tool “FragGeneScan”. The amino acid sequences were then determined and clustered, using the uclust

component of QIIME, on a basis of 90% sequence similarity, maintaining relative abundances for function abundance comparison. Protein identifications were determined for representatives of each cluster using the sBLAT component of the BLAT algorithm. Sequence similarities were identified by comparison to the M5nr non redundant protein database, which contains identities from a range of integrated databases including, but not limited to, GenBank, IMG, KEGG, EBI and NCBI. Abundance profiles were generated for all functions, which can then be scanned for comparison and analysis.

Results

There were a total of 1,326,733 sequences analysed from this run. The mean sequence length was 343 ±105 bp with mean mol % GC content of 51 % ±11 %. After quality control there were 960,943 sequences remaining with a mean length of 360 ±89 bp with a mean GC mol % content of 50 % ±11 %. After processing, the sequences were assigned as such; 36.8 % annotated proteins, 33.7 % unknown proteins, 27.6 % failed quality control and 1.9 % represented ribosomal sequences (**Figure 3.1**). Of the annotated sequences, 96.5 % were of bacterial origin with 1.8 % being eukaryotic, 1.3 % being from archaea and 0.2 % from viruses.

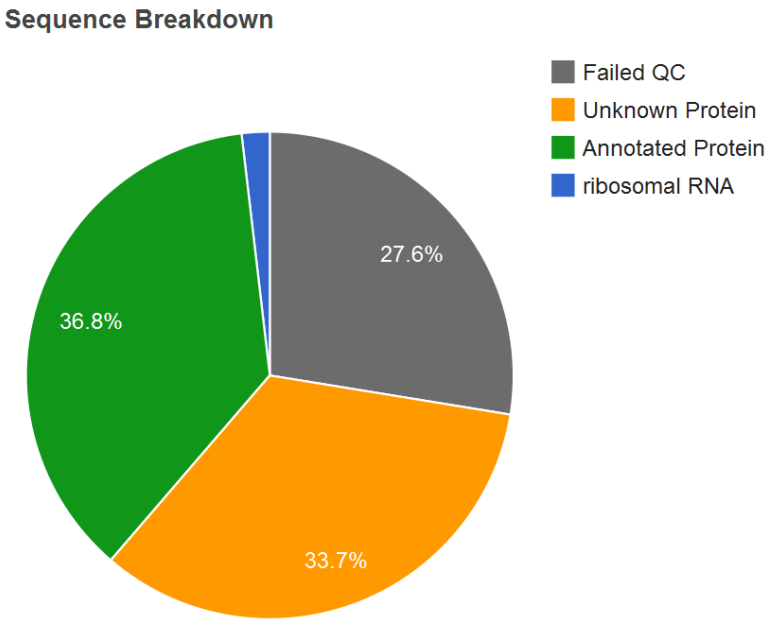


Figure 3.1: A pie chart representing the distribution of sequences after quality control.

After processing and annotation of the sequences with the M5NR database with more stringent annotations, 85 % appeared to be bacterial, 1 % archaeal, 1 % eukaryote, with the remaining being unassigned or other sequences (**Figure 3.2**).

At the phylum level, the DNA sequences were dominated by sequences from *Proteobacteria*, covering 60.2 % of the total annotated sequences followed by bacteroidetes at 12.1 % and firmicutes at 7.6 % (**Table 3.1 Figure 3.3**). The small proportion of archaea are dominated by euryarchaeota (**Figure 3.4**). Surprisingly 2.72 % of the sequences represent cyanobacterial sequences, which would not be expected in an environment such as Movile Cave where there is no light. The cyanobacterial sequences identified here could be close relatives of the canonical photosynthetic

cyanobacteria surviving by utilizing different metabolic strategies other than photosynthesis. The presence of such a large proportion of cyanobacterial sequences may also be an artefact of poorly annotated sequences in reference databases or an incorrect annotation of the sequence. While there is a possibility for contamination in the environment during sampling trips into the cave, the likelihood of survival and proliferation of cyanobacteria in this environment would be limited but not imposible.

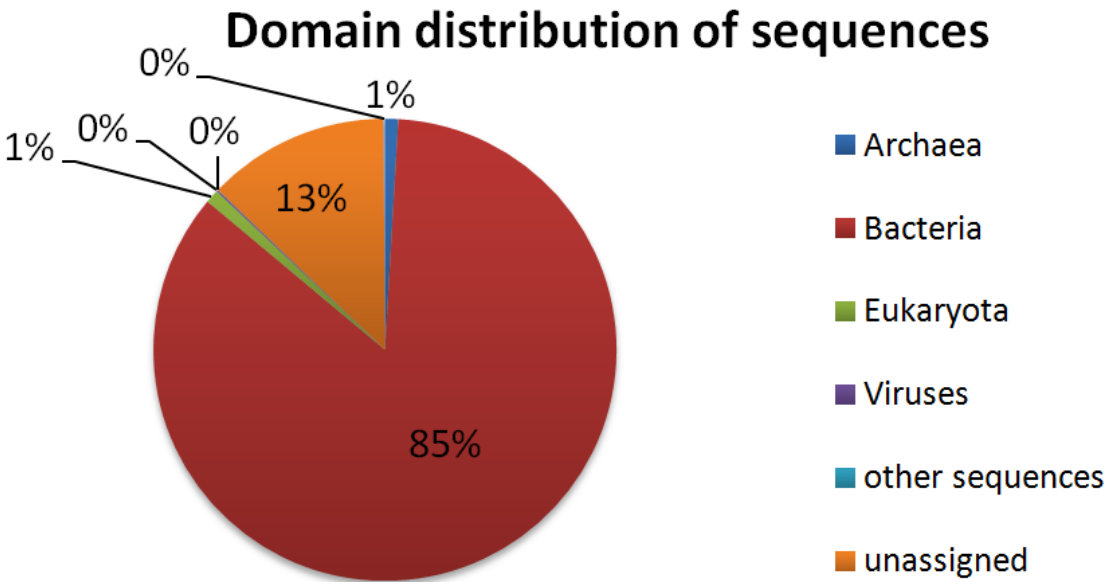


Figure 3.2: Distribution of sequences at the domain level with annotations predicted using the M5NR MG-Rast multi database annotation system. A maximum e-value cutoff of 1 x 10^5 was used with a minimum % identity cut off of 60 % and a minimum alignment length of 15.

Phylum	Number of representative sequences	Percentage of total annotated sequences
Acidobacteria	7905	0.84
Actinobacteria	28045	2.97
Annelida	5	0.00
Apicomplexa	358	0.04
Aquificae	2818	0.30
Arthropoda	2079	0.22
Ascomycota	2525	0.27
Bacillariophyta	216	0.02
Bacteroidetes	114020	12.06
Basidiomycota	499	0.05
Blastocladiomycota	1	0.00
Bryozoa	2	0.00
Candidatus Poribacteria	198	0.02
Chlamydiae	1306	0.14
Chlorobi	14886	1.57
Chloroflexi	14861	1.57
Chlorophyta	787	0.08
Chordata	4233	0.45
Chromerida	1	0.00
Chrysiogenetes	726	0.08
Chytridiomycota	3	0.00
Cnidaria	522	0.06
Crenarchaeota	956	0.10
Cyanobacteria	25723	2.72
Deferribacteres	1736	0.18
Deinococcus-Thermus	4585	0.49
Dictyoglomi	699	0.07
Echinodermata	156	0.02
Echiura	2	0.00
Elusimicrobia	300	0.03
Euglenida	4	0.00
Euryarchaeota	10324	1.09
Fibrobacteres	466	0.05
Firmicutes	69950	7.40
Fusobacteria	2451	0.26
Gemmatimonadetes	839	0.09
Glomeromycota	2	0.00
Hemichordata	62	0.01
Korarchaeota	81	0.01
Lentisphaerae	1908	0.20
Microsporidia	30	0.00
Mollusca	22	0.00
Nanoarchaeota	19	0.00
Nematoda	375	0.04
Neocallimastigomycota	6	0.00
Nitrospirae	2219	0.23
Phaeophyceae	109	0.01
Placozoa	58	0.01
Planctomycetes	15800	1.67
Platyhelminthes	47	0.00
Porifera	10	0.00
Proteobacteria	568691	60.17
Rotifera	9	0.00
Spirochaetes	9150	0.97
Streptophyta	3174	0.34
Synergistetes	1932	0.20
Tenericutes	673	0.07
Thaumarchaeota	137	0.01
Thermotogae	3363	0.36
Verrucomicrobia	13199	1.40
unclassified (derived from Archaea)	552	0.06
unclassified (derived from Bacteria)	3897	0.41
unclassified (derived from Eukaryota)	1855	0.20
unclassified (derived from Fungi)	20	0.00
unclassified (derived from Viruses)	2198	0.23
unclassified (derived from other sequences)	206	0.02
unclassified (derived from unclassified sequences)	1205	0.13

Table 3.1: List of annotated sequences at the phylum level.

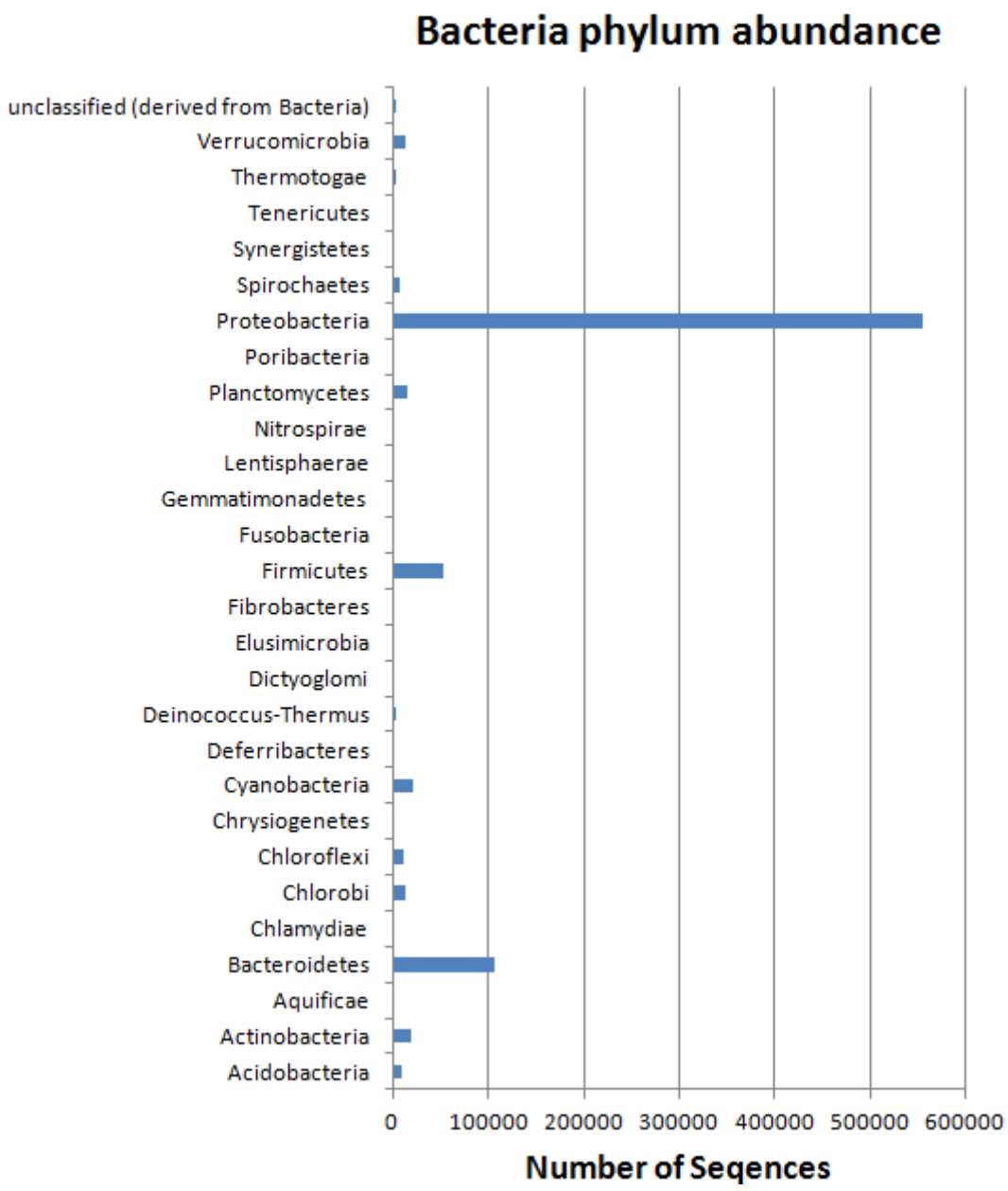


Figure 3.3: Visual representation of bacteria phyla distribution.

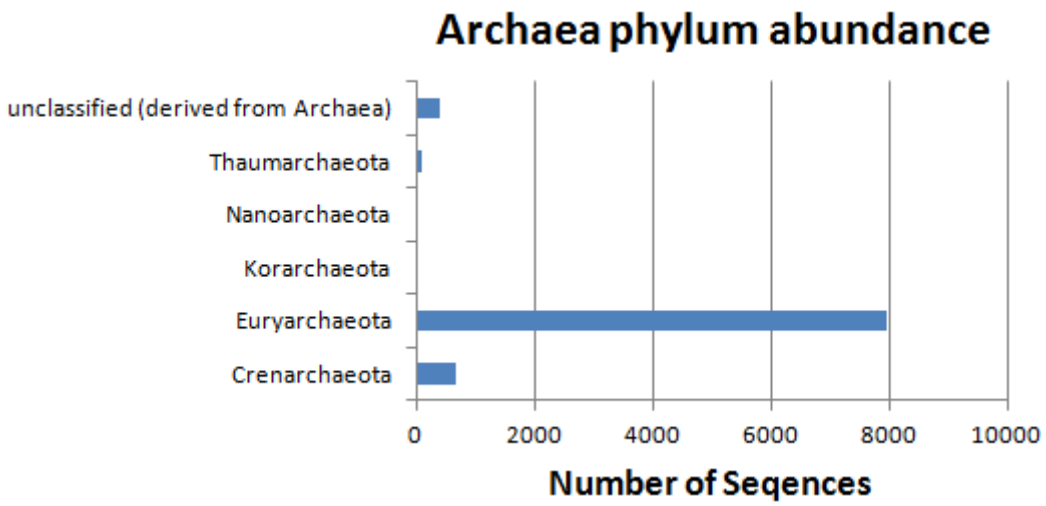


Figure 3.4: Visual representation of archaeal phyla distribution.

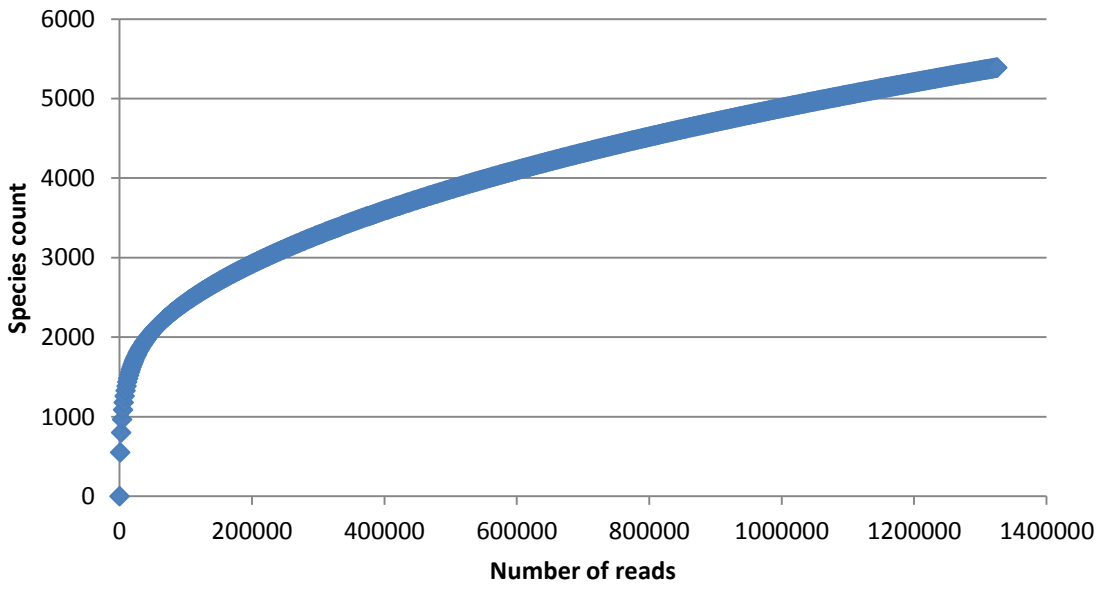
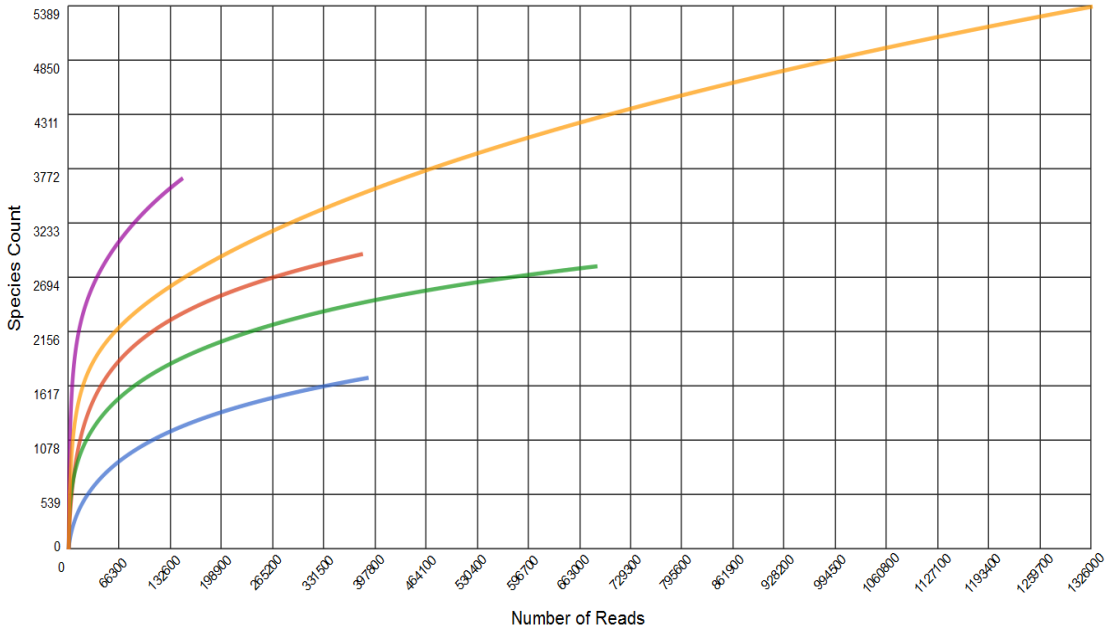


Figure 3.5: Rarefaction curve based on all annotated sequences in the metagenome and their species affiliation.

The rarefaction curve is a display of the number of new species that occur after analysing an increasing number of sequences. To be sure that all the potential species within a sample have been "discovered", one would expect the graph to plateau. In

this case, (**Figure 3.5**), the rarefaction curve is heading to a plateau but does not reach it, indicating that this sequencing effort has potentially not captured the entire community composition present in Movile Cave mat. When compared with metagenomic data from other ecosystems, the Movile Cave biofilm has a rather high microbial diversity (**Figure 3.6**). The metagenomic data from Movile Cave mats was compared with those from different environments: mine sediment, fish pond water, Antarctic water and a Galapagos mangrove. There were no other metagenomes from a similar environment to Movile Cave found in the MG RAST database.



Metagenome	Rarefaction curve
Movile Cave (4482825.3)	
Black Soudan mine sediment (4440281.3)	
Tilapia pond water (4440413.3)	
Antarctic water (Ace Lake) metagenome (4443680.3)	
Mangrove on Isabella Island (Marine water) – Ecuador (4441598.3)	

Figure 3.6: Rarefaction curves based on metagenomic data from different environments. All metagenomes were obtained from the MG-Rast public database, Accession numbers provided. Image produced using MG-Rast.

As there were no metagenomes available from environments similar to Movile Cave, the database was scanned for metagenomes from a range of different environments for comparison.

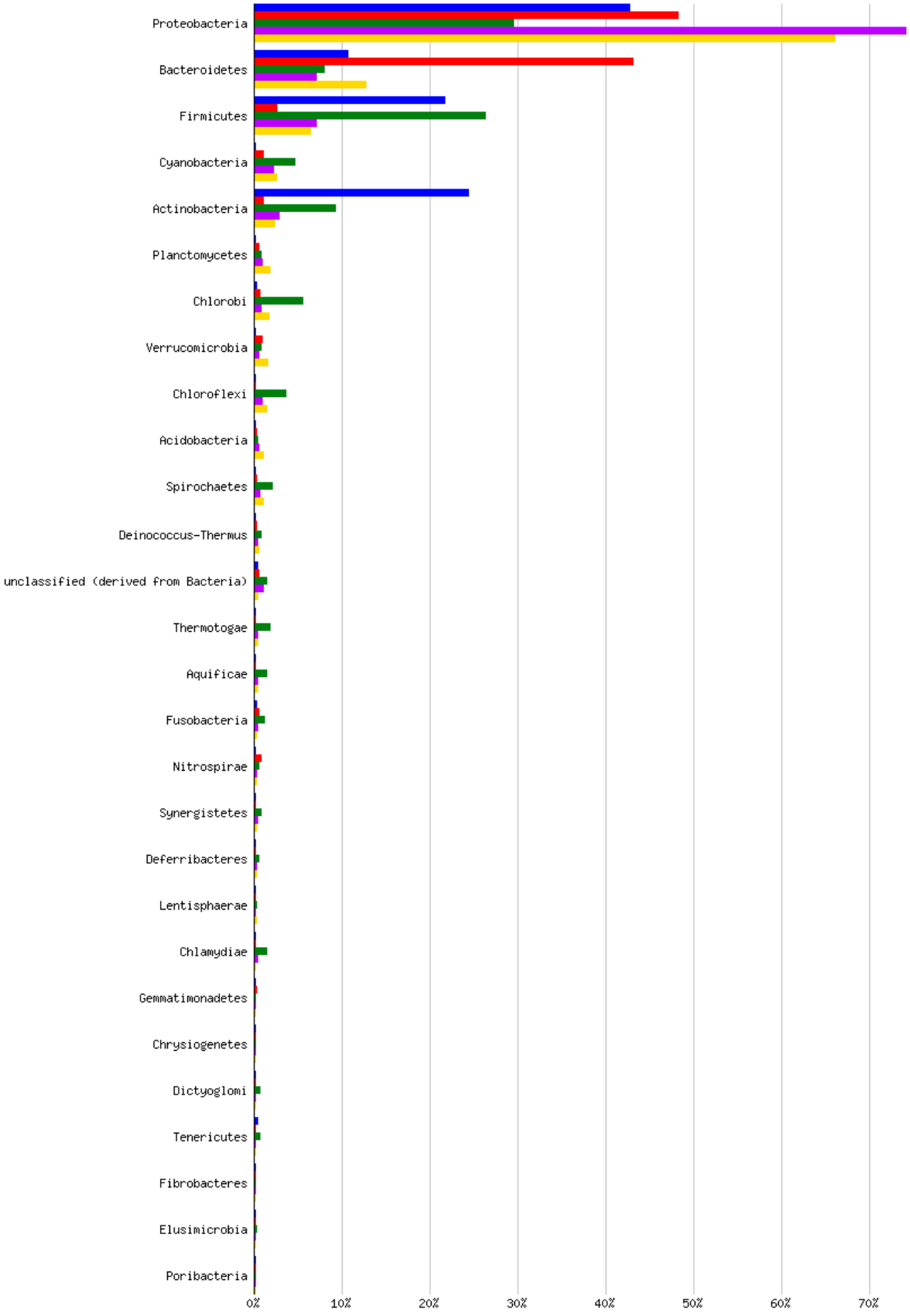


Figure legend on next page.

Figure 3.7: Relative abundance of bacterial phyla across the compared metagenomes. Colours represent the same metagenome as in the rarefaction curve above (Yellow: Movile Cave, Blue: Black Soudan mine sediment, Red: Tilapia pond water, Green: Antarctic water and Purple: Mangrove on Isabella Island).

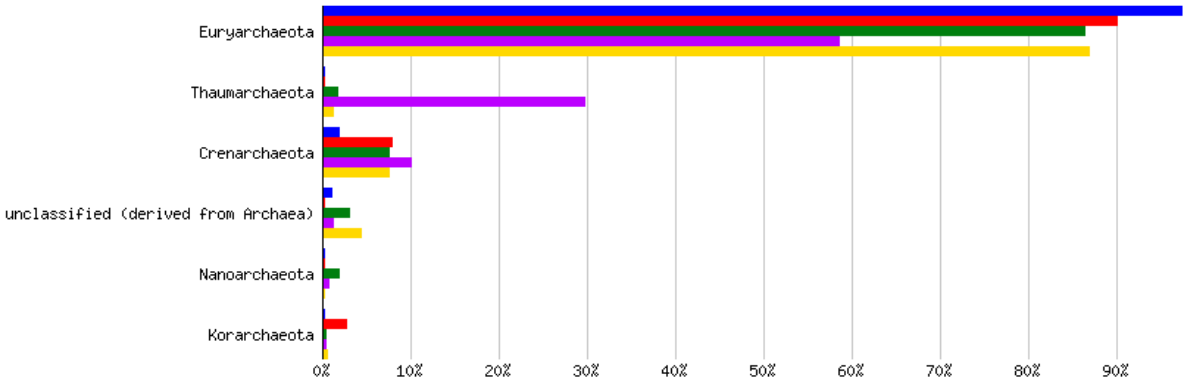


Figure 3.8: Relative abundance of archaeal phyla across the compared metagenomes. (Yellow: Movile Cave, Blue: Black Soudan mine sediment, Red: Tilapia pond water, Green: Antarctic water and Purple: Mangrove on Isabella Island).

The Movile Cave rarefaction curve is steeper than the curves of the pond, Antarctic and mine samples, showing that more organisms have been “discovered” with fewer sampled sequences, thus indicating a higher diversity of microorganisms. The rarefaction curve for the mangrove marine water sample is steeper than that of Movile Cave, but the sequence data set was not large enough to discover more species than was found within the Movile Cave metagenome.

All of the metagenomes compared at the relative abundance of bacterial phyla level shared similar profiles with proteobacteria being the most abundant across all metagenomes, with the mangrove metagenome (4441598.3) having the highest percentage of the metagenome being represented by proteobacteria. The Black Soudan mine sediment (4440281.3) and the Antarctic water (4443680.3) metagenomes were similar in that, compared to the other metagenomes, they had relatively high abundances of firmicutes and actinobacteria whereas the Movile Cave (4482825.3) and Tilapia fish pond (4440281.3) metagenomes had relatively higher bacteroidetes. In fact the Tilapia fish pond metagenome had almost as many bacteroidetes sequences as it had proteobacteria. The Movile Cave bacteria domain relative abundance profile appears to most closely correlate with the profile of the mangrove metagenome.

Similarly to the bacterial phyla distribution, the archaeal phyla distribution saw a single phylum as most abundant across all metagenomes, euryarchaeota. The mangrove metagenome had quite a large relative abundance of thaumarchaeota, whereas the other metagenomes had very little thaumarchaeota. The other four metagenomes had crenarchaeota, as the next most abundant archaeal domain which was less than the thaumarchaeota presence in the mangrove.

3.2. The methanotroph community structure in Movile Cave mat

3.2.1 Methane monooxygenase analysis

Methane monooxygenase is the first enzyme in the oxidation pathway of methane (Trotsenko and Murrell, 2008). Methane oxidising bacteria in the Movile Cave biofilm were examined by screening the functional annotation database of the metagenome for genes annotated as encoding the methane monooxygenase enzyme. All annotations in the metagenome were set at a threshold of having a maximum e-value cutoff of 1×10^{-5}, a minimum identity cut off of 60 % and a minimum alignment length of 15. The screening was done using the keyword search of function to search all annotated sequences for methane monooxygenase sequences. (25) Representative sequences, out of 238 sequences, from the metagenome were obtained (**Table 3.2**). Interestingly most of the annotated sequences were related to soluble methane monooxygenase, highlighted blue in **Table 3.2**. This is contrary to what was found when using specific PCR primers to identify both particulate and soluble methane monooxgenase gene sequences from ^{13}C labelled SIP-DNA (see **chapter 5**).

Table 3.2: DNA sequences annotated as methane monooxygenase in the Movile Cave metagenome. sMMO sequences are highlighted in blue.

Closest hits	Accesion number	Function	Abundance in metagenome	Mean e-value	Mean % identity	Mean seq length (AA)
Methylococcus capsulatus str. Bath	AAU92736.1	methane monooxygenase, alpha subunit, hydroxylase component. *mmoX*	73	-26.4	87.0	63
Methylosinus trichosporium OB3b	EFH04545.1	methane monooxygenase, alpha subunit, hydroxylase component. *mmoX*	22	-34.6	82.4	81
Methylococcus capsulatus	AAB62391.2	methane monooxygenase, Component C, the iron-sulfur flavoprotein. *mmoC*	20	-22.1	67.2	70
Methylococcus capsulatus str. Bath	AAU92722.1	methane monooxygenase, Component C, the iron-sulfur flavoprotein. *mmoC*	20	-22.1	67.2	70
Methylocella silvestris BL2	ACK50231.1	methane monooxygenase, alpha subunit, alpha chain. *mmoX*	19	-34.4	76.2	82
Methylocella silvestris BL2	ACK50232.1	methane monooxygenase, alpha subunit, beta chain. *mmoX*	12	-12.3	73.2	48
Methylococcus capsulatus	AAF04158.2	methane monooxygenase, regulatory protein B. *mmoB*	8	-19.5	81.6	57
Methylococcus capsulatus str. Bath	AAU92726.1	methane monooxygenase, regulatory protein B. *mmoB*	8	-19.5	81.6	57
Methylosinus trichosporium OB3b	EFH04546.1	methane monooxygenase, protein A-beta subunit. *mmoY*	7	-20.4	68.8	74
Methylococcus capsulatus	AAB62392.3	methane monooxygenase, alpha subunit, hydroxylase component. *mmoX*	6	-51.3	87.0	103
Methylocystis sp. M	AAC45290.1	methane monooxygenase, protein A-beta subunit. *mmoY*	5	-32.7	67.0	95
uncultivated type I methanotroph	AAM22463.1	methane monooxygenase, alpha subunit, hydroxylase component. *mmoX*	4	-60.0	84.1	132
Methylocella palustris	AAC46173.1	methane monooxygenase, alpha subunit, hydroxylase component. *mmoX*	4	-66.0	74.7	138
Methylococcus capsulatus str. Bath	AAU91113.1	methane monooxygenase, subunit C. *pmoC*3	4	-23.4	67.2	74
Methylococcus capsulatus str. Bath	AAU92181.1	methane monooxygenase, subunit C. *pmoC*1	4	-23.4	67.2	74
Methylocystis sp. M	AAC45289.1	methane monooxygenase, alpha subunit, hydroxylase component. *mmoX*	3	-18.3	84.4	52
uncultivated bacterium	ABQ10690.1	methane monooxygenase, alpha subunit, hydroxylase component. *mmoX*	2	-34.0	88.0	77
uncultivated bacterium	AAR04314.1	methane monooxygenase, alpha subunit, hydroxylase component. *mmoX*	2	-26.5	79.8	70
Methylocystis hirsuta	ABG56535.1	methane monooxygenase, alpha subunit, hydroxylase component. *mmoX*	2	-17.0	71.2	52
Methylocystis sp. M	AAC45294.1	methane monooxygenase, Component C, the iron-sulfur flavoprotein. *mmoC*	2	-14.0	68.1	56
Methylocystis sp. M	AAC45291.1	methane monooxygenase, regulatory protein B. *mmoB*	2	-16.0	84.0	50
uncultivated bacterium	AAZ06199.1	methane monooxygenase, alpha subunit, hydroxylase component. *mmoX*	1	-6.0	96.0	25
Methylobacter tundripaludum SV96	EFO03473.1	methane monooxygenase, subunit A. *pmoA*	1	-22.0	84.5	58

By far the most abundant sequence (31 % of total methane monooxygenase sequences) was annotated as being closely related to *mmoX* from *Methylococcus capsulatus* Bath, sharing 87 % amino acid similarity to the annotated sequence over an average of 63 amino acids. Genes encoding other soluble methane monooxygenase subunits related to *Methylococcus capsulatus* Bath (*mmoY*, *mmoC*, *mmoB*) were also abundant. As a result, 45 % of the annotated sequences were from the same organism, *Methylococcus capsulatus* Bath. This includes 8 sequences that were annotated as particulate methane monooxygenase sequences related to *pmoA* of *Methylococcus capsulatus* Bath. It is odd that the particulate methane monooxygenase represents such a low proportion of the annotated methane monooxgenase sequences, when the same organism that contains the most highly abundant methane monooxygenase sequence is likely to have the genes for both soluble and particulate methane monooxygenase in its genome. One would therefore expect to see similar representation of both soluble and particulate methane monooxygenase gene sequences in the metagenomic data set, at least represented from the same organism. *Methylococcus capsulatus* strain Bath was isolated by Whittenbury et al., (1970) and is an obligate methanotroph. Strain Bath was isolated from the Roman spa bath house in Bath, England. *Methylococcus capsulatus* Bath has been the focus of many research projects since its isolation the genome of this methanotroph was sequenced in 2004 (Ward et al., 2004).

The next most abundant sequence, representing 22 sequences, is closely related to *Methylosinus trichosporium* OB3b. Again these are soluble methane monooxygenase gene sequences with 82 % sequence identity (over a mean of 81 amino acids). The *Methylosinus trichosporium* OB3b sequences represent 9.2 % of the methane

monooxygenase sequences in this metagenome. *Methylosinus trichosporium* OB3b was isolated by Whittenbury et al., (1970) from a soil sample exposed to gas leaks from natural gas pipelines and natural seeps (soil used in the study by Coty et al., 1967). Again this strikes resonances with the Movile Cave environment, as there are natural gas seeps only a few hundred meters away and there is methane dissolved in the thermal waters entering the cave.

The next two most abundant methanotroph sequences affiliate to *Methylococcus capsulatus* again. They account for 20 sequences each and are both homologous to mmoC, component C of the soluble methane monooxygenase. The sequences differ in their similarity to the mmoC sequences from two different ecotypes of *Methylococcus capsulatus*. The two different sequences are likely from the same organism within the cave, but covering slightly different positions over the same gene. When compared to the genes from the similar strains, one end of the gene may be more similar to one strain and the other end of the gene more similar to the second strain. Alternatively, there could be several closely related strains of *Methylococcus capsulatus* in the cave.

There are 19 and 12 hits for two different regions of the soluble methane monooxygenase of *Methylocella silvestris* BL2. The similarity of the query sequences to their respective hit sequences are very close, being 76 % and 73 %. This could indicate that both query sequences came from the same organism. Had they been largely different, it could be argued there may be more than one strain in the cave that is closely related to *Methylocella silvestris* BL2. *Methylocella silvestris*

BL2 was isolated from acidic (pH 3.8-4.3) forest soil outside of Marburg, Germany (Dunfield et al., 2003). Subsequently, *Methylocella* has been found in many environments at a range of pHs including the sediment of the alkaline Lonar Lake (Rahman et al., 2011). *Methylocella* is an unusual methanotroph as it does not possess particulate methane monooxygenase, nor does it contain any intracytoplasmic membranes to which particulate methane monooxygenase associates, which are found in all other known proteobacterial methanotrophs (Dunfield et al., 2003). It is also the first fully authenticated facultative methanotroph, being able to grow on multi-carbon compounds such as acetate (Theisen et al., 2005).

There are again more *Methylococcus capsulatus* related sequences, 16 in total, here being annotated as *mmoB*, encoding protein B of soluble methane monooxygenase. These have quite high similarity to the query sequence at 81.6 % similarity over 57 amino acids. There is another gene from *Methylosinus trichosporium* OB3b present. These sequences relate to the mmoY gene products, but the similarity to the query sequence is much lower than for *the Methylosinus trichosporium* OB3b *mmoX* gene product, being only 68.8 % similar over 74 amino acids. There are considerably fewer copies of another *Methylococcus capsulatus*-like *mmoX* sequence (6) than there were for the strain Bath *mmoX* (73), but the similarity of 87 % over a the derived amino acid sequence is the same. There are 5 sequences that are annotated as having 67 % similarity (over 95 amino acids) to the *mmoY* of a *Methylocystis* sp. M. This bacterium was isolated from a consortium that was found to be able to degrade trichloroethylene by Uchiyama et al., (1989).

The sequence identified as “uncultivated type 1 methanotroph” that is similar to 4 sequences in the metagenome is very similar to the *mmoX* sequence of several *Methylomonas* species. When a protein Blast search was performed on the NCBI website, the top hit with 100 % similarity was the uncultivated methanotroph sequence as it was the query sequence. However, there are sequences related to *Methylomonas mmoX* sequences that share 99 % similarity to the query sequence. Therefore, it is likely the *mmoX* sequence originates from an uncultivated *Methylomonas* strain or the *Methylomonas* strain isolated in this study.

There are few particulate methane monooxygenase sequences in the metagenome from Movile Cave. There are two sequences that have 4 representatives each that are annotated as being two of the three *Methylococcus capsulatus* Bath pmoC homologues (Stolyar et al., 1999). It could be that low sequence coverage gives a miss representation of the true abundance of all of the methane monooxygenase sequences that may be present within Movile Cave. Interestingly, there are two copies of the particulate methane monooxygenase gene cluster found in the genome of the *Methylomonas* LWB isolate found in this study (one of which is putative owing to a rare gene arrangement). PCR with *pmoA*-specific primers using the same DNA sample yields a positive result, so possibly both soluble methane monooxygenase and particulate methane monooxygenase are being used to oxidise methane in Movile Cave. This warrants further study in the future using, for example, a transcriptomics approach (mRNA sequence analysis).

3.2.2 Methanol dehydrogenase analysis

Another potential gene marker for identifying methane oxidising bacteria within the Movile Cave metagenome is *mxaF*, the gene encoding methanol dehydrogenase, the second enzyme involved in the methane oxidation pathway. Methanol dehydrogenase is also the first enzyme in the oxidation pathway of methanol found in most methylotrophic organisms. Methylotrophs will be important in the Movile Cave carbon flow as they will grow on C_1 compounds that are released from other organisms (such as methanol being released from methane oxidisers). The methanol dehydrogenase sequences found in the Movile Cave metagenome can be found in **Table 3.3**.

Table 3.3: Methanol dehydrogenase gene sequences annotated as such in the Movile Cave metagenome. Known methane oxidiser *mxaF* sequences are highlighted in blue.

Closest hits	id	function	Abundance in Metagenome	Mean eValue	Mean % identity	Mean sequence length (AA)
Methylophilus methylotrophus	AAA83765.1	methanol dehydrogenase large subunit, (*mxaF*)	20	-38.7	90.4	79.9
Methylobacillus flagellatus KT	ABE50311.1	methanol dehydrogenase large subunit, (*mxaF*)	15	-37.5	87.9	80.6
Methylococcus capsulatus str. Bath	AAU92935.1	methanol dehydrogenase large subunit, (*mxaF*)	9	-16.0	77.4	47.1
Methylobacterium organophilum	AAA50289.1	methanol dehydrogenase large subunit, (*mxaF*)	9	-23.6	80.6	64.0
Methylobacterium extorquens PA1	ABY32516.1	methanol dehydrogenase small subunit, (*mxaI*)	8	-18.1	77.6	52.5
Methylobacterium chloromethanicum CM4	ACK85291.1	methanol dehydrogenase small subunit, (*mxaI*)	8	-18.1	77.6	52.5
Methylobacterium extorquens AM1	ACS42166.1	methanol dehydrogenase small subunit, (*mxaI*)	8	-18.1	77.6	52.5
Methylobacillus flagellatus KT	ABE50304.1	Ca2+ insertion into methanol dehydrogenase, (*mxaC*)	6	-25.6	70.5	84.2
Beta proteobacterium KB13	EDZ63907.1	methanol dehydrogenase large subunit, (*mxaF*)	4	-36.5	76.1	93.0
Methylobacterium nodulans ORS 2060	AAG49450.1	methanol dehydrogenase large subunit, (*mxaF*)	4	-41.0	77.5	98.3
Methylobacterium nodulans ORS 2060	ACL63034.1	methanol dehydrogenase large subunit, (*mxaF*)	4	-41.0	77.5	98.3
Rhodopseudomonas palustris BisB18	ABD87467.1	methanol dehydrogenase small subunit, (*mxaI*)	4	-17.4	67.6	58.5
Cytophaga hutchinsonii ATCC 33406	ABG57482.1	methanol dehydrogenase regulator, (CDS)	4	-20.6	69.2	69.9

Closest hits	id	function	Abundance in Metagenome	Mean eValue	Mean % identity	Mean sequence length (AA)
Xanthomonas campestris pv. campestris str. ATCC 33913	AAM42752.1	methanol dehydrogenase large subunit, (*mxaF*)	3	-34.3	66.5	94.5
Xanthomonas campestris pv. campestris str. 8004	AAY47758.1	methanol dehydrogenase large subunit, (*mxaF*)	3	-34.3	66.5	94.5
Acaryochloris marina MBIC11017	ABW30237.1	methanol dehydrogenase regulator, (CDS)	3	-18.3	75.3	63.0
Methylocystis sp. ATCC 49242	EFX98596.1	methanol dehydrogenase small subunit, (*mxaI*)	2	-12.0	72.1	43.0
Flavobacteriales bacterium ALC-1	EDP69957.1	methanol dehydrogenase regulatory protein, (*moxR*)	2	-42.5	82.6	103.0
Methylococcus capsulatus str. Bath	AAU90462.1	methanol dehydrogenase large subunit, (*mxaF*)	2	-37.0	79.2	93.0
Methylophilales bacterium HTCC2181	EAV46784.1	methanol dehydrogenase large subunit, (*mxaF*)	2	-16.5	70.3	57.0

The sequences annotated as methanol dehydrogenase genes present in the Movile Cave metagenome are dominated by *mxaF* from known methylotrophs, as would be expected. There are only two methane oxidising organisms that have representative methanol dehydrogenase sequences in the metagenome. The most abundant methanotroph *mxaF* sequence is that from *Methylococcus capsulatus* Bath. This is not suprising given the relatively high abundance of *Methylococcus capsulatus* Bath methane monooxygenase sequences. These *mxaF* sequences shared 77 % identity to the *mxaF* of *Methylococcus capsulatus* Bath, but was a relatively short sequence representing only 47 amino acids on average. This sequence was one of the most abundant of all of the methanol dehydrogenase sequences, but still fewer than half of the most abundant sequence. The second methanotroph *mxaF* sequence is annotated as belonging to a *Methylocystis* species. This was only represented by two sequences. A similar situation occurred with *Methylocystis* methane monooxygenase genes, with only 5 sequences being found in the metagenome. The derived *mxaF* sequences in the metagenome share 72 % sequence identity over an average of 43 amino acids to the *mxaF* from *Methylocystis*.

It would have been expected there would be more methanotroph representatives among the methanol dehydrogenase sequences given there is a rather large diversity of methane monooxygenase sequences. All methane oxidising bacteria should contain the genes encoding methanol dehydrogenase. Therefore, all genomes that contain a methane monooxygenase should contain a methanol dehydrogenase. The number of methanol dehydrogenase sequences observed might be lower than those of the methane monooxygenase, as some methanotrophs can have multiple gene copies of the methane monooxygenase (Stolyer et al., 1999). However, even though the

abundance of methanol dehydrogenase would be less, a similar diversity should still be seen for both methanol dehydrogenase and methane monooxygenase sequences. This also suggests that the metagenome coverage is not sufficent to determine the full diversity of methanotrophs present in Movile Cave.

The most abundant methanol dehydrogenase sequence, with 20 representatives, was related to *mxaF* from a *Methylophilus methylotrophus*. This *mxaF* sequence has a very high similarity to the annotation, having 90.4 % similarity over an average of 80 amino acids. *Methylophilus methylotrophus* is a restricted facultative methylotroph with the ability to grow on methanol and a limited range of more complex organic compounds (Jenkins et al., 1987). With methanotrophic bacteria in Movile Cave, there may be "leakage" of one-carbon compounds, such as methanol which can be utilised by methylotrophs such as *Methylophilus methylotrophus*.

The next most abundant *mxaF* gene sequence was related to *mxaF* sequences from *Methylobacillus flagellatus* KT. There are 15 of these sequences in the metagenome, with a high similarity of 80 % to the annotated sequence over an average of 81 amino acids. *Methylobacillus flagellatus* KT is an obligate methanol and methylamine utilising methylotroph. It was isolated from a metropolitan sewer system (Govorukhina et al., 1987), and is suited to industrial application due to its high growth rate on, and tolerance of, methanol and its high yield coefficient of turning methanol into biomass (Chistoserdova et al., 2007). There may be micro niches within the Movile Cave biofilm, where there are concentrated pockets of methanol or even formaldehyde where a *Methylobacillus flagellatus* KT-like bacterium would most likely thrive.

There are multiple *mxaF* sequences annotated as belonging to different strains of *Methylobacterium*, the total of which exceeds that of *Methylophilus* related sequences. *Methylobacterium* strains are facultative methylotrophs and are found in a variety of environments. The first *Methylobacterium* strain named *Bacillus extorquens* was isolated by Bassalik, (1913) from the gut contents of an earthworm. The current lab "work horse" strain is *Methylobacterium extorquens* AM1 (originally *Pseudomonas* AM1), which was isolated by Peel and Quayle (1961). Initially, a presumed airborne contaminant on a 0.1 M methylamine agar plate, the organism was subcultured and isolated. There are currently 44 different species of *Methylobacterium* that have validated names according to the J.P Euzéby website (http://www.bacterio.cict.fr/). *Methylobacterium* species are ubiquitous in nature (Lidstrom, 2006), so it is no suprise they are present in Movile Cave.

There are 3 sequences annotated as a methanol dehydrogenase sequence that only shares 67 % identity over an average of 95 amino acids to that of *Xanthomonas campestris*. This bacterium is a plant pathogen and, like *Methylobacterium*, colonises leaves of terrestrial plants (Shaad et al., 2005). It might oxidise methanol released from the leaf in the same manner as *Methylobacterium*. There are another 3 sequence hits annotated as being closely related to the cyanobacterium *Acaryochloris marina*. The sequences share 75 % sequence identity over an average of 63 amino acids to that of *Acaryochloris marina*. The number of representative sequences for this organism is fairly low, but it would be very unlikely that this photosynthetic organism would be able to thrive in the Movile Cave environment in the absence of light. There may be a chance that these organisms have been brought into Movile Cave as contaminants by researchers entering the cave. In the case of the

Acaryochloris, it might suggest that the water in Movile Cave is linked to the water of the Black Sea, which is only around 2 km from the cave. One could imagine that if the bodies of water are in some way connected, there may be a path for a photosynthetic organism to make its way into Movile Cave (although this rather unlikely).

3.2.3 Formate dehydrogenase analysis

Formate dehydrogenase is the enzyme converting formate into carbon dioxide. For methylotrophic organisms, this is the last step of the complete oxidation pathway of one carbon compounds. The genes encoding formate dehydrogenase can often be found in multiple copies in some methylotrophs, such as *Methylobacterium extorquens* (Chistoserdova et al., 2004). Formated dehydrogenase is a valuable enzyme in biosythetic processes, such as the dehydrogenase based production of optically active compounds, and is used for its high throughput of NADH regeneration (Tishkov and Popov, 2006). The putative formate dehydrogenase sequences found in this metagenome are shown in **Table 3.4**.

Table 3.4: Formate dehydrogenase sequences annotated as such in the Movile Cave metagenome. Known methane oxidiser sequences highlighted in blue. There were 368 unique sequences annotated as formate dehydrogenase. A selection of representative sequences are shown.

Closest hits	id	function	Abundance in Metagenome	Mean eValue	Mean % identity	Mean sequence length (AA)
Syntrophobacter fumaroxidans MPOB	ABK19180.1	formate dehydrogenase, alpha subunit	14	-20.3	72.8	62.7
Methylococcus capsulatus str. Bath	AAU92551.1	formate dehydrogenase, alpha subunit	10	-18.4	73.2	60.3
Methylococcus capsulatus str. Bath	AAU92353.1	formate dehydrogenase, alpha subunit	9	-36.8	83.6	93.2
Methylobacillus flagellatus KT	ABE48989.1	formate dehydrogenase beta subunit	8	-23.3	79.1	66.4
Methylobacillus flagellatus KT	ABE48988.1	formate dehydrogenase alpha subunit	8	-38.0	80.8	88.6
Methylibium petroleiphilum PM1	ABM94131.1	formate dehydrogenase large subunit precursor	7	-48	86.6	103
Sinorhizobium meliloti AK83	EFN28969.1	formate dehydrogenase, alpha subunit	6	-43.3	82.0	98.6
Methylotenera versatilis 301	ADI30808.1	formate dehydrogenase family accessory protein FdhD	4	-21.2	69.5	71.9
Methylococcus capsulatus str. Bath	AAU92707.1	formate dehydrogenase, iron-sulfur subunit	3	-42	81.6	90.8
Methylocella silvestris BL2	ABQ35207.1	formate dehydrogenase alpha subunit	3	-20.7	89.6	52.5
Ruegeria pomeroyi DSS-3	AAV95075.1	formate dehydrogenase, alpha subunit, putative	2	-28	81.2	70.5

The most abundant formate dehydrogenase sequence from a known methane oxidising bacterium is related to that of *Methylococcus capsulatus* Bath. There are 10 sequences that share 73 % sequence identity over 63 amino acids of the *Methylococcus capsulatus* Bath sequence. There are a further 9 occurances of another sequence that shares 83.6 % sequence identity over an average of 93 amino acids of the same *Methylococcus capsulatus* Bath sequence. There are also a further two sequences related to *Methylococcus capsulatus* Bath that share 82 % sequence identity over an average of 91 amino acids. As seen with other genes of the methylotrophic pathway, *Methylococcus capsulatus* Bath formate dehydrogenase sequences are the most abundant in the Movile Cave metagenome.

The only other methane oxidising bacterium with a formate dehydrogenase represetative sequence was that of *Methylocella silvestris* BL2. There are 2 sequences that share 90 % sequence identity over an average of 53 amino acids to the sequence annotated as *Methylocella silvestris* BL2. *Methylocella silvestris* BL2 annotated sequences made up a large number of the soluble methane monooxygenase sequences, so it was likely it would have representative sequences of the other genes involved in the metabolism of methane. However, there were no methanol dehydrogenase sequences annotated as being related to *Methylocella silvestris* BL2, and here we only see 2 representative sequences. As mentioned previously, the disproportion in abundance of different genes from the organisms that should have even representatives of genes from a given metabolic pathway, is evidence to suggest that the coverage and annotation of this metagenome may not be adequate.

The most abundant formate dehydrogenase present in this metagenome shared 72.8 % sequence identity with that of *Syntrophobacter fumaroxidans* MPOB over an average of 63 amino acids. *Syntrophobacter fumaroxidans* MPOB is a non-methylotrophic sulfate reducer that can grow independently or syntrophically on propionate. This strain is known to reduce fumarate into succinate using formate as an electron donor (Harmsen et al., 1998).

As with methanol dehydrogenase, there were a relatively large number of sequences for the formate dehydrogenase sequences that are annotated as being related to formate dehydrogenase from *Methylobacillus flagellatus* KT. There were 16 sequences in total, with 8 sequences sharing 79 % sequence identity over 66 amino acids and the other 8 sequences sharing 81 % sequence identity over 89 amino acids. *Methylobacillus flagellatus* KT is likely one of the dominant methylotrophs in the Movile Cave environment.

There were 7 sequences related to the formate dehydrogenase sequence of *Methylibium petroleiphilum* PM1. These sequences share nearly 87 % sequence identity over an average of 103 amino acids. *Methylibium petroleiphilum* PM1 is a betaproteobacterial methylotroph first isolated from a compost biofilter by Nakatsu et al., (2006). It was isolated from a mixed bacterial culture being grown on methyl tert-butyl ether (MTBE). *Methylibium petroleiphilum* PM1 can grow on methanol (Kalyuzhnya et al., 2008), but there were no methanol dehydrogenase sequences related to *Methylibium* species found in this metagenome.

Of the formate dehydrogenase sequences, 6 shared 82 % sequence identity over an average of 99 amino acids to the formate dehydrogenase sequence of *Sinorhizobium meliloti*. *Sinorhizobium meliloti* is a reclassification of the organism *Rhizobium meliloti* which was first isolated by Jordan, (1984) according to Lajudie et al., (1994). *Sinorhizobium* species are root nodulating bacteria, so it is odd to find them in Movile Cave.

There are 2 sequences annotated as being closely related to the formate dehydrogenase sequence of *Ruegeria pomeroyi* DSS-3. The sequences share 81 % sequence identity over an average of 71 amino acids. *Ruegeria pomeroyi* DSS-3 was originally isolated as a DMSP degrader from marine water and named *Silicibacter pomeroyi* (González et al., 2003). The organism was then renamed *Ruegeria pomeroyi* DSS-3 after combining two closely related taxa (Yi et al., 2007).
This is another case of a known marine organism being found among the Movile Cave sequences, which gives more evidence that there may be some connection between the cave water and the marine water of the Black Sea.

3.2.4 Hexulose-6-phosphate synthase analysis

There are two major pathways used by methylotrophic organisms for the assimilation of formaldehyde produced by the oxidation of C_1 compounds. One of the assimilation pathways is the Ribulose mono phosphate pathway (RuMP) (**Figure 3.9**). The RuMP pathway first involves the addition of formaldehyde and Ribulose-5-phosphate. After a rearrangement of the molecule and addition of another phosphate from ATP, the resultant Fructose1-6-bisphosphate is cleaved with one of the products being dihydroxyacteonephosphate. This is then converted into pyruvate which then feeds into the TCA cycle for cellular synthesis. Here the gene encoding the first enzyme of the RuMP pathway, Hexulose-6-phosphate synthase, is used as a marker of organisms able to carry out this metabolic pathway within Movile Cave and focusing on the methylotrophic sequences. The hexulose-6-phosphate synthase sequences can be found in **Table 3.5**.

Figure 3.9: The ribulose monophosphate pathway redrawn from Anthony, (1982).

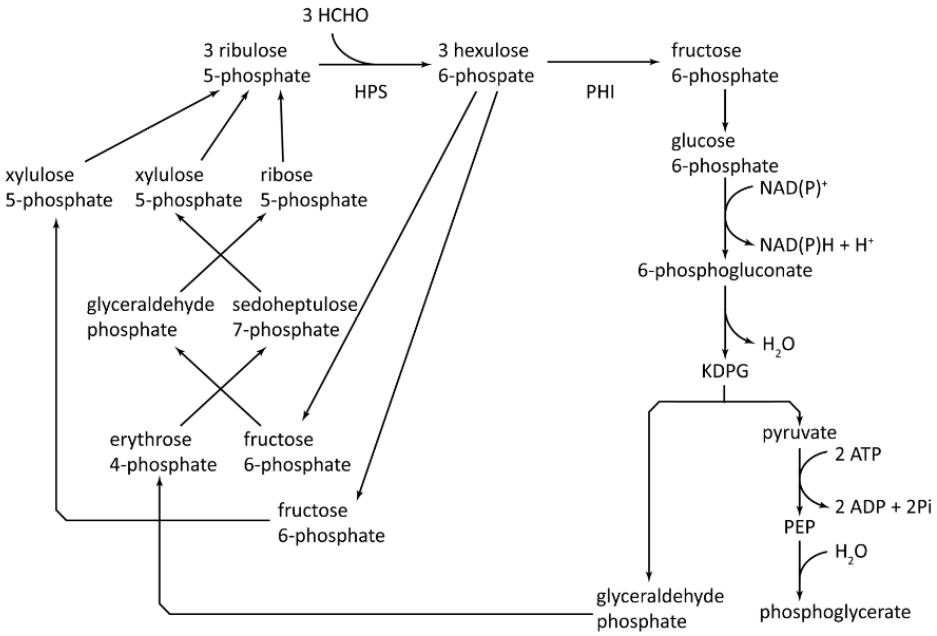


Table 3.5: Hexulose-6-phosphate synthase sequences in the Movile Cave metagenome. Known methane oxidiser sequences are highlighted in blue.

Closest hits	id	function	Abundance in Metagenome	Mean eValue	Mean % identity	Mean sequence length (AA)
Methylobacillus flagellatus KT	ABE48521.1	3-hexulose-6-phosphate synthase	17	-27.4	81.8	76
Methylobacillus flagellatus KT	ABE49922.1	3-hexulose-6-phosphate synthase	16	-20.3	90.4	54.9
Methylococcus capsulatus str. Bath	AAU91180.1	putative hexulose-6-phosphate synthase/SIS domain protein	15	-25.9	72.0	78.9
Betaproteobacterium KB13	EDZ64934.1	3-hexulose-6-phosphate synthase	9	-29.7	78.6	85.3
Methylovorus glucosetrophus SIP3-4	ACT49517.1	3-hexulose-6-phosphate synthase	5	-16	89.1	47.2
Methylovorus sp. MP688	ADQ84716.1	3-hexulose-6-phosphate synthase	4	-9.8	86.6	38.2
Methylococcus capsulatus str. Bath	AAU90883.1	hexulose-6-phosphate synthase	4	-18.7	81.9	55.7
Methylobacter tundripaludum SV96	EFO06236.1	3-hexulose-6-phosphate synthase	3	-6	72.1	35.5
Methylophilales bacterium HTCC2181	EAV47244.1	hexulose-6-phosphate synthase	3	-20.2	81.8	59.6

In total, there are 19 hexulose-6-phosphate synthase annotated sequences, similar to that of *Methylococcus capsulatus* Bath; 15 of these sequences share 72 % sequence identity over an average of 79 amino acids. The other 4 sequences share 82 % sequence identity over 56 amino acids. The *Methylococcus capsulatus* Bath hexulose-6-phosphate synthase sequences are one of only two methane oxidising bacteria representatives of the gene found in this metagenome. Similar to other genes observed, *Methylococcus capsulatus* Bath sequences by far dominate the methanotroph sequences observed.

The second methane oxidising bacterium hexulose-6-phosphate synthase sequence was annotated as being closely related to that of *Methylobacter tundripaludum* SV96. There were only 3 sequences in the metagenome that matched this organism, which shared 72 % sequence identity over an average of 36 amino acids. There was a similarly low abundance of the particulate methane monooxygenase sequences related to this organism, being only 1 annotated sequence. *Methylobacter tundripaludum* SV96 was isolated by Wartiainen et al., (2006) from the Arctic wetland soil of Svalbard.

The most abundant hexulose-6-phosphate synthase sequences were annotated as being closely related to that of *Methylobacillus flagellatus* KT. In total, there were 33 sequences annotated with 17 of the sequences sharing 82 % sequence identity over an average of 76 amino acids, and a further 16 sequences sharing 90 % sequence identity over an average of 55 amino acids. *Methylobacillus flagellatus* KT annotated sequences appear with relatively high frequency for several of the C_1 oxidation

pathway genes. Therefore, it is likely to be one of the more abundant methylotrophs in Movile Cave.

There are 9 sequences that share 77 % sequence identity over an average 85 amino acids to an unknown betaproteobacterium KB13. There are many betaproteobacterial methylotroph species, including *Methylobacillus*, *Methylobacterium* and *Methylotenera*. The betaproteobacterium KB13 is believed to be a coastal marine and estuarine isolate, and had its genome sequenced as part of a Gordon and Betty Moore Foundation marine microbial genome sequencing project carried out at the J. Craig Venter Institute. (https://moore.jcvi.org/moore/SingleOrganism.do?speciesTag=KB13).

There are 9 *Methylovorus* species hexulose-6-phosphate synthase sequences present in the metagenome. Of the 9, there are 5 sequences annotated as *Methylovorus glucosetrophus* SIP3-4 sharing 89 % sequence identity over an average of 47 amino acids. There are also 4 sequences that share 87 % sequence identity over an average of 37 amino acids annotated as *Methylovorus* sp. MP688. *Methylovorus glucosetrophus* SIP3-4 was isolated by Lapidus et al., (2011) from Lake Washington in Seattle WA. Its genome was sequenced as part of a comparative study into the metabolic diversity of 3 methylotrophic isolates from Lake Washington. *Methylovorus* sp. MP688 was isolated from a soil sample by Xiong et al., (2011), and had its genome sequenced due to an interest in the high levels of pyrroloquinolone quinine that the organism produced.

There are 3 hexulose-6-phosphate synthase sequences related to the sequence from *Methylophilales* bacterium HTCC2181. The sequence shares 82 % sequence identity over an average of 60 amino acids. On the JCVI website (https://moore.jcvi.org/moore/SingleOrganism.do?speciesTag=MB2181), it is noted that this organism is one of the most abundant betaproteobacterial representatives in coastal marine 16S rRNA gene clone libraries.

3.2.5 Hydroxypyruvate reductase analysis

The second of the two major pathways, used by methylotrophic organisms for the assimilation of formaldehyde, produced by the oxidation of C_1 compounds, is the serine cycle (Hanson and Hanson, 1996) (**Figure 3.10**). Formaldehyde is first combined with a molecule of glycine by serine transhydroxymethylase to produce serine, which is then converted into hydroxypyruvate by serine-glyoxylate aminotransferase. Hydroxypyruvate is then converted into glycerate by the enzyme hydroxypyruvate reductase. Glycerate is phosphorylated into phosphoglycerate by glycerate kinase and then converted into phosphoenolpyruvate (PEP) by enolase. The addition of CO_2 to the PEP is carried out by PEP carboxylase, producing oxaloacetate which is then converted into malate by malate dehydrogenase. Malyl-CoA is then produced through the use of malate thiokinase. The malyly-CoA is then broken into acetyl-CoA, which leads into the TCA cycle for cellular synthesis and glyoxylate which is used to begin the cycle again. Here the *hpr* gene encoding for the hydroxypyruvate reductase was used to identify organisms present in the Movile Cave metagenome, which are able to use the serine cycle for their metabolism of carbon. The hydroxypyruvate reductase sequences from the metagenome are listed in **Table 3.6**.

Figure 3.10: The serine cycle. Courtesy of Professor Chris Anthony.

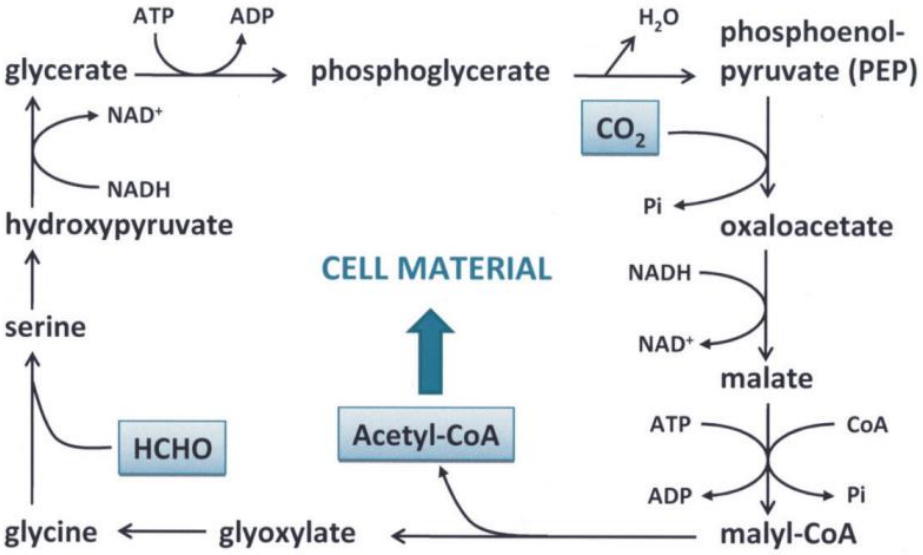


Table 3.6: Hydroxypyruvate reductase sequences in the Movile Cave metagenome.

Closest hits	id	function	Abundance in Metagenome	Mean eValue	Mean % identity	Mean sequence length (AA)
Meiothermus silvanus DSM 9946	ADH63318.1	Hydroxypyruvate reductase	4	-13	59.1	64
Verminephrobacter eiseniae EF01-2	ABM56706.1	Hydroxypyruvate reductase	4	-30.7	70.9	95.8
Delftia acidovorans SPH-1	ABX34755.1	Hydroxypyruvate reductase	3	-26.3	71.9	83.9
Roseiflexus castenholzii DSM 13941	ABU59323.1	Hydroxypyruvate reductase	3	-7	73.3	37.8
Chloroflexus aurantiacus J-10-fl	ABY33859.1	Hydroxypyruvate reductase	3	-7.87	56.9	55.3
Thiomonas intermedia K12	ADG32195.1	Hydroxypyruvate reductase	3	-13.6	72.7	56.7
Nitrosomonas sp. AL212	EET33322.1	Hydroxypyruvate reductase	2	-5.7	57.7	44.3

Compared with the other genes identified here, there are relatively few sequences annotated as hydroxypyruvate reductase. No hydroxypyruvate reductase gene sequences found in the Movile Cave metagenome were annotated as sequences related to known methane oxidising organisms.

There are 4 sequences annotated as being related to the hydroxypyruvate reductase of *Meiothermus silvanus* DSM 9946. The sequence shares only 59 % sequence identity over an average of 64 amino acids to the annotation. *Meiothermus silvanus* DSM 9946 is an interesting organism, as they are found to produce coloured biofilms on machines in the paper production industry (Ekman et al., 2007). It could be likely that a similar organism may play a role in the production of the biofilms found in Movile Cave.

Four of the hydroxypyruvate reductase sequences were annotated as being related to *Verminephrobacter eiseniae* EF01-2 (71 % identity over an average of 96 amino acids). *Verminephrobacter eiseniae* EF01-2 was isolated from the nephridia excreatory organ of the earthworm *Eisenia foetida* by Pinel et al., (2008). Nematodes are known to live within and consume the microbial mats in Movile Cave (Muschiol et al., 2007; 2008a,b; 2009). This organism may have been in the excretory products from a nematode and been present in the mat sample that was used to prepare DNA for this metagenome.

There are 3 sequences annotated as being similar to the hydroxypyruvate reductase gene of *Delftia acidovorans* SPH-1. The sequence shares 72 % sequence identity over an average of 84 amino acids. *Delftia acidovorans* SPH-1 was isolated as part of

a consortia of organisms from a German sewage treatment plant that are able to degrade linear alkylbenzenesulfonate, which is a laundry surfactant used globally (Schleheck et al., 2004).

Roseiflexus castenholzii DSM 13941 is the annotation of 3 of the hydroxypyruvate reductase sequences (73 % sequence identity over 38 amino acids). *Roseiflexus castenholzii* DSM 13941 is a thermophilic filamentus photosynthetic organism capable of growing under anaerobic conditions in the light. It is also capable of growing chemoheterotrophically under aerobic conditions in the dark, which would be suitable for growth in Movile Cave (Hanada et al., 2002).

Chloroflexus aurantiacus J-10-fl is a similar organism to *Roseiflexus castenholzii* DSM 13941, as it is an anoxygenic photoheterotroph able to grow chemoheterotrophically under aerobic conditions. The organism is found in hot thermal springs rich in sulfide similar to the Movile Cave environment (Pierson and Castenholz, 1974). This *hpr* sequence shares 57 % sequence identity to the *hpr* gene of *Chloroflexus aurantiacus* J-10-fl over an average of 55 amino acids.

There are 3 sequences annotated as being closely related to the hydoxypyruvate reductase gene from *Thiomonas intermedia* K12 (73 % sequence identity over an average of 57 amino acids). *Thiomonas intermedia* K12 was isolated from a sewage pipe in Hamburg, Germany (Milde et al., 1983). The organism was originally a *Thiobacillus* before being reclassified. *Thiomonas intermedia* K12 is known to be a sulfur oxidiser and capable of growth on polythionates (Wentzien and Sand, 2004).

There are 2 sequences annotated as being closely related to the hydroxypyruvate reductase gene sequence of *Nitrosomonas* sp. AL212 (58 % sequence identity over an average of 44 amino acids). *Nitrosomonas* sp. AL212 is an obligate chemolithotrophic ammonia-oxidiser isolated from an activated sludge sample.

3.2 Analysis of Movile Cave mat DNA using a *pmoA* Microarray

A *pmoA* microarray (Bodrossy et al., 2003) has been used in several studies to analyse the diversity of particulate methane monooxygenase gene sequences from DNA samples from a range of environments including saline soil, pond water and landfil cover soil (e.g. see Saidi-Mehrabad et al., 2013; Bisset et al., 2011; Kumaresan et al., 2011). Functional gene microarray studies can identify organisms at a species level resolution without the need for cultivation. They also provide semi-quantitive data on relative abundances of the different gene sequences in a given sample (Stralis-Pavese et al., 2011).

The *pmoA* microarray is a tool for fingerprinting the gene, encoding the particulate methane monooxygenase enzyme alpha-subunit from a community of bacteria. Most methanotrophs contain the genes encoding the particulate methane monooxygenase, so the *pmoA* microarray provides a suitable screening method of methanotrophs from a given community. The *pmoA* microarray was used here as another approach to identify the methane oxidising bacteria of Movile Cave. The microarray was performed in conjunction with the metagenome sequencing to fully illustrate the methane oxidising bacterial community, as it would exist in Movile Cave and without any enrichment or substrate bias acting on the community. It was thought that the use of this polyphasic approach would provide robustness to data produced, as opposed to carrying out either of the techniques individually which have limitations on how much information they can reliably produce.

DNA extracted from the Movile Cave microbial mat was sent to Dr Levente Bodrossy's lab for *pmoA* microarray analysis. There were no replicates of the microarray produced. The *pmoA* microarray was carried out by Dr. D. Kumaresan at the CSIRO laboratories, Hobart, Tasmania, Australia, under guidance of Dr Levente Bodrossy. The *pmoA* microarray was performed according to the procedure described by Stralis-Pavese et al., (2011). The Movile Cave DNA sample was amplified with 2 different sets of primers targeting the *pmoA* gene, sharing the 189 forward primer with either the mb661 reverse primer, specifically targeting methanotrophs (Costello and Lidstrom, 1999) or the A682 reverse primer that targets *pmoA* and *amoA* from methane and ammonia oxidisers respectively, along with other *pmoA*-like sequences identified in different environments (Bourne et al., 2001). There was also a third product used by creating a semi-nested PCR product, using both sets of PCR primers. These were used to obtain full coverage of *pmoA* genes present in the amplified PCR products, and were hybridised onto the microarray probes. A full list of hybridisation probes used in this study can be found in Appendix 1.

Figure 3.11: *pmoA* microarray heat map indicating hybridisation of *pmoA* PCR products amplified from the Movile Cave microbial mat to various probes. Hybridisation was carried out with three different sets of PCR products. The top track PCR products were amplified with A189f and A682r. The middle track PCR products were amplified with A189f and mb661r. The bottom track PCR products were produced from a semi-nested approach using A189f with both A682r and mb661r. The colour of each probe represents relative hybridisation signal intensity, where red is maximum relative intensity, yellow is about 10% hybridisation intensity and blue is no signal. Only the section of probes with hybridisation is shown. The heat map was created by Dr Levente Bodrossy, as he had the software available to create it.

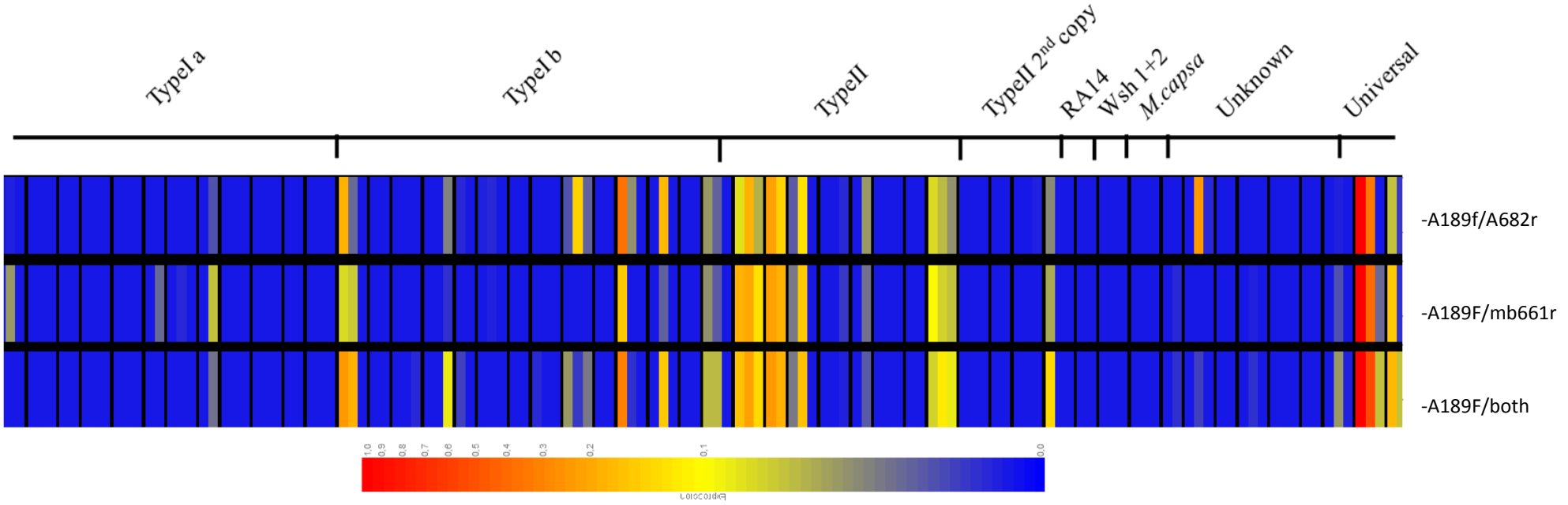


Table 3.7 contains a highlighted version from the the pmoA microarray heat map of **Figure 3.11.** It can be observed that there was only one Type Ia probe that gave any significant signal with the three microarray analyses. This was with PCR generated products with the mb661 reverse primer and hybridising probe Mm275 targeting *pmoA* sequences from genus *Methylomonas*. There were signals across all 3 microarrays for the probe SWI1-377, targerting *pmoA* from Type Ia probes and represents a marine sediment clone.

A significant proportion of the stronger hybridisation signals came from the Type 1b methanotroph *pmoA* probes. There were strong hybridisation signals across all 3 microarrays for probes 501-375 and 501-286. These probes target *pmoA* sequences from *Methylococcus* found in both marine and fresh water sediment clone libraries. There were signals for probe Mcl404, which is a *Methylocaldum pmoA*, in both A189f/A682r and semi-nested PCR amplifications. There were also relatively weak hybridisation signals for the probes RSM1-419, JHTY2-562 and JHTY2-578 across the A682 and semi-nested PCR product microarrays. There were strong hybridisation signals across all three microarrays for the probe LW21-391, which is assumed to be a *pmoA* from a Type 1b clone from the Lake Washington studies. There was a hybridisation product for probe M90-574 on the A682 microarray, which is another probe targeting *Methylococcus* and *Methylocaldum*-like *pmoA* sequence. There were hybridisation signals on the A682 and nested product microarrays for the probe Mha-500, targerting *pmoA* from *Methylohalobius* and *Methylothermus*. Finally, there are hybridisation signals across all 3 microarrays for the probes Ib453 and Ib559 which targer *pmoA* from *Methylococcus*, *Methylothermus* and *Methylocaldum*.

Most of the stronger hybridisation signals represent Type II methanotroph *pmoA* sequences. There are strong and some of the strongest hybridisation signals with most of the *Methylocystis* probes (Mcy255, Mcy459, Mcy264, Mcy270, Mcy413 and Mcy233). There was a rather weak signal across all three microarrays for probe Msi423, which is representative of a *Methylosinus pmoA*-like sequence. There were relatively weak hybridisation signals across all three microarrays for probe Msi232, also targeting *pmoA* from *Methylosinus*. Finally, there were strong hybridisation signals across all three microarrays for the probes II509 and II630, which are both generic probes targeting *pmoA* from Type II methanotrophs, which is to be expected given the strong signals seen with the other more specific Type II probes.

There was a relatively low hybridisation signal across all three microarrays for probe NMsiT-271, which is representative of Type II second copy of a *pmoA* sequence from Type II methanotrophs, which targets a novel copy of the *pmoA* gene found in *Methylosinus*. There was a very strong hybridisation signal in the A682 microarray for probe pmoAMO3-486, which is just labelled as clone pmoAMO3 with from an unidentified organism or location. There is a rather weak hybridisation signal for the probe TUSC409 on the nested product microarray, which is a probe based on the Tropical Upland Soil Cluster 2 sequences (Kneif et al., 2005).

	relative probe intensities			
Probes with strong hybridisation signal	A682r	mb661r	nested	Identity of related organism
501-375	11	5	14	*Methylococcus*
501-286	3	5	11	
Mcl404	3		6	*Methylocaldum*
RSM1-419	2		4	*pmoA environmental* clones
JHTY2-562	8			
JHTY2-578	2		3	
LW21-391	35	13	29	type IB environmental clone
M90-574	12			*M'coccus/M'caldum* sediment clone
Mha-500	10	2	9	*Methylohalobius*
Ib453	12	12	15	type IB
Ib559	6	5	11	
Mcy255	8	16		*Methylocystis*
Mcy459	24	28	29	
Mcy264	19	30	33	
Mcy270	14	15	16	
Mcy413	24	32	33	
Mcy522	5	7	7	
Mcy233	9	11	12	
Msi423	4	3	2	*Methylosinus*
Msi232	5	6	5	*M.sinus+ most M.cystis*
II509	7	8	10	Type II
II630	8	10	12	
NMsiT-271	4	5	8	Novel pmoA copy of *M.sinus*
pmoAMO3-400	28			clone pmoAMO3-400
TUSC409		2	4	Tropical Upland Soil Cluster #2
Mm275		16		*Methylomonas*

Table 3.7: List of *pmoA* microarray probes that displayed significant hybridisation along with relative intensity values for each of the 3 amplificaion products, using the primer sets A189f/A682r, A189f/mb661r and A189f/nested.

There were no hybridisation signals seen for any of the *amoA* probes on A682 microarray, and there were no signals for any of the novel monooxygenase probes.

In summary, the *pmoA* microarray data suggest that the most abundant *pmoA* sequences are from *Methylocystis*-like organisms closely followed by sequences related to *pmoA* clones obtained from lake washington. There is an average representation of *Methylococcus*-like *pmoA* sequences and a more modest representation of the *Methylosinus*- and *Methylocaldum*-like sequences. There is a rather low representation of *Methylomonas* sequences, and an apparently less Type Ia methanotroph representation when compared to Type Ib and Type II representation.

3.3 Discussion

The ability to analyse the genetic potential of all bacteria in a given environmental sample simultaneously, allows one to gain a better understanding of how the bacteria as a whole can function together as a sustainable community. One of the most thorough ways of achieving a snapshot of genetic potential within bacterial communities, is to carry out high throughput metagenomic sequencing. Not only can this provide great coverage of the genetic potential of a given community, but it also rules out sequence misrepresentation often seen with PCR based sequencing techniques that often carry primer bias. By removing the primer bias, it is more likely that novel sequences will be found. Often the downside to this is not being able to annotate a novel sequence correctly due to it never being seen before. However, despite potential drawbacks, metagenomics is being used to identify novel biosynthetic enzymes that would otherwise be missed due to the lack of cultivation of most bacteria (Wilson and Piel, 2013).

The purpose of this section of work was to determine the population of methane oxidising bacteria in the Movile Cave microbial floating mat without enrichment, as this would represent the natural methanotroph profile. Two independent approaches, metagenomics and functional gene microarray, were used to determine the methane oxidising bacterial community.

The metagenome obtained here appears to have had reasonable coverage of the microbial community that is present in Movile Cave mat. When looking at the rarefaction curve of the metagenomic sequences, the curve does appear to be reaching a plateau. The total number of sequences from the Movile Cave

metagenome is also much higher than in the other metagenomes compared in this analysis. Of the sequences that passed quality control, nearly half of the predicted proteins had unknown function. If the sequences had unknown function, then they likely had unknown phylogeny, which could change the shape of the rarefaction curve. If the sequences of unknown origin came from organisms already identified, then the rarefaction curve would probably reach a more distinct plateau.

The large number of cyanobacterial sequences observed could be representative of the microbial population. In the study by Ortiz et al., (2014), a similar number (2 %) of sequences from a metagenome of stalactites in Kartchner cavers were attributed to cyanobacteria. A relative of cyanobacteria, melainabacteria, has been identified from genomes reconstructed from gut and ground water metagenomes (Di Rienzi et al., 2013). These melainabacteria are non-photosynthetic and, unlike cyanobacteria, instead of producing oxygen, melainabacteria produce hydrogen. The melainabcteria also contain homologs to light response regulators (Di Rienzi et al., 2013). With that in mind, it may be that the melainabacteria are present in Movile Cave and the sequences being identified are being misannotated as cyanobacterial due to how similar and closely related the organisms are.

With a more comprehensive data set, the mangrove metagenome would likely have a higher diversity than that of Movile Cave. It is interesting to see that there is such a high diversity of microorganisms in Movile Cave. With the cave being isolated and relatively stable and being isolated for so long, it would be expected that some dominant organisms would take over and any rare biosphere would be out-competed, possibly to extinction. Instead, we see there is a complex and high diversity within the biofilm, indicating there must be a delicate balance in which these rare organisms

play an important role within the floating microbial mat. This could also indicate the presence of ecological niches, allowing the rarer microorganisms to survive. This was the case in Frassasi cave; it was found that many sulfur oxidising species were found to co-exist due to the niche partitioning of counter reduced sulfur species and oxygen gradients (Macalady et al., 2008).

The metagenome was predominantly used to identify which methanotrophs and other methylotrophs were present in Movile Cave. The metagenome was analysed for any sequences annotated as soluble or particulate methane monooxygenase. The soluble methane monooxygenase sequences that were found, far outnumbered the particulate methane monooxygenase sequences identified. Of the methane monooxygenase sequences, most were related to genus *Methylococcus.* Therefore, it is assumed that *Methylococcus* may be the most abundant methane oxidising bacterium in Movile Cave. Despite many efforts, *Methylococcus* was not isolated from any Movile Cave sample. It may not be too suprising that a methane oxidising bacterium, like *Methylococcus capsulatus* Bath, is able to proliferate and dominate in this environment, as there are several similarities between Movile Cave and the Roman Baths where *Methylococcus capsulatus* Bath was isolated (Whittenbury et al., 1970). The environment from which strain Bath was isolated contains water at 42 °C that is rich in sulfur compounds and methane. Hutchens et al., (2004) found both *mmoX* and *pmoA* gene clones relating to *Methylococcus capsulatus* Bath from heavy fractions of a DNA-Stable Isotope Probing experiment, proving that this organism is indeed active in Movile Cave.

The methane monooxygense gene sequences were dominated by soluble methane monooxygenase gene sequences. All methanotrophs except *Methylocella* and *Methyloferulla* have particulate methane monooxygenase, so it is odd that there would be more soluble methane monooxygenase gene sequences (Theisen et al., 2005; Vorobev et al., 2011). This suggests that the metagenome coverage is not good enough, as one would expect to see similar representation of both soluble and particulate methane monooxygenase.

The *pmoA* microarray indicated there was much more diversity of the particulate methane monooxygense gene in Movile Cave than was observed from the metagenome. The microarray suggests that the most abundant *pmoA* sequences are from the genus *Methylocystis*, as opposed to the *Methylococcus* as seen in the metagenome. Relatively, the hybridisation to the *Methylococcus* probes were some of the lowest hybridisation signals observed. This does not mean that *Methylococcus* is less abundant than the other methanotrophs, because when analysing the microarray data the potential for primer bias in amplification of the *pmoA* gene from the sample DNA needs to be taken into account. Primer bias could occur if the *pmoA* sequences in Movile Cave differ in sequence enough at the positions where the primers are designed to anneal. Two primer sets are used along with a nested approach, using both sets to try and account for either of the sets, missing any target sequences and also to cover the various *pmoA* sequences that are known. *pmoA* gene PCR products and hybridisation probe efficiency may also be weak if the target sequences are novel or have a slight sequence variation to a matching probe. There is also the possibility that there isn't a probe designed that would hybridise with any potentially novel *pmoA* gene sequences.

When looking at the diversity of methanotrophs in general, rather than of a particular gene, both metagenome and microarray suggest a similar level of diversity. The metagenome highlighted organisms related to *Methylococcu*s, *Methylosinus*, *Methylocella*, *Methylocystis* and *Methylobacter*. The *pmoA* microarray highlighted *pmoA* sequences related to genera *Methylomonas*, *Methylococcus*, *Methylocalcdum*, *Methylohalobius*/*Methylothermus*, *Methylocystis* and *Methylosinus*. The microarray could not identify *Methylocella* or *Methyloferulla*, as they don't have a particulate methane monooxygenase.

Methanotroph species	Abundnce in metagenome	Abundance in *pmoA* microarray
Methylococcus	+++	++
Methylocystis	+	++
Methylomonas	-	+
Methylosinus	++	+
Methylocella	++	-
Methylobacter	+	-
Methylocaldum	-	+
Methylohalobius / Methylothermus	-	+

Table 3.8: Comparison of relative occurrence of different methanotroph species observed from the metagenome and microarray analyses. "+++" is very high occurrence, "++" is high occurrence, "+" is low occurrence and "-" is absent.

From this it can be assumed that *Methylococcus* species are likely to be the most abundant in the microbial floating mat, as they are highly represented in both the metagenome and the *pmoA* microarray. *Methylocystis* and *Methylosinus* species are both represented in the metageome and *pmoA* microarray, and are also likely to be relatively abundant in the Movile Cave microbial floating mat. *Methylobacter* species

were only present in the metagenome, not as abundant in comparison to the other methanotroph species, but present nonetheless. *Methylomonas*, *Methylocaldum* and *Methylohalobius/Methylothermus* were present in the *pmoA* microarray, but not the metagenome and none with a particularly high abundance, but again there were still representatives of these methanotrophs in the Movile Cave microbial floating mat.

With these findings it can be hypothesised that *Methylococcus* species are the most abundant and active methanotrophs in Movile Cave microbial floating mat. It can also be hypothesised that there is a diverse community of methanotrophs active in the Movile Cave microbial floating mat.

There was little more to be gained in terms of methanotroph diversity from the other functional genes analysed from the metagenome. The same methane oxidising organisms were being highlighted, particularly *Methylococcus* which had representative sequences in the methanol dehydrogenase, formate dehydrogenase and hexulose-6-phosphate synthase tables. Unfortunately, there were no hydroxypyruvate reductase genes from methanotrophs identified. It is again assumed that this is due to a lack of sequence coverage or poor annotation.

Metagenomics has no doubt been a valuable tool in this study, but there are concerns over how much information can be taken from it. As the cost of metagenomic sequencing comes down drastically, there is a huge increase in the number of uncurated sequences (Temperton and Giovannoni, 2012). This then has knock-on effects for the annotation of future metagenomic studies. A sequence could be

assigned based on a sequence, which itself could be an uncurated annotated sequence. For now, all that can be done is to treat the output of metagenomics with caution and use the data as an indication of potential microbes and metabolism in a given environment from which hypotheses can be drawn and subsequently tested.

Chapter 4

Isolation of the methanotroph: Candidatus: *Methylomonas* LWB from Movile Cave

4.1 Introduction

To gain a more in-depth understanding of the organisms carrying out methane oxidation in Movile Cave, enrichment and isolation of methanotrophs was carried out. This process is used to gain better understanding of individual organisms from which conclusions may be drawn as to why they succeed in a given niche. Methane oxidising bacteria have been isolated and characterised for more than a century. The first described *Methylomonas* species, then named *Bacillus methanicus*, appeared in a communication by Söhngen (1906). Since then there have been many methane oxidising bacteria from the Proteobaceria and Verrucomicrobia phyla isolated and characterised (Hanson and Hanson, 1996; Pol et al., 2007; Dunfield et al., 2007; Trotsenko and Murrell, 2008).

Bacterial isolation is conducted so that individual organisms can be characterised based on how they react to any given stimulus. This is an important facet of microbiology, understanding how the individual components (in this case, bacteria) contribute to the overall output or function of a given ecosystem (in this case, Movile Cave). The function of the environment is a sum of the individual components. Bacterial isolation facilitates the finer detail and understanding of the individual components. Once characterisation of the individual components has been done, models and predictions can be made as to what may happen if a given environmental sample experiences different stimuli. Here isolation of methanotrophs was attempted in order to characterise them and to gain a better understanding of their function in Movile Cave.

Characterisation of methane oxidising bacteria has typically involved monitoring the growth of an organism in bacterial cell culture under varying conditions. More recently with the availability of high-throughput DNA sequencing, it has been possible to sequence the entire genomes of a number of methane oxidising species such as *Methylococcus capsulatus* Bath, *Methylocystis parvus* OBBP, *Methylosinus trichosporium* OB3B and *Methylomonas methanica* MC09 (Ward et al., 2004; del Cerro et al., 2012; Stein et al., 2010 and Boden et al., 2011). Genome sequences do not dictate physiology in the environment. They do, however, provide a metabolic blueprint of how organisms grow and cope with certain environmental stresses. Genomes also provide longer regions of DNA sequence from which gene phylogenies can be determined. Phylogeny comparisons are more robust when longer DNA sequences are compared. DNA sequences obtained from whole genome sequence will likely be longer than the shorter sequences obtained from PCR-based gene analysis, therefore improving the robustness of the sequence comparison conclusions.

The genome of a *Methylomonas* species isolated in this study was sequenced. The genome was sequenced to identify genetic traits that make this organism suitable to living in the conditions of Movile Cave. Genome comparison can also be carried out with the genomes of closely related organisms to identify differences and similarities that are dictated by the environments from which they are isolated. For example, genome size and an allowance for genetic redundancy may vary between two organisms if they are from environments with varying nutrient availability (Giovannoni et al., 2008; Grote et al., 2012).

The aim of this section of work was to:

- Isolate and identify methanotrophic bacteria from Movile Cave
- Compare methanotrophic isolates obtained with closely related species
- Characterise methanotrophic isolates obtained at the genomic level

4.2 Enrichment, isolation and characterisation

Method

A sample of water taken from the lake in Movile Cave was used to enrich and isolate methane oxidising bacteria. The enrichment culture was set up in a 120 ml serum vial with 20 ml of lake water. The air in the vial was flushed with oxygen-free-nitrogen to remove all other gases and then to amend with to contain 7 % O_2 and 3.5 % CO_2, as would be found in the cave atmosphere (Riess et al., 1999). The serum vial was then spiked with 10 ml of CH_4 as carbon source for enrichment of obligate methanotrophs.

After two weeks, 50 µl of the enrichment culture was spread onto DBS agar plates together with a 1:10 and 1:100 dilutions of the culture. The spread plates were then incubated in plastic boxes containing ~10 % CH_4 in air. Plates were incubated for two weeks and monitored for microbial colony formation. A selection of colonies that had grown was streaked out onto fresh DBS agar plates. After several sub-cultures of single colonies, the cultures were deemed to be pure. Further purity checks included microscope examination of cells from colonies under phase contrast microscopy (x1000) and lack of growth on R2A agar plates.

Results

Initial identification of the methanotroph isolates was determined by 16S rRNA gene sequencing. The 16S rRNA gene sequence was obtained from the isolates by colony PCR and sequencing of the PCR product. One of the isolates appeared to be a genuine methanotroph. Isolate LWB 16S rRNA gene sequence was used as a query sequence to search the GenBank database using nucleotide BLAST. The closest hits to the isolate sequence all appeared to be 16S rRNA genes of *Methylomonas* species. The phylogeny of isolate LWB was determined from the 16S rRNA gene sequence obtained from the LWB genome (**Figure 4.1**).

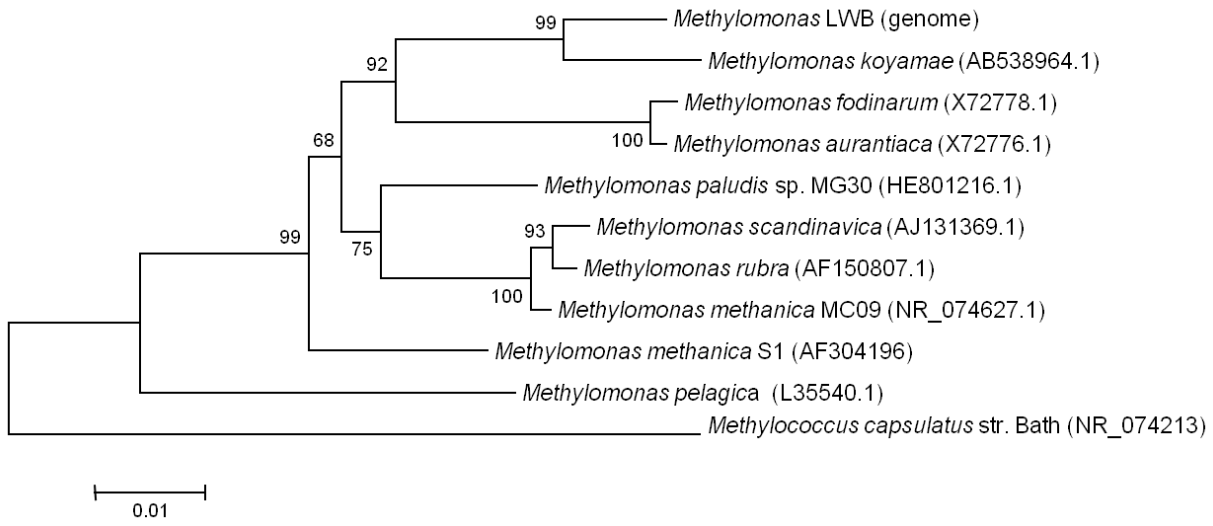


Figure 4.1: Neighbour-joining phylogenetic tree of *Methylomonas* 16S rRNA gene sequences including the 16S rRNA gene sequence from the isolate *Methylomonas* LWB. The sequence alignment was determined using ClustalW with the sequences covering 1,300 nucleotide positions. The scale bar represents 1 % sequence divergence. Bootstrap values calculated over 1000 replicates are represented at the nodes. The sequence at the root of the tree is the 16S rRNA gene sequence of *Methylococcus capsulatus* Bath (NR_074213). The phylogenetic tree was created using MEGA5.

The 16S rRNA gene sequence of isolate LWB confirmed the isolate to be a *Methylomonas* species as determined by BLAST of the sequence against the GenBank database. The *Methylomonas* LWB 16S rRNA gene sequence branched separately from other *Methylomonas* species along with the sequence of *Methylomonas koyamae*. *Methylomonas koyamae* was isolated by Ogiso et al., (2012) from floodwater of a Japanese rice paddy field. When observing the phylogenetic

distance between the different species of *Methylomonas* on the 16S rRNA gene phylogenetic tree, there appeared to be a greater sequence divergence between *Methylomonas* LWB and *Methylomonas koyamae* than there is between other *Methylomonas* species. *Methylomonas* LWB and *Methylomonas koyamae* share 97 % sequence identity of the 16S rRNA gene, whereas *Methylomonas fondinarum* and *Methylomonas aurantiaca* share 99 % sequence identity over the same region and are deemed to be different species. This is good evidence to suggest that *Methylomonas* LWB may in fact be a new species of the genus *Methylomonas*.

4.3 Growth of *Methylomonas* LWB in the presence of tetrathionate ($S_4O_6^{2-}$)

Movile Cave water contains high concentrations of sulfide (240 µM) (Porter et al., 2009). Some bacteria including species of *Thiobacillus* (Podgorsek and Imhoff,1999) can produce tetrathionate from sulfide. There are polythionates in Movile Cave (measured at 250-500 µM in airbell 2 with tetrathionate being around 70 µM of the total polythionate (Boden, personal communication, unpublished)). *Thiobacillus* species were identified in Movile Cave by Chen et al., (2008) and likely benefit from the source of tetrathionate. Organisms such as the obligate chemolithoautotroph *Thermithiobacillus tepidarius* (Lu and Kelly, 1988) can gain energy from tetrathionate as an energy source. It was hypothesised that isolates from Movile Cave might gain energy from substrates such as tetrathionate. Pure cultures of *Methylomonas* LWB were grown on methane plus increasing concentrations of tetrathionate to observe its effect on growth (**Figure 4.2**).

Method

Methylomonas LWB cultures were set up in 120 ml serum vials containing 10 % (v/v) methane in air. Cultures were grown in ANMS medium in a total volume of 20 ml with an inoculum of 2 ml. Tetrathionate was added to the cultures from a 100 mM stock solution to give a range of concentrations of 0, 0.5, 1, 5 and 10 mM. All cultures were set up in triplicate. Growth of the cultures was monitored by measuring the optical density at 440 nm (OD_{440}).

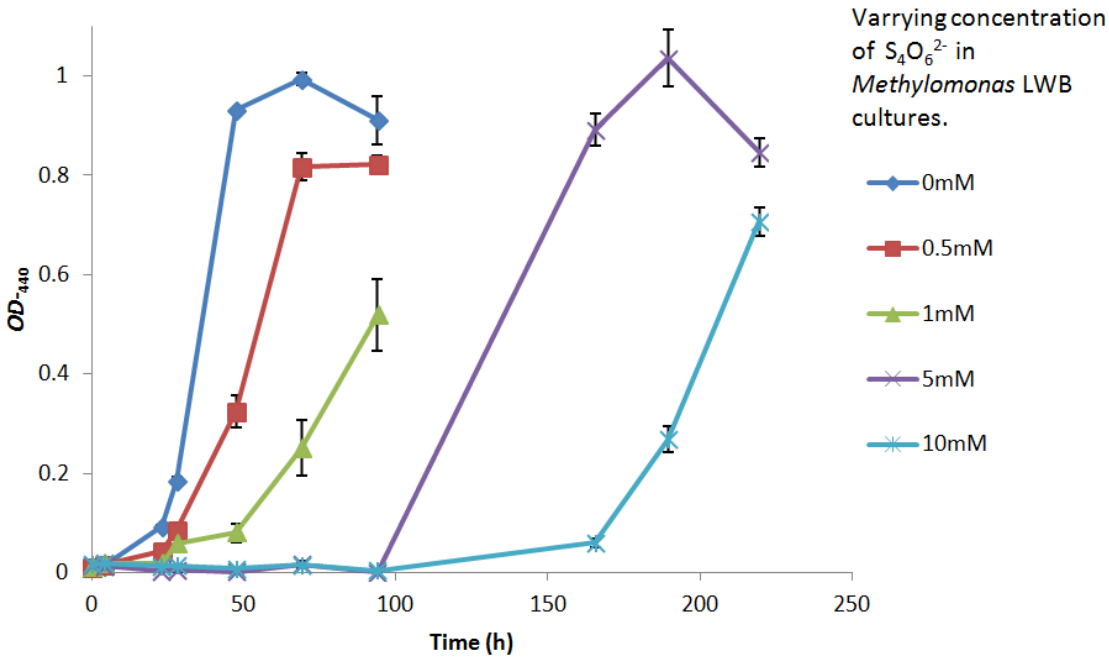


Figure 4.2: Growth curves of *Methylomonas* LWB grown in the presence of increasing concentrations of $S_4O_6^{-2}$. Culture growth was monitored by measuring optical density of the cultures at 440 nm (OD_{440}). Measurements for cultures containing 5 mM and 10 mM $S_4O_6^{2-}$ were taken at 165 hours to 225 hours when it was noticed they had started to grow. Growth curves were conducted in triplicate; the error bars represent standard deviation from the mean.

Results

Increasing concentrations of tetrathionate in the culture medium resulted in an increasing lag phase before growth was observed (**Figure 4.2**). *Methylomonas* LWB culture with no addition of tetrathionate had reached its maximum OD by 69 h. The OD then appears to fall. It was noticed at this point that the culture had begun to flocculate and form a pellicle around the edge of the serum vial. There was no significant difference in growth yield between the cultures grown without tetrathionate and those grown in the presence of 5 mM tetrathionate. With these data, it is not known what the final yield OD would have been observed with the 1mM and 10 mM tetrathionate cultures. It is likely that the 1 mM tetrathionate would have given a similar yield to that of no tetrathionate and 5 mM tetrathionate cultures. It was assumed that the 5 mM and 10 mM tetrathionate cultures were not going to grow by the 94 h time point. It was noticed later that they had begun to grow; it is not known at what point the 5 mM tetrathionate culture began to grow. From this data set, it looks very unlikely that *Methylomonas* LWB can grow whilst using tetrathionate as a supplementary energy source. There is no increase observed in final yield in the presence of tetrathionate, and growth rate appeared to be slower than the control.

4.4 The genome of *Methylomonas* strain LWB

Method

A 50 ml batch culture of *Methylomonas* LWB was grown and the DNA extracted as described in section **2.4.1**. The DNA sample was sent to The Genome Analysis Center (TGAC), Norwich for genomic sequencing. The DNA sample was sequenced using an Illumina MiSeq benchtop sequencer.See section 2.11 for more detail. The assembled data were then uploaded to the RAST website for annotation and analysis (http://rast.nmpdr.org). Further analysis was also carried out by uploading the assembled genome to the IMG genome analysis website (http://img.jgi.doe.gov/).

Result

The *Methylomonas* LWB genome consisted of 5,365,682 bp over 102 contigs. There were a total of 5296 predicted genes. For further details see section **4.4.3.**

4.4.1 Analysis of *Methylomonas* strain LWB methane functional gene sequences

The *Methylomonas* LWB genome was screened for methane monooxygenase genes encoding the first enzyme in the methane oxidation pathway. There were two sets of genes encoding particulate methane monooxygenase found along with a single soluble methane monooxygenase gene cluster. One of the sets of particulate methane monooxygenase genes conformed to the expected gene arrangement of *pmoCAB.* However, the second set had the gene arrangement *pmoABC* (**Figure 4.3**). The *pmoA* gene from the *pmoCAB* operon was too short (219 bp, 73 aa, **Table 4.1**) to be

included in the following PmoA alignment, but the *pmoA* sequence from the *pmoABC*, designated PxmA, was present at full length and included in the alignment for the phylogenetic tree of **Figure 4.4**. The *pmoA* from the *pmoCAB* operon along with flanking sequence would not align with the other *pmoA* or *pxmA* sequences.

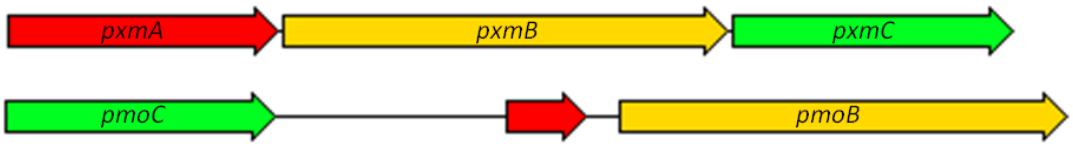


Figure 4.3: Arrangement of the two particulate methane monooxygenase operons found in the *Methylomonas* LWB genome. *pmoC* is represented by the green arrow, *pmoA* is represented by the red arrow and *pmoB* is represented by the yellow arrow. The *pmoA* gene from the *pmoCAB* operon (bottom) has an incomplete *pmoA* gene sequence identified.

Table 4.1: Size of particulate methane monooxygenase genes as annotated by RAST.

Gene	*Methylomonas* LWB Pxm	*Methylomonas* LWB Pmmo	*Methylomonas methanica* MC09 Pmmo
pmoA/pxmA	753 bp – 251 aa	219 bp – 73 aa	750 bp – 250 aa
pmoB/pxmB	1239 bp 413 aa	1245 bp – 415 aa	1245 bp – 415 aa
pmoC/pxmC	774 bp 258 aa	753 bp – 251 aa	213 bp – 71 aa

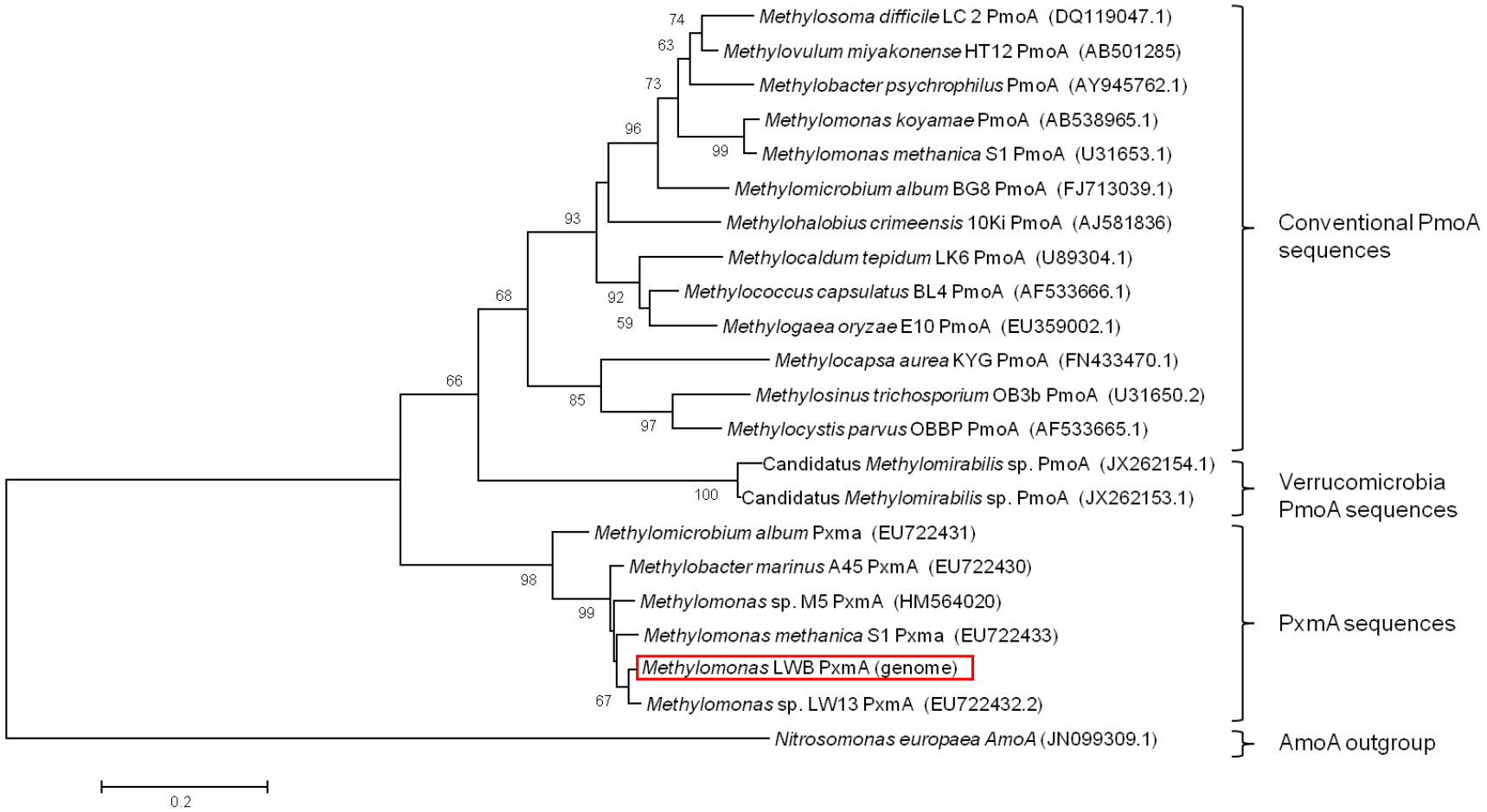


Figure 4.4: Figure legend, see next page.

Figure 4.4: Neighbour-joining phylogenetic tree of PmoA and PxmA derived sequences showing the relationship of the *Methylomonas* LWB PxmA sequence among other particulate methane monooxygenase alpha-subunit sequences obtained from GenBank (accession numbers in brackets). The tree was created at the amino acid level (128 amino acid residues) derived from nucleotide sequences. Bootstrap values were calculated from 1000 replicates. Bootstrap values <50 were removed. The tree is rooted with the AmoA derived amino acid sequence from *Nitrosomonas europaea* (Winogradsky, 1892). The scale bar represents 0.2 substitutions per amino acid. The phylogenetic tree was created using Mega5.

The putative second copy of the *pmoA* gene from the *pmoABC* operon did not group with any of the derived PmoA sequences. Instead, the derived amino acid sequence of the gene clustered tightly with a group of sequences which have been annotated as being PxmA. The *pxm* genes belong to a divergent copy of the particulate methane monooxygenase gene set, which has the gene orientation of *pxmABC* (Tavormina et al., 2011). The *pxm* genes have only been observed in Gammaproteobacterial methanotrophs.

The *Methylomonas* LWB genome was shown to contain a set of genes that encode a soluble methane monooxygenase enzyme. Other *Methylomonas* species including MC09, LW13 and M5 all have soluble methane monooxygenase genes as well as the genes encoding the particulate methane monooxygenase (Boden et al., 2011; Auman et al., 2000 and Kip et al., 2011). The phylogeny of the derived MmoX sequence of *Methylomonas* LWB was compared to other derived MmoX sequences obtained from

the Genbank database (**Figure 4.5**). The MmoX sequence of *Methylomonas* LWB clustered among the other Type I methanotroph MmoX sequences as expected. It is well known that there is a "copper switch" in methane oxidising bacteria which contain both a particulate and soluble methane monooxygenase. This copper switch operates depending on the copper to biomass ratio during growth of cells (Murrell et al., 2000; Semrau et al., 2013). When the copper to biomass ratio is high, the particulate methane monooxygenase is expressed. When the copper to biomass ratio is low, the soluble methane monooxygenase is expressed. This may be an indication that *Methylomonas* LWB may encounter situations where copper availability in Movile Cave is low and, therefore, it may be a competitive advantage for strain LWB to have sMMO as well as pMMO.

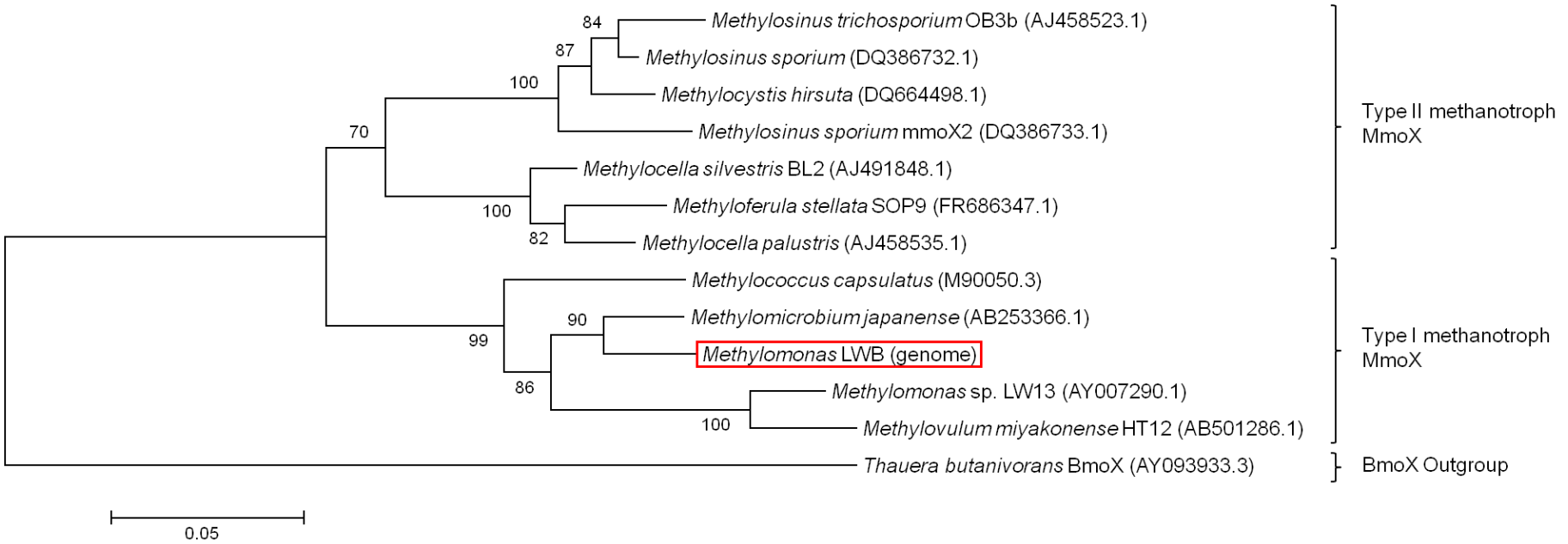


Figure 4.5: Neighbour-joining phylogenetic tree of MmoX derived sequences of *Methylomonas* LWB and other MmoX sequences encoding the soluble methane monooxygenase, alpha subunit, obtained from GenBank (accession numbers in brackets). The tree was created at the amino acid level (367 amino acid residues) derived from nucleotide sequences. Bootstrap values were calculated from 1000 replicates. The tree is rooted with the BmoX derived amino acid sequence from *Thauera butanivorans* (Takahashi et al., 1980), encoding the homologous alpha subunit of butane monooxygenase. The scale bar represents 0.5 substitutions per amino acid. The phylogenetic tree was created with Mega5.

Methylomonas LWB has the genes encoding the second enzyme in the methane metabolic pathway, methanol dehydrogenase. As mentioned earlier, not all methanotrophs contain the genes encoding either or both forms of the methane monoogygenase enzyme (pMMO and sMMO). All methanotrophs identified to date do however have the genes encoding methanol dehydrogenase. Lau et al., (2013) showed that methanotroph 16S rRNA gene phylogeny was almost mirrored by the phylogeny of the *mxaF* gene sequences (**Figure 4.6**) Therefore, the identity of *Methylomonas* LWB can therefore be inferred from the phylogeny of its *mxaF* gene sequence. In the phylogenetic tree of methanotroph *mxaF* gene sequences in **Figure 4.6**, the *mxaF* gene sequence of *Methylomonas* LWB is most closely related to the *mxaF* gene sequence of another *Methylomonas* species.

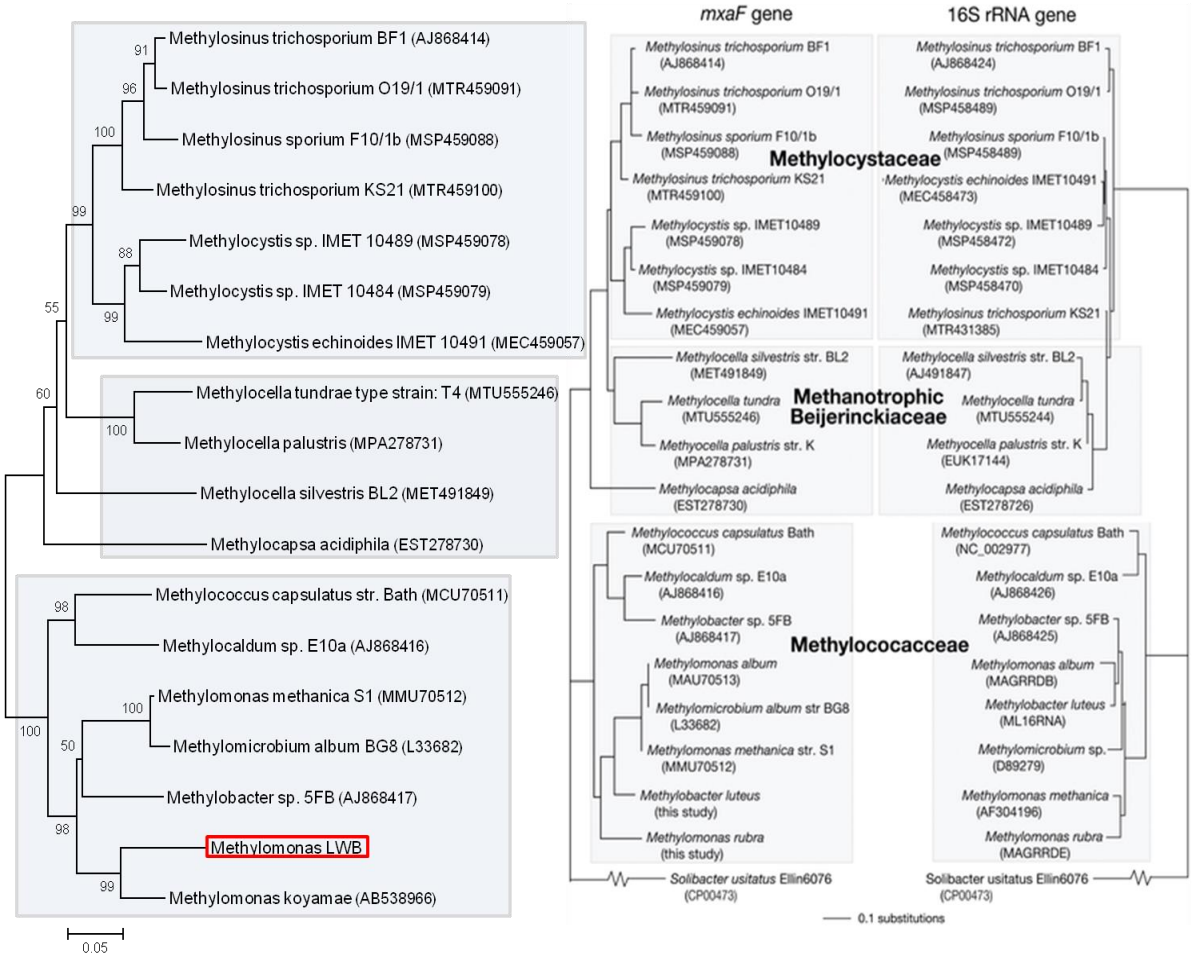


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Figure 4.6: Left, is a neighbour-joining phylogenetic tree of *mxaF* gene sequences obtained from several genera of methanotroph. Sequences were obtained from the Genbank database (accession numbers in brackets). The tree was constructed at the nucleotide level comparing 496 nucleotide positions. The alignment was constructed using ClustalW, while evolutionary distance was calculated using the Jukes-Cantor method. Bootstrap values were calculated from 1000 replicates. Right, is a figure taken from Lau et al. 2013 illustrating the similarity of methanotroph phylogeny when determined using either the *mxaF* gene sequence or the 16S rRNA gene sequence.

4.4.2 Metabolic pathway analysis for *Methylomonas* LWB

The potential metabolic pathways of *Methylomonas* LWB were determined using the Kegg recruitment maps through The Seed Viewer on the rast.nmpdr.org website. These were used to determine if the *Methylomonas* LWB genome contained the relevant genes encoding enzymes for specific metabolic pathways. It should be noted that the *Methylomonas* LWB genome was not closed, which leaves the potential for a number of genes to not be present and, therefore, will not be present on the Kegg recruitment plots. **Figure 4.7** displays all potential enzymes in the methane oxidation pathway. All of the enzymes required to oxidise methane through to carbon dioxide via methanol, formaldehyde and formate (methane monooxygenase, methanol dehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase) are present in the *Methylomonas* LWB genome.

There are two routes for formaldehyde assimilation in methanotrophs, the ribulose monophosphate pathway or the serine cycle (Hanson and Hanson, 1996). The gene for the enzyme formaldehyde transketolase (2.2.1.3) is present, which produces D-glyceraldehyde-3-phosphate from formaldehyde and D-xylulose-5-phosphaste. The D-glyceraldehyde-3-phosphate then feeds into the ribulose monophosphate pathway. The *Methylomonas* LWB genome also contains the genes encoding serine hydroxymethyltransferase (2.1.2.1), which is used to combine formaldehyde bound to methylene-tetrahydrofolate with glycine to produce the serine which feeds into serine metabolism.

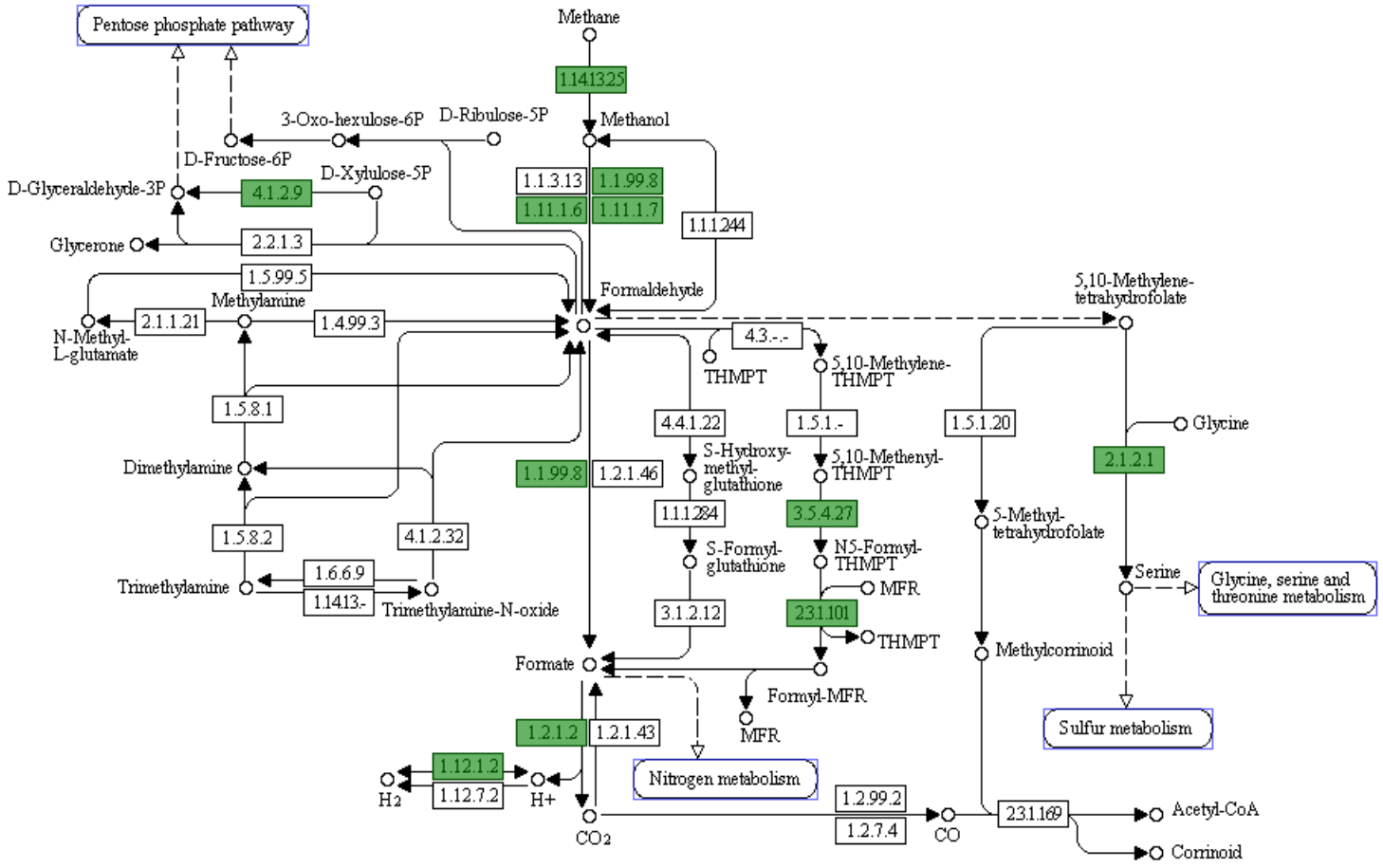


Figure 4.7: Kegg recruitment plot of the genes from the *Methylomonas* LWB genome that are involved in methane metabolism. Enzyme positions highlighted in green indicate that the gene encoding that enzyme is present in the *Methylomonas* LWB genome.

D-glyceraldehyde-3-phosphate enters the ribulose monophosphate pathway (**Figure 4.8**, bottom left) after the condensation of formaldehyde and D-xylulose-5-phosphate. There are many of the genes involved in the production of enzymes for the ribulose monophosphate pathway present in the *Methylomonas* LWB genome. The D-glyceraldehyde-3-phosphate is converted into β-D-fructose-6-phosphate via fructose –bisphosphate aldolase and fructose-bisphosphatase (4.1.2.13 and 3.1.3.11 respectivley). The β-D-fructose-6-phosphate can be broken down to produce more D-xylulose-5-phosphate by a transketolase enzyme (2.2.1.1), or it can be converted into α-D-glucose-6-phosphate by the enzyme glucose-6-phosphate isomerase (5.3.1.9). The α-glucose-6-phosphate is converted into 6-phospho-D-gluconate via 3 enzymes (5.3.1.9, 1.1.1.49 and 3.1.1.31); the genes encoding all of these enzymes are present in the *Methylomonas* LWB genome. Genes encoding the enzyme that converts 6-phospho-D-gluconate into D-ribulose-5-phosphate, phosphogluconate dehydrogenase (1.1.1.44), are not present in the *Methylomonas* LWB genome. There are, however, genes encoding enzymes that are used to utilise D-Ribulose-5-phosphate. These include ribulose-phosphate 3-epimerase (5.1.3.1) used to produce D-Xylulose-5-phosphate and ribose-5-phosphate isomerase (5.3.1.6), which is used to produce D-ribose-5-phosphate for further reactions in the cycle. It may be that the phosphogluconate dehydrogenase (1.1.1.4) is missing due to the LWB genome not being complete.

Alternatively, the 6-phospho-D-gluconate can be converted into 2-dehydro-3-deoxy-D-gluconate-6-phosphate by the enzyme phosphogluconate dehydrogenase (4.2.1.12); the gene encoding this enzyme is present in the *Methylomonas* LWB genome. The next enzyme in the pathway, which also has the corresponding gene

present in the genome, is 2-dehydro-3-deoxy-phosphogluconate aldolase (4.1.2.14) ; this is used to split the 2-dehydro-3-deoxy-D-gluconate-6-phosphate into pyruvate and D-glyceraldehyde-3-phosphate, which both feed into glycolysis. The gene encoding ribose-phosphate pyrophosphokinase (2.7.6.1) is also present in the *Methylomonas* LWB. D-ribose-5-phosphate is converted into 5-phospho-α-D-ribose 1-diphosphate which feeds into purine, pyrimidine and histidine metabolic pathways.

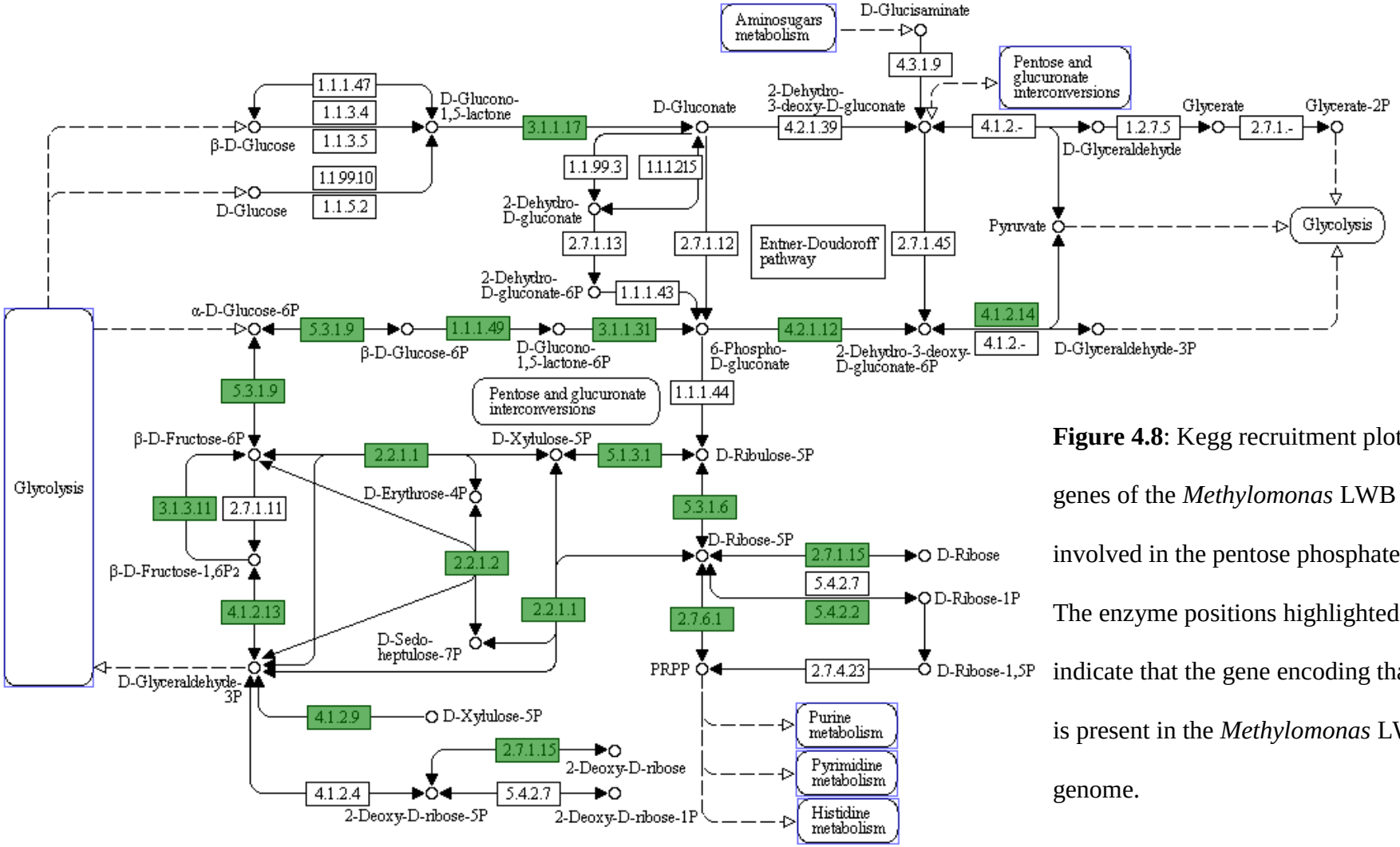


Figure 4.8: Kegg recruitment plot of the genes of the *Methylomonas* LWB genome involved in the pentose phosphate pathway. The enzyme positions highlighted in green indicate that the gene encoding that enzyme is present in the *Methylomonas* LWB genome.

The *Methylomonas* LWB genome has the gene required to produce the enzyme which makes serine from glycine and formaldehyde (2.1.2.1). The serine then feeds into the serine cycle (**Figure 4.9**). Pyruvate can be produced directly from serine by the enzyme L-serine ammonia-lyase (4.3.1.17), for which the corresponding gene is present in the *Methylomonas* LWB genome. There are genes present for the enzymes required to metabolise serine into D-glycerate-3-phosphate (3.1.3.3, 2.6.1.52 and 1.1.1.95), which leads into the glycolysis pathway. There is also the gene encoding serine-glyoxylate transaminase (2.6.1.45) which produces hydroxypyruvate further leading into glyoxylate metabolism. Glyoxylate metabolism via hydroxypyruvate does not appear to be an option for *Methylomonas* LWB (**Figure 4.10**). There are not sufficient genes present to produce the enzymes that can facilitate glyoxylate production from hydroxypyruvate. Therefore, it is likely that the ribulose monophosphate pathway (**Figure 4.8**) is the major route for formaldehyde assimilation in *Methylomonas* LWB.

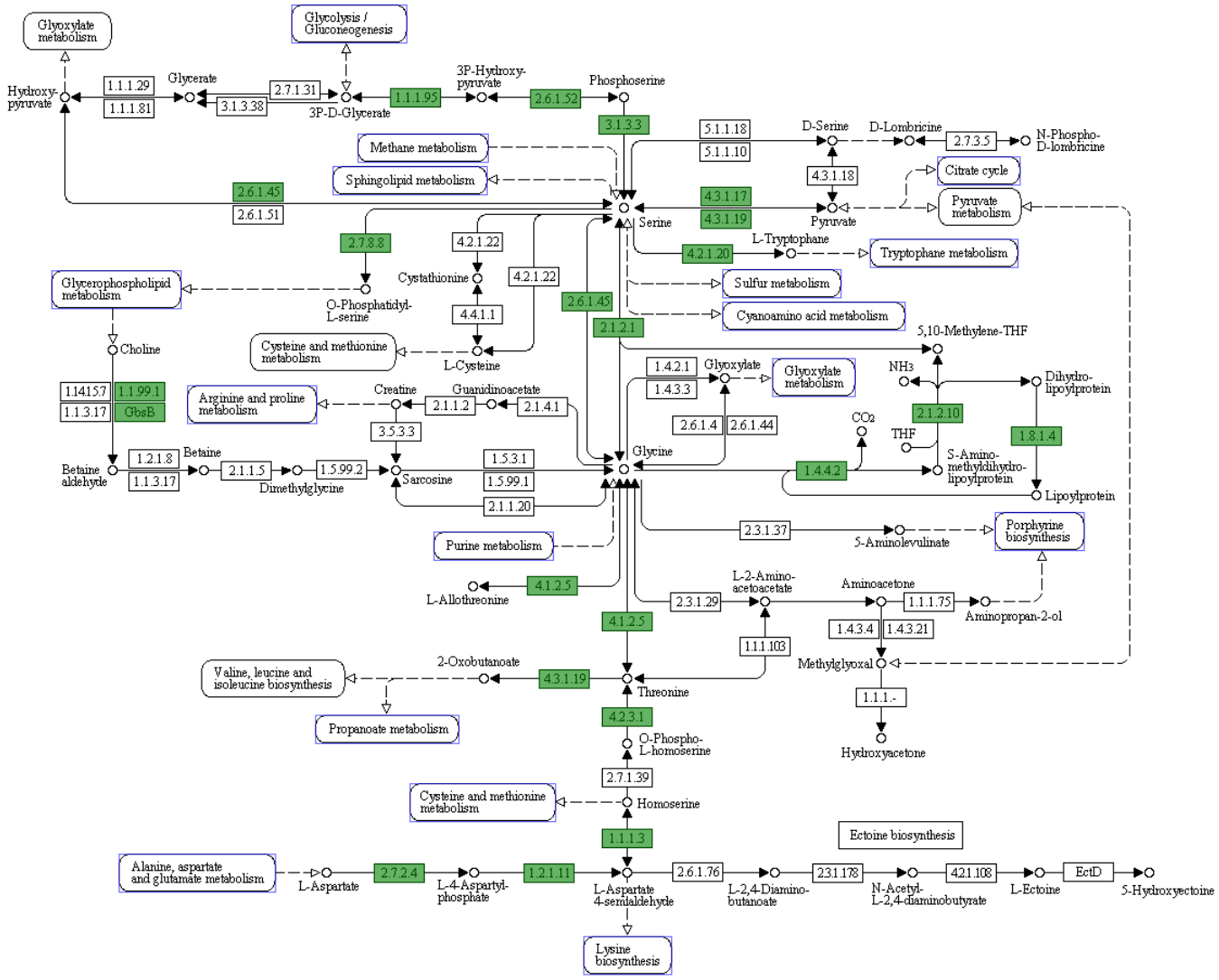


Figure 4.7: Kegg recruitment plot of the genes involved in the serine, glycine and threonine metabolic pathways from the *Methylomonas* LWB genome. The enzymes highlighted in green indicate that the corresponding gene is present in the genome.

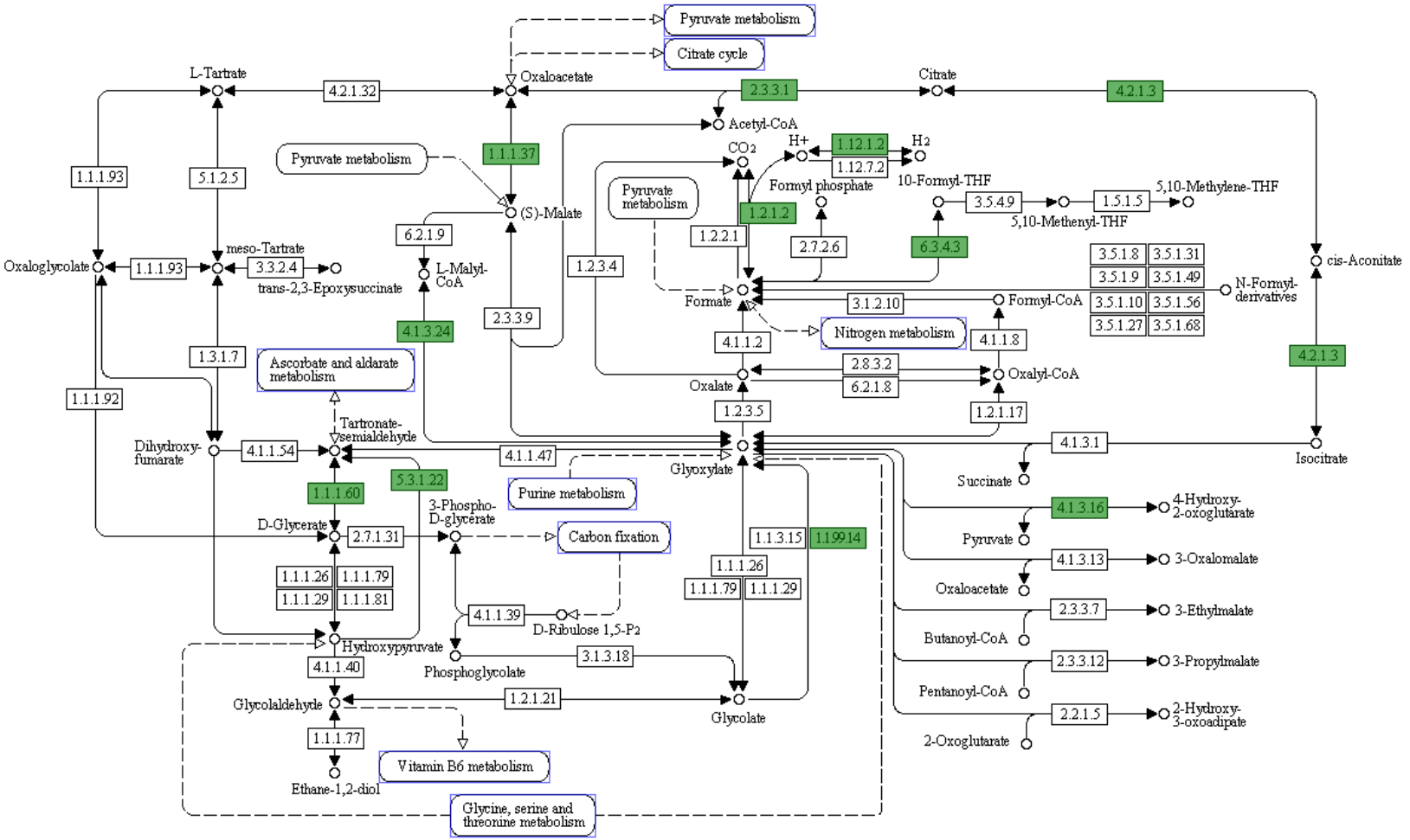


Figure 4.8: Kegg recruitment plot of the genes involved in glyoxylate metabolism from the *Methylomonas* LWB genome. The enzymes highlighted in green have corresponding genes in the genome.

Both pyruvate and D-glyceraldehyde-3-phosphate produced by the ribulose monophosphate pathway and partly by the serine pathway lead into the glycolysis pathway (**Figure 4.11**). There is nearly a full suite of genes in the *Methylomonas* LWB genome to facilitate glycolysis. Genes are present for enzymes that are required for glucose metabolism into α-D-glucose-6-phosphate (2.7.1.2, 5.3.1.9), which can feed into the ribulose monophoshate pathway or convert into D-glyceraldehyde-3-phosphate. The D-glyceraldehyde-3-phosphate, either from glycolysis or the Ribulose monophosphate pathway, can be converted into pyruvate by the enzymes 1.2.1.12, 2.7.2.3, 5.4.2.1, 4.2.1.11 and 2.7.1.40; the corresponding genes of which are present in the *Methylomonas* LWB genome. In the case of pyruvate metabolism, all genes are present to facilitate the production of the enzymes (1.2.4.1, 2.3.1.12 and 1.8.1.4), which are needed to feed pyruvate into the TCA cycle. Interestingly there are genes present for some of the enzymes (6.2.1.1, 1.2.1.3, 1.1.1.1 and 1.1.99.8) that are used to ferment acetyle-CoA, produced from the pyruvate, via acetate and acetaldehyde into ethanol. The genes for the enzyme required to convert pyruvate directly into acetaldehyde (4.1.1.1) is not present.

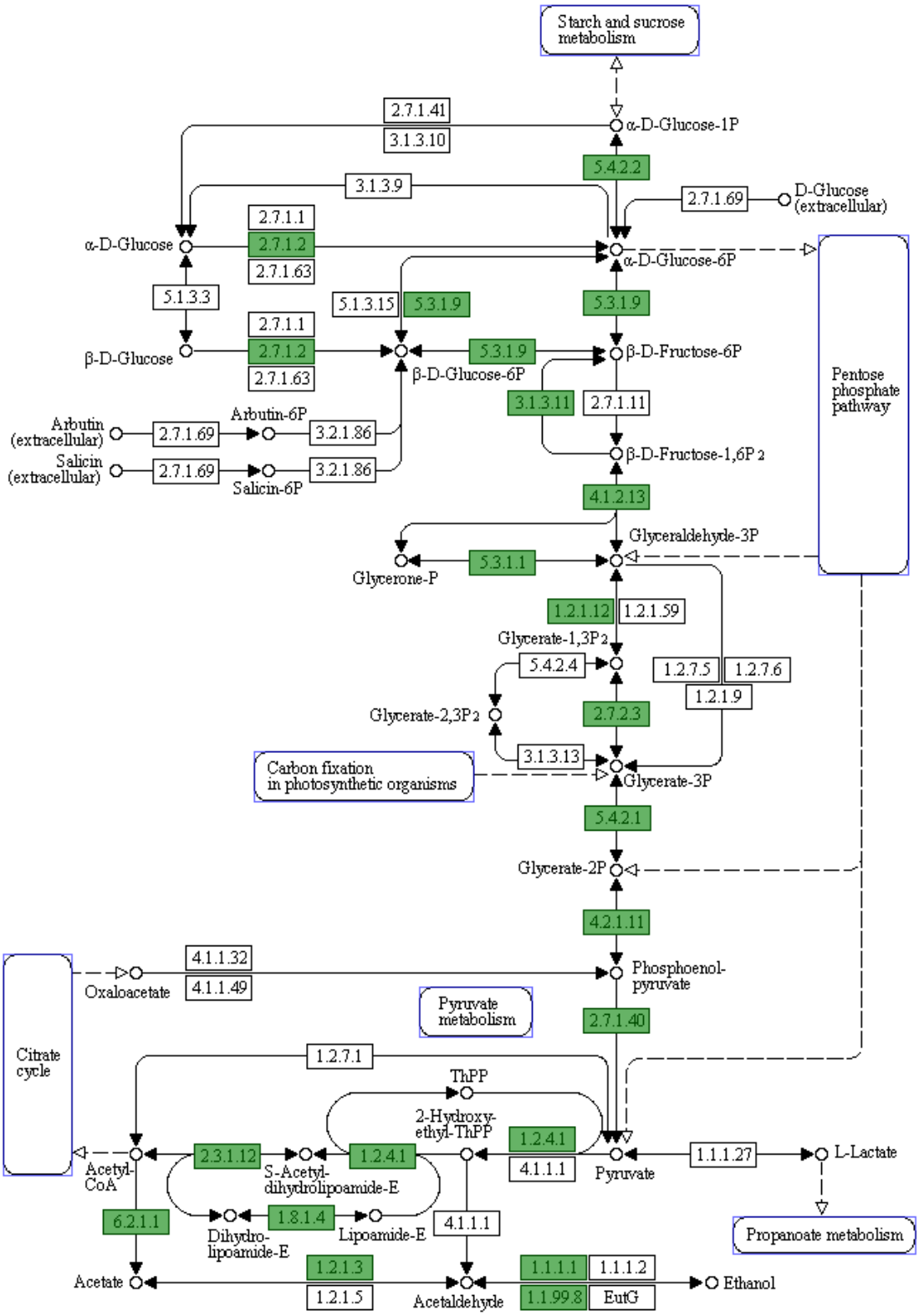


Figure 4.11: Kegg recruitment plot of the genes involved in glycolysis that are present in the *Methylomonas* LWB genome. The enzymes highlighted in green indicate that corresponding genes can be found in the *Methylomonas* LWB genome.

Pyruvate produced from either the ribulose monophosphate pathwathway or glycolysis feeds into the TCA (tricarboxylic acid) or Krebs cycle (**Figure 4.12**). The TCA cycle is the major metabolic powerhouse of bacteria producing reducing equivalents to facilitate dependant metabolic reactions. Pyruvate is converted into oxaloacetate by pyruvate carboxylase (6.4.1.1), with the addition of malate by malate dehydrogenase (1.1.1.37) or into acetyle-CoA as mentioned previously. Both enzymes have corresponding genes in the *Methylomonas* LWB genome to be facilitated. Both oxaloactate and acetyl-CoA feed into the TCA cycle to produce citrate. There are genes present in the *Methylomonas* LWB genome that encode for all of the necessary enzymes (4.2.1.3, 1.1.1.41, 1.1.1.42, 1.2.4.2, 2.3.1.61, 1.8.1.4, 6.2.1.5, 1.3.99.1, 4.2.1.2 and 1.1.1.37), that are required for the TCA cycle to convert citrate into malate and oxaloacetate and to produce reducing equivalents.

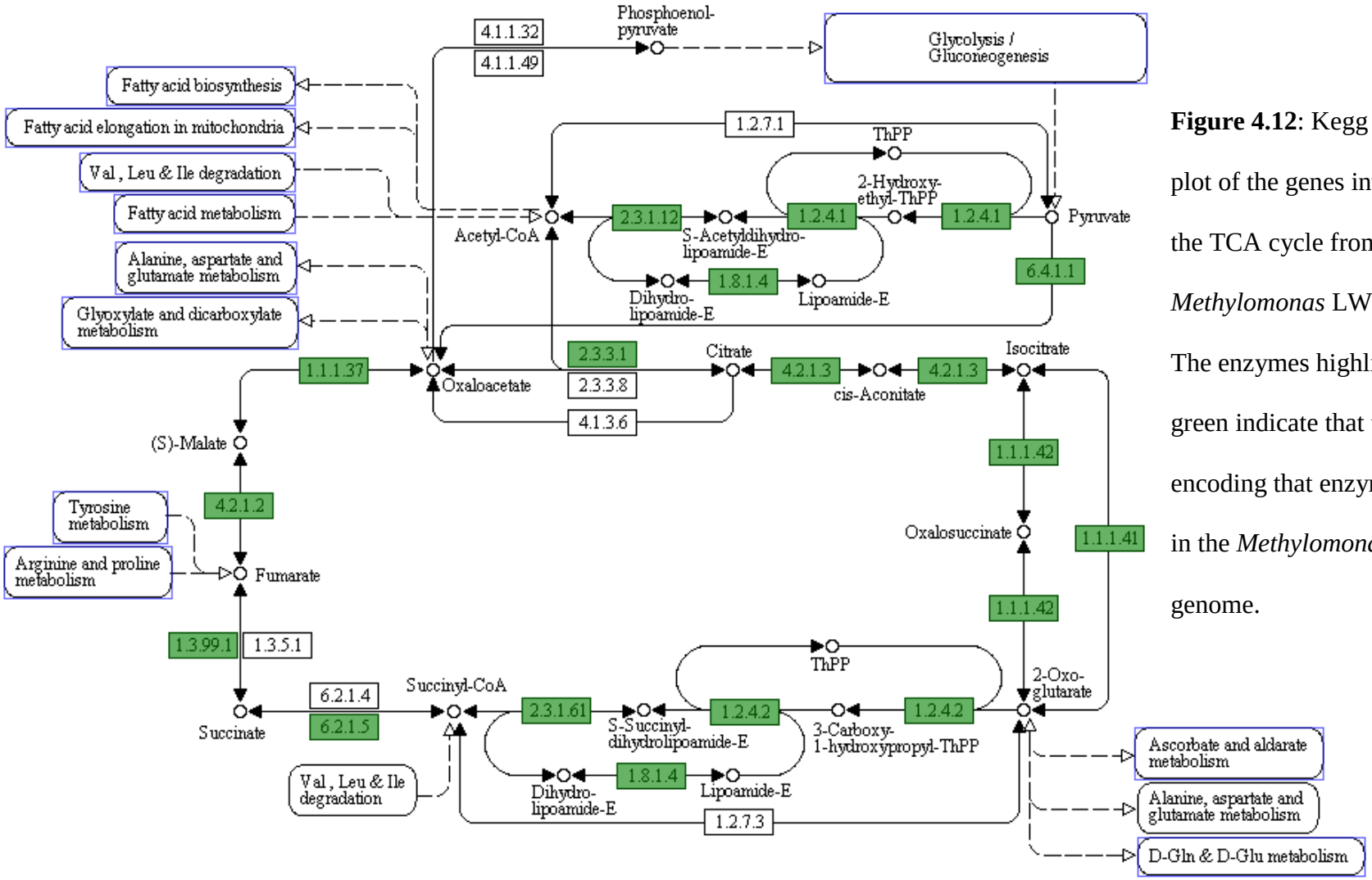


Figure 4.12: Kegg recruitment plot of the genes involved in the TCA cycle from the *Methylomonas* LWB genome. The enzymes highlighted in green indicate that the gene encoding that enzyme is present in the *Methylomonas* LWB genome.

The *Methylomonas* LWB genome contains the genes encoding the enzyme nitrogenase (1.18.6.1) which is used for the fixation of di-nitrogen (N_2) gas into ammonia (**Figure 4.13**). There is also the presence of the genes encoding the enzymes assimilatory nitrate reductase and nitrite reductase (1.7.99.4 and 1.7.1.4 respectively), which can produce ammonia from nitrate or nitrite. The *Methylomon*as LWB genome does not contain the genes for the enzymes (1.7.3.4, 1.7.1.10 and 1.7.99.1) that allow for ammonia oxidation to nitrite via hydroxylamine. There is also the lack of genes encoding the enzymes (1.7.2.1, 1.7.99.7 and 1.7.99.6) required to metabolise nitric oxide. There are several genes present in the *Methylomonas* LWB genome encoding enzymes for utilisation of ammonia; some of which are involved in amino acid production via glycine (2.1.2.10), the production of glutamine and glutamate (6.3.1.2, 1.4.1.13 and 6.3.5.4) and for the production of cyclic amides (6.3.1.5). Many genes for enzymes involved in obtaining ammonia from amino acids or nitrogenous compounds (1.4.3., 4.3.1., 1.14.13.35, 1.14.12.1, 4.4.1.1, 4.4.1.2 and 4.4.1.8) are not present in the *Methylomonas* LWB genome.

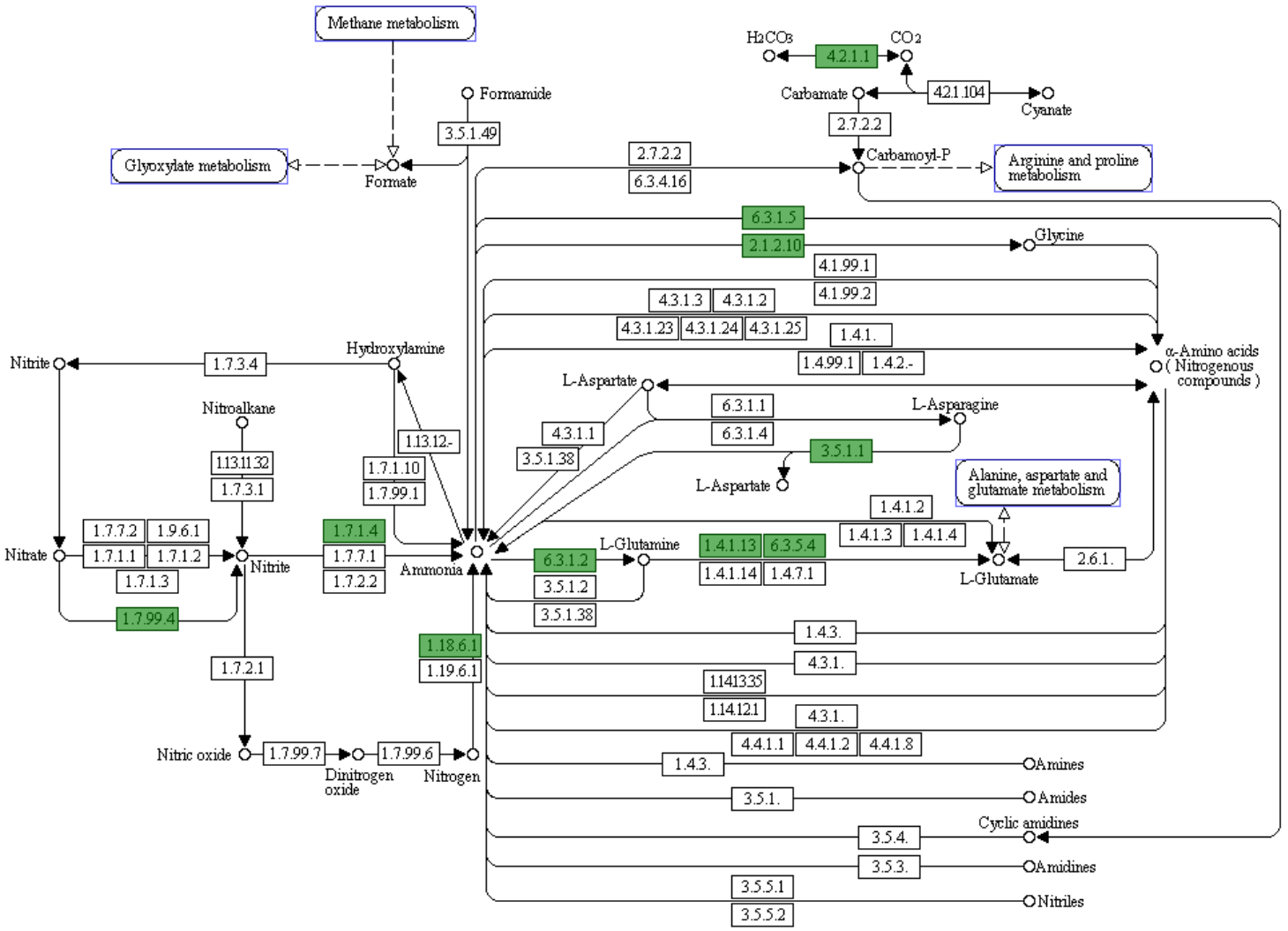


Figure 4.13 Kegg recruitment plot of the genes involved in nitrogen metabolism found in the *Methylomonas* LWB genome. Enzymes highlighted in green indicate that the gene encoding that enzyme is present in the *Methylomonas* LWB genome.

4.4.3 Genome comparison

The genome of *Methylomonas* LWB was uploaded to the IMG server for analysis. Of the 5,365,682 bp, 87.09 % is predicted to contain coding sequence with a G+C content of 55.92 %. A total of 5296 genes were predicted, with 5225 identified to be protein coding of which 3552 were identified to have a function. 71 RNA genes were identified including 3 x 5S rRNA, 6 x 16S rRNA, 9 x 23S rRNA and 53 x tRNA genes.

Data output from the RAST analysis server was slightly different to the output from the IMG output, but was used for genome comparison. The compared genomes here were both analysed after processing through the same pipeline. The genome of *Methylominas* LWB was compared to the genome of the most closely related organism with a fully sequenced genome, *Methylomonas methanica* MC09. It must be reiterated that the genome of *Methylomonas* LWB is not a closed genome and any genes that are absent, but present in the MC09 genome, may not be representative of the true nature of the *Methylomonas* LWB genome. A comparison of the major genome features can be found in **Table 4.2**.

Table 4.2: A comparison of genome features between the genomes of *Methylomonas* LWB and *Methylomonas methanica* MC09.

Feature	*Methylomonas* LWB	*Methylomonas methanica* MC09
Genome size	5,365,682 bp	5,051,681 bp
Number of Contigs	102	1
Number of predicted coding sequences	4825	4748
Number of functional annotations	1986	2071
Number of unique functional annotations	93	178
Number of shared functional annotations	1893	1893
Number of metabolism specific annotations		
Methane metabolism	11	11
Nitrogen metabolism	10	15
Sulfur metabolism	8	9
Pentose phosphate pathway	17	17

Despite being far from complete, the *Methylomonas* LWB genome has more predicted coding sequences than that of *Methylomonas* MC09. The two genomes are of relatively similar size (just over 5 million bp), but the *Methylomonas* LWB genome could be much larger once complete. The two genomes share a large number for functional annotations (1893). *Methylomonas* LWB has 93 unique functional annotations and *Methylomonas* MC09 has 179 unique functional annotations. On a functional basis, the two genomes share all the genes for methane metabolism, however *pxmABC* was absent from the *Methylomonas* MC09 genome. When the full

pxmA gene sequence was used as a query to BLAST against the *Methylomonas* MC09 genome, the top hit was the *pmoA* gene of *Methylomonas* MC09. However, the largest identity between the sequences was only over 30 bases. The *Methylomonas* MC09 genome was searched for the ABC orientation of particulate methane monooxygenase genes indicative of the pxm operon, but this was absent from the genome with only a single copy of the *pmoCAB* operon being present. Where nitrogen metabolism is concerned, the functions found in the *Methylomonas* LWB are also all present in the *Methylomonas* MC09 genome. The *Methylomonas* MC09 has additional genes for the metabolism of nitrite to nitric oxide and dinitrogen oxide along with additional genes involved with the metabolism between glutamine and glutamate. These include a second glutamate synthase and an asparagine synthase which are essential for ammonium assimilation. Therefore, it is likely that these are missing from the *Methylomonas* LWB genome due to the incomplete sequence. The only difference in sulfur metabolism function was the *Methylomonas* MC09 genome having a component of the enzyme alcohol sulfotransferase, conferring the ability for metabolism between 3'Phosphoadenylylsulfate and Adenosine3',5'-bisphosphate.

4.5 Discussion

Isolate LWB, is a methane oxidising bacterium that belongs to the genus *Methylomonas*. The 16S rRNA gene sequence of *Methylomonas* LWB groups closely with the other members of the *Methylomonas* genus. Based only on the 16S rRNA gene phylogenetic tree, *Methylomonas* LWB appears to be a new species sharing only 97 % identity to the closest organism *Methylomonas Koyamae*. The PxmA sequence observed provides more evidence for isolate LWB belonging to the *Methylomon*as genus, as PxmA genes have so far only been identified from Gammaproteobacterial methanotrophs, most of which have come from *Methylomonas* species. The PxmA gene sequence of *Methylomonas* LWB is most closely related to the PxmA sequence of *Methylomonas* LW13 (Auman et al., 2000). *Methylomon*as LW13 does not have a valid name with standing in nomenclature and has not been fully characterised. The *pxm* genes belong to a divergent copy of the particulate methane monooxygenase gene set, which has the gene orientation of *pxmABC*). It was indicated by Tavormina et al., (2011) that there was sufficient amino acid representation and similarity of the PxmA to the PmoA to suggest that the Pxm monooxygenase would also be a membrane bound enzyme. Tavormina et al., (2011) were able to show the presence of *pxmA* mRNA in pure cultures of *Methylomonas* species strains LW13 and S1 grown on methane and also RNA extracted from freshwater creek sediments. This showed that the *pxm* genes were probably of ecological and functional significance and may represent an alternative pMMO system in some methanotrophs.

It was speculated by Tavormina et al., (2011) that the pXMO could have a primary substrate other than methane, as the function of pXMO was not categorically proven. It was shown that other Pxm proteins grouped more closely with proteins involved in ethane or ammonia oxidation, rather than methane oxidation (Tavormina et al., (2011). It could be that the pXMO enzyme is capable of oxidising a broad range of substrates, including ethane and ammonia but further investigation is required for this to be confirmed. The MmoX sequence of *Methylomonas* LWB grouped separately from the MmoX sequence of *Methylomonas* LW13. The MmoX of *Methylomonas* LWB grouped separately with that of a *Methylomicrobium* species, whereas that of LW13 grouped more closely to that of *Methylovulum* species. This indicates some divergence between the MmoX sequences of the two *Methylomonas* species.

In order to determine if *Methylomonas* LWB is in fact a new species more in-depth characterisation is required including DNA-DNA hybridisation with closely related species such as *Methylomonas Koyamaea*, the most closely related organism at the 16S rRNA gene level and also *Methylomonas methanica* the type species of the *Methylomonas* genus. Also more in depth physiological characterisation, such as profiling growth with regards to temperature and pH, examining intracytoplasmic membranes, cell morphology and growth tests on a range of substrates.

The growth of *Methylomonas* LWB in pure culture is not enhanced by the presence of tetrathionate as hypothesised, and no significant improvement in growth rate or final yield were observed. The presence of increased concentrations of tetrathionate to 10 mM in the *Methylomonas* LWB culture resulted in increased lag phase and an

apparent reduction in growth rate. *Methylomonas* LWB is not able to utilise tetrathionate as an additional energy source.

The genome of *Methylomonas* LWB was not closed, which presents some difficulty in characterisation as the genes missing obviously present an unknown. It was established that the *Methylomonas* LWB contained all enzymes required for methane oxidation, with the exception of having an incomplete *pmoA* gene sequence in the standard pMMO operon. It was apparent that *Methylomonas* LWB assimilates formaldehyde by the ribulose monophosphate pathway, as do other *Methylomonas* species including *Methylomon*as *methanica* MC09 (Boden et al., 2011). *Methylomonas* LWB has all genes required for glycolysis and a TCA cycle. The *Methylomonas* LWB genome has the genes encoding the proteins that make up the nitrogenase enzyme, which allow an organism to fix nitrogen. Nitrogen fixation has been shown to occur in many obligate methanotrophs, including *Methylococcus capsulatus* bath (Murrell and Dalton, 1983) and several strains of *Methylomonas* (Auman et al., 2001). The annotated genome of *Methylomonas* LWB also indicated that the bacterium would be able to utilise the inorganic nitrogen sources nitrate, nitrite and ammonia.

A direct comparison was made between the genomes of *Methylomonas* LWB and the closest related, fully sequenced genome of *Methylomonas methanica* MC09 (Boden et al., 2011). The *Methylomonas* LWB genome was incomplete and consisted of 102 contigs compared with the single completed genome sequence of *Methylomonas* MC09. With this in mind, there may be gene sequences present in the genomes of

both organisms but not present in this version of the *Methylomonas* LWB genome. Despite the genome of *Methylomonas* LWB being incomplete, it is still larger than that of *Methylomonas* MC09. There are also more predicted coding sequences in the *Methylomonas* LWB genome, though slightly less functionally annotated genes. These observations may be explained through the environment from which both of the organisms were isolated. Here *Methylomonas* LWB was isolated from a relatively nutrient rich environment, allowing it to offset having a larger genome as nutrients are not so limiting to the survival of the organism. The *Methylomonas* MC09, however, is a marine organism from an environment where nutrients are at a premium. This will have a streamlining affect on the organism's genome (Giovannoni et al., 2008; Grote et al., 2012), as can be seen by the larger number of functionally annotated genes within a smaller sized genome. The larger genome of *Methylomonas* LWB containing as yet, less functionally annotated genes indicate that it may not be suited to surviving in such a low nutrient environment.

Both the *Methylomonas* LWB and *Methylomonas* MC09 genomes contain the same functional genes involved in methane metabolism. Both genomes contain genes required for the production of both soluble and particulate methane monooxygenase enzymes. However, it was found that the *Methylomonas* MC09 did not contain any additional particulate methane monooxygenase operons as found with the additional *pxm* operon found in the *Methylomonas* LWB genome. This could again be explained by the streamlining of the *Methylomonas* LWB genome in comparison to the *Methylomonas* LWB genome. The *Methylomonas* MC09 smaller genome may not permit the functional redundancy of a second similar copy of the methane

monooxygenase enzyme system, assuming that the *pxm* operon is indeed involved in the metabolism of methane (Tavormina et al., 2011).

There were 5 genetic functions present in the *Methylomonas* MC09 genome involved in nitrogen metabolism that were absent from the *Methylomonas* LWB genome. Two of these genes were involved in nitrite reduction metabolism between nitrite and dinitrogen oxide, encoding the enzymes nitrite reductase and nitric-oxide reductase. The *Methylomonas* MC09 genome also has two more functional genes involved in nitrogen metabolism involved in the metabolism between glutamine and glutamate. Perhaps the extra nitrogen processing enzymes help *Methylomonas* MC09 to survive in a more nitrogen limited environment. Alternatively, the genes for these functions are missing from the *Methylomonas* LWB genome.

Chapter 5

Stable Isotope Probing

5.1 Stable Isotope Probing

One of the key advances in molecular microbial ecology in the 21st century so far was the advent of Stable Isotope Probing, initially with the introduction of Phospholipid Fatty Acid Stable Isotope Probing (PLFA-SIP) (Boschker et al.,1998), followed by DNA Stable Isotope Probing (DNA-SIP) by Radajewski et al., (2000). Stable Isotope Probing stands out from other molecular microbiology tools because it allows one to focus in on the active proportion of a microbial community. This is achieved by using ^{13}C-labelled substrate (or alternate stable isotopes such as ^{15}N or ^{18}O) that is metabolised by the target community and into the cellular material. In the case of DNA-Stable Isotope Probing, the metabolised ^{13}C substrate will result in the target bacteria incorporating the ^{13}C label into their DNA. The DNA from the total microbial community can then be extracted. Then the heavy ^{13}C labelled DNA, which will consist of the active community, can be separated from the lighter ^{12}C DNA that represents the microbial community incapable of utilising the ^{13}C labelled substrate (see section **1.4.2.5** for more detail).

DNA-Stable Isotope Probing has been used successfully in a number of studies with a wide variety of ^{13}C label substrates including methane, methanol, methylamine, bicarbonate and many multi-carbon compounds (Morris et al., 2002; Radajewski et al., 2002; Neufeld et al., 2007; Chen et al., 2009; Murrell and Whiteley, 2011). In these studies, the active microbial community capable of utilising the ^{13}C substrate has been identified. Use of this technique allows researchers to focus in on a specific niche-group of microorganisms rather than making assumptions based on analysis of the whole microbial community.

Potential methane oxidisers in Movile Cave have been identified through metagenomics, microarray analysis and the isolation of a methane oxidising bacterium from the environment (see Chapters 3 and 4). In this study, DNA Stable Isotope Probing was used to identify the active microbial community responsible for the metabolism of methane in Movile Cave microbial mats.

Aims of this section of work:

- Identify the active microbial population metabolising methane in Movile Cave.
- Use a time-course labelling regime to follow succession of the ^{13}C label throughout the Movile Cave microbial community.
- Identify any potential cross feeders of the carbon metabolised by methanotrophs.

5.2 Methods: Sampling and Experimental set up

Samples from Movile Cave for these experiments were obtained by Dr Alexandra Hillebrand-Voicilescu, Mr Vlad Voicilescu, Prof Colin Murrell and Dr Rich Boden on the 13th April 2011. A sample of water and floating microbial mat was pooled from several regions of the air-water interface in air bell 2 of Movile Cave, and was subsequently aliqoted into 120 ml serum vials inside the cave environment just a few minutes after being sampled. The microcosms each contained 20 ml of the mat plus cave water. As far as was practical, each 20 ml of the mat sample contained approximately the same amount of biomass.

The microcosms were spiked at the time of sampling with 2 ml of either ^{12}C methane or ^{13}C methane, resulting in a headspace concentration of 2.0 % (v/v) methane. There was no way of measuring the methane concentration in the microcosm until the samples had arrived back to the lab at Warwick University, 48 hours after sampling. Headspace methane concentration was measured by gas chromatograph (GC). On arrival into the lab, the microcosms were processed. 5 ml mat and water was removed from each microcosm 48 hours after sampling. The vial was resealed and methane was injected to 2.0 % (v/v). Subsequently, the methane concentration in the headspace was monitored by GC (**Figure 5.1**). The microcosm was sampled again at 165 hours when another 5 ml of mat and water was removed. The serum vial stopper was replaced and the microcosm was spiked with methane, again to 2.0 % (v/v). The methane concentration in the serum vial headspace was monitored by GC until the microcosm was harvested at 261 hours. All samples were immediately stored at -20 ^{O}C until DNA extraction was carried out.

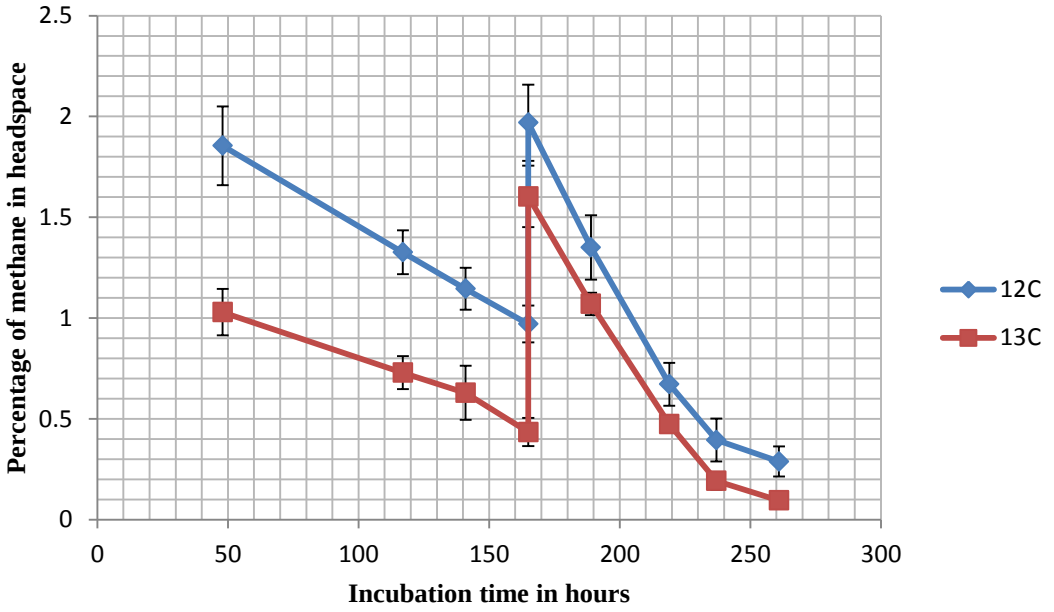


Figure 5.1: Consumption of methane in microcosms over time.

Plots indicate the consumption of methane over time from the DNA-SIP microcosms. Error bars represent 1 standard deviation from the mean of triplicate microcosms.

DNA was extracted from t=48, t=165 and t=261 samples and was quantified using the Nano-drop 1000. The DNA was then subject to isopycnic density gradient ultracentrifugation, as outlined in the Neufeld et al., (2007) DNA SIP protocol paper. In brief, 3 µg of DNA was added to an ultracentrifuge tube along with gradient buffer and CsCl to a density of 1.725 g/ml^{-1}. This was then inserted into a 5.1 ml ultracentrifuge tube, the tube was heat sealed and tubes were spun in a Beckman Vti 65.2 rotor on a Beckman L-90K ultracentrifuge at 177,000 g_{av}, at 20 ^{O}C for 40 hours under vacuum. Samples were then fractionated directly from the centrifuge tube by inserting a needle and tubing that was hooked up to a peristaltic pump. A second hole was made at the bottom of the centrifuge tube and the peristaltic pump turned on to

feed water into the top of the tube. This allowed the CsCl to drain at a controlled rate so as not to disturb the gradient, and fractions of ~425 µl were collected every minute. To ensure a gradient had formed, the CsCl density of each fraction was measured using a Reichart AR200 digital refractometer (**Figure 5.2**). Good gradients formed in both centrifuge tubes. The density of heavy DNA (1.725 g/ml^{-1}) was found in fractions 6 and 7. The very low density of fraction 12 from both samples is due to a small amount of the displacement water mixing with the CsCl at the end of the fractionation process. DNA was precipitated from the fractionated samples using polyethylene glycol 6000 (Neufeld et al., 2007). Precipitated DNA was then stored at -20 ^{O}C until processed.

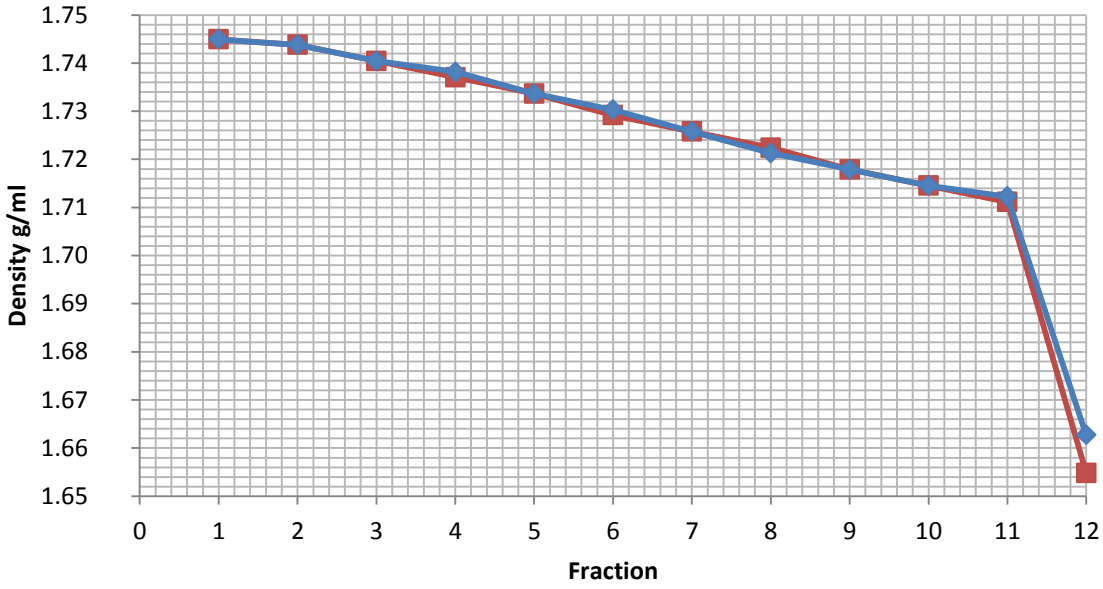


Figure 5.2: Density gradients of CsCl measured from each fraction of the $^{12}CH_4$ and $^{13}CH_4$ incubated samples after fractionation. ^{12}C Blue trace, ^{13}C Red trace.

In order to determine which microorganisms had assimilated the $^{13}CH_4$, and also to observe the shift in microbial community between heavy and light DNA, PCR

amplification of 16S rRNA genes of DNA from each of the fractions was carried out. The PCR primers were designed with GC clamps in order analyse the PCR products using DGGE. The PCR products were then run on a DGGE polyacrylamide gel with a denaturing gradient of 30-70 % urea and formamide (**Figure 5.3**). Bands from the DGGE profiles were excised using a razor blade after visualisation using a UV lamp table. Excised bands were placed into 20 µl of PCR grade water and left overnight at 4 ^{O}C for the DNA to dissolve in the water. This solution was then used as template for 16S rRNA gene PCR amplification using standard 341F 907R primers without the GC clamp. The PCR reactions that yielded PCR products were sent for DNA sequencing.

A clone library targeting the methane monooxygenase *pmoA* gene was constructed to analyse the methanotroph community labelled by the DNA-SIP process. The *pmoA* gene PCR products were cloned using the Promega p-GEM-T Easy vector cloning kit. In brief, the *pmoA* gene PCR products were ligated into the p-GEM-T vector, which was then transformed into JM109 *E.coli* competent cells. The transformed cells were screened on LB agar with ampicillin, IPTG and X-Gal. White colonies were picked, re-streaked and used for whole cell colony PCR using the M13 primer pair (Messing, 1983).

5.3 Results of the DNA-SIP experiment

16S rRNA gene analysis

After fractionation of the DNA samples, only the fractions obtained from a T^3 sample gave PCR products when amplified with the DGGE 16S rRNA gene primers. Where weak PCR products were obtained for some replicates and other time points, the DGGE profiles were not visible. A DGGE profile was obtained from the T3 sample and dominant bands were excised. The 16S rRNA gene sequences obtained from the T^3 DGGE bands were used as BLAST query sequences against the GenBank database. The 16S rRNA gene sequences obtained, together with some of the closest hits from GenBank, were compared using Mega5 to create a phylogenetic tree of 16S rRNA genes obtained from these DNA-SIP experiments (**Figure 5.4**).

Figure 5.3: DGGE images of 16S rRNA genes obtained by PCR of DNA from all fractions of the microcosm containing $^{12}CH_4$ (left) and the microcosm incubated with $^{13}CH_4$ (right). Excised bands that gave retrievable 16S rRNA gene sequences are highlighted. Marker lanes are labelled "L".

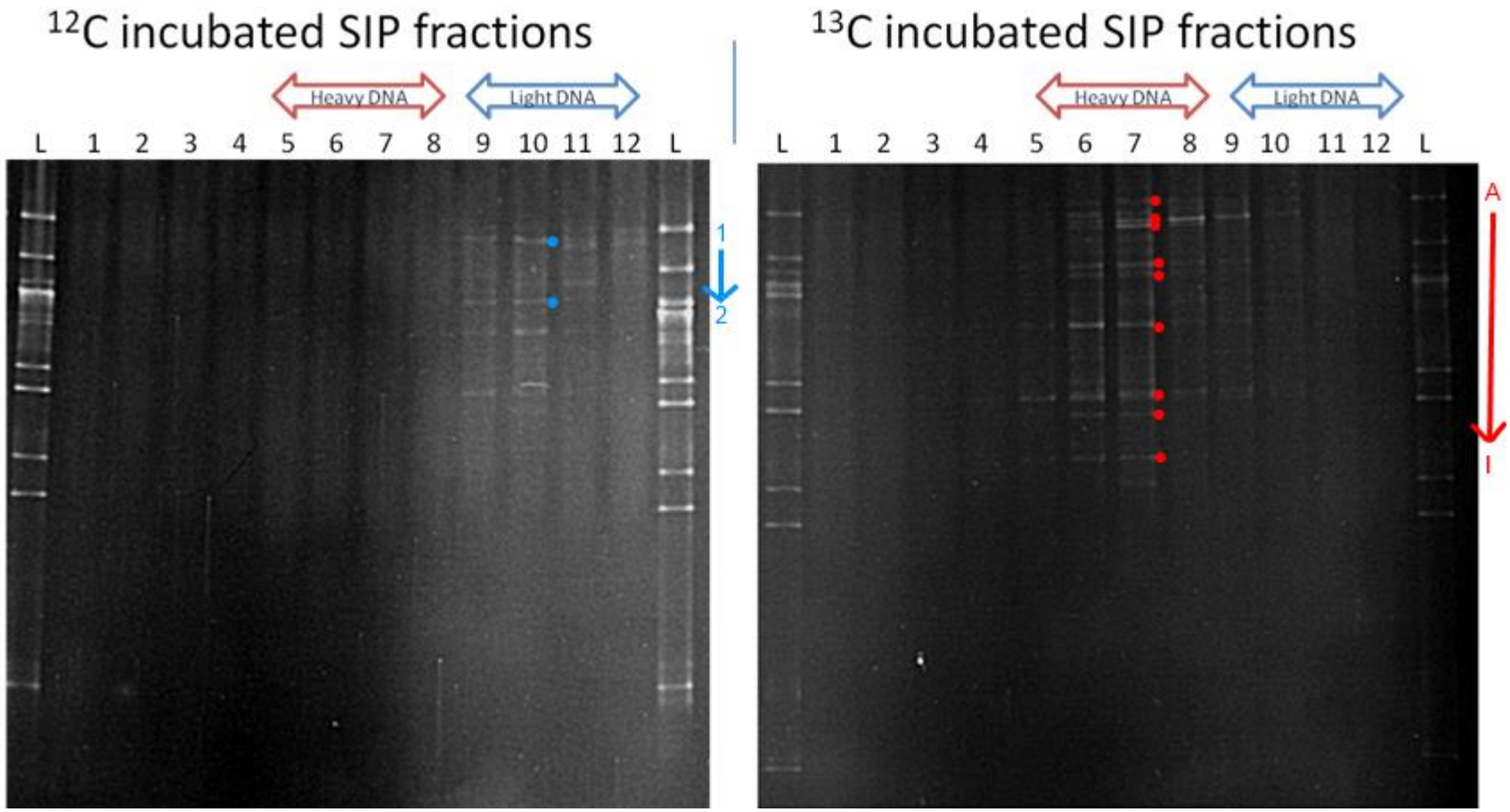
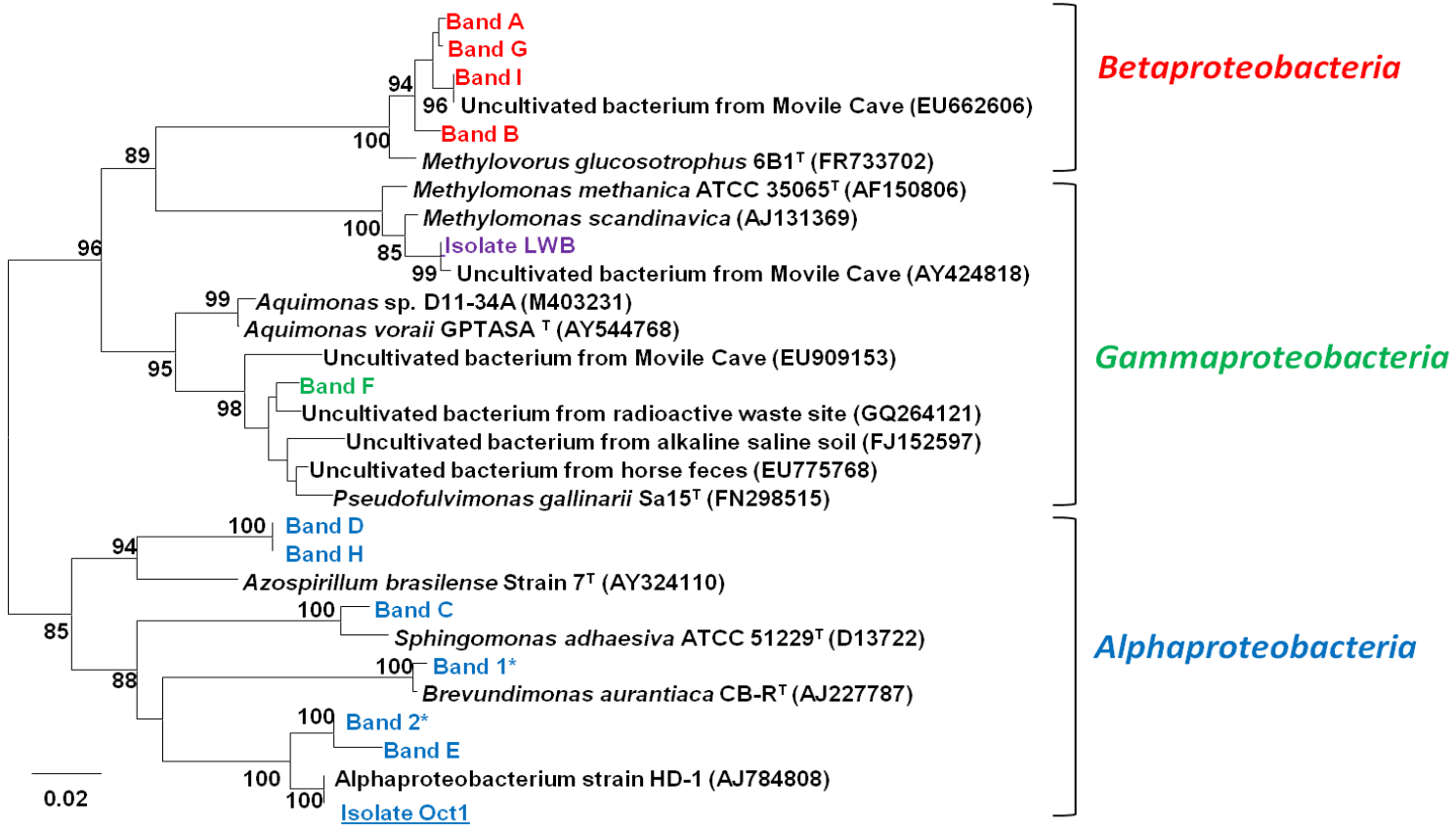


Figure 5.4: Phylogenetic tree of the 16S rRNA gene sequences obtained from bands excised from the 16S rRNA gene DGGE gel. The phylogenetic tree was constructed using Mega5 with the neighbour-joining algorithm with bootstrap values calculated from 1000 replicates. Bands marked with letters are from the heavy fractions of the ^{13}C incubation while the bands marked with numbers are from the ^{12}C incubated microcosm. The *Methylomonas* LWB isolate 16S rRNA gene sequence is highlighted in purple.



Most DGGE bands visible, particularly the brightest, were excised as these would represent the most abundant sequences after PCR amplification with the DGGE primers. All of the excised bands from the DGGE analysis from which retrievable sequences were obtained, contained 16S rRNA genes from Proteobacteria. None of the sequences were closely relate to any 16S rRNA gene from known methane oxidisers. Band 1 from the ^{12}C incubated microcosm was closely related to the bacterium *Brevundimonas aurantiaca*, which is a reclassification of the organism *Caulobacter henricii* sub. sp. *Aurantiacus* isolated in 1964 from a fresh water sample by Poindexter, (1964) (Abraham et al., 1999).

Band 2, also from the ^{12}C incubated microcosm, was closely related to band E from the ^{13}C incubated microcosm. The closest affiliated 16S rRNA gene sequence to these sequences was that of *Oleomonas sagaranensis*. This bacterium was originally isolated by Kanamori et al., (2002) from a Japanese oil field and is able to grow on long chain aliphatic hydrocarbons. A similar organism was also isolated in this study, isolate *Oleomonas* OCT1 (data not shown) as a contaminant in the *Methylomonas* LWB isolation process. It was also able to grow on aliphatic hydrocarbons from hexane and longer chain molecules.

Bands A, B, G and I were all 16S rRNA gene sequences from Betaproteobacteria that shared similarity to 16S rRNA gene of *Methylovorous glucosotrophus*, and also to a 16S rRNA gene sequence from an uncultivated organism obtained from a previous study on Movile Cave (Hutchens et al., 2004). *Methylovorous glucosotrophus* is a

facultative methylotroph that is able to metabolise methanol and was isolated from waste water at Alma-ata (USSR), now Kazakhstan (Doronina et al., 2005).

The 16S rRNA gene sequence obtained from Band F was a Gammaproteobacterial 16S rRNA gene sequence that was mostly related to uncultivated organism 16S rRNA gene sequences, interestingly from a range of environments including a radioactive waste site, an alkaline saline soil and also from horse faeces. The closest related sequence to the 16S rRNA gene sequence of B and F is the heterotroph *Pseudofluvimonas gallinarii*. This organism was isolated from a sample of air from a duck barn by Kämpfer et al., (2010)

The 16S rRNA gene sequence of Band C from the ^{13}C incubated microcosm was very closely related to the 16S rRNA sequence of *Sphingomonas adhaesiva*. *Sphingomonas* species are chemoheterotrophs that grow on a broad range of organic compounds. They are also known to form part of biofilms in association with metheylotrophs such a *Methylobacterium* on shower curtains. In the study by Kelly et al., (2004), 16S rRNA gene sequence clones that were very closely related to *Sphingomonas adhaesiva* were also found from a sample of biofilm growing on a shower curtain.

Bands D and F shared identical 16S rRNA gene sequences, and no closely related 16S rRNA gene sequences could be identified. The closest BLAST hit that matched these was the 16S rRNA gene sequence from the bacterium *Azospirillum brasilense*.

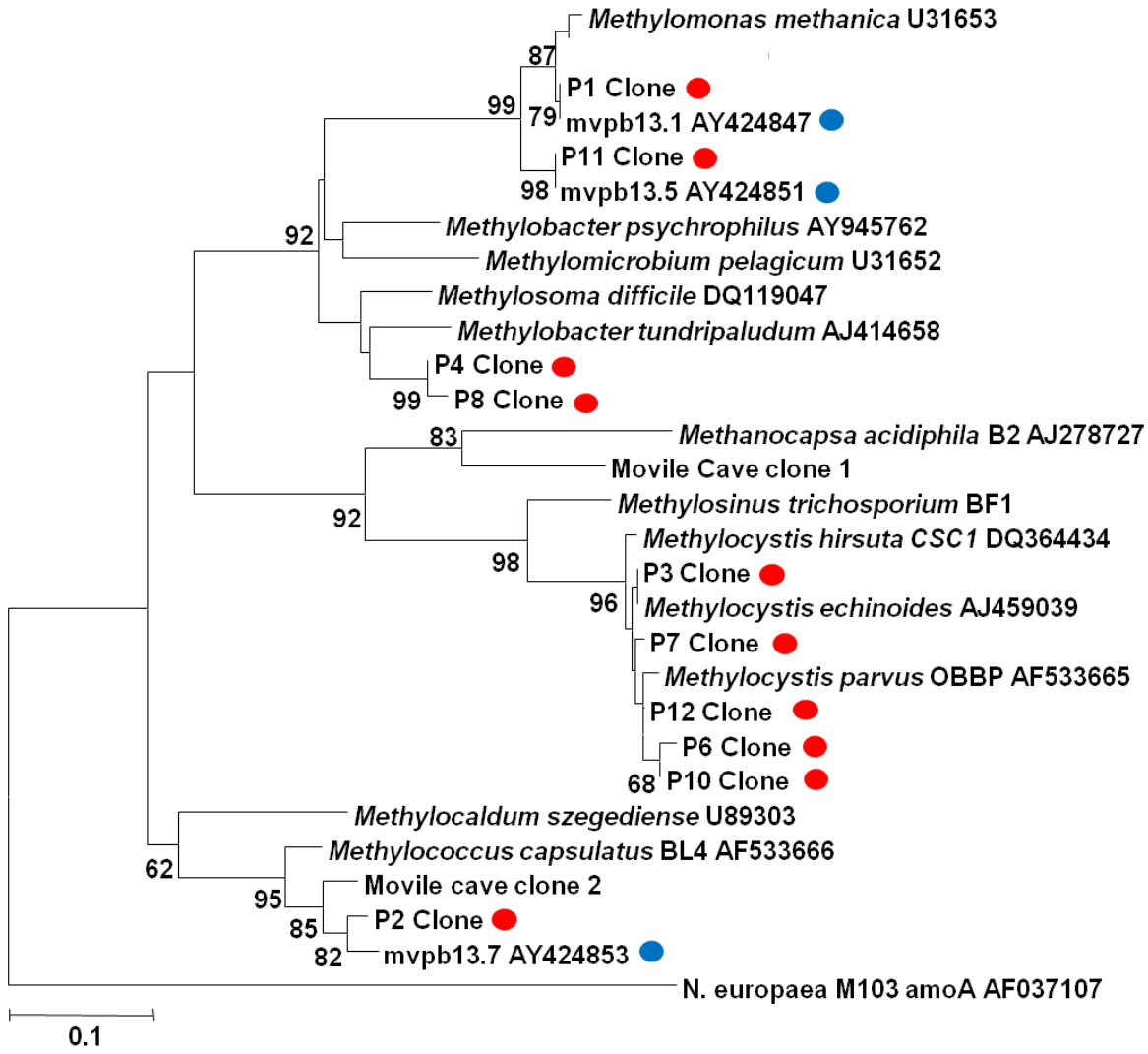
Azospirillum brasilense is a root-associated, nitrogen fixing bacterium that grows on glucose as sole carbon source in nitrogen-free media (Tarrand et al., 1978).

Functional gene analysis

As the 16S rRNA gene analysis did not highlight any methane oxidisers, despite a clear indication of metabolism and incorporation of the ^{13}C label, a small clone library targeting the particulate methane monooxygenase gene (*pmoA*) was constructed. DNA from fraction 7 of the T^3 ^{13}C incubated microcosm was used as template in PCR to construct a *pmoA* gene clone library, using the primers 189F and mb661R (Costello and Lidstrom, 1999). 15 clones were chosen for sequencing and subject to colony PCR, using the M13 primer set to amplify the cloned insert.

PCR products of the correct length were sent for sequencing with the M13F primer. Sequences retrieved were trimmed of excess sequence from the backbone of the p-GEM-T vector. The obtained sequences were used as BLAST query sequences to search the GenBank sequence database. The *pmoA* gene sequences were then compared to the closest BLAST hits using Mega5. The derived polypeptide (PmoA) sequences were compared at the amino acid level (**Figure 5.5**).

Figure 5.5: Neighbour-joining phylogenetic tree of derived PmoA sequences from *pmoA* genes obtained by PCR from the heavy fraction of the ^{13}C DNA SIP microcosm. Bootstrap values were calculated from 1000 replicates. The tree was constructed at the amino acid level (146 amino acid residues) derived from nucleotide sequences. The phylogenetic tree was constructed using Megas5. *pmoA* clones obtained from the "heavy" DNA of the $^{13}CH_4$ incubated sample are highlighted in red. The *pmoA* sequences highlighted in blue are clones from the Hutchens et al., (2004) study on Movile Cave methanotrophs.



The *pmoA* clone library indicated that there were indeed methane oxidiser sequences in the heavy fraction of the DNA from the ^{13}C incubated microcosm. Of the 15 clones sequenced, there were 10 unique sequences identified. There were four known methane oxidising bacteria species that were shown to have incorporated the ^{13}C label into their DNA. These were *Methylomonas*, *Methylobacter*, *Methylocystis* and *Methylococcu*s.

Clone P1 was identical to a previous *pmoA* clone obtained from another Movile Cave study by Hutchens et al., (2004) and Clone P11 was also identical to another *pmoA* sequence that was identified by Hutchens et al., (2004). The PmoA sequence from a validated methanotroph that was most closely related to sequences derived from *pmoA* clones P1 and P11 was the *pmoA* sequence of *Methylomonas methanica*. *Methylomonas methanica* was first identified in 1906 by Söhngen (Söhngen, 1906) according to website List of prokaryotic names with standing in nomenclature (http://www.bacterio.net/m/methylomonas.html) and was then reclassified by Whittenbury and Krieg, (1984) in their entry of the Bergey's Manual of Systematic Bacteriology First Edition, Volume 1 (International Journal of Systematic Bacteriology, 1984). The *Methylomonas* genus was then re-defined by Bowman et al., (1993). The genome of *Methylomonas methanica* MC09 (Boden et al., 2011) was the first genome of an aerobic marine methanotroph to be sequenced and published. The majority of *Methylomonas* species however are terrestrial isolates. The two clones P1 and P11 are both different from the *pmoA* sequence obtained from the *Methylomonas* LWB strain isolated from Movile Cave in this study, suggesting that there may be several species of *Methylomonas* in Movile Cave.

Clones P4 and P8 were most closely related to each other, with the closest PmoA sequence from a validly named methanotroph being *Methylobacter tundripaludum*. This organism was isolated by Wartiainen et al., (2006) from an arctic wetland soil sample taken from Svalbard, Norway. The *Methylobacter* genus currently contains 8 validly published species (http://www.bacterio.net/m/methylobacter.html). Four of the original species were reclassifications by Bowman et al., (1993) of *Methylococcus* and *Methylomonas* species that grouped together. The *Methylobacter* genus is mostly populated by organisms that were isolated from marine environments. Some are psychrophilic in nature (Wartiainen et al., 2006).

Five unique *pmoA* clones, P3, P6, P7, P10 and P12 were all closely related to *pmoA* from *Methylocystis*. The derived amino acid sequence from clone P3 was identical to the PmoA sequence of *Methylocystis echinoides*, a methanotroph described as having a spiky appearance, originally isolated by Gal'chenko et al., (1977) and reclassified by Bowmen et al., (1993). Clones P6, P7 and P10 were all similar to the PmoA sequence of *Methylocy*stis parvus, while Clone P12 was identical to the PmoA sequence of *Methylocystis parvus*. *Methylocystis parvus* is the type species of the genus (http://www.bacterio.net/m/methylocystis.html). *Methylocystis parvus* strain OBBP was originally isolated by Whittenbury et al., (1970). In a more recent study, a strain of *Methylocystis parvus* was isolated from a methane-fed bioreactor and was shown to produce poly-β-hydroxybutyrate when under microaerobic conditions (5-10 % oxygen). The poly-β-hydroxybutyrate could then be fermented under anaerobic conditions when other exogenous carbon sources were not available (Vecherskaya et al., 2009).

Clone P2 was most closely related to another clone that was identified in the Hutchens et al., (2004) study. These sequences were most closely related to the PmoA sequence of *Methylococcus capsulatus*. *Methylococcus capsulatus* was isolated by Foster and Davies, (1966) and has been the focus of many studies of methanotrophic microbiology over the past 50 years. The most notable strain, *Methylococcus capsulatus* Bath, was isolated from the Roman spa bath houses in Bath, England by Whittenbury et al., (1970). Studies such as those by Colby and Dalton, (1976) allowed identification of a soluble methane monooxygenase, as well as a membrane-bound form of the enzyme (Hanson and Hanson 1996, Trotsenko and Murrell, 2008). The genome of *Methylococcus capsulatus* Bath was sequenced by Ward et al., (2004). Now that much more of the molecular biology of the organism is known, more research is focusing on how the organism regulates methane oxidation, methane monooxygenase gene expression and how *Methylococcus* regulates copper homeostasis using chalkophores such as methanobactin (reviewed in Balasubramanian and Rosenzweig, 2008; Kenney and Rosenzweig, 2012; Semrau et al., 2013).

As part of the DNA-SIP study, it was planned to directly monitor the amount of ^{13}C label incorporated into individual cells. Unfortunately, this aim was not completed, but it was shown that the relative amount of ^{13}C incorporation into cells could be determined using Raman spectroscopy. These data can be found in appendix 2.

5.4 Discussion

Stable Isotope Probing has been used to identify organisms that were capable of methane oxidation in the Movile Cave environment. It was also shown that the carbon fixed by the methane oxidisers is used by other organisms in the cave that are capable of a chemolithoheterotrophic life style.

The 16S rRNA gene sequences from the DNA-SIP fractions were analysed in order to identify the organisms that had been able to utilise the ^{13}C-labelled methane. None of the sequences identified were closely related to the 16S rRNA gene sequences of any known methane oxidising bacteria. The 16S rRNA gene sequences that were identified were all proteobacterial 16S rRNA gene sequences. There were very few identifiable bacteria that were closely related to the sequences found, but those that were tend to be heterotrophs.The bacteria identified from this 16SrRNA gene sequencing are found in a wide range of environments, not sharing significant environmental similarities such as location or type of environment.

Unlike previous DNA-SIP studies which highlight only the organisms that utilise the substrate of interest, here it was shown that the carbon metabolised by methanotrophs feeds subsequent trophic layers of the Movile Cave microbial community. The 16S rRNA gene sequences obtained from the heavy fractions of DNA from the DNA-SIP must have incorporated the ^{13}C label in order to be present at that position along the CsCl gradient. These highlighted organisms, which have not been shown to metabolise methane must be feeding on other metabolites produced by the

methanotrophs, such as the $^{13}CO_2$ that will be released from the complete oxidation of the $^{13}CH_4$.

Not finding methane oxidiser sequences among the 16S rRNA gene sequences could be due to a number of reasons. The sequences were obtained from bands that appeared after DGGE analysis. Not all bands that were excised yielded useable sequence, and there is the possibility that the methane oxidiser 16S rRNA gene sequences may have been among those. Another possible reason for not finding methane oxidisers could be due to primer bias, or simply low numbers of methanotroph sequences in the Movile Cave mat DNA. There may also be conditions within the microcosm where a build up of potentially toxic compounds to the methanotrophs occurs. This may result in the methanotrophs oxidising more methane to generate energy for cooxidation reactions which then remove harmful compounds (rather than channelling the carbon into building new cell materials). This would result in a larger output of $^{13}CO_2$ which could be used by autotrophs. If the bacteria feeding on the $^{13}CO_2$ are able to utilise the carbon for building cellular material faster than the methanotroph is able to use the $^{13}CH_4$, then the secondary organisms might well begin to outcompete the methane oxidisers. Perhaps one way of getting around this problem would have been to analyse the heavy ^{13}C- DNA from the $^{13}CH_4$ incubated microcosm by metagenomic sequencing. The low quantity of DNA in the heavy fraction would probably not be enough for metagenomic sequencing, but this could be solved using multiple displacement amplification (Arakaki et al., 2010) to generate enough DNA for analysis.

DNA-SIP has been used to analyse the methane oxidising population of Movile Cave in a previous study (Hutchens et al., 2004). In this study *pmoA* clones similar to those identified by Hutchens et al. were identified along with some otherwise unidentified *pmoA* clones. Clones P1 and P11 were similar to two of the clones from the Hutchens et al., (2004) study and are closely related to PmoA from *Methylomonas*. With the isolation of clones P1 and P11, it has been shown that *Methylomonas* species are active at metabolising methane in the Movile Cave environment, as they are found in the heavy fraction of the ^{13}C incubated sample.

The two clones P4 and P8 are most closely relate to the PmoA sequence of *Methylobacter tundripaludum*. However, they are somewhat different to the *M. tundripaludum* PmoA sequence and may originate from a different, but, closely related organism. Clone P8, in particular, is almost the same distance phylogenetically from the *M. tundripaludum* as the phylogenetic distance between Clone P3 and *Methylosinus trichosporium* BF1 sequence yet P3 is a *Methylocystis* Clone. It may be likely then that clones P4 and P8 are from a yet unidentified methanotroph.

Clones P3, P6, P7, P10 and P12 are all very closely related. They form a tight group clustering within the *Methylocystis* PmoA clade. Clones P3 and P7 appear to be more closely related to PmoA sequence of *Methylocystis echinoides* (Clone P3 is identical). Clones P6, P10 and P12 are more closely related to the PmoA sequence of *Methylocystis parvus*. The Hutchens et al., (2004) study did not have as many *pmoA* clones as closely related to the *Methylocystis* sequences found here. When analysing

the Movile Cave metagenome, *Methylocystis* was very under-represented among the methane monooxygenase sequences (see **chapter 3**). It could be an artefact of the microcosm that favours the growth of methanotrophs such as *Methylocystis*, whereas in the Movile Cave environment, *Methylocy*stis represent the less active methanotrophs present. However, the opposite is suggested by the *pmoA* microarray (see section **3.3**). Some of the strongest signals observed on the microarray were found with *Methylocystis* probes. Therefore, it looks more likely that it could be a primer bias that favours more *Methylocystis*-like sequences since PCR is used for both the microarray and for amplification from the heavy ^{13}C labelled DNA, whereas there is no amplification involved with the metagenomic sequencing.

Clone P2 was most closely related to Clone mvpb13.7 from the Hutchens et al., (2004) study. Both Clone P2 and mvpb13.7 are relatively low in number among the representative clones from both studies. The most closely related PmoA sequence to that of Clone P2 is that of *Methylococcus capsulatus* BF4. Rather the opposite was seen with *Methylococcus* clones compared with those of *Methylocystis*. *Methylococcus* was the most represented methanotroph among the methane monooxygenase sequences in the metagenome and yet there was relative low representation of this gene among the heavy ^{13}C DNA *pmoA* clones. The *pmoA* clone library presented here was very small as only 15 clones were sequenced, with 5 of the sequences being identical. With such a low sample number across the potential *pmoA* clones, it is unlikely that a true representation of the diversity among the *pmoA* clones was observed. It may be that *Methylococcus* is the most abundant methanotroph but it cannot be determined from this data set. What is known though, is that there are *Methylococcus* species that actively metabolise methane in Movile Cave.

In order to observe the full diversity among the *pmoA* gene sequences in the heavy ^{13}C DNA, a much larger clone library would need to be constructed or, alternatively, a sample of the *pmoA* PCR products could be sent for high-throughput sequencing. This would give much deeper coverage of the clones and give insights to the true diversity of the active population of methanotrophs. It would also be beneficial to repeat the *mmoX* PCR amplification using the ^{13}C heavy DNA again. It had been attempted several times in this study with little success. The organisms that have been identified from the *pmoA* clones are known to have representatives among their genera that contain both particulate and soluble methane monooxygenase. If the *pmoA* genes from these organisms have been labelled with the ^{13}C label then the *mmoX* genes should also have been labelled. It is surprising that PCR assays of *mmoX* were not successful given that the methane monooxygenase sequences in the metagenome were dominated by soluble methane monooxygenase gene sequences (see section **3.2.1**).

From *pmoA* analysis, it is clear that methane oxidising bacteria are indeed active at metabolising methane in Movile Cave. Methanotrophs of the genera *Methylomonas*, *Methylocystis* and *Methylococcus* are among the most active, along with a bacterium closely related to *Methylobacter*. More in-depth sequence coverage is required to elucidate the true diversity and relative abundance among the active methanotrophs.

The data identifying the active methanotrophs in this study do not add to current knowledge of what exists, but it does complement and back up what was found in the Hutchens et al., (2004) study. This study does however further the understanding of the importance of the methanotroph community in the Movile Cave food web, as it is

evident that carbon from methane metabolised by the methanotrophs provides a significant carbon source for organisms further along the food chain. This highlights the Movile Caves methanotrophs as key primary producers in this environment and potentially one of the foundations on which this rare ecosystem is built upon.

Chapter 6

Final discussion and future perspectives

6.1 Discussion and future perspective

Movile Cave is an isolated ecosystem that harbours a thriving community of organisms that are not supported by photosynthetically fixed carbon. Instead, primary producers including chemolithoautotrophic and methanotrophic bacteria are believed to provide the organic carbon supporting the cave inhabitants. The aim of this project was to determine the presence of methane oxidising bacteria in Movile Cave, to determine which, if any, actively oxidise methane in the cave, to identify if methanotrophs provide a carbon source for other organisms in the Movile Cave environment and to isolate and characterise any methane oxidising bacteria. The study here has been able to fulfil all aims stated above through the use of cultivation and cultivation independent methods.

A metagenomic analysis was carried out on DNA extracted from microbial floating mat and water obtained from air bell 2 in Movile Cave. The sample was frozen only a few hours after been taken in order to maintain the original community of organisms present at the time of sampling. DNA extraction took place when the sample was first thawed. The metagenome was screened for several functional genes associated with methane oxidising bacteria. Methane monooxygenase genes required for the production of both soluble and particulate methane monooxygenase were identified. Almost all of the methane monooxygenase sequences identified were genes encoding soluble methane monooxygenase components. By far the most abundant methane monooxygenase sequences identified from a single organism related very closely to the species *Methylococcus capsulatus* Bath. It is likely that the *Methylococcus capsulatus* Bath like organism is the most prolific methane oxidising bacterium in

Movile Cave. Methane monooxygenase gene sequences closely relate to the organism *Methylosinus trichosporium* OB3B were also rather abundant among the MMO sequences suggesting that it too may be one of the more prolific methanotrophs in Movile Cave. Other functional genes screened for including methanol dehydrogenase, formate dehydrogenase and hexulose-6-phosphate synthase indicated *Methylococcus capsulatus* species gene sequences to be among the most highly represented. Interestingly, there were no gene sequences involved in the oxidation of methane related to *Methylomonas* species despite this being the only methanotroph to be isolated from the cave. More work could be done with the comparison of this metagenome with other metagenomes, more specifically metagenomes from other cave environments to find trends in community structure linked with this type of environment. As the methane monooxygenase sequences were heavily biased towards soluble methane monooxygenase it indicated that coverage of the community was not sufficient as there should have been more particulate methane monooxygenase sequences. This would likely warrant the re-sequencing of the Movile Cave metagenome on a larger scale with the aim of increased coverage.

The *pmoA* microarray gave a good indication of the *in situ* diversity among methanotrophs. The tool is limited as it only targets the particulate methane monooxygenase which means it would miss organisms like *Methylocella* and *Methyloferulla* (Theisen et al., 2005; Vorobev et al., 2011). Contrary to the metagenome data set, the *pmoA* microarray identified that metanotrophs with particulate methane monooxygenase were present in Movile Cave and that there was diversity among them. It also suggested that it might be *Methylocystis* species that are

the most abundant methanotroph (owing to the semi-quantitative nature of the microarray), however, *Methylococcus* species were also highly represented. *Methylomonas* was highlighted on the *pmoA* microarray although quite low in abundance compared with the other organisms. Having conducted both the metagenome sequencing and the *pmoA* microarray, and both giving different insights into the diversity of methane oxidising bacteria in Movile Cave, it highlights the importance of having multiple sources of evidence as drawing conclusions from either of the techniques alone would have resulted in inaccurate conclusions being drawn. Ideally, these studies should be conducted as replicates to add robustness to the diversity of methanotrophs observed. It would also be advisable in future to conduct a longitudinal study to determine if the methanotroph community is generally stable or perhaps more dynamic.

Methylomonas LWB was the only methane oxidising bacterium to be isolated from Movile Cave. This organism showed no apparent tolerance to tetrathionate, a possible toxic compound found in Movile Cave due to the high amount of sulfurous compounds present. The genome of *Methylomonas* LWB was sequenced to gain a more in depth understanding of the potential metabolisms that the organism employs to survive in the Movile Cave environment. *Methylomonas* LWB displayed characteristic metabolic pathways for a Type I methane oxidising bacterium including the genes required for formaldehyde assimilation by the ribulose monophosphate pathway. The *Methylomonas* LWB genome contained genes for expression of both the soluble and particulate form of methane monooxygenase. More interestingly, the *Methylomonas* LWB genome contained genes required for the putative particulate methane monooxygenase enzyme encoded by the pXM genes

pxmABC. The orientation of the genes in the pXM operon differs to pMMO as the pMMO operon genes are found in the order *pmoCAB*. The *pxmA* gene branches separately from the *pmoA* genes on a phylogenetic tree, grouping with the few *pxmA* genes that have recently been identified. The function of pXM has not been determined categorically but transcripts of the *pxmA* gene have been found during the growth of *Methylomonas* LW13 growing on methane as a sole carbon and energy source (Tavormina et. al., 2011). The *Methylomonas* LWB isolate may well be a new species but further characterisation experiments are required to determine this. In order to fully understand the potential metabolic processes of *Methylomonas* LWB, the genome will need to be completed.

DNA-Stable Isotope Probing was carried out using a sample of floating microbial mat and water from airbell 2. A microcosm was set up with the Movile Cave sample containing $^{13}CH_4$ in order to label the DNA of any active methane oxidising bacteria. Analysis of 16S rRNA gene sequences from ^{13}C-labelled heavy DNA did not indicate any methane oxidising bacteria having incorporated the ^{13}C-label. Most of the bacteria identified from the 16S rRNA genes from the heavy DNA were heterotrophic *Proteobacteria*. This suggested that the ^{13}C-label had been incorporated by methane oxidisers and cross-fed into these other organisms, thus highlighting methanotrophs as primary producers supporting the growth of other organisms in Movile Cave. Functional gene analysis by way of a clone library targeting the *pmoA* gene from the heavy ^{13}C-labelled DNA indicated that *Methylomonas*, *Methylocystis* and *Methylococcus* along with a bacterium closely related to *Methylobacter* were actively oxidising methane in the DNA-SIP experiment. There were a number of different clones identified for *Methylomonas*, *Methylocystis* and *Methylobacter* like

sequences suggesting multiple species of each genus are present and active in Movile Cave. The clone library carried out here was too small to determine the true diversity of methane oxidising bacteria from the heavy DNA. If the experiment was to be repeated either a larger clone library would be constructed or high throughput sequencing would be used to analyse the *pmoA* PCR products. The *mmoX* analysis would need to be repeated to ensure that they were definitely not present in the heavy DNA. Knowing that the *Methylomonas* LWB isolate contains the putative particulate methane monooxygenase *pxm* genes, the heavy DNA should be analysed for the *pxmA* functional gene as proxy for a potential third methane monooxygenase. Initially, in this study it was planned to carry out a time course experiment with the DNA-SIP but complications meant that this never happened, one of the factors being a lack of labelling in the first time point. This could be overcome by carrying out RNA-SIP as RNA molecules are labelled much faster than DNA as cell replication is not required as prerequisite for labelling. The multiple time point SIP, if realised, would potentially give higher resolution of the incorporation of the ^{13}C- label into the methanotrophs and the cross-feeding into the other organisms in the environment.

Continuation of the project

Should the project be continued over the next few years an effort should be put into isolating more methanotrophs from Movile Cave. *Methylomonas* species were shown to be active methane oxidisers but were one of the lesser abundant methanotrophs present from the in situ community studies. Ideally, isolation and characterisation of *Methylococcus* and *Methylocystis* species should be a priority. Characterisation of the molecular biology of the pXM protein from *Methylomonas* LWB needs further

attention. Too little is known about its precise function and yet it could be the main enzyme system LWB uses to oxidise methane in Movile Cave. It would be good to start with transcription studies to see if the genes are used during the oxidation of methane. Purification of the pXM protein and characterisation of kinetics with a range of substrates would be needed to identify substrate specificity and potential activity. A good set of functional gene primers targeting the *pxmA* gene specifically would be useful to identify if *Methylomonas* LWB is the only methanotroph with this enzyme system in Movile Cave.

Much more work with the Raman spectroscopy is needed to carry out the cross-feeding experiment. A standard curve of *Methylomonas* LWB with varying ^{13}C ratios incorporated would be useful. The cross-feeding experiment could be taken a lot further with the addition of other isolates from Movile Cave being added to the mix to see if a stable community with the methane oxidiser as primary producer can be produced in vitro and to monitor the flow of the ^{13}C label through the organisms present. This experiment could be taken to the next level by combining it with a stable-isotope probing experiment. Live bacterial floating mat could be incubated with ^{13}C methane. The sample could be monitored by DNA SIP in conjunction with Raman FISH. DNA SIP would identify the key organisms in action, FISH probes could be designed for those organisms to then look within the mat structure at the single cell level to confirm uptake of ^{13}C label by the identified organisms and to get an idea of the macrostructure to determine if there is any structure to the microbial floating mats e.g. with secondary feeders surrounding the methanotrophs.

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Appendicies

Appendix 1: List of probes used in the *pmoA* microarray study. See next page.

Name	Intended specificity	Sequence 5' → 3'	L	GC%	T_m
MbA557	*Methylobacter*	CAATGGCATGATGTTCACTCTGGCT	25	48.0	61.5
MbA486	*Methylobacter*	AGCATGACATTGACAGCGGTTGTT	24	45.8	61.6
Mb460	*Methylobacter*	GACAGTTACAGCGGTAATCGGTGG	24	54.2	60.9
Mb_LW12-211	*Methylobacter*	CGTCTTTGGGTTACTGTTGTGCC	23	52.2	60.0
Mb_SL#3-300	*Methylobacter*	GGCGCTGTTGTTTGTGTATTGGGT	24	50.0	62.2
Mb_SL299	soda lake *Methylobacter* isolates and clones	GGGGTGCAACTCTGTGTATCTTAGG	25	52.0	60.5
Mb_SL#1-418	soda lake *Methylobacter* isolates and clones	GCGATCGTATTAGACGTTATCCTGATG	27	44.4	58.6
MmbB284	*Mmb. Buryatense*	ATCGCATCGCTTGGGGTGCAA	21	57.1	62.5
Jpn284	clone Jpn 07061	ACCGTATCGCATGGGGTG	18	61.1	58.0
BB51-302	*Methylobacter*	CGGTTGTTTGTGTCTTAGGTCTG	23	47.8	57.2
Mb267	*Methylobacter*	GCATGCTTGTGGTTCCGTTAC	21	52.4	58.1
Mb292	*Methylobacter*	CCGTTACCGTCTGCCTTTCG	20	60.0	59.1
Mb282	*Methylobacter*	TTACCGTCTGCCTTTCGGC	19	57.9	58.6
Mb_URC278	*Methylobacter*	GTTCCGTTACAGACTGCCTTTCGG	24	54.2	61.3
511-436	*Methylobacter 511 group*	GTTTTGATGCTGTCTGGCAG	20	50.0	55.5
511-436L	*Methylobacter* 511 group	GUUUUGAUGCUGUCUGGCAGCA	22	50.0	60.0
LP10-424	*Methylobacter* LP 10 group	GTACTTGATTGTATCTTGATGCTGTCAG	28	39.3	55.7
LF1a-456	*Methylobacter* LF 1a group	CATGGTATTGACTGCTGTTATCGGTG	26	46.2	57.7
Mb_C11-403	*Methylobacter*	CAAACTTCATGCCTGGTGCTATCGT	25	48.0	61.4
Mb380	*Methylobacter* group A (broad specificity probe)	CAGTAAATTTCTGCTTCCCTTCAAATCT	28	35.7	55.8
Mb271	*Methylobacter*	TTGTGGTGGCGTTACCGT	18	55.6	58.0
S14m2-270	Marine type Ia cluster, S14m#2	CTTATGGTACCGTTACAGATTGCCTTA	27	40.7	56.4
S14m2-406	Marine type Ia cluster, S14m#2	TTAATTCCTGGTGCAATTGCACTTGAC	27	40.7	58.3
PS80-291	clone PS-80	ACCAATAGGCGCAACACTTAGT	22	45.5	58.3
MS1-440	Marine type Ia cluster, Marine sediment #1	TGATGTTGTCTGGTAGCTTCACATTAAC	28	39.3	57.1
Mm_pel467	*Methylomicrobium pelagicum*	ACTGCGGTAATCGATGGTTTGGC	23	52.2	61.6

Kuro18-205	Marine type Ia cluster, Kuro18	AGACGTTTGTGGGTGACAGTTGC	23	52.2	60.0
DS1-401	Deep sea cluster #1	GCGCGGTAGTTTGTGTTATGGCT	23	52.2	61.7
Mm531	*Methylomonas*	CTCCATTGCACGTGCCTGTAGA	22	54.5	60.7
Mm_M430	*Methylomonas*	TGGACGTGATTTTGATGTTGGGCAA	25	44.0	61.6
Mm_RS311	*Methylomonas methanica*, RS clade	CTGTTGTTGCTCTGATGCTGGG	22	54.5	58.6
Mm_ES294	*Methylomonas*	CCAATCGGTGCAACAATTTCTGTAGT	26	42.3	59.8
Mm_ES543	*Methylomonas*	GTGCCAGTTGAGTATAACGGCATGA	25	48.0	60.9
Mm_ES546	*Methylomonas*	CCAGTTGAGTATAACGGCATGATGAT	26	42.3	58.7
Mm_MV421	*Methylomonas*	CTATCGTGCTGGATACAATCCTGATGT	27	44.4	60.0
Mm451	*Methylomonas*	CTGATGTTGGGTAACAGCATGACT	24	45.8	58.8
Mm275	*Methylomonas*	GTGGTGGAGATACCGTTTGCC	21	57.1	59.2
Alp7-441	Alpine soil *Methylomonas*, Alp#7	GATGTTAGGTAACAGCATGACACTGAC	27	44.4	57.4
peat_1_3-287	*Mehtylomonas*-related peat clones	AACTGCCTTTAGGCGCTACC	20	55.0	58.6
Est514	*Methylomicrobium*-related clones	AATTGGCCTATGGTTGCGCC	20	55.0	59.9
Mmb259	*Methylomicrobium album* + Landfill *M.microbia*	CTGTTCAAGCAGTTGTGTGGTATCG	25	48.0	59.8
Mmb303	*Methylomicrobium album*	CAATGCTGGCTGTTCTGGGC	20	60.0	60.3
Mmb304	*Methylomicrobium album* + Landfill *M.microbia* and related	ATGCTGGCTGTTCTGGGCTTG	21	57.1	60.6
LW14-639	*Methylomicrobium* LW14 group	AAAAGGUACUUGGAGAACCUUCGGU	25	44.0	60.0
Mmb_RS2-443	*Methylomicrobium*, Mmb_RS2	TGCTGGGCAACAGCATGCAGT	21	57.1	62.8
Mmb562	*Mmb. album* and *Methylosarcina*	ATGGTAATGACCCTGGCTGACTTG	24	50.0	60.6
Mm229	Deep-branching *Methylomonas* group (WHmb3 related)	CCAATCGTTGGAATCACTTTCCCAGC	26	50.0	60.2
MsQ290	*M.sarcina quisquilliarum* related	TGCCATTCGGCGCTGTAATTTCAGTA	26	46.2	60.8
MsQ295	*M.sarcina quisquilliarum*	CGGCGCGGTTCTTTCTGTACTG	22	59.1	60.6
LP20-644	*Methylomicrobium*-related clones	GTACACTGCGTACTTTCGGTAA	22	45.5	56.0
LP20-607	LP20 group (Type Ia, deep branching-*Methylomicrobium*?)	ACTGGTATGCCTGAATACATCCGTA	25	44.0	57.4
Ia193	Type I a (*M.bacter-M.monas-M.microbium)*	GACTGGAAAGATAGACGTCTATGGG	25	48.0	57.8
Ia575	Type I a (*M.bacter-M.monas-M.microbium-M.sarcina)*	TGGCTGACTTGCAAGGTTACCAC	23	52.2	61.3
Bsed516	Marine sediment #2, Bsed	AACTGGCCAATGGTTGCTCCA	21	52.4	59.9
SWI1-375	Marine sediment #2, SW#1	TGCTGGCGCTATGGGTTGG	19	63.2	60.9

SWI1-377	Marine sediment #2, SW#1	TGGCGCTATGGGTTGGGGTT	20	60.0	62.1
Nc_oce426	*Nitrosococcus oceani*	CTTGGATGCCATGCTTGCGA	20	55.0	59.8
DS2-287	Deep sea #2, subgroup (N.coccus and Deep sea Type Ia 10-298)	GAATCCCATTTGGCGCGACTTTGTG	25	52.0	61.0
AIMS1-442	Deep sea #2, AIMS#1	TTGTTGACAGGTAGCTATTTGGCAAC	26	42.3	57.7
DS2-220	Deep sea #2, subgroup	ACGGTGACTCCGATTGTGTGTAT	23	47.8	58.2
DS2-626	Deep sea #2, subgroup	ATTGCTGGTCTGCATCAGCCTG	22	54.5	60.2
USCG-225	Upland soil cluster Gamma	CTGACGCCGATCATGTGCAT	20	55.0	59.1
USCG-225b	Upland soil cluster Gamma	CTGACGCCGATCATGTGCATCA	22	54.5	61.2
JR2-409	JR cluster #2 (California upland grassland soil)	TTATTCCCGGCGCTATCATGATCG	24	50.0	60.5
JR2-468	JR cluster #2 (California upland grassland soil)	ACAGCCATAATTGGACCATTCTTCTG	26	42.3	59.2
JR3-505	JR cluster #3 (California upland grassland soil)	TGTATCCTACCAATTGGCCTCATCTG	26	46.2	60.1
JR3-593	JR cluster #3 (California upland grassland soil)	CTATCAGTATGTGCGGACAGGC	22	54.5	58.6
501-375	*Methylococcus*- related marine and freshwater sediment clones	CTTCCCGGTGAACTTCGTGTTCC	23	56.5	61.3
501-286	*Methylococcus*- related marine and freshwater sediment clones	GTCAGCCGTGGGGCGCCA	18	77.8	66.7
USC3-305	Upland soil cluster #3	CACGGTCTGCGTTCTGGC	18	66.7	59.5
Mc396	*Methylococcus*	CCCTGCCTCGCTGGTGCC	18	77.8	64.4
MclT272	*Methylocaldum tepidum*	GGCTTGGGAGCGGTTCCG	18	72.2	61.9
MclG281	*Methylocaldum gracile*	AAAGTTCCGCAACCCCTGGG	20	60.0	61.5
MclS402	*Methylocaldum szegediense*	GCGCTGTTGGTTCCGGGT	18	66.7	61.8
MclS394	*Methylocaldum szegediense* and related	TTCCCGGCGCTGTTGGTTCC	20	65.0	63.3
MclS400	*Methylocaldum szegediense* and related	CGGCGCTGTTGGTTCCGGGT	20	70.0	65.7
MclE302	*Methylocaldum* E10	CGCAACCATGGCCGTTCTG	19	63.2	60.3
Mcl404	*Mcl.tepidum-Mcl. Gracile-Mcl.Szeg* and related	TTTTGGTTCCGGGTGCGATTT	21	47.6	58.0
Mcl408	*Methylocaldum*	GGTTCCGGGTGCGATTTTG	19	57.9	57.8
fw1-286	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones	ATCGTCAACCGTGGGGCG	18	66.7	61.1
fw1-639	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones	GAAGGGCACGCTGCGTACG	19	68.4	62.0
fw1-641	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones	AGGGCACGCTGCGTACGTT	19	63.2	63.3

JHTY1-267	*JH-TY#1*	TTGGTTGTGGGAAAACTTCCGT	22	45.5	57.4
JRC4-432	Japanese rice cluster #4	GACGTTGTCCTGGCTCTGAG	20	60.0	58.3
OSC220	Finnish organic soil clones and related	TCACCGTCGTACCTATCGTACTGG	24	54.2	60.8
OSC300	Finnish organic soil clones and related	GGCGCCACCGTATGTGTACTG	21	61.9	61.4
JRC3-535	Japanese Rice Cluster #3	CGTTCCACGTTCCGGTTGAG	20	60.0	59.3
LK580	fw-1 group + Lake Konstanz sediment cluster	CCGACATCATTGGCTACAACTATGT	25	44.0	58.7
RSM1-419	*RSM#1*	CCATTCTGCTCGACGTGGTTCT	22	54.5	59.4
JHTY2-562	*JH-TY#2*	ATGCTGTTGTCGATCGCCGACTTGC	25	56.0	63.6
JHTY2-578	*JH-TY#2*	CCGACTTGCAAGGCTACAACTATGTC	26	50.0	59.5
JRC2-447	Japanese Rice Cluster #2	CTGAGCACCAGCTACCTGTTCA	22	54.5	60.2
LW21-374	LW21 group	CTACTTCCCGATCACCATGTGCT	23	52.2	60.2
LW21-391	LW21 group	TGTGCTTCCCCTCGCAGATC	20	60.0	60.5
M90-574	*M.coccus-M.caldum* related marine and freshwater sediment clones	ATCGCCGACCTGCTGGGTTA	20	60.0	62.2
M90-253	*M.coccus-M.caldum* related marine and freshwater sediment clones	GCTGCTGTACAGGCGTTCCTG	21	61.9	61.7
Mth413	*Methylothermus*	CACATGGCGATCTTTTTAGACGTTG	25	44.0	58.3
Mha-500	*Methylohalobius - M.thermus and related ?*	TGATGTACCCGGGCAACTGGC	21	61.9	62.3
DS3-446	Deep sea cluster #3	AGCTGTCTGGCAGTTTCCTGTTCA	24	50.0	62.5
PmoC640	*PmoC*	AAGGGAACGCTTCGTACGTTTGG	23	52.2	59.8
PmoC308	*PmoC*	CCTGTGTGCTGGCGATTCTGCT	22	62.3	59.1
Ib453	Type I b (*M.thermus-M.coccus-M.caldum* and related)	GGCAGCTACCTGTTCACCGC	20	65.0	61.7
Ib559	Type I b (*M.thermus-M.coccus-M.caldum* and related)	GGCATGCTGATGTCGATTGCCG	22	59.1	62.5
McyB304	*M.cystis B (parvus/echinoides/*strain M)	CGTTTTCGCGGCTCTGGGC	19	68.4	62.7
Mcy255	*M.cystis B (parvus/echinoides/*strain M)	GGCGTCGCAGGCTTTCTGG	19	68.4	62.3
Mcy459	*Methylocystis*	GTGATCACGGCGATTGTTGGTTC	23	52.2	60.2
Mcy264	*Methylocystis*	CAGGCGTTCTGGTGGGTGAA	20	60.0	61.0
Mcy270	*Methylocystis*	TTCTGGTGGGTGAACTTCCGTCT	23	52.2	61.8
Mcy413	*Methylocystis*	TTCCGGCGATCTGGCTTGACG	21	61.9	63.2
Mcy522	*Methlocystis* A + peat clones	GGCGATTGCGGCGTTCCA	18	66.7	62.3
Mcy233	*Methylocystis*	ATTCTCGGCGTGACCTTCTGC	21	57.1	60.9

McyM309	*M.cystis strain M and related*	GGTTCTGGGCCTGATGATCGG	21	61.9	61.0
Peat264	peat clones	GGCGTTTTTCTGGGTCAACTTCC	23	52.2	60.3
MsS314	*Methylosinus sporium*	GGTTCTGGGTCTGCTCATCGG	21	61.9	60.8
MsS475	*Methylosinus sporium*	TGGTCGGCGCCCTGGGCT	18	77.8	68.3
Msi263	*Methylosinus sporium* + 1 *Msi.trichosporium* subclaster	GGCGTTCCTGTGGGAGAACTTC	22	59.1	61.2
Msi423	*Methylosinus*	CTGTGGCTGGACATCATCCTGC	22	59.1	61.4
MsT214	*Methylosinus trichosporium* OB3b and rel.	TGGCCGACCGTGGTTCCG	18	72.2	63.5
Msi520	*Methylosinus trichosporium*	GCGATCGCGGCTCTGCA	17	70.6	61.6
Msi269	*Methylosinus trichosporium*	TCTTCTGGGAGAACTTCAAGCTGC	24	50.0	60.6
Msi294	*Methylosinus*	GTTCGGCGCGACCTTCGC	18	72.2	62.5
ARC2-518	Deep branching type II clade ARC2	GGCCGGCGATTGGTCAGTATCA	22	59.1	61.7
Msi232	*M.sinus*+ most *M.cystis* -considered as additional type II probe	ATCCTGGGCGTGACCTTCGC	20	65.0	63.3
II509	Type II	CGAACAACTGGCCGGCGAT	19	63.2	61.7
II630	Type II	CATGGTCGAGCGCGGCAC	18	72.2	62.4
Alp8-468	Type II novel pmoA, Alpine cluster Alp#8	CGCGCTCCTTGGCTCGTTGG	20	70.0	64.0
xb6-539	Novel *pmoA* copy of type II and related environmental clones	AGGCCGCCGAGGTCGAC	17	76.5	63.0
LP21-190	Novel *pmoA* copy of type II and related environmental clones	ATCGACTTCAAGGATCGCCG	20	55.0	58.2
LP21-260	Novel *pmoA* copy of type II and related environmental clones	CGCAGTCCTTCTTCTGGACG	20	60.0	58.6
NMcy1-247	Novel *pmoA* copy of *M.cystis #1* (?)	TCGACATCGTGCTGATGATCTCGG	24	54.2	62.1
NMsi1-469	Novel *pmoA* copy of *M.sinus*	GCGCTGGTCGGCTCCATGG	19	73.7	64.3
NMcy2-262	Novel *pmoA* copy of *M.cystis #2* (?)	CAGTCCTTCTTCTGGCAGAAGTTCC	25	52.0	60.9
LP21-436	*Mcy + Msi* novel pmoA #1 groups	GTGCTGATGATGTCGGGCAGCTGGC	25	64.0	66.1
NMsiT-271	Novel *pmoA* copy of *M.sinus trichpsporium* (?)	AGCGCTTCCGTCTGCCGAT	19	63.2	62.9
LP21-232	Novel *pmoA* copy of type II and related environmental clones	ATCGTCGCCATGTGCTTCGC	20	60.0	61.9
RA14-299	RA14 related clones	GCGCGACGTTCCTTTGTGTC	20	60.0	59.5
RA14-594	RA14 related clones	CCACAACGTTCGTACCTCGA	20	55.0	57.9
RA14-591	RA14 related clones	GGCTTCCACAACGTTCGTACCT	22	54.5	60.9
Wsh1-566	Watershed + flodded upland cluster 1	GCTCATGAGCTTGGCCGACATC	22	59.1	61.8
Wsh2-491	Watershed + flodded upland cluster 2	TCATTTGGCCAACCTCTCTCATTCC	25	48.0	60.9
Wsh2-450	Watershed + flodded upland cluster 2	CAAGAGCTGGATCATCACGATG	22	50.0	56.8

B2rel251	*Methylocapsa*-related clones	CCGCCGCGGCCCAGTATTA	19	68.4	63.4
B2-400	*Methylocapsa*	ACCTCTTTGGTCCCGGCTGC	20	65.0	63.4
B2-261	*Methylocapsa*	TCAGGCCTATTTCTGGGAAAGCT	23	47.8	58.3
B2all343	*Methylocapsa* and related clones	AACCGCTACACCAATTTCTGGGG	23	52.2	61.2
B2all341	*Methylocapsa* and related clones	TCAACCGCTACACCAATTTCTGGG	24	50.0	61.1
pmoAMO3-400	clone pmoA-MO3	ACCCAGATGATCCCGTCGGC	20	65.0	62.6
pmoAMO3-486	*MO3 group*	ggGATGGGGCCTTCTCATGTACC	23	60.9	61.5
pmoAMO3-511	*MO3 group*	AGCAACTGGCAGGTCCTCG	19	63.2	60.2
Ver330	*Verrucomicrobia*, all pmoA1+pmoA2	TGGTCAGTGGATGAATAGGTATTGGA	26	42.3	57.3
Ver307	*Verrucomicrobia*, all pmoA2	TTCAGCTGTGCCGGATTGTTTT	22	45.5	57.9
Ver285	*Verrucomicrobia, Ma.fum pmoA2+Ma.kam. pmoA2*	TAAAGCGCCTATAGGAGCAACCT	23	47.8	58.0
Ma_F1-355	*Ma.fum. pmoA1*	AACTTCTGGGGTTGGGGCACTT	22	54.5	61.5
Ma_F1-594	*Ma.fum. pmoA1*	TGAATACATCCGGACTTCTACCCC	24	50.0	57.9
Ma_I1-312	*Ma.inf. pmoA1*	AACCGTTGGGCTTTTCTTTGGC	22	50.0	59.1
Ma_I1-401	*Ma.inf. pmoA1*	AAACATTAATTCCCCAGGCTGTCGT	25	44.0	58.9
Ma_F3-638	*Ma.fum. pmoA3*	AAAGTGGGACTCTTCGGACCTT	22	50.0	58.1
Ma_F3-542	*Ma.fum. pmoA3*	AACCCTTAGAAGCCTTAGGCCA	22	50.0	58.1
ESR-579	ESR (Eastern Snake River) cluster	GACCTGATCGGATTCGAGAACATC	24	50.0	58.5
M84P22-514	environmental clones of uncertain identity	AACTGGGCCTGGCTGGG	17	70.6	61.0
TUSC409	Tropical Upland Soil Cluster #2	CGATCCCGGGCGCGATTC	18	72.2	61.8
TUSC502	Tropical Upland Soil Cluster #2	TCTTCTACTTCGGCAACTGGC	21	52.4	58.3
mtrof173	Universal	GGbGACTGGGACTTCTGG	18	66.7	57.4
mtrof362-I	Methanotrophs	TGGGGCTGGACCTACTTCC	19	63.2	59.5
mtrof661	Methanotrophs	GGTAARGACGTTGCKCCGG	19	63.2	60.4
mtrof662-I	Methanotrophs	GGTAAGGACGTTGCGCCGG	19	68.4	61.9
mtrof656	Methanotrophs	ACCTTCGGTAAGGACGT	17	52.9	53.2
NmNc533	*Nitrosomonas-Nitrosococcus*	CAACCCATTTGCCAATCGTTGTAG	24	45.8	58.6
Nsm_eut381	*Nitrosomonas eutropha*	CCACTCAATTTTGTAACCCCAGGTAT	26	42.3	59.0

PS5-226	*Nitrosomonas-Nitrosococcus* related clones	ACCCCGATTGTTGGGATGATGTA	23	47.8	59.9
Pl6-306	*Nitrosomonas-Nitrosococcus* related clones	GGCACTCTGTATCGTATGCCTGTTAG	26	50.0	60.5
NsNv207	*Nitrosospira-Nitrosovibrio*	TCAATGGTGGCCGGTGG	17	64.7	58.5
NsNv363	*Nitrosospira-Nitrosovibrio*	TACTGGTGGTCGCACTACCC	20	60.0	59.6
SV308	Svalbard clade	TGAGCATCTCTGGGCTTGTCGT	22	54.5	60.7
SVrel583	Svalbard clade and related	TACATGGGATTCACATTTGTGAGGAC	26	42.3	57.0
Nit_rel471	AOB related clones/probably methanotrophs	CGTTCGCGATGATGTTTGGTCC	22	54.5	60.1
Sed585	Ssedi#1	GGGCATTCGCGATGATGTTTTATCCGA	27	48.1	61.2
Sed422	Ssedi#1 and related	TGATCCTAGACTGCACCCTGTTG	23	52.2	58.5
Nit_rel223	AOB related clones/probably methanotrophs	GTCACACCGATCGTAGAGGT	20	55.0	56.9
Nit_rel417	Arctic soil related #1, subgroup	CGCGTTGATCTTTGATTGCACCCTGTT	27	48.1	61.8
Nit_rel419	Arctic soil related #1, subgroup	CGTTGATCCTTGATTGCACCCTGTT	25	48.0	59.8
Nit_rel526	JRC#1+CCd#1 groups	GCCATCAACCATTGGTTGCGGA	22	54.5	60.8
Nit_rel652	Arctic soil MOB	CGTACATTCGGTGGTCACACTG	22	54.5	57.9
ARC529	AOB related clones/probably methanotrophs	TAAGCAGCCGATGGTCGTGGAT	22	54.5	62.2
Nit_rel470	AOB related clones/probably methanotrophs	CGATATTCGGGGTATGGGCG	20	60.0	58.4
Nit_rel351	AOB related clones/probably methanotrophs	GTTTGCCTGGTACTGGTGGG	20	60.0	59.2
gp17-438	environmental clones of uncertain identity - gp17	ACTCTTATTGACCAGGAATTGGACCTTG	28	42.9	58.5
Nit_rel304	AOB related clones/probably methanotrophs - *Crenothrix* and related	CGCTCTGCATTCTGGCGCT	19	63.2	61.8
NLw303	environmental clones of uncertain identity - NL wetland	AACGATCACTATTCTGGCTCTTGCCTTT	28	42.9	60.1
M84P105-451	environmental clones of uncertain identity	AACAGCCTGACTGTCACCAG	20	55.0	58.1
WC306_54-385	environmental clones of uncertain identity	AACGAAGTACTGCCGGCAAC	20	55.0	59.2
WC306-54-516	environmental clones of uncertain identity	AACTGGCCGATTTTTGGCATGTT	23	43.5	58.4
gp23-454	environmental clones of uncertain identity	AACGCGCTGCTCACTGCG	18	66.7	62.3
MR1-348	environmental clones of uncertain identity	AATCTTCGGTTGGCACGGCT	20	55.0	61.1
gp619	environmental clones of uncertain identity	CGGAATATCTGCGCATCATCGAGC	24	54.2	61.5
gp391	environmental clones of uncertain identity	ATCTGGCCGGCGACCATG	18	66.7	61.1

gp2-581	environmental clones of uncertain identity	ACATGATCGGCTACGTGTATCCG	23	52.2	60.0
RA21-466	clone RA21 - environmental clone of uncertain identity	CGGCGTTCTTGGCGGCAT	18	66.7	62.4
hyaBp	spiking control (*hyaB* gene of E.coli)	GATTACGCGCATCGAAGGC	19	57.9	57.5

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Appendix 2: Raman spectroscopy

Introduction

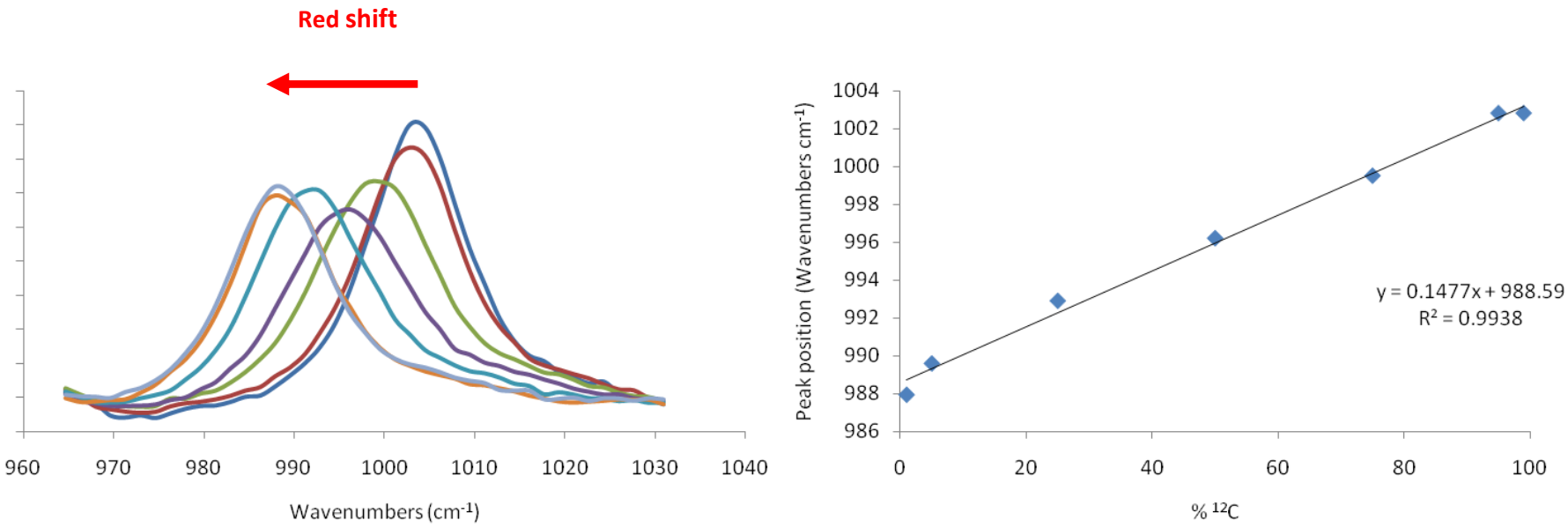
Raman spectroscopy was used to measure different ratios of ^{12}C and ^{13}C that had been incorporated into single cells grown on carbon source with a range of ratios of the two isotopes. The Raman spectroscopy was carried out by Dr Daniel Read at the Center for Ecology and Hydrology Wallingford. Raman spectroscopy is described in detail in the chapter Raman FISH by Read et al., (2010).

Single cell studies using Raman microscopy.

A pure culture of a *Methylobacterium* strain isolated from Movile Cave by D. Wischer was grown in batch culture with methanol as the sole carbon source. Seven cultures were set up with the same concentration of methanol but with different isotope ratios of ^{13}C-methanol (1%, 5%, 25%, 50%, 75%, 95% and 99%). The *Methylobacterium* grows on the ^{13}C-labelled methanol and the components of these cells should contain ^{13}C depending on the ration of ^{12}C to ^{13}C methanol in the growth medium. Samples of each of the *Methylobacterium* cultures were fixed in 4% paraformaldehyde and were sent to Dr Daniel Read at the Center for Ecology and Hydrology, Wallingford. The samples of Methylobacterium containing between 1% and 99% ^{13}C were analysed using a Raman spectrophotometer at the single cell level. The Raman spectra for each sample were overlaid. Focusing on one of the sharper peaks of the Raman spectra that is indicative of phenylalanine, a "red-shift" could be observed (See Intro 1.4.2.6) (**Figure 5.6**). The shift of the peak position has been shown to be directly

proportional to the ^{12}C to ^{13}C ratio within the cells with an R^2 value of 0.99. This shows that it is possible to estimate the relative amount of ^{13}C label within single cells.

Figure 5.6: (Left) Phenylalanine peaks of the Raman spectra from the labelled *Methylobacterium*. (Right) Plot of wave number position for the phenylalanine peak against the relative %^{12}C in the cells. The red line indicates the "red-shift" of peak wave number as the wave length becomes shorter with increased ^{13}C labelling of the *Methylobacterium* cells.



With the data obtained from the Raman spectra of *Methylobacterium* grown with different ratios of ^{12}C and ^{13}C methanol, it was shown that there is a direct correlation between the relative abundance of label within the bacterium and a "red shift" in key peaks of a Raman spectrum. This means that it is possible to measure the realative percentage of ^{13}C label that has been incorporated into single cells. Unfortunately this was as far as the study went but points to potential methods for future experiments to determine if single cells have incorporated the ^{13}C label. The Raman spectroscopy has been used with Fluorescence *In Situ* Hybridisation (FISH) in order to identify organisms that have incorporated a ^{13}C label (Huang et al., 2007). More recently, Raman FISH was used to generate data similar to what is presented here, indicating that there is a direct link between the shift of particular Raman spectra peaks and the amount of label incorporated (Li et al., 2012).

Disscusion

Raman spectroscopy was used to create a standard curve with a *Methylobacterium* isolate from Movile Cave with different ratios of ^{13}C incorporated into the cells. This means that single cells from ^{13}C-enriched sample can be identified having taken up the ^{13}C-label and the extent to which the ^{13}C-lable makes up the organic molecules in the cell can be determined. It was planned to use this data to be able to directly follow the flow of a ^{13}C label between two isolates from Movile Cave. A *Methylomonas* culture would be grown with $^{13}CH_4$ as the sole carbon source. The *Methylomonas* culture would then be inoculated with the *Methylobacterium* isolate from Movile Cave. The only way in which the *Methylobacterium* growth would be

able to be supported would be from any carbon released by the *Methylomonas* be it methanol, formate, formaldehyde etc. Raman FISH would then have been use to identify single cells and to obtain Raman spectra from the *Methylobacterium* cells. If the Raman spectra from any *Methylobacterium* cells displayed a "red shift" then they must have gained that carbon from the *Methylomonas*. This would directly illustrate the first step of carbon flow from the primary consumer (methanotroph) into the secondary consumer (methylotroph). It would only be representative of one possible route of carbon flow but would show that it is possible between two of the Movile Cave isolates. Hopefully this experiment or similar experiments will be carried out in near the future.