Molecular Ecology of

Facultative Methanotrophs

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A thesis submitted to the Department of Biological Sciences in fulfilment of the requirements for the degree of Doctor of Philosophy

July 2010

University of Warwick
Coventry, UK

Dedicated to Lily and my Family, who were not here at that very moment, when I needed them!

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Abbreviations

ANME	Anaerobic methanotrophs
ANMS	Ammonium nitrate mineral salts
bp	Base pairs
cDNA	Complementary DNA
Cy3	Cyanine 3
Cy5	Cyanine 5
cRNA	Complementary RNA
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNMS	Diluted nitrate mineral salts
EDTA	Ethylene diamine tetraacetic acid
FISH	Fluorescent *in situ* hybridisation
GC	Gas chromatography
IPCC	Intergovernmental Panel on Climate Change
kDa	kilo dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Lysogeny broth
MIAME	Minimum Information About a Microarray Experiment
MPN	Most probable number
mRNA	Messenger RNA
MS	Mass spectrometry

NMS	Nitrate mineral salts
OTU	Operational taxonomic unit
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
pMMO	Particulate methane monooxygenase
ppmv	Part per million (by volume)
PQQ	Pyrroloquinoline quinone
qPCR	Quantitative real-time polymerase chain reaction
RDP	Ribosomal database project
RFLP	Restriction fragment length polymorphism
RPM	Revolutions per minute
RuMP	Ribulose monophosphate
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase PCR
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SIP	Stable isotope probing
sMMO	Soluble methane monooxygenase
sp.	Species
spp.	Species (pl.)
TCA	Tricarboxylic acid
TE	Tris EDTA
T-RFLP	Terminal restriction fragment length polymorphism

Tris	Tris (hydroxymethyl) methylamine
USC	Upland soil cluster
µ	Micro

Acknowledgements

I am very grateful to Prof J. Colin Murrell for his scholastic support and guidance during my PhD. There is no doubt at all that without his encouragement, patience and effort this thesis would have been completed. One could not wish for a better supervisor and I feel privileged and proud to have worked under his guidance.

I thank Chen and Andrew, with whom I worked alongside in this project, for their guidance and help all the time (I wonder how they could know everything!). I wish I could make frequent visit to Oxford on weekend at Andrew's place to be with him and Jan for an absolute relax one could ever have! I thank Hélène for making me critical about science, of course for teaching me the French (Je redige ma these), the duck and the Une Galette Bretonne! Wish Hélène and Fred all the very best for the regatta. I also thank Deepak dada, Rich, Ashraf and Julie for their help and critical discussions throughout his project. I have really learnt a lot from them. I would like to thank everyone in the Colin Murrell lab, both past and present, Hendrick, Natalila, Michael, Elizabetta, Jason, Daniela, Antony, Kristin, Gez and Cathy for their help and cooperation during my stay here at the lab. I wish really lose my emotion, and don't miss these wonderful people, who were always around me here!

I also want to thank my collaborators Niall, Nancy and Levente for their excellent help with experiments.

Thanks to Colin and all the members of his family as well so much for allowing me to be with them for the wonderful time during Christmas holidays, when there was no one around me here.

I acknowledge the University of Warwick and the Department of Biological Sciences for financial assistance. I also thank very much all my colleagues at the Bangladesh Agricultural University for their help and cooperation during my stay at UK.

Finally, I am extremely thankful and forever indebted to my wife Mukti, my son Rabith, my parent, my brother Amin and Taufique, Labue bhi, and Deepa for their understanding, endless support and encouragement when it was most required. Mukti get ready for the Katus Kutus:) Rabith, darling I promise, you don't ever have to miss me again anymore. I am your now, totally. Simply can't wait any more to be with you all.

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 here has been previously submitted for any other degree.

Abstract

Methylocella spp. are facultative methanotrophs, able to grow not only on one carbon molecules *e.g.,* methane or methanol, but also on multi-carbon compounds *e.g.,* acetate, succinate and malate.

PCR-based molecular techniques were developed and validated to target the 16S rRNA genes of all the species of the genus *Methylocella* present in environmental samples. DNA extracted from a variety of environmental samples screened by PCR to determine the environmental distribution of *Methylocella* spp. *Methylocella* were found to be widely distributed, not only in acidic environments, but also in neutral and alkaline environments. New primers targeting the *mmoX* gene of *Methylocella* spp. that encoded the α-subunit of methane monooxygenases were designed. A SYBR® green-based real-time quantitative PCR assay was developed and validated using these *Methylocella mmoX* gene-targeting primers. The abundance of *Methylocella* spp. present in selected environmental samples was quantified using the newly developed real-time quantitative PCR assay

A series of $^{13}CH_4$ DNA-SIP experiments were carried either in the presence or absence of ^{12}C acetate in microcosms containing Moor House peat soil to investigate the effect of acetate (0.5 mM) on the ability of *Methylocella silvestris* to oxidize methane. Methane oxidation data indicated that acetate repressed the ability of *Methylocella silvestris* to oxidize methane in peat soil microcosms. $^{13}CH_4$ DNA-SIP experiments revealed that in presence of acetate, *Methylocella silvestris* did not utilize methane as a carbon source, suggesting that acetate might be the alternative source of carbon. However, when ^{13}C-labelled acetate DNA-SIP experiments were carried out, *Methylocella* spp. were not found to be dominant acetate utilizers in the peat soil microcosms. *Methylocella* spp. seem to have been outcompeted by more efficient acetate utilizers such as *Brevundimonas* and *Burkholderia.*

To identify genes that might be involved in the utilization of methane or acetate, *Methylocella silvestris* whole genome transcriptomics experiments were carried out. All the genes of the sMMO gene cluster were found to be highly upregulated during growth on methane. In addition to the sMMO gene cluster, a gene encoding Fur was also found to be highly upregulated during growth on methane. During growth on acetate, a gene cluster encoding glycine dehydrogenase was found to be highly upregulated. Microarray experiments carried out here provided potential candidate genes for further characterization by gene knockout based studies. Further work is also required to validate the microarray findings.

A study was carried out on forest soils derived from Swedish islands that were at different successional stages. All islands were found to be positive for the presence of *Methylocella* spp., and identity of composition of other methanotrophs were determined using a *pmoA*-diagnostic microarray. All the islands were dominated by *Methylococcus, Methylosinus* and methanotrophs of the uncultivated RA14 clade. The diversity of methanotrophs was higher in late successional islands compared to mid and early successional islands. In addition the diversity of methanotrophs decreased as the soil depth increased.

Chapter 1

Introduction

1.1 Methane as an important greenhouse gas

Methane (CH_4) is an important greenhouse gas having an effect on the Earth's temperature and climate system (Houghton *et al.*, 2001). The concentration of methane in the atmosphere is ~1.7 ppmv (IPCC., 2001). Although methane has a short life time in the atmosphere *e.g.,* 9 to 15 years and its concentration in the atmosphere is 200 times lower than carbon dioxide (CO_2), its potential to absorb infrared radiation is 20 to 30 times more efficient than CO_2 (Rodhe, 1999). Recently an increase (at a rate of about 1% annually) in the concentration of methane has been observed across the globe, from both anthropogenic and natural sources (Blake & Rowland, 1988 ; IPCC, 2001). Calculations suggest that a two-fold increase in methane content in the atmosphere may cause a global increase in temperature of 0.2–0.4° C (Rasmussen & Khalil, 1981).

1.1.1 Sources and sinks of methane

Methane is emitted into the atmosphere from a variety of anthropogenic and natural sources. The major anthropogenic sources include rice cultivation, biomass burning, waste management and animal husbandry *e.g.,* enteric fermentation in livestock and manure management. In fact, it has been estimated that more than 60% of global methane emissions are related to human activities (IPCC, 2007). The major natural sources of methane include wetlands, gas hydrates, termites, oceans, freshwater bodies, non-wetland soil, and wildfires. Wetlands are the major sources of atmospheric methane, accounting for 110 Tg yr^{-1} (1Tg = 10^{12}g) (Wahlen, 1993;

IPCC, 2001). Although anaerobic production of methane by the methanogenic *Archaea* was considered as the only source of microbial methane production, recently Keppler *et al*. (2006) and Wang *et al*. (2009) reported that some plants *e.g.*, European ash (*Fraxinus excelsior* L.), European beech leaves (*Fagus sylvatica* L.) and buffalo grass (*Anthoxanthum odoratum* L.) can produce methane *in situ* under oxic conditions, the mechanism of which is not known. Further experiments need to be carried out to understand the mechanism by which plants produce methane and their contribution to the global methane cycle.

Once methane is released into the atmosphere, it is converted into CO_2 and H_2O in the troposphere by OH radicals. This accounts for more than 85% of the global removal of methane (Wahlen, 1993). Methane is also removed by methane-oxidizing bacteria and *Archaea* that utilize methane as their carbon source. Soils are the major site for the biological sink of methane and remove 20 to 60 Tg of methane per year and contribute 10% of the global methane sink (King, 1992; Knief *et al.*, 2003). Natural forests and upland soils also contribute to the oxidation of methane from the atmosphere (Holmes *et al.*, 1999; Bull *et al.*, 2000; Knief *et al.*, 2003; Kolb *et al.*, 2005; Lau *et al.*, 2007). There are reports suggesting that methane oxidation also takes place in extreme environments such as deserts (Striegl *et al.*, 1992; Angel & Conrad, 2009), tundra soils (Whalen & Reeburgh, 1990), the surface water of the oceans (Conrad & Seiler, 1988) and hot acidic environment such as volcano mudpots, geothermal field soils and acidic hot springs (Dunfield *et al.*, 2007, Islam *et al.*, 2008; Pol *et al.*, 2007).

1.2 The role of microorganisms in methane cycling

Biological methane is produced in the environment by methanogenic *Archaea* under anoxic conditions. These organisms use acetate, or CO_2 and hydrogen (H_2) as the substrates for the production of methane (Whiticar *et al.*, 1986; Thauer & Shima, 2006). Methanogenic *Archaea* are distributed in a wide range of ecosystems ranging from the human gut to deep, high-temperature (>100 $^{\circ}$C) and high-pressure hydrothermal vents. Methane produced by methanogens and methane released from gas and coal mines or deep sea vents can be oxidized by either *Bacteria* (methanotrophs and ammonia oxidizers) or *Archaea* (coupled with sulfate reduction, or denitrification) (Hinrichs *et al.*, 1999; Raghoebarsing *et al.*, 2006).

Microbial methane oxidation can occur under both anaerobic and aerobic conditions. Anaerobic methane oxidation has been detected in marine sediments, particularly in methane seeps and vents and in anoxic waters (Orcutt *et al.*, 2008; Knittel & Boetius, 2009). So far microorganisms that are involved in anaerobic methane oxidation have not yet been cultivated. Studies have reported coupling of anaerobic methane oxidation with sulfate reduction (Hinrichs *et al.*, 1999) and denitrification (Raghoebarsing *et al.*, 2006). Microorganisms involved in anaerobic methane oxidation process have been identified as anaerobic methanotrophic *Archaea* (ANME) *e.g.*, ANME-1(Hinrich *et al.*, 1999), ANME-2 (Boetius *et al.*, 2000) and ANME-3 (Knittel *et al.*, 2005).

Microorganisms performing aerobic methane oxidation include methanotrophs belonging to the *Alphaproteobacteria, Gammaproteobacteria* and *Verrucomicrobia.* Further, methane may be oxidized by the ammonia oxidizers (which can perform co-oxidation of methane using the ammonium monooxygenase) (reviewed in Trotsenko & Murrell, 2008).

A significant proportion of the methane produced by methanogens is oxidized by methanotrophs in the environment. Methanotrophs limit the efflux of methane produced in flooded soils and wetlands and consume atmospheric methane directly in aerated upland soils, thus helping to reduce the greenhouse effect of methane producers (Trotsenko & Murrell, 2008).

1.2.1 Classification of methanotrophs

Methanotrophs have been classified in many ways, based upon certain criteria such as i) the concentration of methane they require for growth: high affinity and low affinity methanotrophs, ii) aerobic and anaerobic methanotrophs, based on the requirement of oxygen, iii) obligate methanotrophs and facultative methanotrophs based on their ability to use multi-carbon compounds as a carbon source in addition to methane, iv) Alphaproteobacterial methanotrophs, Gammaproteobacterial methanotrophs and Verrucomicrobial methanotrophs based on their phylogeny and v) Type I and Type II methanotrophs, primarily based on their morphology, physiology and biochemistry.

High affinity methanotrophs can oxidize methane at atmospheric concentrations (~1.7 ppmv), while the low affinity methanotrophs require a higher concentration of methane *e.g.,* greater than 100 ppmv methane (Bender & Conrad, 1992). So far no high affinity methanotrophs have been isolated, but they are detected in the environment using specific biomarkers such as phospholipid fatty acids (PLFA) and *pmoA* (encoding the alpha subunit of particulate methane monooxygenase, pMMO) sequences. The ability of methanotrophs to oxidize atmospheric concentrations of methane was demonstrated by Maxfield *et al.* (2006). These authors incubated forest soil with $^{13}CH_4$ and found enrichment of ^{13}C-labeled 18:1ω7c containing methanotrophs. This 18:1ω7c PLFA is mainly found in *Methylocella* and *Methylocapsa*-related species (Dedysh *et al.,* 2002), suggesting that these species could be potential atmospheric methane oxidizers. The 18:1 PLFAs were earlier suggested to be the major labeled PLFAs in a forest soil from Wales (Bull *et al.,* 2000), forest soils from USA and Denmark (Holmes *et al.,* 1999) and in a number of forest soils from around the world (Roslev *et al.,* 1997) that were also capable of taking up atmospheric concentrations of methane. *pmoA* sequences of the RA14 group (also known as upland soil cluster-α (USCα)) retrieved from these forest soils are considered to belong to the high affinity methanotrophs (Holmes *et al.,* 1999). *pmoA* sequences of the RA14 group are phylogenetically distantly related to *pmoA* sequences from extant *Alphaproteobacteria* methanotrophs (Holmes *et al.,* 1999). *pmoA* transcripts of these uncultured *Alphaproteobacteria* have been detected from forest soils from Germany, suggesting that these high affinity methanotrophs were

actively involved in the oxidation of methane at atmospheric concentration in forest soil (Kolb *et al.,* 2005). In addition, *Methylocystis*- and *Methylosinus*-like methanotrophs are also considered to be able to oxidize atmospheric concentrations of methane (Kravchenko *et al.,* 2010). Knief *et al.* (2003) demonstrated the ability of *Gammaproteobacteria* to oxidize atmospheric concentrations of methane. These authors found 16:1 PLFAs (16:1ω7c) that are signature biomarkers of *Gammaproteobacteria* as the major labeled PLFA when forest soil from Germany was incubated with atmospheric concentrations of $^{13}CH_4$. In addition, these authors also detected *pmoA* sequences (USCγ) distantly related to *pmoA* sequences from Type I (*Gammaproteobacteria*) methanotrophs as the most abundant *pmoA* sequences from these soils (Knief *et al.,* 2003). Low affinity methanotrophs have been well studied and a number of strains such as *Methylococcus capsulatus* and *Methylosinus trichosporium* have been isolated in pure culture. This group of methanotrophs is the most well studied and a number of strains have been characterized in detail at the physiological, biochemical and molecular level (Trotsenko & Murrell, 2008; Semrau *et al.,* 2010).

Classical aerobic methanotrophs are classified into either Type I or Type II methanotrophs, based on their carbon assimilation pathways, cell morphology, GC content of their DNA, phospholipid fatty acid profiles, intracytoplasmic membrane arrangements, nitrogen-fixing capability and resting stages (reviewed in Trotsenko & Murrell, 2008) (**Table 1.1**).

Table 1.1. Methanotroph genera and their main characteristics (Adapted from Chen, 2008d).

Genus name	Phylogeny	MMO type	C_1 assimilation	ICM type	(mol % GC content)	Major PLFA	Trophic niche	References
Methylobacter	*Gammaproteobacteria*	pMMO	RuMP	Type I	49-54	16:1	Some psychrophilic	1, 2, 3, 4
Methylosoma	*Gammaproteobacteria*	pMMO	not known	Type I	49.9	16:1	Not extreme	22
Methylomicrobium	*Gammaproteobacteria*	pMMO sMMO	RuMP	Type I	49-60	16:1	Halotolerant; alkaliphilic	7, 8, 9, 10, 11, 12, 13
Methylomonas	*Gammaproteobacteria*	pMMO sMMO	RuMP	Type I	51-59	16:1	some psychrophilic	1, 2, 14, 15, 16
Methylosarcina	*Gammaproteobacteria*	pMMO	RuMP	Type I	54	16:1	Not extreme	17
Methylosphaera	*Gammaproteobacteria*	pMMO	RuMP	ND	43-46	16:1	Psychrophilic	18
Methylococcus	*Gammaproteobacteria*	pMMO sMMO	RuMP/ Serine	Type I	59-66	16:1	Thermophilic	1, 5
Methylocaldum	*Gammaproteobacteria*	pMMO	RuMP/ Serine	Type I	57	16:1	Thermophilic	6
Methylothermus	*Gammaproteobacteria*	pMMO	RuMP	Type I	62.5	18:1/16:0	Thermophilic	23
Methylohalobius	*Gammaproteobacteria*	pMMO	RuMP	Type I	58.7	18:1	Halophilic	24
Methylocystis	*Alphaproteobacteria*	pMMO sMMO	Serine	Type II	62-67	18:1	Some acidophilic	1, 2
Methylosinus	*Alphaproteobacteria*	pMMO sMMO	Serine	Type II	63-67	18:1	Not extreme	1, 2
Methylocella	*Alphaproteobacteria*	sMMO	Serine	NA	60-61	18:1	Acidophilic	19, 20
Methylocapsa	*Alphaproteobacteria*	pMMO	Serine	Type II	63.1	18:1	Acidophilic	21
Crenothrix	*Alphaproteobacteria*	pMMO	ND	Type I	ND	ND	Not extreme	25
Cloonothrix	*Alphaproteobacteria*	pMMO	ND	Type I	ND	ND	Not extreme	26
"Methylokorus"	*Verrucomicrobia*	pMMO	Serine,	ND	ND	ND	Thermoacidophilic	27
"Acidimethylosilex"	*Verrucomicrobia*	pMMO	RuMP?	ND	ND	C18:0	Thermoacidophilic	28
"Methyloacida"	*Verrucomicrobia*	pMMO	ND	ND	ND	ND	Thermoacidophilic	29

ND, not determined; NA, not applicable; ICM, intra cytoplasmic membrane. 1. (Whittenbury *et al.*, 1970); 2. (Bowman *et al.*, 1993a); 3. (Omelchenko *et al.*, 1996); 4. (Kalyuzhnaya *et al.*, 1998); 5. (Malashenko *et al.*, 1975); 6. (Bodrossy *et al.*, 1997); 7. (Bowman *et al.*, 1995); 8. (Sieburth *et al.*, 1987); 9. (Fuse *et al.*, 1998); 10. (Khmelenina *et al.*, 1997) 11. (Kalyuzhnaya *et al.*, 1999a); 12. (Sorokin *et al.*, 2000); 13. (Kaluzhnaya *et al.*, 2001); 14. (Whittenbury & Krieg, 1984); 15. (Omelchenko *et al.*, 1996); 16. (Kalyuzhnaya *et al.*, 1999b) ; 17. (Wise *et al.*, 2001); 18. (Bowman *et al.*, 1997); 19. (Dedysh *et al.*, 2000); 20. (Dunfield *et al.*, 2003); 21. (Dedysh *et al.*, 2002); 22. (Rahalkar *et al.*, 2007); 23. (Tsubota *et al.*, 2005); 24. (Heyer *et al.*, 2005); 25. (Stoecker *et al.*, 2006); 26. (Vigliotta *et al.*, 2007); 27. (Pol *et al.*, 2007); 28. (Dunfield *et al.*, 2007); 29. (Islam *et al.*, 2008).

The genera *Methylokorus, Acidimethylosilex* and *Methyloacida* have recently been unified under the genus *Methylacidiphilum* (Op den Camp *et al.*, 2009)

The Type I methanotrophs belonging to the *Gammaproteobacteria* include the genera *Methylobacter, Methylomonas, Methylomicrobium, Methylocaldum, Methylosphaera, Methylothermus, Methylosarcina, Methylohalobius, Methylosoma* and *Methylococcus*. The Type II methanotrophs include the genera *Methylocystis, Methylosinus, Methylocella* and *Methylocapsa*, belonging to the *Alphaproteobacteria* (**Figure 1.1**).

In addition, recently two filamentous methane-oxidizers *Crenothrix polyspora* (Stoecker *et al.*, 2006) and "*Clonothrix fusca*" (Vigliotta *et al.*, 2007) have been described that are also able to use methane as a carbon source. *Crenothrix polyspora* is characterized by the presence of a novel *pmoA* sequence (Stoecker *et al.*, 2006), while "*Clonothrix fusca*" has a more conventional *pmoA* sequence (Vigliotta *et al.*, 2007). Both of these bacteria belong to the *Gammaproteobacteria* and are phylogenetically closely related to the Type I methanotrophs (**Figure 1.1**). Morphological characteristics of methanotrophs are presented in **Figure 1.2 and 1.3.**

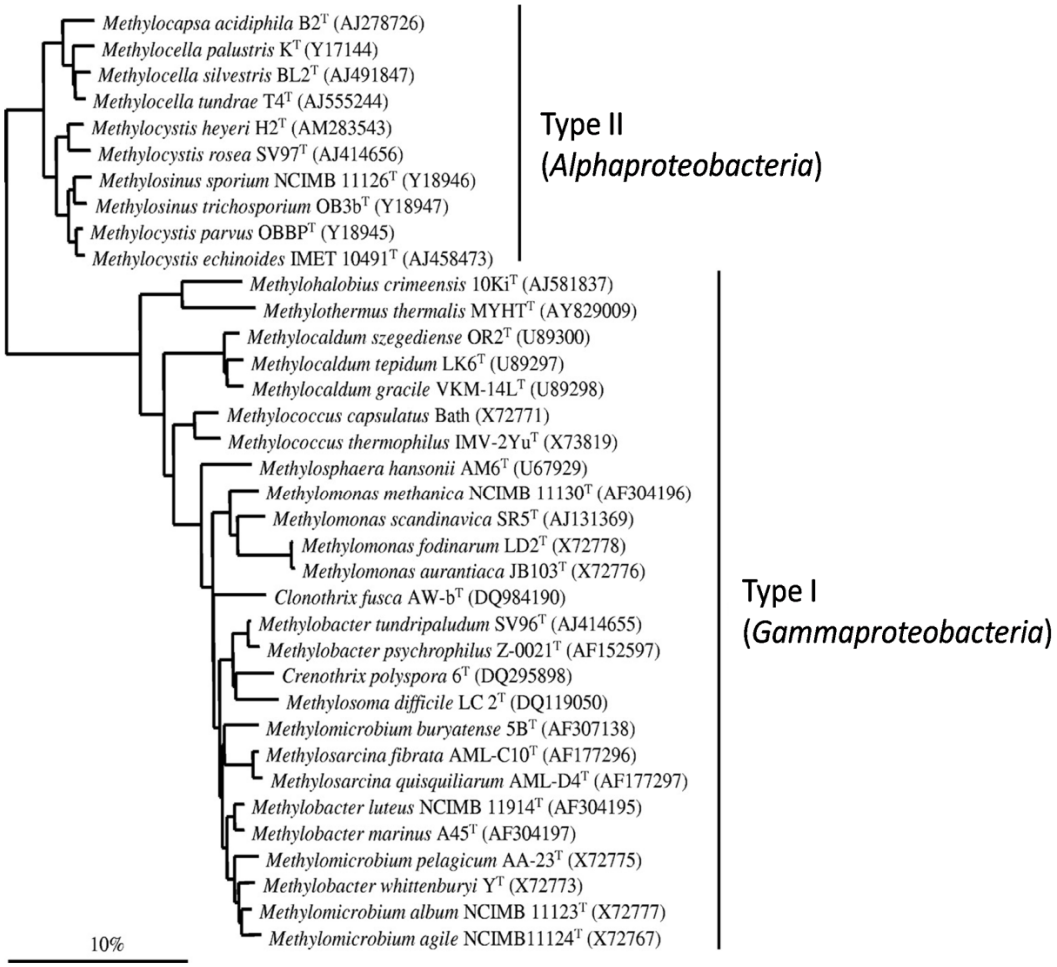


Figure 1.1. Phylogenetic tree based on the analysis of the 16S rRNA gene sequences of the type strains of Type I and II methanotrophs. The dendrogram was produced using the Neighbor-joining method based on 1245 base pairs of aligned sequence. The phylogenetic tree was rooted to *Methylobacterium extorquens* (AF531770). The bar represents 0.1 substitution per nucleotide position (taken from McDonald *et al.*, 2008).

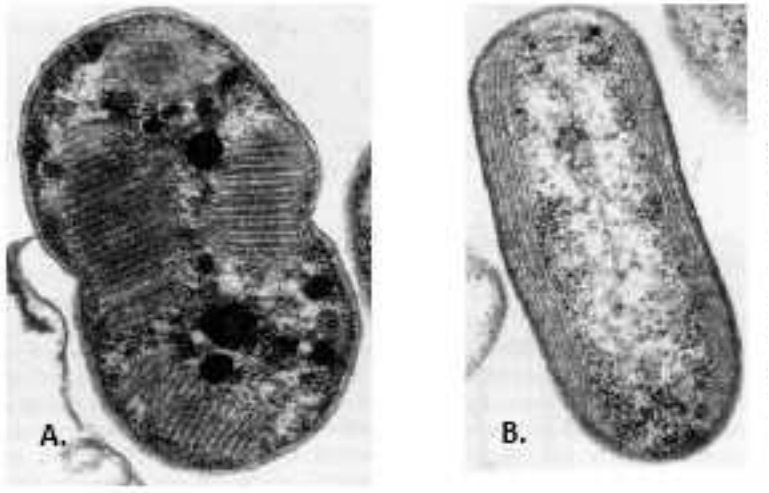


Figure 1.2. Electron micrographs of methanotrophs showing the different types of membrane arrangement. A: Section of *Methylomonas methanica* showing the membrane system found in Type I methanotrophs. B: Section of *Methylocystis parvus* showing the membrane system found in Type II methanotrophs (taken from Green, 1992).

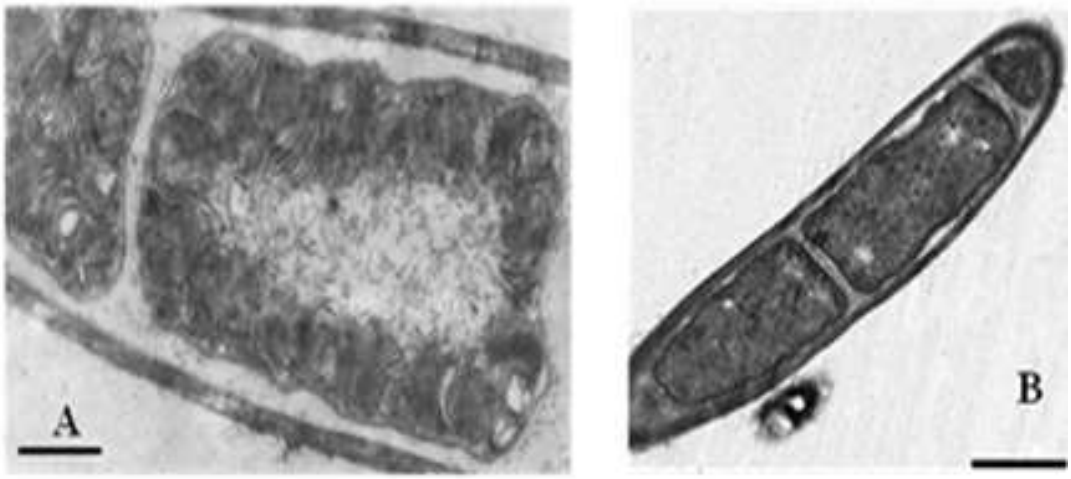


Figure 1.3. Electron micrographs of novel methanotrophs. **A.** Section of a sheathed *Crenothrix polyspora* filament. (bar 0.5 µm) (taken from Stoecker *et al.*, 2006). **B.** Section of "*Clonothrix fusca*" (Bar 1 µm) (taken from Vigliotta *et al.*, 2007).

Recently, three isolates from the *Verrucomicrobia* phylum namely "*Methylacidiphilum infernorum*" strain V4 (Dunfield *et al.*, 2007), "*Methylacidiphilum fumarolicum*" strain SolV (Pol *et al.*, 2007) and "*Methylacidiphilum kamchatkense*" strain Kam1 (Islam *et al.*, 2008) have been isolated from a methane-emitting geothermal field soil in the North Island of New Zealand, from a Solfatara volcano mudpot in Italy and from an acidic hot spring in Kamchatka, Russia, respectively. These Verrucomicrobial methanotrophs are thermoacidophilic in nature and can grow at temperatures higher than 50° C and at a pH lower than 5.0 (Semrau *et al.*, 2008).

Most *Proteobacteria* methanotrophs contain two similar *pmoCAB* operons, however, Verrucomicrobial methanotrophs contain three divergent *pmoCAB* operons, which encode the particulate methane monooxygenase and are quite unusual compared to other methanotrophs. These Verrucomicrobial methanotrophs may have diverged from Proteobacterial methanotrophs a long time ago and it is unlikely that the *pmoA* genes of Verrucomicrobial methanotrophs were acquired by horizontal gene transfer (Dunfield et *al.*, 2007). The environmental, genomic and taxonomic perspectives on the Verrucomicrobial methanotrophs have recently been discussed in detail in a review by Op den Camp *et al.* (2009).

1.2.2 Ecology of methanotrophs

Methane-oxidizing bacteria have been detected from a wide variety of environments, including wetlands, rice paddies, fresh and ocean water and sediments, peat soil, forest soil, coal mines and landfill cover soils (Hanson & Hanson, 1996; Han *et al.*, 2009). The distribution of methanotrophs in the environment is influenced by several parameters *e.g.*, moisture content, vegetation, the availability of oxygen and nitrogen (N) and other environmental factors, such as pH and temperature (Amaral & Knowles, 1995; Bussmann *et al.*, 2004; Han *et al.*, 2009). Although most of the methanotroph isolates are mesophilic, some of them are thermophilic *e.g.*, *Methylococcus capsulatus* and *Methylothermus thermalis* (growing at 45 – 55^{o} C). There are also reports on moderately thermophilic methanotrophs such as *Methylocaldum* spp. (Medvedkova *et al.*, 2007). Members of the genus *Methylohalobius* are considered to be halophilic methanotrophs (Heyer *et al.*, 2005) isolated from hypersaline lakes in the Crimean Peninsula of Ukraine. These halophilic methanotrophs are able to tolerate NaCl concentrations from 0.2 M to 2.5 M (1.2-15%). Lin *et al.* (2004) detected *Methylobacter, Methylomonas, Methylosinus* and *Methylocapsa* in soda lake sediments (pH 9.5) and also some sequences of the genera are considered to be alkalophilic methanotrophs. Psychrophilic methanotrophs such as *Methylobacter psychrophilus*, *Methylosphaera hansonii* and *Methylomonas scandinavica*, have been isolated from Atlantic lakes and groundwater with Sweden (Bowman *et al.*, 1997: Kalyuzhnaya *et al.*, 1999). These psychrophilic methanotrophs belong to the *Gammaproteobacteria* and can grow between 5 - 15^{o} C (Whittenbury et al., 1970; Pacheco-Oliver *et al.*, 2002). In addition, the recently identified Verrucomicrobial

methanotrophs (Dunfield *et al.,* 2007; Pol *et al.,* 2007; Islam *et al.,* 2008) belong to the thermoacidophilic group and can grow at extremely low pH *e.g.,* pH 1.0 and at a high temperature *e.g.,* 65^{o} C (Op den Camp *et al.,* 2009).

1.2.3 Facultative methanotrophs

Earlier it was thought that methanotrophy is an obligate trait, and that methanotrophs are not able to grow on substrates containing carbon-carbon bonds (Anthony, 1982). The reason for the obligate nature of methanotrophy is a long-standing scientific mystery. However, the only known exception is the recently discovered facultative methanotroph *Methylocella* spp. Using low nutrient media, *Methylocella palustris*, *Methylocella silvestris* and *Methylocella tundrae* were isolated from acidic peat, forest and tundra soils, respectively (Dedysh *et al.,* 1998; Dedysh *et al.,* 2000; Dunfield *et al.,* 2003). These *Methylocella* spp. are facultative methanotrophs able to grow not only on methane, but also on multi-carbon compounds such as acetate, succinate, pyruvate, malate and ethanol (Dedysh *et al.,* 2005). In fact, *Methylocella* spp. have a higher growth rate and carbon conversion efficiency when grown on multi-carbon substrates (such as acetate) than on methane. When they are exposed to both methane and acetate in excess, they preferentially utilize acetate and shut down methane oxidation (Dedysh *et al.,* 2005; Theisen *et al.,* 2005). Phylogenetically, *Methylocella* spp are members of the family *Beijerinckiaceae* of the class *Alphaproteobacteria.* These new methanotrophs, together with *Methylocapsa acidophila,* form a distinct clade of Type II methanotrophs (Dunfield *et al.*, 2003). Their phylogeny is such that they group with the well-known

genera *Methylosinus* and *Methylocystis* in the *Alphaproteobacteria.* However, *Methylocella* species are more phylogenetically related to the non-methanotrophic heterotroph *Beijerinckia indica* (Dunfield *et al.*, 2003). Facultative methanotrophs have had a somewhat chequered history in the field of methanotrophy over the last 30 years. For example, it was claimed that *Methylobacterium organophilum* and *Methylomonas* strain 761M grew on methane or glucose, but this capacity to grow on methane was either lost or results were not substantiated in other laboratories. In the case of cultures of *Methylobacterium ethanolicum*, this was found to be a very tight syntrophic association between a methanotroph and a heterotrophic *Xanthobacter* spp. (Lidstrom O' Connor *et al.*, 1983). Fortunately, doubts about the existence of facultative methanotrophs seem finally to have been dispelled by the meticulous experiments performed by Dedysh *et al.* (2005). Quantitative real-time PCR (qPCR) targeting *mmoX* in *Methylocella silvestris* BL2, showed a parallel increase of copies of *mmoX* and microscopic cell counts in both methane and acetate-grown cultures. Fluorescence *in situ* hybridization (FISH) using strain- and genus-specific oligonucleotide probes and most probable number (MPN) dilution experiments ruled out the possibility of contaminants. This organism is also unique among the all other methanotrophs in being able to oxidize methane via only a soluble methane monooxygenase (sMMO) enzyme (Theisen *et al.*, 2005). *Methylocella* spp. lack the particulate methane monooxygenase (pMMO) enzyme and associated intracellular membrane systems, whereas pMMOs present in all other methanotrophs thus far tested.

Recently Dunfield *et al.* (2010) isolated a new species of facultative methanotroph named *Methylocapsa aurea* from a forest soil in Germany. This isolate belongs to the family *Beijerinckiaceae* of the class *Alphaproteobacteria* and is most closely related to the obligate methanotroph *Methylocapsa acidiphila* BT2. However, unlike *Methylocapsa acidiphila*, which grows only on methane and methanol, *Methylocapsa aurea* also grows on acetate. It shares this facultative ability with methanotrophs of the genus *Methylocella*, although *Methylocella* spp. do not have pMMO. Another important characteristic of *Methylocapsa aurea* is that it lacks sMMO and oxidizes methane using pMMO only. Very recently Belova *et al.*, (2010) reported the ability of *Methylocystis* sp. H2s to grow and use acetate as the sole carbon source. *Methylocystis* sp. H2s is mildly acidophilic member of the genus *Methylocystis*, containing both the soluble and particulate methane monooxygenase, isolated from *Sphagnum* peat collected on the bank of the bog lake Teufelssee in north-eastern Germany (Dedysh *et al.*, 2007). The capability of these facultative methanotrophs to utilize acetate represents an important part of their survival strategy in the environments where methane availability is variable or limited.

1.3 Methane oxidation pathways

The oxidation of methane to methanol is catalyzed by the enzyme methane monooxygenase (MMO) (Reviewed in Murrell *et al.*, 2000; Semrau *et al.*, 2010). Two types of MMO are found in methanotrophs: (i) particulate methane monooxygenase (pMMO) (ii) soluble methane monooxygenase (sMMO). pMMO is a membrane-bound

enzyme with a narrower substrate affinity such as short chain alkanes, alkenes (up to five carbons) and ammonia (Trotsenko & Murrell, 2008) compared to sMMO. sMMO is a cytoplasmic enzyme that has attracted attention due to its broader substrate specificity compared to pMMO. It can oxidize short-chain alkanes, alkenes, alicyclic and aromatic hydrocarbons and hence is of great interest in bioremediation and biotransformation (Fishman *et al.*, 1997). sMMO enzymes are also able to co-oxidize environmental pollutants such as the halogenated hydrocarbons trichloroethylene and chloroform and thus may contribute to reducing environmental pollution (Sullivan *et al.*, 1998). Although sMMO can oxidize these compounds, methanotrophs do not use them as energy sources. They cannot grow on these pollutants (Murrell *et al.*, 2000). Once methanol is produced from methane, it is converted into formaldehyde by methanol dehydrogenase (Anthony, 1982). Formaldehyde can be either oxidized to formate to produce reducing power and energy or assimilated into biomass *via* the ribulose monophosphate pathway (RuMP) or the serine pathway (reviewed in Hanson & Hanson, 1996; Trotsenko & Murrell, 2008). Formate is further oxidized by formate dehydrogenases to produce CO_2 and H_2O **(Figure 1.4)**.

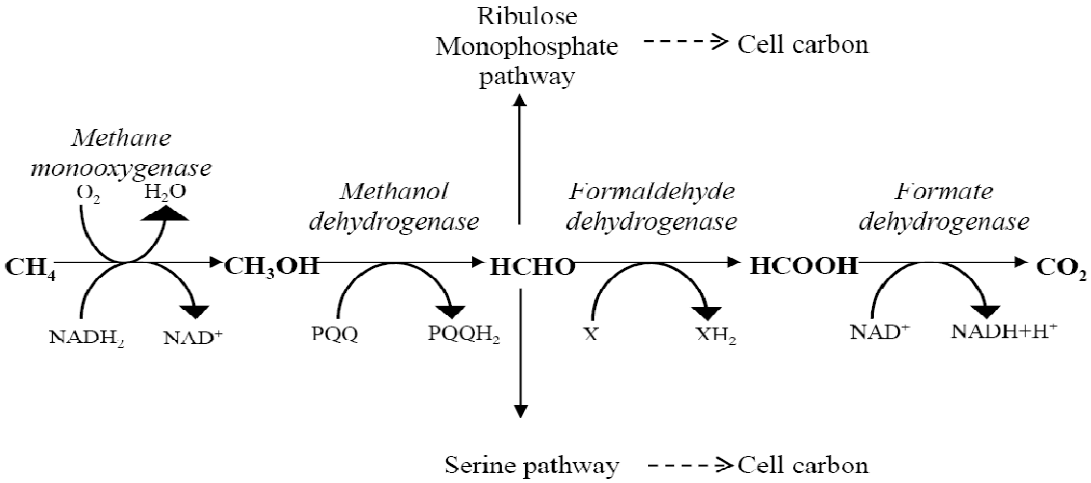


Figure 1.4. Methane (CH_4) oxidation pathway in methanotrophs. Methane is oxidized to methanol (CH_3OH) by methane monooxygenase. Methanol is then converted into formaldehyde (HCHO) by methanol dehydrogenase. Formaldehyde can be assimilated into cell carbon either through the ribulose monophosphate pathway or the serine pathway. Alternatively, formaldehyde is converted into formate (HCOOH) by formaldehyde dehydrogenase, which is finally converted into CO_2 by formate dehydrogenase. Taken from Kumaresan (2009).

1.4 Methane monooxygenases

1.4.1 Particulate methane monooxygenase

pMMO is an integral membrane metallo-enzyme responsible for methane oxidation in all known methanotrophs except for members of the genus *Methylocella* (Dedysh *et al.*, 2000; Theisen *et al.*, 2005). Due to the difficulties associated with the purification of pMMO in its active form, it is less studied compared to sMMO. Smith and Dalton (1989)

were the first to partially purified and characterized pMMO from *Methylococcus capsulatus* Bath grown in medium containing a relatively high concentration of copper. The pMMO complex consists of two components, the hydroxylase (pMMOH) and a reductase (pMMOR).

The pMMO hydroxylase component consists of three subunits protein α, β and γ with approximate masses of 47, 24 and 22 kDa respectively, while the reductase (pMMOR) component consists of 63 and 8 kDa polypeptides (Zahn & DiSpirito, 1996; Basu *et al.*, 2003; Balasubramanian *et al.*, 2010). The genes encoding pMMO are clustered on the genome in the order of *pmo*CAB with a σ^{70} promoter located 5′ of *pmoC* (**Figure 1.5**) (Gilbert *et al.*, 2000b). *pmoCAB* operons have been cloned and sequenced from a number of methanotrophs, including *Methylococcus capsulatus* Bath, *Methylobacterium album* BG8 (Semrau *et al.*, 1995), *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M (Gilbert *et al.*, 2000b).

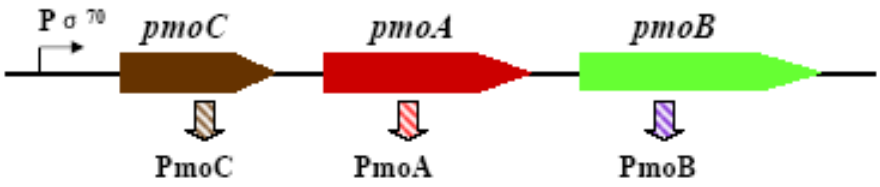


Figure 1.5. Structure of the *pmoCAB* operon. Adapted from Theisen (2006).

There are two virtually identical copies (13 base pair changes over 3,183 bp of *pmoCAB*) present in the genome of *Methylococcus capsulatus* Bath, and a third copy of *pmoC* has also been identified (Stolyar *et al.*, 1999). Similar findings have been noted in *Methylosinus trichosporium* OB3b (Gilbert *et al.*, 2000). However, in *Methylocystis* sp. SC2 although there are two copies of pMMO, they are highly divergent in sequence (Dunfield *et al.*, 2002). Recently Banni and Liesack (2008) have shown that these two copies of pMMO (pMMO1 and pMMO2) in *Methylocystis* sp. SC2 have different affinities for methane. pMMO1, encoded by the *pmoCAB1* gene cluster, oxidizes methane only at mixing ratios >600 ppmv, while pMMO2 encoded by the *pmoCAB2* gene cluster, is constitutively expressed and oxidizes methane at lower, even atmospheric, concentrations (~2 ppmv). The filamentous methanotrophs *Crenothrix polyspora* (Stoecker *et al.*, 2006) and "*Clonothrix fusca*" (Vigliotta *et al.*, 2007) and methanotrophs from the *Verrucomicrobia* phylum (Dunfield *et al.*, 2007, Pol *et al.*,2007, Islam *et al.*,2008) are also known to have highly divergent *pmoA* sequences.

1.4.2 Soluble methane monooxygenase

sMMO is a relatively stable and extensively studied enzyme involved in methane oxidation (Trotsenko & Murrell, 2007). sMMO is present in the methanotrophs of the genera *Methylocella, Methylosinus, Methylocystis, Methylococcus, Methylomicrobium* and *Methylomonas* (Dedysh *et al.*, 1998; Fuse *et al.*, 1998; Shigematsu *et al.*, 1999). sMMO enzymes present in *Methylocella* spp. are similar to those of Type II methanotrophs (Dedysh *et al.*, 2000) and have been well characterized by Theisen *et al,*

(2005). The crystal structure of the hydroxylase of sMMO has been resolved from *Methylococcus capsulatus* Bath (Rosenzweig *et al.*, 1993) and *Methylosinus trichosporium* OB3b (Elango *et al.*, 1997). sMMO is a multi-component enzyme consisting of a hydroxylase (a dimer of three subunits: $\alpha_2\beta_2\gamma_2$), a coupling protein (MmoB) and a reductase (MmoC).

The *mmoXYBZDC* operon that encodes the different components of sMMO has been cloned and sequenced from *Methylococcus capsulatus* Bath (Stainthorpe *et al.*, 1990), *Methylosinus trichosporium* OB3b (Cardy *et al.*, 1991), *Methylocystis* sp. strain M (McDonald *et al.*, 1997), *Methylocystis* sp. strain W114 (Grosse *et al.*, 1999) and *Methylomonas* sp. strains KSPIII and KSWIII (Shigematsu *et al.*, 1999) and *Methylocella silvestris* BL2 (Theisen *et al.*, 2005) . *mmoX*, *Y* and *Z* encode the α (61 kDa), β (45 kDA) and γ (20 kDa) subunits of the hydroxylase, respectively. *mmoB* encodes the coupling protein B, *mmoC* encodes the reductase and *mmoD* encodes a polypeptide of uncertain function. *mmoD* is the least conserved gene in the sMMO gene cluster. Merkx and Lippard (2002) proposed that MmoD may play an important role in the assembly of the diiron centre. In some methanotrophs, *mmoR* and *mmoG* have been identified either upstream (5′) or downstream (3′) of *mmo*XYBZDC cluster. *mmoR* encodes a σ^{54}-dependent transcriptional regulator MmoR and *mmoG* encodes a homolog of the chaperone GroEL (Csáki *et al.*, 2003; Stafford *et al.*, 2003). All the genes in the *mmoXYBZDC* operon are under the control of a σ^{54} promoter. In *Methylosinus trichosporium* OB3b, both MmoR and MmoG are required for the transcription of

sMMO (Stafford *et al.*, 2003; Scanlan *et al.*, 2009). Unlike pMMO, sMMO is only present in one copy, however a single duplication of *mmoX* has been found in *Methylosinus sporium* (Ali *et al.*, 2006). The organization of *mmoXYBZDC* gene clusters and *mmoR* and *mmoG* from several methanotrophs is presented in **Figure 1.6.**

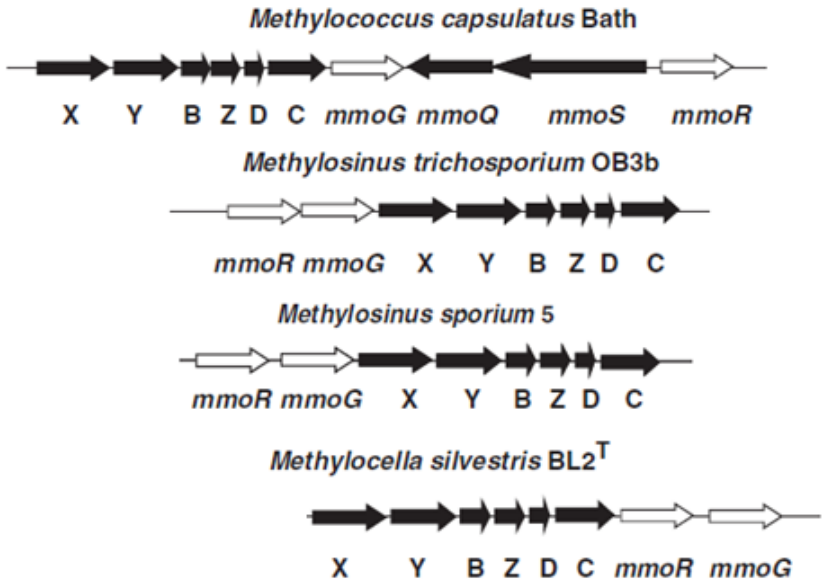


Figure 1.6. Gene organization of sMMO gene clusters in methanotrophs. *Methylococcus capsulatus* Bath (Accession No: AE017282), *Methylosinus trichosporium* OB3b (accession no: X55394), *Methylosinus sporium* (accession no: DQ386732) and *Methylocella silvestris* BL2 (accession no: AM072757). Taken from Scanlan *et al.* (2009).

1.5 Techniques for studying methanotrophs

1.5.1 Enrichment, isolation and cultivation

Traditionally methanotrophs have been studied from a variety of environments in laboratory using cultivation-based strategies. Both nitrate mineral salts and ammonium nitrate mineral salts (Whittenbury *et al.*, 1970) were used for enrichment and isolation of methanotrophic bacteria. From time to time, modifications to the conventional culturing methods, such as alteration of concentrations of mineral salts or pH of the medium were made to isolate particular types of methanotrophs. For example, dilution of the concentration of the mineral salts and lowering the pH of the medium contributed to the isolation of methanotrophs *Methylocella* and *Methylocapsa*, which prefer to grow on acidic medium and do not tolerate high mineral salts concentrations (Dedysh *et al.*, 2002; Dunfield *et al.*, 2003). Altering the pH of the medium and growth temperature also allowed the isolation of acidophilic thermophilic Verrucomicrobial methanotrophs (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008).

1.5.2 Cultivation-independent methods for studying methanotrophs

It has been estimated that the vast majority of microbes (>99%) in the natural environment are currently unculturable using traditional culture-dependent approaches (Amann *et al.*, 1995; Felske *et al.*, 1999; Huang *et al.*, 2009b). The media used to isolate microorganisms often introduceing a selection bias (Gray & Head, 2001). Methanotrophs are difficult to culture. But to overcome the problems associated with their isolations,

several cultivation-independent methods have been developed to study these microorganisms, including methanotrophs present in the environment (reviewed in Murrell & Radajewski, 2000; McDonald *et al.*, 2008; Zengler, 2009).

1.5.2.1 16S rRNA gene-based methods

Due to the presence of large numbers of 16S rRNA gene sequences in the public database and its conserved nature, the 16S rRNA gene has been targeted as the marker for detecting methanotrophs present in the environment. Primers and probes targeting different genera or species of methanotrophs have been designed, validated and used comprehensively in combination with PCR-based clone library analyses, denaturing gradient gel electrophoresis (DGGE) analysis and fluorescent *in situ* hybridisation (FISH) analysis to study the methanotrophs (**Table 1.2 and 1.3**). However, none of these primers or probes can cover the entire methanotroph community, because of the presence of highly diverse methanotrophs in both the *Alphaproteobacteria* and *Gammaproteobacteria.* Moreover, continuous discovery of novel methanotrophs demands continuous updating of these primers and probes. One of the major drawbacks with the use of 16S rRNA gene targeting methods to study methanotrophs is that some of these primers target 16S rRNA genes of non-methanotrophs. Therefore, more attention in interpreting the results obtained with these primers is required.

Table 1.2. 16S rRNA-targeted oligonucleotide probes that can be used in FISH for the detection of methanotrophic bacteria (taken from Dedysh, 2009).

Probe	Probe sequences (5′ - 3′)	Specificity	References
MG-64	CCGAAGGCCTRTTACCGTTC	Type I methanotrophs	(Bourne *et al.*, 2000)
MA-221	GGACGCGGGCCGATCTTTCG	*Methylosinus* spp., some *Methylocystis* spp.	(Bourne *et al.*, 2000)
Mc-1029	CCTGTGTCTTGGCTCCCGAA	*Methylococcus* spp.	(Bourne *et al.*, 2000)
M-450	ATCCAGGTACCGTCATTATC	Type II methanotrophs	(Eller *et al.*, 2001)
M-84	CCACTCGTCAGCGCCCGA	Type I methanotrophs	(Eller *et al.*, 2001)
M-705	CTGGTGGTTCCTTCAGATC	Type I methanotrophs	(Eller *et al.*, 2001)
Mcell-1026	GTTCTCGCCACCCGAAGT	*Methylocella palustris*	(Dedysh *et al.*, 2001)
Mcaps-1032	CACCTGTGTCCCTGGCTC	*Methylocapsa acidiphila*	(Dedysh *et al.*, 2001)
Mcells-1024	TCCGGCCAGCCTAACTGA	*Methylocella silvestris*	(Dedysh *et al.*, 2005)
Mcells-1445	GCCTCTCTCCTTGCGGTT	*Methylocella silvestris, Methylocella palustris*	(Dedysh *et al.*, 2005)
Mcellt-143	TTCCCCGAGTTGTTCCGA	*Methylocella tundrae*	(Dedysh *et al.*, 2004)
Msint-1268	TGGAGATTTGCTCCGGGT	*Methylosinus trichosporium*	(Dedysh *et al.*, 2003)
Msins-647	TCTCCCGGACTCTAGACC	*Methylosinus sporium*	(Dedysh *et al.*, 2003)
Mcyst-1432	CGGTTGGCGAAACGCCTT	*Methylocystis* spp.	(Dedysh *et al.*, 2003)
Mcyst-1261	TTGCTCGAGGTCGCCCTT	Peat-inhabiting subgroup of *Methylocystis* spp.	(Dedysh *et al.*, 2003)

Table 1.3. 16S rRNA gene probes targeting methanotrophs. Adapted from Chen (2008d).

Name	Sequence (5′ - 3′)	Target	References
Type I methanotroph probes			
10γ	GGTCCGAAGATCCCCCGCTT	RuMP pathway methylotrophs	(Tsien *et al.*, 1990)
1035-RuMP	GATTCTCTGGATGTCAAGGG	RuMP pathway methanotrophs	(Brusseau *et al.*, 1994)
Mb1007[a]	CACTCTACGATCTCTCACAG	*Methylobacter* (*Methylomicrobium*)[a]	(Holmes *et al.*, 1995b)
Mc1005	CCGCATCTCTGCAGGAT	*Methylococcus*	(Holmes *et al.*, 1995b)
Mm1007	CACTCCGCTATCTCTAACAG	*Methylomonas*	(Holmes *et al.*, 1995b)
MethT1dF	CCTTCGGGMGCYGACGAGT	Type I methanotrophs	(Wise *et al.*, 1999)
MethT1bR	GATTCYMTGSATGTCAAGG	Type I methanotrophs	(Wise *et al.*, 1999)
Type 1b	GTCAGCGCCCGAAGGCCT	Type I methanotrophs	(Auman *et al.*, 2000)
Gm633	AGTTACCCAGTATCAAATGC	*Methylobacter* and *Methylomicrobium*	(Gulledge *et al.*, 2001)
Gm705[c]	CTGGTGTTCCTTCAGATC	γ-methanotrophs except *Methylocaldum*	(Gulledge *et al.*, 2001)
Mlb482	GGTGCTTCTTCTAAAGGTAATGT	*Methylobacter*	(Gulledge *et al.*, 2001)
Mlb662[d]	CCTGAAATTCCACTCTCCTCTA	*Methylobacter*	(Gulledge *et al.*, 2001)
Mmb482	GGTGCTTCTTCTATAGGTAATGT	*Methylomicrobium*	(Gulledge *et al.*, 2001)
Mlm482	GGTGCTTCTTGTATAGGTAATGT	*Methylomonas*	(Gulledge *et al.*, 2001)
Mlm732a	GTTTTAGTCCAGGGAGCCG	*Methylomonas*	(Gulledge *et al.*, 2001)
Mlm732b	GTTTGAGTCCAGGGAGCCG	*Methylomonas*	(Gulledge *et al.*, 2001)
Mlc123	CACAACAAGGCAGATTCCTACG	*Methylococcus*	(Gulledge *et al.*, 2001)
Mlc1436	CCCTCCTTGCGGTTAGACTACCTA	*Methylococcus*	(Gulledge *et al.*, 2001)
Mcd77	GCCACCCACCGGTTACCCGGC	*Methylocaldum*	(Gulledge *et al.*, 2001)
Mγ84	CCACTCGTCAGCGCCCGA	Type I methanotrophs	(Eller *et al.*, 2001)
Mγ669[d]	GCTACACCTGAAATTCCACTC	*Methylobacter* and *Methylomonas*	(Eller *et al.*, 2001)
Mγ983	TGGATGTCAAGGGTAGGT	Type I methanotrophs	(Eller *et al.*, 2001)
Mγ993	ACAGATTCTCTGGATGTC	Type I methanotrophs	(Eller *et al.*, 2001)
Mγ1004[a]	TACGATCTCTCACAGATT	*Methylomicrobium*	(Eller *et al.*, 2001)
Mh996r	CACTCTACTATCTCTAACGG	*Methylosphaera*	(Kalyuzhnaya *et al.*, 2002)
Type IF	ATGCTTAACACATGCAAGTCGAACG	Type I methanotrophs	(Chen *et al.*, 2007)
Type IR	CCACTGGTGTTCCTTCMGAT	Type I methanotrophs	(Chen *et al.*, 2007)

Type II methanotroph probes			
9α	CCCTGAGTTATTCCGAAC	Serine pathway methylotrophs	(Tsien *et al.,* 1990)
1034-Ser	CCATACCGGACATGTCAAAAGC	Serine pathway methanotrophs	(Brusseau *et al.,* 1994)
Ms1020	CCCTTGCGGAAGGAAGTC	*Methylosinus*	(Holmes *et al.,* 1995b)
Type 2b	CATACCGGRCATGTCAAAAGC	Type II methanotrophs	(Costello & Lidstrom 1999)
MethT2R	CATCTCTGRCSAYCATACCGG	Type II methanotrophs	(Wise *et al.,* 1999)
Am455[b]	CTTATCCAGGTACCGTCATTATCGTCCC	Alphaproteobacterial methanotrophs	(Gulledge *et al.,* 2001)
Am976	GTCAAAAGCTGGTAAGGTTC	Alphaproteobacterial methanotrophs	(Gulledge *et al.,* 2001)
Mα464	TTATCCAGGTACCGTCATTA	Type II methanotrophs	(Eller *et al.,* 2001)
Mcell-1026	GTTCTCGCCACCCGAAGT	*Methylocella palustris*	(Dedysh *et al.,* 2001)
AcidM-181	TCTTTCTCCTTGCGGACG	*Methylocella palustris* and *Methylocapsa acidiphila*	(Dedysh *et al.,* 2001)
Mcaps-1032	CACCTGTGTCCCTGGCTC	*Methylocapsa acidiphila*	(Dedysh *et al.,* 2003)
Msint-1268	TGGAGATTTGCTCCGGGT	*Methylosinus trichosporium*	(Dedysh *et al.,* 2003)
Msins-647	TCTCCCGGACTCTAGACC	*Methylosinus sporium*	(Dedysh *et al.,* 2003)
Mcyst-1432	CGGTTGGCGAAACGCCTT	All *Methylocystis* spp.	(Dedysh *et al.,* 2003)
Mcell-1024	TCCGGCCAGCCTAACTGA	*Methylocella silvestris*	(Dedysh *et al.,* 2005)
Mcell-1445	GCCTCTCTCCTTGCGGTT	*Methylocella silvestris, Methylocella palustris*	(Dedysh *et al.,* 2005)
Type IIF	GGGAMGATAATGACGGTACCWGGA	Type II methanotrophs	(Chen *et al.,* 2007)
Type IIR	GTCAARAGCTGGTAAGGTTC	Type II methanotrophs	(Chen *et al.,* 2007)

[a]Also called Mmb1007 and primer Mγ1004 has an identical 15 bp overlap with Mb1007; [b]Primer Mα450 is identical to Am455; [c]Primer Mγ705 is identical to Gm705; [d]Primer Mγ669 has an identical 15 bp overlap with Mlb662.

1.5.2.2 Functional gene-based methods

To minimize the problems associated with the application of 16S rRNA gene primers, such as the amplification of non-specific targets, PCR primers have been developed to detect methanotrophs by targeting their functional genes such as *mmoX* (**Table 1.4**), *pmoA* (**Table 1.5**), *mxaF* (encoding the large subunit of methanol dehydrogenase) (**Table 1.6**) and *nifH* (encoding the nitrogenase reductase) (Auman *et al.,* 2001). Use of functional marker genes has a clear advantage of being exclusively specific for the particular functional group of organisms and thus allows detection of putative uncultivated members of this group based on the presence of a homologous gene sequence (McDonald *et al.,* 2008). Among these functional genes, PCR primers targeting *pmoA* have been widely used to target methanotrophs including the filamentous methanotrophs *Crenothrix polyspora* (Stoecker *et al.,* 2006) and "*Clonothrix fusca*" (Vigliotta *et al.,* 2007) and Verrucomicrobial methanotrophs (Dunfield *et al.,* 2007; Pol *et al.,* 2007) as well as the high affinity atmospheric methane oxidizers (Holmes *et al.,* 1999; Kolb *et al.,* 2005). However, these primers could not be applied to study facultative methanotrophs of the genus *Methylocella*, since *Methylocella* spp. do not contain pMMO (Dedysh *et al.,* 2005; Theisen *et al.,* 2005). In addition to *pmoA*-targeting primers, *mmoX* gene-specific primers have also been developed, validated and used to detect methanotrophs present in various environmental samples. Among these, primer set 206f/886r (Hutchens *et al.,* 2004) was reported to have broader target specificity compared to other *mmoX* primers. However, these primers should be used in combination with either 16S rRNA or *pmoA* primers to study the diversity of methanotrophs in the

environment, because the data set of *mmoX* sequences is relatively small. Moreover, only a few methanotrophs contain sMMO. Primer sets targeting *mxaF*, which is present in methanotrophs, and nearly all Gram negative methylotrophs, have also been applied to study the diversity of methanotrophs (**Table 1.6**). However, these primers does not detect some methylotrophic strains *e.g.*, *Methyloversatilis* (Kalyuzhnaya *et al.*, 2006) and *Methylibium* (Kane *et al.*, 2007), because they lack a conventional methanol dehydrogenase and have an alternative methanol oxidation system (Kalyuzhnaya *et al.*, 2006).

Other functional genes such as *nifH* (encoding dinitrogen reductase, a key component of the nitrogenase enzyme complex) (Auman *et al.*, 2001; Dedysh *et al.*, 2004) and *fhcD* (encoding the D subunit of the formyl transferase/hydrolase complex, part of the H_4MPT-linked C_1-transfer pathway) (Kalyuzhnaya *et al.*, 2004) have been used to detect methanotrophs in environmental samples. The advantages and disadvantages of different 16S rRNA gene and functional gene based primers designed to detect methanotrophs in the environment have been reviewed in detail by McDonald *et al.* (2008).

Table 1.4. PCR primers used for the amplification of *mmoX* genes from environmental samples. Adapted from Chen (2008d).

Name[a]	**Sequence (5′ - 3′)**	**Product (bp)**	**References**
mmoXf882	GGCTCCAAGTTCAAGGTCGAGC	535	(McDonald *et al.,* 1995)
mmoXr1403	TGGCACTCGTAGCGCTCCGGCTCG		
mmoX1	CGGTCCGCTGTGGAAGGGCATGAAGCGCGT	369	(Miguez *et al.,* 1997)
mmoX2	GGCTCGACCTTGAACTTGGAGCCATACTCG		
536f	CGCTGTGGAAGGGCATGAAGCG	341	(Fuse *et al.,* 1998)
877r	GCTCGACCTTGAACTTGGAGCC		
mmoXr901	TGGGTSAARACSTGGAACCGCTGGGT	396[b]	(Shigematsu *et al.,* 1999)
A166f	ACCAAGGARCARTTCAAG	1230	(Auman *et al.,* 2000)
B1401r	TGGCACTCRTARCGCTC		
534f	CCGCTGTGGAAGGGCATGAA	863	(Horz *et al.,* 2001)
1393r	CACTCGTAGCGCTCCGGCTC		
met1	ACCAAGGAGCAGTTC		(Baker *et al.,* 2001)
met4	TCCAGAAGGGGTTGTT		
mmoX206f	ATCGCBAARGAATAYGCSCG	719	(Hutchens *et al.,* 2004)
mmoX886r	ACCCANGGCTCGACYTTGAA		
mmoXLF	CCCAATCATCGCTGA AGGAGT	450	(This study)
mmoXLR	GAAGATTGG GGCGGCATC TG		

[a] Primer *mmoX1* was located 2008-2037 and *mmoX2* was located 2347 – 2376. Primers A166f and B1401r are also known as mmoXA and mmoXD. [b] When used in PCR with primer *mmoX1*.

Table 1.5. PCR primers used for the amplification of *pmoA* genes from environmental samples. Adapted Chen (2008d).

Name[a]	Sequence (5′ - 3′)	Product (bp)	References
A189f/	GGNGACTGGGACTTCTGG	525	(Holmes *et al.*, 1995b)
A682r	GAASGCNGAGAAGAASGC		
mb661	CCGGMGCAACGTCYTTACC	510[b]	(Costello & Lidstrom 1999)
pmof1/	GGGGGAACTTCTGGGGITGGAC	330	(Cheng *et al.*, 1999)
pmor	GGGGGRCIACGTCITTACCGAA		
pmof2/pmor	TTCTAYCCDRRCAACTGGCC	178	(Cheng *et al.*, 1999)
pmoA206f/	GGNGACTGGGACTTCTGGATCGACTTCAAGGATCG	530	(Tchawa Yimga *et al.*, 2003)
pmoA703b	GAASGCNGAGAAGAASGCGGCGACCGGAACGACGT		
A650r	ACGTCCTTACCGAAGGT	478[b]	(Bourne *et al.*, 2001)
mb661r_nd	CCGGCGCAACGTCCTTACC	510[b]	(Lin *et al.*, 2005)
*pmoA*for/	TTCTGGGGNTGGACNTAYTTYCC	281	(Steinkamp *et al.*, 2001)
*pmoA*rev	CCNGARTAYATHMGNATGGTNGA		
f326/r643	TGGGGYTGGACCTAYTTCC	358	(Fjellbirkeland *et al.*, 2001)
	CCGGCRCRACGTCCTTACC		
Mb601 R[c]	ACRTAGTGGTAACCTTGYAA	432[b]	(Kolb *et al.*, 2003)
Mc468 R[c]	GCSGTGAACAGGTAGCTGCC	299[b]	(Kolb *et al.*, 2003)
II 223 F[c]/ II646 R[c]	CGTCGTATGTGGCCGAC	444	(Kolb *et al.*, 2003)
	CGTGCCGCGCTCGACCATGYG		
Mcap630	CTCGACGATGCGGAGATATT	461[b]	(Kolb *et al.*, 2003)
Forest675 R[c]	CCYACSACATCCTTACCGAA	506[b]	(Kolb *et al.*, 2003)
mb661 R[c]	GGTAARGACGTTGCNCCGG	491[b]	(Kolb *et al.*, 2003)
USCα-346f	TGGGYGATCCTNGCNC	185 [d]	(Degelmann *et al.*, 2010)

[a] Primer A189f is also known as A189gc.
[b] When used in PCR with primer A189f.
[c] These primers were designed for real-time PCR quantification of subsets of methanotrophs.
[d] When used in PCR with primer A682r.

Table 1.6. PCR primer sets for the amplification of *mxaF* genes from environmental samples. Taken from Chen (2008d).

Name	Sequence (5′ - 3′)	Product (bp)	References
1003F/	GCGGCACCAACTGGGGCTGGT	550	(McDonald & Murrell, 1997)
1561R	GGGCAGCATGAAGGGCTCCC		
mxaF_for/	TGGAACGAGACCATGCGTC	455	(Moosvi *et al.*, 2005)
mxaF_rev	CATGCAGATGTGGTTGATGC		
mxaF-f769	TGGGAGGGCGAYGCCTGGAA[b]		(Dedysh *et al.*, 2005)
mxaF-1392	CTTSGGGCCCGGATACATG		(Dedysh *et al.*, 2005)
mxaF-1585	CTTCCASAGNAGKTCRCCNGTGTC		(Dedysh *et al.*, 2005)
mxaF-1690	CCCGGCCARCCGCCGAC		(Dedysh *et al.*, 2005)
1555R	CATGAABGGCTCCCARTCCAT	544[a]	(Neufeld *et al.*, 2007a)

[a] When used with primer 1003F.

[b] mxaf-f769/1561r were used to amplify an *mxaF* fragment from *Albibacter methylovorans* (Dedysh *et al.*, 2005).

1.5.2.3 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a powerful technique that allows rapid profiling of dominant members of microbial communities, microbial diversity and microbial community dynamics based on the separation of double stranded PCR products of similar length, but different sequence composition (Muyzer *et al.*, 1993). The technique also permits one to analyze multiple samples simultaneously in a short period of time. Several studies have developed primers and protocols to selectively amplify and analyze by DGGE methanotroph-specific 16S rRNA genes from the environment (Henckel *et al.*, 1999; Henckel *et al.*, 2000; Eller & Frenzel, 2001; Chen *et al.*, 2007). In addition to 16S rRNA genes, DGGE primers and protocols have been developed to analyze methanotrophs by targeting their functional genes, such as *pmoA* (Henckel *et al.*, 1999; Horz *et al.*, 2001; Knief *et al.*, 2003; Lin *et al.*, 2005), *mmoX* (Iwamoto *et al.*, 2000) and *mxaF* (Henckel *et al.*, 1999; Fjellbirkeland *et al.*, 2001). One of the major concerns in using DGGE with functional genes is that the degenerate primers used to amplify the corresponding gene products may generate duplex bands during electrophoresis. Thus they may not represent the true microbial community profile (McDonald *et al.*, 2008). In addition, low abundance members of the microbial community may not be detected in the DGGE profile.

1.5.2.4 Microbial diagnostic microarrays

This term ‘microbial diagnostic microarray’ in microbial ecology was first introduced by Bodrossy and Sessitsch (2004). A microbial diagnostic microarray allows one to analyze complex gene mixtures in a single assay. The first oligonucleotide microarray applied to microbial ecology utilized nine 16S rRNA gene probes that comprised 15–20 bp

oligonucleotides for the discrimination of nitrifying bacteria (Guschin *et al.*, 1997). Later, Wu *et al*. (2001) developed a functional gene array that targeted functional genes involved in nitrogen cycling, such as *nirS, nirK, amoA* and also *pmoA*. These two studies were the first demonstration of the potential use of diagnostic microarrays to study microbial community composition in the environment. The first microbial diagnostic microarray designed to target methanotrophs was developed by Bodrossy *et al.* (2003). This microarray was spotted with 59 validated oligonucleotide probes (short oligonucleotides *i.e.,* 18 to 27 bp) targeting *pmoA* genes of all known methanotrophs and *amoA* of the ammonia-oxidizing bacteria**.** This microarray has been upgraded and spotted with 138 probes (Stralis-Pavese *et al.*, 2004a). The list of these *pmoA* probes and their specificity to target specific groups of methanotrophs are presented in **Appendix 1.** Subsequently Bodrossy *et al.* (2006) developed a protocol for an mRNA-based *pmoA* microarray to study the active methanotroph composition. A schematic representation of different stages involved in the *pmoA* diagnostic microarray is presented in **Figure 1.7.** From time to time, new *pmoA* sequences retrieved from various novel methanotrophs and environments are being continuously added into this *pmoA* microarray. Several studies have applied this *pmoA* microarray technique to study the diversity of methanotrophs in various environmental samples, such as in landfill cover soil (Stralis-Pavese *et al.*, 2006; Cébron *et al.*, 2007a; Gebert *et al.*, 2008; Héry *et al.*, 2008; Kumaresan *et al.*, 2009), peat soil (Chen *et al.*, 2008b; Chen *et al.*, 2008a), Chinese coal mine soil (Han *et al.*, 2009) and the marine environment (Moussard *et al.*, 2009). *pmoA* microarray analyses provide data on the diversity and semi-quantitative information on the proportion of phylogenetically distinct *pmoA* sequences belonging to different phylogenetic groups of methanotrophs.

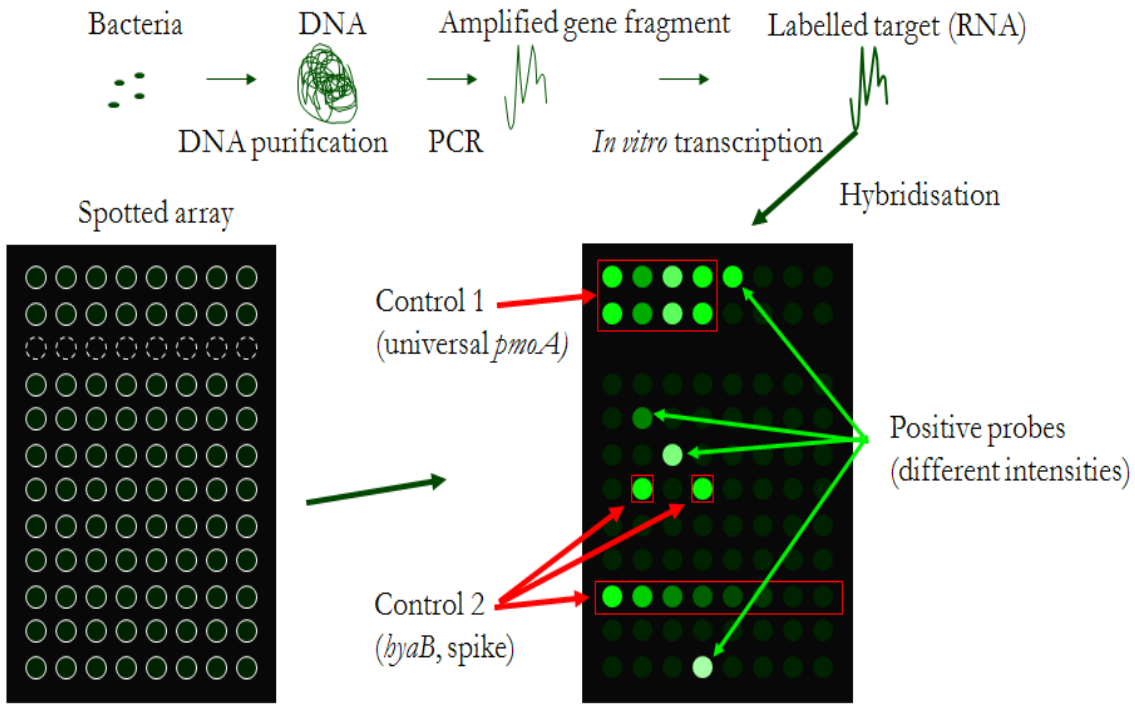


Figure 1.7. Schematic representation of *pmoA* diagnostic microarray protocol. (Levente Bodrossy, *personal communication).*

1.5.2.5 Gene expression microarrays

The DNA microarray technique is one of the most powerful techniques to study whole genome transcriptomics (study of the total RNA of an organism) (Kothapalli *et al.*, 2002). The significant advantage of microarrays over other techniques is that transcription of all genes in an organism can be studied simultaneously in a single experiment (Hinton *et al.*, 2004). This technique has enabled unraveling of the complexities of the host-pathogen interaction, response of an organism to particular substrate or certain type of stimuli and metabolic switch, to the comparative genomics of an organism and genomic diversity (Wu *et al.*, 2008; Hardin *et al.*, 2009; Sauer *et al.*, 2009; Tai *et al.*, 2009; Zhu *et al.*, 2009; Borneman *et al.*, 2010). A gene is usually transcribed only when and where its function is

required (Dorrell *et al.*, 2001; Chalabi *et al.*, 2007; Borneman *et al.*, 2010), therefore, determining the locations and the conditions under which a gene is transcribed allows inferences about its function. However, the disadvantage of the microarray technique is that it does not reflect any possible post-transcriptional events. Moreover, validation of the microarray results by reverse transcription quantitative PCR (RT-qPCR) is required to make the microarray findings meaningful.

A DNA microarray consists of a series of DNA targets (PCR products or oligonucleotide probes) immobilized on a solid substrate. These are then hybridized with fluorescently labelled target made from nucleic acids in the test sample, so as to allow analysis of the relative concentrations of mRNA (Schena *et al.*, 1996). The size of the PCR products may vary from 250 to 500 bp, whereas synthetic oligonucleotide probes are often between 25 to 75 bp in length (Rhodius *et al.*, 2002). In general, when comparing DNA transcription under two different conditions, RNA is prepared from the two samples to be compared (test and control), and labeled cDNA/cRNA is prepared by reverse transcription, incorporating either Cyanine 3 (Cy3) or Cyanine 5 (Cy5) fluorescent dyes. The two labeled cDNAs are mixed and hybridized to the microarray (two colour microarray), and the slide is washed, dried and scanned. In single or one-colour microarrays, the arrays provide intensity data for each probe or probe set, indicating a relative level of hybridization with the labeled target generated from a single condition only. With the use of image analysis software, signal intensities are determined for each dye for each spot of the array, and the logarithm of the ratio of Cy5 intensity to Cy3 intensity is calculated. Finally the data are analyzed by a variety of standard softwares such as, GeneSpring® (Agilent Technologies, USA), to determine the transcription pattern of genes under different conditions. A schematic representation of processes involved in the gene expression microarray is presented in **Figure 1.8**.

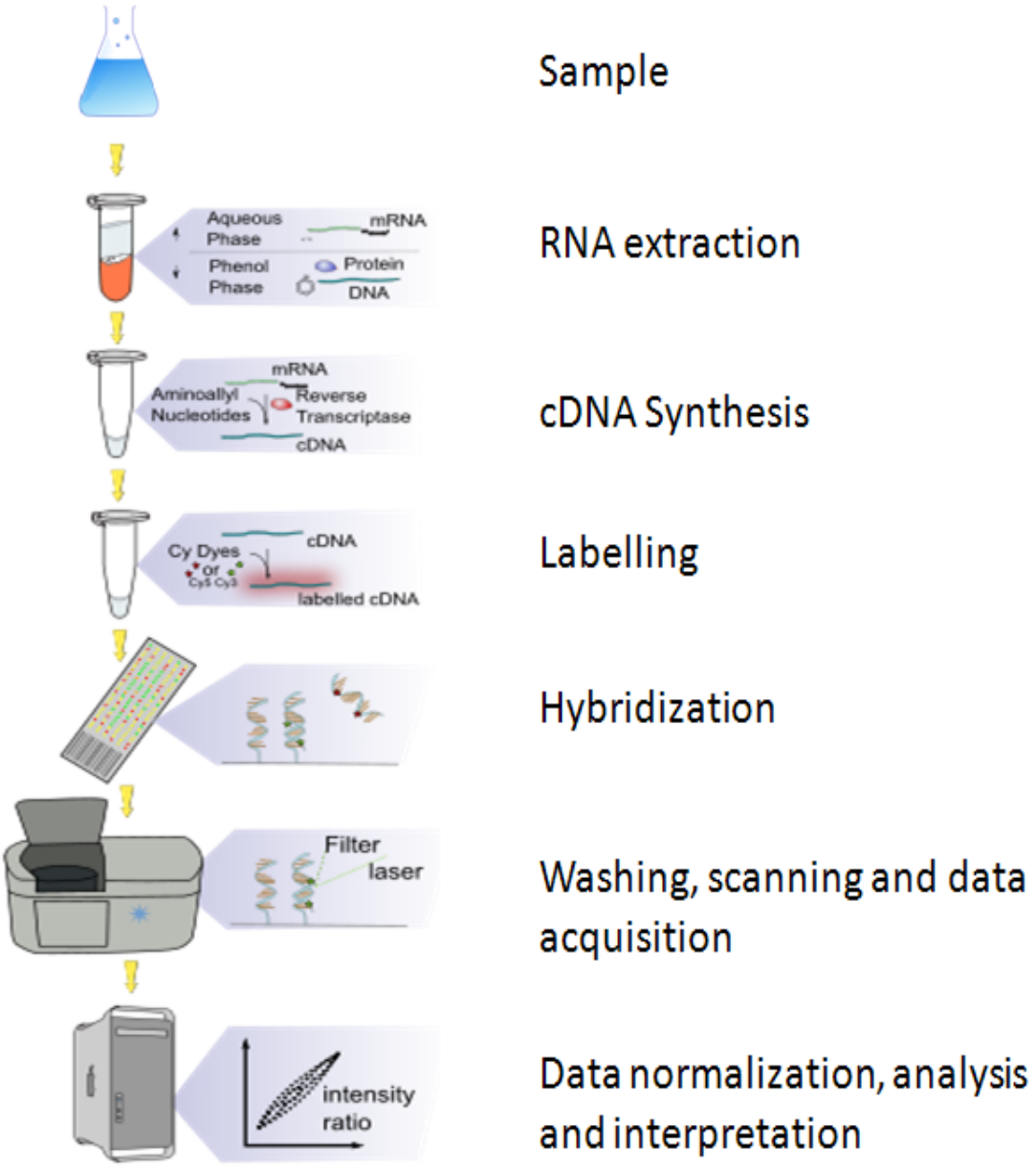


Figure 1.8. Schematic representation of processes involved in gene expression microarray. Adapted from: www.en.wikipedia.org/wiki/DNA_microarray_experiment

Microarray analysis has become a widely used tool for the generation of gene expression data on a genomic scale; however, one limitation is the lack of standards for presenting and exchanging microarray data. To overcome this problem, a scientific standard called

Minimum Information About a Microarray Experiment (MIAME) has been established (Brazma *et al.*, 2001). MIAME describes the minimum information required to ensure that data generated from microarray experiments can be easily interpreted and that results derived from its analysis can be independently verified. The ultimate goal of MIAME is to establish a standard for recording and reporting microarray-based gene expression data, to facilitate the establishment of databases, public repositories and enable the development of data analysis tools.

Microarray data normalization and analysis has been an area of considerable discussion and has been one of many factors that have led to the considerable expansion of the field of bioinformatics (Fundel *et al.*, 2008b; Fundel *et al.*, 2008a). The very first step in microarray data analysis is background correction, where background fluorescence signals are removed from the spot fluorescence signals to have the actual signals coming from the hybridized target cDNA/cRNA. Any probes where the fluorescence signals are below the background are often removed from the analysis. However, in such a case genes expressed at low levels are missed. Data are normalized before analysis so that data obtained from different arrays in a particular experiment can be compared. Normalization compensates for technical differences between chips. There are several ways to normalize, such as using housekeeping genes, the expression of which remains quite stable under different experimental conditions (Reid *et al.*, 2000; Quackenbush, 2002; Jandu *et al.*, 2009). If a housekeeping gene is not available, whole genomic DNA can be used for the purpose, with an assumption that in a whole genome, relatively few genes are expressed and the number of genes transcribed up and down are about equal (Padmanabhan *et al.*, 2003). Since most of the genes remain unchanged, the mean transcript levels should be the same for each condition. Based on this theory, data are often normalized using the “total intensity-based global normalization” where the ratios

of intensity of both (fluorescent dye) channels are made equal across the array grid. In GeneSpring® the data are normalized based on a percentile distribution. In the default normalization settings in GeneSpring®, all the data are ranked according to their intensity. These data are first transformed into $\log_2$, and then a percentile shift to the 75th percentile for each array is carried out. This value is subtracted from all of the values on the array. Percentile normalization is based on the assumption that a certain level of expression values should be equal for all arrays. When the 75th percentile is used, it is assumed that the expression level, below which 75% of the expression values are found, should be the same for all samples. A multiplicative factor is also applied to the data so that the chosen percentile is at the same expression value for all arrays (Fundel *et al.*, 2008b).

Microarray gene expression studies are based on the hypothesis that gene expression changes are 'biologically important' (Kendall *et al.*, 2004). What level of change in the expression of a gene will be considered as biologically important is an issue in interpreting the findings of microarray experiments. The commonly used criterion is that the expression of a gene is considered as biologically significant if the expression ratio is 2 or more (Butcher, 2004). However, whether an expression ratio of less than 2 is biologically insignificant remains a question for most microarray experiments, and is best addressed by statistical analysis, followed by further characterization *in vivo*. Using a figures of "2" or similar as a cut-off arbitrary and is intuitively less useful than ensuring reproducibility of the data by appropriate replication and analysis.

Gene expression microarrays have been applied successfully to compare the whole genome transcriptional profiling of *Azorhizobium caulinodans* ORS571 grown under free-living and symbiotic conditions (Tsukada *et al.*, 2009), the response of *Bacillus subtilis* to salt stress adaptation (Hahne *et al.*, 2010), the effects of carbon dioxide on

growth of *Clostridium botulinum* (Artin *et al.*, 2010), the response of *Listeria monocytogenes* to iron limitation (Ledala *et al.*, 2010) and the response of *Pyrococcus furiosus* grown on carbohydrates or peptides (Schut *et al.*, 2003). The microarray technique has also been applied successfully to study genes transcribed differentially across the whole genome of the methylotroph *Methylibium petroleiphilum* PM1 grown on methyl tert-butyl ether or ethanol (Hristova *et al.*, 2007). In addition, genes transcribed differentially in *Methylobacterium extorquens* AM1 grown on methanol and succinate were also studied by the microarray technique (Okubo *et al.*, 2007).

1.5.2.6 Quantification of methanotrophs

The most probable number (MPN) technique has long been used in the past for quantifying methanotrophs in the environment. However, MPN-based analyses allows one to quantify only the population of methanotrophs that are cultivated in a specific medium. To overcome these problems, cultivation-independent quantitative methods, such as fluorescent *in situ* hybridization (FISH) and real-time quantitative PCR (qPCR) have been used to quantify methanotrophs in environmental samples.

FISH relies on the specific annealing of a fluorescently labeled oligonucleotide probe to its complementary sequence in a fixed microbial sample, which can be visualized using fluorescence or confocal laser scanning microscopy (Delong, 1989). FISH targeting the 16S rRNA genes of methanotrophs has been successfully used to analyze methanotroph communities in the environment (Dedysh *et al.*, 2001; Dedysh *et al.*, 2003; Raghoebarsing *et al.*, 2005). FISH has proved a powerful tool for quantifying the presence and localization of bacteria based on 16S rRNA sequences, but these are dependent upon the availability of the suitable discriminating probes (Amann & Fuchs, 2008). FISH probes available for the detection and quantification of methanotrophs are

presented previously in **Table 1.2.** However, the difficulty in using FISH in soil could be due to high background auto-fluorescence of soil materials and the potential difficulty of low numbers of ribosomes (Rogers *et al.*, 2007).

qPCR is an advanced and sensitive methods to quantify microbial population present in a particular sample. SYBR-green based qPCR assays currently available for quantifying methanotrophs were firstly developed by Kolb *et al.* (2003) targeting *pmoA*. The authors designed six qPCR assays: MBAC, MCOC, TYPEII, MCAP, FOREST and MTOT that target specific groups of methanotrophs. Assay MBAC was designed to target the *Methylobacter/Methylosarcina* group, MCOC for the *Methylococcus* group, TYPEII for the *Methylosinus* group, MCAP for the *Methylocapsa*, FOREST for the forest clones and MTOT for the *Methylobacter/Methylosarcina, Methylococcus, Methylosinus* group, *Methylocapsa, Nitrosococcus* (Kolb *et al.*, 2003). These *pmoA*-targeting qPCR assays have been extensively used for the quantification of methanotrophs from environmental samples (Kolb *et al.*, 2003; Kolb *et al.*, 2005; Knief *et al.*, 2006; Rahalkar & Schink, 2007; Chen *et al.*, 2008a; Colwell *et al.*, 2008; Rahalkar *et al.*, 2009; Vorob'ev *et al.*, 2009). It has been suggested that the *pmoA* primer set (A189F/mb661r) which is commonly used to quantify methanotrophs may underestimate methanotroph populations and hence is not ideal for qPCR (Kolb *et al.*, 2003). Recently Tuomivirta *et al.* (2009) have developed another *pmoA*-targeting SYBR-green based qPCR assay to detect specific groups of *pmoA*-containing methanotrophs that could not be detected by the method developed by Kolb *et al.* (2003). Very recently Degelmann *et al.* (2009) have developed a new SYBR-green based qPCR assay (Q-USCα, Q-C7, Q-AOB) to quantify the abundance of *pmoA* genes of USCα, *pmoA/amoA* genes of cluster 7, and *amoA* genes of ammonium-oxidizing bacteria within the *Betaproteobacteria*.

1.5.2.7 Stable isotope probing

The discovery of the enormous diversity of microorganisms in the environment raises the question: what are the functions of these microorganisms *in situ*? Microorganisms are crucial to the function of all ecosystems and are probably more diverse than any other organisms. Although techniques such as clone library analyses, DGGE, FISH, qPCR and microbial diagnostic microarrays provide information on microorganism abundance, diversity and identity, these techniques do not allow one to identify and study the active organisms contributing to key processes in ecosystems. Recently advances have been made in developing techniques to link microbial diversity with a particular function.

Stable-isotope probing (SIP) is a powerful technique to directly identify microbial populations actively involved in the utilization of a particular substrate *in situ* (Radjewski *et al.*, 2000). The technique is based on the incorporation of a ‘‘heavy’’ isotope (such as ^{13}C, ^{15}N, ^{18}O) into cellular biomarkers such as DNA (Radajewski *et al.*, 2000), RNA (Manefield *et al.*, 2002), PLFA (Boschker *et al.*, 1998) or protein (Jehmlich *et al.*, 2008) by microbes that actively transform it. In SIP, these ‘‘heavy’’ cellular biomarkers are separated from unlabelled “light” cellular biomarkers by density gradient centrifugation. Molecular techniques such as DGGE, clone library analyses and diagnostic microarray are then applied to identify members of the active community that have utilized the labeled substrate (Radjewski *et al.*, 2000) (**Figure 1.9**). **Table 1.7** lists a number of publications using SIP for analyzing a variety of microorganisms involved in different processes.

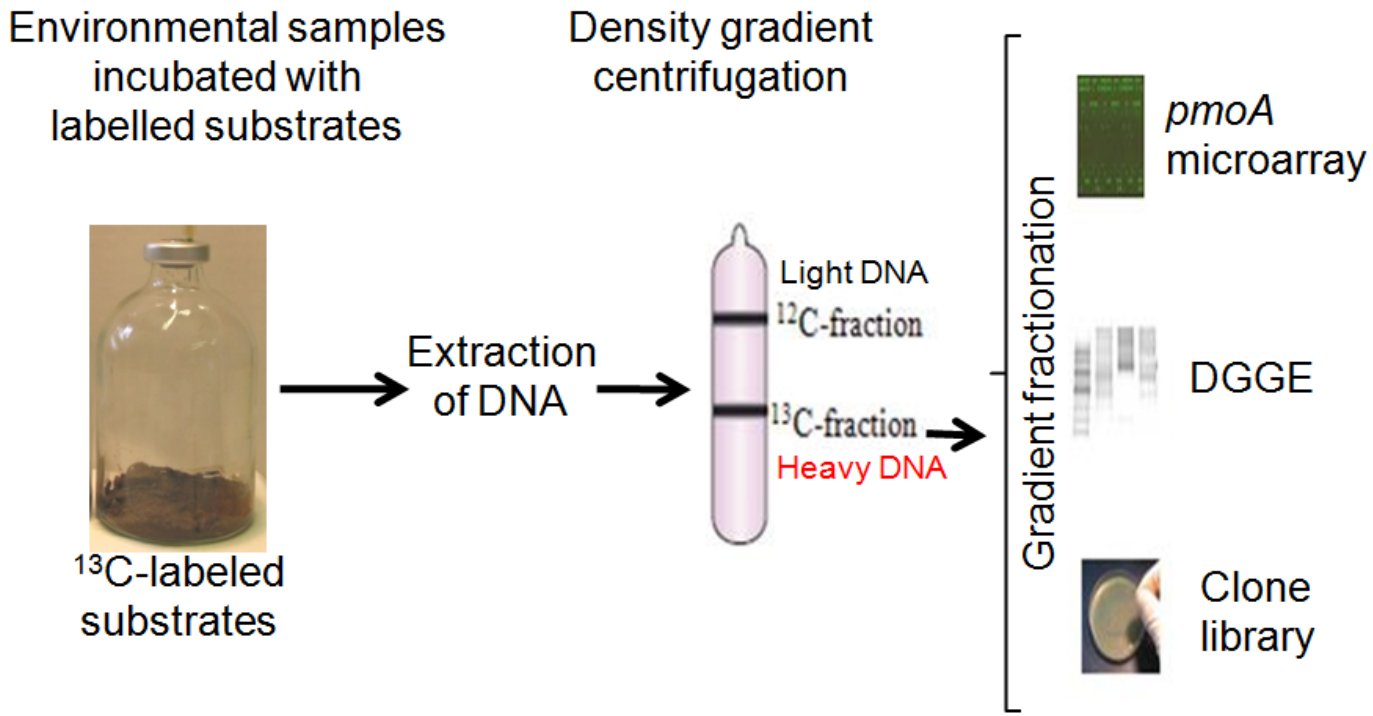


Figure 1.9. Schematic representation of a DNA-stable isotope probing experiment to study active microorganisms.

Table 1.7. Recent studies using DNA/RNA-SIP for identifying active microorganisms from diverse habitats. Adapted from Kumaresan (2009).

Substrate	Habitat	Phylogenetic groups identified	Marker genes	References
$^{3}CH_4$	Rice root	*Methylocystaceae*	*pmoA*	(Qiu *et al.*, 2009)
$^{13}CH_4$	Coal mine soil	*Methylosinus/Methylocystis, Type I methanotrophs related to Methylobacter/Methylosoma and Methylococcus*	16S rRNA; *pmoA*; *mxaF*	(Han *et al.*, 2009)
$^{13}CH_4$	Forest soil	Upland soil cluster-α	*pmoA*	(Bengtson *et al.*, 2009)
$^{13}CH_4$	Landfill soil originally from a peat bog	*Methylobacter*; *Methylomonas*; *Methylocystis*; *Methylocella*	16S rRNA; *pmoA*; *mmoX*	(Cébron *et al.*, 2007b)
$^{13}CH_4$	Landfill soil originally from a peat bog	*Methylobacter*; *Methylomonas*; *Methylocystis*; *Methylocella*	16S rRNA; *pmoA*; *mmoX*	(Cébron *et al.*, 2007a)
$^{13}CH_4$	Landfill soil	*Methylobacter*; *Methylomicrobium*; *Methylocystis*	16S rRNA; *pmoA*	(Cébron *et al.*, 2007a)
$^{13}CH_4$	Rice field soil	*Methylococcaceae*; *Methylocystaceae*; *Lobosea*; *Heterolobosea*; *Colpodea*; *Cercozoa*	16S rRNA; 18S rRNA	(Murase & Frenzel, 2007)
$^{13}CH_4$	Forest soil	*Methylocystis*	16S rRNA, *pmoA*	(Dumont *et al.*, 2006)
$^{13}CH_4$	Cave water and microbial mat	Type I and Type II methanotrophs; *Hyphomicrobium*; *Bdellovibrio*; *Thiobacillus*, *Methylophilus*	16S rRNA; *pmoA*; *mmoX*; *mxaF*	(Hutchens *et al.*, 2004)
$^{13}CH_4$	Soda lake sediment	Type I methanotrophs; *Methylophilaceae*	16S rRNA; *pmoA*; *mmoX*;	(Lin *et al.*, 2004)
$^{13}CH_4$	Peat soil	*Methylosinus/Methylocystis*; RA-14 group; *Methylobacter/Methylomonas*; novel *Bettaproteobacteria*	16S rRNA; *pmoA*; *mmoX*; *mxaF*	(Morris *et al.*, 2002)
$^{13}CH_3OH$	Estuary sediment	*Methylophaga*	16S rRNA	(Moussard, *et al* 2009)
$^{13}CH_3OH$	Coastal sea water	*Methylophaga*	16S rRNA; *mxaF*	(Neufeld *et al.*, 2008)
$^{13}CH_3OH$, ^{13}C-methylamine	Coastal sea water	*Methylophaga*; novel *Gammaproteobacteria*	16S rRNA; *mxaF*	(Neufeld *et al.*, 2007a)
$^{13}CH_3OH$	Activated sludge	*Methylophilaceae*; *Hyphomicrobiaceae*	16S rRNA; *nirS*; *nirK*	(Osaka *et al.*, 2006)
^{13}C-labelled methanol, methylamine, formaldehyde,	Lake sediment	*Methylophylaceae*; *Sphingomonadales Methylophylaceae*; *Methylophylaceae*; *Holophaga /Geothrix*	16S rRNA; *pmoA*; *fae*	(Nercessian *et al.*, 2005)

formate		*Xanthomonadaceae*; *Holophaga /Geothrix*; *Gemmatimonadetes*		
$^{13}CH_3OH$	Rice field soil	*Methylobacterium*; *Methylophilaceae*	16S rRNA	(Lueders *et al.*, 2004b)
$^{13}CH_3OH$	Active sludge	*Methylophilaceae*	16S rRNA	(Ginige *et al.*, 2004)
$^{13}CH_3OH$	Forest soil	*Methylocella*; *Methylocapsa*; *Methylocystis*; *Rhodoblastus*; *Acidobacterium*	16S rRNA; *mxaF*	(Radajewski *et al.*, 2002)
$^{13}CH_3OH$	Forest soil	*Methylocella/Methylocapsa*; *Acidobacterium*-related	16S rRNA; *mxaF*	(Radajewski *et al.*, 2000)
$^{13}CH_3Cl$	Soil	*Hyphomicrobium*; *Aminobacter*	*cmuA*	(Borodina *et al.*, 2005)
$^{13}CH_3Br$	Soil	*Burkholderia*	16S rRNA; *cmuA*	(Miller *et al.*, 2004)
$^{13}CH_3Cl$	Soil	*Rhodobacter*; *Lysobacter*; *Nocardioides*	16S rRNA; *cmuA*	(Miller *et al.*, 2004)
$^{13}K_2CO_3$	Lake sediment	*Nitrosomonas*	16S rRNA	(Whitby *et al.*, 2001)
$^{13}HCO_3^-$	Water sediment	*Nitrosomonas*; *Nitrospira*	16S rRNA	(Freitag *et al.*, 2006)
$^{15}N_2$	Soil	*Rhizobiales*; *Methylosinus*; *Methylocystis*; novel bacteria	*nifH*	(Buckley *et al.*, 2007a)
$^{15}N_2$	Soil	*Rhizobiales*; *Actinobacteria*; *Alphaproteobacteria*	16S rRNA, *nifH*	(Buckley *et al.*, 2007b)
$^{13}CO_2$	Plant root	Extremely diverse Bacteria and Fungi	16S rRNA; 18S rRNA	(Vandenkoornhuyse *et al.*, 2007)
$^{13}CO_2$	Rhizosphere soil with *Festuca rubra* *Crepis arenaria*	*Pseudomonas, Burkholderia*	16S rRNA	(Drigo *et al.*, 2010)
$^{13}CO_2$	Potatoes plant	*Acinetobacter* and *Acidovorax*	16S rRNA	(Rasche *et al.*, 2008)
$^{13}CO_2$	Rice root	*Azospirillum*; *Burkholderiaceae*; *Clostridim*; *Comamonas*	16S rRNA	(Lu *et al.*, 2006)
$^{13}CO_2$	Rice soil	Rice cluster-1 *Archaea*; *Methanosarcineae*; *Methanomicrobiaceae*; *Methanosaetaceae*	16S rRNA	(Lu & Conrad, 2005)
$^{13}CO_2$	Upland grassland soil	*Sphingomonas*; *Mycobacterium*; *Sistotrema*; *Rhodotorula*	16S rRNA	(Rangel-Castro *et al.*, 2005)
^{13}C-acetate	Estuary sediment	*Epsilonproteobacteria, Gammaproteobacteria* , Crenarchaeotic Group *Archaea.*	16S rRNA	(Webster *et al.*, 2010)
^{13}C-acetate	Activated sludge	Peritrich ciliate *Epistylis galea*	18S rRNA	(Moreno *et al.*, 2010)
^{13}C-acetate	Groundwater	*Proteobacteria,* Firmicutes	16S rRNA	(Longnecker *et al.*, 2009)

^{13}C-acetate	Lake sediment	*Methanosaeta concilii*; *Rhodocyclales; Nitrosomonadales; 'Magnetobacterium bavaricum, Thermodesulfovibrio yellowstonii*	16S rRNA	(Schwarz *et al.*, 2007b)
^{13}C-acetate	Rice field soil	*Geobacter*; *Anaeromyxobacter*	16S rRNA	(Hori *et al.*, 2007)
^{13}C-acetate	Arsenic contaminated aquifer sediments	*Sulfurospirillum*; *Desulfotomaculum*; *Geobacter*	16S rRNA; *arrA*	(Lear *et al.*, 2007)
^{13}C-acetate	Soil	*Syntrophus*; *Propionibacterium*; *Geobacter*; *Methanosaeta*; *Methanosarcina*	16S rRNA	(Chauhan & Ogram, 2006)
^{13}C-acetate	Activated sludge	*Comamonadaceae*; *Rhodocyclaceae*; *Rhodobacteraceae*	16S rRNA; *nirS*; *nirK*	(Osaka *et al.*, 2006)
^{13}C-acetate	Activated sludge	*Comamonadaceae*; *Rhodocyclaceae*	16S rRNA	(Ginige *et al.*, 2005)
^{13}C-pyrene	PAH[a]-contaminated soil	Uncultivated *Gammaproteobacteria*	16S rRNA	(Jones *et al.*, 2008)
^{13}C-acetate + perchloroethene	Pristine river sediment	*Dehalococcoides*	16S rRNA	(Kittelmann & Friedrich, 2008)
^{13}C-polychlorinated biphenyls	Pine tree soil	*Pseudonocardia*; *Kirbella*; *Nocardiodes*; *Sphingomonas*	16S rRNA; ARHD[b]	(Leigh *et al.*, 2007)
^{13}C-biphenyl	PCB-contaminated river sediment	*Achromobacter, Pseudomonas*	16S rRNA, *bphA*	(Sul *et al.*, 2009)
^{13}C-phenanthrene,^{13}C-pyrene	PAH-contaminated soil	*Acidovorax*	16S rRNA	(Singleton *et al.*, 2007)
^{13}C-labelled 2,4-dichlorophenoxyacetic acid	Agriculture soil	*Bettaproteobacteria* related to *Ramlibacter* (*Comamonadaceae*)	16S rRNA	(Cupples & Sims, 2007)
$^{13}C_6$-benzene	Gasoline Contaminated groundwater	*Azoarcus*	16S rRNA	(Kasai *et al.*, 2006)
$^{13}C_6$-benzene	Benzene-degrading sulfidogenic consortium enrichment	An uncultivated bacterium from the family *Desulfobacteraceae*	16S rRNA	(Oka *et al.*, 2008)
$^{13}C_6$-benzene	Anaerobic benzene-degrading enrichment culture	C*ryptanaerobacter, Pelotomaculum,* uncultivated *Epsilonproteobacteria*	16S rRNA	(Herrmann *et al.*, 2009)

$^{12}C_6$ salicylate; ^{13}C naphthalene phenanthrene	Bioreactor treating PAH-contaminated soil	*Acidovorax*.; *Pseudomonas*; *Ralstonia*	16S rRNA	(Singleton *et al.*, 2005)
^{13}C-labelled naphthalene and glucose	Soil	*Acidovoras*; *Pseudomonas*; *Intrasporangium*	16S rRNA	(Yu & Chu, 2005)
^{13}C-labelled glucose	Estuary sediment	*Gammaproteobacteria*, Marine Group 1 *Archaea*	16S rRNA	(Webster *et al.*, 2010)
^{13}C-labelled glucose	Soil	*Bacillus subtilis*	16S rRNA	(Nicholson *et al.*, 2009)
^{13}C-phenol	Activated sludge	*Acidovorax*	16S rRNA	(Manefield *et al.*, 2005)
^{13}C-pentachlorophenol	Pristine grassland soil	*Pseudomonas*; *Burkholderia*; *Sphingomonas*	16S rRNA	(Mahmood *et al.*, 2005)
^{13}C-naphthalene	Ground water	*Pseudomonas fluorescens, Pseudomonas putida, Acidovorax* sp.	16S rRNA, naphthalene dioxygenase gene	(Huang *et al.*, 2009b)
$^{13}C_6$ naphthalene	Coal tar waste contaminated aquifer	*Polaromonas naphthalenivorans*	16S rRNA	(Jeon *et al.*, 2003)
^{13}C-phenol	Soil	*Pseudomonas, Pantoea, Acinetobacter, Enterobacter, Stenotrophomonas*; *Alcaligenes*	16S rRNA	(Padmanabhan *et al.*, 2003)
$^{13}C_6$ naphthalene		*Pseudomonas, Acinetobacter*; *Variovorax*		
^{13}C-caffeine		*Acinetobacter, Enterobacter, Stenotrophomonas*; *Pantoea*		
^{13}C-phenol	Bioreactor	*Thauera*	16S rRNA	(Manefield *et al.*, 2002)
$^{13}C_6$-toluene	Oil-contaminated aquifer sediment	*Clostridium*	16S rRNA	(Winderl *et al.*, 2010)
Ring-$^{13}C_6$-toluene	Agriculture soil	*Candidate phylum* TM7	16S rRNA	(Luo *et al.*, 2009)
^{13}C-cellulose	Soil	*Dyella, Mesorhizobium, Sphingomonas, Myxobacteria*	16S rRNA	(Haichar *et al.*, 2007)
^{13}C-cellulose	Municipal soil waste	*Firmicutes, Bacteroidetes, Gammaproteobacteria*	16S rRNA	(Li *et al.*, 2009)
^{13}C-labelled wheat residue	Copper contaminated soil	*Betaproteobacteria*	16S rRNA	(Bernard *et al.*, 2007; Bernard *et al.*, 2009)

[a] Polycyclic aromatic hydrocarbon
[b] Aromatic ring hydroxylating dioxygenase.

SIP experiments were first carried out to detect ^{13}C-labelled PLFAs in methane- and acetate-utilizing bacteria from lake sediments (Boschker *et al.*, 1998). Subsequently, Radjewski *et al.* (2000) developed the DNA-SIP technique to identify methanol utilizing bacteria in a forest soil. The method offers access to genomic information of novel organisms involved in a particular ecosystem function (Dumont *et al.*, 2006; Neufeld *et al.*, 2008). DNA-SIP experiments often involves the use of high concentrations of labeled substrate to ensure sufficient incorporation of labeled substrate into cellular DNA for the efficient separation of the ‘‘heavy’’ DNA from the background “light” DNA. The use of high concentrations of labeled substrate in DNA-SIP experiments has also been justified by other studies previously (Lueders *et al.*, 2004a; Schwarz *et al.*, 2007a). A few studies have detected “cross-feeding” in the DNA-SIP experiments resulting from the long incubation times with labeled substrates (Hutchens *et al.* 2004; Cébron *et al.*,2007). This problem has been largely overcome by carrying out SIP experiments for short incubation times and with low concentrations of substrates (Cadisch *et al.*, 2005; Chen *et al.*, 2008c; Neufeld *et al.*, 2008).

SIP experiments have been also carried out with RNA. RNA-SIP has a greater sensitivity than DNA-SIP, because RNA synthesis occurs at a faster rate than DNA synthesis (Manefield *et al.*, 2002; Lueders *et al.*, 2004b; Whiteley *et al.*, 2006). Moreover, cells do not need to divide which is a requirement for the DNA-SIP technique for incorporation of stable isotope into DNA. RNA is always synthesized, even in non-dividing cells, to maintain cellular activity. RNA-SIP has been used

extensively to study active bacterial populations in the environment (**Table 1.7**). Since in the RNA-SIP technique, very little isotopes is needed to be incorporated, near *in situ* concentrations of substrate can be used in RNA-SIP experiments, thus allowing one to minimize potential bias caused by high substrate concentrations, such as during earlier DNA-SIP experiments (Radajewski *et al.*, 2000; 2002).

Unlike the DNA-SIP, in RNA-SIP experiments only the 16S rRNA gene-based analysis can be performed, until the recent pioneering work by Huang *et al.* (2009). In this study the authors demonstrated the application of mRNA-SIP and detected *Pseudomonas fluorescens*, *Pseudomonas putida* and *Acidovorax* sp. as the active aerobic naphthalene-degrading bacterial species in groundwater. These authors were able to detect the mRNA of a functional gene encoding the γ-subunits of PAH dioxygenases by RT-PCR from the ^{13}C-labelled mRNA, thus providing a robust method to link the microbial community to its functions.

^{15}N-labelled substrates have also been used in SIP studies that allowed the identification of microbial communities actively involved in the metabolism of various N-compounds (Cadisch *et al.*, 2005; Buckley *et al.*, 2007a, b; Wawrik *et al.*, 2009). As the separation of DNA bands between ^{15}N-DNA and ^{14}N-DNA is only 4 mm compared to the 10 mm separation obtained with ^{13}C substrates, a second round of centrifugation was used for effective separation of the bands (Buckley *et al.*, 2007a, b). However, the limitations of the use of ^{15}N-labelled substrates in DNA-SIP is the requirement of relatively large quantities of DNA to visualize bands (Cadisch

et al., 2005). ^{18}O-labeled H_2O has been applied in *Escherichia coli* and soil to detect actively growing microorganisms and efficient separation of ^{18}O-labeled DNA from the background has been demonstrated (Schwartz, 2007).

1.6 Genome sequencing of *Methylocella silvestris* BL2

The whole genome of *Methylocella silvestris* BL2, the first validated facultative methanotroph has recently been sequenced by the Joint Genome Institute (GenBank accession number CP001280) (Chen *et al.,* 2010). The genome size is 4.305 Mbp with a G+C content of 63%. A total of 3,917 candidate genes have been predicted in the genome in addition to 99 pseudo-genes. About 68% of the genes have functions assigned whereas 32% of the genes could not be assigned with known functions. Based on the BLASTp searches against the KEGG (Kyoto Encyclopedia of Genes and Genomes) database, 3,413 out of 3,917 (87.1%) candidate genes have significant similarity to genes from *Proteobacteria*. In addition, only 11 and 14 genes have best hits with genes in *Archaea* and Eukarya genomes, respectively. In the genome all tRNA encoding regions have been identified including two identical ribosomal RNA operons. Several genes have been identified that are involved in various metabolic pathways and this is described in **Chapter 6.**

1.7 Project aims

1. To develop PCR-based molecular ecological tools for rapid detection of *Methylocella* spp. in the environment and to study their distribution and diversity.

2. To develop a quantitative real-time PCR assay for *Methylocella* spp. to study their abundance in the environment.

3. To study the effect of acetate on the ability of *Methylocella* spp. to oxidize methane as well as to understand if they are able to utilize acetate as carbon source in peat soil microcosms.

4. To identify genes upregulated in *Methylocella silvestris* during growth on methane or acetate using a whole genome DNA microarray.

5. To study the distribution of *Methylocella* in Swedish islands forest soil which are at different successional stages. In addition, the diversity of other methanotrophs in these Swedish islands forest soils was studied by *pmoA* diagnostic microarray (This work was done in collaboration with Niall McNamara and colleagues at CEH, Lancaster).

Chapter 2

Materials and methods

2.1 Bacterial strains

2.1.1 *Escherichia coli*

Escherichia coli strains were cultivated in Lysogeny broth (LB) (ommonly known as Luria broth). LB was incubated on an orbital shaker (150 rpm) at 37° C. LB agar plates were prepared with additional 1.5% (w/v) Bacto agar (BD Diagnostic) before autoclaving. The medium was supplemented with the appropriate concentration of antibiotics if necessary.

2.1.2 Methanotrophs

Methylococcus capsulatus Bath and *Methylosinus trichosporium* OB3b were cultivated in nitrate mineral salt (NMS) medium (Whittenbury *et al.*, 1970). For cultivation of *Methylocella silvestris* a 1:10 dilution of NMS medium was used. When required agar plates were prepared with the addition of 2% (w/v) Noble agar (BD Diagnostic) prior to autoclaving.

Below is the list of methanotroph strains used in this study:

Methanotroph strains	Description	Source
Methylococcus capsulatus	Type strain	Warwick Culture Collection
Methylosinus trichosporium	Type strain	Warwick Culture Collection
Methylocella silvestris	Type strain	Warwick Culture Collection

2.2 Media and solutions

All the media and solutions were made using milli-Q water, unless otherwise stated.

2.2.1. NMS medium

I: 10× Salts solution

Per litre:

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

II: 1000× Iron EDTA solution

Per litre:

Fe-EDTA	3.8 g

III: 1000× Molybdate solution

Per litre:

$NaMoO_4$	0.26 g

IV: 1000× Trace elements solutions

Per litre:

$CuSO_4 \cdot 5H2O$	0.2 g
$FeSO_4.7H_2O$	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

V: 100× Phosphate buffer (pH 6.8)

Per litre:

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

10 ml of phosphate buffer was added to 990 ml of 1 × mineral salt medium. Methanotrophs were cultivated in 1 × NMS medium, which was prepared by diluting 100 ml of the salt solution to 1 liter and adding 1 ml of each solution II to IV. Sterile phosphate buffer was added when the medium cooled down after autoclaving to avoid precipitation of phosphate.

2.2.2 Saline- sodium citrate (SSC) buffer (20 ×)

Per litre:

NaCl	173.3 g
Sodium citrate	88.2 g

The pH was adjusted to 7.0 with 10 M HCl.

2.2.3 Tris-EDTA (TE) buffer (pH 8.0)

Per litre:

Tris-HCl	10 mM
Na_2EDTA	1 mM

Prepared from 1 M Tris-HCl (pH 8.0) and 0.5 M Na_2EDTA (pH 8.0).

2.2.4 Tris-borate-EDTA (TBE) buffer (10 ×)

Per litre:

Tris base	108 g
H_3BO_3	55 g
Na_2EDTA (0.5 M solution (pH 8.0)	40 ml

Dissolve in 800 ml water

2.2.5 Lysogeny broth

Per litre:

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

The pH was adjusted to 7.4 with 10 M NaOH.

2.2.6 Tris-acetate-EDTA (TAE) buffer (50 ×)

Per litre:

Tris	242 g
CH_3COOH	57.1 ml
Na_2EDTA (0.5 M solution; pH 8.0)	100 ml

2.2.7 Polyacrylamide gel

Ingredients	**30%**	**70%**
40% acrylamide	10.0 ml	10.0 ml
50X TAE	1 ml	1 ml
HCOOH	6 ml	14 ml
Urea	6.3 g	14.7 g
Water	to 50 ml	to 50 ml

Solutions were made in a 50-ml Falcon tube and stored at 4^oC for ~ 1month.

2.2.8 Precipitation solution

Polyethylene glycol 6000	20% (w/v)
NaCl	2.5 M

2.3 Nucleic acid extraction, quality assessment, quantification and storage

2.3.1 Extraction of DNA from soil and sediment samples

DNA was extracted from soil and sediment samples using the FastDNA® SPIN Kit for soil (MP Biomedicals, USA) according to the manufacturer's instructions. In brief, 0.4 g of soil, 900 µl sodium phosphate buffer and 100 µl MT buffer were processed in a Hybrid Ribolyser (Hybaid) for 30 s at speed 6.0. After bead-beating, samples were centrifuged (16,000 × g for 5 min) and the supernate (~1000 µl) was transferred into a new tube containing 250 µl PPS (protein precipitating solution) reagent. The mixture was then centrifugated (16,000 × g for 5 min) at room temperature and the supernate was transferred into a 15 ml Falcon tube containing 1 ml binding matrix. The binding matrix was mixed with the supernatant by hand shaking for 2 min. The tube was then placed in a rack for 3 min to allow settling of the matrix. After two min, 1 ml of supernatant was carefully removed and the remaining binding matrix was transferred into a SPIN filter and centrifuged (16,000 × g for 5 min) at room temperature for 1 min. The flow-through was discarded and 500 µl SWES-M solution was added into the SPIN filter and centrifuged at 16,000 × g for 1 min at room temperature. The flow-through was decanted and the SPIN filter was centrifuged at 16,000 × g for 2 min at room temperature to dry the matrix. The

SPIN filter was placed into a 1.5 ml microcentrifuge tube and air dried for 5 min. The DNA was eluted by adding 100 µl nuclease-free water (Ambion) into the SPIN filter, followed by centrifugation at 16,000 × g for 1 min at room temperature.

2.3.2 Extraction of DNA from pure cultures of bacteria

Bacterial liquid culture (1.5 ml, OD_{540} 0.5) was transferred into a 2 ml microcentrifuge tube and centrifuged at 16, 000 × g at 4º C for 5 min to obtain a cell pellet. The pellet was dissolved in 800 µl of lysis buffer and transfered into 15 ml Falcon tubes. Freshly prepared 200 µl of galline egg lysozyme solution (9 mg/ml) was added to the cell suspension and incubated at 37º C for 30 min in a shaker at 100 rpm. Following incubation, 200 µl of 10% (w/v) sodium dodecyl sulfate (SDS) and 100 µl of fungal proteinase K (20 mg/ml) were added to the cell suspension and kept at 55º C on a rotary shaker for 2 hours. The cell suspension was transferred into a phase-lock tube and mixed with 2 ml of phenol-chloroform and centrifuged at 4,000 × g for 5 min at room temperature. The aqueous phase was then transferred into a new 15 ml Falcon tube and mixed with 2 ml of phenol-chloroform (50:50). The tubes were centrifuged at 4,000 × g for 5 min at room temperature. The aqueous phase was transferred into a new Falcon tube and mixed with 2 ml of chloroform and centrifuged at 4,000 × g for 5 min at room temperature. The supernate was transferred into a new 15 ml Falcon tube and the DNA was precipitated by adding 5 µl of glycogen (20 mg/ml) (Roche), 1 ml of 7.5 M NH_4 -acetate and 6 ml of 100% (v/v) ethanol. The tubes were incubated at -20º C for overnight. Tubes were then

centrifuged at 3,000 × g for 30 min at 4º C using a JA-21 rotor. Two ml of ethanol (80% v/v) was added into each tube and centrifuged at 2,000 × g for 20 min at 4º C using a JA-21 rotor. The supernatant was removed and the DNA pellet was air dried for 5 min and dissolved in 250 µl of nuclease-water (Ambion).

2.3.3 DNA extraction from a colony using boiling method

A single colony from an agar plate was placed on a water bath containing boiled water for 15 min in 50 µl of 10 mM EDTA in an 1.5 ml Eppendorf tube. The tube was placed in a boiling water bath for 15 min and after cooling, the tube was centrifuged for 2 min at 12,600 × g. Supernatant (1-5 µl) containing approximately 10 to 20 ng DNA was used as DNA template in subsequent PCR.

2.3.4 Assessment of DNA quality

DNA quality was assayed routinely by running 5 µl of DNA of suspension in a 1% (w/v) agarose gel with 1 x TBE using a Flowgen Minigel Systems (Flowgen). Prior to casting the gel, ethidium bromide (0.5 µg/ml) was added to the gel and DNA was subsequently visualized on a UV trans-illuminator (Syngene, UK).

2.3.5 Quantification and storage of DNA

The concentration of DNA was measured either by running in a 1% (w/v) agarose gel along with 1 kbp DNA ladder standard (Invitrogen) or by running 1.5 µl of DNA suspensions in an NanoDrop Spectrophotometer (ND-1000; NanoDrop™, USA). Extracted DNA was stored either at -20° C or -80° C.

2.3.6 Purification of DNA fragments

DNA fragments were purified by running in a 1% (w/v) agarose gel followed by excision of the band from the gel. A Qiagen QIA Quick Gel Purification Kit (Qiagen) was applied to extract the DNA from the excised band according to the manufacturer's instructions.

2.3.7 Extraction of total bacterial RNA

Total bacterial RNA from batch cultures was extracted according to the method described by Gilbert *et al.,* (2000) with minor modifications. In brief, 700 µl cell suspension (OD_{540} 0.5) was centrifuged at 16,000 × g for 5 min at 4º C to obtain a cell pellet in a 1.5 ml microcentrifuge tube. The cell pellets were resuspended in 200 µl of solution I (0.3 M sucrose and 0.01 M sodium acetate; pH 4.5) and 200 µl of solution II (2% (w/v) sodium dodecyl sulfate and 0.01 M sodium acetate; pH 4.5). The cell suspension was transferred to a blue Ribolyser tube (Hybaid), and 400 µl of phenol (saturated with 50 mM sodium acetate (pH 4.5)) was added. The cells were lysed using a Hybaid Ribolyser (Hybaid) at speed 6 for 30 s. Following these steps, the cells were kept on ice. The cell suspension was centrifuged at 16,000 × g for 5 min at 4º C, and the aqueous phase was transferred to a new microcentrifuge tube. An equal volume of (~ 400 µl) of phenol was added and the tubes were incubated for 4 min at 65° C and frozen in dry ice-ethanol immediately for 10 s, followed by

centrifugation at 16,000 × g for 5 min. The aqueous phase was transferred to a new 1.5 ml microcentrifuge tube. 400 hundred µl of phenol-chloroform (50:50) were added, mixed vigorously for 30 s, and centrifuged at 16,000 × g for 5 min. The aqueous phase was transferred to a new 1.5 ml microcentrifuge tube and mixed with 40 µl of 3 M sodium acetate (pH 4.5) and 900 µl of 96% (v/v) ethanol. The tubes were kept at -20° C for 60 min followed by precipitated of the RNA by centrifugation at 16,000 × g for 15 min at 4º C. The supernatant was removed, and the RNA pellet was washed with 250 µl of 70% (v/v) ethanol, air dried for 5 min and resuspended in 50 µl of nuclease-free water (Ambion). The RNA preparations were finally treated with RNase-free DNase (NEB, USA) for 30 min at 37° C according to manufacturer's instruction. Absence of contaminating genomic DNA in the extracted RNA was confirmed by a PCR performed with 2 µl of RNA as a template with bacterial 16S rRNA gene primers 27f and 1492r as described by Lane (1991). DNA-free RNA was confirmed by the absence of amplification of any 16S rRNA genes from the template RNA. Extracted RNA was stored at -80° C.

2.3.8 Assessment of RNA quality and its concentration

The concentration and quality of the RNA were assayed by using either a NanoDrop Spectrophotometer (ND-1000; NanoDrop™, USA) or a 2100 Bioanalyzer (Agilent Technologies, USA). In addition, 5 µl suspension of RNA was also run in a 1% (w/v) agarose gel to check the quality.

2.3.9 Plasmid extraction from *Escherichia coli*

Plasmid extractions were carried out using the Qiagen Mini Prep Kit (Qiagen) according to the manufacturer's protocol.

2.4 Techniques for nucleic acid manipulation

2.4.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out in a total volume of 50 µl using a Tetrad2 thermal cycling system (Bio-Rad, USA). Each PCR mix consisted of 3 µl $MgCl_2$ (1.5 mM), 5 µl 10 × PCR buffer (KCl + $MgCl_2$) (Fermentas Inc. UK), 0.5 µl dNTPs (25 mM), 1 µl of each forward and reverse primers (10 µM; Invitrogen), 0.5 µl (2.5 U) *Taq* DNA polymerase (Fermentas Inc. UK), 1 µl BSA (20 mg ml^{-1}), 1 µl DNA (10 to 30 ng μl^{-1}) and water to 50 µl. Cycling conditions were initiated at 94° C for 5 min, Cycling conditions were initiated at 94° C for 5 min, followed by 30 or 32 cycles of: 94° C for 1 min, various annealing temperatures as required for 1 min (**Table 2.1**) and extension at 72° C for 1 min with a final extension at 72° C for 10 min. A list of primers used in this studies is given in **Table 2.2.**

Table 2.1. Annealing temperature for different primers used in various PCR protocols.

Primers	**Annealing temperature (° C)**
27f/1492r	56
341F_GC/518R	56
341F_GC/907R	56
M13F/M13R	55
Type IF/Type IR	60
Type IIF/TypeIIR	60
206F/886R	60
Type IIF/Mcell-1445	63
mmoXLF/mmoXLR	68

For amplification of *pmoA* to perform microarray experiments the following PCR protocols was used.

Touchdown PCR: Primer A189f and T7mb682 or A189f and T7mb661

94° C	5 min.	
94° C	1 min.	
62° C	1 min.	Ramp down of -1°C per cycle, 11 cycles until 52° C
72° C	1 min.	
94° C	1 min.	
52° C	1 min.	24 further cycles
72° C	1 min.	
72° C	10 min.	
4° C	pause	

Total: 35 cycles

Nested PCR: First step using the primers A189f and T7mb682

94° C	5 min.	
94° C	1 min.	Ramp down of -1°C per cycle, 11 cycles until 52° C
62° C	1 min.	
72° C	1 min.	
94° C	1 min.	24 further cycles
52° C	1 min.	
72° C	1 min.	
72° C	10 min.	
4° C	pause	

Total: 35 cycles

Second step using primers A189f and T7mb661 (1 µl of PCR product from the first round as the PCR template)

94° C	5 min.	
94° C	1 min.	Ramp down of 1° C per cycle, 11 cycles until 52° C
62° C	1 min.	
72° C	1 min.	
94° C	1 min.	14 further cycles
52° C	1 min.	
72° C	1 min.	
72° C	10 min.	
4° C	pause	

Total: 25 cycles

Direct PCR approach using the primer A189f and T7mb682 or A189f and T7mb661 or A189f and T7mb650

94° C	5 min.	
94° C	1 min.	31 further cycles
56° C	1 min.	
72° C	1 min.	
72° C	10 min.	
4° C	pause	

Total: 32 cycles

The annealing temperature used was 52°C for primers A189f and T7mb682 and primers A189f and T7mb661 and 56° C for primers A189f and T7mb650.

Table 2.2. List of primers used in this study.

Primers	Sequences (5′-3′)	Target	References
27f	AGAGTTTGATCMTGGCTCAG	16S rRNA gene	Lane (1991)
907r	CCGTCAATTCMTTTGAGTTT	16S rRNA gene	Lane (1991)
341F_GC*	CCTACGGGAGGCAGCAG	16S rRNA gene	Muyzer *et al.* (1993)
518F_GC*	CCAGCAGCCGCGGTAAT	16S rRNA gene	Muyzer *et al.* (1993)
1492r	TACGGYTACCTTGTTACGACTT	16S rRNA gene	Lane (1991)
Type IF	ATGCTTAACACATGCAAGTCGAACG	16S rRNA gene (Type I methanotrophs)	Chen *et al.* (2007)
Type IR	CCACTGGTGTTCCTTCMGAT	16S rRNA gene (Type I methanotrophs)	Chen *et al.* (2007)
Type IIF	GGGAMGATAATGACGGTACCWGGA	16S rRNA gene (Type II methanotrophs)	Chen *et al.* (2007)
Type IIR#	GTCAARAGCTGGTAAGGTTC	16S rRNA gene (Type II methanotrophs)	Chen *et al.* (2007)
A189f	GGNGACTGGGACTTCTGG	*amoA*/*pmoA*	Holmes *et al.* (1995)
A682+	GAASGCNGAGAAGAASGC	*amoA*/*pmoA*	Holmes *et al.* (1995)
mb661+	CCGGMGCAACGTCYTTACC	*pmoA*	Costello & Lidstrom (1999)
mb650+	ACGTCCTTACCGAAGGT	*pmoA*	Bourne *et al.* (2001)

Mcell-1445	CCTCTCTCCTTGCGGTT	16S rRNA genes for *Methylocella* spp.	Dedysh *et al.* (2005)
206F	ATCGCBAARGAATAYGCSCG	*mmoX*	Hutchens *et al.* (2004)
886R	ACCCANGGCTCGACYTTGAA	*mmoX*	Hutchens *et al.* (2004)
mmoXLF	CCCAATCATCGCTGAAGGAGT	*mmoX* for *Methylocella* spp.	This study
mmoXLR	GAAGATTGGGGCGGCATCTG	*mmoX* for *Methylocella* spp.	This study
M13F	GTAAAACGACGGCCAG	Cloned insert	Invitrogen
M13R	CAGGAAACAGCTATGAC	Cloned insert	Invitrogen
1003F	GCGGCACCAACTGGGGCTGGT	*mxaF*	McDonald *et al*. (1995)
1555R	CATGAABGGCTCCCARTCCAT	*mxaF*	McDonald & Murrell (1997)

*a GC clamp was attached to the 5′ end of the sequences: CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG

This probe has one mismatch with Am 976 (Gulledge *et al*., 2001).

+ T7 promoter (TAATAC GACTCACTATAG) was added to the 5′ end of the sequences.

2.4.2 Reverse transcription

Reverse transcription (RT) was performed with the SuperScript II Reverse Transcriptase kit (Invitrogen) according to manufacturer's instruction with minor modification. In brief, 200 ng DNA-free RNA was added to either 50 pmol random hexamers (Fermentas) and 1 µl dNTP mix (10 mM each) in a final volume of 12 µl. The mixture was heated to 65° C for 5 min and then immediately chilled on ice. Four µl of 5 × first-strand buffer, 2 µl 0.1 M dithiothreitol (DTT) and 1 µl (200 units) of SuperScript II reverse transcriptase were added to the mixture to a final volume of 20 µl before incubation at 42° C for 40 min, followed by a 15 min incubation at 70° C to inactivate the RT enzyme. The cDNA (5 µl) was used as a template for PCR amplification, as described earlier.

2.4.3 Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was performed using an ABI PRISM ® 7000 Sequence Detection System (Applied Biosystems). The data were analyzed using the ABI 7500 SDS software version 2.1 (Applied Biosystems). The detailed protocol developed to detect *mmoX* of *Methylocella* spp. using the qPCR is described in **Chapter 4.**

2.4.4 Reverse transcription quantitative PCR

To validate results of microarray, SYBR® Green-based reverse transcriptase quantitative PCR (RT-qPCR) was conducted with cDNA derived from the samples. The cDNA was synthesized from 200 ng of total RNA with SuperScript® III First-Strand Synthesis System (Invitrogen) and 50 ng of random primers (Fermentas) per reaction, according to the manufacturer's instruction. Oligonucleotide primers

were designed to have 55 to 60 bp amplicons using the Primer Express software (Applied Biosystems). The qPCR reaction contained 2 ng of cDNA, 0.3 µM of specific primers, and 1× SYBR® Green PCR Master mix (Invitrogen), in a total volume of 20 µl, and was performed on an an ABI PRISM ® 7000 Sequence Detection System (Applied Biosystems). The amplification conditions were as follows: 50° C for 2 min, 95° C for 10 min, 40 cycles of 95° C for 15 sec and 60° C for 1 min, and a dissociation cycle. During dissociation cycle the temperature of the instrument was increased from 60° C to 95° C at approximately 2° C min^{-1}.

Tabel 2.3. List of primer used for the reverse transcription quantitative PCR

Name of gene/function (Gene Id)	**Primers (5′-3′)**
16S rRNA gene Fwd	AAGATTTATCGCCGAAGGATTG
16S rRNA gene Rev	TAGTTGGCGGGGTAATGGC
RNA polymerase (Msil3868) Fwd	CGCAAATTTTTTGGCCACAT
RNA polymerase (Msil3868) Rev	TGAAGTTCAAAAAGCGTCCTATGA
mmoX (Msil1262)Fwd	CACCCTGCTTGACGGATTG
mmoX (Msil1262)Rev	GGAAACCGCGTACATCCG
glcB/Malate synthase (Msil2501) Fwd	CGCAAACCGGGCATGA
glcB/ Malate synthase (Msil2501) Rev	GGAATCAATCTCAAGGTCATGGA
icl/Isocitrate lyase (Msil3157) Fwd	CGGCTGCGAAAACCAT
icl/Isocitrate lyase (Msil3157) Rev	GATCATCGCGGATATCGACG
fur/Ferric uptake regulator (Msil1272) Fwd	TGATCCGCAGTCTGGCAAT
fur/Ferric uptake regulator (Msil1272) Rev	TGATCCGCAGTCTGGCAAT
Glycine dehydrogenase (Msil1215) Fwd	GCCTCCATCCGCATTATGC
Glycine dehydrogenase (Msil1215) Rev	GATGACGGACAATGACGCG

2.5 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed with a DCode™ universal mutation detection system (Biorad, USA). Gels were prepared with 10% (v/v) acrylamide/bisacrylamide with a 30-70% linear denaturant gradient (100% denaturant corresponded to 7.0 M urea and 40% (v/v) formamide). The gel was run in 1 x TAE buffer at 60˚ C at a constant 84 V for 14 hours. Gels were stained with SYBR(R) Gold nucleic acid stain (Invitrogen, UK) for 30 min and destained in water for 10 min before capturing the image using the UV trans-illuminator (Syngene, UK).

2.6 Excision of denaturing gradient gel electrophoresis bands for sequencing

Individual bands of interest were excised from the DGGE gel by using a sterile scalpel blade, washed twice in sterile molecular-grade water and stored overnight in a 1.5 ml microcentrifuge tube containing 20 µl of nuclease-free water (Ambion) at 4° C. The eluted DNA (5 µl) was used as PCR template to re-amplify the DGGE band using the same PCR primers and conditions. PCR products were purified using the QiaQuick PCR purification system (Qiagen, USA) and sequenced at the University of Warwick Molecular Biology Facility.

2.7 Cloning, sequencing and phylogenetic analysis

2.7.1 Construction of clone libraries and restriction fragment length polymorphism analysis

Clone libraries of *mmoX*, *pmoA* and 16S rRNA genes were constructed with gel purified PCR products by ligated the DNA into either pGEMT® (Promega, USA) or

TOPO® (Invitrogen, USA) cloning vectors according to the manufacturer's instructions. Eighteen to 48 clones were randomly selected from each clone library for further analysis. Inserts were amplified from these clones using M13F/M13R primers. When required, clones were grown overnight at 37° C in LB containing the appropriate antibiotics. Plasmids were extracted from the overnight grown culture using the plasmid Miniprep kit (Invitrogen, USA) according to manufacturer's instruction. Restriction fragment length polymorphism analysis (RFLP) analysis was carried out using *Rsa*I of MspI for *mmoX*, *Msp*I for 16S rRNA genes and *Hin*cII or *Pvu*II for *pmoA*. Digested DNA fragments were resolved by electrophoresis in a 2.5% (w/v) agarose gel. Each clone that represented a unique RFLP pattern was assigned to an operational taxonomic unit (OTU). One to two clones from each OTU were selected and sequenced.

2.7.2 DNA sequencing

DNA sequencing was performed at the University of Warwick Molecular Biology Facility by cycle sequencing with a BigDye dideoxy terminator ready reaction kit (Applied Biosystems, Warrington, United Kingdom) and ABI3100 capillary DNA sequencers.

2.7.3 Sequence alignment

Sequence alignment was performed using either the ClustalX program (Thompson *et al.*, 1997). Alignment accuracy was checked manually.

2.7.4 Phylogenetic analysis

Before phylogenetic analysis, all the 16S rRNA genes sequences were checked for chimeras using the Chimera check program at the Ribosomal Data Project II (Maidak *et al.*, 2001) and suspected chimeras were excluded from further analysis. Related gene sequences of 16S rRNA genes and other functional genes from reference strains were obtained from GenBank using the BLAST program version 2.1 (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.*, 1990), and were then aligned with these new sequences using the ClustalX program (Version 1.8) (Thompson *et al.*, 1997). Neighbor-Joining phylogenetic trees were generated using MEGA4 (Tamura *et al.*, 2007) with an appropriate outgroup.

2.8 DNA Stable isotope probing

DNA stable isotope probing was performed as described by Neufeld *et al*. (2007a) and Lueders *et al*. (2004). Briefly, microcosms were set up in triplicate in 120 ml crimp-top serum vials containing 5 g soil. Required amounts of substrates (^{13}C or ^{12}C methane or acetate) were added and the uptake of substrates was measured with standard protocols using either a gas chromatography (GC) or high performance liquid chromatography (HPLC) as described elsewhere in this chapter. All microcosms were incubated at 21° C in the dark. The incubation was stopped once 100 µmol of labelled substrate has been incorporated per g of soil. DNA was extracted from the incubated soil as described earlier using a FastDNA® Spin for soil Kit (MP Biomedicals LLC, USA). Two to four µg of extracted DNA were subjected to caesium chloride gradient ultra-centrifugation and fractionation to separate the ‘‘heavy’’ DNA (density ~1.725 g ml^{-1}) from the “light” DNA (density ~1.710 g ml^{-1}),

followed by precipitation of fractionated ^{12}C- and ^{13}C-DNA as described by Neufeld *et al.,* (2007a). A digital refractometer (Reichert AR2000, Reichert Analytical Instruments, USA) was used to estimate the density of caesium chloride gradient fractions. Gradients were fractionation using a pump (Watson Marlow Ltd, Cat. No. 101U/R) that yielded 12 to 13 fractions of ~ 400 µl each. DNA was precipitated using the precipitation solution (30% PEG 6000 and 1.6 M NaCl) and resuspended in 50 µl of nuclease-free water (Ambion). Five µl of DNA sample from each fraction were quantified by electrophoresis on a 1% (w/v) agarose gel. The purified DNA was stored at -20° C or at -80° C for short or long term storage respectively.

2.9 Quantification of acetate from soil

Quantification of acetate from soil was carried out using high performance liquid chromatography (HPLC) (A1200 series, Agilent Technologies, USA) equipped with a Zorbax SB-Aq column (Agilent Technologies, USA) and an UV detector (210 nm wave length) according to the method described by Nusslein *et al.*, (2001) with minor modifications. In brief, 1.8 ml soil suspension was centrifuged at 16,000 × g for 10 min at 4° C. The aqueous phase supernatant was removed and passed through a cellulose membrane filter (0.2 µm) before injecting (100 µl) into the HPLC. The mobile phase was a mixture of 99% of 20 mM NaH_2PO_4 (pH 2) and 1% (v/v) of acetonitrile. Initially a standard curve for quantifying acetate was generated by running different known concentrations of sodium acetate (0.1 mM to 5 mM) (**Figure 2.1).** In addition, the efficiency of the procedure used to extract acetate from soil slurries was tested by spiking the soil with known concentrations of sodium acetate. The extraction technique retrieved more than 95% of the spiked acetate from the soil slurry (**Figure 2.2**). The limit of detection for acetate using this assay was

around 0.1 mM. The other parameters of the HPLC were as follows: Oven temperature 35° C, injection volume: 100 µl and flow rate: 1 ml/min.

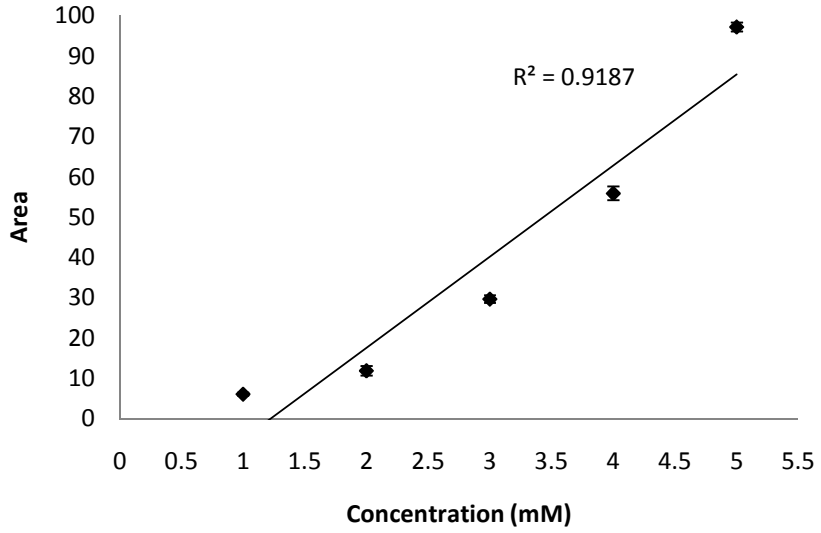


Figure 2.1 Calibration curve for quantification of acetate. Known concentrations of acetic acid solutions were run in triplicate. Values represent mean and SD of three measurements.

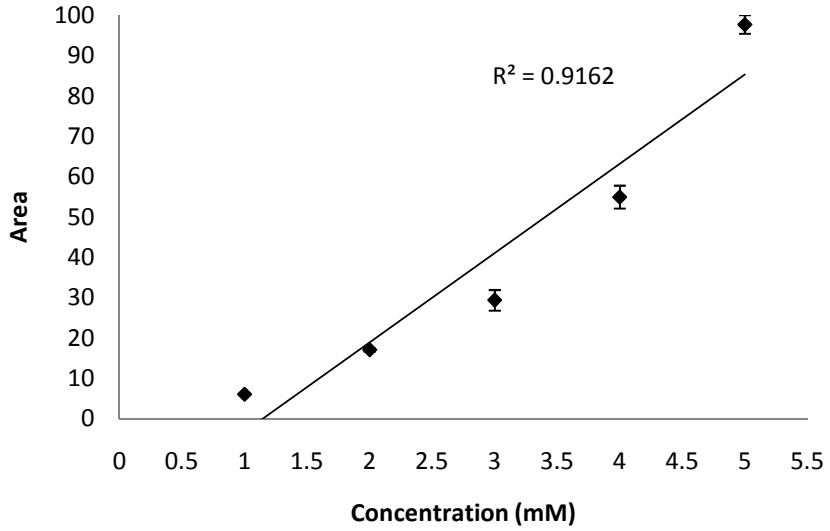


Figure 2.2 Calibration curve for quantification of acetate (spiked soil). Soil samples were spiked with known concentrations of acetic acid solutions in triplicates. Values represent mean and SD of three measurements.

2.10 Quantification of methane

Methane oxidation potentials of soil samples were measured by injecting 0.2 ml of the headspace gas on a gas chromatograph (6890N, Network GC system, Agilent Technologies, USA) equipped with a flame ionization detector as described by Cébron *et al.* (2007).

2.11 Microarray analyses

2.11.1 *pmoA* microarrays

pmoA microarray experiments were performed as described in Stralis-Pavese *et al.*, (2004). *pmoA* genes were amplified from environmental DNA in triplicate using primer set A189f and T7mb650. Amplified PCR products were combined and purified using the QIAquick Spin column (Qiagen) according to manufacturer's instruction. *In vitro* transcription was carried out in a 1.5 ml Eppendorf tube to generate RNA. The final reaction volume was 20 µl containing 1 × T7 RNA polymerase buffer, 10 mM DTT, 20 U RNAsin (Promega), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.25 mM UTP, 1 mM Cy3-UTP, 40 U T7 RNA polymerase (Gibco, BRL) and 400 ng of purified *pmoA* product. The reactions were incubated at 37° C for 4 hours. The RNA produced was immediately purified using the Qiagen RNeasy Kit and eluted in 50 µl of nuclease-free water (Ambion). The concentration of the RNA was determined using a NanoDrop spectrophotometer (ND-1000; NanoDrop™, USA).

Purified RNA was fragmented by incubating at 60° C for 30 min with 10 mM $ZnCl_2$ and 20 mM Tris-HCl (pH 7.4). The fragmentation reaction was stopped by the addition of 10 mM EDTA (pH 8.0) and placing the reaction on ice. 40 U of RNAsin®

(Promega) was added to the fragmented target and the labelled RNA was stored at -20 °C.

Hybridisation was performed overnight on an aluminium block placed on a temperature-controlled Belly Dancer shaker (Stovall Life Sciences, Greensboro, USA). The hybridisation block was preheated to 55° C. Slides that had been pre-spotted with the *pmoA* probes were assembled with 200 µl HybriWell stick on hybridisation chambers and preheated on the hybridisation block for 1 to 2 min. Meanwhile an Eppendorf incubator was also heated to 65° C and hybridisation mixtures were preheated for 1 min. 200 µl hybridisation mixtures were made in 1.5 ml Eppendorf tubes containing 0.01% (w/v) SDS, 1 × Denhardt's reagent (Sigma), 6 × SSC, 124 µl DEPC-treated water and 10 µl target RNA. Preheated hybridisation mixture was added to each slide *via* the open port and incubated at 55° C at 30-40 rpm circulation and maximum bending overnight, in the Belly Dancer shaker.

After hybridization, the array chambers were removed and the slides were immediately washed once for 5 min in 2 × SSC, 0.1% (w/v) SDS at room temperature. The slides were then washed twice for 5 min using 0.2 × SSC, 0.1% (w/v) SDS and finally with 0.1 × SSC for 5 min at room temperature. Slides were dried individually using an airgun before storing in the dark at room temperature. Hybridisation slides were scanned at 10 µm resolution with a Agilent G2565CA Scanner (Agilent technologies, USA) at a wavelength of 532 nm. Results of individual microarrays were normalised and displayed using GeneSpring software (Agilent Technologies, USA). Microarray data were analyzed as described by Bodrossy *et al.,* (2003). List of oligonucleotide probes spotted on the microarray and their intended specificity is presented in **Appendix 1.**

2.11.2 *Methylocella silvestris* whole genome microarray experiments

Methylocella silvestris whole genome differential gene expression microarray experiments were carried out to identify genes transcribed differentially under methane and acetate grown conditions. The Agilent custom-designed microarray platform (single colour) was adopted for the micrioarray experiments. Microarray probes (60-mer oligonucleotide probe) covering the whole genome of *Methylocella silvestris* were designed using the eArray software available at the Agilent Technology web site (https://earray.chem.agilent.com/earray/). A total of 3,917 candidate protein-encoding gene has been identified in the genome of *Methylocella silvestrsi* (Genome size 4.3 Mbp; accession number CP001280) (http://genome.ornl.gov/microbial/msil/). For each gene, three probes were designed (total 11751). The microarray experiments were carried out with RNA extracted from *Methylocella silvestris* grown on a 1:10 dilution of NMS medium (pH 5.5) either in presence of CH_4 (10% (v/v) head space) or acetate (final concentration 5 mM) in batch culture harvested at exponential phase (OD_{540} ~ 0.30). The cultures were incubated at 25° C on a shaker (150 rpm). Three biological replicates of RNA were used for each condition. In addition, one batch culture from each methane and acetate grown conditions were split into two groups to extract RNA separately and was considered as the technical replicate. Total bacterial RNA was extracted as described earlier. Microarray target preparation, hybridization, slide washing, scanning and data acquisition were done by the University of Warwick Molecular Biology Facility. Cy-3 labelled cDNA was synthesized from 200 ng total DNA free RNA using the One-Color Microarray-Based Gene Expression Analysis (Quick Amp Labeling) kit (Agilent Technologies, USA) according to the manufacturer's instruction. However, cRNA was synthesized from the cDNA using T7 IVT primer from Full Spectrum™

MultiStart Primers for the T7 IVT RNA Amplification kit (SBI, Systems Biosciences, USA) as described by Su *et al.*, (2007). The slides were hybridization with Cy-3 labeled cRNA at 65° C for 17 hours, followed by washing in GE Wash Buffer (Agilent Technologies, USA) at room temperature in an Agilent platform as described by the manufacturers. Finally the slides were allowed to dry and scanned in an Agilent G2565CA Scanner (Agilent Technologies, USA). Feature extraction software provided by Agilent (Version 7.5, Agilent Technologies, USA) was used to quantify the intensity of the fluorescent images and to normalize initially the results by subtracting local background fluorescence, according to the manufacturer's instructions. Microarray data were further normalized using GeneSpring software at the 75th percentile level using the GeneSpring GX 11 software package (Agilent Technologies, USA). The *p* value derived by unpaired t test using the GeneSpring software was used to assess the statistical significance of that estimate. The value ranges from 0 to 1, with a smaller *p* value providing more confidence in the regulation pattern. For this study, data with *p* values of <0.05 were generally considered confident and reliable. The biological significance for each comparison was analyzed using the assigned cut-off expression ratio of 2.0 fold-changes and the direction of regulation (*e.g.* an upregulation or downregulation in expression). The functional grouping of the genes were developed based on proposed functions of each gene in the genome annotation page of *Methylocella silvestris*.

Chapter 3

Distribution of *Methylocella* spp. in the environment

3.1 Introduction

One of the goals of microbial ecology is to understand the distribution and diversity of a particular group of organisms within their niches in the environment. Targeting a particular group of organism using molecular ecology techniques requires application of specific primers and probes to selectively detect them in the environment. Physiologically, *Methylocella* species are unique among methanotrophs studied so far. Methanotrophs use one of two types of methane monooxygenase to oxidize methane: the particulate methane monooxygenase (pMMO) or soluble methane monooxygenase (sMMO) encoded by the operons *pmoCAB* and *mmoXYBZDC* respectively (reviewed in Trotsenko & Murrell, 2008). pMMO is present in all known methanotrophs except *Methylocella* spp., whilst the soluble methane monooxygenase is present in relatively few species, including methanotrophic members of the *Alphaproteobacteria* (McDonald *et al.*, 1997). *Methylocella* spp., however, lack particulate methane monooxygenase, and oxidize CH_4 using the soluble monooxygenase (Theisen *et al.*, 2005), therefore, the large *pmoA* database (approximately 60 sequences from methanotrophs and 800 environmental *pmoA* clones) that has been build-up in the last few years could not be used to study the diversity of *Methylocella* spp.

For methanotrophs, there are some group-specific primers to selectively amplify 16S rRNA genes of methanotrophs at the family- or genus-level (Wise *et al.*, 1999; Gulledge *et al.*, 2001; Chen *et al.*, 2007) (**Table 1.3; Chapter 1**). Primer set MethT1dF/MethT1bR and Type IF/Type IR was developed to target the 16S rRNA genes of Type I methanotrophs, while primer MethT2R and Type IIF/Type IIR was designed to target the 16S rRNA genes of Type II methanotrophs. None of these

primers are specific for the 16S rRNA gene of *Methylocella* spp., however, there is a fluorescent *in situ* hybridization (FISH) probe Mcells-1024 (5-TCCGGCCAGCCTAACTGA-3) targeting *Methylocella silvestris* and similarly probe Mcell-1445 (5′-GCCTCTCTCCTTGCGGTT-3′) targeting *Methylocella silvestris* and *Methylocella palustris* 16S rRNA genes (Dedysh *et al.*, 2005). There are no PCR primers and protocols to target *Methylocella* spp. directly from environmental samples, although 16S rRNA genes related to *Methylocella* spp. have been retrieved from diverse environments ranging from peatlands to volcanic glass (Dunfield *et al.*, 2003; Dedysh *et al.*, 2004; Lau *et al.*, 2007; Chen *et al.*, 2008a; Chen *et al.*, 2008b; Cockell *et al.*, 2009; Fabiani *et al.*, 2009b; Han *et al.*, 2009). However, there is little information on the distribution and abundance of *Methylocella* spp. in different the environments.

The aim of the work described in this chapter was to develop PCR-based diagnostic screening techniques for the rapid detection of *Methylocella* genus from DNA extracted directly from environmental samples and to apply these techniques to determine the distribution of this facultative methanotroph in the environment.

3.2 Results

3.2.1 Optimization and validation of a PCR protocol targeting the 16S rRNA genes of *Methylocella* spp.

A PCR protocol was developed to specifically amplify a 1 kbp region of the 16S rRNA genes of *Methylocella silvestris, Methylocella palustris* and *Methylocella tundra* using the primer Type IIF F (5′-GGGAMGATAATGACGGTACCWGGA-3′) and Mcell-1445 (5′-GCCTCTCTCCTTGCGGTT-3′). Initially the PCR conditions were optimized by gradient PCR (annealing temperature 55° C to 70° C) using genomic DNA from pure cultures of *Methylocella silvestris, Methylocella palustris, Methylocella tundrae.* DNA from *Methylocystis parvus, Methylomonas agile, "Methylomonas rubra", Methylomonas methanica, Methylosinus trichosporium* and *Methylosinus sporium* was used as the negative controls. Based on the gradient PCR results it was observed that at an annealing temperature of 63° C, the primers Type IIF and Mcell-1445 were able to amplify 16S rRNA genes from pure culture DNA of all three species of the genus *Methylocella* (*Methylocella silvestris, Methylocella palustris* and *Methylocella tundrae*), but not from DNA of any other methanotrophs tested (**Figure 3.1**).

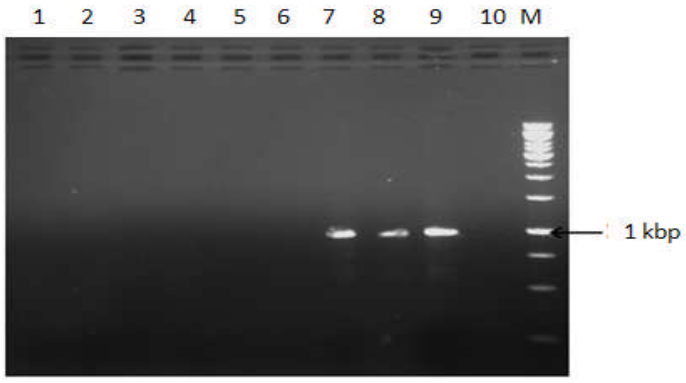


Figure 3.1. Validation of PCR protocol for amplification of 16S rRNA genes of *Methylocella* spp. from genomic DNA from pure culture methanotrophs. Lane 1. *Methylocystis parvus,* Lane 2. *Methylomonas agile,* Lane 3. "*Methylomonas rubra*", Lane 4. *Methylomonas methanica,* Lane 5. *Methylosinus trichosporium,* Lane 6. *Methylosinus sporium,* Lane 7. *Methylocella palustris,* Lane 8. *Methylocella tundrae,* Lane 9. *Methylocella silvestris,* Lane 10. Negative control (Non-template control), M = 1 kbp DNA ladder.

The primers were then tested by a direct PCR approach (at 63°C annealing; 30 cycles) with DNA extracted from environmental samples to see whether they were able to detect *Methylocella* spp. directly from these DNA samples. DNA extracted from Moor House peat soil (UK) was considered as a positive control for *Methylocella* spp., because Chen *et al.* (2008a) previously detected *Methylocella* spp. from DNA extracted from this environment. DNA extracted from Ufton landfill cover soil (UK) was considered as a *Methylocella* negative sample. This was based on the fact that in a 16S rRNA gene clone library generated from DNA extracted

from this soil, Héry *et al.* (2008) could not detect *Methylocella*-related 16S rRNA gene sequences. Direct PCR results indicated that primers Type IIF and Mcell-1445 were able to detect *Methylocella*-like 16S rRNA genes from DNA extracted from Moor House peat soil, while no amplicons were detected from DNA extracted from the Ufton landfill cover soil. The presence of 16S rRNA genes of *Methylocella* spp. in the amplified PCR products were subsequently confirmed by clone library analysis **(**See later in **Figure 3.7)**.

These *Methylocella*-specific 16S rRNA gene primers were applied using direct PCR approach to screen DNA extracted from various environmental samples for 16S rRNA genes from *Methylocella* spp. Amplification of 16S rRNA genes of *Methylocella* spp. from DNA extracted from Cloud forest soil (San Pedro, Peru) and Moor House peat soil (UK) by the direct PCR approach is shown presented in **Figure 3.2.**

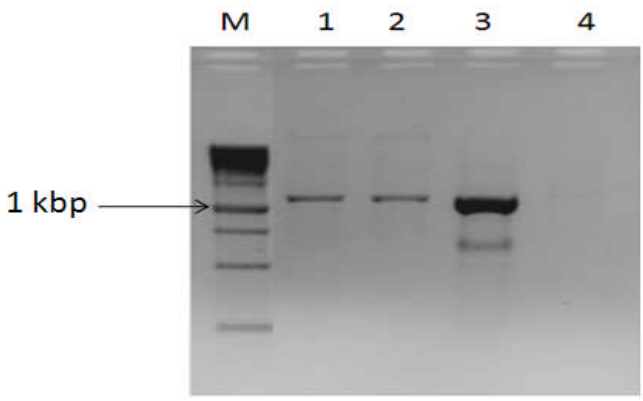


Figure 3.2. Amplification of 16S rRNA genes of *Methylocella* spp. from DNA extracted from Cloud forest soil (San Pedro, Peru) and Moor House peat soil (UK) by the direct PCR approach. Lane 1, Cloud forest soil (San Pedro, Peru). Lane 2, Moor House peat soil (UK). Lane 3, Positive control (*Methylocella silvestris* genomic DNA). Lane 4, Negative control (Non-template control). M = 1 kbp DNA ladder.

However, when any DNA extracted from environmental samples was found to be negative in the direct PCR approach, a nested PCR approach was adopted, where a first round of PCR (30 cycles) was carried out with bacterial universal 16S rRNA gene specific primers 27f and 1492r (Lane, 1991). One µl of the PCR product from the first round was then used as a template for the second round of PCR (30 cycles) using Type IIF and Mcells-1445R primers to detect 16S rRNA genes of *Methylocella* spp. (**Figure 3.3**). DNA samples extracted from various environmental samples collected from around the world and their characteristics are presented in **Table 3.3.** These DNA samples were screened by PCR, both by the direct and the nested PCR approach for the presence of *Methylocella* spp. The results of the PCR based survey for 16S rRNA gene of *Methylocella* spp. are presented in **Table 3.3.**

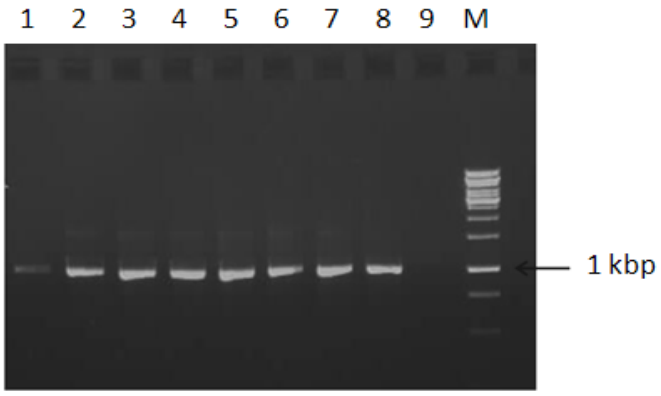


Figure 3.3. Amplification of 16S rRNA genes of *Methylocella* spp. from DNA extracted from environmental samples using the nested PCR approach. Lane 1, Boreal peat (Finland). Lane 2, Moor House peat soil (UK). Lane 3, Lonar lake sediment (India). Lane 4, Cloud forest soil (Peru). Lane 5, Rain forest soil (Peru). Lane 6, Peat soil (Spain). Lane 7, Arable land (Aberdeen, UK). Lane 8, Positive control (*Methylocella silvestris* genomic DNA). Lane 9, Negative control (Non-template control). M = 1 kbp DNA ladder.

3.2.2 Designing primers and optimization of a PCR protocol for *Methylocella* genus-specific *mmoX* genes

New *Methylocella* genus-specific *mmoX* forward primer mmoXLF (5´-GAAGATTGG GGCGGCATC TG -3´) and reverse primer mmoXLR (5´-ACTCCTTCAGCGATGATTGGG -3´) were designed to amplify ~ 450 bp *mmoX* amplicons from all three species of the genus *Methylocella*. The position of the primers targeting *mmoX* of *Methylocella* spp. is presented in **Figure 3.4** along with the other closely related *mmoX* sequences. Initially *mmoX* sequences related to

Methylocella and related organisms (both Type I and Type II methanotrophs) were downloaded from the GenBank database, analysed and aligned using the ARB program (Ludwig *et al.*, 2004). The sequence alignment was manually verified for alignment accuracy. Potential primers were identified and their specificity was tested using the Ribosomal Database Project II Probe Match program (http://rdp.cme.msu.edu/probematch/search.jsp). The primers were analysed for hairpin structures and potential duplex formation using the OLIGO 6 program (http://www.oligo.net/oligo.htm).

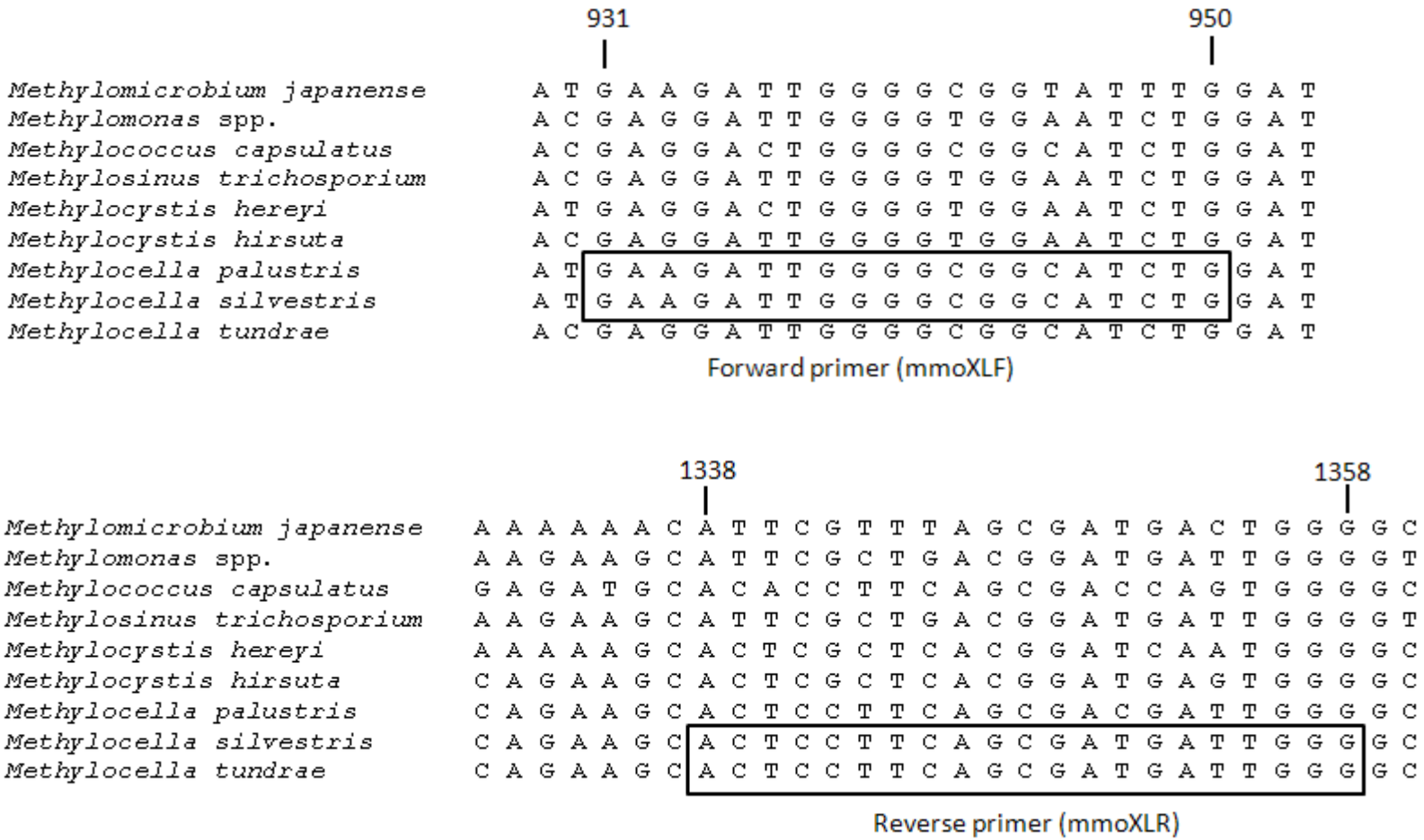


Figure 3.4. Alignment of *mmoX* gene sequences of *Methylocella* spp. and other *mmoX* genes of Type I and Type II methanotrophs. Conserved regions selected to design the *Methylocella* genus specific forward primer (mmoXLF) and reverse primers (mmoXLR) are indicated by the box. Numbers represent the position of the primers with respect to the numbering of nucleotide sequences of *mmoX* from *Methylocella silvestris* BL2.

The PCR conditions were optimized and validated with the newly designed primers to specifically amplify *mmoX* from *Methylocella* spp. Initially the PCR conditions were optimized, by a gradient PCR (annealing temperature 55° C to 70° C) approach using genomic DNA from pure cultures of *Methylocella silvestris, Methylocella palustris*, *Methylocella tundrae*, *Methylocystis parvus*, *Methylomonas agile*, "*Methylomonas rubra*", *Methylomonas methanica*, *Methylosinus trichosporium* and *Methylosinus sporium*. Based on the gradient PCR at various annealing temperature (60° to 70° C), it was observed that an annealing temperature at 68° C, primers mmoXLF and mmoXLR were able to detect specifically *mmoX* from the pure culture genomic DNA of all the three species of the genera *Methylocella,* but not from other *mmoX*-containing methanotrophs pure culture genomic DNA that are available at the University of Warwick Culture Collection *e.g., Methylocystis parvis, Methylosinus trichosporium* and *Methylosinus parvus.* The primers were found to be negative for the negative controls *i.e.,* the non-*mmoX* containing methanotrophs pure culture genomic DNA originating from the *Methylomonas agile* and *"Methylomonas methanica"* (**Figure 3.5**).

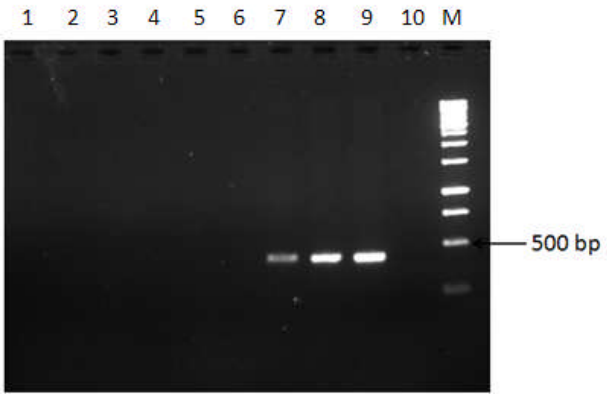


Figure 3.5. Validation of the PCR protocol for the amplification of *mmoX* of *Methylocella* spp. from genomic DNA of pure cultures of methanotrophs. Lane 1, *Methylocystis parvus*. Lane 2, *Methylomonas agile*. Lane 3, "*Methylomonas rubra*". Lane 4, *Methylomonas methanica*. Lane 5, *Methylosinus trichosporium*. Lane 6, *Methylosinus sporium*. Lane 7, *Methylocella palustris*. Lane 8, *Methylocella tundrae*. Lane 9, *Methylocella silvestris*. Lane 10. Negative control (Non-template control). M = 1 kbp DNA ladder.

The primers were then tested (annealing temperature at 68° C; 30 cycles) with DNA extracted from environmental samples to see whether they are able to detect *mmoX* of *Methylocella* spp. from the environment. DNA extracted from Moor House peat (UK) and Ufton landfill cover soil (UK) was then tested to amplify *mmoX* of *Methylocella* spp. The primers were not able to amplify *mmoX* following 30 cycles of PCR. Therefore, a second round of PCR (30 cycles; consecutive PCR) was carried out using the same primer set, where 1 µl of the PCR product generated in the first round was used as the template for the second round of PCR. This approach (total 60

cycles) allowed the detection of *mmoX* from *Methylocella* spp. from DNA extracted from the Moor House peat soil **(Figure 3.6)**.

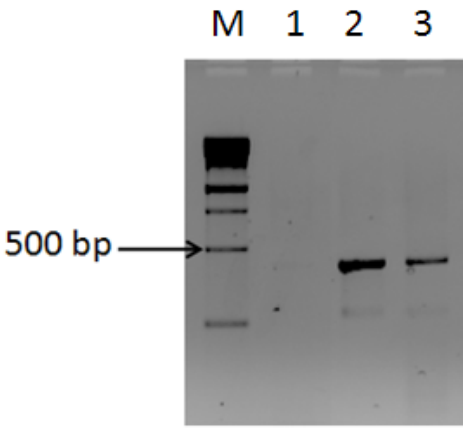


Figure 3.6. Amplification of *mmoX* specific for *Methylocella* spp. from environmental DNA using the two consecutive numbers of PCR approach. Lane 1, Negative control (Non-template control). Lane 2, Positive control (*Methylocella silvestris* genomic DNA). Lane 3, Moor House peat soil (UK). M = 1 kbp DNA ladder.

No amplification of *mmoX* was observed from DNA extracted from the Ufton landfill cover soil. The presence of *mmoX* sequences specific for *Methylocella* spp. in the amplified PCR products originating from the Moor House peat soil was confirmed by clone library analysis.

3.2.3 Specificity of the primers

Specificity of the primers TypeIIF/Mcell-1445 and mmoXLF/mmoXLR used to detect 16S rRNA and *mmoX* genes of *Methylocella* spp. respectively, were tested by PCR using pure culture methanotroph genomic DNA as the template. Results obtained revealed that both the *mmoX* and 16S rRNA gene primers were highly specific to target *mmoX* and 16S rRNA genes specific for all three species of the genus *Methylocella* respectively. No PCR products were amplified from genomic DNA from the other pure cultures of methanotrophs. The specificity of these primers to detect target 16S rRNA and *mmoX* genes from environmental samples were tested by PCR where DNA samples extracted from environmental samples were used as the PCR templates. Clone library analysis of amplified products originating from DNA extracted from environmental samples revealed that, about 82% of the 16S rRNA genes sequences were closely related (>97% identity) to *Methylocella* spp. while the remaining were related to the genera *Methylosinus, Methylobacter, Acinetobacter, Bradyrhizobium, Flexibacter, Hyphomicrobium* and unculturable bacterium (**Table 3.1**). Based on the *mmoX* clone libraries constructed from DNA extracted from environmental samples it is evident that 86% of the sequences were closely related (>97% identity) to *Methylocella* spp. –like *mmoX* sequences and the remaining *mmoX* sequences were related to *mmoX* from *Methylococcus, Methylobacter, Methylomonas* and unculturable bacterium (*i.e.* database sequences of *mmoX*) (**Table 3.2**).

Table 3.1. Percentage distribution of 16S rRNA genes retrieved in clone libraries constructed using *Methylocella* 16S rRNA genes specific primers Type IIF and Mcell-1445 and DNA extracted from various environmental samples.

Sample Name	Total no. of clones analyzed	*Methylocella* spp.	*Methylosinus* spp.	*Methylobacter* spp.	*Acinetobacter* spp.	*Flexibacter* spp.	*Bradyrhizobium* spp.	*Hyphomicrobium* Spp.	Unculturable bacterium
Cloud forest soil, San Pedro (Peru)	48	42	0	4	0	1	0	0	1
Moor House peat (UK)	15	7	6	0	0	0	0	2	0
Forest soil (Hornaven, Sweden)	34	34	0	0	0	0	0	0	0
Arctic soil (Arctic)	24	22	0	0	0	0	1	0	1
Colne estuary (UK)	31	21	0	8	0	0	1	0	1
Lonar lake (India)	34	28	0	6	0	0	0	0	0
Arable land, Aberdeen (UK)	17	14	0	0	3	0	0	0	0
Total (%)	203	168 (82.75 %)	6 (2.95 %)	20 (8.86 %)	3 (1.47 %)	1 (0.49 %)	2 (0.98 %)	2 (0.98 %)	3 (1.48%)

Table 3.2. Percentage distribution of *mmoX* genes retrieved in clone libraries constructed using *Methylocella mmoX* genes specific primers mmoXLF and mmoXLR and DNA extracted from various environmental samples.

Sample Name	Total no. of clones analyzed	*Methylocella* spp.	*Methylococcus* spp.	*Methylobacter* spp.	*Methylomonas* spp.	Unculturable bacterium
Moor House (UK)	42	41	0	0	0	1
Forest soil (Hornaven, Sweden)	27	24	3	0	0	0
Lonar lake (India)	27	19	0	1	7	0
Cloud forest (San Pedro, Peru)	14	11	0	3	0	0
Total	110	95	3	4	7	1
%		86.36%)	(2.72 %)	(3.63%)	(6.36%)	(0.90%)

3.2.4 Surveys for the presence of *Methylocella* spp. in the environment

DNA extracted from various environmental samples was screened by PCR for 16S rRNA genes of *Methylocella* spp. using the newly optimized PCR protocols. Results of the PCR based survey are presented in **Table 3.3.** PCR products of 16S rRNA genes of *Methylocella* spp. were observed with all the DNA samples extracted from soil and sediment, except for DNA extracted from clay soil (Sourhope, UK), Montane cloud forest soil (Wayqecha and Pillahuata, Peru) and Ufton landfill cover soil (UK). In addition, DNA extracted from Atlantic Ocean sea water (three different locations, **Table 3.3**), Movile Cave water (Romania) and the Colne Estuary, Brightlingsea, Essex, UK was found to be negative *i.e.,* no PCR products of 16S rRNA genes of *Methylocella* spp. The presence of 16S rRNA genes of *Methylocella* spp. in DNA extracted from Moor House peat soil (UK), Lonar lake sediment (India), Cloud forest soil (San Pedro, Peru), Arctic soil (Svalbard, Arctic), Colne Estuary sediment (Essex, UK), forest soil (Hornaven, Sweden) and Arable land (Aberdeen, UK) was confirmed by clone library analysis (**Table 3.3**). Representative sequences for the 16S rRNA genes of *Methylocella* spp. detected in these environments are shown in **Figure 3.7**. In addition, DNA samples extracted from Arctic soil (Svalbard, Arctic), Moor House peat soil (UK), Cloud forest soil (San Pedro, Peru), Rain forest (Tono, Peru), Swedish islands forest soil (Uddjaure and Hornaven island, Sweden), Colne Estuary (Essex, UK), and Lonar lake sediment (India) was found to be positive for PCR products of *mmoX* of *Methylocella* spp. The presence of *mmoX* genes related to *mmoX* from *Methylocella* spp. in Moor House peat soil (UK), Lonar lake sediment (India), Hornaven island forest soil (Swedish) and cloud forest soil (San Pedro, Peru) was confirmed by clone library analysis (**Table 3.3**). In addition, *mmoX* sequences retrieved by quantitative real-time PCR from Moor House peat soil

using the primers mmoXL and mmoXLR was also confirmed by clone library analysis (**Figure 3.8)** (Discussed in **Chapter 4**). The phylogenetic affiliation of these *mmoX* sequences retrieved from these environments is shown in **Figure 3.8**.

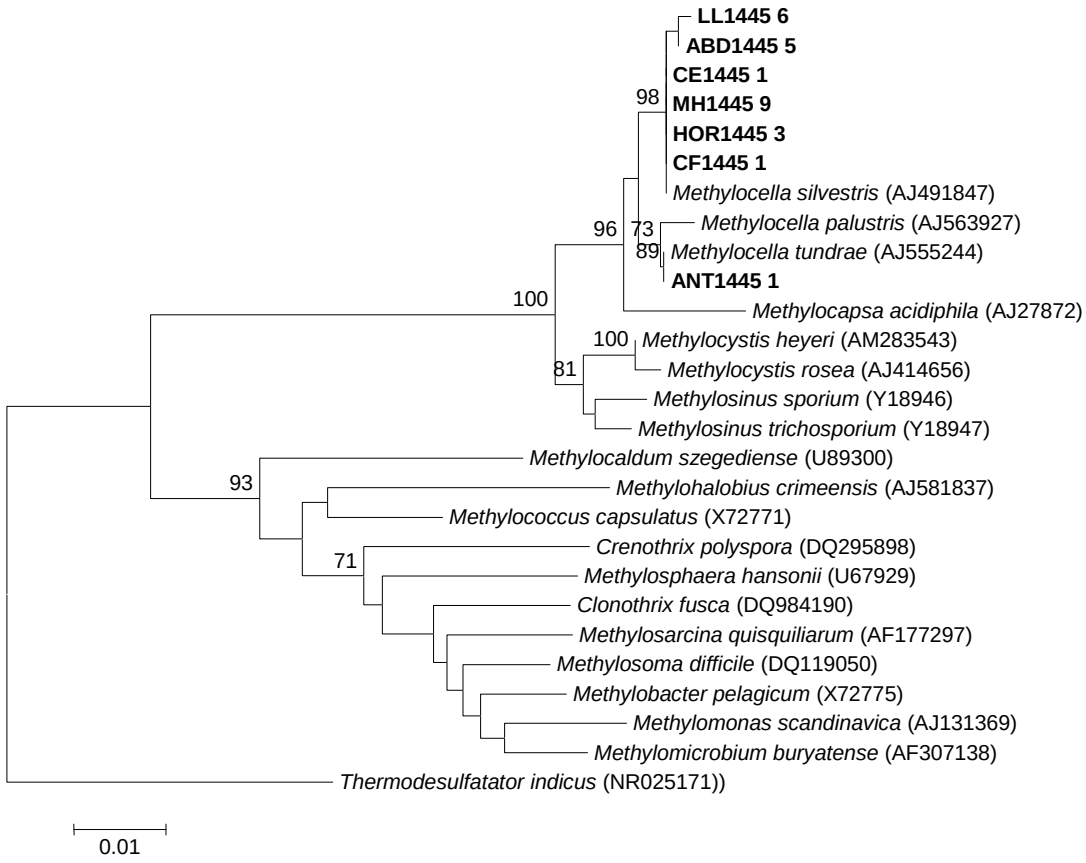


Figure 3.7. Neighbor-joining phylogenetic tree of 16S rRNA gene sequences (in bold) obtained from environmental samples and the closely related 16S rRNA sequences available in the public databases (Accession numbers are in parentheses). The tree was generated with a final data set of 469 nucleotide positions. The scale bar represents 1% sequence divergence. The numbers at the nodes represent bootstrap values (≥70) observed with 1000 replicates (covering the region of 445 to 1445 nucleotide position of *Methylocella silvestris* 16S rRNA gene). The tree is rooted with the 16S rRNA gene sequences from *Thermodesulfatator indicus* (NR025171). The 16S rRNA gene sequences retrieved from the Moor House peat soil (UK) are indicated as as MH, Lonar lake (India) as LL, Arable land, Aberdeen (UK) as ABD, Cloud forest (San Pedro, Peru) as CF, Colne Estuary (UK) as CE, forest soil of Hornaven island (Sweden) as HOR, and Arctic as ANT.

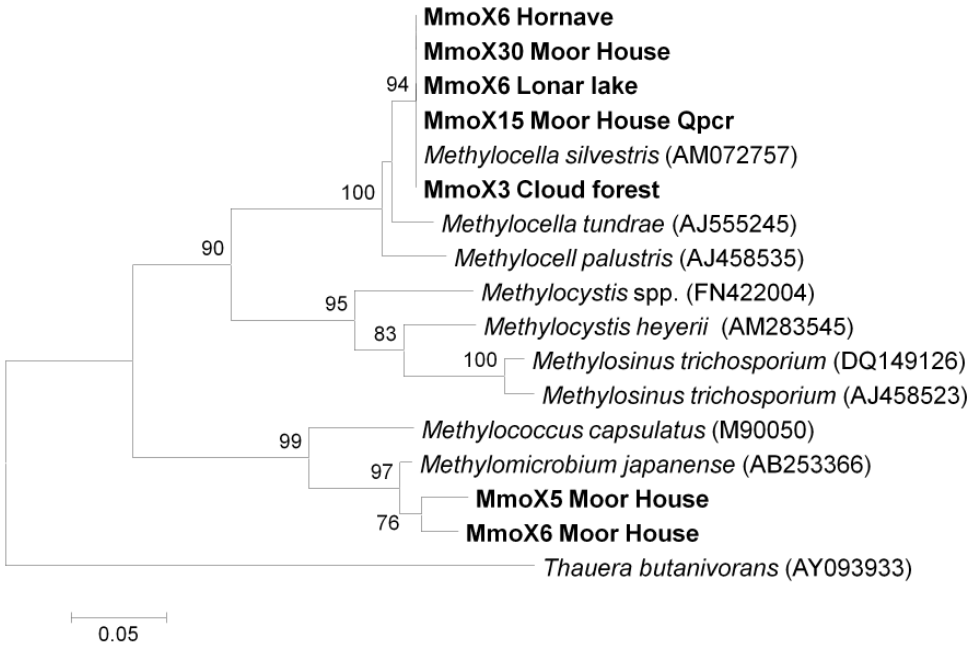


Figure 3.8. Neighbour-joining phylogenetic tree of deduced MmoX sequences (116 amino acids) (in bold) retrieved from the *mmoX* clone library generated from DNA extracted from various environmental samples and the closely related *mmoX* reference sequences available in the public databases (Accession numbers are in parentheses). The scale bar represents 0.05 substitutions per amino acid. The numbers at the nodes represent bootstrap values (≥70) observed with 1000 replicates. The tree is rooted with the partial butane monooxygenase sequence (BmoX) from *Thauera butanovora* (AY093933). The MmoX sequences retrieved from the Moor House peat soil are named as Moor House, Hornave island forest soil (Swedish) named as Hornaven, Lonar lake sediment (India) as Lonar lake, Cloud Forest soil, Peru named as Cloud Forest and the MmoX sequence retrieved by quantitative real-time PCR from Moor House peat soil is indicated as Moor House Qpcr.

Table 3.3. PCR-based survey for *Methylocella* in environmental samples.

Country and location	Latitude and longitude	Vegetation cover	Site description (pH)	*Methylocella* (16S rRNA genes; amplicon size ~ 1 kbp)			*Methylocella* (*mmoX* genes; (amplicon size ~ 450 bp))	
				Direct PCR approach	Nested PCR approach	Confirmed by clone library*	PCR	Confirmed by clone library
Aberdeen, UK	57° 11' N, 2° 12' W	*Solanum tuberosum* L.	Arable land (4.5)	-	+	N.T.	N.T.	N.T.
			Arable land (7.5)	-	+	+	N.T.	N.T.
Ufton landfill, UK	52° 25' N, 1° 44' W	Grassland	Landfill cover soil (7.4)	-	-	N.A.	-	N.A.
Austria	Not recorded	Grassland	Landfill cover soil (7.4)	-	+	N.T.	-	N.A.
Svalbard, Arctic	Not recorded	Not available	Arctic soil (6.2)	+	+	+	+	N.T.
Sourhope, UK	55° 28′ N, 2°14′ W	*Agrostis capillaris* L., *Festuca rubra* L., *Nardus stricta* L.	Upland grassland (7.7)	-	-	N.A.	-	N.A.
Moor House, UK	54° 42′ N, 2° 18′ W	*Sphagnum* spp., *Eriophorum* spp.,	Peatland (4.3)	+	+	+	+	+

		Calluna vulgaris L.						
Xistral, Spain	42° 48′ N, 8° 6′ W	*Eriophorum angustifolium* L., *Carex durieui* L.,, *Carex echinata* L.,, *Molinia caerulea* L.,, *Erica mackaiana* L.,, *Calluna vulgaris* L.	Peatland (4.3)	-	+	N.T.	-	N.A.
Finland	62° 11′ N, 25° 40′ W	*Betula pubescens* Ehrh., *Picea abies* L., *Sphagnum angustifolium* L., *Sphagnum girgensohnii* L., *Polytrichum commune* Hedw., *Carex* spp., *Calamagrostis purpurea* Trin., *Equisetum sylvaticum* L, *Vaccinium myrtillus, Vaccinium vitis-idaea* L.	Boreal forest soil (5.9)	-	+	N.T.	-	N.A.
Wales, UK	52º 26´ N, 3º 46' W	*Sphagnum* spp. L., *Eriophorum* spp. L., *Calluna vulgaris* L.,	Peatland (4.6)	-	+	N.T.	-	N.A.
Peruvian Andes, Peru								

Wayqecha	13° 11′ S , 71°24′ W	*Clusiaceae* spp., *Cunoniaceae* spp.,	Montane cloud forest soil (5.5)	-	-	N.A.	-	N.A.
Pillahuata	13° 7' S, 71° 25′ W	*Cunoniaceae* spp.	Montane cloud forest soil (6.2)	-	-	N.A.	-	N.A.
San Pedro	13°02′ S, 71°32′ W	*Clethraceae* spp.	Cloud forest soil (4.8)	+	+	+	+	+
Tono	12° 53′ S, 71° 33′ W	*Elaeocarpaceae* spp., *Fabaceae* spp.	Highland rainforest soil (4.9)	+	+	N.T.	+	N.T.
Tambopata	12° 49′ S, 69° 16′ W	*Moraceae* spp., *Fabaceae* spp.	Lowland rainforest soil (6.4)	-	+	N.T.	N.T.	N.T.
Sweden								
Hornaven	66° 13′ N, 17° 78′ W	*Vaccinium myrtillus* L., *Vaccinium vitis-idaea* L., *Empetrum hermaphroditum* L., *Pinus sylvestris* L., *Betula pubescens* Ehrh., *Picea abies* L.	Boreal forest soil (4.5)	+	+	+	+	+
Uddjaure	65° 60′ N,	*Vaccinium myrtillus* L., ,	Boreal forest soil	+	+	N.T.	+	N.T.

	17° 48′ E	*Vaccinium vitis-idaea* L., *Empetrum hermaphroditum* L., *Pinus sylvestri* L., *Betula pubescens* Ehrl., *Picea abies* L.	(4.5)					
Movile Cave, Romania	43° 82′ N, 28° 55′ E	N.A.	Fresh water (7.5)	-	-	N.A.	-	N.A.
Lonar lake, India	19° 97′ N, 76° 51′ E	N.A.	Sediment (10.0)	-	+	+	+	+
Colne Estuary, Colchester, Essex, UK	51° 82′ N, 0° 98′ E	N.A.	Marine sediment (7.2)	-	+	+	+	N.T.
Colne Estuary, Brightlingsea, Essex, UK	51° 80′ N, 1° 02′ E		Marine sediment (7.1)	-	-	N.A.	-	N.A.
Atlantic Ocean								
	42° 04′ N, 22° 11′ W	N.A.	Sea water (7.4)	-	-	N.A.	-	N.A.

	2° 47′ S, 26° 51′ W	N.A.	Sea water (7.4)	-	-	N.A.	-	N.A.
	26° 33′ N, 24° 59′ W	N.A.	Sea water (7.4)	-	-	N.A.	-	N.A.

N.A. = Not Applicable, N.T.=Not Tested, + = Presence , - =Absence

* = Number of clones positive for *Methylocella* out of total number of clones analyzed

The salinities of Colne Estuary sediment samples collected from Colchester (Essex, UK), Brightlingsea (Essex, UK) and the Atlantic Ocean sea water were approx 1 g NaCl L^{-1}, 20 g NaCl L^{-1} and 35 g NaCl $^{-1}$ respectively.

3.3 Discussion

In this study, PCR conditions were optimized and validated with specific primers to selectively amplify *mmoX* and 16S rRNA genes of *Methylocella* spp. from DNA extracted from environmental samples. Primer Type IIF primer (Chen *et al.*, 2007) was used as the forward primer to detect 16S rRNA genes of *Methylocella* spp. in combination with reverse primer Mcell-1445 (Dedysh *et al.,* 2005). At an annealing temperature of 63º C, primers Type IIF and Mcell-1445 detected 16S rRNA genes of *Methylocella* spp. from all pure culture genomic DNA of three species of the genus *Methylocella,* indicating the high specificity of these primers for 16S rRNA gene of *Methylocella* spp. (**Figure 3.1**). In addition, these primers were also able to amplify 16S rRNA genes of *Methylocella* spp. from DNA extracted from environmental samples (**Figure 3.2 and 3.3)** both by direct and nested PCR approaches. Clone library analyses of amplified 16S rRNA genes retrieved from DNA extracted from environmental samples indicated that 82% of the retrieved 16S rRNA gene sequences were related to *Methylocella*-like sequences, suggesting a high specificity of these primers for the *Methylocella* spp. However, the remaining 18% of the retrieved 16S RNA gene sequences were related to non-*Methylocella* 16S rRNA genes, suggesting a certain degree of non-specific target amplification by these primers under the PCR conditions developed here. Detection of non-target sequences by genus or group specific primers is not uncommon, particularly from complex environments such as soil. Fierer *et al.* (2005) used DNA extracted from soil to study the specificity of several primers sets that targeted different groups of bacteria and fungi, and observed the detection of a certain level of non-target sequences by some of these primers. In their study they detected between 4% and 40% non-target amplificons using primers designed to target the Firmicutes, *Actinobacteria* and

Alphaproteobacteria. It has been suggested that environmental contaminants can affect both the efficiency and the specificity of PCR and therefore targets that do not exactly match the primer sequences may be amplified (Stach *et al.*, 2001). As the primers and PCR conditions used here can thus detect non-*Methylocella* 16S rRNA genes, the presence of amplified PCR products from DNA extracted from environmental samples should not be considered as absolutely positive for *Methylocella* spp. and results have to confirmed by 16S rRNA gene clone library analysis.

The findings from the survey for the environmental distribution of *Methylocella* spp. are presented in **Table 3.3**. Among the 23 samples originating from diverse locations, *Methylocella*-related 16S rRNA gene PCR products were detected in 15 samples. Of these, six samples were positive for *Methylocella*-related 16S rRNA genes using the direct PCR approach, suggesting a higher relative abundance of *Methylocella* spp. in these samples compared to others, where a nested PCR approach was required. DNA extracted from clay soil (Sourhope, UK), Montane Cloud forest soil (Wayqecha and Pillahuata, Peru) and Ufton landfill cover soil (UK) was negative for *Methylocella*-related 16S rRNA genes, both by direct and nested PCR approaches, suggesting that either there were no *Methylocella* spp. in these environments or they were present in low abundance and could not be detected in the PCR assays. In support of these findings, the diversity of methanotrophs in Ufton landfill cover soil (UK) was analyzed by Héry *et al.*, (2008) and their 16S rRNA gene clone library analyses did not detect any *Methylocella*-related 16S rRNA genes from this environment.

Using PLFA-SIP and DNA-SIP, Chen and colleagues (2008a; 2008b) identified *Methylocella* as one of the active methanotrophs in Moor House peat soil. In this

study, 16S rRNA genes of *Methylocella* spp. were detected in DNA extracted from the Moor House peat soil by both direct and nested PCR approaches, which confirms the earlier observations of Chen *et al.* (2008a; 2008b). In addition, DNA extracted from forest soil originating from the Hornaven and Uddjaure islands of Sweden was found to be positive for the presence of 16S rRNA genes of *Methylocella* spp. *Methylocella*-like 16S rRNA gene sequences have been detected previously in DNA extracted from Harvard forest soil, USA (Lau *et al.*, 2007). The distribution and diversity of methanotrophs in nature has been studied extensively (Bodrossy *et al.*, 1997; Gilbert *et al.*, 2000; Jensen *et al.*, 2000; Radajewski & Murrell, 2002; Radajewski *et al.*, 2002; Dunfield *et al.*, 2003; Hutchens *et al.*, 2004; Lin *et al.*, 2004; Dumont & Murrell, 2005; Cébron *et al.*, 2007b; Cébron *et al.*, 2007a; Chen *et al.*, 2007; Chen *et al.*, 2008a; Héry *et al.*, 2008; Han *et al.*, 2009). Many of these studies revealed that the presence of *Methylocella* spp. is mostly associated with acidic environments such as acidic peatlands and acidic forest soil (Murrell & Radajewski, 2002; Radajewski *et al.*, 2002; Dunfield *et al.*, 2003; Chen *et al.*, 2007; Chen *et al.*, 2008a). In this study, 16S rRNA genes of *Methylocella* spp. were detected not only in DNA extracted from acidic environments *e.g.*, Moor House peat soil (pH 4.3) (UK), Boreal forest soil (pH 5.9) (Finland) and peat soil (pH 4.3) (Spain), but also in DNA extracted from neutral environment *e.g.*, landfill soil (pH 7.4) (Austria) (**Table 3.3**). 16S rRNA genes of *Methylocella* spp. were even detected in alkaline environment *e.g.*, in DNA extracted from Lonar lake sediment (India), which has a pH of ~ 10. Detection of *Methylocella* in alkaline environments as revealed in the present study supports the recent work by Han *et al.* (2009) and Fabiani *et al.* (2009a). Using $^{13}CH_4$ DNA-SIP, Han *et al.* (2009) detected sequences related to *Methylocella* spp. as one of the active methanotrophs in alkaline soil from

a Chinese coal mine (pH 9). Fabiani *et al.* (2009a) recently also detected *Methylocella* spp. as one of the active bacteria in alkaline soil (pH 9) collected from a waste treatment plant, Italy. One of the soil samples analyzed in the present study originated from the Arctic region, which is an extremely cold place on Earth. DNA extracted from Arctic soil was found to be positive for *Methylocella* 16S rRNA. However, it was not a surprise to detect *Methylocella* spp. in such extreme environments. Berestovskaia *et al.* (2002) reported the presence of *Methylocella palustris* in Russian Arctic tundra. In addition, Schütte *et al.* (2009) recently detected *Methylocella*-related 16S rRNA genes sequences from DNA extracted from glacier foreland of the High Arctic through pyrosequencing.

DNA extracted from Movile Cave water (Romania) and Atlantic Ocean sea water (obtained from Professor Dave Scanlan's laboratory, University of Warwick) were also screened by PCR and found to be negative for 16S rRNA genes of *Methylocella* spp. (**Table 3.1**). Chen *et al.* (2009) studied microbial diversity in DNA extracted from Movile Cave (Romania) sediments and water and did not detect any *Methylocella* 16S rRNA genes from such samples. It is interesting to note that in this current study, no 16S rRNA genes for *Methylocella* spp. were detected from high salinity environments (Colne Estuary, Brightlingsea, Essex, UK, and Atlantic Ocean sea water), nor have there been (to our knowledge) any previously published reports of marine *Methylocella* sequences, suggesting that salinity may restrict the diversity of this genus. We found *Methylocella* in soils associated with a wide diversity of vegetation (**Table 3.3**), therefore these data do not permit any conclusions to be drawn regarding particular *Methylocella*-plant associations.

In addition to the *Methylocella* spp.-specific 16S rRNA gene PCR protocol, a new set of primers mmoXLF and mmoXLR, were designed and validated to target *mmoX* genes from all three species of the genus *Methylocella.* These *mmoX* primers were later used to develop a quantitative real-time PCR to quantify populations of *Methylocella* spp. in environmental DNA samples **(discussed in Chapter 4).**

In conclusion, diagnostic PCR protocols were developed and applied to selectively target *mmoX* and 16S rRNA genes of *Methylocella* spp. from DNA extracted from environmental samples. Findings of the *Methylocella* survey work described in this chapter clearly demonstrate that *Methylocella* spp. are more widespread in nature than previously thought and acidic pH is not a limiting factor for their occurrence in the environment. However, the present study was carried out with DNA as the template, and this does not allow one to link the presence of *Methylocella* spp. with their activity. A template derived from environmental mRNA would be ideal to understand their function in such environments.

Chapter 4

Development and validation of a real-time quantitative PCR assay to quantify *Methylocella* spp. from the environment

4.1 Introduction

Members of the genus *Methylocella* are facultative methanotrophs. Recent studies have identified *Methylocella* spp. as an active group of methane-oxidizing bacteria in soil, particularly in peatlands, where they may play a role in methane oxidation (Chen *et al.*, 2008b; Chen *et al.*, 2008c). Under laboratory conditions *Methylocella* spp. can utilize multi-carbon compounds such as succinate, malate, pyruvate and acetate as carbon and energy sources in addition to methane (Dedysh *et al.*, 2005; Theisen *et al.*, 2005). Acetate is a potential nutrient for *Methylocella* spp. present in soil, and indeed, acetate represses the transcription of sMMO of *Methylocella silvestris* when grown in the laboratory (Dedysh *et al.*, 2005; Theisen *et al.*, 2005). It may thus be possible that in soil, *Methylocella* spp. can utilize acetate rather than methane as a carbon source. The effects of acetate present in soil and other soil parameters such as soil type, pH and depth upon the population dynamics of *Methylocella* spp. population and their methane oxidation potential is not known due to the lack of suitably sensitive assays for the quantification of *Methylocella* spp. from environmental samples.

Studying the dynamics of microbial populations present in a complex environment requires specific and efficient detection and quantification of the target organisms. While conventional PCR does not permit quantitative detection of the target organisms, real-time quantitative PCR (qPCR) with genus/species-specific primers can provide precise quantification through measurement of the fluorescence of the amount of PCR products produced in each cycle (Sakamoto *et al.*, 2001). qPCR techniques involve either the application of double-stranded DNA-binding fluorescent dye SYBR® Green I or a fluorescent probe *e.g.*, a TaqMan probe.

TaqMan probe-based approaches are generally more sensitive compared to SYBR® green-based approaches, although there are reports suggesting that the sensitivity of particular SYBR® green-based approaches could be equally sensitive as that of the TaqMan probe-based approach (Papin *et al.*, 2004). However, the TaqMan probe-based approaches are more expensive and involves designing specific probes which could often be difficult (Huang *et al.*, 2009). The major advantage of the SYBR® Green-based qPCR assay is that the available primers used in the traditional PCR detection could be readily transferred to a real-time assay without having to design new primers and probes. In addition, there are reports suggesting that the SYBR® Green-based qPCR assay is more tolerant of polymorphic targets and as a result, it could be beneficial for environmental samples in which the target may show slight genetic variation (Audemard *et al.*, 2004).

An alternative approach to quantify cells is fluorescence *in situ* hybridization (FISH). There are FISH probes such as Mcell-1026, Mcell-1420, Mcell-1445 and H158 which are specific for *Methylocella* spp. (Dedysh *et al.*, 2001; Dedysh *et al.*, 2005), but the difficulty in using FISH technique with soil concern high background auto-fluorescence of soil materials and the potential difficulty of low numbers of ribosomes (Rogers *et al.*, 2007). qPCR assays targeting *pmoA* have been used for the quantification of methanotrophs in environmental samples (Kolb *et al.*, 2003; Tuomivirta *et al.*, 2009). In addition, a *pmoA* diagnostic microarray has been developed, which provides a semi-quantitative method to study methanotrophs in environmental samples (Bodrossy *et al.*, 2003). However, neither of these methods would detect *Methylocella* spp., since *Methylocella* lacks pMMO (Theisen *et al.*, 2005).

The aim of this chapter was to develop a *mmoX*-targeting qPCR assay to enumerate populations of *Methylocella* spp. present in the environment so that the assay can be applied later to study the effect of certain environmental parameters *e.g.,* soil pH and depth and availability of nutrients on the dynamics of *Methylocella* spp. populations in the environment.

4.2. Results

4.2.1. Primer selection

Primers mmoXLF and mmoXLR originally designed and validated to specifically target *mmoX* (amplicon size ~ 450 bp) from all species of the genus *Methylocella* (discussed in **Chapter 3**) validated at the time of the study were selected for the development of a quantitative real-time PCR (qPCR) assay. The use of the *mmoX* gene instead of the 16S rRNA gene narrows down the investigation to the functional group studied, thus enabling a much higher sensitivity of detection in a complex environment (McDonald *et al.*, 2008). The specificity of these primers to target *mmoX* of *Methylocella* spp. are described in **Chapter 3.** Over 86% of the *mmoX* sequences retrieved by these primers from DNA extracted from environmental samples are related to *Methylocella* spp. and although not perfect, could give a good proxy for their abundance in the environment.

4.2.2. Optimization of PCR conditions

A SYBR® green-based *mmoX*-targeting qPCR assay specific to *Methylocella* spp. was developed using a two step qPCR protocol. The qPCR assays were conducted in polypropylene 96-well plates on an ABI PRISM ® 7000 Sequence Detection System (Applied Biosystems). All PCR assays were carried out in triplicate in 25 µl containing 12.5 µl power SYBR® master mix (Applied Biosystems), 1 µl (10 µM; Invitrogen) of each mmoXLF and mmoXLR primers, 0.5 µl of 3.2% (w/v) BSA (Roche, Switzerland), 2 µl template DNA (1/10, 1/50 and 1/100 dilution of the total extracted DNA) and water 8 µl. Non-template controls (NTC) were also run in triplicate during each assay. These contained all of the PCR reagents except the

template DNA and the total volume was adjusted to 25 µl using sterile PCR grade water.

A two-step qPCR protocol was adopted, consisting of an initial denaturation step at 95º C for 5 minutes followed by 45 cycles of denaturation at 95° C for 15 seconds and combined annealing and elongation at 68° C for 1 min (**Table 4.1**). Annealing temperature was selected as 68° C based on earlier observations to ensure that the primers were as specific for *mmoX* of *Methylocella* spp. as they could be (**Chapter 3**).

Table 4.1. . Conditions of qPCR assays for quantitative detection of *Methylocella* spp.

Stages	Repetitions (number of cycles)	Temperature	Time (min)
1	1	94.0° C	5.00
2	1	94.0° C	0.15
3	45	68.0 °C	1.00
4	1	95.0° C	0.15
(Dissociation)		60.0° C	0.20
		95.0° C	0.15

Absolute quantification of *mmoX* copies was achieved by comparing the reaction Ct (threshold cycle) value with a standard curve, made from a dilution series of *Methylocella silvestris* BL2 genomic DNA ranging from 10^2 copies to 10^6 copies per reaction (**Figure 4.1**).

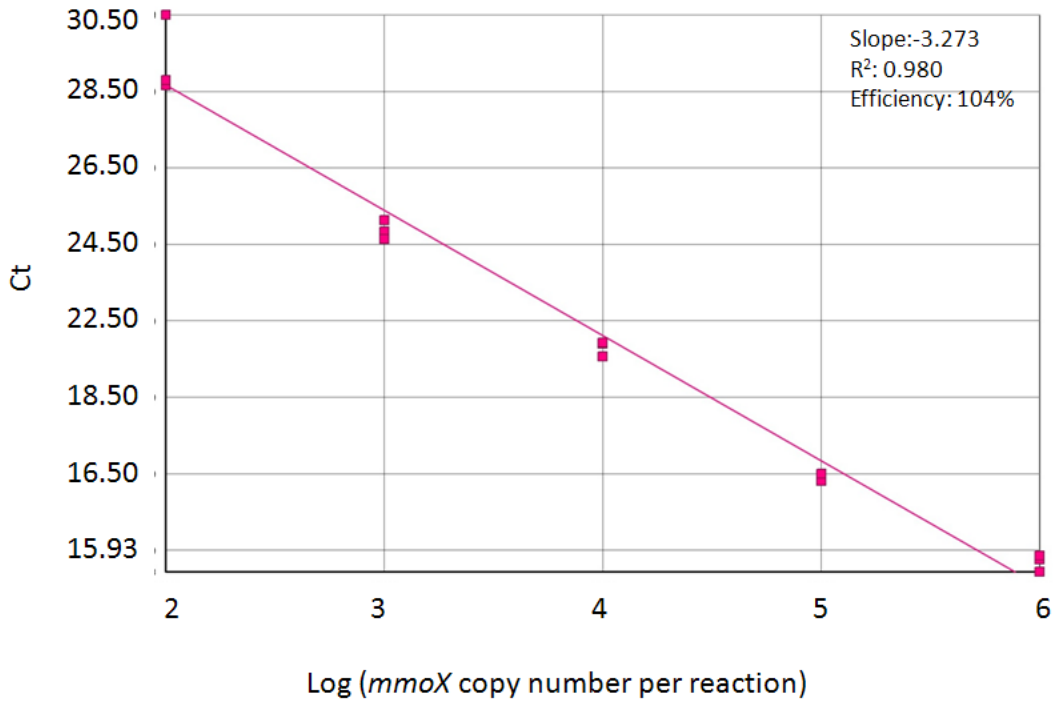


Figure 4.1. Standard curve of the Ct value vs. template DNA mass.

The concentration of genomic DNA was determined by using a ND-1000 NanoDrop spectrophotometer (NanoDrop™, USA) as described earlier. Gene copies were calculated according to the method described by Fogel *et al.* (1999) using the mass of the *Methylocella silvestris* genome (approximately 4.3 Mb). Only one copy of *mmoX* is present in the genome of *Methylocella silvestris* BL2 (accession number CP001280). Therefore we assume that the number of *mmoX* copies present in a particular sample represents the number of cells of *Methylocella* spp. in that sample. For each standard the concentration of DNA was plotted against the cycle numbers at which the fluorescence signal increased above the Ct value. The Ct value is the cycle number at which the fluorescence signal increased above the defined threshold value, calculated by the ABI PRISM ® 7000 Sequence Detection System software

(Applied Biosystems) (Heid *et al.*, 1996). The amplification efficiency of the assay was determined by the real-time instrument software based on the slope of the standard curve. A reaction with 100% efficiency generates a slope of -3.32. The amplification efficiency of the reactions observed in the *mmoX*-targeting qPCR was between 80 and 104% (slope -3.92 to -3.27) (**Figure 4.1**).

4.2.3. Detection limit

The detection limit of the assay was determined during the generation of a standard curve of *Methylocella silvestris* genomic DNA. To prepare template DNA containing known copies of *mmoX,* a 10-fold serial dilution of *Methylocella silvestris* genomic DNA was generated, starting from 10^1 to 10^6 copies of *mmoX* per reaction as described earlier. The detection limit of the assay was 10^2 copies of *mmoX* per reaction. A fluorescent amplification plot generated from one typical standard curve is presented in **Figure 4.2**. The detection efficiency of the assay from the spiked soil was 10^3 copies of *mmoX* per reaction (discussed later).

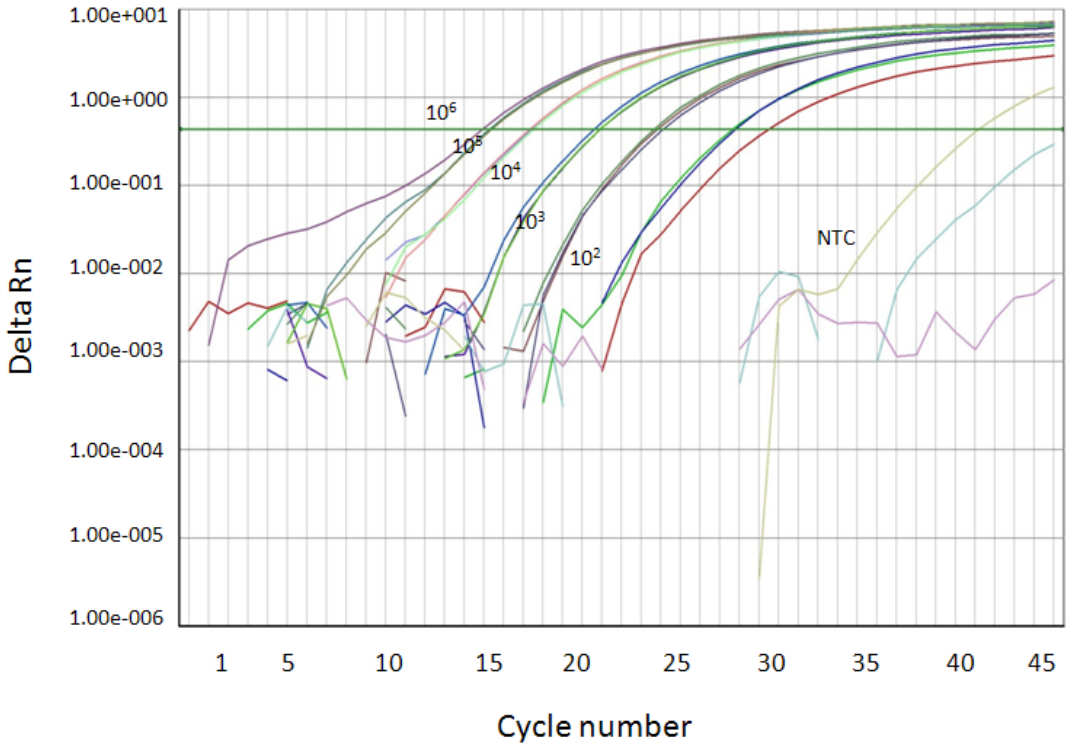


Figure 4.2. Fluorescence amplification plot of 10-fold serial dilutions (10^2 to 10^6 copies of *mmoX*) of *Methylocella silvestris* BL2 genomic DNA and the non-template control (NTC), showing the detection limit of 10^2 copies of *mmoX* per reaction.

4.2.4. Dissociation curve analysis

Amplification of specific *mmoX* amplicons and absence of primer dimer and non-specific amplicon formation in the qPCR assay was confirmed by dissociation curve analysis. During this process the temperature of the instrument was increased from 60° C to 95° C at approximately 2° C min^{-1} at the end of the assay and the loss of SYBR® green fluorescence signals during the heating processes of the PCR amplicons were acquired, processed and presented by the instrument as a graph. The presence of more than one peak in the graph represents non-specific amplification including primer dimer formation, while the presence of a single peak represents amplification of single PCR amplicon (**Figure 4.3**).

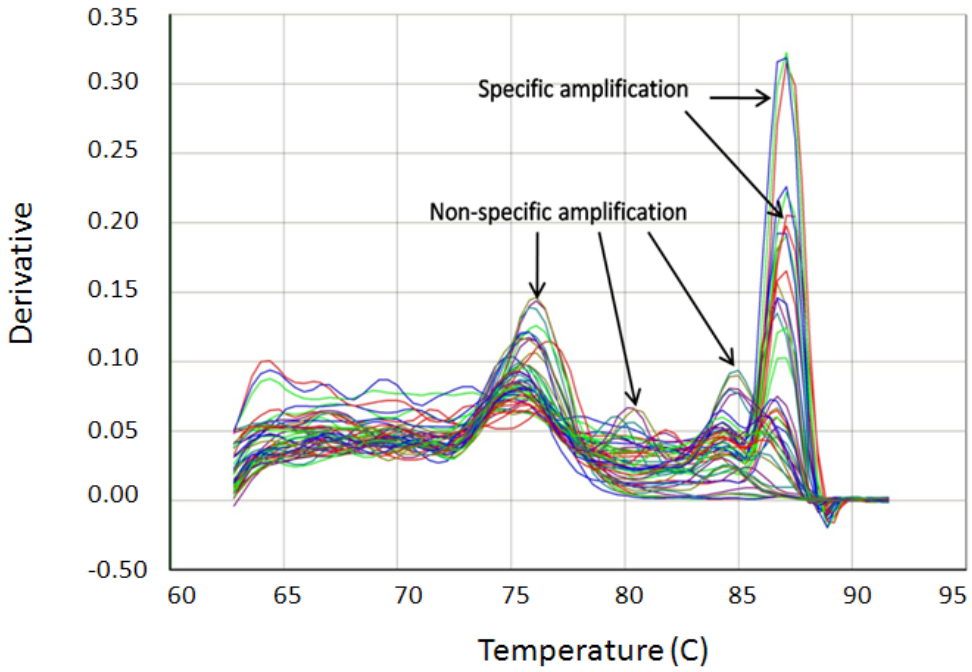


Figure 4.3. Dissociation curve analysis of qPCR products from amplification of *Methylocella silvestris* genomic DNA. Amplification of specific *mmoX* products are indicated by a sharp peak between 87 to 88° C, while non-specific amplification are indicated by different peaks at other temperatures.

Absence of non-specific amplification, including primer dimer formation, during the quantification of *mmoX* from DNA extracted from environmental samples is presented in **Figure 4.4.**

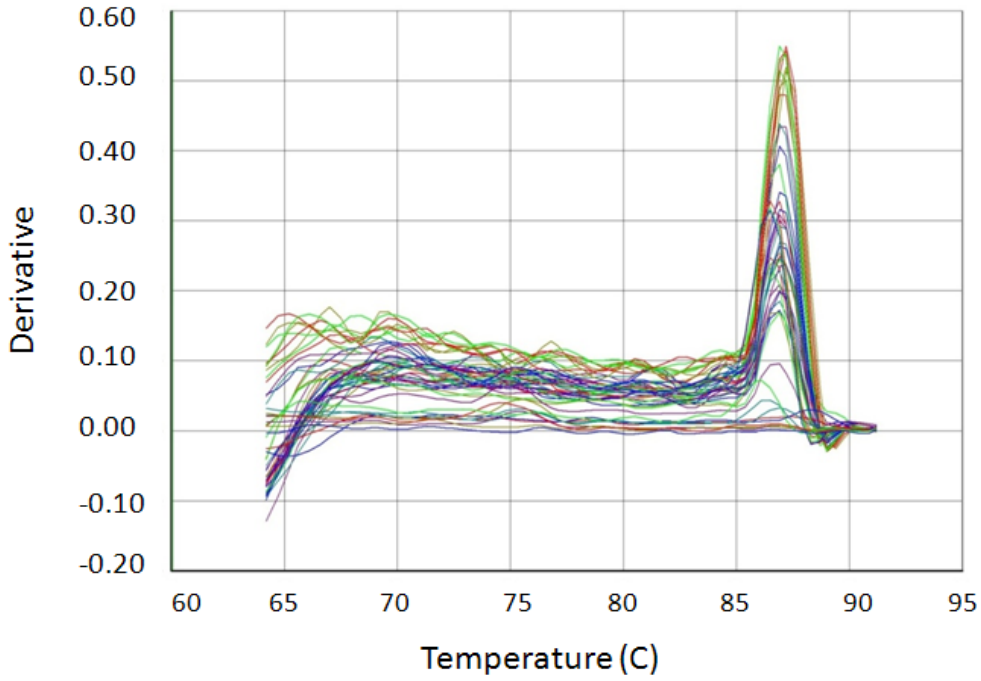


Figure 4.4. Dissociation curve analysis of qPCR products from amplifications of *Methylocella silvestris* genomic DNA and DNA extracted from environmental samples. Amplification of specific *mmoX* products is indicated by a sharp peak between 87° to 88° C.

In addition, qPCR products were run on an agarose in gel to check whether there were single amplicons of the correct size **(Figure 4.5).**

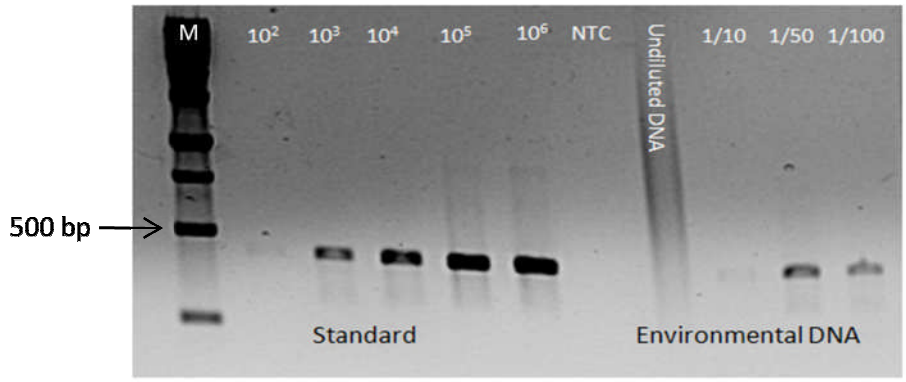


Figure 4.5. Effect of dilution of DNA extracted from soil on qPCR amplification. 1/10, 1/50 and 1/100 dilution of DNA extracted from soil was used as template for PCR amplification of *mmoX*. Standards represent 10^2 to 10^6 copies of *mmoX* of *Methylocella silvestris* per reaction. NTC is the non-template control. M is a 1 kbp DNA ladder. (Amplification of specific *mmoX* amplicon of correct size is evident from the standards).

4.2.5. Optimization of qPCR for template DNA extracted from the environment

One of the major obstacles in the use of soil DNA as PCR template is the frequent presence of PCR inhibitory substances such as humic acids. Humic acids can inhibit PCR, possibly by interacting with the DNA polymerase or preventing the binding of the primers to the template, and thereby greatly reducing the detection limit (Tsai & Olson, 1992). To overcome such inhibition, it is recommended to use BSA (Kreader, 1996). Therefore BSA was added (0.5 µl of 3.2% (w/v) per 25 µl reaction) to the PCR reactions to amplify *mmoX* from the DNA extracted from environmental

samples. However, to overcome this PCR inhibition, the template DNA was diluted (1/10, 1/50 and 1/100) to reduce the contaminating PCR inhibitors including the humic acid. In this study it was observed that 1/50 to 1/100 times dilution of the original soil extracted DNA in combination with BSA overcame this inhibition of PCR. The effect of dilution of template soil DNA on inhibition of PCR is shown in **Figure 4.5.**

4.2.6 Validation of the qPCR assay

The qPCR assay was validated with a study which involved spiking soil from Ufton landfill cover soil (UK) with *Methylocella silvestris*, because no *Methylocella* spp. could be detected from DNA extracted from the Ufton landfill cover soil (Hery *et al.*, 2008). Initially known amounts of *Methylocella silvestris* genomic DNA were added to each reaction (in triplicate) containing DNA extracted from the Ufton landfill cover soil to achieve 10^3, 10^4 and 10^5 copies of *mmoX* per reaction. The results showed a good match with the expected number of *mmoX* copies (**Figure 4.6**). Detection efficiency from the spiked soil was 89 to 104%. Subsequently, 5 g soil was spiked (in triplicates) with known amounts of *Methylocella silvestris* cells ranging from 10^3 to 10^5 cells g^{-1}. Five g soil was spiked (in triplicate) with known amounts of *Methylocella silvestris* cells ranging from 10^3 to 10^5 cells per g of soil. DNA was extracted from spiked soil using the FastDNA® SPIN Kit for soil (MP Biomedicals LLC, Irvine, CA, USA) and quantified using a NanoDrop spectrophotometer as described earlier.

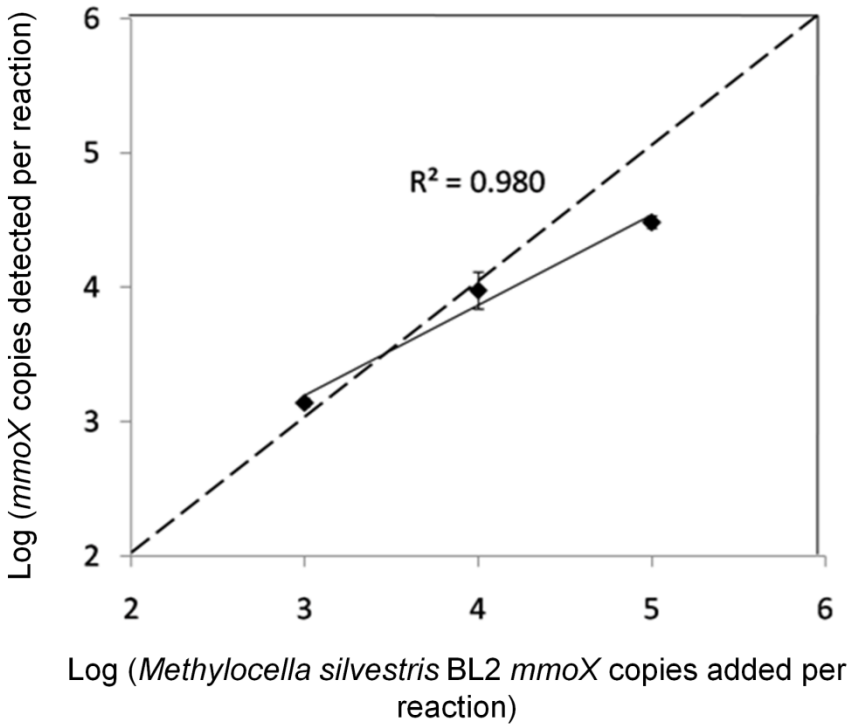


Figure 4.6. Detection of *mmoX* genes from Ufton landfill covered soil DNA spiked with *Methylocella silvestris* genomic DNA, ranging from 10^3 to 10^5 copies of *mmoX* per reaction. The dotted line shows the theoretical 100% detection efficiency. Error bars indicate the standard deviation of triplicate measurements.

The qPCR was done with different dilutions (1/10, 1/50 and 1/100) of the extracted DNA using the primers mmoXLF and mmoXLR to specifically determine the number of *Methylocella* spp. from the spiked soil. This showed a good match with the numbers of added *Methylocella* spp. cells. Detection efficiency from the spiked soil was 88 to 111% **(Figure 4.7)**. The detection limit of the assay from the spiked soil was 10^3 copies of *mmoX* per reaction.

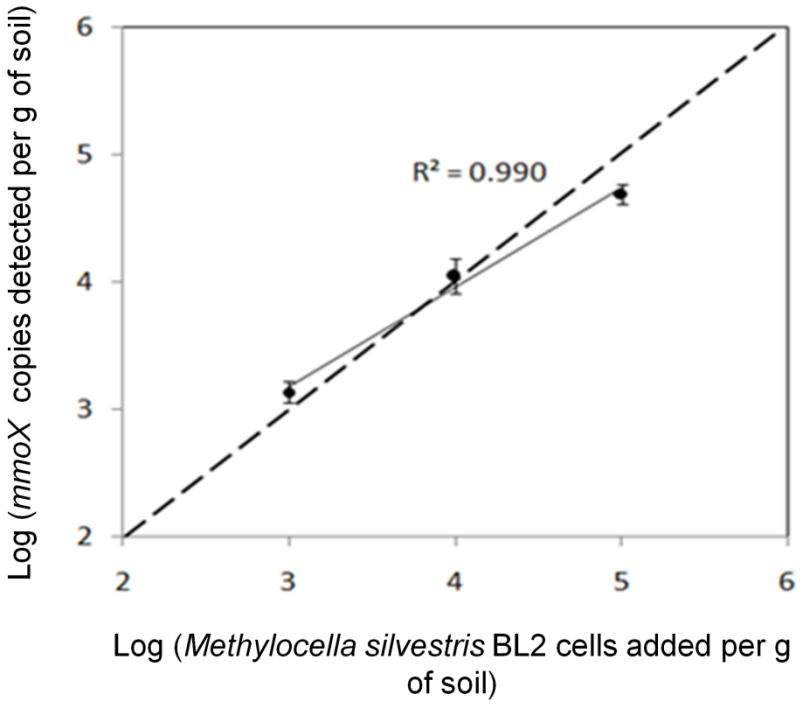


Figure 4.7. Detection of *mmoX* copies from Ufton landfill covered soil spiked with *Methylocella silvestris* cells ranging from 10^3 to 10^5 cells per g of soil. The dotted line shows the theoretical 100% detection efficiency. Error bars indicate the standard deviation of triplicate measurements.

4.2.7 Quantification of *mmoX* from environmental samples by qPCR

Quantification of the total number of *Methylocella* spp. present in Hornaven and Uddjaure island forest soils (Sweden), Moor House peat soil (UK), Colne Estuary sediment (Essex, UK), cloud forest soil (San Pedro, Peru) and rain forest soil (Tono, Peru) was carried out using the qPCR assay. The geographic locations of these samples are presented in **Chapter 3**. One representative amplification plot generated during the qPCR assay used to quantify *mmoX* from DNA extracted from environmental samples is presented in **Figure 4.8.** The abundance of *Methylocella* spp. detected in these selected environmental samples varied from 0.9 (± 0.2) × 10^6 copies of *mmoX* per g of soil (Colne Estuary sediment, Essex, UK) to 3.3 (± 0.6) × 10^6 copies of *mmoX* per g of soil (Hornaven, Sweden) (**Figure 4.9)**. In addition, DNA extracted from Lonar lake sediment (India), and Arctic soil was also subjected to qPCR, however, the data suggest that abundance of *Methylocella* spp. in these samples was below the detection limit of the present assay (*i.e.*10^3 copies of *mmoX* per g of soil).

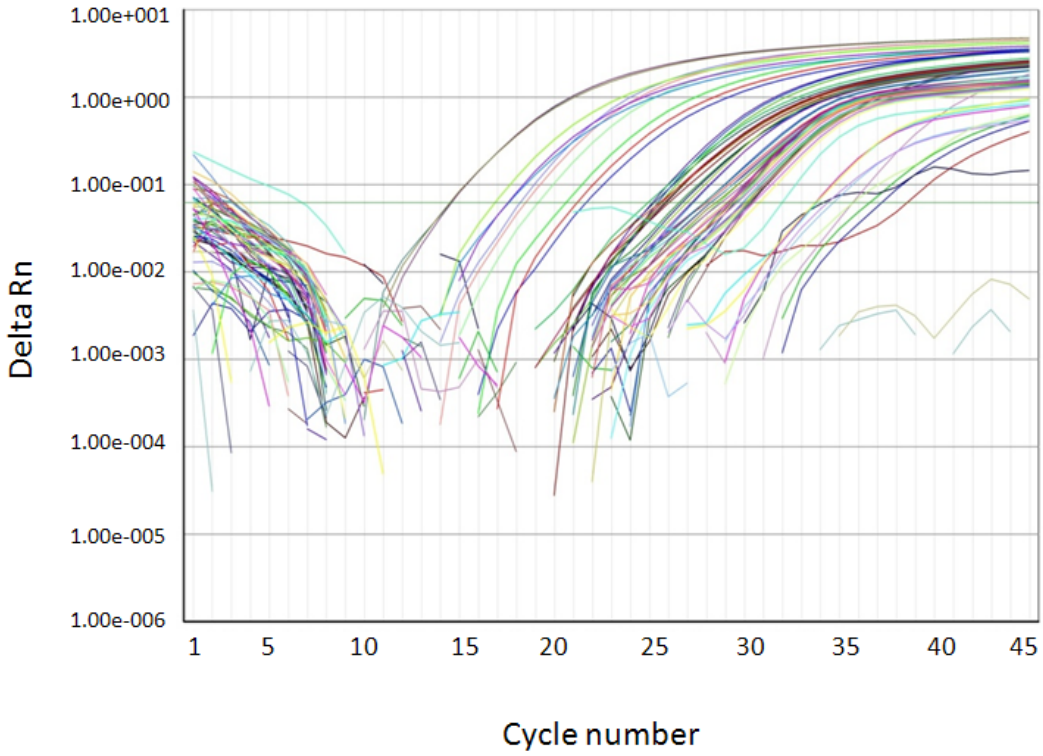


Figure 4.8. Fluorescence amplification plot generated during the quantification of *mmoX* from DNA extracted from environmental samples.

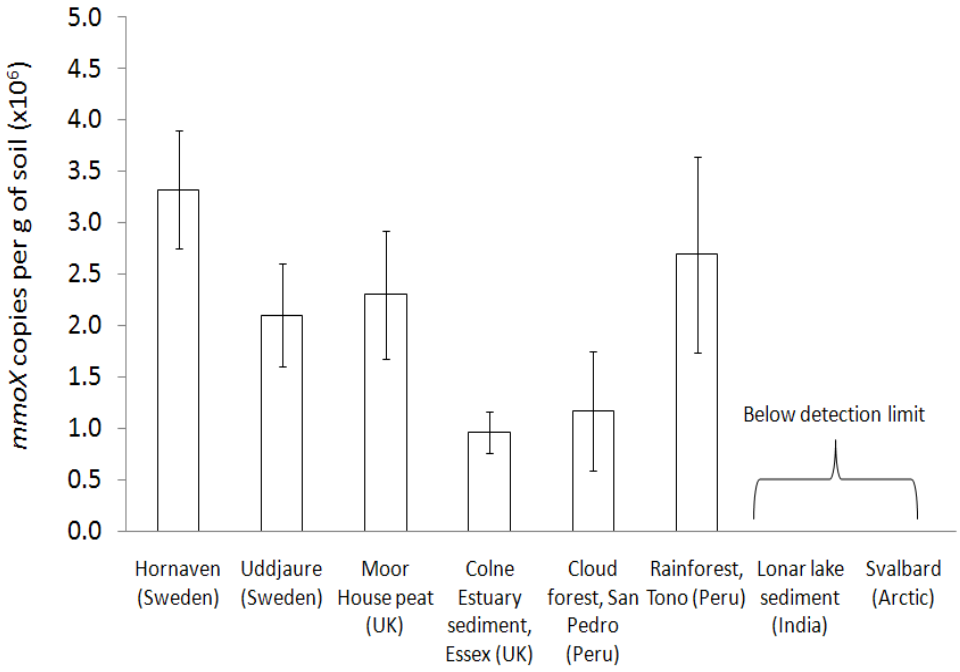


Figure 4.9. Number of *mmoX* copies detected in DNA extracted from soil and sediment. Error bars indicate the standard deviation of triplicate measurements.

4.3 Discussion

The qPCR assays currently available for quantifying methanotrophs targeting *pmoA* were first developed by Kolb *et al.* (2003). Several studies have applied this method for the quantification of methanotrophs from environmental samples (Kolb *et al.*, 2003; Kolb *et al.*, 2005; Knief *et al.*, 2006; Rahalkar & Schink, 2007; Chen *et al.*, 2008a; Colwell *et al.*, 2008; Rahalkar *et al.*, 2009). Recently Tuomivirta *et al.* (2009) have developed another *pmoA*-targeting quantitative PCR assay to detect specific groups of *pmoA*-containing methanotrophs that could not be detected by the method developed by Kolb *et al.* (2003). However, as none of these qPCR assays could be

applied to study *Methylocella* spp. in the present study, a qPCR assay was developed targeting *mmoX* genes of *Methylocella* spp.

The *mmoX*-targeting qPCR assay developed here was validated by two spiking studies. Firstly, DNA extracted from *Methylocella* spp. negative Ufton landfill cover soil was spiked with known amounts of DNA from *Methylocella silvestris* to achieve 1×10^3 to 1×10^5 copies of *mmoX* per reaction. Following the qPCR the detection efficiency of the assay was found to be between 80 and 104% **(Figure 4.6). A** second spiking study was then carried out where the Ufton landfill cover soil was spiked with known numbers of *Methylocella silvestris* cells. DNA extracted from these *Methylocella silvestris* spiked soil was subjected to qPCR. The detection efficiency of the assay in this case was 88 to 111% **(Figure 4.7).** These results suggest that *mmoX*-targeting qPCR assay developed here is quite efficient for detecting *Methylocella* from the spiked DNA and soil. The detection limit of the assay was 10^2 copies of *mmoX* per reaction, as determined during the generation of standard curve from the *Methylocella silvestris* genomic DNA **(Figure 4.2),** which is at the similar range of *pmoA*-targeting qPCR assay developed by Kolb *et al.* (2003). The detection limit of the assay was 10^3 copies of *mmoX* per g of soil as evident from the spiking study.

One of the important limitations of using SYBR® Green dye in qPCR assay is that it may bind to non-specific double-stranded DNA including primer dimer (Zhou *et al.*, 2007). Therefore dissociation curve analysis is necessary during the assay to ensure that the fluorescent signals are actually coming from the correct amplicons, not from any non-specific amplicons including primer dimer. Absence of non-specific

amplicons and primer dimer formation in the assay was confirmed by dissociation curve analysis **(Figure 4.4).** In addition, when amplified qPCR products were run on an agarose gel, only one amplicon of expected size was observed **(Figure 4.5)**. These findings suggest that data generated during these assays are actually coming from the expected amplicons.

DNA extracted from selected environmental samples was analyzed in the qPCR assay to quantify the abundance of *Methylocella* spp. present in those samples **(Figure 4.9).** The abundance of *Methylocella* spp. detected varied from sample to sample, ranging from 0.9 (± 0.2) × 10^6 copies of *mmoX* per g of soil in Colne Estuary sediment, Essex (UK) to 3.3 (± 0.6) × 10^6 copies of *mmoX* per g of soil in Hornaven (Sweden). There are no previous qPCR data describing the abundance of *Methylocella* spp. in the environment; however, a FISH based study carried out by Dedysh *et al*. (2001) on the abundance of *Methylocella* spp. in *Sphagnum* peat was ~ 10^6 copies of *mmoX* per g of soil. The abundance of *Methylocella* spp. that we detected in one peat soil (Moor House peat) was 2.3 (± 0.6) × 10^6 copies of *mmoX* per g of soil, which is in good agreement with these data of Dedysh *et al.* (2001).

In conclusion, a SYBR® green-based qPCR assay was developed targeting the mmoX gene of *Methylocella* spp. The abundance of *Methylocella* spp. present in selected environmental samples was quantified using the assay. This qPCR assay can now be applied to study the effect of environmental parameters such as soil depth, vegetation, pH, moisture content or availability of other nutrients e.g., acetate, on the dynamics of *Methylocella* spp. in microcosms or *in situ*. This will provide

valuable information on how these facultative methanotrophs adapt themselves in nature in response to changing conditions.

Chapter 5

Acetate represses methane oxidation by *Methylocella silvestris* in a peat soil microcosm

5.1 Introduction

Methylocella species are commonly found in acidic soils (Dedysh *et al.*, 2001; Dunfield *et al.*, 2003; Chen *et al.*, 2008a; Chen *et al.*, 2008b). In peatlands, *Methylocella* spp. are thought to play a key role in methane oxidation (Dedysh *et al.*, 2001; Chen *et al.*, 2008a; Chen *et al.*, 2008b). *Methylocella* spp. are facultative methanotrophs, therefore, under certain conditions, it might be possible that acetate rather than methane could be used by *Methylocella* spp. as a preferred carbon source in soil, since a previous study by Theisen *et al.*, (2005) demonstrated that *Methylocella* spp. are able to grow on acetate in both batch and fermenter culture. Moreover, acetate suppresses the transcription of sMMO of *Methylocella silvestris* in culture and therefore reduces its ability to oxidize methane. Acetate is a major intermediate in the degradation of organic matter in soil. Roots of peat-associated plants (such as *Eriophorum*) can exude acetate (Strom *et al.*, 2003) and the accumulation of acetate in the top 20 cm of peatland soil can reach 0.5 mM (Hines et al., 2001), suggesting the availability of enough acetate in the peat soil to be used by *Methylocella* spp.

$^{13}CH_4$ DNA-SIP experiments have identified *Methylocella* spp. as the active methane-utilizing bacteria in the Moor House peat soil (Chen *et al.*, 2008b). PLFA-SIP experiments using $^{13}CH_4$ also identified *Methylocella* spp. as one of the major active methane-utilizing bacteria in those peat soils (Chen *et al.*, 2008a). In addition, Chen *et al.* (2008a) failed to detect transcripts of sMMO from those peat soils using RT-PCR with mRNA extracted directly from the peat soil. This finding may not be surprising, because *Methylocella* species are facultative methanotrophs and acetate

rather than methane could be their preferred carbon source (Dedysh *et al.*, 2005). However, it is not clear why *Methylocella* spp. were still maintained at a relatively large population in these soil, but were not involved in methane oxidation in these peat soils. It is possible that possession of sMMO has been an evolutionary advantage for *Methylocella* spp. in competing with other bacteria during acetate-limiting conditions, such as during winter or after heavy rainfall when peat-associated plants were dead and could not support soil bacteria by supplying sufficient acetate *via* their roots.

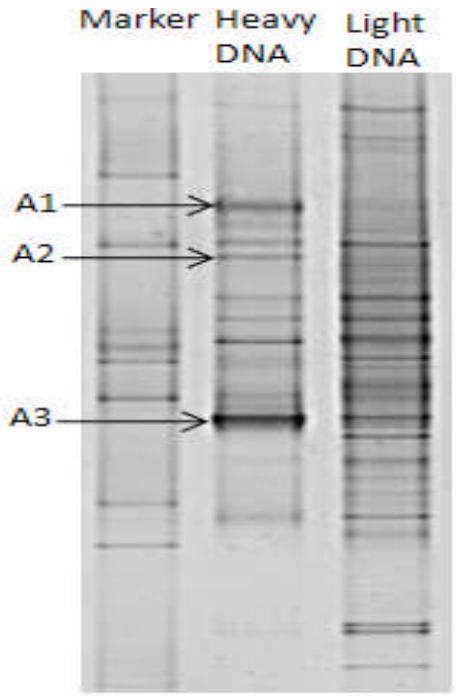
The main aim of the work in this chapter was to study the effect of acetate on the ability of *Methylocella silvestris* to oxidize methane in peat soil by $^{13}CH_4$ DNA-SIP. In addition, the ability of *Methylocella silvestris* to utilize acetate as an alternative carbon source in peat soil was also evaluated by ^{13}C-labelled acetate DNA-SIP. We wished to test the hypothesis that acetate repressed the oxidation of methane by *Methylocella silvestris in vitro* and that in the environment, acetate might offer an alternative supply of carbon and energy for *Methylocella* spp.

5.2 Results and discussion

5.2.1 *Methylocella* spp. do not represent a major group of active methanotrophs in Moor House peat soil

Methylocella spp. are one of the active groups of methanotrophs in Moor House peat soil as revealed by DNA-SIP experiment (2008b). In the current study $^{13}CH_4$ DNA-SIP experiments were carried out to investigate if *Methylocella* spp. were still part of the active methanotroph community in Moor House peat soil. The bacterial 16S rRNA gene-specific DGGE profiles generated from the ‘‘heavy’’ DNA retrieved from the Moor House peat soil that had been incubated with $^{13}CH_4$ revealed that *Methylocystis* spp. are most closely related to strains 5FB1, B3 and H2s (that had previously been detected in upland soil (Knief & Dunfield, 2005), soda lake and peat bog respectively) as the dominant active methanotroph in this peat soil under the DNA-SIP incubation conditions used in this experiment (**Figure 5.1**). No *Methylocella*-like 16S rRNA gene sequences were found to be significantly enriched in the $^{13}CH_4$ “heavy” DNA DGGE profiles.

In addition, $^{13}CH_4$ ‘‘heavy’’ DNA retrieved from this Moor House peat soil was used to construct a clone library using the general methanotroph-specific *mmoX* primers *e.g.,* mmoX206F and mmoX886 designed by Hutchens *et al.* (2004). A total of 26 clones were subjected to RFLP using *Msp*I enzyme and three OTUs (Operational Taxonomic Unit) were obtained. One to two clones from each OTUs were sequenced. Many of these *mmoX* clones retrieved from the Moor House peat soil were phylogenetically related to *mmoX* clones previously detected from this Moor House peat soil by Chen *et al.* (2008a) (**Figure 5.2**). For example *mmoX* clone



A1. *Methylocystis* sp. B3 (DQ496232; 99% identity)
A2. *Methylocystis* sp. H2s (FN422003; 99% identity)
A3. *Methylocystis* sp. 5FB1 (AJ868421; 100% identity)

Figure 5.1. DGGE fingerprint profiles of 16S rRNA gene PCR products of ‘‘heavy’’ and “light” DNA retrieved from the Moor House peat soil incubated with $^{13}CH_4$. Enriched DGGE bands that were excised for re-amplification and sequencing are indicated by arrows.

MH_Mmox_Meth11 (~ 19% of the clone library) is phylogenetically related to *mmoX* from uncultivated bacterium, clone MHPE1 (EF644596; 98% identity) that had earlier been detected from this Moor House peat soil by Chen *et al.* (2008b). Clone MH_Mmox_Meth1 (~ 74% of the clone library) is phylogenetically related to *mmox* from an uncultivated bacterium clone LC2pom (AY781159; 98% identity) detected from Finish peat soil (Jaatinen *et al.*, 2005), while clone

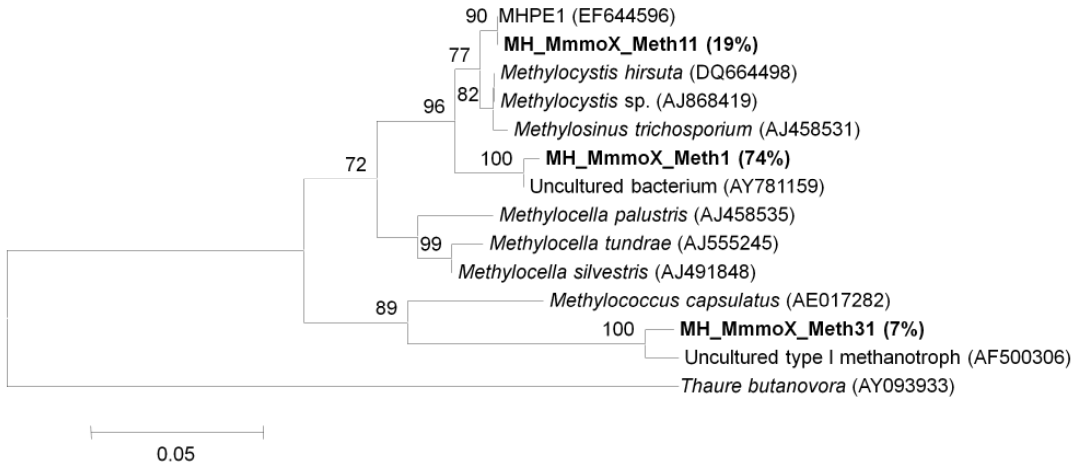


Figure 5.2 Neighbour-joining phylogenetic tree of deduced MmoX sequences (226 amino acids) (in bold) retrieved from the ‘‘heavy’’ DNA of Moor House peat soil incubated with ^{13}C-labelled methane and the closely related sequences available at the public database (Accession numbers are in parentheses). The percentages of clones obtained from each OTU are shown. The scale bar represents 0.05 substitutions per amino acid. The numbers at the nodes represent bootstrap values (≥70) observed with 1000 replicates. The tree is rooted with the partial butane monooxygenase sequence (BmoX) from *Thauera butanovora* (AY093933). The MmoX sequences retrieved from the Moor House peat soil incubated with $^{13}CH_4$ are named as MH_Mmox_Meth. The MHP prefix represents the *mmoX* clones identified from Moor House peat by Chen *et al.* (2008b).

MH_Mmox_Meth31 (~ 7% of the clone library) is phylogenetically related to *mmoX* from uncultivated type I methanotroph clone M (AF500306; 98% identity) detected from Lake Washington sediment (Auman & Lidstrom, 2002). No *Methylocella*-like *mmoX* sequences were retrieved from the $^{13}CH_4$ "heavy" DNA retrieved from the Moor House peat soil, suggesting that *Methylocella* spp. were either not involved in the utilization of methane in Moor House peat soil or were present in such low numbers that they were not detected in this DNA-SIP experiment by *mmoX* clone library analysis. The difference observed in the present study and that of Chen *et al.* (2008a and 2008b) could be related to temporal and special variation. In addition, Chen *et al.* (2008a and 2008b) did not add any water into their microcosms. In the present study water was added into the microcosms for faster incorporation of ^{13}C-labelled substrates, thus to avoid cross feeding of ^{13}C-labelled substrates (Cébron *et al.*, 2007b). Moreover, Chen *et al.* (2008b) carried out their DNA-SIP experiment with soil that had an incorporation of 140 µmol g^{-1} of labelled methane, but in the present study, the DNA-SIP incubation was stopped when 100 µmol g^{-1} of labelled methane was incorporated to prevent the cross feeding.

5.2.2 Acetate represses the ability of *Methylocella silvestris* BL2, added to peat soil microcosms, to oxidize methane

The effect of the addition of 0.5 mM acetate on the ability of the Moor House peat soil to oxidize methane was studied in microcosms. The original concentration of acetate in the Moor House peat soil was found to be below 0.1 mM, which was the level of detection limit of the HPLC assay used in this study (**Chapter 2**, **section 2.9**). The concentration of accumulated acetate in Beech forest soil can reach up to

~ 3.5 mM (Kusel & Drake, 1999). In pore water of peat, the concentration of acetate varies from a few µM to 1 mM (Duddleston & Kinney, 2002), therefore a dose of 0.5 mM acetate was chosen to observe its effect on methane oxidation, which is in the medium range of *in situ* concentrations of acetate observed in peat soil. Based on the ability of the soil in the microcosms to oxidize methane, it is evident that, 0.5 mM acetate had no major impact on methane oxidation by the original soil (non *Methylocella*-spiked soil with acetate) compared to the control soil that did not received any acetate (**Figure 5.3**).

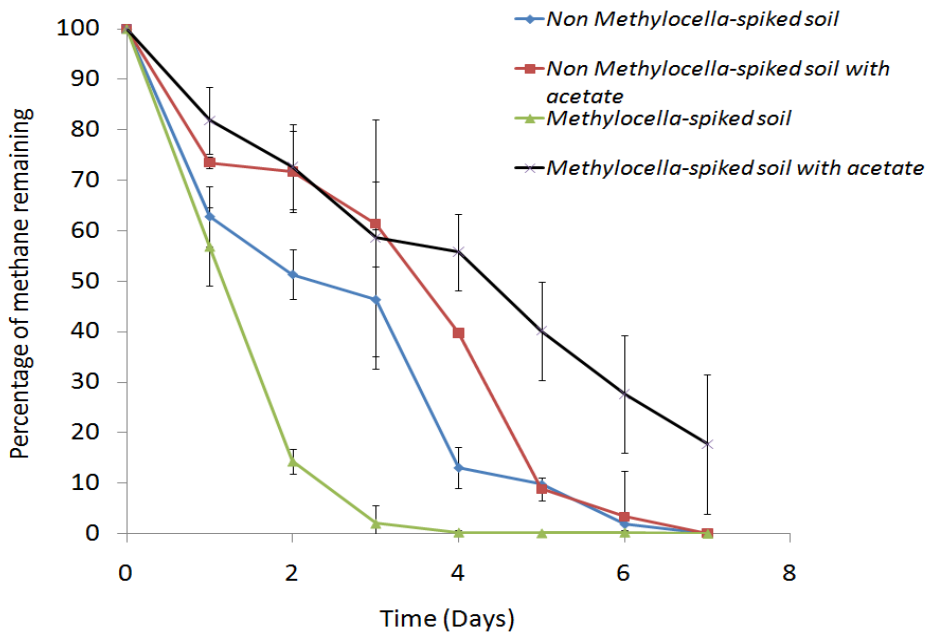
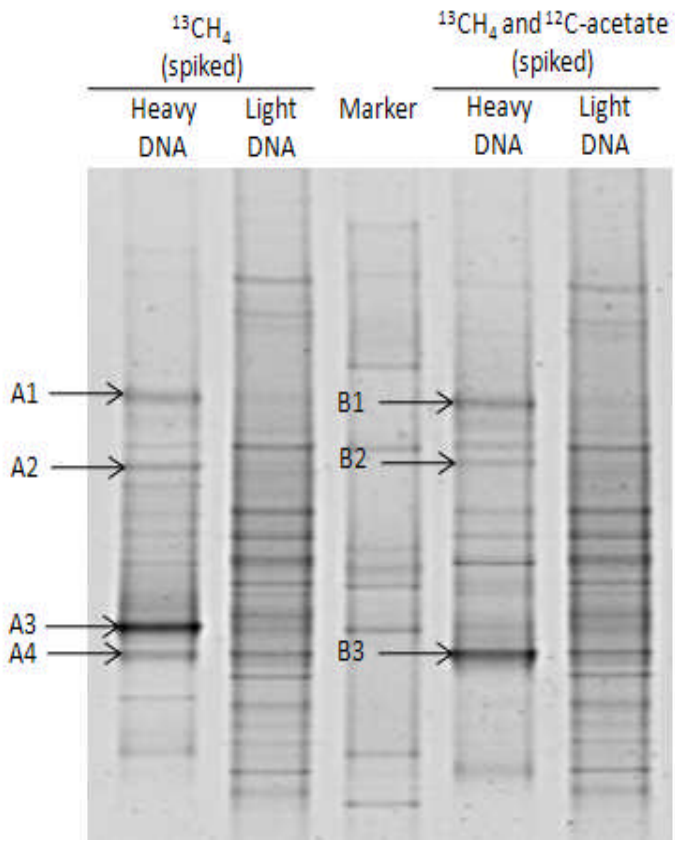


Figure 5.3. Methane oxidation potential in microcosms derived from original non-spiked and *Methylocella silvestris*-spiked Moor House peat soil either in the presence or absence of 0.5 mM sodium acetate. Microcosms were injected with 2% head space (v/v) of methane. The percentage of methane remaining indicate remaining methane from the original 2% head space (v/v) of methane injected into each microcosms. Error bars indicate the standard deviation of triplicate methane concentration measurements.

To determine the effect of 0.5 mM acetate on the ability of *Methylocella silvestris* to oxidize methane, microcosms containing Moor House peat soil were spiked with *Methylocella silvestris* at a the "dose" of 1×10^6 cells g^{-1} of soil, based on the earlier work by Dedysh *et al.* (2001), who enumerated using fluorescence *in situ* hybridization the number of *Methylocella* spp. in *Sphagnum* peat as 1×10^6 cells g^{-1} of soil. The addition of *Methylocella silvestris* to the Moor House peat soil increased the ability of the soil to oxidize methane compared to the original non-*Methylocella*-spiked Moor House peat soil, suggesting that the added *Methylocella silvestris* remained active and contributed to enhanced methane oxidation **(**see previous **Figure 5.3)**. $^{13}CH_4$ DNA-SIP experiments carried out with the *Methylocella*-spiked soil and the bacterial 16S rRNA gene-specific DGGE identified *Methylocella silvestris* BL2 as the dominant active methanotroph in the *Methylocella*-spiked soil (**Figure 5.4**), indicating that *Methylocella silvestris* were actively utilizing methane in the spiked soil microcosms.

However, when 0.5 mM acetate was added, it markedly reduced the ability of the *Methylocella*-spiked soil to oxidize methane compared to the control (*Methylocella* -spiked soil without addition of acetate), suggesting that acetate repressed the ability of *Methylocella silvestris* to oxidize methane in the Moor House peat soil microcosms **(**see previous **Figure 5.3).** Acetate is known to repress the transcription of sMMO in *Methylocella silvestris in vitro i.e.,* laboratory culture (Dedysh *et al.,* 2005; Theisen *et al.,* 2005) and the same effect may have resulted in the observed reduction in methane oxidation by *Methylocella silvestris* in the spiked soil microcosms.



A1 and B1. *Methylocystis* sp. B3 (DQ496232; 99% identity)
A2 and B2. *Methylocystis* sp. H2s (FN422003; 99% identity)
A3. *Methylocella silvestris* BL2 (AJ491847; 100% identity)
A4 and B3. *Methylocystis* sp. 5FB1 (AJ868421; 100% identity)

Figure 5.4 DGGE fingerprint profiles of 16S rRNA gene PCR products of ‘‘heavy’’ and “light” DNA retrieved from the *Methylocella silvestris*-spiked Moor House peat soil incubated with $^{13}CH_4$ either in presence or absence of ^{12}C-acetate. Enriched DGGE bands that were excised for re-amplification and sequencing are indicated by arrows.

To address the hypothesis that acetate represses methane oxidation by *Methylocella silvestris*, DNA-SIP experiments were carried out with *Methylocella* -spiked soil supplied with $^{13}CH_4$ in the presence of 0.5 mM ^{12}C-sodium acetate. If the hypothesis is correct, then in the presence of acetate, *Methylocella silvestris* will not utilize methane. Therefore, the *Methylocella silvestris*-specific enriched DGGE band observed in the ‘‘heavy’’ DNA retrieved from $^{13}CH_4$ incubated microcosms would disappear in the ‘‘heavy’’ DNA retrieved from soil incubated with both $^{13}CH_4$ plus ^{12}C-acetate. The bacterial 16S rRNA gene-specific DGGE profiles retrieved from the $^{13}CH_4$ ‘‘heavy’’ DNA and that of $^{13}CH_4$ plus ^{12}C-acetate ‘‘heavy’’ DNA are presented in previous **Figure 5.4**.

Based on the sequence information of the enriched bacterial 16S rRNA gene-specific DGGE band retrieved from the $^{13}CH_4$ ‘‘heavy’’ DNA, it is evident that *Methylocella silvestris* was the active dominant methanotroph in the spiked-soil without acetate, while its signature 16S rRNA gene band disappeared in the DGGE profile originating from the $^{13}CH_4$ and ^{12}C-acetate ‘‘heavy’’ DNA (see previous **Figure 5.4**). In addition, an *mmoX* clone library was constructed with the ‘‘heavy’’ DNA retrieved from $^{13}CH_4$ plus ^{12}C-acetate microcosms. A total of 36 clones were analyzed by RFLP using *Msp*I and two OTUs were obtained. This identified *mmoX* clones phylogenetically related to *mmoX* clones earlier retrieved from peat soil and other environments (**Figure 5.5**).

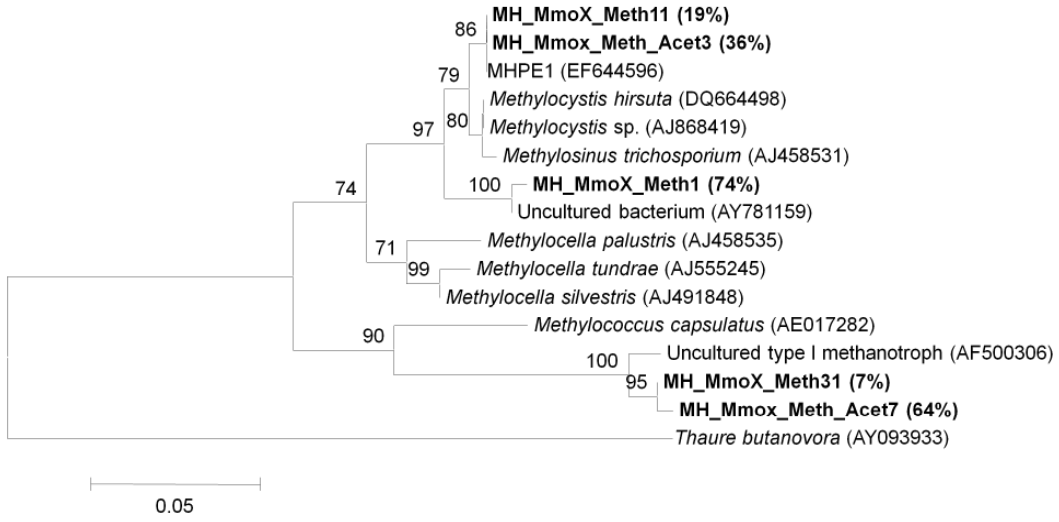
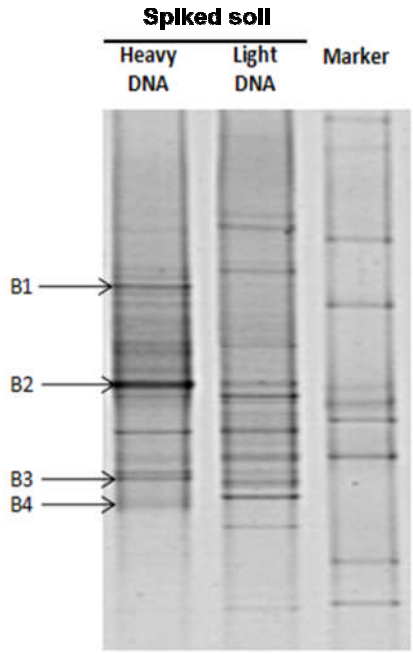


Figure 5.5 Neighbour-joining phylogenetic tree of deduced MmoX sequences (226 amino acids) (in bold) retrieved from the ‘‘heavy’’ DNA of Moor House peat soil incubated with ^{13}C-labelled methane or $^{13}CH_4$ and ^{12}C-acetate and the closely related sequences available at the public database (Accession numbers are in parentheses). The percentages of clones obtained from each OTU are shown. The scale bar represents 0.05 substitutions per amino acid. The numbers at the nodes represent bootstrap values (≥70) observed with 1000 replicates. The tree is rooted with the partial butane monooxygenase sequence (BmoX) from *Thauera butanovora* (AY093933). The MmoX sequences retrieved from the Moor House peat soil incubated with $^{13}CH_4$ are named as MH_Mmox_Meth. The MHP prefix represents the *mmoX* clones identified from Moor House peat by Chen *et al.* (2008b).

For example clone MH_Mmox_Meth_Acet6 (~ 36% of the clone library) is phylogenetically related to *mmoX* of an uncultivated bacterium MHPE1 (EF644596) that had earlier been detected from the Moor House peat soil by Chen *et al.* (2007). While clone MH_Mmox_Meth_Acet7 (~ 64% of the clone library) is phylogenetically related to *mmoX* of uncultivated type I methanotroph clone M (AF500306) detected from Lake Washington sediment (Auman & Lidstrom, 2002). No *mmoX* clones related to *Methylocella* were detected (see previous **Figure 5.5**). Therefore, both 16S rRNA gene-specific DGGE analyses and *mmoX* gene clone library data obtained from the the $^{13}CH_4$ plus ^{12}C-acetate ‘‘heavy’’ DNA demonstrate that in the presence of acetate, *Methylocella silvestris* does not utilize methane in the peat soil microcosms. This observation is congruent with the effect observed in pure culture where acetate was found to repress the transcription of sMMO genes of *Methylocella silvestris* (Theisen *et al.*, 2005).

5.2.3 *Methylocella silvestris* BL2 were not the major active acetate-utilizers in peat soil microcosms

To investigate if *Methylocella silvestris* utilized acetate as the carbon source in this peat soils in the absence of CH_4, ^{13}C-labelled acetate DNA-SIP experiments were carried out with *Methylocella silvestris*-spiked Moor House peat soil. The hypothesis to be tested was that if *Methylocella silvestris* utilized acetate rather than methane *in situ, Methylocella silvestris* 16S rRNA gene should be detected in the ^{13}C-labelled acetate ‘‘heavy’’ DNA as an enriched band. Bacterial 16S rRNA gene-specific DGGE carried out with the ‘‘heavy’’ DNA retrieved from the *Methylocella silvestris*-spiked ^{13}C-labelled acetate soils showed several enriched bands when compared to the banding pattern with the “light” DNA (**Figure 5.6**).



B1. *Burkholderia norimbergensis* (Y09879; 99% identity)
B2. *Brevundimonas bullata* (EU665637; 99% identity)
B3. *Acidovorax* sp. (GQ284468; identity 100%)
B4. *Acidocella aluminiidurans* (AB362219; 99% identity)

Figure 5.6. DGGE fingerprint profiles of 16S rRNA gene PCR products of ‘‘heavy’’ and “light” DNA retrieved from the *Methylocella silvestris*-spiked Moor House peat soil incubated with ^{13}C-labelled acetate. Enriched DGGE bands that were excised for re-amplification and sequencing are indicated by arrows.

Acidocella, *Acidovorax*, *Brevundimonas* and *Burkholderia*-like 16S rRNA gene sequences were enriched in the ^{13}C acetate labelled heavy DNA, indicating them to be the most dominant bacterial genera actively involved in the utilization of acetate in this peat soil under these incubation conditions (**Figure 5.6**). No *Methylocella*-like sequences were detected as enriched bands after 16S rRNA gene DGGE fingerprint analyses. The ^{13}C-acetate labelled “heavy” DNA retrieved from *Methylocella silvestris*-spiked soil was also used to construct a 16S rRNA gene clone library. 34

clones were analyzed by RFLP and five OTUs were obtained (**Figure 5.7**).

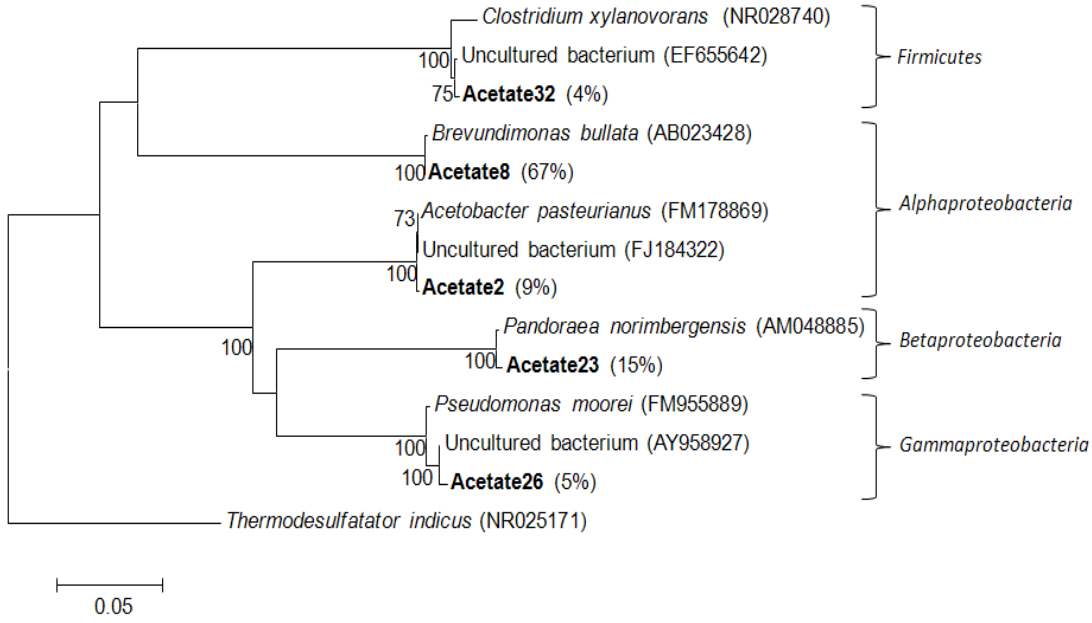


Figure 5.7. Neighbor-joining phylogenetic tree of 16S rRNA gene sequences (in bold) obtained from the "heavy" DNA retrieved from the *Methylocella silvestris*-spiked Moor House peat soil incubated with ^{13}C-labelled acetate and the closely related sequences available in the public database (accession numbers are in parentheses). The tree was generated with a final data set of 1043 nucleotide positions. The scale bar represents 5% sequence divergence. The percentages of clones obtained from the same OTU are shown. The numbers at the nodes represent bootstrap values (≥70) observed with 1000 replicates. The tree is rooted with the 16S rRNA gene sequences from *Thermodesulfatator indicus* (NR025171). The 16S rRNA gene sequences retrieved from the Moor House peat soil incubated with ^{13}C-acetate are named as Acetate2, Acetate8 etc.

This clone library analysis identified *Brevundimonas* spp. as the most dominant bacteria (~ 67% of clones in the library) involved in utilization of acetate in the Moor House peat soil. In addition, 16S rRNA genes phylogenetically related to the 16S rRNA genes of *Pandoraea* (~ 15%), *Acetobacter* (~ 9%), *Pseudomonas* (~ 5%) and *Clostridium* (~ 4%*)* were also identified in this clone library. Several studies have used ^{13}C-labelled acetate to identify bacterial species actively involved in the utilization of acetate in soil and sediment (Scholten & Stams, 2000; Chauhan & Ogram, 2006; Schwarz *et al.*, 2007; Longnecker *et al.*, 2009). *Acetobacter* has been identified in Florida soil as a major acetate-utilizing bacterium in Florida soil (Chauhan & Ogram, 2006). Schwarz *et al.* (2007) identified *Acetobacter* and *Burkholderia* as active genera involved in acetate-utilization in the sediment of Lake Kinneret (Israel). Therefore, our 16S rRNA gene clone library analyses, as well as DGGE fingerprinting analyses, suggested that *Methylocella* spp. were not significant acetate-utilizers even when added to the peat soil microcosm under these present experimental conditions. Probably *Methylocella* spp. were outcompeted by more efficient acetate-utilizers such as *Brevundimonas* and *Burkholderia*. This observation is further supported by the acetate uptake data obtained from the non-spiked and *Methylocella silvestris*-spiked soil. Virtually no difference was observed in the ability of the *Methylocella silvestris*-spiked soil to take up acetate when compared to non-spiked soil in microcosms **(Figure 5.8).**

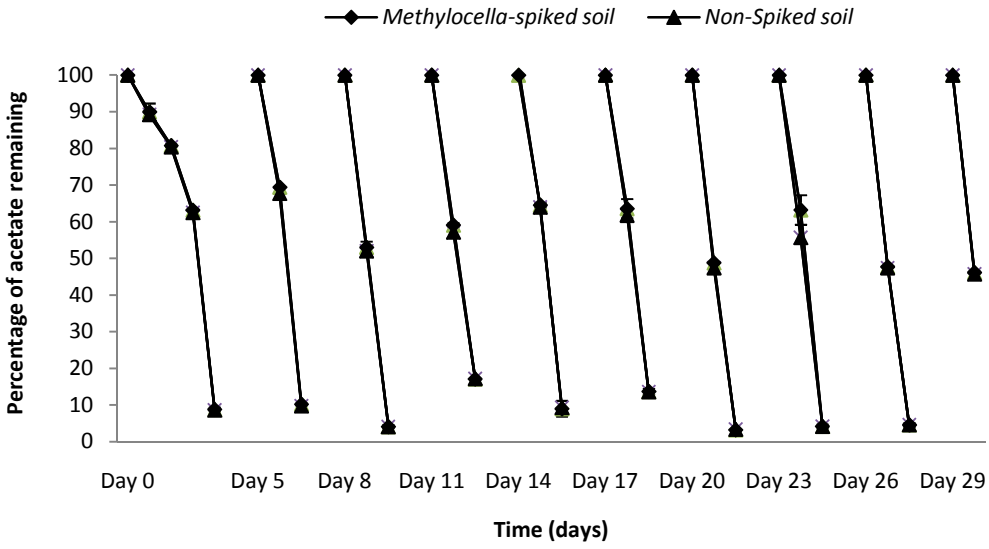


Figure 5.8. The ability of the non-spiked and *Methylocella silvestris*-spiked Moor House peat to take up acetate (0.5 mM final concentration). Error bars indicate the standard error of the mean of triplicate acetate concentration measurements.

In conclusion, $^{13}CH_4$ DNA-SIP experiments indicated that *Methylocella* spp. were not the major methane-utilizers in Moor House peat soil in microcosms under the experimental conditions tested. Spiking of the peat soil with *Methylocella silvestris* increased the ability of the soil to oxidize methane in microcosms. However, more importantly, it was evident that 0.5 mM acetate reduced the ability of the *Methylocella silvestris*-spiked peat soil to oxidize methane probably due to acetate inhibiting methane oxidation by *Methylocella silvestris*. Furthermore, the ^{13}C-labelled acetate DNA-SIP analyses showed that *Methylocella* spp., were not acetate-utilizers in this peat soil microcosm under the conditions used in this study and that they seem to have been outcompeted by more efficient acetate-utilizers. Further experiments are required to investigate the potential competition for acetate between

Methylocella and other acetate-utilizers, such as *Brevundimonas* and *Burkholderia*, and the consequences of this for methane oxidation by facultative *Methylocella* spp. in peat soils. In addition, the recently identified facultative methanotrophs *Methylocapsa aurea* (Dunfield *et al.*, 2010) and *Methylocystis* sp. H2s (Belova *et al.*, 2010) isolated from forest soil and peat soil of Germany respectively can also be tested in the similar way to determine if acetate represses their potential to oxidize methane in soil.

Chapter 6

Methylocella silvestris whole genome transcriptomics

6.1 Introduction

Methanotrophy was thought to be an obligate trait, until the discovery of the genus *Methylocella. Methylocella silvestris* BL2 is the first fully validated facultative methanotroph, able to grow not only on one carbon molecules *e.g.*, methane or methanol, but also on multi-carbon compounds *e.g.*, acetate, succinate, propionate and malate (Dedysh *et al.*, 2005, Theisen *et al.*, 2005). The ability of *Methylocella* spp. to utilize acetate could be a survival strategy of these organisms to maintain their cellular activity and replication in environments where there is a fluctuation in availability of methane or methane is absent. The reason for the obligate nature of methanotrophy is a long-standing scientific mystery. *Methylocella silvestris* provides an opportunity for use as a model organism to understand the obligate nature of other methanotrophs.

Methylocella spp. are unique among all known methanotrophs, because they oxidize methane using only sMMO, since *Methylocella* lack pMMO (Dedysh *et al.*, 2005, Theisen *et al.*, 2005). It has already been established that all the genes encoding the sMMO are upregulated during growth on methane (Theisen *et al.*, 2005). These genes are not transcribed during growth on acetate (Theisen *et al.*, 2005). However, there is no information on the involvement of other genes that may have some direct or indirect role in the utilization of methane or acetate in *Methylocella silvestris.*

Microarray techniques allow one to detect transcriptomics of an organism under particular experimental conditions, such as during growth on a particular substrate. Since a gene is transcribed when its function is required, therefore, the presence of a mRNA transcripts of a gene during growth under a particular substrate indicates that

the protein encoded by the gene is probably involved in the utilization of that particular substrate (Dorrell *et al.*, 2001; Chalabi *et al.*, 2007; Borneman *et al.*, 2010). Microarray techniques have been applied successfully to study genes transcribed differentially across the whole genome of many bacteria during growth on various substrates including methylotrophs, such as *Methylibium petroleiphilum* PM1 during growth on methyl tert-butyl ether or ethanol (Hristova *et al.*, 2007; Kane *et al.*, 2007). The whole genome transcriptome of *Methylobacterium extorquens* AM1 growth on methanol or succinate was also studied using the gene expression microarray during (Okuba *et al.*, 2007). The technique allows one to narrow down the focus area to identify candidate genes that may encode components of key metabolic pathways for further characterization.

This chapter describes preliminary *Methylocella silvestris* whole genome transcriptomics experiments designed to identify genes transcribed differentially during growth on methane (C_1 compound) or acetate (C_2 compound). The hypothesis is that key genes involved in the utilization of methane or acetate will be upregulated during growth on methane or acetate.

6.2. Results

6.2.1. Experimental protocols and data analysis

Gene expression microarray experiments were carried out using a custom synthesized oligonucleotide microarray, using the Agilent platform (Agilent Technologies, USA) which covers the whole genome of *Methylocella silvestris* BL2. In the genome of *Methylocella silvestris*, 3,971 candidate genes have been identified (Chen *et al.*, 2010). Three probes (60 mers) were designed for each gene (Total 11,913 probes). The microarray experiments were carried out using on a one-colour platform, instead of a two colour-platform, because there is a good agreement in the results obtained by these two approaches (Patterson *et al.*, 2006). The microarray experiments were carried out with three biological replicates where RNA was prepared from three independent batch cultures of *Methylocella silvestris* for each experimental condition *i.e.*, methane or acetate. Any spots on the microarray having a signal below the background or saturated or an outlier were not included in the analysis. Microarray data generated from each probe were normalized at the 75th percentile level and analyzed using the GeneSpring® software (Agilent Technology, USA). The *P* value derived from the unpaired *t* test (methane *vs* acetate growth conditions) was used to assess the statistical significance of that fold change. For this study, data with *P* values of <0.05 were considered confident and reliable. Only genes with a ≥ 2 -fold change in expression levels were considered as biologically meaningful as recommended by Butcher, (2004) and used for the functional group analysis. Detailed procedures for the microarray experiments are described in **Chapter 2, section 2.11.2.** The microarray analyses were carried out by comparing mRNA from methane-grown cells with that of acetate grown cells, therefore, any

gene that was found to be upregulated during growth on methane was probably downregulated during growth on acetate and vice versa.

6.2.2. RNA extraction and assessment of RNA quality

Methylocella silvestris BL2 was grown either on methane or on acetate for the microarray experiments (growth conditions are described in **Chapter 2**). Total bacterial RNA was extracted from *Methylocella silvestris* grown on methane or acetate according to the method described earlier (Gilbert *et al.*, 2000) (Described in **Chapter 2; section 2.3.7)**. Initially the extracted RNA was run on a 1% (w/v) agarose gel to check the quality **(Figure 6.1).** The quality of the extracted RNA was further analyzed using the 2100 Bioanalyzer (Agilent Technologies, USA) **(Figures 6.2, 6.3 and 6.4)**.

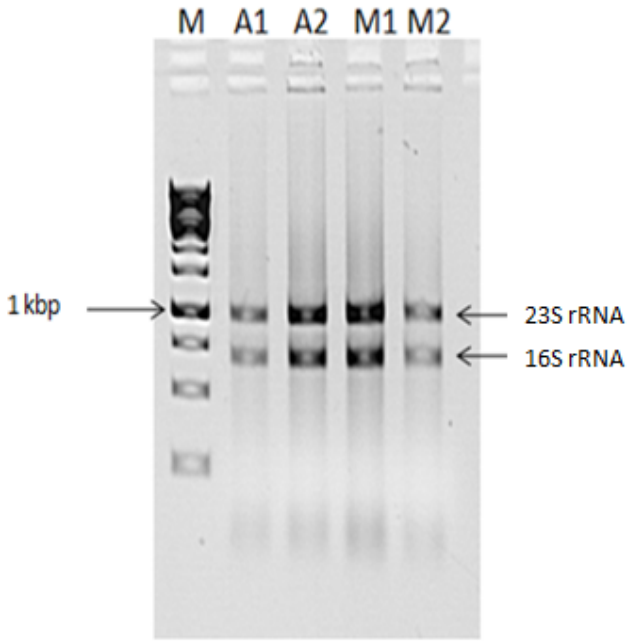


Figure 6.1. Representative gel image of *Methylocella silvestris* RNA extracted from cells grown on methane or acetate. Five µl extracted total bacterial RNA was loaded into each lane in a 1% (w/v) agarose gel. A1, Acetate replicate 1. A2, Acetate replicate 2, M1, Methane replicate 1. M2, Methane replicate 2. M= 1 kbp DNA ladder.

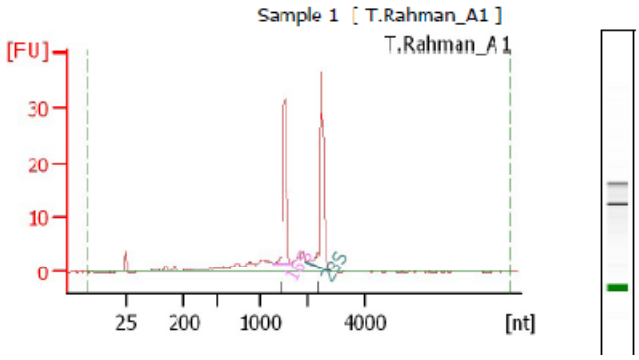


Figure 6.2. Representative electropherogram analysis of *Methylocella silvestris* RNA (extracted from acetate grown cells) generated by a 2100 Bioanalyzer (Agilent Technologies, USA). RNA area: 138.0, RNA concentration: 180 ng/µl. rRNA Ratio (23S/16S): 1.2, RIN: 8.0.

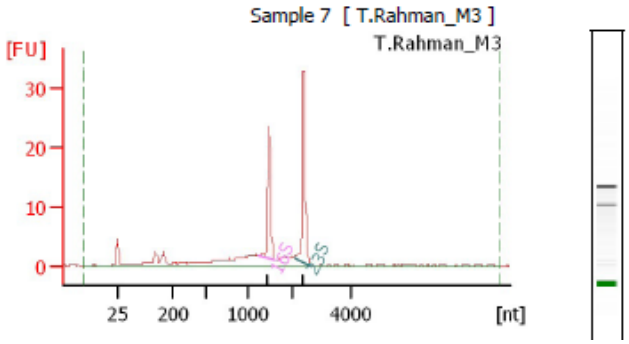


Figure 6.3. Representative electropherogram summary of *Methylocella silvestris* RNA (extracted from methane-grown cells) generated by 2100 Bioanalyzer (Agilent Technologies, USA). RNA area: 111.0, RNA concentration: 145 ng/µl. rRNA Ratio (23S/16S): 1.1, RIN: 8.1.

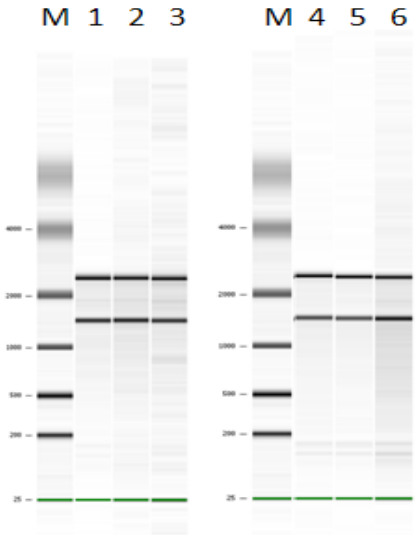


Figure 6.4. False gel image generated by the 2100 Bioanalyzer (Agilent Technologies, USA) with RNA extracted from acetate- (Lane 1-3) or methane- (Lane 4-6) grown *Methylocella silvestris*. M = RNA ladder.

An RNA integrity number (RIN) of 7 and above was taken to indicate as good quality RNA as described by Fleige and Pfaffl (2006). Only RNA with an RIN of 7 and above was used for the microarray experiments.

6.2.3. Technical and biological replicates

Initially microarray data obtained from the two technical replicates of either methane- or acetate-grown cells were compared between themselves. Based on the normalized intensity values of signals of the probes generated from the array, no significant difference were observed between the two technical replicates (**Figure 6.5**). In addition, all three biological replicates originating either from methane- or acetate-grown conditions were also compared among themselves along with corresponding technical replicates. The PCA (Principal Component Analysis) of these replicates experiments showed that, the results obtained from the methane-grown cells from a distinct cluster among themselves in the PCA plot. Similar findings were observed by PCA analysis for the results obtained with different replicates originating from the acetate-grown cells (**Figure 6.6**).

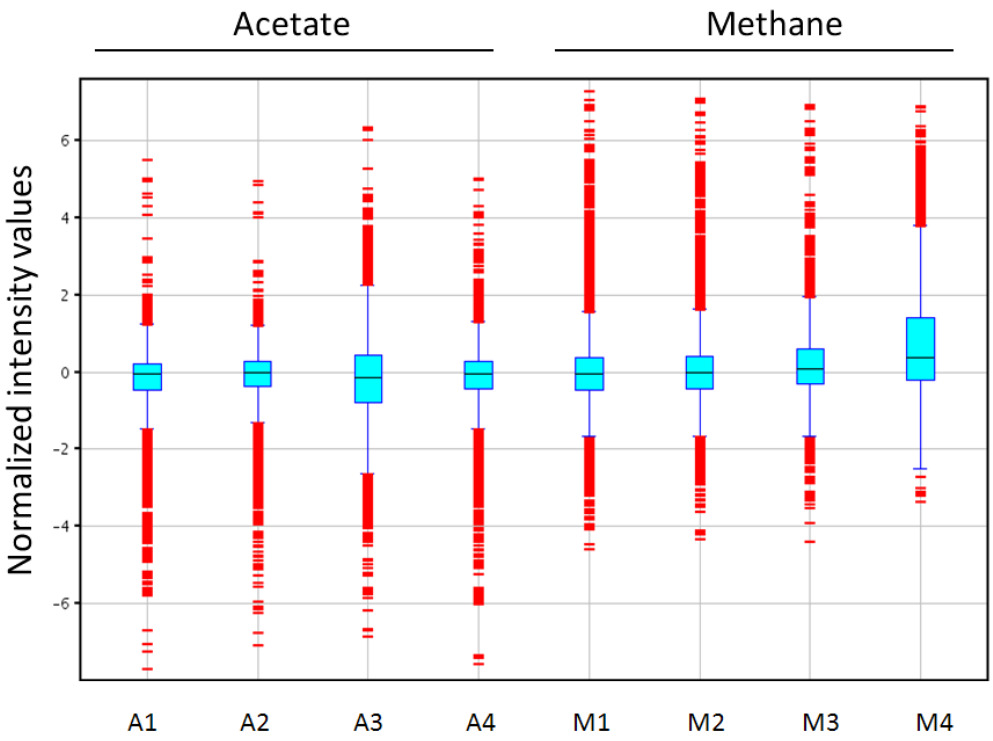


Figure 6.5. A box plot showing the normalized signal intensity values of probes generated from different RNA replicates of either methane- or acetate-grown *Methylocella silvestris.* Here, A1, A2, A3 and A4 represent results obtained from replicates originating from the acetate-grown condition, of which A1 and A2 are technical replicates to each other, while M1, M2, M3 and M4 represent results obtained from replicates originating from the methane-grown condition, of which M1 and M2 are technical replicates to each other.

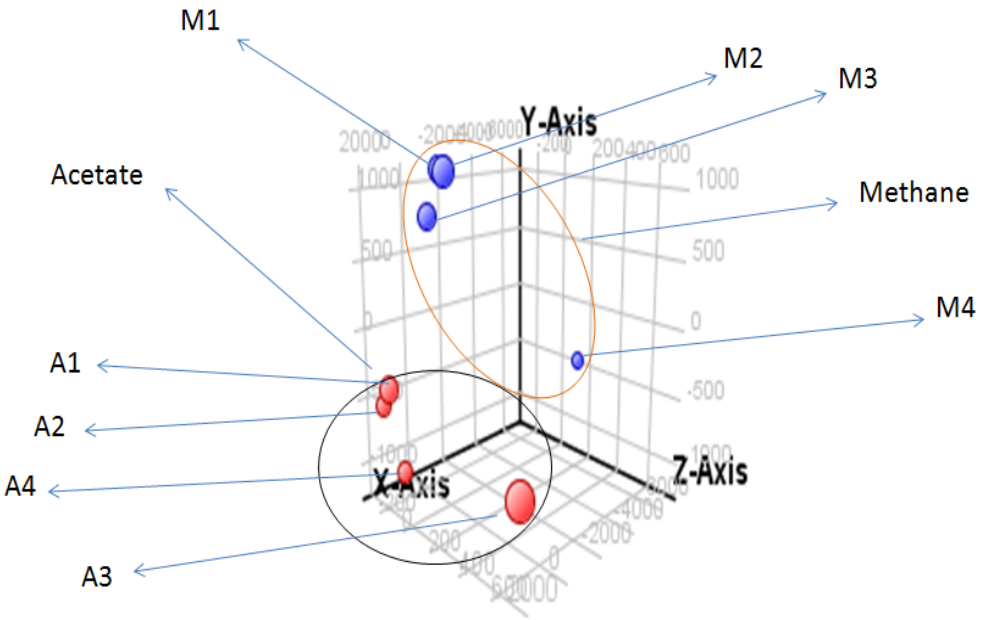


Figure 6.6. Principal component analysis of microarray data generated from technical and biological replicates. Here, A1, A2, A3 and A4 represent results obtained from RNA replicates originating from the acetate-grown condition, of which A1 and A2 are technical replicates to each other, while M1, M2, M3 and M4 represent results obtained from RNA replicates originating from the methane-grown conditions, of which M1 and M2 are technical replicates to each other.

6.2.4 Cluster analysis of genes transcribed significantly

Expression profiles of the genes transcribed in the microarray experiments were subjected to cluster analysis. Cluster analysis is a powerful way to organize genes and conditions in the dataset into clusters based on the similarity of their expression profiles (Eisen *et al.*, 1998). Expression profiles of genes originating from different replicate experiments as observed in the cluster analysis are presented in **Figure 6.7.**

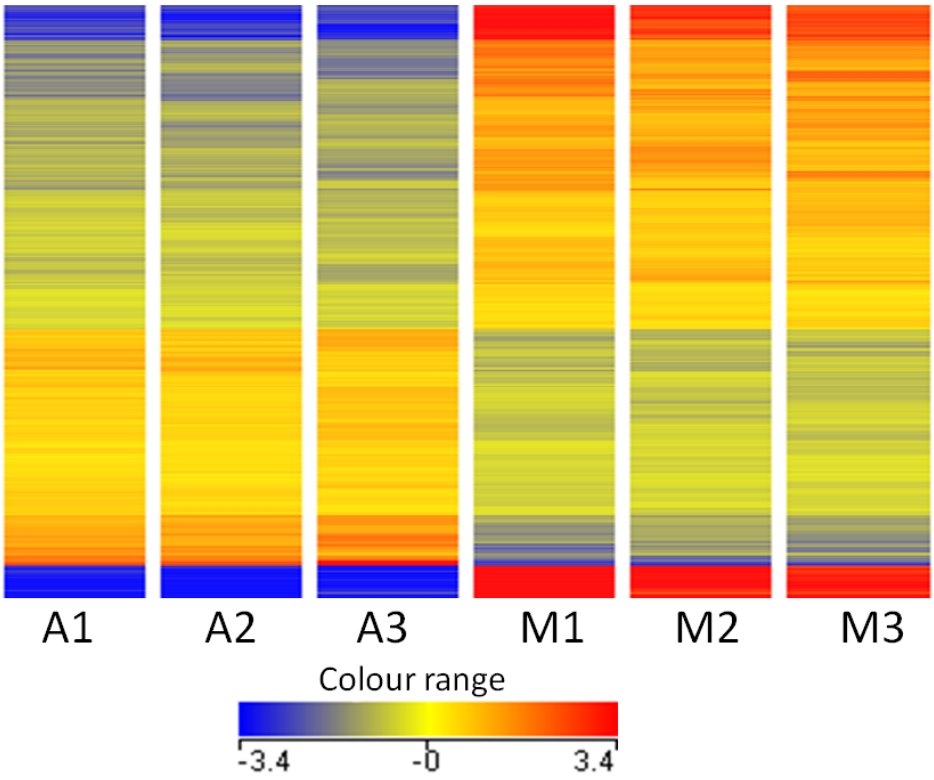


Figure 6.7. Cluster analysis of genes transcribed differentially during growth of *Methylocella silvestris* either on methane or on acetate. Here, A1, A2 and A3 represent three biological replicates originating from the acetate-grown condition, while M1, M2 and M3 represent three biological replicates originating from methane-grown conditions. Expression intensity is indicated by the colour, where the red colour indicates upregulation and the blue colour indicates downregulation.

6.2.5. Genes significantly differentially upregulated in *Methylocella silvestris* during growth on methane

Out of a total of 3,791 genes, 169 were found to be significantly upregulated in *Methylocella silvestris* during growth on methane (*i.e.,* ≥2 fold changes). List of these significantly differentially upregulated genes, their fold changes and *P* values are presented in **Appendix 2.** The fold change and the *P* values of all the genes are presented in **Figure 6.8** as a Volcano plot, which is an easy way to identify significance and magnitude of changes in expression of a set of genes between two conditions (Cui & Churchill, 2003).

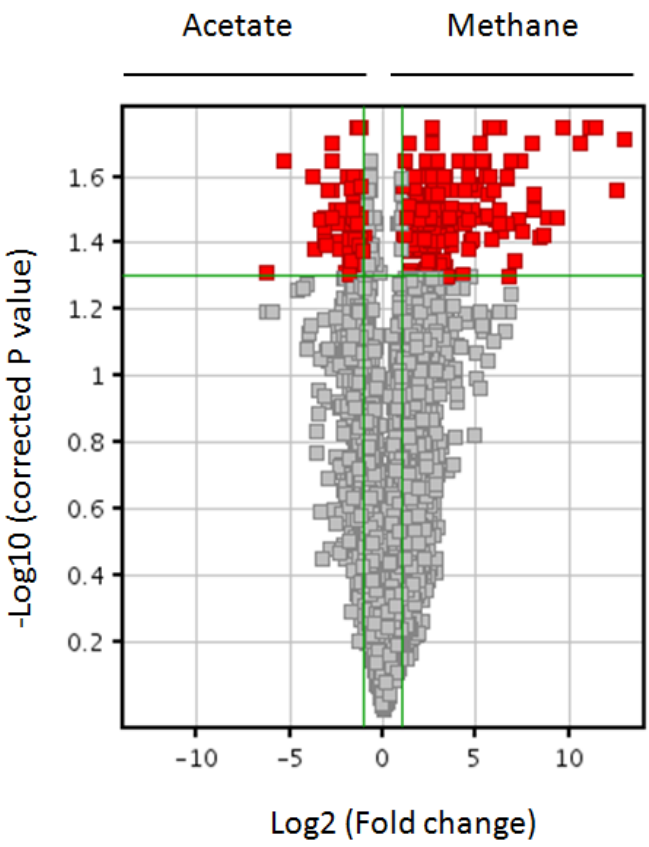


Figure 6.8. A volcano plot showing the significance (corrected *P* value) and magnitude of changes in expression (fold change) in *Methylocella silvestris* during growth on methane and acetate. The 169 genes identified as significantly upregulated 2 of more fold (P≤0.05) on methane are indicated in the upper right corner of the figure as red spots, while the 85 genes significantly upregulated 2 or more fold (P≤0.05) on acetate are indicated in the upper left corner of the figure.

Genes from several functional categories changed in expression level. The functional categories of these genes, their number and percentage distribution are presented in **Table 6.1.**

Table 6.1. The number and percentages of genes belong to different functional categories that were found to be significantly upregulated during growth of *Methylocella silvestris* on methane.

Functional categories	**No. of genes upregulated (%)**
Carbohydrate metabolism	1 (0.59)
Electron transport	11 (6.51)
Fatty acid metabolism	3 (1.78)
Flagella biosynthesis	7 (4.14)
Formate metabolism	2 (1.18)
Iron metabolism	2 (1.18)
Vitamin and cofactors metabolism	4 (2.37)
sMMO operon	9 (5.33)
Nitrogen metabolism	10 (5.92)
PQQ biosynthesis	2 (1.18)
Protein metabolism	5 (2.95)
Putative uncharacterized protein	56 (33.14)
Regulatory functions	11 (6.51)
Secretory function	3 (1.78)
Transport	18 (10.65)
Utilization of lactams	1 (0.59)
Others	24 (14.20)
Total	169 (100)

The major functional category of genes found to be upregulated during growth on methane (~ 33% of the total upregulated genes) is assigned in the genome to encode putative uncharacterized proteins due to lack of a significant identity of these genes with genes in the GenBank database. During growth on methane all the genes of the sMMO gene cluster *e.g., mmoXYBZDC, mmoR, orf2* and *mmoG* were found to be highly upregulated. The other major functional categories of genes found to be upregulated included genes involved in transport functions, regulatory functions, electron transport, N_2 metabolism, iron acquisition and vitamin and cofactor biosynthesis etc.

6.2.6. Genes significantly differentially upregulated in *Methylocella silvestris* during growth on acetate

Out of a total of 3,791 genes, 85 were found to be significantly upregulated in *Methylocella silvestris* during growth on acetate. A list of these genes, their fold changes and *P* values are presented in **Appendix 3.** The fold change and the corrected *P* values of all the genes are presented in previous **Figure 6.8** as a Volcano plot. The functional categories of these genes, their number and percentage distribution are presented in **Table 6.2.** Among them, ~13% of the significantly differentially upregulated genes are annotated in the genome to encode putative uncharacterized proteins. The other major functional categories of genes found to be upregulated included genes electron transport, TCA cycle, glyoxylate cycle, glycine metabolism, fatty acid metabolism, regulatory functions, vitamin and cofactor metabolism etc.

Table 6.2. The number and percentages of genes belonging to different functional categories that were found to be upregulated during growth of *Methylocella silvestris* on acetate.

Functional categories	**No. of genes upregulated (%)**
Carbohydrate metabolism	8 (9.41)
Cell division	3 (3.52)
Electron transport	14 (16.47)
Fatty acid metabolism	3 (3.52)
Glycine metabolism	4 (4.70)
Protein metabolism	8 (9.41)
Putative uncharacterized protein	11 (12.94)
Pyruvate metabolism	2 (2.35)
Ribosome biosynthesis	1 (1.17)
Regulatory functions	3 (3.52)
RNA binding protein	1 (1.20)
Secretory function	1 (1.17)
TCA cycle	2 (2.35)
Vitamin and cofactor metabolism	1 (1.17)
Transport	5 (5.88)
Others	18 (21.17)
Total	85 (100.00)

6.2.7. Preliminary validation of microarray results using reverse transcription quantitative PCR

An attempt was made to validate the microarray results using reverse transcription quantitative PCR (RT-qPCR) with a SYBR® Green-based approach using the Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems). Genes selected for the RT-qPCR were the 16S rRNA gene, RNA polymerase encoding gene (*rpoB*) (Msil3868), *mmoX* (Msil1262), *glcB*/Malate synthase (Msil2501), *icl*/isocitrate lyase (Msil3157), fur/ferric uptake regulator (Msil1272) and glycine dehydrogenase (Msil1215). Detailed procedures of the RT-qPCR are described in **Chapter 2** in **section 2.4.4**. Synthesis of cDNA from RNA extracted from methane or acetate grown *Methylocella silvestris* were confirmed by amplification *mmoX* and 16S rRNA genes from the cDNA by RT-PCR (**Figure 6.9**).

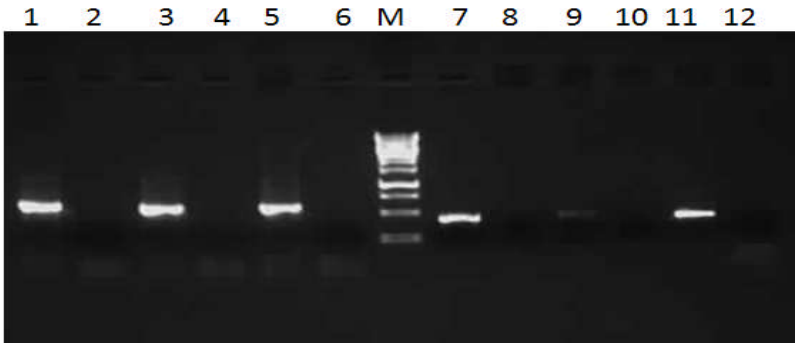


Figure 6.9. Amplification of 16S rRNA gene (Lane 1 to 6) and *mmoX* gene (Lane 7 to 12) from cDNA generated from RNA extracted from *Methylocella silvestris* grown either on methane or acetate. Lane 1 and lane7 = cDNA (methane), lane 2 and lane 8 = RT negative (methane), Lane 3 and lane 9 = cDNA (acetate), Lane 4 and lane 10 = RT negative (acetate), Lane 5 and lane 11 = PCR positive control (*Methylocella silvestris* genomic DNA), Lane 6 and lane 12 = Negative control (Non-template control), M=1 kbp DNA ladder.

However, when the RT-qPCR was carried out with the cDNA, the dissociation curve analysis indicated the formation of non-specific amplicons at the end of the assay. Further attempts were not made in this regard due to time limitations.

6.3 Discussion

The use of high quality RNA is the first step in obtaining meaningful gene expression data. The quality of the RNA used in this study for the microarray experiments were judged by determining the RNA integrity number (RIN) that reflects the integrity of the RNA (Fleige *et al.*, 2006). Total RNA extracted using the hot phenol-chloroform method as described by Gilbert *et al.* (2000) yielded high

quality RNA as revealed by analyses using the 2100 Bioanalyzer (Agilent Technologies, USA).

The technical and biological replicates of RNA used in the microarray experiment did not have any major effect on the findings of the microarray results as shown by the normalized signal intensity values of probes generated from the different replicates (**Figure 6.5**) and the PCA analysis (**Figure 6.6**). However, to make the microarray results more meaningful, only these data obtained from three biological replicates were included in the final analyses. Cluster analyses of the data obtained from the microarray experiments were carried out based upon biological replicates (**Figure 6.7**). Cluster analysis is traditionally used in phylogenetic research and has also been adopted for microarray analysis. It is an also an easy way to find out which genes are differentially transcribed under different growth conditions. The results of the cluster analysis indicated that genes found to be highly upregulated during growth on methane were found to be highly downregulated during growth on acetate, and vice versa, as expected.

To provide global information on gene expression during growth on C_1 or C_2 compounds, microarray experiments were carried out by comparing methane (C_1 compound) with acetate (C_2 compound) growth conditions. During growth on methane, more genes (n=169; 4.25% of the total genome) were found to be upregulated compared to the cells grown on acetate (n=85; 2.14% of the total genome). As expected, all the genes of the sMMO operons, both the structural genes *e.g., mmoXYBZDC* and the regulatory genes *e.g., mmoR, orf2* and *mmoG,* were found to be highly upregulated (~ 40 to ≥ 1000 fold) during growth on methane.

The fold changes in the expression of sMMO gene clusters as observed in the microarray experiment are present in **Figure 6.10.**

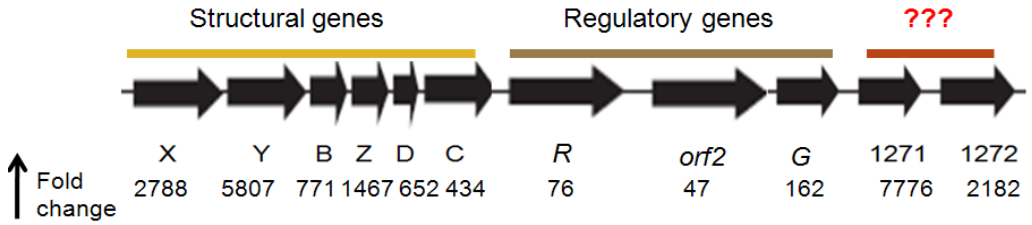


Figure 6.10. The sMMO operon and related genes and their fold changes as observed during growth of *Methylocella silvestris* on methane in the microarray experiment.

The *mmoXYBZDC* gene cluster encodes different subunits of the hydroxylase and associated protein of sMMO, while *mmoR, orf2* and *mmoG* encode the regulatory proteins required for the transcription of sMMO (Please see **Chapter 1 section 1.4.2** for details about the genes located in the sMMO gene cluster and their functions). Upregulation of all of the genes of the sMMO operon in *Methylocella silvestris* during growth on methane is in agreement with the earlier observation of Theisen *et al.* (2005). These authors detected the expression of transcript of the *mmoXYBZDC* operon by RT-PCR with cDNA generated from RNA extracted from *Methylocella silvestris* grown on methane. These authors also demonstrated that acetate represses the transcription of sMMO, which is in agreement with the microarray results observed here. In addition, the RT-PCR result carried out here also indicated that

acetate significantly repressed the transcription of *mmoX* (**Figure 6.9**) in *Methylocella silvestris.*

It is important to note that although all the genes of the sMMO operon are under the control of the same promoter, their transcription level varied from ~ 40 to 1000 fold (**Figure 6.10**) during growth on methane. It is not unexpected and a similar phenomenon has been observed at the transcription and translation level of the *aceBAK* operon in *Escherichia coli* during growth on acetate (Chung *et al.*, 1993). *aceA, aceB* and *aceK* encode isocitrate lyase, malate synthase and isocitrate dehydrogenase kinase/phosphatase respectively (Brice & Kornberg, 1968) . These three genes are also under the control of the same promoter, however, their transcription and translation patterns are highly variable during growth on acetate and is related to premature transcriptional termination (Chung *et al.*, 1993).

It is very interesting to note that two other genes located immediately downstream (3′) of the sMMO operon, *e.g.,* Msil1271 and Msil1272 were also upregulated during growth on methane (> 1000 fold) (**Figure 6.10**). Msil1271 encodes a hypothetical protein with no significant identity to any characterized genes, while Msil1272 is annotated in the genome as a ferric uptake regulator (Fur). Fur is a global transcriptional regulator involved in the regulation of genes responsible for iron acquisition (Hantke, 2001; Rodriguez & Smith, 2003). Iron is an essential component of the α subunit of sMMO since iron is an essential component of the diiron active site of sMMO (Green & Dalton, 1985; Fox *et al.*, 1989). Iron is also required for the activity of formate dehydrogenases (Laukel *et al.*, 2003) and the cytochrome associated with methanol dehydrogenase enzymes which are also

required during growth on methane for the metabolism of the oxidation products arising from methane (Zhang *et al.,* 2005). It may be possible that during growth on methane, *Methylocella silvestris* upregulates the transcription of *fur* so that Fur can in turn induce the transcription of other genes required for the uptake of iron from the medium for the activity of sMMO. For example, two other genes Msil0825 and Msil0826 that encode ExbD and ExbB respectively were found to be upregulated (~ 2 to 3 fold) during growth on methane. ExbD and ExbB are membrane-bound transport proteins possibly involved in iron acquisition. In *Xanthomonas campestris* pv. *campestris* Exb B and ExbD are essential for ferric uptake (Wiggerich *et al.*, 1997). In *Escherichia coli,* ExbB and ExbD are required for the transport of siderophores (Ahmer *et al.*, 1995). In *Methylocella silvestris*, Msil0825 and Msil0826 gene products could also be involved in iron acquisition. In addition, Fur may have some regulatory effect on the transcription of these genes.

During the methane oxidation process, methanol produced from the methane by the methane monooxygenase is converted into formaldehyde by the methanol dehydrogenase (Antony, 1982) (see **Chapter** 1, **section 1.3**, figure 1.4 for methane oxidation pathway). Methanol dehydrogenase is a pyrroloquinoline quinone (PQQ)-linked enzyme encoded by the *mxa* gene cluster *i.e., mxaFJGIRSACKLDEH*. Unusually none of these genes were found to be significantly upregulated during growth on methane, except for two genes Msil1739 and Msil2260 (~ 2 to 160 fold) that are involved in PQQ biosynthesis process. PQQ acts as a prosthetic group for several bacterial enzymes, including methanol dehydrogenase (Anthony, 1982). It was surprising not to see the upregulation of other genes of the *mxa* gene cluster during growth on methane, since methanol dehydrogenase is the second key enzyme

of the methane oxidation pathway. It may be possible that the probes on the microarray designed to target the *mxa* gene cluster were not specific enough/ or hybridization conditions were not conductive to detect the transcripts of other genes of *mxa* gene cluster. Alternatively, methanol dehydrogenase, seems to be expressed in *Methylocella silvestris* under all growth conditions tested (*e.g.*, growth on methane, methanol, succinate, acetate and propane). (Andrew Crombie, *personal communication*). A similar type of results has been observed earlier in microarray experiments, where only one or two genes of an operon were found to be significantly upregulated. For example in *Methylobacterium extorquens* AM1, only one or two genes of the *mxa* operon or the *pqq* operon were found to be significantly upregulated during growth on methanol (Okubo *et al.*, 2007), while the expression of the rest of the genes of the *mxa* operon or *ppq* operon remained unchanged. A gene Msil3655 that encodes the formate dehydrogenase, the last enzyme of the methane oxidation pathway was found to be upregulated (~ 20 fold) during growth on methane. Formate dehydrogenase catalyzes the conversion of formate to CO_2 and supplies reducing power as $NADH+H^+$ for biosynthesis and for initial oxidation of methane by sMMO (Anthony, 1982). Upregulation of these genes suggests that as expected, the methane oxidation pathway was active in *Methylocella silvestris* during growth on methane.

The serine cycle is necessary for the synthesis of cellular biomass in many methanotrophs during growth on C_1 compounds such as methane (Anthony, 1982). The serine cycle is also necessary for the synthesis of cellular biomass during growth on multi-carbon compounds such as succinate by the facultative methylotroph *Methylobacterium extorquens* AM1 (Heptinstall & Quayle, 1970) (please see

Appendix 4 for the serine cycle pathway). Among the serine cycle genes, only one gene, Msil1719 encoding malyl CoA lyase, was found to be upregulated (~ 2 fold) during its growth on methane. Malyl CoA lyase catalyzes the reversible conversion of L-malyl-CoA into glyoxylate and acetyl-CoA (Meister *et al.*, 2005). However, in the serine cycle of *Rhodobacter capsulatus*, the enzyme malyl-CoA lyase is upregulated during growth on acetate (Meister *et al.*, 2005). It was not surprising to see no significant differential transcription of most of the genes of the serine cycle during growth on methane or acetate, since as described above, the serine cycle is active during growth on both the C_1 and C_2 compounds.

The TCA cycle is the central metabolic pathway for all aerobic processes. All the genes involved in the TCA cycle have been identified in the genome of *Methylocella silvestris* (Chen *et al.*, 2010). None of the TCA cycle genes were found to be upregulated during growth on methane. This finding was not surprising, since the TCA cycle is not required for the utilization of C_1 compounds by methanotrophs (Taylor & Anthony, 1976), rather it is required for the utilization of C_2 compounds. A gene knockout based study of the gene encoding the 2-oxoglutarate dehydrogenase in *Pseudomonas* AM1, which is one of the key enzymes of the TCA cycle, Taylor and Anthony (1976) demonstrated that both the wild type and the mutant have the same phenotype, *i.e.* they are both to grown on C_1 compounds, confirming that TCA cycle is not required for the C_1 compound assimilation. However, the mutant did not grown on C_2 compounds, confirming that the TCA cycle is required for the assimilation of C_2 compounds.

Surprisingly among the TCA cycle genes, only one gene *e.g.,* Msil2501encoding the malate dehydrogenase was found to be upregulated (~ 4 fold) during growth on acetate. Malate dehydrogenase catalyzes the conversion of malate to oxaloacetate (Shows *et al.,* 1970). It is difficult to draw a conclusion as to why the other genes of the TCA cycle were not found to be upregulated during growth on acetate. However, one explanation could be that during growth on acetate, *Methylocella silvestris* uses the glyoxylate cycle instead of the TCA cycle for carbon assimilation.

The glyoxylate cycle allows the cells to bypasses several steps of the TCA cycle where carbon is lost as CO_2, thus allowing the cells to conserve carbon (Kornberg & Krebs, 1954) (please see **Appendix 5** for the glyoxylate cycle). This pathway is essential for bacteria when growing on two-carbon compounds (Chung *et al.,* 1988). The first enzyme of the glyoxylate cycle is isocitrate lyase which catalyzes the formation of succinate directly from isocitrate thus bypassing two steps of the TCA cycle to prevent loss of carbon as CO_2 (Kornberg & Krebs, 1954). A gene Msil3157 that encodes the isocitrate lyase in *Methylocella silvestris* was found to be upregulated (~ 5 fold) during growth on acetate, thus suggesting that the glyoxylate cycle was probably functional during growth on acetate. However, surprisingly malate synthase, the second enzyme of glyoxylate cycle, which catalyzes the condensation of acetyl-CoA to glyoxylate to form malate (Höner Zu Bentrup et al., 1999) was not found to be upregulated, and could be related with the specificity of the probes designed to target the transcriptome of malate synthase. It will be interesting to carry out qRT-PCR for a definite answer regarding the transcription of malate synthase during growth on acetate. Similar approaches could be applicable for some of the other genes, such as those of the *mxa* gene cluster that surprisingly were not found to be upregulated during growth on methane (described earlier).

Before being utilized by the cell, acetate is first converted into acetyl-CoaA, which is an essential intermediate at the junction of various anabolic and catabolic pathways and a key player in the central carbon metabolism in all three domains of life (Ingram-Smith *et al.,* 2006). There are few pathways through which acetate is directly converted into acetyl coenzyme A (acetyl-CoA) (Ingram-Smith *et al.,* 2006). For example acetyl-CoA synthetase catalyzes the direct formation of acetyl-CoA from acetate. Acetate can also be converted into acetyl-CoA through acetate kinase and phosphotransacetylase (please see **Appendix 6** for the possible pathways of formation of acetyl-CoA formation from acetate in *Methylocella silvestris* BL2). In the genome of *Methylocella silvestris,* genes encoding the acetyl-CoA synthetase (Msil1226), acetate kinase (Msil2706) and phosphotransacetylase (Msil0083) have been identified. However, surprising none of these genes were found to be upregulated in *Methylocella silvestris* during growth on acetate. Again these could be simply due to poor specificity of the probes, and qRT-PCR will need to be carried out to confirm the expression pattern of these genes during growth on acetate.

Recently Okubo *et al.,* (2010) demonstrated a possible alternative pathway in *Methylobacterium extorquens* AM1 responsible for the consumption of glyoxylate, where glyoxylate is converted into glycine, methylene-H_4F, and serine for subsequent pathways. In *Methylobacterium extorquens* AM1 it has also been suggested that interconversions of glycine and serine play a role in C_2 metabolism (Chistoserdova & Lidstrom, 1994). In the microarray experiment, the formation of glyoxylate by *Methylocella silvestris* during growth on acetate is evident by the upregulation of isocitrate lyase as mentioned earlier. Dedysh *et al.,* (2003) failed to detect the activity of isocitrate lyase in *Methylocella silvestris*, however, a recent

study from Colin Murrell's group showed that isocitrate lyase is functional in *Methylocella silvestris* organism during growth on acetate (Andrew Crombie, *personal communication*), supporting the findings of the microarray result. In addition, microarray experiments have identified genes that are involved in the metabolism of glycine *e.g.*, Msil1214 (glycine dehydrogenase), Msil1215 (probable glycine dehydrogenase subunit 1) and Msil1216 (glycine cleavage system H protein) were also found to be upregulated (~ 2 to 90 fold) during growth on acetate. Glycine dehydrogenase catalyze the reversable conversion of glycine into glyoxylate (Goldman & Wagner, 1962). These findings suggests that like *Methylobacterium extorquens* AM, the alternative pathway might be involved in the utilization of acetate in *Methylocella silvestris.*

The ability to fix N_2 is widely distributed among bacteria (Raymond *et al.*, 2004). Dedysh *et al.*, (2003) observed the ability of *Methylocella silvestris* to fix N_2. Genes involved in N_2 fixation such as Msil3623 (NifQ) and Msil3632 (NifH) were found to be upregulated (~ 6 to one hundred fold) during growth on methane. *Methylocella silvestris* were grown aerobically in DNMS medium which contains KNO_3 as the source of fixed nitrogen. However, it was surprising to see the upregulation of nitrogen fixing genes during growth on methane under aerobic conditions, since nitrogenase proteins are sensitive to oxygen (Dalton & Whittenbury, 1976). Since methane oxidation pathways require oxygen, therefore, it may be possible that for some reason the availability of O_2 in the batch culture dropped to a level, where the cells were able to fix the N_2.

In conclusion, in the microarray experiments, several genes were found to be significantly upregulated during growth on methane or acetate. A substantial fraction of the genes found to be upregulated are hypothetical genes that are assigned in the genome to encode putative uncharacterized proteins **(**33.14% of the total upregulated genes during growth on methane and 12.94% of the total upregulated genes during growth on acetate; **Table 6.1** and **Table 6.2**). These novel genes that do not have any similarity to other well characterized genes in the database pose an unexpected and exciting challenge, not just for functional genomics, but also for biology in general (Galperin, 2001; Galperin & Koonin, 2004). Even in well characterized organisms such as *Escherichia coli* and *Bacillus subtilis,* a large number of genes do not have any assigned functions (Galperin, 2001). It will be very interesting to carry out biochemical and gene knockout-based study to characterize the function of some of these hypothetical genes, such as Msil1271, to understand their role in methane utilization by *Methylocella silvestris.* Similar studies can be carried out for the Fur encoding Msil1272 or the glycine dehydrogenase gene cluster (Msil1214-Msil1216) to reveal their role in methane or acetate utilization respectively by *Methylocella silvestris.*

Chapter 7

Diversity of methanotrophs in the forest soil of Swedish islands as revealed by a *pmoA* diagnostic microarray

7.1 Introduction

The effect of ecosystem succession, vegetation, soil type and soil depth on the diversity of methanotroph communities have received little attention. There are reports suggesting that plant community structure (succession and land-use change) might alter the microbial communities that mediate greenhouse gas fluxes from soils (Reay *et al.*, 2005; Singh *et al.*, 2007) including the diversity and activity of methanotrophs present in soil (Bourne *et al.*, 2001; Knief *et al.*, 2003; Stralis-Pavese *et al.*, 2004; Chen *et al.*, 2008a). The primary aim of the work described in this chapter was to study the effect of soil vegetation, resulting from different successional stages, on the diversity of the methanotroph communities in forest soils from two Swedish islands. In addition, the effect of soil depth on the diversity of the methanotroph community was also studied. This study was undertaken because the opportunity was presented to examine some unusual samples which had been studied in detail with regard to methane oxidation process measurements. It also provided an opportunity to acquire skills in diagnostic microarray analysis. This study was carried out in collaboration with Ruth Gregg and Dr. Niall McNamara, CEH, Lancaster during June, 2008. Sample collection, determination of vegetation and methane flux measurements were carried out by Ruth Gregg at CEH, whereas all the DNA extraction and *pmoA* microarray experiments were carried out by Md.Tanvir Rahman at the Department of Biological Sciences, University of Warwick.

Two freshwater islands located in Uddjaure and Hornaven Lake in the north of Sweden, in a boreal forest region (65° 02′ N, 17° 49′ E) were selected for the study. Geographical locations of these two islands are presented in **Figure 7.1.**

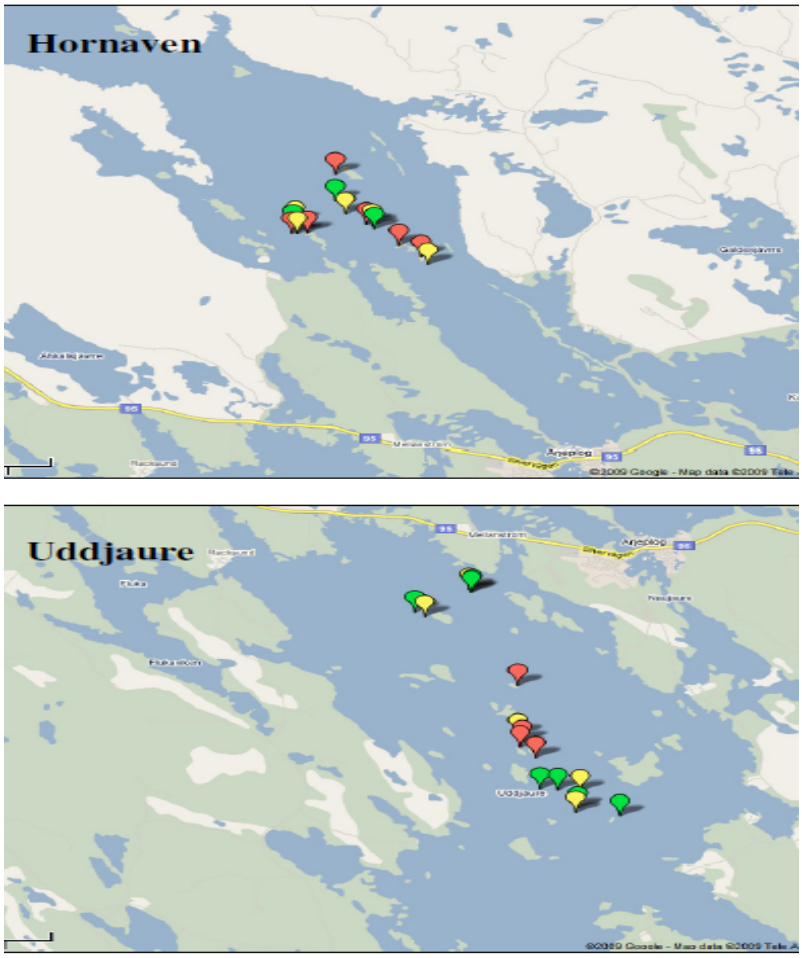


Figure 7.1. Location of islands in Hornaven (top) and Uddjaure (bottom) lakes, Sweden. The successional stage in islands are early successional (586 years; red markers), mid-successional (2220 years; yellow markers) and late successional (3215 years, green markers) (image provided with permission from Ruth Gregg, CEH, Lancaster).

Both of these lakes were formed when the land ice retreated 9000 years ago, leaving behind granite and moraine boulders to form the foundation of the current islands (Niall McNamara, *personal communication*). These islands have the same geological age and experience the same climatic conditions, having a mean annual precipitation of 750 mm and mean temperatures of -14° C in January and +12° C in July (Niall

McNamara, *personal communication*). The major extrinsic factor that differs between these islands is the frequency of wildfires resulting from lightening strike ignition. Large islands are more prone to frequent lightening strikes and wildfires compared to medium and small islands. The burning of vegetation cover on the soil results in the soil being at an early successional stage. The soils of the medium and small islands are comparatively less disturbed, since they are less frequently subjected to lightening strikes and biomass burning, resulting in the soil being at mid and late successional stage respectively. The most recent fire disturbances have been estimated by ^{14}C dating of the uppermost charcoal layer in the humus profiles. The time since the last fire disturbance ranges from 40 to 5330 years (Wardle *et al.*, 1997). Keller *et.al.* (1993) suggested that recovery of soil from disturbance such as vegetation cover burning occurs over long periods, even decades, but little is known about the ability of methanotrophs to colonize natural systems from which they are initially absent *e.g.*, colonization of fire-sterilized surface soil from the deeper layer of soil. The island chronosequence provides an ideal model to assess changes in methanotroph population and diversity during ecosystem succession. The distributions of early, mid and late successional Hornaven and Uddjaure islands are presented in **Figure 7.2. Figure 7.3** shows the examples of typical early, mid and late successional islands with different types of vegetation. Wardle *et al.* (2008) observed changes in the soil properties, including the types of vegetation and soil depth with succession of ages in these islands.

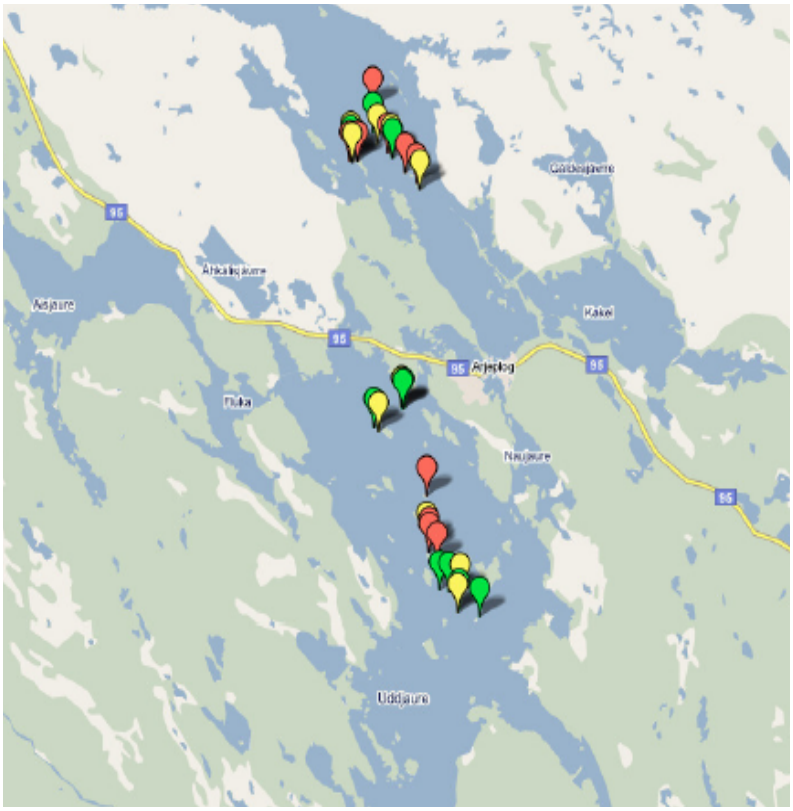


Figure 7.2. The distribution of early (red markers), mid (yellow markers) and late (green markers) successional Hornaven and Uddjaure islands, Sweden. These two groups of islands are adjacent to each other, and were both formed due to the retreat of land ice 9000 years ago (image provided with permission from Ruth Gregg, CEH, Lancaster).

Early successional island. Dominated by Scots pine (*Pinus sylvestris* L.)

Mid successional island. *P. sylvestris* L. still abundant, but downy birch (*Betula pubescens* Ehrh.) are increasing in numbers. A lone Norway spruce (*Picea abies* L.) stands in the centre of the island.

Late successional island. *Picea abies* L. abundant with several *Betula pubescens* Ehrh. Note the thick layer of ericaceous shrubs.

Figure 7.3. Examples of early, mid and late successional islands with different type of vegetation (images provided with permission from Ruth Gregg, CEH, Lancaster).

Detailed information on the vegetation mass, shrub productivity, tree productivity and humus mass etc of these islands are available in the literature (Wardle *et al.*, 1997; Wardle *et al.*, 2008). Unpublished work demonstrated that the rate of methane oxidation by soils was higher in late successional islands that had more time for habitat development compared to mid and early successional islands (Niall McNamara, *personal communication*). As methanotrophs present in these soil utilize methane, the variation in the methane flux observed in these islands soils might be related to the activity of different methanotroph communities occupying different types of soil (early, mid and late successional soil).

The *pmoA* diagnostic microarray is a semi-quantitative method to study the diversity of methanotrophs in environmental samples (Bodrossy *et al.*, 2003; Bodrossy & Sessitsch, 2004) (see **Chapter 2**, **section 2.11.1** for the *pmoA* microarray experiments, and **Appendix 1** for the list of *pmoA* probes and their intended specificity). In this study, the *pmoA* diagnostic microarray technique was applied to study the diversity of methanotrophs community in forest soil of the Swedish islands that are at different successional stages. In addition, the effect of soil depth on diversity of methanotrophs community in these islands was also studied. The sources of soil samples collected from Hornaven and Uddjaure islands and their characteristics are presented in **Table 7.1**.

Table 7.1. Sources of soil samples collected from Hornaven and Uddjaure islands and their characteristics.

Location	Sample/ Island name	Soil depth (cm)	Soil pH	Successional stage	Dominant vegetation
Hornaven, Sweden 66° 13′ N, 17° 78′ E	H28.1	0-20	~ 4.5	Early	*Pinus sylvestris* L.
	H37.1	0-20		Middle	*Pinus sylvestris* L., *Betula pubescens* Ehrh., *Picea abies* L.
	H37.2	20-40	~ 4.5	Middle	*Pinus sylvestris* L., *Betula pubescens* Ehrh., *Picea abies* L.
	H37.3	40-60	~ 4.5	Middle	*Pinus sylvestris* L., *Betula pubescens* Ehrh., *Picea abies* L.
	H43.1	0-20	~ 4.5	Late	*Pinus sylvestris* L., *Betula pubescens* Ehrh., ericaceous shrubs
	H43.2	20-40	~ 4.5	Late	*Pinus sylvestris* L., *Betula pubescens* Ehrh., ericaceous shrubs
	H43.3	40-60	~ 4.5	Late	*Pinus sylvestris* L., *Betula pubescens* Ehrh., ericaceous shrubs
Uddjaure, Sweden 65° 60′ N, 17° 48′ E	U36.1	0-20	~ 4.5	Middle	*Pinus sylvestris* L., *Betula pubescens* Ehrh., *Picea abies* L.
	U36.2	20-40	~ 4.5	Middle	*Pinus sylvestris* L., *Betula pubescens* Ehrh., *Picea abies* L.
	U36.3	40-60	~ 4.5	Middle	*Pinus sylvestris* L., *Betula pubescens* Ehrh., *Picea abies* L.
	U37.1	0-20	~ 4.5	Late	*Pinus sylvestris* L., *Betula pubescens* Ehrh., ericaceous shrubs
	U37.2	20-40	~ 4.5	Late	*Pinus sylvestris* L., *Betula pubescens* Ehrh., ericaceous shrubs
	U37.3	40-60	~ 4.5	Late	*Pinus sylvestris* L., *Betula pubescens* Ehrh., ericaceous shrubs
	U58.1	0-20	~ 4.5	Early	*Pinus sylvestris* L.,
	U58.2	20-40	~ 4.5	Early	*Pinus sylvestris* L.,

7.2 Results

7.2.1 Amplification of *pmoA* genes sequences

Initially attempts were made to amplify *pmoA* from DNA extracted from different forest soil of Swedish islands using the conventional *pmoA* primer A189f along with either T7mb682 or T7mb661 by direct, touchdown or nested PCR approaches (Stralis-Pavese *et al.*, 2004). No *pmoA* amplicons could be obtained using these primers. The absence of PCR inhibitory substances such as humic acids that may be present in these soils, possibly interfering with the amplification of *pmoA*, was ruled out since amplification of bacterial 16S rRNA genes using the 27f and 1492r primer set (Lane, 1991) was successful with the same DNA template (**Figure 7.4**). In addition, when these extracted DNA samples were spiked with genomic DNA of *Methylococcus capsulatus*, it was possible to amplify *pmoA* from the spiked DNA suggesting the absence of PCR inhibitory substances in the extracted DNA. When alternative primer T7mb650 (Bourne *et al.*, 2001) was applied as the reverse primer along with the A189f forward primer, it was possible to obtain *pmoA* amplicons from these DNA samples (**Figure 7.5**), and these were later used for the microarray analyses (the protocol for the *pmoA* microarray experiments is described in **Chapter 2**; **section 2.11.1**).

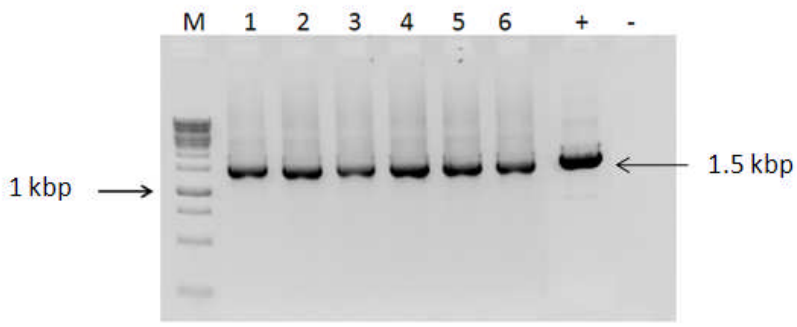


Figure 7.4. Representative figure showing the amplified bacterial 16S rRNA gene products from DNA extracted from forest soil of Swedish islands that are at different successional stages using primers 27f and 1492r. Lane 1-2, Early successional islands. Lane 3-4, Mid successional islands. Lane 5-6, Late successional islands, + = Positive control (*Methylococcus capsulatus* genomic DNA), - = Negative control (non template control), M = 1 kbp DNA ladder,

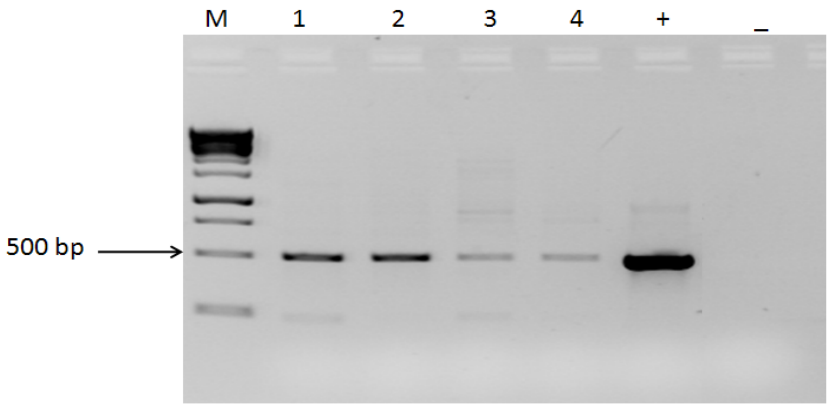


Figure 7.5. Representative figure showing the amplified *pmoA* gene products from DNA extracted from forest soil of Swedish islands that are at different successional stages using the primers A189f and T7mb650. Lane 1-2, Late successional islands. Lane 3, Mid successional island. Lane 4, Early successional island. + = Positive control (*Methylococcus capsulatus* genomic DNA), - = Negative control (non template control), M = 1 kbp DNA ladder.

7.2.2 Diversity of methanotrophs as revealed by a *pmoA* diagnostic microarray

A *pmoA* diagnostic microarray was used to compare the methanotrophs community structures between forest soils from Swedish islands that are at different successional stages. The list of 135 *pmoA* probes spotted on the array and their target groups are listed in **Appendix 1.** Hybridization signals captured by the *pmoA* probes that are specific for specific groups of methanotrophs are presented in **Figure 7.6.**

In general, no hybridisation signals were detected for probes targeting *pmoA* sequences from type Ia methanotrophs in all of the DNA samples extracted from the forest soil of these Swedish islands. Moreover, all of these DNA samples yielded no hybridisation signals for any probes targeting ammonium oxidizers, *Methylocapsa*, Wsh1 (Watershed and flodded upland cluster 1) and Wsh2 (Watershed + flooded upland cluster 2) groups and *pmoA* of uncertain physiology groups. Hybridisation signals for probe Mc396, targeting *Methylococcus*-related *pmoA* sequences (type Ib methanotroph), were detected with varying intensities in all of the DNA samples except for the DNA extracted from islands H28.1, U58.2, U36.1 and U36.2 (characteristics of these island *e.g.,* locations, soil depth, soil pH, successional stage and dominant vegetation are given in **Table 7.1**). However, hybridisation signals for probe 501-375 (*Methylococcus*-related marine and freshwater sediment clones) were detected in DNA extracted from islands H37.1, H37.2, U37.1 and H43.1 (**Figure 7.6**).

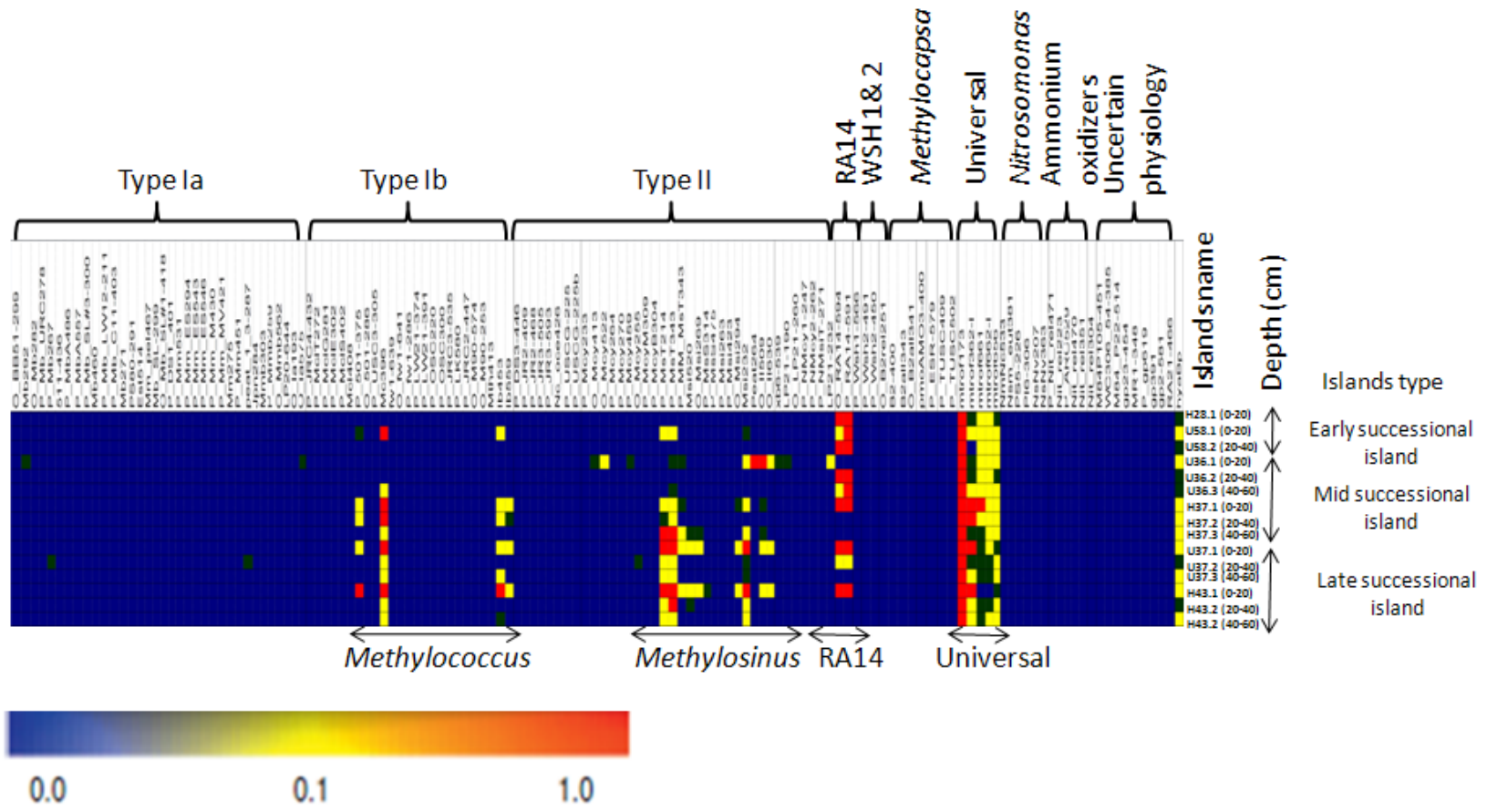


Figure 7.6. *pmoA* microarray analysis representing methanotroph community structure based on hybridization signal patterns of DNA from forest soil of Swedish islands. Colour coding is indicated by the bar below the microarray. A value of 1.0 (red) indicates maximum achievable signal for an individual probe, a value of 0.1 (yellow) indicates that 10% of the total PCR product hybridized to that probe and and a value of 0.0 (blue) indicates that less than 10% of the total PCR product hybridized to that probe. To see the list of individual *pmoA* probes and their targets, please see the **Appendix 1.**

For type II methanotrophs, hybridisation signals were dominated by *Methylosinus trichosporium* OB3b-related *pmoA* sequences (probe MsT214) and were detected with different signal intensities. While stronger signal intensities for probe MsT214 were detected in DNA extracted from islands H37.3, U37.1 and H43.1, weaker signal intensities were detected in DNA extracted from islands U58.1, H37.1, H37.2, U37.2, U37.3, H43.2 and H43.3 (**Figure 7.6**).

In comparison to other DNA samples, DNA extracted from island U36.1 was characterised by a low diversity of methanotrophs and was dominated by *pmoA* sequences related to the *pmoA* peat clones (probe peat264). It should be noted that no hybridisation signal were detected with other DNA samples for the *pmoA* probe peat264. Similarly DNA extracted from islands H28.1, U58.2 and U36.2 were dominated by *pmoA* sequences related to *pmoA* sequences designated as sequence type RA14 (Holmes *et.al.,* 1999) demonstrated by strong signal intensities for *pmoA* probes RA14-594 and RA14-591. Hybridisation signals for these probes were also detected in DNA extracted from islands U58.1, U36.3, H37.1, U37.1, U37.2, and H43.1 (**Figure 7.6**).

In general, the diversity of methanotrophs was higher in mid and late successional islands compared to early successional islands, except for islands U58.2 and U36.1 that were at early and mid successional stages respectively. In addition, the diversity of methanotrophs was higher in the upper most layer of the soil compared to the mid and deeper layers of the soils, especially in the mid and late successional islands (**Figure 7.6**).

7.2.3. *pmoA* and Type II methanotroph 16S rRNA gene clone library analysis

The presence of signals specific for *pmoA* of *Methylococcus* and the upland soil cluster/RA14 clade, as revealed by the *pmoA* microarray, was confirmed by *pmoA* clone library analyses of DNA extracted from island H43.1. A small clone library was constructed with *pmoA* amplicons generated from H43.1 DNA using the primers sets A189f and T7mb650. Five clones were selected randomly and sequenced. The results of BLASTx search of these clones revealed *pmoA* sequences related to *Methylococcus capsulatus* and *pmoA* sequence related to the uncultivated methanotroph RA14 (**Table 7.2**).

Table 7.2. BLASTx results of *pmoA* sequences retrieved from DNA extracted from H43.1 island.

***pmoA* clones**	**BLASTx results (GenBank accession number; amino acid sequence identities)**
Clone 3	*Methylococcus capsulatus* (YP115248; 97%)
Clone 5	*Methylococcus capsulatus* (YP115248; 99%)
Clone 11	*Methylococcus capsulatus* (YP115248; 100%)
Clone 13	Uncultivated methanotroph RA14 (AAD47927; 98%)
Clone 17	*Methylococcus capsulatus* (YP115248; 100%)

In addition, a type II methanotroph 16S rRNA gene clone library was constructed with the same DNA using primers type IIF and type IIR (Chen *et.al.,* 2007). Nucleotides BLAST search of five randomly selected clones identified uncultivated *Methylosinus* sp., *Methylosinus trichosporium*, *Methylocystis* sp. and uncultivated *Alphaproteobacterium* related 16S rRNA gene sequences **(Table 7.3)**.

Table 7.3. Nucleotide BLAST results of 16S rRNA gene sequences retrieved from DNA extracted from H43.1 island.

Type II clones (16S rRNA gene)	**Nucleotide BLAST results (GenBank accession number; nucleotide sequence identities)**
Clone 2, Clone 3	Uncultivated *Methylosinus* sp. (EU359952; 98%)
Clone 5	*Methylocystis* sp. (DQ852351; 97%)
Clone 6	Uncultivated *Alphaproteobacterium* clone FAC13 (DQ451452; 99%) (closest extant methanotroph: *Methylocapsa acidiphila* : 96%)
Clone 11	*Methylosinus trichosporium* (AJ868424; 97%)

7.3 Discussion

This chapter aimed to characterise the diversity of methanotrophs along the successional gradient and soil depth in selected forest soil of Swedish islands, in order to explain the associated changes in the ability of the soil to act as a net methane sink. The methane oxidation rate was found to be higher in late successional islands compared to that of mid and early successional islands (Niall McNamara,

personal communication). This methane oxidation rate was high in the late successional islands, medium at mid successional islands and low at early successional islands (Niall McNamara, *personal communication*). This higher methane oxidation rate in late successional islands soil might be related to less disturbance by lightning strike, which allowed more time for a more favourable habitat development compared to the mid and early successional islands. Perhaps methanotrophs in this system require significant plant and microbial community development and prolonged absence of disturbance before they can colonise these soils. King and Nanba (2008) studied the effect of Hawaiian volcanic deposits on the development of methanotroph communities during ecosystem succession. Based on *pmoA* and *amoA* clone library analyses they observed that methanotrophs colonize volcanic substrates slowly and likely depend on interactions with plants and other microbial communities.

The diversity of methanotrophs along the successional gradient and soil depth in selected Swedish island was analyzed using a *pmoA* diagnostic microarray (**Figure 7.6**). In this study, it was not possible to amplify any *pmoA* gene PCR products from the forest soil of Swedish island using the conventional *pmoA* primer sets A189f and T7mb682 or A189f and T7mb661, which are usually used to target *pmoA* sequences from a variety of environmental samples. However, when reverse primer T7mb650 was applied along with forward primer A189f, it was possible to amplify *pmoA* gene PCR products from the same DNA. Primer T7mb650 was originally designed by Bourne *et al.* (2001) to detect high-affinity methane oxidizers in Danish forest soil, which could not be detected using the conventional *pmoA* primer sets, despite the biases of mb650 primer for *Methylococcus capsulatus* and low coverage in detecting *pmoA* sequences of other methanotrophs.

The *pmoA* microarray analyses of soil from mid and late successional islands revealed *Methylococcus* (probe Mc396 and probe 501-375) and *Methylosinus* (probe MsT214)-like methanotrophs (**Figure 7.6**) to be the dominant methanotrophs in these soil. *pmoA* sequences related to *Methylococcus* had earlier been retrieved from DNA extracted from Norwegian forest soil (Jensen *et al.*, 2000), Craigieburn Range forest soil, New Zealand (Singh & Tate, 2007), forest soil from Marburg, Germany (Mohanty *et al.*, 2007) and forest soil from Harvard, USA (Lau *et al.*, 2007). It is interesting to see *Methylococcus* in these sub-arctic soils, since *Methylococcus* spp. are considered as moderate thermotolerant / thermophilic bacteria, however, there are reports suggesting that some species of *Methylococcus* can grow at much lower temperature, such as at 20° C (Bowman *et al.*, 1993a). Signal intensities for probes targeting *Methylosinus* also increased as more ecosystems developed in mid and late successional island forest soils compared to early successional islands. Mohanty *et al.* (2007) retrieved *Methylosinus*-like *pmoA* sequences from Marburg forest soil, Germany. Roslev and King (1995) reported that *Methylosinus trichosporium* can survive carbon deprivation under anoxic conditions (even though this was for only for six weeks). Moreover, *Methylosinus* are able to form exospores that allow them to remain in a resting stage under harsh environments (Whittenbury *et al.*, 1970b). The presence of more intense signals for *pmoA* from *Methylosinus* spp. in soil from mid and late successional islands could be due to these methanotrophs being able to withstand the long winters during which the soils are frozen and covered in snow. In fact, recently Schütte *et.al.* (2010) using a pyrosequencing approach detected several methanotrophs sequences including *Methylosinus* spp in soils collected from cold glacier foreland of high Arctic. In addition, Chen *et.al.* (2008b) also found

Methylosinus spp. to be the dominant methanotrophs in acidic Moor House peat soils, which are similar to the acidic and organic soils of this study.

Strong signals for *pmoA* probes targeting RA14 clade (probes RA14-594 and RA14-591) were detected in the top layer of all the island soils that were at different successional stages except for island U36.1 (**Figure 7.6**). The RA14 clade consists of *pmoA* sequences from uncultivated *Alphaproteobacteria,* distantly related to *Methylocapsa acidophilia* and are thought to be involved in the utilization of atmospheric concentrations of methane (Holmes *et al.*, 1999). In fact, unpublished data suggested that these island soils were capable of utilizing atmospheric concentrations of methane under *ex situ* conditions (Gregg, 2010). In comparison to other islands, island U36.1 was unique, because it is the only island that was positive for *pmoA* sequences related to peat clones (probe peat264).

The overall diversity of methanotrophs was relatively higher in mid and late successional islands compared to early successional islands. This might be related to the availability of more vegetation and formation of more organic material in the mid and late successional islands. Gregg (2010) also indicated that, when soil samples from mid and late successional islands were incubated under 1,000 ppm methane, the methane uptake rate was found to be higher when compared to the early successional islands. These findings suggest that the methanotrophic community require long periods without disturbance to successfully colonise soils.

Although forest soils have been extensively studied because of their involvement as a biological sink for atmospheric methane, fewer studies have looked at the changes in methanotroph community structure with soil depth. The highest methane oxidation activity in forest soils was measured in subsurface soil layers (Whalen *et*

al., 1992). This localization of methanotrophs in deeper soil layers was attributed to inhibition of methanotrophs by ammonium or terpenes that are released or produced in the organic surface layers of the forest soil (King & Schnell, 1994; Schnell & King, 1994; Amaral & Knowles, 1997). The *pmoA* diagnostic microarray experiment carried out here indicated that the overall diversity of methanotrophs was higher in the top most layer of the soil compared to the mid and bottom layers of the soil, especially in mid and late successional islands **(Figure 7.6),** which might be a result of the lower oxygen diffusion rates into the deep layers of soil.

No hybridisation signals were detected for *pmoA* from type Ia methanotrophs, *Methylocystis, Methylocapsa,* ammonium oxidizers, Wsh1 and Wsh2 groups and *pmoA* of uncertain physiological groups in any of the DNAs tested here. However, it might be possible that methanotrophs related to these groups or species were present in these soils, but below the 5% detection limit of the *pmoA* microarray (Bodrossy *et al.*, 2003). Any population of methanotrophs that represents less than 5% of total bacterial population in these soils is probably not detected by the *pmoA* microarray (Bodrossy *et al.*, 2003).

The diversity of methanotrophs observed here using the *pmoA* microarray was also confirmed by analyses of both *pmoA* and type II methanotroph 16S rRNA gene clone libraries (**Table 7.2 and 7.3**). For example, a *pmoA* clone library constructed with DNA extracted from island H43.1 soil identified sequences phylogenetically related to *pmoA* from *Methylococcus capsulatus* (**Table 7.2**). Hybridization signals for *pmoA* probes targeting *Methylococcus capsulatus* (probe Mc396 and probe 501-375) were also detected in the microarray analysis of the same DNA sample (**Figure 7.6**). *pmoA* clone library analysis also identified *pmoA* sequences related to uncultivated methanotroph RA14. Microarray results revealed signals for *pmoA* of the RA14

clade (probes RA14-594 and RA14-591), which phylogenetically belong to the same clusters that contain upland soil cluster α (Knief *et al.*, 2003). To confirm this, a type II methanotroph 16S rRNA gene clone library was constructed with the same DNA extracted from island H43.1. The 16S rRNA genes obtained were phylogenetically related to uncultivated *Methylosinus* sp., *Methylosinus trichosporium* and *Methylocystis* sp. (**Table 7.3**). Signals for *Methylosinus* (probe MsT214) were also found in the microarray analysis. No signals for the *pmoA* sequences of *Methylocystis* were detected using the *pmoA* microarray, but were observed in the type II methanotrophs 16S rRNA clone library, suggesting that perhaps their numbers could be below the detection limit of the *pmoA* microarray (Bodrossy *et al.*, 2003). Alternatively, lack of detection of signals for *Methylocystis* could be associated with the bias of these primers as mentioned earlier. Surprisingly no clones related to *pmoA* sequence of *Methylococcus capsulatus* was detected in the type II methanotroph 16S rRNA gene clone library. A more extensive *pmoA* and a type II methanotroph 16S rRNA gene clone library will provide further information on the full coverage methanotrophs present in these island soil.

In addition, DNA extracted from all these islands were tested for the presence of *Methylocella* ssp. All the DNA samples were found to be positive for the presence of PCR products of *mmoX* and 16S rRNA gene of *Methylocella* spp. (Discussed in **Chapter 3**).The presence of *Methylocella* in these Swedish island forest soil could be related with the acidic pH of these islands soil, since *Methylocella* are moderately acidophilic methanotrophs (Dedysh *et al.,* 2005). The total abundance of *Methylocella* spp. in island H28.1 and U36.1 was quantified using newly developed qPCR assay that target the *mmoX* genes of *Methylocella* spp. (described in **Chapter 4**; **section 4.2.6).** The abundance of *Methylocella* spp. in island H28.1 and U36.1was

quantified as 3.3 (± 0.6) × 10^6 copies and 2.1 (± 0.5) × 10^6 copies per g of soil respectively. *Methylocella* present in these forest soils might have some contribution in the observed methane oxidation by these island forest soils, along with other *pmoA*-containing methanotrophs detected in the *pmoA* microarray.

In conclusion, the diversity of methanotrophs in forest soils of Swedish islands that are at different successional stages was studied using a *pmoA* diagnostic microarray. Primers A189f and T7mb650 successfully detected *pmoA* sequences related to different methanotrophs. In general, *Methylococcus*, *Methylosinus* and methanotrophs of the uncultivated RA14 clades were the most dominant methanotrophs in these forest soils. A trend in increasing methanotroph diversity was observed as the successional stages progressed with increase in the age of soils. The diversity was higher in late successional islands compared to other successional islands. In addition, the methanotroph diversity decreased as the soil depth increased. The *mmoX* and 16S rRNA gene sequences of *Methylocella* spp. were detected in these island forest soils. It will be interesting to quantify the abundance of *Methylocella* and other methanotrophs present in all of these islands to investigate the correlation between the abundance of methanotrophs and the methane oxidation data. Detailed temporal and spatial studies on the abundance and diversity of methanotrophs in these island soils, with more sensitive techniques such as DNA/RNA-SIP or qPCR, are required in the future to understand the effect of ecosystem succession and soil depth on methanotrophs community development.

Chapter 8

Final summary, discussion and future perspectives

8.1 Final summary, discussion and future perspectives

Methylocella spp. are facultative methanotrophs (Dedysh *et al.* 2005; Theisen *et al.*,2005). There is lack of adequate information on the ecology of these facultative methanotrophs in the environment. This study improves our knowledge of i) the distribution and abundance of *Methylocella* spp. in the environment (**Chapter 3 and Chapter 4**), ii) the effect of acetate on oxidation of methane by *Methylocella silvestris* and their abilities to utilize acetate as an alternative carbon source in microcosms *(***Chapter 5**), iii) the genes which are significantly upregulated in *Methylocella silvestris* during growth on methane or acetate (**Chapter 6**) and iv) the effect of ecosystem succession and soil depth on the diversity of methanotrophs in Swedish islands forest soil that were found to be positive for *Methylocella* spp. (**Chapter 7**).

Although *Methylocella* spp. have been detected in various environments (Chen *et al.*,2008a, Chen *et al.*,2008b, Dedysh *et al.*,2000, Dedysh *et al.*,2004, Dunfield *et al.*,2003), there was little information on the distribution and abundance of these facultative methanotrophs due to the lack of appropriate molecular tools to specifically target them. Use of specific probes had yielded information on the environmental distribution and abundance of these facultative methanotrophs. In this study, I developed and validated a conventional PCR- based assay to target the 16S rRNA genes of all the three species of the genus *Methylocella* (**Chapter 3)**. DNA extracted from a variety of environmental samples was screened by PCR for the presence or absence of *Methylocella* 16S rRNA genes. Based on the screening results and clone library analyses, I have demonstrated that *Methylocella* spp. are

widely distributed in the environment. Results of the PCR-based screening revealed the presence of *Methylocella* spp. not only in acidic environment, but also in neutral and alkaline environment, although *Methylocella* spp. are moderate acidophilic methanotrophs and were originally isolated from acidic peat soil. These findings suggesting that the distribution of *Methylocella* in the environment is not limited by pH. However, *Methylocella* spp. were not detected from high salinity marine environments, suggesting that salinity might restrict their distribution in the environment (**Chapter 3).** Furthermore, I designed a new primer set to selectively amplify the *mmoX* genes of all the three species of the genus *Methylocella*. I also developed a SYBR® Green-based real-time quantitative PCR assay using these new *mmoX* genes-specific primers to enumerate the *Methylocella* populations in the environment (**Chapter 4**). The real-time quantitative PCR assay was validated by a spiking study in which the assay successfully enumerated the abundance of *Methylocella* populations in selected environmental samples. Even though not perfect, the quantitative PCR assay does give us a good proxy for the abundance of *Methylocella* spp. in the environment. The specificity of the primers used in this study to detect *mmoX* and 16S rRNA genes of *Methylocella* spp., were not 100% specific, therefore, I recommend that anyone using them perform a clone library analysis of amplified products to confirm the presence of *Methylocella* spp. in any environmental samples to be studied.

The studies described in **Chapter 3** and **Chapter 4** were carried out using DNA extracted from the environment as the PCR templates. Retrieval of *Methylocella* sequences does not necessarily reflect the activity of *Methylocella* in the environment. Therefore, it will be of interest to carry out an mRNA-based study,

which will provide information on the activity of *Methylocella* spp. in those environments, particularly in alkaline Lonar lake sediment. It will be important to carry out process-based measurements in order to find out the contribution that *Methylocella* makes to methane oxidation in the environment. Similarly, qPCR assay developed in this study could be applied to observe the effect of activity of *Methylocella* spp. in the environment in response to environmental changes such as soil pH and depth, vegetation and availability of other nutrients *e.g.,* acetate. This will give us a better understanding of how adaptable *Methylocella* spp. are in a changing environment.

Acetate present in the soil is a potential nutrient for *Methylocella* spp. In a study using a pure culture, acetate repressed the transcription of the sMMO operon of *Methylocella silvestris* (Theisen *et al.*,2005). However, no studies had focused on understanding the effect of acetate on methane oxidation by *Methylocella silvestris in situ*. It was also not known whether *Methylocella* spp. are able to utilize acetate as a carbon source *in situ.* I hypothesized that *in situ,* in the presence of acetate *Methylocella silvestris* does not utilize methane as a carbon source, because acetate will repress the sMMO. I showed that spiking of Moor House peat soil with *Methylocella silvestris* increased the ability of these soils to oxidize methane (**Chapter 5**). However, when acetate (0.5 mM) was added to these spiked soil acetate markedly repressed the ability of *Methylocella silvestris* to oxidize methane. Application of stable isotope probing allows one to identify active microbial population actively utilizing particular substrates. I carried out series of $^{13}CH_4$ DNA-SIP experiments either in the presence or the absence of acetate and clearly demonstrated that in the presence of acetate, *Methylocella silvestris* did not utilize

methane as the carbon source in peat soil microcosms, similar to the finding observed earlier in pure culture in laboratory by Theisen *et al.*(2005). Then I carried out ^{13}C-labelled acetate DNA-SIP experiments with *Methylocella silvestris*-spiked soil to evaluate whether *Methylocella* are able to utilize acetate *in situ* as an alternative carbon source. Surprisingly the ^{13}C-labelled acetate DNA-SIP experiments did not identify *Methylocella silvestris* as the major acetate utilizers, instead the study identified *Acidovorax, Acinetobacter, Brevundimonas* and *Burkholderia* as the dominant acetate-utilizing bacteria in Moor House peat soil. In conclusions *Methylocella* was outcompeted by these dominant acetate utilizers. It will be of interest to carry out similar experiments using more sensitive methods such as RNA- or PLFA-SIP to investigate whether the findings are the same as shown by the DNA-based approach. In addition, the recently discovered facultative methanotrophs *Methylocapsa aurea* (Dunfield *et al.*,2010) and *Methylocystis* sp. H2s (Belova *et al.*, 2010) can also be subjected to a similar type of study to observe the effects of acetate on their ability to utilize methane in peat soil. In addition, the ^{13}C-labelled acetate "heavy" DNA can be subjected to pyrosequencing to determine whether *Methylocella* and the recently discovered facultative methanotrophs *Methylocapsa aurea* and *Methylocystis* sp. H2s are actively involved in the utilization of acetate in the peat soil.

Genes located on the sMMO operon are known to be directly involved in the utilization of methane by *Methylocella silvestris* (Theisen *et al.*,2005). However, no studies have focused on the involvement of other genes that may also contribute directly or indirectly to methane oxidation by *Methylocella silvestris.* Moreover, no information is available on the involvement of genes that are essential for the

utilization of acetate by *Methylocella silvestris.* To attempt to identify such genes, I carried out *Methylocella silvestris* whole genome transcriptomics on mRNA from cells grow on methane or acetate (**Chapter 6).** Several genes were significantly upregulated during growth on methane or acetate. As expected all genes of the sMMO operon were significantly upregulated during growth on methane. Two genes located immediately downstream of these sMMO operon, however, were also found to be highly upregulated during growth on methane. One of these genes (Msil1272) encodes a Fur family global transcriptional regulator, which might be involved in the acquisition of iron by *Methylocella silvestris.* Iron is essential for the activity of sMMO during growth on methane, since sMMO contains a diiron centre at its active site. During growth on acetate, I found that isocitrate lyase and a gene cluster involved in the glycine metabolism was highly upregulated. These genes are probably involved in the utilization of acetate by *Methylocella silvestris.* Gene knock-out based studies can be carried out to determine the function of these genes in the utilization of methane or acetate by *Methylocella silvestris.* Notably, one of the draw backs of microarray experiments is that they do not provide any information about post-transcriptional events. Therefore, a proteomics-based study can be carried out alongside this in order to make the microarray findings more meaningful. Before that however, the microarray results need to be fully validated by reverse transcription quantitative PCR, since there was not sufficient time to carry out sufficient reverse transcription quantitative PCR assays. The probes used in the microarray also need to be validated to check their specificity by hybridization with *Methylocella silvestris* genomic DNA.

In **Chapter 7,** I focused on the distribution of methanotrophs in forest soil collected from two Swedish islands. This work was carried out in collaboration with Dr. Niall McNamara of CEH, Lancaster. These Swedish islands are unique ecosystems, because they are at different successional stages (early, mid and late successional stages) and have different vegetation. All the soil samples originating from these two islands and collected from different depths were found to be positive for the presence of 16S rRNA genes of *Methylocella* using the PCR assay that I developed (**Chapter 3)**. The abundance of *Methylocella* in two of these islands were also determined by the realtime PCR I developed (**Chapter 4**). Then I wanted to know what the other major methanotrophs present in this unique ecosystems were. Using a DNA-based *pmoA* microarray, I found that all of these islands were dominated by *Methylococcus*, *Methylosinus* and RA14 clade methanotrophs. The community composition of methanotrophs increased with the progression of successional stages as evident by higher diversity in late successional islands. These findings suggest that less disturbed mature soil with more vegetation favoured the colonization of methanotrophs. Based on the *pmoA* microarray, I have demonstrated that the diversity of methanotrophs decreased as the soil depth increased, probably due to low availability of oxygen in the deeper layer of soil. It will be of interest to carry out *in situ* mRNA-based *pmoA* microarray experiments for a better understanding on the role of ecosystem succession on the diversity of active methanotrophs, rather than focussing on methanotrophs present based on the analysis of DNA carried out here. The methane oxidation data generated by Dr. Niall McNamara of CEH, Lancaster indicated that these Swedish islands forest soil were taking up atmospheric concentration of methane *in situ.* Using the *pmoA* microarray, I detected signals for *pmoA* sequence related to RA14 clades methanotrophs, which are capable of

oxidizing atmospheric concentrations of methane. So far, no methanotrophs has been isolated in pure culture capable of oxidizing atmospheric concentrations of methane (Conrad, 1996). Therefore, future studies should focus on culture based work to isolate RA14 clade-like methanotrophs from these Swedish island forest soils.

Publications

Part of the work described in Chapter 3 and Chapter 4 is under revision for publication in the ISME Journal (Publication 1). Manuscripts describing part of the work mentioned in Chapter 5 and Chapter 6 are under preparation for submission to Environmental Microbiology as Publications 2 and 3 respectively. In addition, I was also involved in the annotation of *Methylocella silvestris* BL2 genome and the ability of this organism to metabolize methylamine which has been published recently as Publication 4 and 5 respectively.

1. **Rahman, M.T**., Crombie, A., Chen, A., Stralis-Pavese, N., Bodrossy, L., McNamara, N.P., and Murrell, J.C. (2010). Distribution and abundance in the environment of the facultative methanotroph *Methylocella.* The ISME Journal (in revision)

2. **Rahman, M.T**., Crombie, A., Moussard, H., Chen, Y., and Murrell, J.C. (2010). Acetate represses methane oxidation by *Methylocella silvestris* BL2 in an acidic peat soil microcosm. (in preparation)

3. **Rahman, M.T.,** Gregge, R., McNamara, N.P., and Murrell, J.C. (2010). Diversity of methanotrophs in Swedish island forest soil that are at different successional stages. (in preparation)

4. Chen, Y., Crombie, A., **Rahman, M.T.,** Dedysh, S.N., Liesack, W., Stott, M.B., Alam, M., Theisen, A.R., Murrell, J.C., and Dunfield, P.F. (2010). Complete genome sequence of the aerobic facultative methanotroph *Methylocella silvestris* BL2. *J Bacteriol* **192**: 3840-3841.

5. Chen, Y., Scanlan, J., Song, L., Crombie, A., **Rahman, M.T**., Schäfer, H., Murrell, J.C. (2010) γ-glutamylmethylamide is an essential intermediate in the metabolism of methylamine by *Methylocella silvestris*. *Appl Environ Microbiol.* **76**: 4530–4537.

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Appendices

Appendix 1

Oligonucleotide probe set for *pmoA*-microarray

Probe Name	Intended specificity	Sequence 5' → 3'	Length	GC%	T_m
BB51-302	*Methylobacter*	CGGTTGTTTGTGTCTTAGGTCTG	23	47.8	57.2
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
Mb267	*Methylobacter*	GCATGCTTGTGGTTCCGTTAC	21	52.4	58.1
511-436	*Methylobacter*	GTTTTGATGCTGTCTGGCAG	20	50.0	55.5
MbA486	*Methylobacter*	AGCATGACATTGACAGCGGTTGTT	24	45.8	61.6
MbA557	*Methylobacter*	CAATGGCATGATGTTCACTCTGGCT	25	48.0	61.5
Mb_SL#3-300	*Methylobacter*	GGCGCTGTTGTTTGTGTATTGGGT	24	50.0	62.2
Mb460	*Methylobacter*	GACAGTTACAGCGGTAATCGGTGG	24	54.2	60.9
Mb_LW12-211	*Methylobacter*	CGTCTTTGGGTTACTGTTGTGCC	23	52.2	60.0
Mb_C11-403	*Methylobacter*	CAAACTTCATGCCTGGTGCTATCGT	25	48.0	61.4

Mb271	*Methylobacter*	TTGTGGTGGCGTTACCGT	18	55.6	58.0
PS80-291	Clone PS-80	ACCAATAGGCGCAACACTTAGT	22	45.5	58.3
Est514	*Methylomicrobium*-related clones	AATTGGCCTATGGTTGCGCC	20	55.0	59.9
Mm_pel467	*Methylomicrobium pelagicum*	ACTGCGGTAATCGATGGTTTGGC	23	52.2	61.6
Mb_SL-299	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
DS1_401	Deep sea cluster #1	GCGCGGTAGTTTGTGTTATGGCT	23	52.2	61.7
Mm531	*Methylomonas*	CTCCATTGCACGTGCCTGTAGA	22	54.5	60.7
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_M430	*Methylomonas*	TGGACGTGATTTTGATGTTGGGCAA	25	44.0	61.6
Mm_MV421	*Methylomonas*	CTATCGTGCTGGATACAATCCTGATGT	27	44.4	60.0
Mm275	*Methylomonas*	GTGGTGGAGATACCGTTTGCC	21	57.1	59.2
Mm451	*Methylomonas*	CTGATGTTGGGTAACAGCATGACT	24	45.8	58.8
peat_1_3-287	*Methylomonas*-related peat clones	AACTGCCTTTAGGCGCTACC	20	55.0	58.6

Jpn284	Clone Jpn 07061	ACCGTATCGCATGGGGTG	18	61.1	58.0
Mmb303	*Methylomicrobium album*	CAATGCTGGCTGTTCTGGGC	20	60.0	60.3
Mmb259	*Methylomicrobium album* + Landfill *Methylomicrobium*	CTGTTCAAGCAGTTGTGTGGTATCG	25	48.0	59.8
Mmb562	*Mmb. album* and *Methylosarcina*	ATGGTAATGACCCTGGCTGACTTG	24	50.0	60.6
LP20-644	*Methylomicrobium*-related clones	GTACACTGCGTACTTTCGGTAA	22	45.5	56.0
Ia193	Type I a (*Methylobacter- Methylomonas- Methylomicrobium)*	GACTGGAAAGATAGACGTCTATGGG	25	48.0	57.8
Ia575	Type I a (*Methylobacter- Methylomonas- Methylomicrobium- Methylosarcina)*	TGGCTGACTTGCAAGGTTACCAC	23	52.2	61.3
JRC4-432	Japanese rice cluster #4	GACGTTGTCCTGGCTCTGAG	20	60.0	58.3
MclT272	*Methylocaldum tepidum*	GGCTTGGGAGCGGTTCCG	18	72.2	61.9
MclG281	*Methylocaldum gracile*	AAAGTTCCGCAACCCCTGGG	20	60.0	61.5
MclE302	*Methylocaldum* E10	CGCAACCATGGCCGTTCTG	19	63.2	60.3
MclS402	*Methylocaldum szegediense*	GCGCTGTTGGTTCCGGGT	18	66.7	61.8
Mcl408	*Methylocaldum*	GGTTCCGGGTGCGATTTTG	19	57.9	57.8
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
fw1-639	Fw-1 group: *Methylococcus*-*Methylocaldum* related marine and freshwater sediment clones	GAAGGGCACGCTGCGTACG	19	68.4	62.0
fw1-641	Fw-1 group: *Methylococcus*-*Methylocaldum* related marine and freshwater sediment clones	AGGGCACGCTGCGTACGTT	19	63.2	63.3
fw1-286	Fw-1 group: *Methylococcus*-*Methylocaldum* related marine and freshwater sediment clones	ATCGTCAACCGTGGGGCG	18	66.7	61.1
LW21-374	LW21 group	CTACTTCCCGATCACCATGTGCT	23	52.2	60.2
LW21-391	LW21 group	TGTGCTTCCCCTCGCAGATC	20	60.0	60.5
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	CCGACATCATTGGCTACAACTATGT	25	44.0	58.7

	sediment cluster				
JRC2-447	Japanese Rice Cluster #2	CTGAGCACCAGCTACCTGTTCA	22	54.5	60.2
M90-574	*Methylococcus-Methylocaldum* related marine and freshwater sediment clones	ATCGCCGACCTGCTGGGTTA	20	60.0	62.2
M90-253	*Methylococcus-Methylocaldum* related marine and freshwater sediment clones	GCTGCTGTACAGGCGTTCCTG	21	61.9	61.7
Mth413	*Methylothermus*	CACATGGCGATCTTTTTAGACGTTG	25	44.0	58.3
Ib453	Type I b (*Methylothermus-Methylococcus-Methylocaldum* and related)	GGCAGCTACCTGTTCACCGC	20	65.0	61.7
Ib559	Type I b (*Methylothermus-Methylococcus-Methylocaldum* and related)	GGCATGCTGATGTCGATTGCCG	22	59.1	62.5
DS3-446	Deep sea cluster #3	AGCTGTCTGGCAGTTTCCTGTTCA	24	50.0	62.5
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
Nc_oce426	*Nitrosococcus oceani*	CTTGGATGCCATGCTTGCGA	20	55.0	59.8
USCG-225	Upland soil cluster Gamma	CTGACGCCGATCATGTGCAT	20	55.0	59.1
USCG-225b	Upland soil cluster Gamma	CTGACGCCGATCATGTGCATCA	22	54.5	61.2
Mcy233	*Methylocystis*	ATTCTCGGCGTGACCTTCTGC	21	57.1	60.9
Mcy413	*Methylocystis*	TTCCGGCGATCTGGCTTGACG	21	61.9	63.2
Mcy522	*Methylocystis* A + peat clones	GGCGATTGCGGCGTTCCA	18	66.7	62.3
Mcy264	*Methylocystis*	CAGGCGTTCTGGTGGGTGAA	20	60.0	61.0
Mcy270	*Methylocystis*	TTCTGGTGGGTGAACTTCCGTCT	23	52.2	61.8
Mcy459	*Methylocystis*	GTGATCACGGCGATTGTTGGTTC	23	52.2	60.2
Mcy255	*Methylocystis B (parvus/echinoides/*strain M)	GGCGTCGCAGGCTTTCTGG	19	68.4	62.3
McyM309	*Methylocystis strain M and related*	GGTTCTGGGCCTGATGATCGG	21	61.9	61.0
McyB304	*Methylocystis B (parvus/echinoides/*strain M)	CGTTTTCGCGGCTCTGGGC	19	68.4	62.7
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
MsS314	*Methylosinus sporium*	GGTTCTGGGTCTGCTCATCGG	21	61.9	60.8
MsS475	*Methylosinus sporium*	TGGTCGGCGCCCTGGGCT	18	77.8	68.3
Msi263	*Methylosinus sporium* + 1 *Methylosinus trichosporium subcluster*	GGCGTTCCTGTGGGAGAACTTC	22	59.1	61.2
Msi423	*Methylosinus*	CTGTGGCTGGACATCATCCTGC	22	59.1	61.4
Msi294	*Methylosinus*	GTTCGGCGCGACCTTCGC	18	72.2	62.5
Msi232	*Methylosinus* + most *Methylocystis* -considered as additional type II probe	ATCCTGGGCGTGACCTTCGC	20	65.0	63.3
Peat264	peat clones	GGCGTTTTTCTGGGTCAACTTCC	23	52.2	60.3
II509	Type II	CGAACAACTGGCCGGCGAT	19	63.2	61.7
II630	Type II	CATGGTCGAGCGCGGCAC	18	72.2	62.4
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 *Methylocystis*	TCGACATCGTGCTGATGATCTCGG	24	54.2	62.1
NMcy2-262	Novel *pmoA* copy of *Methylocystis*	CAGTCCTTCTTCTGGCAGAAGTTCC	25	52.0	60.9
NMsiT-271	Novel *pmoA* copy of *Methylosinus trichosporium*	AGCGCTTCCGTCTGCCGAT	19	63.2	62.9
LP21-232	Novel *pmoA* copy of type II and related environmental clones	ATCGTCGCCATGTGCTTCGC	20	60.0	61.9
RA14-594	RA14 related clones	CCACAACGTTCGTACCTCGA	20	55.0	57.9
RA14-591	RA14 related clones	GGCTTCCACAACGTTCGTACCT	22	54.5	60.9
Wsh1-566	Watershed + flooded upland cluster 1	GCTCATGAGCTTGGCCGACATC	22	59.1	61.8
Wsh2-491	Watershed + flooded upland cluster 2	TCATTTGGCCAACCTCTCTCATTCC	25	48.0	60.9
Wsh2-450	Watershed + flooded 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
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

ESR-579	ESR (Eastern Snake River) cluster	GACCTGATCGGATTCGAGAACATC	24	50.0	58.5
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
Nit_rel471	AOB related clones/probably methanotrophs	CGTTCGCGATGATGTTTGGTCC	22	54.5	60.1

Nit_rel223	AOB related clones/probably methanotrophs	GTCACACCGATCGTAGAGGT	20	55.0	56.9
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
Nit_rel304	AOB related clones/probably methanotrophs	CGCTCTGCATTCTGGCGCT	19	63.2	61.8
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
M84P22-514	Environmental clones of uncertain identity	AACTGGGCCTGGCTGGG	17	70.6	61.0
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

Appendix 2

Genes upregulated significantly during growth of *Methylocella silvestris* on methane

Primary Accession Number	p-value	Fold changes	Assigned functions	Putative proposed functions
Msil0014	0.002809775	2.23	ABC transporter related	Transport
Msil0024	0.00171712	99.88	Flagellar basal body rod protein FlgB	Flagella biosynthesis
Msil0027	0.002497164	20.06	Flagellar basal-body rod protein FlgG	Flagella biosynthesis
Msil0028	0.00331853	9.39	Flagella basal body P-ring formation protein FlgA	Flagella biosynthesis
Msil0029	0.002740683	7.97	Flagellar P-ring protein	Flagella biosynthesis
Msil0032	0.002208488	6.45	Flagellar basal body-associated protein FliL	Flagella biosynthesis
Msil0036	0.002876923	4.49	Putative uncharacterized protein	Putative uncharacterized protein
Msil0037	0.003451239	9.97	OmpA/MotB domain protein	Secretory function
Msil0041	0.002516725	7.08	Putative two component transcriptional regulator	Regulation
Msil0048	0.001821477	25.45	Flagellar biosynthetic protein FliQ	Flagella biosynthesis
Msil0052	0.001977784	46.87	Putative uncharacterized protein	Putative uncharacterized protein
Msil0059	0.003357686	5.56	Molybdate ABC transporter, inner membrane subunit	Transport

Msil0060	0.002809775	5.70	Molybdate ABC transporter, ATPase subunit	Transport
Msil0164	0.00264092	4.19	Cytochrome B561	Electron transport
Msil0176	0.001878169	20.48	Putative uncharacterized protein	Putative uncharacterized protein
Msil0258	0.002516725	97.06	Putative uncharacterized protein	Putative uncharacterized protein
Msil0267	0.002205977	10.88	Putative uncharacterized protein	Putative uncharacterized protein
Msil0290	0.003062422	3.64	Putative uncharacterized protein	Putative uncharacterized protein
Msil0307	0.00170967	2.90	AMP-dependent synthetase and ligase	Fatty acid metabolism
Msil0346	0.002671339	81.25	Putative uncharacterized protein	Putative uncharacterized protein
Msil0347	0.003439185	7.81	Putative uncharacterized protein	Putative uncharacterized protein
Msil0456	0.002205977	6.34	Putative uncharacterized protein	Putative uncharacterized protein
Msil0608	0.001878169	22.08	Putative uncharacterized protein	Putative uncharacterized protein
Msil0609	0.002809775	8.80	TOBE domain protein	Transport
Msil0632	0.003661893	2.97	Septum site-determining protein MinC	Other
Msil0725	0.001519132	7.66	Putative uncharacterized protein	Putative uncharacterized protein
Msil0825	0.002373591	2.29	TonB system transport protein ExbD	Transport
Msil0826	0.001977784	3.33	TonB-system energizer ExbB	Transport
Msil0854	0.003633231	3.32	RNA polymerase, sigma-24 subunit, ECF subfamily	Regulation

Msil0914	0.001836699	2.08	General secretion pathway protein I	Secretory function
Msil0976	0.002723787	6.40	Molybdenum cofactor synthesis domain protein	Other
Msil1011	0.002197378	12.52	Putative uncharacterized protein	Putative uncharacterized protein
Msil1019	0.003014333	3.89	Putative uncharacterized protein	Putative uncharacterized protein
Msil1157	0.003285949	6.16	Periplasmic glucan biosynthesis protein MdoG	Other
Msil1262	0.001153113	2766.44	Methane monooxygenase	mmoX
Msil1263	0.001878169	5807.98	Methane monooxygenase	mmoY
Msil1264	0.001153113	771.08	Monooxygenase component	mmoB
Msil1265	0.00129915	1467.21	Methane monooxygenase	mmoZ
Msil1266	0.002373591	652.73	Putative uncharacterized protein	mmoD
Msil1267	0.002373591	434.52	Oxidoreductase FAD/NAD(P)-binding domain protein	mmoC
Msil1268	0.001153113	76.35	GAF modulated sigma54 specific transcriptional regulator, Fis family	mmoR
Msil1269	0.002304884	47.64	Putative uncharacterized protein	ORF2
Msil1270	0.001513263	162.57	Chaperonin Cpn60/TCP-1	mmoG
Msil1271	0.001257954	7776.49	Putative uncharacterized protein	Putative uncharacterized protein
Msil1272	0.001153113	2182.31	Ferric uptake regulator, Fur family	Regulation

Msil1386	0.002516725	3.51	LamB/YcsF family protein	Other
Msil1392	0.002208488	27.12	Putative uncharacterized protein	Putative uncharacterized protein
Msil1393	0.002208488	27.01	Putative uncharacterized protein	Putative uncharacterized protein
Msil1469	0.003524143	4.74	Aromatic-ring-hydroxylating dioxygenase beta subunit	Other
Msil1488	0.003062422	2.59	Conjugal transfer protein; TrbL	Other
Msil1506	0.002373591	50.66	PpiC-type peptidyl-prolyl cis-trans isomerase	Protein metabolism
Msil1508	0.002392926	151.58	Nitrate reductase molybdenum cofactor assembly chaperone	Nitrogen metabolism
Msil1509	0.002208488	268.13	Nitrate reductase, beta subunit	Nitrogen metabolism
Msil1510	0.00170967	99.18	Nitrate reductase, alpha subunit	Nitrogen metabolism
Msil1511	0.00170967	24.06	Transcriptional regulator, Crp/Fnr family	Regulation
Msil1512	0.002205977	75.20	Nitrite transporter	Transport
Msil1513	0.001702142	45.65	Cytochrome c oxidase subunit III	Electron transport
Msil1515	0.003729948	105.71	Nitric-oxide reductase	Nitrogen metabolism
Msil1516	0.00331853	135.46	Nitric-oxide reductase	Nitrogen metabolism
Msil1546	0.00286521	2.25	Peptidase S49	Vitamins and Cofactor metabolism
Msil1582	0.002205977	5.30	Putative uncharacterized protein	Putative uncharacterized protein
Msil1655	0.002516725	72.48	Putative uncharacterized protein	Putative uncharacterized protein

Msil1656	0.001878169	30.29	Flagellar basal body rod protein	Flagella biosynthesis
Msil1719	0.002485588	2.09	Citrate (Pro-3S)-lyase	Other
Msil1738	0.002789234	4.34	Ferredoxin, 2Fe-2S (AaFd4)	Electron transport
Msil1739	0.001519132	166.52	Coenzyme PQQ biosynthesis protein C	PQQ biosynthesis
Msil1744	0.002876923	2.83	Putative uncharacterized protein	Putative uncharacterized protein
Msil1802	0.001153113	51.24	Putative uncharacterized protein	Other
Msil1807	0.00170967	3.91	Putative uncharacterized protein	Putative uncharacterized protein
Msil1812	0.001153113	6.00	Putative uncharacterized protein	Putative uncharacterized protein
Msil1817	0.003521404	6.08	4-oxalocrotonate tautomerase	Other
Msil1822	0.002208488	6.24	Poly-beta-hydroxybutyrate polymerase domain protein	Other
Msil1824	0.002800406	55.32	Response regulator receiver protein	Regulation
Msil1827	0.001153113	6.03	Alcohol dehydrogenase GroES domain protein	Carbohydrate metabolism
Msil1828	0.00129915	5.90	Luciferase-like monooxygenase	Other
Msil1868	0.0029001	3.55	Putative uncharacterized protein	Putative uncharacterized protein
Msil1887	0.002358262	2.67	Adenylate/guanylate cyclase	Other
Msil1909	0.002178866	2.46	Protein-(Glutamine-N5) methyltransferase, release factor-specific	Protein metabolism

Msil1931	0.00241115	6.70	Periplasmic binding protein	Transport
Msil1933	0.002373591	15.52	ABC transporter related	Transport
Msil1934	0.00241115	63.87	Antibiotic biosynthesis monooxygenase	Other
Msil2133	0.003661893	3.79	Dethiobiotin synthase	Vitamins and cofactor metabolism
Msil2134	0.00286521	3.77	Transcriptional regulator, TetR family	Regulation
Msil2138	0.003014333	2.70	Binding-protein-dependent transport systems inner membrane component	Transport
Msil2260	0.003590304	2.78	PQQ-dependent dehydrogenase, methanol/ethanol family	PQQ biosynthesis
Msil2261	0.002876923	6.25	Putative uncharacterized protein	Putative uncharacterized protein
Msil2322	0.002875901	12.71	4 TPR repeat-containing protein	Other
Msil2406	0.002373591	2.27	Transcriptional coactivator/pterin dehydratase	Other
Msil2412	0.002741542	6.52	Ethanolamine ammonia-lyase	Fatty acid metabolism
Msil2413	0.002740683	2.08	Ethanolamine ammonia lyase large subunit	Other
Msil2514	0.002800406	2.96	Putative uncharacterized protein	Putative uncharacterized protein
Msil2544	0.001874087	3.16	Putative uncharacterized protein	Putative uncharacterized protein
Msil2555	0.001513263	38.68	Oxygen-independent coproporphyrinogen III oxidase	Other
Msil2561	0.001519132	2.21	Alpha/beta hydrolase fold protein	Other

Msil2626	0.002516725	2.38	Putative uncharacterized protein	Putative uncharacterized protein
Msil2629	0.002208488	19.75	Putative uncharacterized protein	Putative uncharacterized protein
Msil2630	0.00170967	8.10	Putative uncharacterized protein	Putative uncharacterized protein
Msil2633	0.002373591	2.30	Glutamate synthase alpha subunit domain protein	Nitrogen metabolism
Msil2642	0.0027227	4.41	Putative uncharacterized protein	Putative uncharacterized protein
Msil2657	0.002800406	27.75	Putative uncharacterized protein	Putative uncharacterized protein
Msil2752	0.002392926	4.95	Putative uncharacterized protein	Putative uncharacterized protein
Msil2759	0.002809775	5.07	Putative uncharacterized protein	Putative uncharacterized protein
Msil2792	0.00170967	3.22	Putative uncharacterized protein	Putative uncharacterized protein
Msil2809	0.002516725	17.76	Putative uncharacterized protein	Putative uncharacterized protein
Msil2859	0.002205977	6.23	Putative uncharacterized protein	Putative uncharacterized protein
Msil2906	0.001878169	9.01	Putative uncharacterized protein	Putative uncharacterized protein
Msil2908	0.002800406	12.69	Putative uncharacterized protein	Putative uncharacterized protein
Msil2951	0.002208488	4.24	Secretion protein HlyD family protein	Secretory function
Msil3047	0.002392926	3.58	Putative uncharacterized protein	Putative uncharacterized protein
Msil3055	0.002392926	3.59	Putative uncharacterized protein	Putative uncharacterized protein
Msil3066	0.0027227	3.55	Putative uncharacterized protein	Putative uncharacterized protein

Msil3067	0.003014333	3.84	Putative uncharacterized protein	Putative uncharacterized protein
Msil3068	0.003661893	2.93	MOSC domain containing protein	Electron transport
Msil3082	0.002741542	2.21	Putative uncharacterized protein	Putative uncharacterized protein
Msil3105	0.002541074	2.45	Putative uncharacterized protein	Putative uncharacterized protein
Msil3175	0.003014333	5.68	Putative uncharacterized protein	Putative uncharacterized protein
Msil3176	0.001878169	10.56	Undecaprenyl-phosphate glucose phosphotransferase	Transport
Msil3177	0.00171712	5.83	Putative uncharacterized protein	Putative uncharacterized protein
Msil3178	0.001513263	41.63	Putative uncharacterized protein	Putative uncharacterized protein
Msil3195	0.003398863	5.24	Malonate transporter, MadM subunit	Transport
Msil3315	0.001977784	7.70	Putative uncharacterized protein	Putative uncharacterized protein
Msil3316	0.001153113	60.70	RNA polymerase, sigma-24 subunit, ECF subfamily	Regulation
Msil3317	0.001878169	59.00	PepSY-associated TM helix domain protein	Transport
Msil3318	0.002671339	172.44	L-lysine 6-monooxygenase (NADPH)	Protein metabolism
Msil3319	0.001977784	261.07	Amino acid adenylation domain protein	Iron metabolism
Msil3320	0.002740683	401.28	Putative siderophore biosynthesis protein	Iron metabolism
Msil3321	0.001513263	114.11	MATE efflux family protein	Transport
Msil3322	0.00129915	254.69	Putative uncharacterized protein	Protein metabolism

Msil3323	0.00129915	36.67	RNA polymerase, sigma-24 subunit, ECF subfamily	Regulation
Msil3324	0.00170967	53.53	TonB-dependent siderophore receptor	Transport
Msil3329	0.002516725	5.20	Permease	Transport
Msil3457	0.002392926	5.01	Cobalamin synthesis protein P47K	Vitamins and cofactor metabolism
Msil3506	0.002373591	10.32	Putative uncharacterized protein	Putative uncharacterized protein
Msil3545	0.003014333	22.84	Methionine synthase	Protein metabolism
Msil3606	0.00129915	2.54	Amino acid permease-associated region	Transport
Msil3623	0.001878169	6.49	NifQ family protein	Nitrogen metabolism
Msil3624	0.002304884	9.57	Ferredoxin III, nif-specific	Electron transport
Msil3625	0.001513263	4.87	Putative uncharacterized protein	Putative uncharacterized protein
Msil3630	0.002373591	5.59	Nitrogenase molybdenum-iron protein beta chain	Nitrogen metabolism
Msil3631	0.001519132	25.48	Nitrogenase protein alpha chain	Nitrogen metabolism
Msil3632	0.00170967	10.11	NifH, Nitrogenase iron protein	Nitrogen metabolism
Msil3643	0.00170967	22.87	Tetratricopeptide TPR_2	Other
Msil3645	0.001928084	13.04	4Fe-4S ferredoxin iron-sulfur binding domain protein	Electron transport
Msil3649	0.00170967	6.01	Putative uncharacterized protein	Putative uncharacterized protein
Msil3650	0.002205977	14.62	Globin	Trsnsport

Msil3651	0.002373591	33.78	Ferric uptake regulator, Fur family	Regulation
Msil3654	0.002358262	17.66	NADH dehydrogenase (Ubiquinone) 24 kDa subunit	Electron transport
Msil3655	0.002373591	27.69	NADH dehydrogenase (Quinone)	Electron transport
Msil3657	0.002392926	23.89	Formate dehydrogenase family accessory protein FdhD	Formate metabolism
Msil3658	0.0027227	10.90	Putative NAD-dependent formate dehydrogenase	Formate metabolism
Msil3680	0.002536109	2.72	GYD family protein	Other
Msil3748	0.003302289	5.09	Hydrogenase accessory protein HypB	Other
Msil3749	0.002392926	15.10	Hydrogenase expression/synthesis HypA	Other
Msil3750	0.001977784	5.85	Hydrogenase expression/formation protein HypE	Vitamins and cofactor metabolism
Msil3753	0.001878169	9.59	(NiFe) hydrogenase maturation protein HypF	Other
Msil3755	0.003661893	18.72	Rieske (2Fe-2S) domain protein	Electron transport
Msil3756	0.001878169	17.59	Putative uncharacterized protein	Putative uncharacterized protein
Msil3757	0.002358262	11.52	Putative uncharacterized protein	Putative uncharacterized protein
Msil3758	0.003727393	11.03	Putative uncharacterized protein	Putative uncharacterized protein
Msil3761	0.003230289	5.16	Putative uncharacterized protein	Putative uncharacterized protein
Msil3763	0.002809775	25.48	Nickel-dependent hydrogenase large subunit	Other
Msil3764	0.001878169	28.13	NADH ubiquinone oxidoreductase 20 kDa subunit	Electron transport

Msil3765	0.003014333	3.76	Putative uncharacterized protein	Putative uncharacterized protein
Msil3766	0.001702142	9.39	Two component, sigma 54 specific, transcriptional regulator, Fis family	Regulation
Msil3778	0.001519132	16.43	Ferredoxin	Electron transport
Msil3803	0.001472999	16.57	Putative uncharacterized protein	Putative uncharacterized protein
Msil3806	0.002800406	4.67	4'-phosphopantetheinyl transferase	Fatty acid metabolism
Msil3857	0.003661893	2.54	DNA protecting protein DprA	Other
Msil3905	0.002779906	347.77	Two component transcriptional regulator, winged helix family	Regulation

Appendix 3

Genes upregulated significantly during growth of *Methylocella silvestris* on acetate

Primary Accession Number	p-value	Fold changes	Assigned functions	Putative proposed function
Msil0697	0.002800406	2.33	Polysaccharide deacetylase	Carbohydrate metabolism
Msil2712	0.002373591	2.01	dTDP-4-dehydrorhamnose reductase	Carbohydrate metabolism
Msil3697	0.002273026	2.78	UTP-glucose-1-phosphate uridylyltransferase	Carbohydrate metabolism
Msil0522	0.001977784	3.12	Dihydrolipoyl dehydrogenase	Carbohydrate metabolism
Msil0521	0.00354868	3.45	Pyruvate dehydrogenase complex dihydrolipoamide acetyltransferase	Carbohydrate metabolism
Msil1375	0.002373591	9.08	Phosphoenolpyruvate carboxykinase (ATP)	Carbohydrate metabolism
Msil1194	0.003302289	2.04	Fructose-bisphosphate aldolase, class II, Calvin cycle subtype	Carbohydrate metabolism
Msil1406	0.003014333	2.44	Glucose/sorbosone dehydrogenase-like protein	Carbohydrate metabolism
Msil0327	0.002341006	2.70	Cell divisionFtsK/SpoIIIE	Cell division
Msil3480	0.00170967	2.53	UDP-N-acetylmuramyl-tripeptide synthetase	Cell division
Msil3488	0.002516725	2.79	UDP-N-acetylmuramate--L-alanine ligase	Cell division

Msil0829	0.00129915	6.86	Putative DNA helicase related protein	DNA binding protein
Msil1905	0.003336701	2.50	DNA gyrase subunit B	DNA topology
Msil0204	0.002205977	4.12	FAD dependent oxidoreductase	Electron transport
Msil0205	0.001153113	2.74	Aldo/keto reductase	Electron transport
Msil2427	0.002809775	2.37	Sulfite reductase (NADPH) flavoprotein, alpha chain	Electron transport
Msil2446	0.00241115	2.86	Aldo/keto reductase	Electron transport
Msil2920	0.001878169	7.19	NADH-quinone oxidoreductase	Electron transport
Msil2921	0.002208488	5.96	NADH dehydrogenase I, D subunit	Electron transport
Msil2925	0.002809775	9.50	NADH dehydrogenase (Quinone)	Electron transport
Msil2926	0.002208488	6.17	NADH-quinone oxidoreductase subunit I	Electron transport
Msil2929	0.002208488	4.71	Proton-translocating NADH-quinone oxidoreductase, chain L	Electron transport
Msil2930	0.002373591	7.18	Proton-translocating NADH-quinone oxidoreductase, chain M	Electron transport
Msil2931	0.002892723	8.40	Proton-translocating NADH-quinone oxidoreductase, chain N	Electron transport
Msil3663	0.002800406	3.61	Cytochrome c oxidase accessory protein CcoG	Electron transport
Msil3799	0.003729948	3.68	Cytochrome c1	Electron transport
Msil3800	0.001878169	8.20	Cytochrome b	Electron transport
Msil2480	0.001153113	2.55	Short-chain dehydrogenase/reductase SDR	Fatty acid metabolism

Msil2975	0.002609724	9.49	Short-chain dehydrogenase/reductase SDR	Fatty acid metabolism
Msil3611	0.002373591	2.18	Short-chain dehydrogenase/reductase SDR	Fatty acid metabolism
Msil1214	0.001513263	40.61	Glycine dehydrogenase (Decarboxylating)	Glycine metabolism
Msil1215	0.003631434	82.37	Probable glycine dehydrogenase [decarboxylating] subunit 1	Glycine metabolism
Msil1216	0.00170967	14.11	Glycine cleavage system H protein	Glycine metabolism
Msil1259	0.002208488	2.12	Homoserine kinase	Glycine metabolism
Msil1208	0.001878169	2.21	Histidinol-phosphate aminotransferase	Other
Msil2781	0.00331853	3.66	Manganese containing catalase	Other
Msil1012	0.002208488	3.13	3-hydroxybutyryl-CoA dehydrogenase	Other
Msil1275	0.001977784	2.14	(2Fe-2S)-binding domain protein	Other
Msil3070	0.002800406	2.85	Histidinol dehydrogenase	Other
Msil3246	0.002516725	5.41	Hopanoid biosynthesis associated RND transporter like protein HpnN	Other
Msil2744	0.003043154	3.19	Hydrogenase accessory protein HypB	Other
Msil3255	0.002516725	3.38	NAD(P)(+) transhydrogenase (AB-specific)	Other
Msil2580	0.003230289	2.14	ErfK/YbiS/YcfS/YnhG family protein	Other
Msil2618	0.003631434	4.14	Glutathione S-transferase domain protein	Other

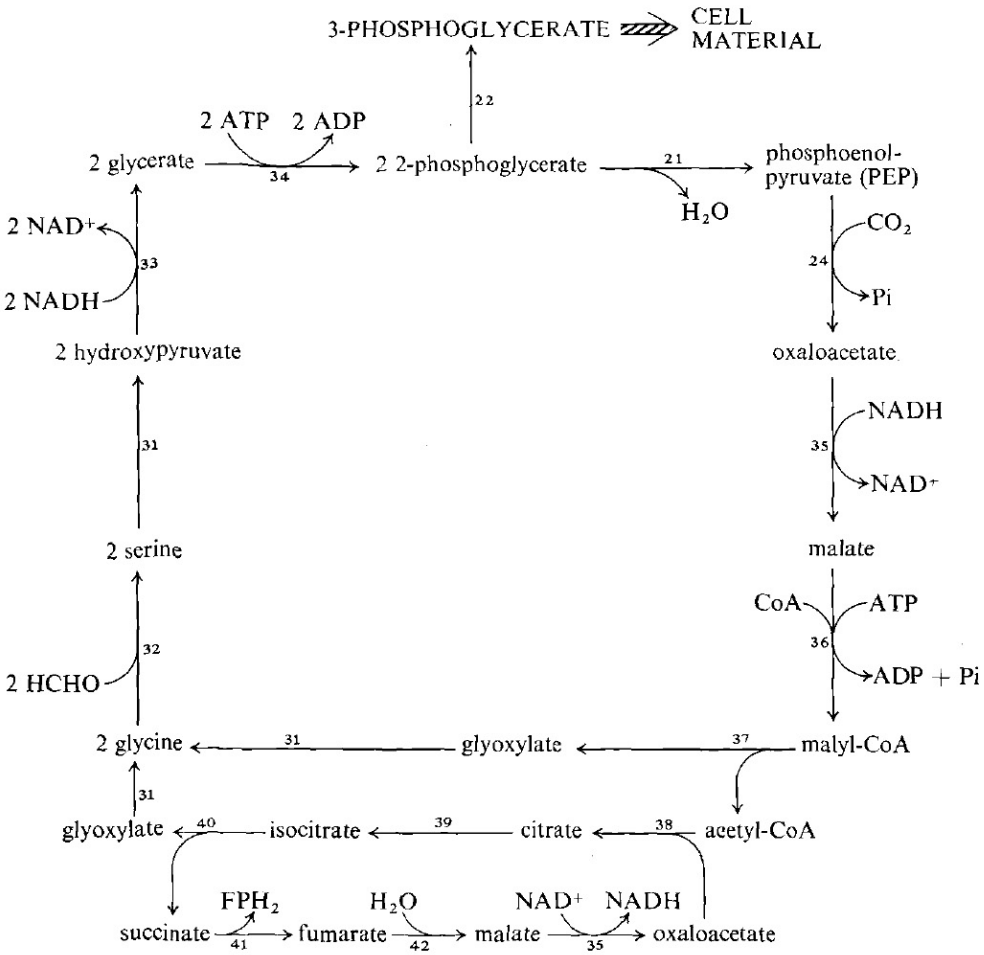
Msil2733	0.002911714	2.54	Coagulation factor 5/8 type domain protein	Other
Msil2734	0.003014333	2.41	Methyltransferase FkbM family	Other
Msil2426	0.003043154	2.21	Sulfite reductase (NADPH) hemoprotein, beta-component	Other
Msil2241	0.001854177	2.31	2-polyprenylphenol 6-hydroxylase	Other
Msil2917	0.002392926	3.86	Xanthine permease	Other
Msil0166	0.001928084	2.25	ErfK/YbiS/YcfS/YnhG family protein	Others
Msil3798	0.002373591	2.09	Lysine tRNA ligase	Protein metabolism
Msil1209	0.002373591	2.33	Arogenate dehydrogenase	Protein metabolism
Msil0759	0.003077552	2.17	CDP-diacylglycerol/serine O-phosphatidyltransferase	Protein metabolism
Msil1095	0.002373591	3.10	Adenylosuccinate lyase	Protein metabolism
Msil2091	0.002373591	2.25	Catalase domain protein	Protein metabolism
Msil1616	0.002876923	3.22	Tyrosyl-tRNA synthetase	Protein metabolism
Msil0210	0.001779737	2.78	Transglutaminase domain protein	Protein metabolism
Msil0605	0.001513263	6.91	HflC protein	Protein metabolism
Msil0139	0.002741542	2.71	Putative uncharacterized protein	Putative uncharacterized protein
Msil0419	0.003661893	3.69	Putative uncharacterized protein	Putative uncharacterized protein
Msil0601	0.002740683	3.14	Putative uncharacterized protein	Putative uncharacterized protein

Msil1006	0.002788429	2.08	Putative uncharacterized protein	Putative uncharacterized protein
Msil3850	0.002809775	2.62	Putative uncharacterized protein	Putative uncharacterized protein
Msil1096	0.002358262	2.44	Putative uncharacterized protein	Putative uncharacterized protein
Msil2599	0.002373591	2.04	Putative uncharacterized protein	Putative uncharacterized protein
Msil3355	0.003679724	2.89	Putative uncharacterized protein	Putative uncharacterized protein
Msil3491	0.001702142	3.99	Putative uncharacterized protein	Putative uncharacterized protein
Msil3736	0.00331853	2.73	Putative uncharacterized protein	Putative uncharacterized protein
Msil3844	0.002208488	4.02	Putative uncharacterized protein	Putative uncharacterized protein
Msil3000	0.00293667	3.60	Succinate CoA transferase	Pyruvate metabolism
Msil0520	0.002373591	4.17	Transketolase central region	Pyruvate metabolism
Msil1213	0.002373591	4.44	Putative transcriptional regulator, CopG family	Regulation
Msil2307	0.003302289	3.27	RNA polymerase sigma factor	Regulation
Msil1627	0.003439185	2.03	RNA polymerase sigma factor	Regulation
Msil0577	0.00170967	3.08	50S ribosomal protein L2	Ribosome biosynthesis
Msil2293	0.0033985	2.82	tRNA pseudouridine synthase B	RNA binding
Msil2695	0.003014333	5.17	Type II and III secretion system protein	Secretory function
Msil3157	0.002818684	5.10	Isocitrate lyase	TCA cycle

Msil2501	0.002809775	4.31	Malate dehydrogenase	TCA cycle
Msil0992	0.002205977	3.51	Signal peptidase I	Transport
Msil1359	0.001153113	2.39	Sodium/calcium exchanger membrane region	Transport
Msil1860	0.00241115	10.67	Ammonium transporter	Transport
Msil3789	0.001874087	3.29	ABC-2 type transporter	Transport
Msil3771	0.003014333	13.35	Major facilitator superfamily MFS_1	Transport
Msil2999	0.003230289	2.00	Thiamine biosynthesis protein ThiC	Vitamin and cofactor metabolism

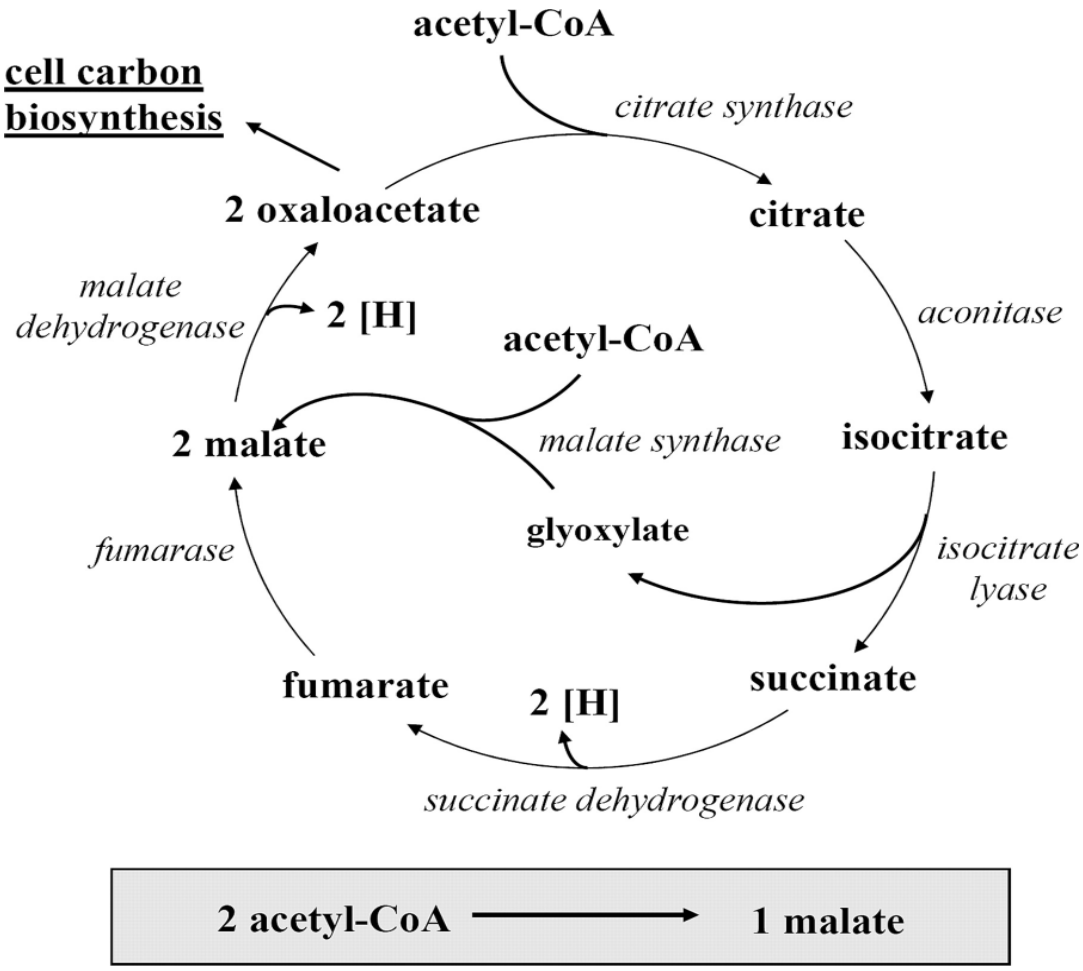
Appendix 4

The serine cycle pathways (Antony, 1982). Here the enzyme numbers refer to those in the text: (21) enolase; (22) phosphoglycerate mutase; (24) phosphoenolpyruvate carboxylase; (31) serine-glyoxylate aminotransferase; (32) serine transhydrogenase; (36) malate thiokinase; (37) malyl-CoA lyase; (38) citrate synthase; (39) aconitase; (40) isocitrate lyase; (41) succinate dehydrogenase; (42) fumarase. (Taken directly from Antony, 1982)



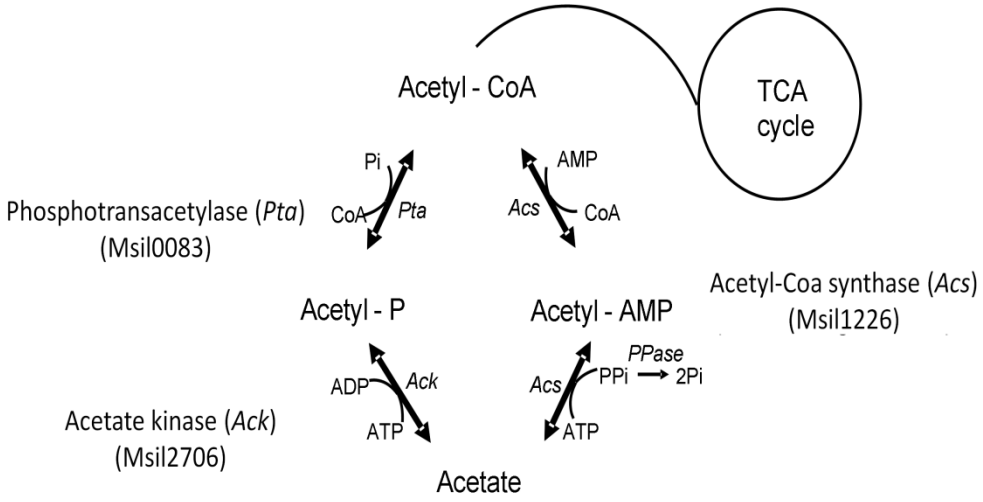
Appendix 5

The glyoxylate cycle as proposed by Kornberg and Krebs 50 years ago (Kornberg & Krebs, 1954). The citric acid cycle is modified to bypass the two decarboxylation steps by the action of its two key enzymes isocitrate lyase and malate synthase. This allows the net synthesis of malate from two molecules of acetyl-CoA. (Taken directly from Erb *et al.*, 2007).



Appendix 6

The possible pathways of formation of acetyl-CoA from acetate in *Methylocella silvestris* BL2 (Adapted from Chen, 2008d)



JOURNAL OF BACTERIOLOGY, July 2010, p. 3840–3841 Vol. 192, No. 14
0021-9193/10/$12.00 doi:10.1128/JB.00506-10

Complete Genome Sequence of the Aerobic Facultative Methanotroph *Methylocella silvestris* BL2▽

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Received 3 May 2010/Accepted 7 May 2010

***Methylocella silvestris* BL2 is an aerobic methanotroph originally isolated from an acidic forest soil in Germany. It is the first fully authenticated facultative methanotroph. It grows not only on methane and other one-carbon (C_1) substrates, but also on some compounds containing carbon-carbon bonds, such as acetate, pyruvate, propane, and succinate. Here we report the full genome sequence of this bacterium.**

Methylocella spp. are abundant in acidic soils and wetlands and help attenuate methane emissions from these habitats (2). They are unique in several ways compared to all other known aerobic methanotrophs. Notably, they lack extensive internal membrane systems and also appear to lack the particulate methane monooxygenase (pMMO) enzyme found in all other methanotrophs (6). Instead, they use only a soluble methane monooxygenase (sMMO) for methane oxidation. In addition, *Methylocella* spp. are not limited like other methanotrophs to growing on one-carbon (C_1) compounds but also utilize a number of multicarbon compounds (3). The genome of *Methylocella silvestris* BL2 (4) was sequenced, assembled, and annotated by the Joint Genome Institute (U.S. Department of Energy; http://www.jgi.doe.gov/sequencing/strategy.html). A total of 38,459 reads (~6× coverage), including 32,993 paired-end shotgun Sanger reads, 5,040 Roche 454 reads, and 580 finishing reads were included in the final assembly. Three lanes of Solexa data were used to polish the project.

The genome size is 4.3 Mbp. The G+C content is 63%. In total, 3,917 candidate genes were predicted and 99 pseudogenes were found. Functionality was assigned to 67.9% of the genes, while 30.9% of the genes could not be assigned any known function. Based on BLASTP searches against the KEGG (Kyoto Encyclopedia of Genes and Genomes) database, 3,413 out of 3,917 (87.1%) candidate genes have significant similarity to genes from *Proteobacteria*. Only 11 and 14 genes have best hits to genes from *Archaea* and *Eukarya*, respectively. All tRNA-encoding regions were identified, and two identical rRNA operons were found.

The absence of any *pmoCAB* genes encoding a pMMO enzyme that is present in all other genera of methanotrophs is now conclusively verified by the genome sequence. A complete operon encoding sMMO (*mmoXYBZDC*) was verified, as was a complete operon encoding methanol dehydrogenase (*mxaFJGIRSACKLDEH*) and all genes necessary for fixation of methane-derived carbon via the serine cycle. Genes encoding key enzymes in both the tetrahydrofolate and the tetrahydromethanopterin-mediated formaldehyde oxidation pathways were found.

M. silvestris can grow on two-carbon compounds, particularly acetate. Acetate kinase- and phosphotransacetylase-encoding genes are present, allowing acetate to be fed into the tricarboxylic acid (TCA) cycle. Genes encoding glyoxylate bypass enzymes (i.e., isocitrate lyase and malate synthase) have been identified. This pathway is essential for bacteria when growing on two-carbon compounds (1). The bacterium can also grow on C_3 and C_4 compounds, and a full gene set encoding enzymes of the TCA cycle is present, including genes encoding α-ketoglutarate dehydrogenase, which are lacking in some methanotrophs. Interestingly, a gene cluster encoding di-iron-containing multi-component propane monooxygenase is also present.

The genome sequence of *M. silvestris* is the first genome available for an alphaproteobacterial methanotroph. It joins the gammaproteobacterial methanotroph *Methylococcus capsulatus* Bath (7) and the verrucomicrobial methanotroph "*Methylacidiphilum infernorum*" (5). More detailed analyses of the genome as well as comparative analysis with obligate methanotrophs will provide deeper insight into the metabolism of this fascinating bacterium.

Nucleotide sequence accession number. The genome sequence and annotation have been deposited in GenBank under accession no. CP001280.

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▽ Published ahead of print on 14 May 2010.

This work was performed under the auspices of the U.S. Department of Energy's Office of Science, Biological and Environmental Research Program, and by the University of California, Lawrence Livermore National Laboratory, under contract no. W-7405-Eng-48, Lawrence Berkeley National Laboratory under contract no. DE-AC02-

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Applied and Environmental Microbiology, July 2010, p. 4530–4537 Vol. 76, No. 13
0099-2240/10/$12.00 doi:10.1128/AEM.00739-10

γ-Glutamylmethylamide Is an Essential Intermediate in the Metabolism of Methylamine by *Methylocella silvestris*▽

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Received 24 March 2010/Accepted 5 May 2010

***Methylocella silvestris* BL2, a facultative methane utilizer, can grow on monomethylamine (MMA) as a sole carbon and nitrogen source. No activity of MMA dehydrogenase was detectable. Instead, this bacterium utilizes a methylated amino acid pathway (γ-glutamylmethylamide [GMA] and *N*-methylglutamate [NMG]) for MMA metabolism. The activities of the two key enzymes in this pathway, GMA synthetase and NMG dehydrogenase, were found when the bacterium was grown on MMA. GMA was detected by high-performance liquid chromatography–mass spectrometry only when the bacterium was grown on MMA but not when it was grown on methanol. Proteomic analysis of soluble and membrane fractions of the proteome from MMA- and methanol-grown cultures revealed that an eight-gene cluster (Msil2632 to Msil2639) was induced by MMA and cotranscribed as an operon, as shown by reverse transcription-PCR. GMA-dissimilating enzyme activity was also detected when it was grown on MMA. Formaldehyde and ammonium production from GMA was dependent on glutamate but not on α-ketoglutarate. Marker exchange mutagenesis of a putative GMAS gene homologue (*gmas*, Msil2635) within this eight-gene cluster, with a kanamycin gene cassette, abolished growth of *M. silvestris* on MMA as either a sole carbon or a sole nitrogen source. Overall, our results suggest that *gmas* is essential in MMA metabolism by *M. silvestris*.**

Monomethylamine (MMA) is ubiquitous in the environment. For example, putrefaction of proteins (14a, 17) and degradation of many nitrogen-containing pesticides and herbicides can release MMA (5, 16b, 18). In the marine environment, MMA is released from the degradation of quaternary amines, such as betaine, carnitine, choline, and trimethylamine *N*-oxide, which are used as osmolytes by many marine organisms (3, 6). Once released, MMA can be used by some microorganisms as a sole carbon and nitrogen source through different pathways. Methanogenic archaea, such as *Methanosarcina* and *Methanomicrobium*, can use MMA anaerobically as a substrate to produce methane via a methyltransferase system (28). Gram-positive bacteria, such as *Arthrobacter*, metabolize MMA aerobically via an oxidase, which breaks down MMA into formaldehyde and ammonium (39). Gram-negative bacteria such as *Methylobacterium extorquens* and *Paracoccus denitrificans* utilize MMA dehydrogenase, a multisubunit enzyme that generates formaldehyde and ammonium from MMA aerobically (9, 16). Many other Gram-negative bacteria, such as *Aminobacter aminovorans* (previously known as strain MA and strain MS), can use MMA as a sole carbon and nitrogen source aerobically; however, they lack MMA dehydrogenase. It has been shown that in these microorganisms, two unusual amino acids, γ-glutamylmethylamide (GMA) and *N*-methylglutamate (NMG), are involved in MMA metabolism (1, 23, 33). In strain MA, an enzyme proposed as "NMG synthase" ("NMGS") converted MMA to NMG, which was subsequently oxidized to formaldehyde, regenerating glutamate (Glu) by a membrane-bounded particulate, NMG dehydrogenase (NMGDH) (33). The reactions carried out by these enzymes are summarized below. In strain MS, GMA was found to be a key metabolite in MMA metabolism (23). The synthesis of GMA was proposed to be carried out by a glutamine synthetase-like protein, and the reaction was dependent on ATP and Mg^{2+}. However, the fate of GMA in such a GMA-dependent MMA pathway is not clear.

$$\text{"NMG synthase" ("NMGS"): Glu + MMA} \rightarrow \text{NMG + } NH_4^+$$

$$\text{NMG dehydrogenase (NMGDH): NMG} \rightarrow \text{HCHO + Glu}$$

$$\text{GMA synthetase (GMAS): Glu + MMA + ATP} \rightarrow \text{GMA + ADP + } P_i$$

Although enzymes in these methylated amine-mediated pathways have been purified earlier, such as "NMGS" (31), NMGDH (2), and GMAS (21, 25, 37), the genes involved in these pathways have been studied only recently (24, 38).

Methylocella silvestris BL2 is a facultative methane-oxidizing bacterium belonging to the *Alphaproteobacteria* (11, 13, 35). It can grow on methylamine as a sole carbon and nitrogen source. Here we report the characterization of a gene cluster in *M. silvestris* BL2 involved in MMA metabolism and demonstrate that *gmas* is essential in MMA metabolism by this bacterium.

MATERIALS AND METHODS

Cultivation of *Methylocella silvestris*. Cells were grown at 25°C in a fed-batch mode in a 5-liter fermentor using diluted nitrate mineral (DNMS) medium, as

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▽ Published ahead of print on 14 May 2010.

TABLE 1. List of oligonucleotides used in this study

Target	Sequence (5′–3′)		Product size (bp)	Annealing temp (°C)
	Forward primer	Reverse primer		
Msil2632	GCGGCATAGTTGGATTGTTT	CATTCCAGCAAGATCGAACC	412	55
Msil2633	CTGCGAAAATCTTCGACCTC	GATCGAGCCTTTGACGAGAA	400	55
Msil2634	CTGAAAATTCCCGTCACCAT	TTCTCCCAATCGGTGATTTC	421	55
Msil2635	CAAAGAGTGTGGCGTCAAGA	TCTCGGTGATGACGTCGTAG	460	55
Msil2636	CGTGCTCAATCTCTGCCATA	GAATCCCTTGGTGGTTTCAA	327	55
Msil2638	GGCCGCAATATTTCCCTATT	TCGCAGATTGTGATCTCAGC	510	60
Msil2639	GACCGTGTTTGACGTCTCG	TCCGTCAACGCATGATAGAG	250	60
Msil2632/Msil2633	CCGATCAATATGTCGCCTTC	TGACGTCGCCTTTCACATAG	423	55
Msil2633/Msil2634	GCGCCTTGTATCATTTCCAC	GCGCTTGGAGATTTTCTGAC	598	55
Msil2634/Msil2635	CGCCTTGCAAACTACCTTTC	TATCAAGCCATGTCGCAAAG	411	55
Msil2635/Msil2636	CATCAGGAATGGCAGGATTT	CGAATTCCTTGGCGAGATAA	333	55
Msil2636/Msil2638	AATCGAAGGGCTGTTCTTCA	CGTGTCGCCGAAATATCC	591	60
Msil2638/Msil2639	CGCTGAACGATCGCAAAC	GAGCGACGCCAACAGCTC	547	60
Construction of *gmas* mutant	CCTGAGAATTCATGATCATGTCGGCATCC	CTGGAAGGATCCTCAGCAGTCGAGCGTCTGCTC	1,308	52
Amplification of *kan* gene cassette	GGTAGGTCGACGCATGCGAGCTCGGAAAGCCACGTTGTGTCTC	GGTAGGTCGACGCATGCGAGCTCAAGGTGTTGCTGACTCATAC	1,184	55
Confirmation of *gmas*::*kan* mutant	GCCTTGCAAACTACCTTTCG	CGAATTCCTTGGCGAGATAA	2,591 (for mutant), 1,665 (for wild type)	55

described by Theisen and colleagues (35). A total of 10 mM methylamine hydrochloride or 10 mM methanol plus 2 mM NH_4Cl was used as a carbon and nitrogen source. Cells were harvested at late exponential phase, resuspended in 10 mM 1,4-piperazinediethanesulfonic acid (PIPES) buffer (pH 7.6), and then stored at −80°C. To test whether sarcosine could be used as a substrate for *M. silvestris*, 10 mM sarcosine was used with or without 10 mM methanol.

To test if the *gmas* mutant could grow on MMA, a nitrogen-free DNMS medium was used. MMA was added at 5, 10, or 20 mM as the sole carbon and nitrogen source. A control, wild-type *M. silvestris*, was grown on either DNMS medium with methanol or nitrogen-free DNMS medium with 10 mM MMA. All growth experiments were set up in triplicate using 120-ml serum vials containing 20 ml medium, with an inoculum size of 5%. The serum vials were incubated at 25°C in a shaker (150 rpm).

HPLC-MS analyses of GMA. GMA was extracted from 100 μl frozen cells by vigorously vortexing them in 1 ml methanol for 1 min. Cells were then removed by centrifugation. The supernatant was heated at 60°C to remove methanol by evaporation. The residual powder was dissolved in 20 μl of water, and the solution was kept at −20°C prior to mass spectrometry (MS) analyses, which were carried out using an Agilent 1100 high-performance liquid chromatography (HPLC) system, with a diode array detector (DAD) coupled to a Bruker HCT-plus (high-capacity ion trap) mass spectrometer. The HPLC column was obtained from Agilent (Zorbax reverse-phase HPLC column Rx-C_{18}, 150 by 4.6 mm and particle size of 5 μm with C_{18} guard cartridge). Samples were filtered through a 0.2-μm microspin filter before injection. The mobile phases used were water with 0.1% trifluoroacetic acid (TFA) (A) and methanol with 0.1% TFA (B). Gradient settings used were 0 to 2 min at 100% A, 2 to 25 min from 100% A to 100% B, 25 to 30 min at 100% B, 30 to 35 min of equilibration to 100% A, and 35 to 45 min at 100% A. The flow rate was 1 ml min^{-1}, and 10% of the flow was diverted to MS. The DAD setting was 210 nm, 254 nm, and 280 nm. The MS setting was full scan, with electrospray ionization in positive mode, nebulizer gas at 10 liters min^{-1}, dry gas at 300°C and 40 lb/in^2, and capillary exit at 65 V. GMA was identified based on the elution time, and molecular mass was compared to synthesized standards, which were prepared and purified according to a previously published method (27).

Protein analyses. Cells were broken for protein analyses and enzyme assays by passing them three times through a French pressure cell (American Instrument Co.) at 110 MPa. Cell debris was removed by centrifugation at 6,000 × *g* for 15 min. Where necessary, soluble and membrane protein fractions were further separated by ultracentrifugation at 150,000 × *g* for 2 h, and the membrane fraction was washed once with buffer (10 mM PIPES, pH 7.6), followed by ultracentrifugation for 1 h under the same conditions. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad). One-dimensional protein analyses were carried out using a precast NuPage Bis-Tris gel (10%), according to the manufacturer's protocol (Invitrogen). Gels were stained with Coomassie brilliant blue R-250, and bands were excised, digested with trypsin, and analyzed using matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS) and tandem mass spectrometry at the Biological Mass Spectrometry and Proteomics Facility, Department of Biological Sciences, University of Warwick, as described previously (32).

RNA extraction and reverse transcription (RT)-PCR. RNA was extracted from frozen cells using either an RNeasy mini kit (Qiagen) or the method described by Gilbert and colleagues (14). Trace DNA contaminants in the RNA were further removed by DNase I (Qiagen) digestion, and RNA was then recovered using the RNeasy minikit (Qiagen). Reverse transcription was performed using the SuperScript II system (Invitrogen). Reverse transcription and PCR were carried out using a Bio-Rad thermocycler. Primers used are listed in Table 1.

Enzyme assays. All enzyme assays were carried out in triplicate using cell-free crude extract in 10 mM PIPES buffer (pH 7.6) at room temperature (22°C), unless otherwise stated. GMA synthetase (MMA + glutamate + ATP → ADP + P_i + GMA) activity was measured by quantifying the production of γ-glutamylhydroxamate from glutamate and hydroxylamine in the presence of ATP and Mg^{2+} by monitoring the change in absorbance at 540 nm. The assay was originally developed to quantify the ability of glutamine synthetase to form glutamine (4) but later adapted to quantify GMAS activity (24, 37). The following concentrations of substrates were used: glutamate (50 mM), hydroxylamine hydrochloride (15 mM), $MgCl_2$ (20 mM), and ATP (5 mM).

NMGDH activity was measured using 2,6-dichlorophenolindophenol (DCPIP) as an artificial electron acceptor with or without KCN (1 mM), as described by Bamforth and Large (2). The initial rate of decrease in A_{600} was recorded continuously for 5 min (within the linear phase). MMA dehydrogenase was assayed in the same way, except that methylamine hydrochloride (final concentration, 10 mM) was used as the substrate to initiate the reaction. In addition, NMGDH activity was also measured by quantifying NMG-dependent formaldehyde production using the method described below.

The activity of GMA dissimilation enzymes was measured using the following two methods: (i) GMA-dependent formaldehyde production and (ii) GMA-dependent ammonium production. Formaldehyde concentration was determined colorimetrically using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald reagent; Sigma). The reaction was started by adding GMA, and absorbance at 550 nm was recorded before and after incubation for 10 min to measure the initial rate of conversion within the linear phase (12). Prior to assaying ammonium, the cell-free enzyme extract was dialyzed to remove ammonium and MMA from the culture using a Slide-A-Lyzer 3.5-kDa-molecular-mass-cutoff dialysis cassette (Thermo Scientific), dialyzing for 2 h against 50 mM PIPES buffer (1 liter, pH 7.6) at 4°C. GMA-dependent ammonium released before and after 30 min of incubation was determined colorimetrically using Nessler's reagent (Sigma), measuring the change of absorbance at 440 nm (7). A decrease in absorbance was observed when α-ketoglutarate was present in the assay. This

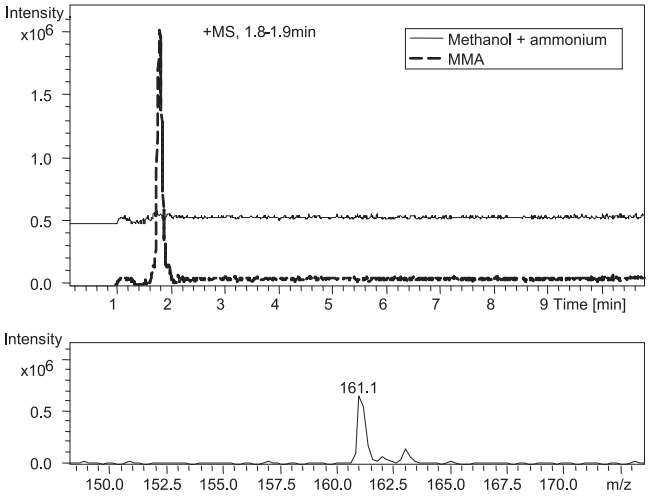

FIG. 1. (Top) Comparison of extracted ion chromatograms (EIC; *m/z* 161) of methanol extract of *Methylocella silvestris* cells grown on methylamine (dashed line) and methanol plus ammonium (solid line). (Bottom) The peak identified with a mass-to-charge ratio of 161.1 is γ-glutamylmethylamide.

was due to the glutamine synthetase/glutamate synthase activity in the presence of residual ammonium after dialysis (data not shown).

"NMGS" activity was measured indirectly by quantifying glutamate and MMA-dependent formaldehyde production as described previously (24). The assay was initiated by adding either glutamate (final concentration, 10 mM) or MMA (final concentration, 10 mM).

Bioinformatics. Sequence similarity searches were performed with GenBank using the BLASTP program against the nonredundant protein sequence database and the Swiss-Prot protein sequence database. Protein sequences of type I, type II, and type III glutamine synthetases were downloaded from GenBank and aligned using the ClustalX program (36). Phylogenetic analysis was performed using the MEGA4 program (34).

Marker exchange mutagenesis of *gmas*. To construct a *gmas* mutant of *M. silvestris*, the *gmas* gene was amplified by PCR and inserted into pUC19 under the EcoRI and BamHI sites. The 258-bp SacI fragment from the *gmas* gene was then removed and replaced with a kanamycin gene cassette, which was amplified from the plasmid pCM184 by PCR using the primers listed in Table 1 (30). The plasmid was cut with EcoRI and BamHI, and the 2.2-kb fragment containing the kanamycin gene cassette was then gel purified and electroporated into *M. silvestris* using the method of Kim and Wood (19), with minor modifications. A total of 500 ng of DNA was added to 100 μl of cells. The electroporation settings used were resistance at 400 Ω, voltage at 2.2 kv, and a cuvette of 1 mm. Cells were recovered at 25°C overnight with DNMS medium containing methanol (10 mM) before plating. Potential mutants were selected on DNMS (20 μg ml^{-1} Kan) agar plates, with methanol (10 mM) as the sole carbon source. Mutation of the *gmas* gene was confirmed by diagnostic PCR and subsequent DNA sequencing.

RESULTS

MMA metabolism by *Methylocella silvestris* involves GMA. *M. silvestris* can utilize MMA as the sole carbon and nitrogen source (13). Its genome is available in GenBank (accession number CP001280), and the genome does not contain any candidate coding sequences for MMA oxidase or MMA dehydrogenase. In addition, dye-linked MMA dehydrogenase assays were performed, and no activity was found using cell extract from cells grown on MMA as a sole carbon and nitrogen source.

Experiments were then carried out to investigate if *M. silvestris* used the methylated amino acid pathway for MMA metabolism. HPLC-MS analysis showed that when *M. silvestris* was grown on MMA, GMA could be detected, whereas no GMA was found when *M. silvestris* was grown on methanol (Fig. 1). No NMG was detected in either MMA-grown cultures or methanol-grown cultures by HPLC-MS analysis. Assays were then carried out to determine activities of key enzymes involved in the GMA- and NMG-mediated metabolic pathways (Table 2). The γ-glutamylhydroxamate assay showed that GMAS/glutamine synthetase activities were approximately 3-fold higher in MMA-grown cultures than in methanol-grown cultures. The GMAS/glutamine synthetase activity detected by the γ-glutamylhydroxamate assay in methanol-grown cultures was due to glutamine synthetase rather than GMAS, since GMAS is not expressed under these conditions (see below). Furthermore, assays for NMGDH were carried out using two methods. Dye-linked NMGDH activity was found only when the bacterium was grown on MMA and not when it was grown on methanol plus ammonium. This activity was relatively low (4.4 ± 0.6 nmol min^{-1} mg $protein^{-1}$). Dye-linked NMGDH activity was approximately 4-fold lower without KCN (final concentration, 1 mM), added in order to inhibit cytochrome oxidases, as suggested by Bamforth and Large (2). A more robust assay for NMGDH was used to quantify the production of formaldehyde from NMG. This activity was, again, detectable only in MMA-grown cultures but was higher (16.6 ± 0.7 nmol min^{-1} mg $protein^{-1}$) than that obtained using the dye-linked NMGDH assay, indicating that DCPIP was a poor artificial electron acceptor for this enzyme. Assays using cytochrome *c* from horse heart did not yield any activity for NMGDH from *M. silvestris*. In addition, no activity of "NMGS" was detected in either MMA-grown or methanol-grown cultures using the method described by Latypova and colleagues (24).

Identification and characterization of a gene cluster involved in MMA metabolism. A proteomic analysis was carried out to investigate the proteins involved in MMA metabolism by *M. silvestris*. By comparing polypeptide profiles of cells grown on methanol or MMA, it was obvious that several proteins were highly induced by MMA in both the soluble fraction and membrane fraction of MMA-grown cells (Fig. 2A). These polypeptides were cut from the gel and sequenced by tandem mass spectrometry. The results indicated that proteins encoded by a gene cluster were highly induced in MMA-grown

TABLE 2. Key enzyme activities in cells grown on MMA or methanol

Assay	Activity (nmol min^{-1} mg $protein^{-1}$) in cells grown on[a]:	
	MMA	Methanol plus ammonium
GMAS	31.1 ± 0.4	11.9 ± 0.9
NMGDH		
DCPIP	4.4 ± 0.6	ND
Formaldehyde	16.0 ± 0.7	ND
GMA dissimilation activity		
Formaldehyde	4.74 ± 0.12	ND
Ammonium	7.98 ± 0.49	ND

[a] Values are means ± standard deviations from three assays. ND, not detectable.

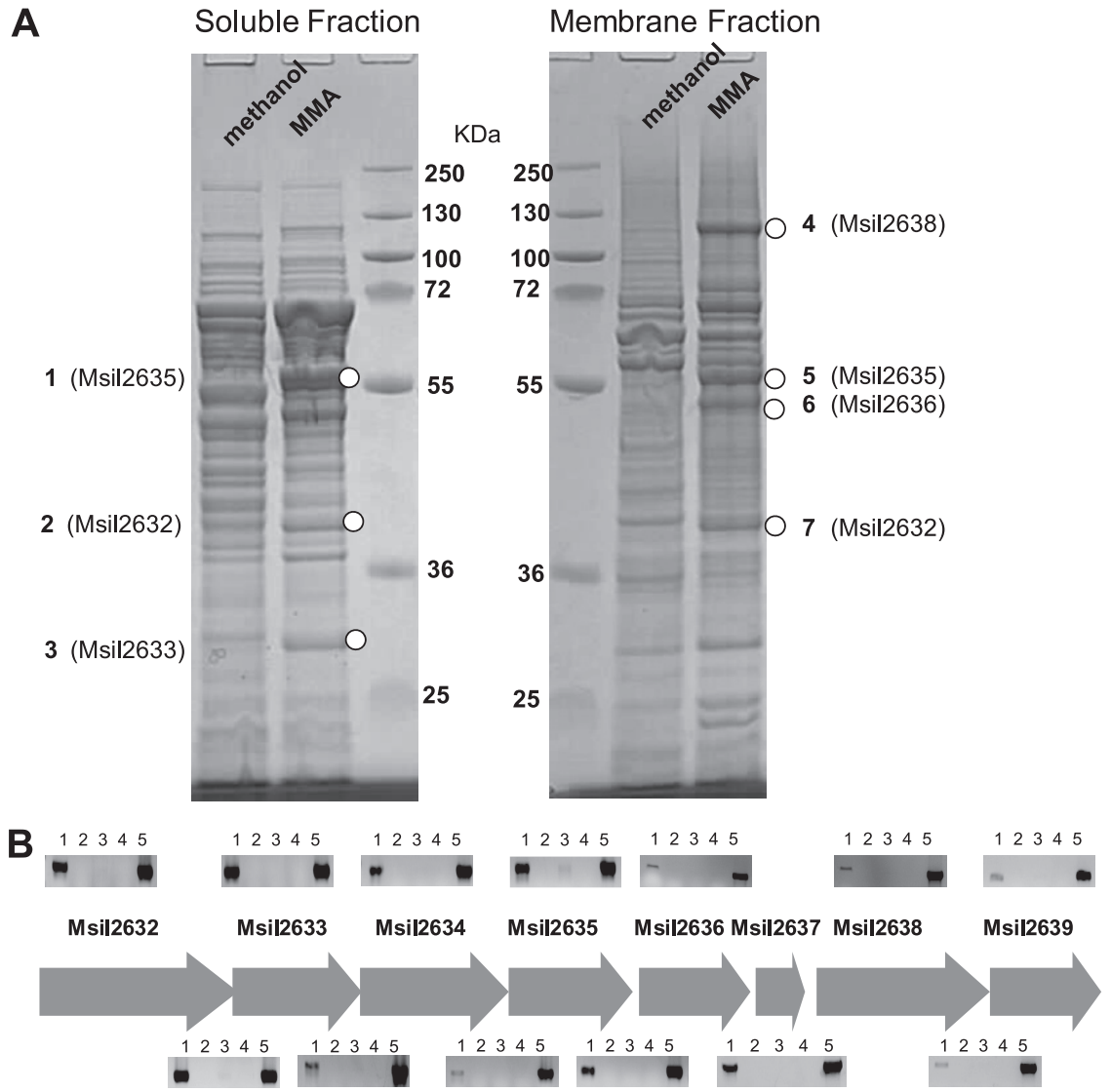

FIG. 2. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of soluble and membrane proteins from cells grown on MMA alone and methanol plus ammonium. Bands highlighted (1 to 7) were cut from the gel and analyzed by matrix-assisted laser desorption ionization–mass spectrometry and tandem mass spectrometry. (B) Agarose gel photos of RT-PCR products showing the eight-gene cluster, which was induced by MMA. Agarose gel photos above each gene show the induction of each gene by MMA only, and gel photos above intergenic regions show that this gene cluster is cotranscribed. RT-PCR products using mRNA extracted from MMA-grown cells and methanol-grown cells are shown in lanes 1 and 3, respectively. Corresponding controls without reverse transcriptase are shown in lanes 2 and 4, respectively. Lane 5 is the positive control for PCR using genomic DNA from *Methylocella silvestris*.

cells and were, therefore, likely to be involved in MMA metabolism by *M. silvestris* (Table 3; Fig. 2A).

The results from BLAST searches for each gene in this cluster are shown in Table 4. The first three open reading frames (ORFs; Msil2632 to Msil2634) encode polypeptides with high similarities to individual domains of the glutamate synthase large subunit, containing a glutamine amidotransferase domain, a GXGXG motif, and a highly conserved flavin mononucleotide (FMN)-binding domain, respectively. All the BLASTP hits matched with significant identities are putative genes with no known experimentally validated functions. ORF Msil2635 encodes a glutamine synthetase-like protein; however, sequence analysis indicated that this gene

TABLE 3. Identification of polypeptides induced by MMA

Band	ORF identification	Calculated molecular mass from protein sequence (kDa)	No. of polypeptides detected	Sequence coverage (%)
1	Msil2635	48.4	5	21
2	Msil2632	32.0	5	21
3	Msil2633	24.7	4	24
4	Msil2638	104.3	7	10
5	Msil2635	48.4	8	17
6	Msil2636	44.8	6	18
7	Msil2632	32.0	4	20

TABLE 4. Gene description and BLAST search results

ORF	Length (aa)	Top BLASTP match (identity [%])	Gene assignment[a]		Note
			a	b	
Msil2632	299	Glutamine amidotransferase, class II (*Azorhizobium caulinodans* ORS 571 [67]) Glutamine amidotransferase, class II (*Xanthobacter autotrophicus* Py2 [65])	*gltB1*	*mgsA*	Contains a GlxB-like glutamine amidotransferase domain
Msil2633	235	Glutamate synthase domain 3-like protein (*Methylobacterium populi* BJ001 [72]) Glutamate synthase family protein (*Xanthobacter autotrophicus* Py2 [66])	*gltB3*	*mgsB*	Contains a GXGXG motif commonly found in glutamate synthase
Msil2634	444	Putative glutamate synthase GltB2 subunit (*Methylobacillus flagellatus* KT [87]) Glutamate synthase domain 2-like protein (*Azorhizobium caulinodans* ORS 571 [86])	*gltB2*	*mgsC*	Contains a highly conserved FMN-binding domain in GltS
Msil2635	435	Putative type III glutamine synthetase (*Rhodopseudomonas palustris* HaA2 [69]) γ-Glutamylmethylamide synthetase (*Methylovorus mays* [41])	*gltIII*	*gmas*	Does not contain key residues for ammonium binding
Msil2636	416	Putative sarcosine oxidase β subunit (*Azorhizobium caulinodans* ORS 571 [75]) Sarcosine oxidase β subunit (*Corynebacterium* sp. P-1 [49])	*soxB*	*mgdA*	
Msil2637	95	Putative sarcosine oxidase, δ subunit (*Bradyrhizobium* sp. BTAi1 [55]) Sarcosine oxidase δ subunit (*Corynebacterium* sp. P-1 [37])	*soxD*	*mgdB*	
Msil2638	984	Putative sarcosine oxidase α subunit (*Methylobacterium populi* BJ001 [55]) Sarcosine oxidase α subunit (*Corynebacterium* sp. P-1 [36])	*soxA*	*mgdC*	Contains two domains of the glycine cleavage T-protein
Msil2639	209	Putative sarcosine oxidase γ subunit (*Rhodopseudomonas palustris* HaA2 [37]) Sarcosine oxidase γ subunit (*Corynebacterium* sp. P-1 [31])	*soxG*	*mgdD*	

[a] a, assigned based on BLASTP matches; b, assigned based on the similarity between Msil2632 to Msil2639 and the gene cluster of *Methyloversatilis universalis* (24).

is lacking a conserved ammonia-binding site (D^{50}, S^{53}, and Y^{179}), which is commonly found in glutamine synthetases (26). This gene also shows 41% identity to a newly characterized GMAS from *Methylovorus mays* (38) (Fig. 3). In both Msil2635 and GMAS from *Methylovorus mays*, the three key ammonium-binding residues are nonpolar rather than polar ($D^{50} \rightarrow G^{50}$, $S^{53} \rightarrow A^{53}$, and $Y^{179} \rightarrow C^{179}$). The last four ORFs in this gene cluster showed 35 to 75% sequence identities to putative heterotetrameric sarcosine oxidase subunits. Msil2636 and Msil2638 had 36% and 49% identity, respectively, to characterized sarcosine oxidase subunits of *Corynebacterium* sp. P-1 (10). However, when tested, *M. silvestris* failed to grow on sarcosine supplied as either a sole carbon source or a sole nitrogen source.

To further investigate whether this gene cluster is induced by MMA, transcriptional analyses were performed with RNA extracted from MMA- and methanol-grown cultures. The results (Fig. 2B) indicated that the genes in this cluster were induced by MMA, whereas no transcripts could be detected in methanol-grown cells. Furthermore, RT-PCR targeting the intergenic region yielded products of the expected sizes, indicating that the genes were cotranscribed as a single operon.

The activity of the GMA-dissimilating enzyme is dependent on glutamate. The fate of GMA in microorganisms which use the methylated amine pathway for MMA metabolism is controversial. We therefore decided to further explore whether GMA could be dissimilated by *M. silvestris*. The results are shown in Fig. 4. Formaldehyde production was seen when GMA was incubated with cell-free crude extract, and this was stimulated by glutamate. Ammonium production from GMA was also observed. This was also dependent on glutamate. These experiments indicated that GMA, via the presence of glutamate and release of ammonium, may be transformed into NMG, which is further oxidized by NMG dehydrogenase to yield formaldehyde.

A *gmas* mutant cannot grow on MMA as either a sole carbon source or a sole nitrogen source. To investigate if the putative *gmas* gene, which was induced by MMA, is essential in MMA metabolism by *M. silvestris*, we constructed a mutant by marker exchange. This mutant can grow on methanol in the presence of ammonium but failed to grow on MMA as a sole carbon and nitrogen source at 5, 10, or 20 mM (Fig. 5). In addition, unlike wild-type *M. silvestris*, this mutant cannot grow on MMA as a sole nitrogen source in the presence of methanol using nitrogen-free DNMS medium (data not shown).

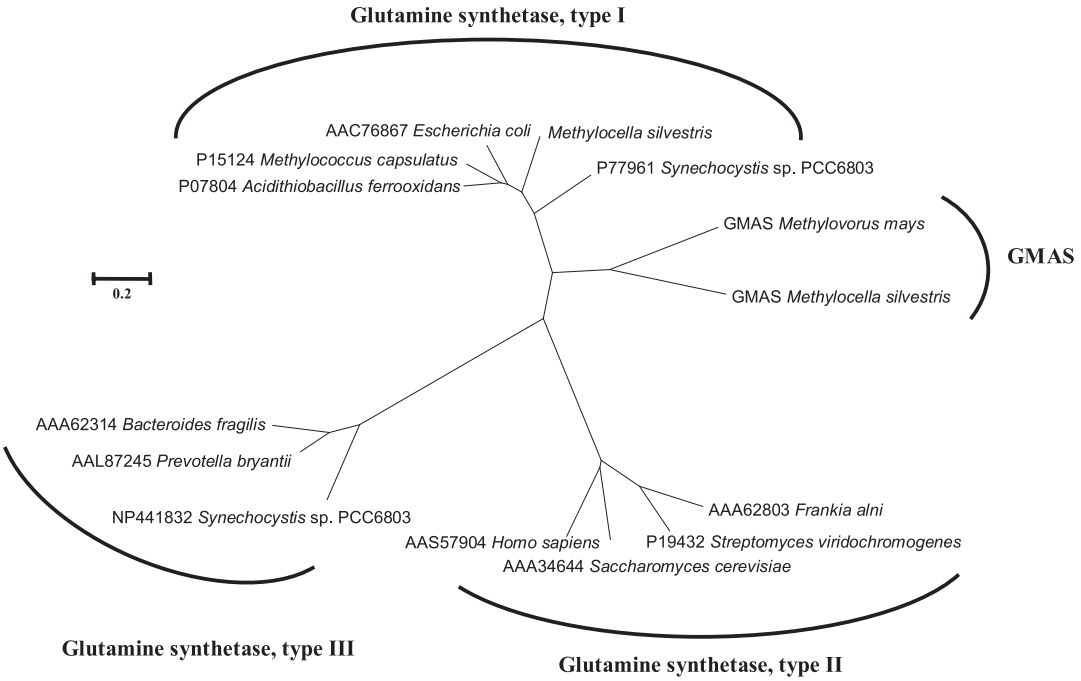

FIG. 3. An unrooted phylogenetic tree showing the relationship between γ-glutamylmethylamide synthetases (GMAS) and three types of glutamine synthetases. Amino acid sequences were aligned using the ClustalX program, and the tree was constructed using MEGA4 (~350 amino acids [aa] for γ-glutamylmethylamide synthetases, ~370 aa for type I glutamine synthetases, ~335 aa for type II glutamine synthetases, and ~440 aa for type III glutamine synthetases). The scale bar represents 2 substitutions per 10 amino acids.

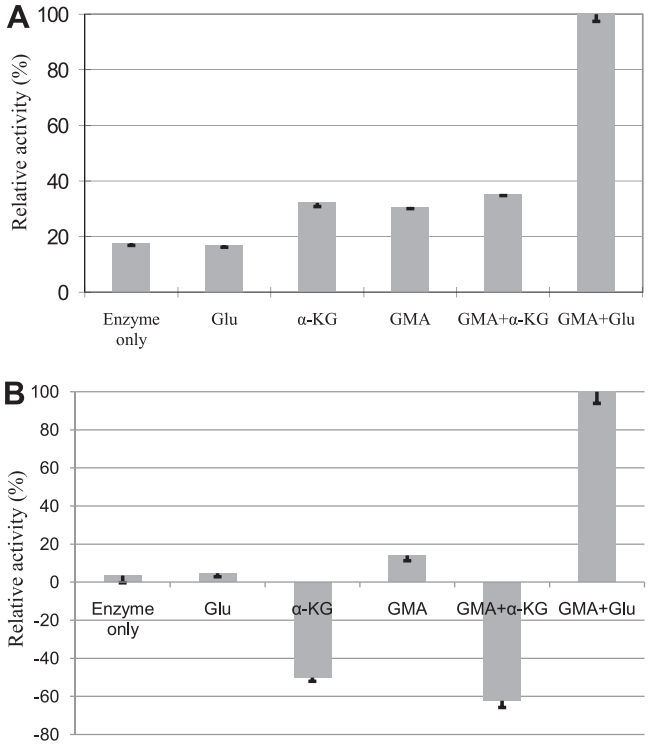

FIG. 4. GMA-dissimilating enzyme activities determined by quantifying GMA-dependent formaldehyde production (A) or ammonium production (B). The activities with GMA and Glu were chosen as 100%, which were 4.74 ± 0.12 nmol min^{-1} mg $protein^{-1}$ for the formaldehyde assay and 7.98 ± 0.49 nmol min^{-1} mg $protein^{-1}$ for the ammonium assay. Glu, glutamate; α-KG, α-ketoglutarate; GMA, γ-glutamylmethylamide. Means and standard deviations of the results from three replicates are shown.

DISCUSSION

Through comparative proteomic analyses, we revealed that a cotranscribed cluster of eight genes is involved in MMA metabolism by *M. silvestris*. Recently, a similar gene cluster was identified in *Methyloversatilis universalis* and was designated NMGS (*mgsABC*), GMAS, and NMGDH (*mgdABCD*), respectively (24). No attempt was made to elucidate the functions of putative *mgsABC* (Msil2632 to Msil2634) and *mgdABCD* (Msil2636 to Msil2639) identified in *M. silvestris* in this study; however, it is likely that they are NMGS and NMGDH, respectively.

GMA, which is induced by MMA, was found as an early product in the metabolism of MMA by *Aminobacter aminovorans* strain MS (23), suggesting a key role for GMA in MMA metabolism by this bacterium. It was also found that the enzyme, which was responsible for GMA synthesis, was similar to, but distinct from, glutamine synthetase. GMAS and glutamine synthetase from *Aminobacter aminovorans* strain MS were subsequently purified, and it was demonstrated that GMAS was more specific for GMA (25). In *M. silvestris*, a separate bona fide type I glutamine synthetase gene is present in the genome, and this enzyme is expressed in both methanol- and methylamine-grown cells (data not shown). The *gmas* homolog (Msil2635), which is induced by MMA (Fig. 2B) in *M. silvestris*, is likely to be GMAS. The translated amino acid of this protein showed high sequence identity to a characterized GMAS from *Methylovorus mays* (38), and phylogenetic analyses showed that these two proteins, which are related to type I glutamine synthetases, form a separate branch (Fig. 3). Moreover, similar to GMAS from *Methylovorus mays*, GMAS from

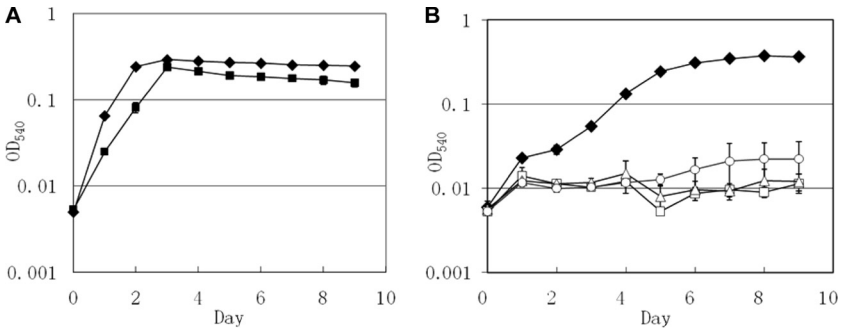

FIG. 5. (A) Growth curves of the wild type (filled diamonds) and the *gmas*::*kan* mutant (filled squares) of *Methylocella silvestris* grown on methanol (10 mM) with ammonium (2 mM). (B) Growth curves of the wild type (filled diamonds, 10 mM) and the *gmas*::*kan* mutant (open triangles, 5 mM; open squares, 10 mM; open circles, 20 mM) of *Methylocella silvestris* grown on MMA alone at different concentrations. Means and standard deviations of the results from three replicates are shown. OD_{540}, optical density at 540 nm.

M. silvestris lacks key residues that are crucial in the binding of ammonium, suggesting that these enzymes do not prefer ammonium and thus are likely to be GMAS rather than glutamine synthetase.

The fate of GMA in bacterial MMA metabolism is controversial. Latypova and colleagues found that GMA was a key intermediate in *Methyloversatilis universalis* when it was grown on MMA, but no GMA-dissimilating enzyme activity could be detected (24). Different observations were made in other studies. For example, using ^{14}C-labeled GMA, Loginova and colleagues demonstrated that GMA was converted to NMG in *Hyphomicrobium vulgare* and that the reaction was dependent on glutamate (29). GMA dissimilation activity was also found in a *Methylophaga* strain, and the reaction was dependent on α-ketoglutarate and ammonium (20, 22). In *M. silvestris*, we were unable to show a direct conversion of GMA to NMG (data not shown); however, we demonstrated that there is glutamate-dependent, but not α-ketoglutarate-dependent, GMA-dissimilating enzyme activity which releases formaldehyde and ammonium from GMA (Fig. 4). This is similar to what has been found in *Hyphomicrobium vulgare*, where the conversion of GMA to NMG was also dependent on the presence of glutamate. The demonstration of α-ketoglutarate- plus ammonium-dependent, but not glutamate-dependent, GMA dissimilation enzyme activity in *Methylophaga* spp. is probably misleading, since the assay was carried out in a buffer containing 100 mM ammonium (20), which might have inhibited the glutamate-dependent GMA dissimilation activity. In fact, high glutamine synthetase/glutamate synthase activity was found in this bacterium when it was grown on MMA (20), and α-ketoglutarate and ammonium may have been converted to glutamate, which might have served as a true substrate for the GMA-dissimilating enzyme in this *Methylophaga* strain. GMAS in *M. silvestris* seems to be essential, and mutation of the corresponding gene (*gmas* homologue, Msil2635) resulted in a mutant which failed to grow on MMA as either a sole carbon source or a sole nitrogen source (Fig. 4B).

We therefore propose the pathway of MMA metabolism by *M. silvestris* shown in Fig. 6, in which MMA is metabolized, via GMA and NMG, to formaldehyde and ammonium. Formaldehyde produced is either further oxidized to CO_2 to generate energy and reducing equivalents or assimilated into cell biomass, probably through the serine cycle. Ammonium produced in this pathway is used as a nitrogen source which is probably assimilated through bona fide glutamine synthetase and glutamate synthase (genes encoding glutamate dehydrogenase or alanine dehydrogenase are not present in the genome). However, we were unable to detect NMG from MMA-grown cells of *M. silvestris*. This is probably due to the fact that accumulation of GMA or NMG is related to growth state, as demonstrated previously (16a). Although it was suggested that GMA-dependent and NMG-dependent MMA metabolism by bacteria involved two different pathways (1, 24), our data and those of others suggest that it is more likely that these two may indeed be the same pathway (8, 29). It has been shown that glutamate-dependent ammonium and formaldehyde production from GMA also occurs in *Aminobacter aminovorans* strain MA when it is grown on MMA (8). This strain has been studied extensively, and it uses the NMG-mediated pathway for MMA metabolism (15, 31, 33). The NMG-mediated pathway for MMA metabolism was proposed, based mainly on studies using this strain. The key lies in whether GMA or MMA is the true substrate for this so-called "NMG synthase" ("NMGS"). The purified "NMGS" from strain MA is specific for glutamate but not for MMA, and indeed, a number of amines can substitute for MMA (31). There is, therefore, an urgent need to purify "NGMS" and to reexamine the specificity of this en-

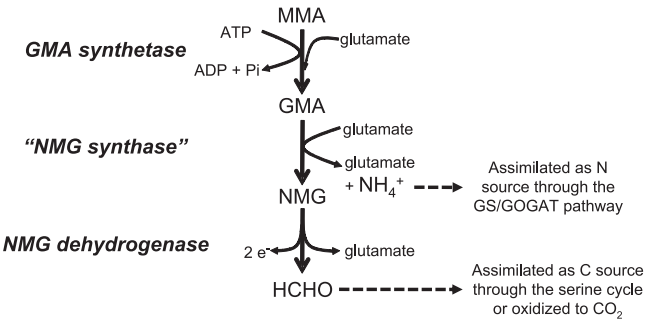

FIG. 6. Proposed pathway of MMA metabolism by *Methylocella silvestris*. Formaldehyde produced from this pathway is either assimilated as a carbon source through the serine cycle or further oxidized to CO_2, and the ammonium produced is assimilated as a nitrogen source through the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway. e^-, electron.

zyme. Kinetic studies are also required to finally resolve the controversial roles of the GMA- and NMG-dependent pathways for MMA metabolism by bacteria.

ACKNOWLEDGMENTS

We thank M. Kalyuzhnaya, S. Yamamoto, S. Dedysh, P. Dunfield, D. P. Kelly, P. J. Large, and C. Anthony for advice on enzyme assays and helpful discussions and R. Boden for his advice on GMA synthesis. S. Slade is acknowledged for helping with the MALDI-MS analyses.

This work was supported by NERC research grant NE/E016855/1 to J. C. Murrell. We are grateful to the Joint Genome Institute for providing the genome sequence of *M. silvestris* BL2 (community sequencing program 2007; ID 4023905).

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