Characterisation of methylotrophs in the rhizosphere

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Abstract

Methanol is the second most abundant volatile organic compound in the atmosphere, with the majority of this methanol being produced as a waste metabolic by-product of the growth and decay of plants. There is a large disparity between the amount of methanol estimated as being produced and that which enters the atmosphere. This disparity is believed to be due to the utilisation of methanol by plant associated methylotrophs. The diversity and activity of methylotrophs associated with the root and rhizosphere of pea and wheat plants was assessed through a range of cultivation independent and dependent approaches.

Enrichments performed with a range of environmental samples supplemented with methanol resulted in the isolation of several strains of methylotrophic bacteria, including two novel species of methylotroph belonging to the family *Methylophilaceae,* whose genomes were sequenced and their physiological capabilities assessed.

The diversity and abundance of methanol dehydrogenase encoding genes in bulk soil and the pea and wheat rhizosphere was assessed through 454 sequencing and qPCR respectively. Sequencing showed high levels of diversity of methylotrophic bacteria within the bulk soil and also showed a shift in this diversity between the bulk soil and the plant associated soils, in spite of no shift in the abundance of these genes occurring.

Active methylotrophs present in the bulk and plant associated soils were identified by DNA stable isotope probing using ^{13}C labelled methanol. Next generation sequencing of the 16S rRNA genes and construction of metagenomes from the ^{13}C labelled DNA revealed members of the *Methylophilaceae* as highly abundant in all of the soils. A greater diversity of the *Methylophilaceae* and the genus *Methylobacterium* were identified as active in the plant associated soils relative to the bulk soil.

A $^{13}CO_2$ stable isotope probing experiment identified methylotrophs as utilising plant exudates in the pea and wheat root and rhizosphere communities. Several methylotrophic genera were identified as exudate utilising, in addition to heterotrophic genera and Actinomycetes. The specific ^{13}C labelled genera were shown to vary between both the wheat and pea plants and between the rhizosphere and root communities.

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Chapter 1: Introduction

1.1 Methylotrophic bacteria

Methylotrophs are organisms that utilise reduced carbon substrates with either no carbon-carbon bonds or one carbon atom as their sole source of carbon and energy (Anthony 1983; Chistoserdova 2011a). Methylotrophs are studied because of their impact on several important environmental processes, including the cycling of nitrogen, carbon, sulfur and phosphorous and compounds with climate relevant impacts such as dimethyl sulfide, methane and methanol (Boden *et al.*, 2010; Chistoserdova, 2011b). There are also industrial and biotechnological applications of methylotrophic bacteria (Chistoserdova 2011a; Beck et al. 2014), with methylotrophs cultivated to convert C1 compounds to industrial products. This research project has focused on the methanol utilising methylotrophs, organisms that utilise methanol as their sole carbon and energy source.

1.1.1 Basic characteristics

Methylotrophs have been shown to be broadly distributed throughout most environments, including in soil, seawater, in association with plants (leaf and root) and in more extreme environments, including hot springs and volcanic mudpots (Stacheter et al. 2013; Neufeld et al. 2007b; Jourand et al. 2005; Hutchens et al. 2003; Stephenson 2014.; Antony et al. 2010; Knief et al. 2012; Doronina et al. 2017; Pol et al. 2013). Most methylotrophs prefer moderate growth conditions e.g. neutral pH (Kolb, 2009; Stacheter *et al.*, 2013). However, there are some methylotrophs, e.g. *Methyloversatilis thermotolerans* and *Methylomirabilis oxyfera*, that are capable of growth in more extreme environments with regards to parameters such as temperature and pH respectively (Anvar *et al.*, 2014; Doronina *et al.*, 2014).

Methylotrophy is found in a range of phylogenetic groups, including the Alpha, Beta and Gamma-proteobacteria, Verrucomicrobia, NC10, Firmicutes, Bacteroidetes and Actinobacteria (Kolb 2009; Chistoserdova 2011a; Keltjens et al. 2014 and references therein). With a greater understanding of the pathways involved in the utilisation of C1 compounds it is proposed that methylotrophy may be more widespread than previously considered (Taubert et al. 2015; Beck et al. 2015; Kalyuzhnaya et al. 2008). The phylogenetic diversity encompassing methylotrophs is expanding, with the detection of

methanol dehydrogenase genes within the genomes of members of recently created phyla, including the *Tectomicrobia*, *Gemmatimonadetes* and *Rukobacteria* (Wilson *et al.*, 2014; Butterfield *et al.*, 2016).

The process of methylotrophy can broadly divided into three stages (Chistoserdova 2011a). 1) The oxidation of the C1 substrate to formaldehyde, 2) the oxidation of formaldehyde and 3) the incorporation of C1 units into biomass. The incorporation of C1 units into biomass can occur at the step of formaldehyde for the ribulose monophosphate pathway and the serine cycle, and at the point of carbon dioxide (CO_2) for the ribulose bisphosphate cycle and serine cycle (Anthony 1983) (Figure 1.1).

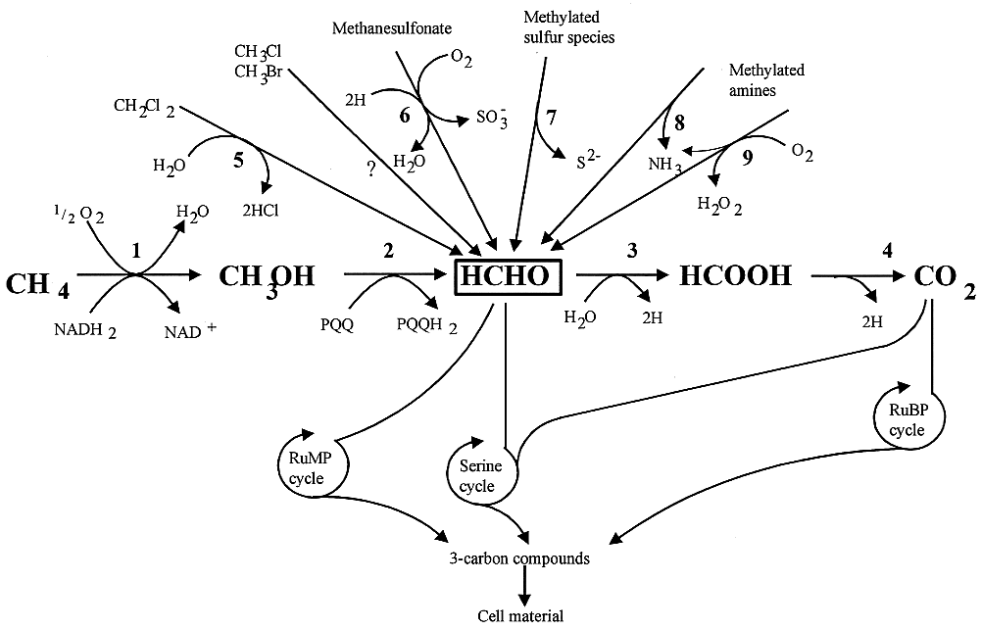


Figure 1.1 Generalised schematic of the metabolism of C1 compounds by aerobic methylotrophic bacteria (taken from Murrell and McDonald (2000)). Enzymes: 1, methane monooxygenase; 2, methanol dehydrogenase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase; 5, dichloromethane dehalogenase; 6, methanesulfonic acid monooxygenase; 7, methylated sulfur dehydrogenases or oxidases; 8, methylated amine dehydrogenases; 9, methylamine oxidase.

There are also multiple oxidation pathways for formaldehyde, with some organisms possessing more than one. The presence of multiple formaldehyde oxidation pathways is believed to prevent the accumulation of excess formaldehyde that would prove toxic to the cell (Chistoserdova *et al.*, 2000). These pathways include the binding of formaldehyde to tetrahydromethanopterin (Marx *et al.*, 2003) and tetrahydrofolate, the

cyclic oxidative pathway of the ribulose monophosphate pathway (Anthony, 1983; Chistoserdova *et al.*, 2000) and the glutathione dependent formaldehyde dehydrogenase (Goenrich et al. 2002; Vorholt 2002 and references therein). XoxF is also capable of oxidising formaldehyde to formate (Schmidt *et al.*, 2010).

Methylotrophs can also be broadly categorised into two functional groups, obligate and facultative (Dedysh and Dunfield 2011; Chistoserdova et al. 2009; Anthony 1983). Obligate methylotrophs, e.g. *Methylobacillus flagellatus* and *Methylovorus mays* (Chistoserdova *et al.*, 2007; Doronina *et al.*, 2016), can only utilise C1 compounds for carbon and energy, whereas facultative methylotrophs, e.g. *Methylobacterium extorquens* and *Hyphomicrobium facile*, are capable of utilising multicarbon compounds in addition to C1 compounds (Anthony 1986; Chistoserdova 2011a). The causes of obligate methylotrophy are not fully understood. However, enzymatic lesions in the TCA cycle (with some obligate methylotrophs lacking succinate dehydrogenase, a-ketoglutarate dehydrogenase and malate dehydrogenase) are proposed to be the reason in some phylogenetic groups such as the *Methylophilaceae* (Chistoserdova *et al.*, 2007). However, the genomes of some obligate methylotrophs possess complete TCA cycles and some species that do not possess complete TCA cycles have been shown to be capable of weak growth on multicarbon compounds such as glucose and fructose (Lapidus *et al.*, 2011b; Doronina *et al.*, 2016). Facultative methylotrophs can be further subdivided into restricted facultative, that can only grow on a limited range of multicarbon compounds e.g. *Hyphomicrobium* (Moore, 1981), and unrestricted, with the metabolic capability of utilising a broad range of multicarbon compounds e.g. *Variovorax* (Anthony 1983; Kolb 2009 and references therein). The majority of methanol utilising methylotrophs that have been isolated are facultative and genera that contain obligate methylotrophs typically also contain facultative methylotrophs (Bosch *et al.*, 2009; Doronina *et al.*, 2016).

1.1.2 Methanotrophic methylotrophs

A great deal of research has focused on methanotrophic bacteria. Methanotrophs can grow on methane as a sole carbon source, oxidising it to methanol and then formaldehyde for subsequent oxidation to CO_2 or incorporation into biomass (Figure 1.1) (Anthony 1983; Chistoserdova 2011a). Methanotrophs oxidise methane to

methanol using the enzyme methane monoxygenase (Chistoserdova 2011a; Murrell et al. 2000), either a membrane bound periplasmic methane monoxygenase (*pMMO*) or a soluble cytoplasmic enzyme with a diiron centre (*sMMO*) (McDonald *et al.*, 2005). The relative activities of these two enzymes is copper dependent, with the *pMMO* actively expressed and the *sMMO* repressed in the presence of high concentrations of copper (Farhan Ul-Haque et al. 2015; Murrell and Smith 2010). Methanotrophs are also capable of efficient growth with methanol supplied as a sole carbon source (Leak *et al.*, 1986). Methanotrophs receive high levels of attention due to the environmental importance of methane and the value of the oxidation products of methane.

1.2 Methanol utilising methylotrophs and methanol dehydrogenases

The oxidation of methanol to formaldehyde requires the enzyme methanol dehydrogenase. There are several methanol dehydrogenases that have been characterised in the different classes of methylotrophic organism. These methanol dehydrogenases vary to a great extent in their relative phylogenetic distribution, with some distributed between phyla and others restricted to a specific class of bacteria (Kolb *et al.*, 2013; Keltjens *et al.*, 2014; Taubert *et al.*, 2015). There are also additional enzymes enabling the utilisation of methanol as a carbon and/or energy source.

1.2.1 The classic methanol dehydrogenase (MxaFI)

The classic methanol dehydrogenase enzyme is heterotetrameric in structure, with *mxaF* and *mxaI* encoding the large and small subunits (Figure 1.2) (Morris *et al.*, 1994; Anthony *et al.*, 2003). The large subunit contains a PQQ cofactor and a calcium ion. The calcium ion acts as a Lewis acid and maintains the PQQ cofactor in the correct configuration (Morris *et al.*, 1994; Mcdonald *et al.*, 1997; Anthony *et al.*, 2003). The small subunit is proposed to coordinate the calcium ion in the large subunit (Keltjens *et al.*, 2014). The function and expression of this methanol dehydrogenase in *Methylobacterium extorquens* AM1 requires 25 genes in total (Keltjens et al. 2014 and references therein) (Table 1.1).

Table 1.1 Genes involved in the function and expression of the *mxaFI* encoded methanol dehydrogenase in *Methylobacterium extorquens* AM1

Gene	Proposed function	Reference
mxaACDLK	The insertion of calcium into the large subunit of MxaFI	Richardson and Anthony 1992
mxaB	Response regulator for methanol oxidation	Springer 1998
mxaW	A methanol regulated gene of unknown function	Springer 1998
mxaRSEH	Proteins involved in methanol dehydrogenase maturation	Nunn and Lidstrom 1986
mxaG	Cytochrome C_L	
mxaF	Large subunit of the methanol dehydrogenase	
mxaI	Small subunit of the methanol dehydrogenase	
mxaJ	Periplasmic solute binding protein	Kim 2012
mxbDM	Two component response regulator	Springer 1997
mxcQE	Two component response regulator	Lidstrom 1994
pqqABCDE	PQQ biosynthesis genes	Goosen 1992
pqqFG	PQQ biosynthesis genes	Gilese 2010

Methanol is oxidised by MxaFI through the following reaction: The PQQ located in the large subunit is reduced by methanol. This results in the release of formaldehyde and the transfer of two single electrons to cytochrome C_L (Anthony and Williams 2003). The electrons are transferred to an additional cytochrome (C_H) and then to a molecule of oxygen by a terminal oxidase (Anthony 1992). Protons are produced following the reaction of MxaFI with cytochrome C_L, which combine with the oxygen in the terminal oxidase reaction (Anthony 1992).

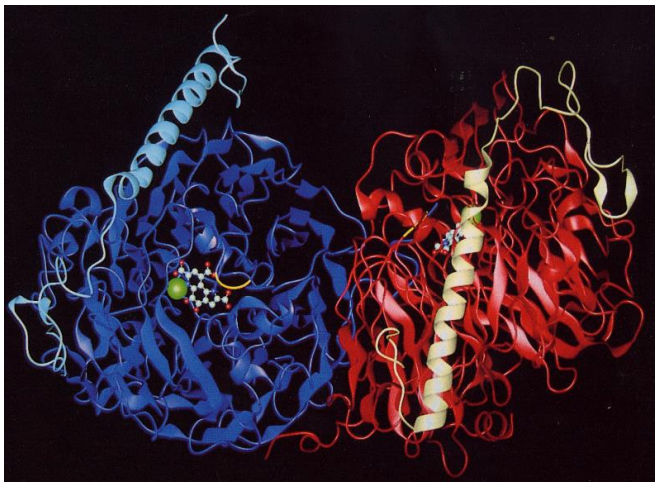


Figure 1.2 Crystal structure of methanol dehydrogenase, MxaFI, from *Methylobacterium extorquens* AM1. Figure taken from Ghosh et al. (1995). The α subunits encoded by *mxaF* are in dark blue and red. The smaller β subunits encoded by *mxaI* are in light blue and pale yellow. The calcium ion at the active site is seen in green, next to the PQQ prosthetic group.

1.2.2 The alternate methanol dehydrogenase (XoxF)

First identified as a putative methanol dehydrogenase in 1997 in *Methylobacterium extorquens* AM1 (Chistoserdova and Lidstrom 1997), it was not until much more recently that the function of the *xoxF* methanol dehydrogenase encoding gene was clarified. Uncertainties remain with regards to aspects of the functioning of the *xoxF* genes and enzymes. However, a great deal has been learnt through a range of deletion studies (Kalyuzhnaya *et al.*, 2008; Wilson *et al.*, 2008; Skovran *et al.*, 2011; Nakagawa *et al.*, 2012). Some of these deletion studies produced conflicting results, leading to several proposals as to the genuine role of *xoxF*. Following the absence of a phenotypic change in a *xoxF* deletion mutant of *Methylobacterium extorquens* AM1 it was suggested that *xoxF* did not encode a methanol dehydrogenase (Chistoserdova and Lidstrom 1997). A later study characterising the methanol dehydrogenases in species of *Methyloversatilis* also showed no change in phenotype in a *xoxF* deletion mutant relative to the wild type (Kalyuzhnaya *et al.*, 2008). The first proof of the role of *xoxF* as a methanol dehydrogenase was shown in a study where a *xoxF* deletion mutant of *Rhodobacter sphaeroides*, which only possesses one copy of *xoxF*, lost the ability to grow on methanol and showed no methanol dependent oxygen uptake (Wilson et al. 2008). The function

of *xoxF* as a methanol dehydrogenase encoding gene was further supported following the reassessment of the phenotype of a *xoxF* deletion mutant of *Methylobacterium extorquens* AM1 (Chistoserdova *et al.*, 1997), which showed a 30 % decrease in growth rate on methanol and a reduction in the ability to colonise and survive on inoculated *Arabidopsis* seedlings relative to the wild type (Schmidt *et al.*, 2010). A mutant strain of *Methylobacterium extorquens* AM1 with null mutations in both *xoxF* genes was unable to grow on methanol and showed a nearly complete loss of methanol dehydrogenase activity (Skovran et al. 2011). It was subsequently proposed that *xoxF* functions as an environmental signal for regulating genes involved in methanol oxidation, as the loss of both *xoxF* genes reduced the expression of the two component response systems *mxbDM* and *mxcQE* involved in the expression of *mxaFI* (Skovran *et al.*, 2011). Further support for the function of *xoxF* as a methanol dehydrogenase came from *Methylotenera mobilis,* a species that lacks the classic methanol dehydrogenase, being capable of growth on methanol. Although growth on methanol by this species was reported as variable or weak (Kalyuzhnaya *et al.*, 2006; Mustakhimov *et al.*, 2013), this growth was entirely abolished following the deletion of both *xoxF* genes (Mustakhimov *et al.*, 2013).

A series of studies showed that the methanol oxidising activity of XoxF was induced through the supplementation of lanthanides in *Methylobacterium radiodurans*, *Methylobacterium extorquens* and *Bradyrhizobium japonicum* (Fitriyanto *et al.*, 2011; Hibi *et al.*, 2011; Nakagawa *et al.*, 2012). Furthermore, it was shown that *xoxF* contained lanthanum at the active site (Nakagawa *et al.*, 2012). Long presumed to be irrelevant to molecular biology due to their low solubility, the first elements within the lanthanide series (lanthanum, cerium, praseodymium and neodymium) were shown to convey methanol oxidation activity in the XoxF enzymes (Fitriyanto *et al.*, 2011; Hibi *et al.*, 2011; Nakagawa *et al.*, 2012). The impact of the members of the lanthanide series varies, with heavier lanthanides producing less of an impact on methanol oxidation (Fitriyanto *et al.*, 2011; Vu *et al.*, 2016). A greater understanding of the impact of lanthanides on the functioning of *xoxF* was gained following the isolation of *Methylacidiphilum fumarilocum* SolIV from an acidic volcanic mudpot (Pol *et al.*, 2013). The ability of this strain to grow on methane was dependent on water from the same mudpot from which

it was isolated. The key growth-enabling component of the water was identified as the lanthanides present. The crystal structure of the XoxF of this organism was obtained, identifying a cerium ion at the active site where the calcium ion is located in MxaFI (Pol *et al.*, 2013). XoxF is proposed to be homodimeric in structure (Schmidt *et al.*, 2010; Nakagawa *et al.*, 2012; Pol *et al.*, 2013), lacking the small subunit present in MxaFI.

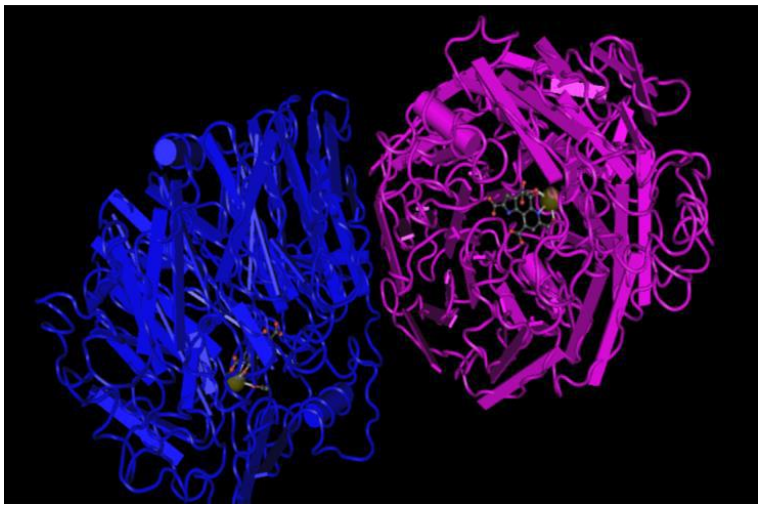


Figure 1.3 Crystal structure of XoxF from *Methylacidiphilum oxyfera* SolV (taken from Pol et al. 2014). The two subunits are seen in purple and blue. The cerium ion is seen in green, next to the PQQ prosthetic group.

The XoxF methanol dehydrogenase has also fewer associated genes than the MxaFI methanol dehydrogenase, with *xoxG* and *xoxJ*, encoding a cytochrome C_L and a periplasmic solute binding protein respectively (Keltjens *et al.*, 2014) (Figure 1.4).

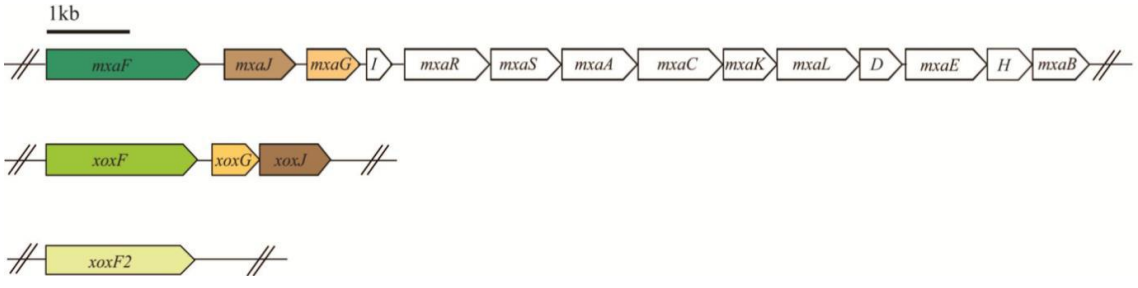


Figure 1.4 Gene order of the *mxa* and *xox* operon in the genome of *Methylobacterium extorquens* AM1 (Figure taken from Schmidt et al 2010). *mxa* genes are detailed in table 1.1.

Research using *Methylobacterium extorquens* AM1, *Methylomicrobium buryatense* and *Methylosinus trichosporium* OB3b has shown that the transcription of *mxaF* is downregulated and *xoxF* upregulated with an increasing supply of lanthanides (Farhan Ul-Haque *et al.*, 2015; Chu and Lidstrom, 2016; Vu *et al.*, 2016). Copper has been shown to ameliorate the suppression of *mxaF*, but this effect has only been shown in *Methylosinus trichosporium* OB3b (Farhan Ul-Haque *et al.*, 2015; Chu and Lidstrom, 2016). It was also shown in *Methylomicrobium buryatense* that the lanthanide controlled regulation of the *mxaF* and *xoxF* genes was partially controlled by the response regulator *mxaB*, but further testing identified that the lanthanide mediated control of gene expression was performed by the histidine kinase *mxaY* (Chu and Lidstrom, 2016; Chu, Beck, *et al.*, 2016). Other studies have indicated that *mxaY* affects *mxaF* expression in *Paracoccus denitrificans* but it is not indispensable for the expression of *mxaF* (Yang 1995). Therefore, the role of *mxaY* and regulation of the expression of the *mxa* and *xox* genes appears to vary between phylogenetic groups.

There are other proposed roles for XoxF in addition to methanol oxidation. These include a role in stress response, with a *xoxF* deletion mutant of *Methylobacterium dichloromethanicum* DM4 having a reduced ability to grow on dichloromethane and a reduced capacity to tolerate oxidative, osmotic and heat related stresses (Firsova *et al.*, 2015). Furthermore, a *xoxF* deletion mutant of *Methylotenera mobilis* has been shown to produce a reduced amount of nitrous oxide when grown on methylamine and methanol, indicating the XoxF enzymes may function as electron donors to the denitrification pathway (Mustakhimov *et al.*, 2013).

Our ability to detect methylotrophic bacteria in the environment is enhanced through our improved understanding of the role of *xoxF.* A greater understanding of *xoxF* has resulted in genera being confirmed to be capable of methanol oxidation and several additional genera being identified as potentially methylotrophic (Fitriyanto *et al.*, 2011; Hibi *et al.*, 2011; Keltjens *et al.*, 2014). The *Comamonadaceae* is an example of a family that contains several species that possess *xoxF* genes (*Ramlibacter, Acidovorax, Leptothrix, Comamonas, Pelomonas, Serpentimonas*) but contains a low number of species confirmed to be capable of methylotrophy (*Variovorax paradoxus* and *Hydrogenophaga* sp.) (Anesti *et al.*, 2004; Eyice *et al*., 2015b), which is potentially the

result of these species originally being tested for methanol oxidation in the absence of lanthanides.

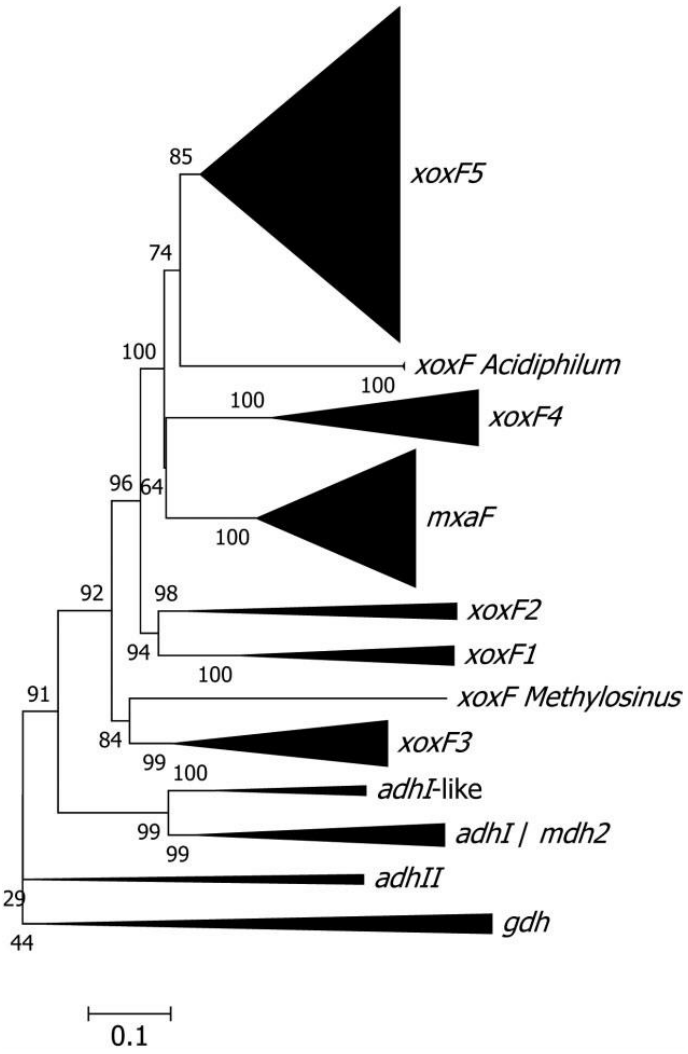


Figure 1.5 Phylogenetic relationship between the different clades of *xoxF* genes, *mxaF* genes and genes encoding other PQQ-dependent dehydrogenases (Taken from Taubert et al. 2015). The tree was constructed using the neighbour-joining method for clustering and the maximum composite likelihood method for computing evolutionary distances. Numbers at branches are bootstrap values of 500 replicates. Scale bar: 1 nucleotide substitution per 10 nucleotides.

xoxF is more genetically diverse than *mxaF*, forming five distinct clades (Chistoserdova, 2011a; Keltjens *et al.*, 2014) (Figure 1.5). These *xoxF* clades vary in their relative genetic diversity and phylogenetic distribution, with some clades being much more broadly distributed than others.

1.2.2.1 xoxF1

xoxF1 is found within species of *Xanthomonas*, genera within the *Beijerinkaceae* and within the genome of the sole representative of the NC10 phylum, *Methylomirabilis oxyfera*.

1.2.2.2 xoxF2

xoxF2 is also limited in its phylogenetic distribution, being found in members of the NC10 phylum and Verrucomicrobia. There is also a *xoxF2* sequence located on a fosmid produced from DNA extracted from the sediment of Lake Washington.

1.2.2.3 xoxF3

xoxF3 is broadly distributed across several phylogenetic group. It is found within members of the *Rhizobiales* in the Alphaproteobacteria, *Methylococcales* in the Gammaproteobacteria, *Methylophilaceae* and *Comamonadaceae* in the Betaproteobacteria and *Solibacter* in the Acidobacterium.

1.2.2.4 xoxF4

xoxF4 is the most restricted with regards to phylogenetic distribution, as it is only located within members of the bacterial family *Methylophilaceae* within the Betaproteobacteria.

1.2.2.5 xoxF5

xoxF5 is the most broadly distributed of the *xoxF* clades, within representatives of the Alpha, Beta and Gamma-Proteobacteria in addition to a member of the Firmicutes (Watanabe *et al.*, 2015).

1.2.2.6 xoxF outgroups

There are two outgroups to the *xoxF* clades. One of these outgroups clusters outside of the *xoxF3* clade and contains the *xoxF* from *Methylosinus trichorporium* OB3b. The second outgroup clusters outside of the *xoxF5* clade and is represented by a *xoxF* from the *Acidiphilum* within the Alphaproteobacteria.

1.2.3 The other alternate methanol dehydrogenase (Mdh2)

An additional PQQ methanol dehydrogenase was characterised in 2008 (Kalyuzhnaya). The *mdh2* encoded methanol dehydrogenase was shown to have low identity to the classic methanol dehydrogenase (~35 %). Based on the sequence identity of the enzyme it was proposed to be an alcohol dehydrogenase II as opposed to a highly divergent *mxaF*

or *xoxF* (Kalyuzhnaya *et al.*, 2008). The *mdh2* methanol dehydrogenase was confirmed to encode a functional methanol dehydrogenase through the creation of a series of *mdh2* deletion mutants of species of *Methyloversatilis* and *Methylibium* (Kalyuzhnaya *et al.*, 2008). The transcription of this gene was shown to be upregulated in the presence of methanol (Lu *et al.*, 2012). The *mdh2* gene is only found within these two genera, indicating that the phylogenetic distribution of this methanol dehydrogenase is more restricted than that of *mxaF* and *xoxF* (Keltjens *et al.*, 2014). This could potentially explain the low number of publications describing attempts to further characterise this methanol dehydrogenase and the lack of primers for the amplification of this gene from an environmental sample for the assessment of its diversity. It is also interesting to note that all currently described *mdh2* containing organisms also possess *xoxF* methanol dehydrogenases.

1.2.4 NAD(P)+ methanol dehydrogenase

NAD(P)+ dependent methanol dehydrogenases have previously been detected in species of *Bacillus* (Arfman et al. 1992; Arfman et al. 1997) and have more recently been detected and shown to be functional in a species *Cupriavidus*, the first report of a methanol dehydrogenase found in both Gram positive and Gram negative bacteria (Wu *et al.*, 2016). As opposed to the periplasm, where the PQQ methanol dehydrogenases are located, these enzymes are located in the cytoplasm. These methanol dehydrogenases have been classified as metal containing group III alcohol dehydrogenases (Müller *et al.*, 2014; Wu *et al.*, 2016). In *Bacillus* this type of methanol dehydrogenase has been shown to require an endogenous activator protein, but no such requirement was detected in the *Cupriavidus* (Arfman *et al.*, 1989; Wu *et al.*, 2016). It is interesting to note that this gene is not the sole methanol dehydrogenase gene in *Curpiavidus*.

1.2.5 N,N9-dimethyl-4-nitrosoaniline (DMNA)-dependent nicotinoprotein methanol:DMNA oxidoreductase

Actinobacteria have been shown to possess a distinct methanol dehydrogenase gene from that found in Gram negative bacteria and the firmicutes. This type of methanol dehydrogenase encoding gene has been shown to occur in species of *Amycolatopsis*, *Rhodococcus* and *Mycobacterium* (Dijkhuizen et al. 1989; Park et al. 2010). This methanol dehydrogenase is an N,N9-dimethyl-4-nitrosoaniline (DMNA)-dependent

nicotinoprotein methanol:DMNA oxidoreductase. This enzyme is of a similar structure to that of the NAD(P)+ methanol dehydrogenase gene described above, but this enzyme is DMNA-dependent as opposed to NAD(P) dependent (Park *et al.*, 2010). This enzyme has been confirmed to be functional in representatives of the three mentioned genera (Ekimova et al. 2015 and references therein).

1.2.6 Eukaryotic methanol dehydrogenase

Eukaryotic organisms, including some members of the Ascomycota, moulds and some yeasts possess alcohol dehydrogenases enabling growth on methanol (Kolb 2013 and references therein). This enzyme is a flavin adenine nucleotide-dependent alcohol oxidase (Nakagawa 2006). This enzyme is used in an assay for measuring the concentration of methanol, with the enzyme converting all methanol to formaldehyde and a subsequent reaction with acetyl acetone providing a colorimetric assay (Klavons *et al.*, 1988). This enzyme has been shown to be functional, however these are homologues of this gene that have not been confirmed to encode functional methanol dehydrogenases (Nakagawa 2006).

1.2.7 Methylotrophic and methyl reducing Archaea

In addition to the highly characterised acetoclastic and hydrogenotrophic methanogenic archaea, there are also methylotrophic and methyl reducing methanogens (Garcia *et al.*, 2000; Sorokin *et al.*, 2017). The methylotrophic methanogens are capable of the dismutation of methanol and methylamine to CO_2 in order to provide the reducing compounds for methanogenesis (Vanwonterghem *et al.*, 2016; Sorokin *et al.*, 2017). The methyl reducing methanogens instead use C1 compounds as electron acceptors and hydrogen as the electron donor for the process of methanogenesis (Sorokin *et al.*, 2015, 2017). Key genes involved in the methyl reducing methanogens are methanol transferase (*mtaA*) and methyl-CoM reductase, with *mtaA* also proposed to be enable reduction of methylamine (Lang *et al.*, 2015). The pathway enabling oxidation of methanol to CO_2 is either non-functional (Fricke *et al.*, 2006) or absent (Borrel *et al.*, 2014) in the methyl reducing archaea. The pathway for the complete oxidation of methanol involves the transfer of the methyl group from coenzyme M to tetrahydromethanopterin (H4MPT), with the subsequent oxidation of the methyl group

to CO2 and the restoration of H4MPT (Blaut, 1994), as occurs in methylotrophic bacteria (1.1.1).

1.3 The Global Methanol Budget

Methanol is the second most abundant organic gas (0.1-10ppb) after methane (1800 ppb) (Oikawa 2011). Methanol in the troposphere has a lifespan of approximately ten days (Sargeant, 2013). During this period, depending on the prevailing concentration of NO_x, methanol and its products through atmospheric interaction will act as either a net source or a net sink for radicals (Galbally 2002). Methanol can be converted to formic acid by photochemical reactions that can enhance the formation of acid rain (Jacob, 1986).There are multiple sources and sinks of methanol in both the terrestrial and marine environments (Figure 1.6 and Table 1.2). There are also uncertainties in the amounts of methanol produced from the different sources due to the difficulties of accurately measuring the concentration of methanol over time and variation in the models used to produce the estimates (Dixon et al. 2013; Galbally 2002).

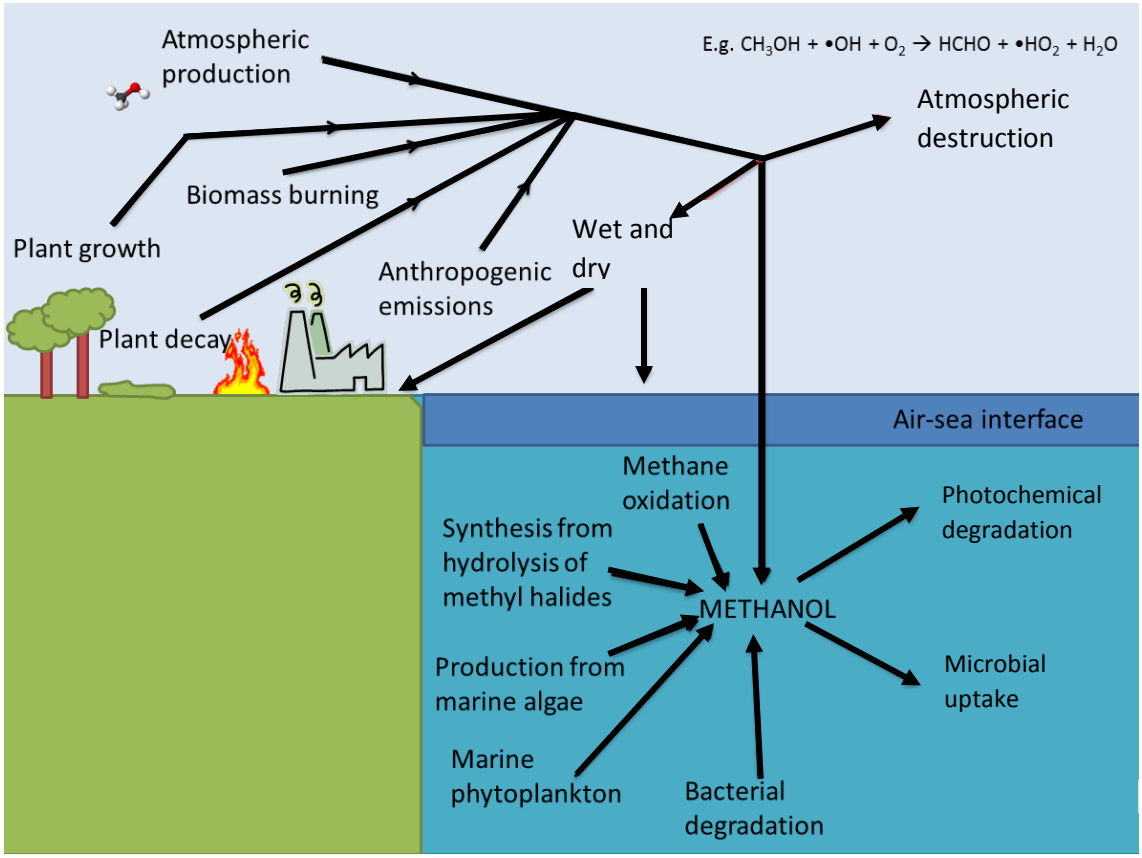


Figure 1.6 Simplified overview of methanol production and degradation in the atmosphere and in the terrestrial and marine environments. Modified from Sargeant 2013.

Table 1.2 Global atmospheric methanol budget (Taken from Sargeant 2013)

Reference	Singh et al (2000)	Heikes et al (2002)	Galbally and Kirstine (2002)	Tie et al (2003)	von Kuhlmann et al (2003)	Jacob et al (2005)	Millet at al (2008)
			Sources (Tg yr^{-1})				
Plant growth	75 (50 - 125)	280 (50 - >280)	100 (37 - 212)	104 - 312	77	128 (100 - 160)	80
Plant decay	20 (10 - 40)	20 (10 - 40)	13 (5 - 31)			23 (5 - 40)	23
Biomass burning	6 (3 - 17)	12 (2 - 32)	13 (6 - 19)		15	13 (10 - 20)	12
Urban	3 (2 - 4)	8 (5 - 11)	4 (3 - 5)		2	4 (1 - 10)	5
Atmospheric production	18 (12 - 24)	30 (18 - 30)	19 (14 - 24)	31	28	38 (50 - 100)	37
Ocean		0 - 80	<0.1				85
Total source	***122 (75 - 210)***	***350 (90 - 490)***	***149 (83 - 260)***	***135 - 343***	***123***	***206 (170 - 330)***	***242***
			Sinks (Tg yr^{-1})				
Gas-phase oxidation by OH		100 (25 - 150)	109 (60 - 203)	59 - 149	77	129	88
In-cloud oxidation by OH (aq)		10 (5 - 20)	5 (2 - 15)			<1	<1
Dry deposition (land)		70 (35 - 210)	24 (11 - 43)	32 - 85	37	55	40
Ocean uptake		50 (-20 - 150)	0.3 (0.2 - 0.6)			10	101
Wet deposition		10 (4 - 36)	11 (5 - 20)	16 - 50	9	12	13
Total sinks	***40 – 50***	***270 (160 - 570)***	***149 (82 - 273)***	***107 - 284***	***123***	***206***	***242***

1.3.1 Production of methanol in the marine environment

There is no consensus on whether the marine environment represents a net source or sink of methanol, with studies conflicting on the concentration of methanol produced and utilised (Sargeant, 2013). This is partially the result of the concentration of methanol in seawater being hard to quantify, which then impacts on the ability to estimate the relative exchange between seawater and the air (Sargeant 2013; Dixon et al. 2013; Beale et al. 2013). Further to this, the long hypothesised role of phytoplankton as a source of methanol has been confirmed, with species of phytoplankton confirmed to produce methanol, using a significant portion of their total carbon pool (Mincer *et al.*, 2016). This is in addition to synthesis of methanol from methyl halides (Rowland, 1995) and the release of methanol by methanotrophic bacteria (Krause *et al.*, 2017), which are both proposed to represent comparatively small sources of methanol in the marine environment (Sargeant, 2013).

1.3.2 Production of methanol in the terrestrial environment

The terrestrial environment represents a net source of methanol, with a great deal of variation in the total amounts of methanol contributed by the different sources (Figure 1.6, Table 1.2). There are also inputs of methanol into the terrestrial environment, with the wet and dry deposition of methanol resulting in nanomolar concentrations of methanol being contributed to the soil (Jacob *et al.*, 2005; Stacheter *et al.*, 2013).

1.3.2.1 Plants growing and decaying

Growing plants represent the greatest terrestrial and global source of methanol production (Galbally *et al.*, 2002; Oikawa *et al.*, 2011, 2013). The majority of the methanol produced by growing plants arises through the action of pectin methyl esterase enzymes restructuring pectin in the cell walls (Kutschera, 2007). As the plant grows, the pectin in the cell walls is restructured to increase the stability of the cells (Oikawa *et al.*, 2011). This liberates methoxy groups that are then converted to methanol. A small portion of this methanol is oxidised to formaldehyde by alcohol oxidase enzymes possessed by the plant, but the majority is released from the plant as methanol (Oikawa *et al.*, 2013). Methanol has been shown to be released through the stomata of the leaves, however there are also studies using PTR-MS that confirm that

methanol is also released from the roots of *Arabidopsis* and sugar beet plants (Steeghs *et al.*, 2004; Tsurumaru, 2015). In fact, 0.1 % of the total photosynthate is estimated as being converted to methanol (Kolb 2009 and references therein). The flux of methanol released from the plant has also been shown to vary with herbivory, temperature and the growth stage of the plant (Fall and Benson 1996; Oikawa and Lerdau 2013 and references therein). There has also been day-night variation proposed in the release of methanol by plants due to the opening and closing of stomata (Harley *et al.*, 2007; Oikawa *et al.*, 2011).

Decaying plant material represents another significant source of methanol. Some of this methanol is also produced through pectin methyl esterase enzymes, that are capable of demethylating pectin in the tissue of dead plants (Galbally *et al.*, 2002; Heikes, 2002; Oikawa *et al.*, 2011). Additional methanol is produced from lignin. Lignin represents a major percentage of woody tissues, but its conversion to methanol is inhibited by the presence of oxygen and therefore most of the lignin within plant tissues is instead released as carbon dioxide (Galbally *et al.*, 2002).

1.3.2.2 Anthropogenic activity

Methanol is also produced through a range of anthropogenic activities (Figure 1.6). It is produced as a result of biomass burning, with the smouldering stage of burning resulting in the pyrolysis of methoxy groups. Methanol is also produced intentionally through industry for a range of industrial purposes, including as a fuel, solvents or the chemical production of organic compounds (Galbally *et al.*, 2002). 4 – 8 Tg yr^{-1} of methanol is produced through anthropogenic activities, which is dwarfed by the collective contributions of the biological sources in the terrestrial environment (Galbally *et al.*, 2002; Heikes, 2002; Sargeant, 2013).

1.3.3 Disparity in the methanol budget

The production of methanol by plants has led to the suggestion that the rhizosphere soil contains a higher methanol concentration than bulk soils (Kolb, 2009). Although the role of the terrestrial environment as a source of methanol is clearly defined, the disparity between the estimated plant produced methanol and the methanol entering the atmosphere (Galbally and Kirstine 2002; Dixon et al. 2013) and the extent to which this

is a result of the methanol oxidation by plant associated microbes relative to other processes requires further study.

1.4 Methylotrophs in the soil environment

Methylotrophs are key organisms in the turnover of methanol in the terrestrial environment (Kolb 2009), with the equally ubiquitous presence of methanol in this environment due to input from multiple sources (Table 1.2).

1.4.1 *Hyphomicrobium*

Hyphomicrobium is a diverse genus, comprised of appendaged bacteria that divide through budding (Moore, 1981 and references therein). The genus has been shown to possess facultative and obligate methylotrophs, capable of utilising methanol, methylamine and dimethyl sulfide (DMS) in addition to a range of halogenated compounds such as chloromethane and dichloromethane (Urakami *et al.*, 1995; Yang *et al.*, 1995; Anesti *et al.*, 2004; Vuilleumier *et al.*, 2011, Bringel *et al.,* 2017). Some strains of *Hyphomicrobium* have been shown to possess methanol dehydrogenases with very low *Km* values, indicating that they are able to exploit methanol as a resource in the soil environment even at very low concentrations (Nojiri *et al.,* 2006). Incorporation of C1 compounds into biomass is achieved through the serine cycle (Anthony 1983). Strains of *Hyphomicrobium* are frequently isolated due to the widespread distribution of the genus and their rapid growth on a range of compounds (Hayes *et al.*, 2010). Species of *Hyphomicrobium* have been linked to the deposition of iron and manganese oxides, impacting on nutrient availability in the soil (Moore, 1981), in addition to some species being confirmed to be capable of complete denitrification (Urakami *et al.*, 1995).

1.4.2 *Methylophilaceae*

The family *Methylophilaceae* is comprised of four genera, *Methylovorus, Methylophilus, Methylobacillus* and *Methylotenera*. Species within these genera have been isolated from a range of environments, including both natural and artificial, and terrestrial and marine environments (Doronina et al. 2015; Gogleva et al. 2011; Kaparullina et al. 2017). Several species have been shown to be plant associated, having been isolated from meadow grass (Doronina *et al.*, 2004), willow buds, silverweed (Gogleva *et al.*, 2011) and red peppers (Madhaiyan *et al.*, 2013). All genera comprising this family include species of obligate and facultative methylotrophs, except *Methylobacillus* which only contains

obligate methylotrophic bacteria (Doronina *et al.*, 2016). C1 compounds used by these species include methanol and methylamine, with several species showing high growth rates on these compounds (Beck et al. 2014; Chistoserdova 2011b). There are also species of *Methylotenera* that are capable of truncated denitrification, producing nitrous oxide as the final compound (Mustakhimov *et al.*, 2013), and it is proposed that the *Methylophilaceae* may be involved in the utilisation of DMS (Eyice *et al.*, 2015). Cultivation independent studies indicate that there is a large range of uncultivated diversity that remains within this family (Kalyuhznaya *et al.*, 2009; Lapidus *et al.*, 2011a; Beck *et al.*, 2014; Chistoserdova, 2015).

1.4.3 *Methylobacterium*

Methylobacterium is a widespread genus with great relevance to the soil environment that has been shown to dominate the *mxaF* profile of certain soils (Kolb, 2009; Kolb *et al.*, 2013). Consistently found in association with plants (Iguchi et al. 2015 and references therein), *Methylobacterium* is abundant in the phyllosphere and present in the stems, rhizospheres and roots of several plant species (Chistoserdova *et al.*, 2003; Delmotte *et al.*, 2009; Vuilleumier *et al.*, 2009; Knief *et al.*, 2012; Minami *et al.*, 2016). This association has been shown to occur in the field, in growth rooms and even after the harvesting and distribution of plants for retail (Iguchi *et al.*, 2015). Some species are endophytic, forming root nodules in symbiosis with *Crotalaria podocarpa* (Sy *et al.*, 2001). Further to this, *Methylobacterium* are incredibly widespread, with strains detected from freshwater and sea waters in addition to being associated with humans and even in the dust surrounding the international space station (Anesti *et al.*, 2004; Egamberdieva *et al.*, 2015; Mora *et al.*, 2016). *Methylobacterium extorquens* AM1 has become the workhorse for the study of methanol oxidation and methylotrophy, with several studies using this strain as the model strain for molecular genetics and the study of metabolism (Anthony 1983; Vuilleumier et al. 2009; Chistoserdova et al. 2003).

1.4.3.1 Plant growth promoting traits confirmed in species of Methylobacterium

Species within the genus have been shown to possess a broad array of plant growth promoting traits including the production of indole acetic acid and cytokinins, reducing the concentration of ethylene, the immobilisation of heavy metals and the solubilisation of phosphate (Iguchi et al. 2015; Madhaiyan et al. 2005; Madhaiyan et al. 2010; Abanda-

Nkpwatt et al. 2006). Species of *Methylobacterium* have also been shown to be capable of nitrogen fixation, both in nodules and free living in the soil (Jourand *et al.*, 2005; Ardley *et al.*, 2009; Madhaiyan *et al.*, 2009).

1.5 Methods to study the microbial communities

1.5.1 Cultivation dependent approaches

Before the advent of PCR and the decrease in the costs of sequencing, classical microbiology depended on the isolation and characterisation of bacteria from environmental samples. The cultivation-dependent approach has many known disadvantages, including the large disparity between the number of microbial species visible through a microscope when analysing an environmental samples relative to the number of microbes that can be successfully isolated (Dini-Andreote 2012 and references therein). Enrichment regimes for the isolation of methanol utilising bacteria tend to favour the enrichment of specific methylotrophs (Lu *et al.*, 2005; Hayes *et al.*, 2010). Furthermore, there are often physiological reasons for the inability of organisms to be enriched under certain strategies, such as a nutrient requirement that is unknown (Pol *et al.*, 2013). This issue is also further complicated by syntrophy, with some organisms depending on the presence and metabolism of other organisms to enable growth e.g. *Syntrophobacter* and methanogenic archaea (Harmsen *et al.*, 1998). However, there is still a clear need for cultivation dependent work as it is through isolations and work with pure cultures that the physiology of organisms and evolutionary processes can be assessed (Prosser 2012; Dini-Andreote 2012). It is also interesting to observe the isolation of organisms in pure culture that were previously considered uncultivable or syntrophic (Wallrabenstein and Hauschild 1995; Stewart 2012 and references therein). Single cell genomics (Hutchinson *et al.,* 2006) and the production of metagenomes from DNA extracted from environmental samples enables the inference of function in the absence of a pure culture, but it is hard to draw direct conclusions from the presence of a gene within a genome.

There is a clear need to use varied approaches when attempting to isolate bacteria to maximise the diversity of organisms that are cultivated. Multiple variables have been shown to impact on the growth and relative competitive ability of methylotrophs, including oxygen concentration (Hernandez *et al.*, 2015), the use of solid or liquid media

(Mustakhimov *et al.*, 2013; Vorobev *et al.*, 2013), the presence of other organisms (Ho *et al.*, 2014) and the supply of lanthanides (Pol *et al.*, 2013). These variables also need to be considered for the characterisation of methylotrophic bacteria, as certain traits will not be expressed under certain conditions.

1.5.2 Low resolution approaches

Prior to the development of high throughput sequencing technologies, one way of analysing diversity was through the creation and Sanger sequencing of clone libraries of 16S rRNA genes and genes that encode for key enzymes (functional genes). Although low throughput, cloning still plays an important role in molecular biology e.g. testing novel primer sets. There are additional low resolution profiling techniques that are utilised for characterising microbial communities, namely denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) (Muyzer *et al.*, 1993; Liu *et al.*, 1997). There are instances where the resolution of community profiles analysed using these techniques may not be sufficient. However, depending on the research question being asked, both of these techniques still have applicability in modern molecular biology e.g. screening of ^{13}C and ^{12}C labelled fractions from a stable isotope probing experiment (Neufeld *et al.,* 2007) or assessing differences between soil treatments (Marileo *et al.*, 2016).

1.5.3 Next generation sequencing

The advent of high throughput sequencing technologies, such as 454 and Illumina, has led to a rapid expansion in the volume of sequences produced when attempting to characterise the diversity of a community. The ability to amplify the 16S rRNA gene and functional genes from environmental samples and subsequently sequence these genes enhances the characterisation of microbial diversity. However, biases inflicted by the PCR amplification and the region of the 16S rRNA gene or functional gene that the selected primers amplify has been shown to have a strong impact on the diversity captured (Acinas *et al.*, 2005). The amplification of genes is also typically Kingdom specific, with the exception of some 16S rRNA gene primers that can amplify variable regions within both bacteria and archaea. There are also major issues with the use of the 16S rRNA gene to infer function within an environment, with this shown clearly with regards to methylotrophy. Firstly, there are some bacteria where methylotrophy is

present within members of that phylogenetic group but it is not present within all members, such as the *Bacillus* (Arfman *et al.*, 1992; Müller *et al.*, 2014). In particular instances, the methylotrophic members of a specific phylogenetic group are atypical of the commonly characterised metabolic capabilities, such as the methanol oxidising species of the genus *Flavobacterium* (Eyice and Schäfer 2015; Madhaiyan et al. 2010). Therefore the detection of a genus as present through sequencing of the 16S rRNA gene cannot be used as the sole means through which the diversity of methylotrophs is characterised, necessitating the sequencing of functional genes relating to methanol oxidation and additional techniques.

1.5.4 Omics-based approaches

The increased performance in sequencing technologies has also seen an expansion in the sequencing of metagenomes from environmental samples. This approach overcomes several of the limitations involved with the use of PCR and is capable of capturing sequences from representatives of all Kingdoms. However, the ability of a metagenome to characterise the diversity within an environment is reduced as the complexity of the environment increases. Metagenome construction from complex environments will produce an abundance of data but can prevent the assignment of phylogeny to sequences and the binning of sequences into genomes (Chen *et al.*, 2008a).

1.5.5 Stable Isotope Probing

The technique of stable isotope probing is based on the supply of a substrate that is enriched with a stable isotope to an environmental sample. The organisms within this environmental sample that are capable of utilising the substrate will incorporate the stable isotope into their biomass, including their PLFAs, DNA, RNA and protein (Dumont and Murrell 2005; Neufeld et al. 2007c). SIP experiments have tended to favour the use of ^{13}C, but additional stable isotopes have also been utilised e.g. ^{15}N and ^{18}O (Buckley *et al.*, 2007; Schwartz, 2007; Taubert *et al.*, 2017). Stable isotope labelled DNA or RNA can be separated from the un-labelled DNA or RNA through ultracentrifugation. The labelled DNA or RNA can be subsequently assessed through a range of molecular techniques in order to identify the organisms and processes involved in the utilisation of the specific substrate.

Since its inception, SIP has been used to characterise a range of metabolic processes. This technique was applied for the first time to identify active methylotrophs in acidic forest soil (Radajewski *et al.*, 2000). This experiment revealed a dominance of methylotrophs following enrichment and provided direct evidence of methylotrophy being present within the *Acidobacterium*. This experiment showed the potential for SIP to identify novel groups involved in functional processes. Previous SIP experiments have typically used low resolution profiling techniques or the low throughput sequencing technique of cloning in order to characterise the labelled community. However, for the substrate based SIP experiments this has consistently resulted in the ability to detect the ^{13}C labelled organisms. This has been shown in incubations using environmental material from both marine and terrestrial environments. Examples of these experiments include ^{13}C labelled methanol incubations with seawater from the L4 sampling station at Plymouth, which consistently identified *Methylophaga* as the key methylotroph in this environment (Neufeld et al. 2007b; Neufeld et al. 2008; Grob et al. 2015). Incubations performed using soil indicated the activity of *Methylobacterium* and *Methylophilaceae* in the utilisation of methanol in this environment, albeit at a concentration of 22 mM (Lueders *et al.*, 2003). Further SIP experiments have characterised the key organisms involved in the utilisation of more recalcitrant substrates. Sediment from tidal flats incubated with ^{13}C labelled toluene identified *Desulfuromonas* as the dominant organism involved in the biodegradation of this compound (Kim *et al.*, 2014). SIP incubations performed with ^{13}C labelled benzene also identified the dominant organisms involved in the utilisation of this substrate in both groundwater (Kasai *et al.*, 2006) and oil tar contaminated sediments (Liou *et al.*, 2008).

Increasingly, SIP experiments are utilising high throughput sequencing and omics based approaches to characterise the ^{13}C labelled community, which provides greater characterisation of the active organisms. The labelling of DNA of the organisms involved in a specific metabolic process also enables the construction of focused metagenomes, which is useful when attempting to characterise communities in complex environments (Chen and Murrell, 2010). Focused metagenomes can be produced through the acquisition of sufficient labelled DNA or by multiple displacement amplification (Binga et al. 2008; Neufeld et al. 2007a; Chen et al. 2008). Both approaches have disadvantages,

with MDA having been shown to inflict an amplification bias (Binga *et al.*, 2008) and long term SIP incubations biasing the enriched community (Chen *et al.*, 2008a). Examples of the application of omics in combination with SIP are provided in two studies which incubated marine samples with ^{13}C labelled methanol and ^{15}N labelled methylamine (Grob *et al.*, 2015; Taubert *et al.*, 2017). The labelled samples of DNA were used to create metagenomes alongside additional proteomic analysis of labelled peptides. This approach enabled the creation of genomes of the dominant methylotrophs through the binning of the metagenomic data and the subsequent confirmation of specific pathways detected in the genomes as active.

1.5.5.1 Identification of exudate utilising bacteria in the rhizosphere through the supply of $^{13}CO_2$

Changes in the bacterial community in the soil due to the presence of a plant are hard to delineate, with some groups being enriched through direct utilisation of exudates released from the plant whereas others are enriched due to the priming effect, the enhanced rate of breakdown of soil organic matter, or the subsequent acquisition of carbon from the primary utilisers (Bernard *et al.*, 2007; Ai *et al.*, 2015).

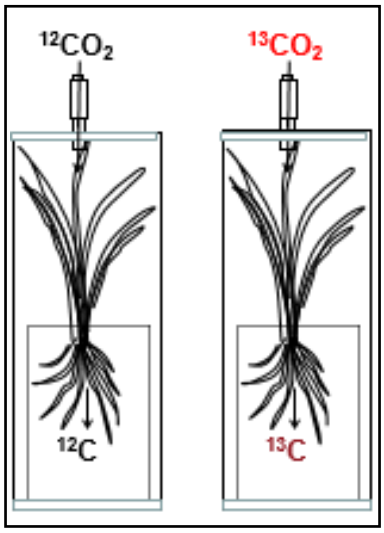


Figure 1.7 Schematic figure of the containers used in this work and additional rhizosphere SIP experiments. Figure is adapted from (Lu and Conrad, 2005)

There have been a range of SIP studies seeking to identify the active exudate utilising bacteria within the rhizosphere, defined as the region directly affected by the roots of a plant (Hiltner 1904), of different plant species (Table 1.3). Rhizosphere SIP experiments

typically utilise a similar design of container in which the plants are supplied with ^{13}C labelled CO_2 (Figure 1.7).

Table 1.3 Rhizosphere SIP studies identifying exudate utilising bacteria in the rhizosphere through the supply of $^{13}CO_2$

Plant	SIP technique	Analysis	Reference	Key finding
Grassland	PLFA	PLFA analysis	Treonis 2004	Bacterial and fungal members of the soil community were utilising carbon from the grass. Liming had no effect on the rate of uptake
Grassland	RNA	16S rRNA DGGE profiling	Griffiths 2004	The microbial uptake of labelled carbon in a natural grassland system is low and requires optimisation
Limed and unlimed grassland	RNA	16S rRNA DGGE profiling 18S rRNA DGGE profiling	Rangel-Castro 2005	Exudate utilising communities of the limed soil were more complex and active
Rice	DNA	T-RFLP Cloning	Lu 2005	RICE cluster-1 archaea dominated under N_2/CO_2 incubations whilst *Methanosarcina* dominated under H_2/CO_2 incubations
Rice	RNA	T-RFLP Cloning	Lu 2006	*Azospirillium* and *Burkholderiaceae* were the most active exudate utilisers
Rice	PLFA	PLFA analysis	Lu 2007	Gram negative bacteria and eukaryotes dominated the exudate utilising community

Wheat Maize Rape Barrel clover	DNA	16S rRNA DGGE profiling Cloning Sequencing	Haichar 2008	The exudate utilising communities of bacteria are distinct between the four plant species. The exudate utilising communities of maize and wheat are less distinct from the bulk soil and light fractions than rape and barrel clover
Arabidopsis thaliana cultivars	DNA	16S rRNA DGGE profiling 18S rRNA DGGE profiling	Bressan 2009	The genetic modification of an *Arabidopsis thaliana* cultivar resulting in greater production and exudation of glucosinate resulted in an altered exudate utilising community
Arabidopsis thaliana	DNA RNA	qPCR rt-qPCR	Haichar 2012	Genes involved in denitrification and ethylene metabolism are expressed by members of the exudate utilising community and the general bulk soil community
Potato cultivars	RNA PLFA	T-RFLP rt-qPCR PLFA analysis	Hannula 2012	*Ascomycota* and *Glomeromycota* utilised plant exudates from an earlier time point, whilst *Basidomycota* appeared later in the enrichment. There was variation in the community between cultivars
Potato cultivars	RNA PLFA	16S rRNA DGGE profiling Cloning PLFA analysis	Dias 2013	Gram negative bacteria (*Pseudomonas* and *Burkholderia*) dominated the exudate utilisers. The exudate utilisers varied over time and between cultivar

Carex arenaria *Festuca rubra*	RNA PLFA	rt-qPCR 16S rRNA DGGE profiling 18S rRNA DGGE profiling PLFA analysis	Drigo 2013	Arbuscular mycorrhiza dominated the exudate utilising community, supplying the labelled carbon to the additional members of the fungal and microbial community. An elevated concentration of CO_2 resulted in a delayed supply of this ^{13}C label
Rice	RNA	454 sequencing of 16S rRNA genes	Hernandez 2015	Proteobacteria and Verrucomicrobia dominated the exudate utilising community. The root showed a greater proportion of the microbial community to be labelled than the rhizosphere
Wheat	DNA	454 sequencing of 16S rRNA genes	Ai 2016	Actinobacteria and Proteobacteria dominated the exudate utilising portion of the rhizosphere. Diversity of the exudate utilising community decreased with soil fertilisation.

There is variation in the methodology of these studies with regards to the age of the plant at the start of pulsing, the duration of the pulsing and the specific plant species tested. Similarities between the experiments include the concentration of CO_2, which is typically maintained at an ambient concentration (350 ppmv), due to an above ambient concentration of CO_2 altering the rhizosphere community (Drigo *et al.*, 2010, 2013). A higher concentration of CO_2 in this type of experiment can be used solely to characterise the impact of an elevated CO_2 atmosphere on the rhizosphere community of the plant (Drigo *et al.*, 2013).

1.5.5.2 Flaws in the designs of SIP experiments

There are aspects of stable isotope probing that necessitate careful experimental design. Firstly, there must be sufficient incorporation of the ^{13}C label into the DNA to enable successful separation of the heavy and light DNA. For soil the suggested substrate concentration is 50 µmol ^{13}C incorporated per gram of environmental sample (Chen and Murrell, 2010). In order to gain sufficient incorporation of ^{13}C, the concentrations of substrate used may be higher than ambient concentrations and therefore the identified substrate utilisers may not be representative of the natural community (Radajewski *et al.*, 2000). However, depending on the particular research question, e.g. "which organisms within an environment are capable of utilising this substrate?", it can provide useful information by enriching organisms of interest (Taubert *et al.*, 2017). Furthermore, organisms that are enriched at higher concentrations of a labelled substrate may still be active and involved in its utilisation at more environmentally relevant concentrations e.g. *Methylophaga* and methanol in the marine environment (Stacheter *et al.*, 2013; Grob *et al.*, 2015). Sufficient incorporation of ^{13}C can require long incubation times, which could result in production of a lab-adapted sample or cross feeding of ^{13}C (McDonald *et al.*, 2005; Cébron *et al.*, 2007; Chen *et al.*, 2008a). Upon utilisation of a ^{13}C labelled substrate, this is converted into metabolic intermediates that are converted to biomass or further oxidised to CO_2 to generate reducing power and energy. These intermediates and biomass represent a source of ^{13}C that can result in the labelling of the DNA of non-target organisms unable to use the supplied substrate and this labelling is termed cross feeding (Hutchens *et al.*, 2003; Lueders *et al.*, 2003, 2006). To improve the activity of organisms involved in the utilisation of the labelled substrate, e.g. methanol, some studies supply nutrients to the environmental samples, which can

overcome the issues of nutrient limitation but reduce the observed diversity within the sample (Cébron *et al.*, 2007), and bias the detection of organisms to more rapidly growing strains (Haichar *et al.*, 2008).

RNA SIP and protein SIP both possess the advantage over DNA SIP of being more sensitive, as labelling with ^{13}C does not require replication of the cell to occur (Manefield *et al.*, 2002). The majority of RNA SIP experiments have focused on rRNA (Lueders et al. 2016 and references therein), but labelling and subsequent analysis of mRNA is also possible (Huang *et al.*, 2009; Dumont *et al.*, 2011). A previous SIP incubation analysing both DNA and RNA identified active ammonia oxidising through successfully labelled rRNA whilst detecting no labelled DNA (Pratscher *et al.*, 2011), showing the greater sensitivity of RNA-SIP. However, the disadvantages of RNA SIP include the instability of RNA and the limited information that can be gained if only rRNA or mRNA is processed (Lueders *et al.*, 2016). Protein SIP is the most recently developed of the SIP techniques (Jehmlich *et al.,* 2008). In the instances where it has been applied, it has provided a wealth of information on the utilisation of the labelled substrate e.g. the identification of the proteome of a *Methylophaga* enriched with ^{13}C labelled methanol (Grob *et al.*, 2015). However, this technique requires metagenomic data to enable assignment of taxonomy to the labelled peptides (reviewed in Lueders *et al.*, 2016).

Studies utilising stable isotope probing of the rhizosphere encounter the same problem as other stable isotope probing experiments as there has to be sufficient time to achieve ^{13}C labelling (Neufeld et al. 2007a). Another major issue specific to SIP of the rhizosphere is that the quantity and composition of exudates from the plant will change with the developmental stage of the plant. This has been shown in *Arabidopsis*, pea, wheat and sugar beet (Chaparro et al. 2013; Houlden et al. 2008; Haichar et al. 2012) and therefore the microbial community of exudate utilising bacteria is dynamic. As the exudation pattern changes over time there will be transient labelling of some members of the rhizosphere community, such as the slow growers, and these may therefore be excluded from further analysis (Haichar *et al.*, 2008). Several rhizosphere SIP studies did not utilise next generation sequencing, instead analysing communities through DGGE profiling (Rangel-Castro *et al.*, 2005; Haichar *et al.*, 2008; Dias *et al.*, 2013; Drigo *et al.*, 2013). Additionally, some studies either do not process or sequence an ambient or ^{12}C control,

complicating the detection of labelling (Ai *et al.*, 2015). There are many factors to consider when performing a SIP experiment, including the possibility of a shift in the gradients that is undetected, and failure to account for these issues can result in false positives and incur challenges when attempting to analyse results.

1.6 The Plant microbiome

1.6.1 The phyllosphere

The phyllosphere represents a nutrient poor environment with high levels of variation in environmental selection pressures (Lindow *et al.,* 2003). These selection pressures include exposure to ultraviolet light, heat, desiccation and the presence of radicals. Furthermore, there is a greater impact of the day and night cycle on the microbial community of the phyllosphere than in the plant associated soil environment (Brigel 2015). Amongst the carbon compounds available on the leaves are some sugars and volatiles, including methanol (Fall *et al.*, 1996) and isoprene (MacDonald *et al.*, 1993), which are emitted through the stomata. *Sphingomonas*, *Pseudomonas* and *Methylobacterium* have been shown to be key players with regards to the phyllosphere of rice plants (Knief *et al.*, 2012). Methylotrophy has been suggested to be advantageous in the phyllosphere environment, with high levels of *mxaF* and *xoxF* expression detected in the phyllospheres of rice plants, soybean and clover (Delmotte *et al.*, 2009; Knief *et al.*, 2012) and *xoxF* exclusively detected in the phyllosphere of *Arabidopsis* plants (Delmotte *et al.*, 2009). Methylotrophs have also been shown to be present in the stem of soybeans, which would expose microbes to a similar array of selection pressures as the phyllosphere (Minami *et al.*, 2016).

1.6.2 The rhizosphere

Soil environments are typically oligotrophic, carbon poor, with the available carbon resources typically comprised of more recalcitrant material. This is proposed to result in the selection of more K-strategist organisms (Bulgarelli *et al.*, 2012; Turner *et al.*, 2013; Ai *et al.*, 2015). The rhizosphere (Figure 1.8), represents a more carbon rich, or copiotrophic, environment (Fierer, 2007) due to the carbon exuded by the plant. Compounds exuded from plant roots include alcohols, sugars, fatty acids, hormones, vitamins, growth factors and organic acids (Dennis *et al.,* 2010 and references therein; Chaparro *et al.,* 2013). The exudation profile of pea plants is altered between sterile plants and plants inoculated with bacteria (Turner, 2013), complicating the

characterisation of the exudate profiles which are relevant to the soil. Furthermore, in the rhizosphere environment, the release of carbon by the plant also enhances the breakdown of soil organic matter in the soil, termed priming, which can also impact on the microbial community and cause changes in the relative abundance of specific genera (Kuzyakov 2002 and references therein).

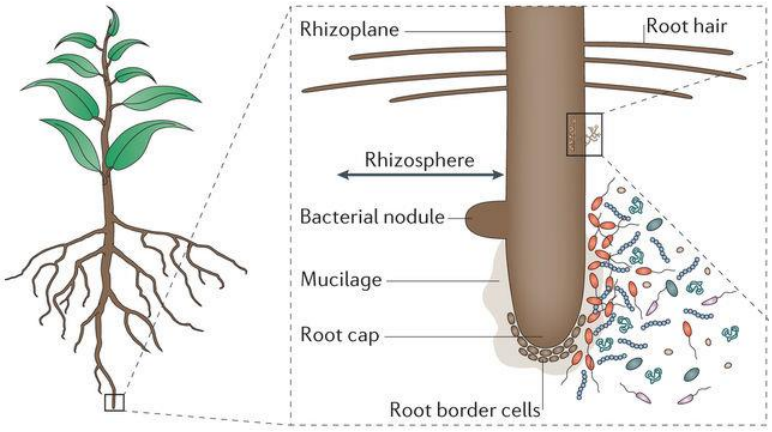


Figure 1.8 Schematic of the rhizosphere and rhizoplane**.** (Figure adapted from Phillipott et al 2013).

1.6.2.1 Root and rhizoplane colonising bacteria

The root environment is comprised of epiphytic bacteria on the exterior of the root surface (rhizoplane) and endophytic bacteria within the root (Figure 1.8). The root has been shown to be more selective an environment than the rhizosphere soil, which may be due to many reasons, including the release of compounds by the plant discriminating against specific groups of bacteria (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012), the preferential recruitment of bacteria through the release of signalling compounds (Prosser *et al.*, 2006; Jones *et al.*, 2009) and the immune system of the plant (Lundberg *et al.*, 2012). The root colonising bacteria may also be more competitively efficient, due to the higher concentration of exuded carbon compounds the closer to the root (Gao *et al*., 2011). The roots also provide a structure on which the bacteria can attach, affecting growth dynamics of the species on the root and allowing more direct access to structural material (Bulgarelli *et al.,* 2012).

1.6.2.2 Impacts of the rhizosphere community on the plant

The rhizosphere community has also been shown to have an impact on the plant (Badri *et al.*, 2013; Chaparro *et al.*, 2013; Zolla *et al.*, 2013). The microbial community can

benefit the plant host through the suppression of pathogenic bacteria and fungi within the rhizosphere through the production of antimicrobial and antifungal compounds (Sanguin *et al.*, 2009; Berendsen *et al.*, 2012). A broad diversity of bacteria have been shown to play a role in improving plant health through pathogen suppression and the induction of the plants immune system, including the Actinomycetes (Badji *et al.*, 2006; El-Tarabily, 2006; Merzaeva *et al.*, 2006), *Pseudomonas* (Bakker *et al.*, 2007; Jousset *et al.*, 2011; Mendes, 2011)and the *Myxococcaceae* (Zafriri *et al.*, 1981; Lueders *et al.*, 2006). Members of the rhizosphere community can also benefit plant growth through a variety of interactions, including the improved supply and cycling of nutrients to the plant. This includes nitrogen related compounds, such as ammonia which is produced through the fixation of nitrogen by diazotrophic bacteria (Galloway 1995 and references therein). This also includes the production of nitric oxide through truncated denitrification, which is proposed to play a role in antimicrobial interactions (Turner, 2013) and has been shown to enhance root proliferation through inducing auxin responses (Wendehenne *et al.*, 2001). The concentration of phosphorous, often limiting in soil (Chabra *et al.*, 2013), can be increased by microbes in the rhizosphere through the solubilisation of insoluble phosphorous containing minerals. Sulfur cycling in the soil can also be enhanced by bacteria through desulfonation, a process by which a sulphur group is liberated from a substrate (Schmalenberger *et al.*, 2007; Satola *et al.*, 2013). Further to altering the availability of nutrients in the soil, rhizosphere-occupying bacteria have been shown to impact plant growth through manipulating the concentration of plant hormones present in the soil (Compant *et al.*, 2010; Glick, 2014 and references therein). Examples of this interaction include the degradation of ethylene, which inhibits the stress response of the plant and encourages growth of the plant roots (Glick, 2014 and references therein) and the catabolism and anabolism of indole acetic acid by bacteria, enabling control over the concentration of this plant growth promoting hormone (Glick, 2014 and references therein).

1.6.3 Methylotrophs in the rhizosphere

Methylotrophy is proposed as an advantageous trait to possess in the colonisation of both the phyllosphere and rhizosphere (Sy *et al.*, 2005), with a methylotrophy deficient mutant strain of *Methylobacterium extorquens* being shown to be competitively inferior to the wild type with regards to the colonisation of the leaves and roots of *Medicago*

truncatula (Sy *et al.*, 2005). One of the proposed reasons for this difference in fitness is the wild type being able to exploit methanol as an additional carbon source which the mutant is incapable of metabolising. Furthermore, some studies indicate that methylotrophic bacteria may be enriched in the rhizospheres of certain plant species. This has been indicated through several studies, including through the detection of an increased relative abundance of specific bacteria in the rhizosphere relative to the bulk soil, such as the families *Methylobacteraceae* and *Hyphomicrobiaceae* in the rhizosphere of *Arabidopsis* plants (Lundberg *et al.*, 2012). The potential enrichment of methylotrophs in the rhizosphere is also indicated by the detection of *mxaF* and *xoxF* methanol dehydrogenase genes and the presence of methanol dehydrogenase enzymes in the rhizosphere of rice plants in addition to the phyllosphere, where methylotrophy has been previously characterised as present (Knief *et al.*, 2012). In a study of a grassland site, which applied proteomics and metagenomics, XoxF was actually shown to be the most abundant protein (Butterfield *et al.*, 2016). Further evidence is produced through the production of *Methylobacterium* genomes following the binning of metagenomes produced from DNA extracted the rhizosphere of soybean plants (Tsurumaru *et al.,* 2015). Soils in association with *Arabidopsis thaliana* have also been shown to have higher rates of methanol dissimilation than non-plant associated soils (Stacheter *et al.*, 2013).

Methylotrophs are suggested to provide the benefit to plants of detoxification of the methanol produced through plant growth, which at a sufficiently high concentration would inhibit the growth of the plant (Abanda-Nkpwatt *et al.*, 2006). Furthermore, several studies have shown that some methylotrophic bacteria possess plant growth promoting capabilities and some studies show a benefit to the growth of plants following inoculation with methylotrophic bacteria. This has been shown with wheat plants, which displayed enhanced germination and seedling growth, and white mustard, tomato, wild strawberry and tobacco plants having a higher seedling weight and shoot length relative to controls when inoculated with *Methylobacterium extorquens* or other strains of *Methylobacterium* (Abanda-Nkpwatt *et al.*, 2006; Meena *et al.*, 2012). Additional inoculation studies used *Methylobacterium oryzae* in combination with species from additional genera (*Azosporillium* and *Burkholderia*) and these studies

showed a benefit to the growth of rice, tomato and red pepper plants (Madhaiyan *et al.,* 2010; Chung and Sa 2012). Inoculation studies also showed that *Methylobacterium* sp. were capable of inducing resistance in potatoes against the plant pathogen *Pectobacterium* (Kozyrovska *et al.*, 2012). However, it is interesting to note that inoculation of plants with *Methylobacterium* sp. yields inconsistent results between plant species, with maize and sunflowers showing no impact on growth following inoculation with *Methylobacterium* (Abanda-Nkpwatt *et al.*, 2006; Kutschera 2007).

A recent metatranscriptomic study showed an increase in the relative abundance of several methylotrophic genera following the growth of a plant. The methylotrophic genera that increased in abundance varied between cereal (wheat and oat) and legume (pea) plants (Turner 2013). Members of the *Methylophilaceae, Beijerinckaceae* and *Varivorax* increased in abundance in the pea rhizosphere and members of the *Methylophilaceae, Methylibium* and *Methylocaldum* increased in abundance in the wheat rhizosphere (Turner, 2013). This suggests that in addition to plants potentially enriching methylotrophic bacteria in the rhizosphere, there is variation in the specific genera enriched between the different species of plant. However, it is not possible to delineate between the possible reasons for this increase in relative abundance in the rhizosphere through analysis of the total community (e.g. exudate utilisation or priming). This metatranscriptomic study was performed using soil from a grassland site called Church Farm in Bawburgh (Norfolk, United Kingdom) (52.6276 N 1.1786 E), and therefore this site was the primary site for sample collection used in this research.

Taken together, several studies have shown that following plant growth there is an increase in the relative abundance of genera that possess a functional trait enabling them to utilise a carbon source that other competing members of the rhizosphere community cannot. The specific genera increasing in abundance have been shown to vary between the plant species. Furthermore, several of these genera possess plant growth promoting traits and a specific genus has been shown to benefit plant growth in inoculation studies. However, what has not been studied in depth is whether these methylotrophic genera are increasing in abundance due to the utilisation of carbon directly from the plant or due to priming and whether the genera that are changing in abundance are actually utilising methanol. Furthermore, there have been no studies

that have characterised the greater diversity of methylotrophs present within soil environments through the sequencing of methanol dehydrogenase genes other than *mxaF* i.e. *xoxF*. There has also been limited research attempting to enrich and cultivate methylotrophs from the soil environment with the additional supplementation of lanthanides now their role in the function of *xoxF* has been shown.

1.7 Project aims

The hypothesis being tested in this project was that methylotrophic bacteria are enriched in the rhizospheres of pea and wheat plants, and different methylotrophic genera are enriched between these plant species. The aims of the work described here were:

1) To isolate and characterise methylotrophs from a range of terrestrial environments to screen for methylotrophs in genera where methylotrophy had not been detected, to isolate novel species and to enable the expansion of the reference sequence database of methanol dehydrogenase genes
2) To investigate the diversity of methylotrophic bacteria within the Church Farm soil by sequencing methanol dehydrogenase genes (*mxaF*, *xoxF* and *mdh2*) and assess whether there is a shift in their diversity in the pea and wheat rhizosphere relative to the bulk soil
3) To investigate any potential impact of plant growth on the active methylotrophs within the soil environment through stable isotope probing experiments performed with Church Farm soil, pea rhizosphere soil and wheat rhizosphere soil supplemented with ^{13}C labelled methanol
4) To identify the bacteria in the pea and wheat rhizosphere and root communities that are actively utilising carbon exuded from the plant by stable isotope probing with the supply of $^{13}CO_2$ to pea and wheat plants

Chapter 2 Materials and Methods

2.1 Chemicals and reagents

Analytical grade reagents used in this research were from Sigma-Aldrich (MIS, USA), Melford laboratories (Ipswich, UK), Fisher Scientific (Loughborough, UK). Molecular biology grade reagents were from ThermoFisher (MA, USA), Promega UK (Southampton, UK), Quiagen (Germany) and Roche (Switzerland). Gases were supplied by BOC (UK). $^{13}CO_2$ and ^{13}C labelled methanol was supplied by Cambridge Isotope Laboratories (MA, USA). All ultracentrifuge work involved using tubes, rotors and ultracentrifuges from Beckman Coulter (CA, USA). Additional reagents and suppliers are specified in the text.

2.2 Growth of bacterial strains

2.2.1 Bacterial strains

Table 2.1 Organisms used in this study

Strain	Reference	Location
Escherichia coli Top 10	Invitrogen	Murrell lab strain
Methylobacterium extorquens AM1		Murrell lab strain
Methylocella silvestris BL2	(Dunfield *et al.*, 2003)	Murrell lab strain
Variovorax paradoxus S110	(Davis *et al.,* 1969)	DSMZ collection 30034
Methylibium sp. Root1272	(Bai *et al.*, 2015)	DSMZ collection 102455
Variovorax paradoxus MM1	This study	Church Farm soil
Methylovorus methylotrophus MM2	This study	Church Farm soil
Methylobacillus denitrificans MM3	This study	Church Farm soil
Methylophilus flavus CF1	This study	Church Farm soil
Burkholderia terricola CF2	This study	Church Farm soil
Hyphomicrobium denitrificans CF3	This study	Church Farm soil
Methylobacterium pseudosasae CF4	This study	Church Farm soil
Methyloversatilis discipulorum LF1	This study	Landfill soil
Hydrogenophaga pseudoflava LF3	This study	Landfill soil
Oharaeibacter diazotrophicus LF4	This study	Landfill soil
Starkeya koreensis LF6	This study	Landfill soil
Azohydromonas australica LF	This study	Landfill soil
Methylobacterium extorquens BR2	This study	Norfolk Broads water
Starkeya koreensis BR13	This study	Norfolk Broads water
Methylophilus TWE2 BR10	This study	Norfolk Broads water
Methylophilus leisingeri BR11	This study	Norfolk Broads water
Burkholderia sartisoli BR14	This study	Norfolk Broads water

2.2.2 Growth media and culturing of organisms

All bacteria except *E. coli* were routinely cultured on dNMS media (Theisen *et al.*, 2005), detailed below (Table 2.2). Glassware was acid washed with 10 % nitric acid for all cultures grown for the purpose of nucleic acid extraction, protein extraction, or growth curves.

Table 2.2 Components of dNMS media for growth of methylotrophic bacteria

Components added before autoclaving	Volume per 1 L (ml)		
	Standard dNMS	Modified dNMS	Standard dANMS
Solution 1 $MgSO_4.7H_2O$ (43.82 mM)	10	10	10
Solution 2 $CaCl_2.2H_2O$ (17.68mM)	10	10	10
$FeCl_2$ (100 mM)	0.1	0.1	0.1
Trace elements (SL10) (Widdel *et al.*, 1983)	0.5	0.5	0.5
KNO_3 (1 M)	1	1	1
NH_3Cl_2 (1 M)	0	0	1
Components added after autoclaving			
Phosphate buffer	10	10	10
Vitamin Solution	0.5	0.5	0.5
Lanthanum chloride ($LaCl_3$) /Cerium chloride ($CeCl_3$) solution (10 mM)	0	0.5	0

dNMS was modified to include 5 µM lanthanum or cerium. This medium was designated modified dNMS. The composition of dNMS was also altered to include ammonium as an additional nitrogen source, and this was designated dANMS. Nitrate was also substituted with ammonium and urea to assess growth on these as a sole nitrogen source.

Cultures were maintained at 30°C in a shaking (150 rpm) or static incubator. All cultures with the exception of *Escherichia coli* TOP10 were maintained on methanol (10 mM) as the sole carbon source. Additional carbon compounds were tested as potential sole carbon sources (5 – 10 mM). The optical density (OD) of a culture was measured using a UV-1800 spectrophotometer (Shimadzu, Japan) at 540 nM.

The standard temperature (30°C), salinity (0%), and pH (7) were altered to assess optimal growth conditions. The salinity of dNMS was adjusted through the addition of sodium chloride to adjust salinity across a range of 0-4% (w/v). The pH of dNMS was altered through the addition of phosphate buffers of the desired pH. The temperature was altered across a range, from 4-42°C. Vitamin B12 dependency was assessed through inoculation of strains into dNMS with a modified vitamin solution which did not contain vitamin B12.

R2A medium was supplied in the form of dehydrated media and prepared according to manufacturer's instructions.

LB media supplemented with X-GAL and ampicillin, and SOC medium (Hanahan, 1983) was used for transformations involving *Escherichia coli* TOP10.

Testing for starch hydrolysis, acetoin production and catalase and oxidase activity were performed by Sean Jenkins (University of East Anglia). The functioning of the denitrification pathway (with the exception of the reduction of nitrate to nitrite) was confirmed to occur under anaerobic conditions using gas chromatography by Alexander Goodchild (University of East Anglia).

2.2.2.1 Nitrate reduction

The ability to reduce nitrate to nitrite was testing using cultures grown in nitrate broth (meat extract 5 g/L, KNO_3 3 g/L, NaCl 3 g/L). Media with KNO_2 (3 g/L) as the sole nitrogen source was also produced. Phosphate buffer (20 mM), lanthanum (5 µM), MAMs vitamin solution and 0.2 % agar were added after autoclaving. The medium (10 ml) was aliquoted into 40 ml test tubes and inoculated from liquid culture (5 % inoculum). *E. coli* was used as a positive control. 20 mM methanol was supplied to each tube before sealing with suba seals. The tubes were statically incubated at 30 ^{o}C for 14 days. Greiss reagent was added to each sample to test for the reduction of nitrate to nitrite. 5 mg of Zinc powder was subsequently added to the cultures to assess whether any produced nitrite has been further reduced.

2.2.2.2 Siderophore production

Siderophore production was tested using modified dNMS plates supplemented with CAS solution (chrome azurol S/iron(III)/hexadecyltrimethylammonium bromide) (100 µM final concentration). Modified dNMS plates were prepared, half of the plate removed under aseptic conditions and modified dNMS with CAS solution poured into the empty half of each plate. Streak plates were produced for each isolate tested, with *Methylosinus trichosporium* OB3b streak plates for a positive control. A shift in colour of the CAS reagent from blue to red signified a positive result.

2.2.2.3 Indole acetic acid production

The production of indole acetic acids (IAA) was tested using cultures grown on modified dNMS and modified dNMS supplemented with 1 µM tryptophan. Cultures were incubated at 30 °C with shaking at 150 rpm. 1 ml Samples were harvested from the growing cultures, centrifuged at 13, 000 g for five min and the supernatant collected. R2 Salkowski reagent (Glickmann *et al.*, 1995) was added to the supernatant and incubated for 30 min in the dark prior to measuring of the OD at 540 nM.

2.2.2.4 Gelatinase assay

Nutrient gelatin plates were produced (4 g/L Peptone, 1 g/L yeast extract, 12 g/L Gelatin) and supplemented with the same lanthanum and phosphate concentration as the modified dNMS. The plates were stab inoculated from single colonies in triplicate. The

plates were incubated at 30 °C with methanol. A positive result was indicated by a clear halo forming around the colonies.

2.2.2.5 Voges-Proskauer assay for acetoin production

Modified dNMS was supplemented with glucose at a concentration of (20 mM) and dispensed into test tubes. *E.coli* was used as a positive control. Methanol was added to a final concentration of 20 mM and the test tubes sealed with suba seals. The tubes were incubated at 30 °C for seven days. 25 µL of Barrits A (1.25 g α-naphthol in 25 ml ethanol) and Barrits B (10 g KOH in 25 ml H_2O) were added to the test tubes. A positive result was indicated by the production of a red colour.

2.2.2.6 Starch hydrolysis

Modified dNMS plates containing 0.5 % starch were inoculated using spots of liquid culture. The plates were incubated at 30 °C and incubated with methanol. The plates were incubated for seven days and then flooded with iodine solution. A positive result was indicated by a region of the inoculated portion of the plate not containing dye.

2.2.2.7 Catalase and Oxidase testing

The catalase test was performed by the addition of 3 % (v/v) hydrogen peroxide to colonies grown for one week on modified dNMS plates. A colony was tested on 1 % N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (Kovac's oxidase reagent) on filter paper for the oxidase test.

2.2.2.8 Polyhydroxybutyrate production

Modified dNMS plates were spot inoculated from liquid culture. The plates were incubated with methanol for seven days. An ethanolic solution of 0.02 % Sudan Black B was used to flood the plate which was then incubated for 30 minutes. The Sudan Black B solution was then washed off with 96 % ethanol.

2.2.2.9 Motility

Modified dNMS plates were made containing 0.3 % (w/v) agar to test for swarming motility, 0.5 % for swimming motility and 1 % (w/v) agar to test for twitching motility. 5 µL of liquid culture was spot inoculated onto 0.3 % agar plates. Liquid culture was stab inoculated into the 0.5 % and 1 % agar plates. Plates were incubated for seven days at 30 ˚C and checked for motility. Water was used as a negative control.

2.2.2.10 Antibiotic sensitivity

Modified dNMS plates were produced containing specific concentrations. The antibiotics tested were (µg ml^{-1}) Gentamycin (10), Neomycin (30), Streptomycin (10), Nalidixic acid (30), Novobiocin (5), Kanamycin (30), Tetracycline (10), Ampicillin (100), Lincomycin (2) and Chloramphenicol (10). Liquid cultures were spread on the plates, which were then incubated for two days.

2.2.3 Enrichment and isolation of methanol degrading bacteria

Several isolation experiments were performed in order to obtain novel isolates. Soil samples from the landfill site and Church Farm (Section 2.3.1) were enriched with methanol, over a range of concentrations of methanol (2 – 100 mM), for variable lengths of time (one – twenty days) and with different media types (Section 2.2.2). Water from the Norfolk Broads at Hickling (Section 2.3.1) was also enriched with methanol.

2.2.3.1 ***Enrichment and isolation of methylotrophs using soil from CF using dNMS modified with lanthanides***

Enrichment cultures were established in 120 ml serum vials using 0.5 g CF soil, with the addition of 20 ml of modified dNMS and 10 mM methanol. These enrichments were then incubated at 30 °C for a period of ten days. After ten days, the enrichments were plated onto modified dANMS plates and incubated with methanol in a gas tight chamber. The plates were incubated for ten days, and the methanol was replenished in the gas tight chamber every two days. Several colonies developed on the methanol enriched plates. Individual colonies from these plates were streaked onto new dANMS plates to produce pure streak plates for each culture. Single colonies from the streak plates were then used to inoculate 20 ml of modified dANMS, which was supplemented with 10 mM methanol. Single colonies from the streak plates were also used in a colony PCR to amplify their 16S rRNA genes.

2.2.3.2 ***Enrichment and isolation of methylotrophs using soil from CF using dNMS and sloppy agar***

Enrichments were established using fresh CF soil (wet and un-sieved) and 20 ml of autoclaved RO water. These enrichments were supplemented with either 2 mM, 5 mM or 10 mM of methanol, and incubated at 30°C in a static incubator for three days. 15 ml of 0.3 % agar containing modified dNMS media were pipetted into sterile 20 ml test tubes. 100 µL of inoculant from the methanol-enriched soil was added to the test tubes

using a dilution series of 10^{-2} to 10^{-8}. The test tubes were then supplemented with 10 mM methanol and sealed using suba-seals (Sigma-Aldrich). The tubes were incubated at room temperature for five days. Colonies were picked from these tubes using glass pipettes and re-inoculated into test tubes containing the same media and incubated for four days. Colonies were picked with glass pipettes from these test tubes, and the samples were plated onto modified dNMS plates. Single colonies were used to establish liquid cultures with 20 ml modified dNMS and 5 mM methanol in 120 ml serum vials.

2.2.3.3 ***Enrichment and isolation of methylotrophs using soil from CF using repeated pulsing of methanol***

CF soil was continually supplied with ^{13}C labelled methanol at a concentration for a final concentration of 250 µM. The methanol was respiked upon depletion, over the course of 20 days (2.8.1). A dilution series of this soil was plated on modified dNMS plates supplemented with methanol. As above (2.2.3.1), individual colonies were restreaked to ensure the purity of individual cultures and to produce single colonies for the inoculation of liquid cultures.

2.2.3.4 ***Enrichment and isolation of methylotrophs using water from Norfolk Broads and soil from Marburg Forest and Strumpshaw Landfill***

Water from the Norfolk Broads in Hickling (5 ml) or soil from a forest in Marburg and Strumpshaw landfill (1 g) was placed in 120 ml serum vials, made up to a volume of 20 ml with 5% modified dNMS and supplied with methanol (5 mM). The enrichments were incubated at 30 °C for five days. The enrichments were subcultured three times into 5 % modified dNMS with 5 mM methanol and each subculture was left for five days. After the third subculture, the samples were plated onto modified dNMS plates and single colonies were used to inoculate liquid cultures. The 16S rRNA genes of isolates that grew on methanol as a sole carbon source were PCR amplified and sequenced.

2.3 Environmental sampling

2.3.1 Collection of environmental material

Soil was collected from undisturbed former grassland from the Antirrhinum wall at Church Farm in Bawburgh, (Norfolk, United Kingdom) (52.6276 N 1.1786 E). Soil was collected in March 2014, April 2015 and September 2016. The top 10 cm of soil was removed from a 1 m^2 section of wild grassland. Soil was collected down to 20 cm below

the removed layer. Samples of this soil were analysed for their physical parameters, and samples were also frozen and stored at -20°C and -80°C for subsequent molecular analysis. The collected soil was air-dried in the University of East Anglia greenhouses for three days and then sieved through 10 mm^2 and 5 mm^2 sieves. Stones, roots, insects, amphibians, and all other forms of detritus were manually removed from the soil. Samples of the sieved, dried soil were stored at -20 °C and -80 °C for subsequent extraction of nucleic acids. The remaining soil was either used to grow plants (Section 2.3.2) or stored at 4 °C.

Strumpshaw landfill soil (52.6106 N 1.4702 E) was collected at a depth of five cm below the surface and supplied by Elliot Brooks (University of East Anglia).

Water from Hickling Broad in the Norfolk Broads (52.7462 N 1.5704 E) was collected in a 50 ml Falcon tube and supplied by Dr. Jennifer Pratscher (University of East Anglia).

2.3.2 Plant seed sterilisation, germination and planting

Paragon wheat seeds (*Triticum aestivum* var Paragon) were sterilised by washing the seeds in 5% (v/v) sodium hypochlorite solution for one minute. Seeds were then rinsed in autoclaved ddH_2O. Seeds were then placed onto a filter paper disk in a petri dish. The filter paper was moistened with autoclaved ddH_2O.

Pea seeds (*Pisum sativum* var. Avolar) were sterilised through washing the seeds in 95% (v/v) ethanol for one minute. Seeds were then washed with autoclaved ddH_2O, soaked in 2% sodium hypochlorite for five minutes, and washed a second time in autoclaved ddH_2O. Pea seeds were placed in a petri dish on filter paper disks saturated with autoclaved ddH_2O.

The petri dishes containing pea and wheat seeds were covered with aluminium foil and left in the dark for three days to germinate. Germinated seeds were manually inspected for fungal contamination. Germinated seedlings were planted in 500 ml pots under short, medium or long day regimes in plant growth rooms at 22 °C. Moisture levels of soils were monitored using an SM300 soil moisture sensor (DELTA-T, UK), and moisture levels were maintained at 5 % soil moisture content with autoclaved ddH_2O.

Plants were harvested after four or six weeks of growth. The wheat and pea plants were manually removed from the 500 ml pots. Roots were cut from the plant at the base of

the stem using a flame-sterilised razor blade. Roots were separated from bulk soil by shaking three times over a gloved hand in order to remove loose soil from the roots (Bulgarelli *et al.*, 2012). Soil which remained attached to the roots after shaking was defined as rhizosphere soil. To collect the rhizosphere soil, the roots of each plant were individually transferred to Falcon tubes and submerged in phosphate buffered saline (PBS) (10 mM Na_2HPO_4, 1.8 mM NaH_2PO_4). The Falcon tubes were then vortexed for 30 seconds. After vortexing, the roots were transferred to new Falcon tubes. The used PBS was centrifuged at 3,200x g for 15 min to pellet the soil. Three separate 0.5 g aliquots of soil from each plant were transferred to lysing matrix E tubes (MP Biomedicals, CA, USA). For each sample, one lysing matrix E-tube was snap frozen in liquid nitrogen and stored at -80 °C. The other two lysing matrix E tubes were frozen at -20 °C.

Roots were washed two subsequent times in fresh PBS. The roots were then placed in Petri dishes, and soil particles attached to the roots manually removed using flame sterilised tweezers. Roots were washed in PBS for a fourth time, before being snap-frozen in liquid nitrogen and stored at -80 °C

2.3.3 Measuring soil pH

10 g of soil was mixed with 10 ml of ddH_2O using a magnetic stirrer. The pH of the solution was then measured using a calibrated Jenway 3505 pH meter (Jenway, UK). The pH reading of the soil was also measured using pH indicator paper.

2.3.4 Measuring soil water content of soils

10 g aliquots of fresh, non-dried soil were transferred into glass vessels of known weight. The vessels were then baked at 110 °C and the weight periodically recorded until it ceased to change. The difference in weight of the soil before and after heating was used to calculate the moisture content of the soil.

2.4 Extraction of nucleic acids

2.4.1 Extraction of nucleic acids from soil

DNA and RNA were extracted from soil using a cetytrimethyl ammonium bromide (CTAB) based method (Griffiths *et al.*, 2000). 0.5 g of soil was weighed into a 2 ml lysing matrix E tube and either frozen for later processing, or processed immediately. 500 µL of CTAB (equal volumes of 10 % (w/v) CTAB, 0.7 M NaCl, 240 mM potassium phosphate buffer,

pH 8.0) and 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8) were added to the lysing matrix E tube. The lysing matrix E tubes were loaded into a Fast Prep bead beating machine (MP Biomedicals, CA, USA) run at 5.5 m/s for 30 seconds. The tubes were then centrifuged at 16,000x g at 4°C for 5 min. The supernatant was transferred to a new 1.5 ml microcentrifuge tube, and an equal volume (~500 µL) of chloroform:isoamyl alcohol (24:1). The microcentrifuge tubes were briefly vortexed before being centrifuged at 16,000x g at 4 °C for 5 min. The supernatant was then transferred to a new 1.5 ml microcentrifuge tube. Nucleic acids were precipitated through the addition of 1 ml of polyethylene glycol 6000-NaCl solution (30 % polyethylene glycol, 1.6 M NaCl solution), followed by inversion and incubation at room temperature for 2 hours. Following incubation, samples were centrifuged at 18,000x g at 20 °C for 30 min to pellet the nucleic acids. The supernatant was discarded and pelleted nucleic acids washed in 70 % (v/v) ice-cold ethanol. The samples were centrifuged at 18,000x g at 20 °C for 15 min and the supernatant discarded. The pellets were then left to air dry for 10 min before being resuspended in 30-100 µL of nuclease-free water.

2.4.2 Additional RNA extraction techniques

2.4.2.1 Hot-phenol RNA extraction

All solutions used in this RNA extraction technique were diethyl pyrocarbonate (DEPC) treated or made using DEPC treated water. Extractions were performed using an adapted version of an established protocol (Gilbert *et al.*, 2000). Briefly, 0.5 g of soil was resuspended in solution 1 (0.3 M sucrose, 0.01 M sodium acetate, pH 4.5), and 200 µL of solution 2 (2 % (w/v) SDS, 0.01 M sodium acetate, pH 4.5) was added. The mixture was then transferred to a lysing matrix B tube and 400 µL of acid phenol (pH 4.3) added. Samples were loaded into a Fast Prep bead beating machine run at 6 m/s for 30 seconds, and cooled on ice. Samples were then centrifuged for 5 min at 14,000x g at 4 °C. The upper aqueous phase was transferred to 400 µL of acid phenol in a 1.5 ml microcentrifuge tube and mixed by inversion. The samples were incubated at 65 °C and then cooled in dry ice mixed with ethanol. Samples were left to thaw before centrifuging at 14,000x g for 5 min at 4 °C. The upper aqueous phase was then transferred to 400 µL of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8) in a 1.5 ml microcentrifuge tube. The tubes were shaken vigorously and centrifuged at 14,000x g for 5 min at 4 °C. The

upper aqueous phase was transferred to chloroform:isomayl alcohol (24:1) and centrifuged at 14,000x g for 5 min at 4 °C. The upper aqueous phase was transferred to a 1.5 ml microcentrifuge tube, and RNA precipitated with 0.1 x volume of 3 M sodium acetate (pH 4.5) and 2 x volume of ice-cold ethanol. Samples were incubated at -20 °C for 30 min and centrifuged at 14,000x g at 4 °C for 20 min. Pelleted RNA was washed in 150 µL 70% (v/v) ethanol and centrifuged at 14,000x g at 4°C for 20 min. The ethanol was aspirated and the pellets air-dried for 5 min. Pellets were then resuspended in 87.5 µL of nuclease-free water.

2.4.2.2 RNA extraction using the Modified Burgmann method

Extractions were performed using an adapted version of an established protocol (Burgmann *et al.*, 2003; Pratscher *et al.*, 2011). 0.5 g of soil was transferred to lysing matrix E tubes, to which 1 ml of SDS extraction buffer (2.5 % (w/v) SDS, 200 mM sodium phosphate pH 8.0, 100 mM NaCl, 50mM EDTA pH 8) was added. The samples were lysed through bead beating in a Fast Prep bead beating machine run at 5.5 m/s for 45 seconds. Samples were centrifuged at 14,000x g for 5 min at 4 °C. The supernatant was transferred to 2 ml microcentrifuge tubes and 850 µL of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8) added. The samples were mixed by inversion and centrifuged at 14,000x g for 5 min at 4 °C. The supernatant was transferred to a new 2 ml microcentrifuge tube and 800 µL of chloroform:isoamyl alcohol (24:1) added. The samples were mixed by inversion and centrifuged at 14,000x g for 5 min at 4°C. The supernatant was transferred to a new 2 ml microcentrifuge tube and 1 ml of precipitation solution (20 % polyethylene glycol, 1.6 M NaCl solution) added. Samples were incubated at room temperature for an hour and centrifuged for 30 min at 14,000x g at 20 °C. The supernatant was discarded and the pellet washed using cold 75% (v/v) ethanol. The samples were centrifuged for 10 min at 14,000x g at 4 °C. The ethanol was aspirated and the samples air dried for five min. Pellets were resuspended in 100 µL of nuclease-free water.

2.4.3 Processing and storage of DNA

The quality of extracted DNA was visualised using agarose gel electrophoresis (Section 2.5.1). DNA was quantified using 1 µL on an ND-1000 Nanodrop (Nanodrop Technologies Inc., DE, USA) or using a broad range DNA assay for Qubit fluorometric quantitation

(ThermoFisher). DNA aliquots were stored long term at -20 °C and were kept on ice when out of storage.

2.4.4 Processing and storage of RNA

The quality of RNA extracted was visualised using agarose gel electrophoresis (Section 2.5.1). RNA aliquots were treated using DNAse I and RNeasy columns (Quiagen, Germany), per manufacturer's instructions detailed below (Table 2.3).

Table 2.3 Protocol for DNase treatment of RNA

DNase Treatment	Volume for a single reaction (µL)
RNA solution	<87.5
Buffer RDD	10
DNase I stock solution	2.5
Water to 100 µL	
10 min at 20-25 °C	
Buffer RLT (+ 2-mercaptoethanol)	350
Ethanol	250
Transfer the sample to an RNeasy Mini spin column in a collection tube	
Centrifuge for 15 seconds at 8,000x g and discard the flow through	
Buffer RPE	500
Centrifuge for 15 seconds at 8,000x g and discard the flow through	
Buffer RPE	500
Centrifuge for 120 seconds at 8,000x g and discard the flow through	
Transfer the RNeasy Mini spin column to a 1.5 ml microcentrifuge tube	
RNase free water	30
Centrifuge for 60 seconds at 8,000x g	
Rnase free water	30
Centrifuge for 60 seconds at 8,000x g	

RNA was quantified by Qubit fluorometric quantitation, using 2-5 µL with a high-sensitivity RNA assay. RNA aliquots were stored long term at -80 °C and were kept on ice when out of storage.

2.4.5 Genomic DNA extraction

Genomic DNA from bacterial cultures was extracted using a CTAB based technique (Ausubel *et al.*, 2003). DNA was extracted from 50 ml of culture, with cells pelleted through centrifugation at 10,000x g for 10 min at 20 °C. Pelleted cells were resuspended in 567 µL of Tris-EDTA (TE). 30 µL proteinase K in SDS (10 mg ml^{-1} proteinase K, 0.5 % (w/v) SDS) and 7 µL RNase A (10 mg / ml) was added to the samples, which were then incubated for one hour at 37 °C. 100 µL of 5 M NaCl and was added and the samples mixed by pipetting. 80 µL of CTAB/NaCl solution (10 % CTAB in 07 M NaCl) was added, the sample inverted and incubated for 10 min at 65 °C. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the mixture. The samples were mixed through shaking and then incubated at 60 °C for 10 min. The samples were mixed and centrifuged at 8,000x g for 5 min. The supernatant was transferred to a new tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added. The samples were centrifuged at 8,000x g for 5 min and the supernatant transferred to a new tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the tubes, which were centrifuged at 8,000x g for 5 min.The supernatant was transferred to a new tube and 0.6 x vol isopropanol added to precipitate the DNA. The tube was centrifuged at 17,000x g for 5 min at 20 °C. The supernatant was discarded and the pellet washed in 70 % (v/v) ethanol. The tubes were then centrifuged at 17,000x g for 15 min, the ethanol aspirated and the DNA pellets were air-dried for 10 min. The pellets were resuspended in 100 µL of nuclease-free water.

2.4.6 Extraction of RNA from pure culture

RNA was extracted from 50 ml of culture. Cells were pelleted by centrifugation and processed using the hot-phenol RNA extraction technique (Section 2.4.2.1).

2.5 Nucleic acid manipulation techniques

2.5.1 Agarose gel electrophoresis

Extracted nucleic acids and PCR products were analysed using gel electrophoresis. Samples were combined with 6 x loading dye (30 % (v/v) glycerol, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF), and loaded into 1 % (w/v) agarose gels containing ethidium bromide (0.5 µg ml^{-1}) in 1 x TBE buffer. GeneRuler 1kb DNA ladder (ThermoFisher) was used for estimation of product size and nucleic acid integrity. Agarose gels were analysed and imaged using the Bio-Rad Gel Doc XR imager (Bio-Rad, CA, USA).

2.5.2 Polymerase chain reaction (PCR)

Amplification of specific products through PCR was performed in 25 µL or 50 µL reaction volumes. The PCR machine used was a BIORAD Tetrad 2 peltier thermal cycler. The reaction mixture was 1 x Master Mix (PCR BIO, United Kingdom), 0.4 µM forward primer and 0.4 µM reverse primer. The non-template control for all PCR reactions was the nuclease-free water used in the Master Mix.

Table 2.4 PCR primers used in this study

Primers	Target gene	Sequence	Reference	Positive Control	Amplicon length (bp)	Annealing temperature (°C)	Cycles
27F	16S rRNA gene	AGAGTTTGATCMTGGCTCAG	(Lane 1991)	Any bacterial DNA	1465		
1492R		TACGGYTACCTTGTTAGGACTT					
341F-GC		CGCCCGCCGCGCGCGGCGGGCGGGGCGGG	(Muyzer *et al.*, 1993)		177	70-60 Touchdown	30
		GGCACGGGGGGCCTACGGGAGGCAGCAG					
518R		ATTACCGCGGCTGCTGG					
27F MOD		AGRGTTTGATCMTGGCTCAG			492	60	30
519R MODBIO		GTNTTACNGCGGCKGCTG					
520F		AYTGGGYDTAAAGNG	(Klindworth *et al.*, 2013)	*Eschericha coli* Top 10	282	57	40
802R		TACNVGGGTATCTAATCC					

xoxF1F	*xoxF1*	TAYGCCGAYGGCAAGSTGST	(Taubert *et al.*, 2015)	*Methylocella silvestris* BL2	600	65- 55 Touchdown	30
xoxF1R		CCGTCRTARTCCCAYTGRTCGAA					
xoxF2F	*xoxF2*	GGCYTAYCAGATGACBCCNTGG		Confirmed *xoxF2* containing clone	620	62- 52 Touchdown	30
xoxF2R		GCCTTRAACCAKCCRTCCA					
xoxF3F	*xoxF3*	GGHGAGWCCATSACVATGGC		*Methylocella silvestris* BL2	1000	62- 52 Touchdown	30
xoxF3R		TCCATSGTKCCGTAGAA					
xoxF4F	*xoxF4*	TTYCCHAAYAACGTNTAYGC		*Methylobacillus flagellatus* KT	660	58- 48 Touchdown	30
xoxF4R		GGRTTRCCHGTHCCGTAGTA					
xoxF5F	*xoxF5*	GAYGAVTGGGAYTWYGACGG		*Methylocella silvestris* BL2	370-390	62- 52 Touchdown	30
xoxF5R		GGYTCVTARTCCATRCA					
1003F	*mxaF*	GCGGCACCAACTGGGGCTGGT	(Neufeld, *et al.,* 2007b)	*Methylobacterium extorquens* AM1	552	65- 55 Touchdown	30
1555R		CATGAABGGCTCCCARTCCAT					
mauAF1	*mauA*	ARKCYTGYGABTAYTGGCG	(Neufeld, *et al.,* 2007b)	*Methylobacterium extorquens* AM1	310	50	30
mauAR1		GARAYVGTGCARTGRTARGTC					
557F	*gmaS*	GARGAYGCSAACGGYCAGTT	(Chen 2012)	*Methylocella silvestris* BL2	775	60-55 Touchdown	30
1332R		GTAMTCSAYCCAYTCCATG					

M13F	Insert-flanking regions of pGEM®-T Easy Vector	GTAAAACGACGGCCAG	Invitrogen		Insert size + 200bp	56	35
M13R		CAGGAAACAGCTATGAC					
mdh2F	*mdh2*	TGGCAGACCGCSTCGTTCGA	This work	*Methyloversatilis discipiluorum* LF1	516	52	35
mdh2R		CAGTTGGTGCCGCCSAGGAA					

2.5.2.2 Reaction mixtures and protocols

Table 2.5 Reaction conditions for PCR

Component	Stock Concentration	Final concentration	Volume in 25 µL reaction	Volume in 50 µL reaction
Forward primer	10 µM	0.4 µM	1	2
Reverse primer	10 µM	0.4 µM	1	2
PCR BIO mastermix	2 x	1 x	12.5	25
Nuclease free water	-	-	10	20
DNA	>20 ng/µL	5 - 80 pg	0.5	1

2.5.2.1 Optimisation of PCR amplification of mxaF and xoxF genes

The established amplification protocols (Taubert *et al.,* 2015) were used for the initial screening of environmental DNA samples with the *xoxF*1-5 and *mxaF* primers. Secondary bands were produced during the amplification of *mxaF* and *xoxF1* genes from DNA extracted from the CF soil. An optimised touchdown PCR protocol was developed which reduced but did not eliminate non-specific product formations, so gel extraction was necessary to allow sequencing and cloning of amplified *xoxF1* genes.

The majority of the amplified products were confirmed to belong to the correct clade through the construction of small clone libraries and Sanger sequencing (5 sequences per library). The sequencing of the cloned products confirmed an issue with cross-specificity for each of the primer sets. The degree of cross-specificity varied between the primer sets and is detailed further below (Table 2.6). However, the PCR primers could amplify sequences of the correct clade of methanol dehydrogenase, and the PCR products from the CF soil DNA were therefore sequenced using the Roche 454 platform.

Table 2.6 Cross specificity of the *mdh2, xoxF* and *mxaF* PCR primers

Primers	Products						
	xoxF1	*xoxF2*	*xoxF3*	*xoxF4*	*xoxF5*	*mxaF*	*mdh2*
xoxF1	✓	✓			✓		
xoxF2	✓	✓					✓
xoxF3	✓	✓	✓				✓
xoxF4				✓	✓	✓	
xoxF5	✓	✓	✓	✓	✓	✓	
mxaF					✓	✓	
mdh2							✓

2.5.2.3 mdh2 primer design

Primers were designed to amplify *mdh2* methanol dehydrogenase genes (Kalyuzhnaya *et al.*, 2008). These primers were based on conserved regions in *mdh2* gene sequences. The muscle algorithm in MEGA6 was used to align five *mdh2* sequences at the amino acid level (Chapter 3) in order to identify conserved regions. The alignments were manually searched at the nucleotide level for 15-20 nucleotide regions, allowing for a maximum of three degenerate nucleotides. The *mdh2* gene sequences used for the alignment were selected as they were confirmed to encode functional methanol dehydrogenases (See Introduction and Chapter 3). A gradient of annealing temperatures was initially used for optimisation of the protocol for PCR amplification of *mdh2* genes. The primers designed to amplify the *mdh2* gene were tested for specificity through PCR on DNA extracted from a range of isolates and DNA extracted from environmental samples (See Chapter). The environments tested include Church Farm soil, pea rhizosphere soil, water from the Norfolk Broads and landfill soil. Amplified products of ~500bp were used to produce clone libraries (Section 2.5.5), which were then screened through RFLP (Section 2.5.7).

2.5.3 Quantitative PCR

The copy number of 16S rRNA, *mxaF* and *xoxF5* genes in DNA and cDNA samples was estimated using quantitative PCR (qPCR). The qPCR machine was an Applied Biosystems Step one plus real-time PCR system (ThermoFisher, MA, USA). Primers and reaction volume are listed in Tables 2.7 and 2.8.

Table 2.7 Reaction set-up for qPCR

Component	Stock Concentration	Final concentration	Volume (12.5 µL)	Volume (25 µL)
Forward primer	10 µM	0.4 µM	0.5	1
Reverse primer	10 µM	0.4 µM	0.5	1
SYBR Green PCRBIO 2x Taqmix (ThermoFisher)	2x	1x	6.25	12.5
BSA			0.1	0.2
Nuclease free water	-	-	5	10
DNA	-	-	0.25	0.5

Table 2.8 Amplification protocol for qPCR

Temperature (°C)		Time (seconds)		Stage	Cycle number
16s rRNA genes	*mxaF* and *xoxF5*	16s rRNA genes	*mxaF* and *xoxF5*		
96	95	600	180		
96	95	30	55	Cycling	40 x
52	55	30	10		
72	72	60	20		
96	95	15	15	Melt curve	
75	60	60	60		
95	95	10	15		

2.5.3.1 Optimisation of the xoxF5 and mxaF qPCR assays

The qPCR assays for the quantification of *xoxF5* and *mxaF* copy number were tested using *xoxF5* and *mxaF* PCR products amplified from DNA extracted from *Methylocella silvestris* BL2. These PCR products were purified and diluted to produce a series of standards with a copy number of 10^8 to 10^1 per µL. These standards were made from a frozen stock of 10^9 copy number per µL.

After the initial tests of each qPCR assay, the reaction mixtures were amended to increase the concentration of primer used in the assay (400 nM) and BSA was also added in order to improve the efficiency of amplification for both genes. The efficiency of the amplification was increased to 98% for *mxaF* and 83% for *xoxF5*. These samples were used as standards in further qPCR assays with environmental DNA.

2.5.4 Reverse transcription of RNA

RNA was reverse transcribed to cDNA to enable further molecular analysis. The protocol is detailed below (Table 2.9). Superscript III reverse transcriptase was used throughout.

Table 2.9 Reaction set-up and protocol for reverse transcription

	Volume for a single reaction (µL)
Random primers (200 ng)	0.4
10 mM dNTP mix	1
RNA	5
Water to 13 µL	
65°C for 5 min	
Ice 1 minute	
5 x F5 buffer	4
0.1M DTT (5 mM)	1
Superscript III reverse transcriptase (Thermofisher)	1
Protector RNase inhibitor (Sigma Aldrich)	1
25°C for 5 min	
50°C for 45 min	
70°C for 15 minute	

2.5.5 Cloning of PCR products

All cloning was performed with the Promega pGEM-T Easy vector system according to manufacturer's instructions. PCR products were ligated into the pGEM-T Easy vector using T4 DNA ligase. The concentration of PCR product used in the ligation reaction was

altered to be in a 1:1 ratio with the vector. The final volume of the ligation reaction was equalised to 10 µL per sample using nuclease-free water. Ligation reactions were left at room temperature for one hour or overnight at 4 °C.

Ligated vectors were transformed into *Escherichia coli* TOP10 cells using heat shock (55 °C for 50 sec), and were spread onto LB plates supplemented with ampicillin (100 µg/ml) and XGAL (80 µg/ml). Using white/blue selection colonies containing vectors with inserts were picked for colony PCR with the M13F and M13R primers (Section 2.5.2). Picked colonies were patched onto new LB plates supplemented with ampicillin and X-GAL.

2.5.6 Purification and Gel extraction of PCR products

PCR products were purified using NucleoSpin Gel and PCR clean-up columns (Macherey-Nagel, Germany) according to manufacturer's instructions. For gel extraction of DNA, the PCR products were loaded onto an agarose gel and ran for sufficient time to separate the band of interest from other bands. The band of interest was then excised with a sterilised razor blade. DNA excised from agarose gels was purified using the recommended instructions for the Nucleospin columns. PCR products were alternatively purified using the PEG:NaCl precipitation technique (Section 2.8.2).

2.5.7 RFLP analysis of cloned PCR products

Cloned PCR products of interest were amplified through PCR using the M13 primers (Section 2.5.5). These PCR products were purified using NucleoSpin columns (Section 2.5.6), and the purified PCR products digested using restriction enzymes. Selection of restriction enzymes was based on the online tool NEBCUTTER (http://nc2.neb.com/NEBcutter2/) (New England Biolabs, MA, USA). The restriction enzymes used are detailed in table 2.10. RFLP profiles were analysed through gel electrophoresis using 2% agarose gels.

Table 2.10 Restriction enzymes for RFLP profiling of cloned PCR products

Gene	Restriction enzymes		
xoxF1	*Rsa*I	*Cla*I	*Eco*RI
xoxF3	*Rsa*I	*Alu*I	
xoxF4	*Rsa*I	*Hin*cII	*Eco*RI
xoxF5	*Rsa*I	*Hin*cII	*Eco*RI
mxaF	*Rsa*I	*Hin*cII	*Eco*RI
mdh2	*Hin*dIII		*Eco*RI

2.5.8 Sanger sequencing of PCR products and bioinformatic analysis

Purified PCR products were sent for Sanger sequencing by the companies Source Bioscience (United Kingdom) or MWG Eurofins (Germany). Products were diluted to 1 ng/µL per 100 bp sequence length. Chromatograms of sequences were analysed using Bioedit (Hall, 1999, 2011) to assess sequence quality. Sequences were analysed and aligned using the program MEGA6 (Tamura *et al.*, 2013). High-throughput sequencing of the *mxaF* gene amplified from DNA extracted from the wheat rhizosphere and CF soil collected in 2015 failed, so the diversity of this gene in these particular environments could not be characterised.

2.5.9 Next generation sequencing of PCR products and bioinformatic analysis

Purified PCR products were sent for sequencing by 454 (3,000 reads) and Illumina Hiseq (20,000 reads) by the company Molecular Research LP (Texas, USA). 16S rRNA genes were processed by Molecular Research LP through their independent pipeline.

Reads of sequenced functional genes were analysed using a modified version of a published protocol (Taubert *et al.*, 2015). SFF files were processed using Mothur (Schloss *et al.*, 2009) to convert the raw files into flowgrams, which were then translated to nucleotide sequences. Sequences of poor quality were identified and removed. USEARCH (Edgar *et al.*, 2011) was used on the files for the identification and removal of chimeric sequences. Sequences were clustered into OTUs using USEARCH (Edgar, 2010),

using similarity values of 80% and 90%. Using the program MEGA6 and MEGA7, OTUs were aligned using the Muscle algorithm against a database containing representative sequences from different clades of PQQ dehydrogenase (Keltjens *et al.*, 2014). OTUs which clustered with the correct clade were re-aligned at the amino acid level using a database of sequences specific to that clade. OTUs which clustered with an incorrect clade were removed from subsequent phylogenetic analysis. This was performed for the OTUs at an 80% level of similarity.

Phylogenetic trees were then produced using the alignment of the OTUs clustered at the 80% level of similarity. Phylogenetic trees were produced using the maximum likelihood and neighbour joining algorithms with bootstrap values of 500 in order to assign phylogeny to the sequences.

There were issues with sequence quality following 454 sequencing of the *xoxF3* amplicon and the *xoxF3* data was not of sufficient quality to study the diversity of the *xoxF3* gene within this environment. 1,459 sequences were retained following quality control, but over 1,000 of these sequences were either *xoxF1* sequences or were not PQQ methanol dehydrogenase sequences. Furthermore, all identified *xoxF3* sequences were below 100 bp in length. A clone library of 100 clones made from the *xoxF3* PCR product amplified from the DNA extracted from the CF soil was screened through RFLP to assess the diversity of this gene in this environment. Representatives of each profile were then sent for sequencing.

2.5.10 Genome sequencing and analysis

Genome sequencing was provided by MicrobesNG (http://www.microbesng.uk), which is supported by the BBSRC (grant number BB/L024209/1). Cultures of *Variovorax paradoxus* MM1, *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3 were sent for sequencing. The sequencing was performed using the Illumina Miseq platform, producing paired-end reads 250bp in length. Trimmed sequences were assembled using SPAdes version 3.7.1 (Bankevich *et al.*, 2012) by Microbes NG. Genome annotation was performed using the RAST annotation server (Aziz *et al.*, 2008; Brettin *et al*., 2015.; Overbeek *et al.*, 2014). The genomes were also screened for genes of interest using local Blast against a nucleotide database constructed from the genome sequence. KEGG (Kyoto Encyclopaedia of genes and genomes) maps produced by Blast

Koala were also used to direct further analysis and assess the metabolic capability of the strains.

2.5.11 Denaturing gradient gel electrophoresis (DGGE)

16S rRNA genes were amplified using PCR (Section 2.5.2). The forward primer used in the amplification has a GC clamp (Table 2.4). A 10% (w/v) polyacrylamide gel with a 30-70% (w/v) linear denaturant gradient was produced (Table 2.11), with a top up gel of 0% denaturant. The amplified 16S rRNA gene PCR products were combined with 6 x loading dye, and loaded into the wells of the top up gel.

Table 2.11 Composition of solutions for denaturing gradient gels

50 ml solution	10% acrylamide gel		Stacking gel
Linear denaturant gradient	30%	70%	0%
40% (w/v) acrylamide/bis (37:5:1) (ml)	12.5	12.5	0.75
50X TAE (ml)	1	1	0.1
Formamide (ml)	6	14	-
Urea (g)	6.3	14.7	-
ddH_2O (ml)	To 50 ml		4.1
10% (w/v) Ammonium persulfate (µL)	500	500	50
Tetramethylethylenediamine (TEMED) (µL)	50	50	5

The electrophoresis system was run using 1 x TAE as a buffer. Electrophoresis was performed at 75 V, with a maximum current of 200 mA for 16 hours, and the tank heated constantly at 60 °C. Gels were stained in 400 ml of 1 x TAE buffer with 4 µL of SYBR Gold Nucleic Acid Gel stain for one hour in the dark. After staining, the gels were washed using ddH_2O, and imaged using a Bio-Rad Gel Doc XR imager. Bands of interest were stabbed with a sterile pipette tip, which was left overnight in nuclease free water. This sample was used as a template for a PCR reaction using the 341F-GC and 518R primers (Section 2.5.2).

2.5.12 Metagenome sequencing and analysis

DNA from the heavy fractions of a SIP experiment (Section 2.8.2) was pooled, quantified, and sent for metagenome sequencing by the Centre for Genomic Research at the University of Liverpool. Sequencing was performed using paired-end sequencing (2 x 150 bp) on an Illumina Hiseq 4000. Subsequent bioinformatic processing of the metagenomes was performed by Dr. Jennifer Pratscher. Short sequences and sequences of poor quality were excluded from the files using the program Trimmomatic (Bolger *et al.*, 2014). Trimmed metagenomes were phylogenetically analysed using the program Metaphlan (Segata *et al.*, 2012). The metagenome sequences were assembled into contigs using the program Megahit (Li *et al.*, 2015) and annotated using myRast (da Rocha *et al.*, 2009). The metagenome sequences were then binned into genomes using MetaBat (Kang *et al.*, 2015).These files were also used to create blast databases. The tblastn function of blast+ was used to run amino acid sequences from proteins of confirmed function against the sequences. Contigs containing genes of interest were then annotated and matched to the corresponding binned genomes.

2.6 Gas chromatography

2.6.1 Measurement of methanol using gas chromatography

Methanol in the headspace of serum vials was measured by gas chromatography (GC) on an Agilent 7820A instrument, using a flame ionisation detector, a Poropak Q column (6 ft x 1/8 ” x 2.1 mm film) and nitrogen carrier gas. The following settings were used:

Injector temperature: 300 °C

Detector temperature: 300°C

Column temperature: 115 °C

Oven temperature: 115 °C

The injection volume was initially 100 µL, but this was increased to 250 µL to increase sensitivity. The run time of the protocol was four min, with the retention time of methanol at 2.9 min. Standards were prepared in 120 ml serum vials and the same media or water as used in the relevant experiment. The detection limit for methanol was around 100 µM.

2.6.2 Measurement of carbon dioxide by gas chromatography

Carbon dioxide was measured by gas chromatography (GC) on an Agilent 7890A instrument, using a flame ionisation detector, a Poropak Q column (6ft x 1/8") HP plot/Q (30 m x 0.530 mm, 40 µM film), a nickel catalyst, and nitrogen carrier gas. The following settings were used:

Injector temperature: 250 °C

Detector temperature: 300 °C

Column temperature: 115 °C

Oven temperature: 50 °C

The injection volume was 250 µL. The run time was five min, with the retention time of carbon dioxide at 3.4 min. Standards were prepared in 120 ml serum vials that were flushed with nitrogen.

2.7 Enrichment of methylotrophs from Church Farm soil

2.7.1 Enrichment of methylotrophs from Church Farm soil with methanol and lanthanides

Enrichments were established with 5 g of Church farm soil in 5 ml of 1% dNMS in 120 ml serum vials. The enrichments were supplied with 3 mM methanol. Enrichments were established in triplicate and were supplemented with either 5 µM lanthanum chloride, 5 µm cerium chloride, or no lanthanides. The concentration of methanol in the aqueous solution was inferred through measuring the concentration of methanol in the headspace of the cultures and a series of controls using gas chromatography (Section 2.7.1). Following depletion of the methanol, DNA was extracted from the soil samples (Section 2.3.1). 16S rRNA genes were amplified through PCR for 16S rRNA gene DGGE profiling (Section 2.5.12).

2.7.2 Identification of active methylotrophs in Church Farm soil using DNA stable isotope probing and ^{13}C methanol

Wheat and pea plants and unplanted controls were kept under long day growth conditions in Church Farm soil for four weeks before collection of soil from the rhizosphere of each plant (Section 2.3.2). 2 g of soil from each environment and 40 ml of autoclaved ddH_2O were aliquoted into serum vials. The serum vials were then

supplemented with 250 µM ^{13}C methanol and sealed. Parallel enrichments with ^{12}C methanol were established. The enrichments were done in triplicate. The serum vials were incubated at 30°C, without light, in a shaking incubator (120 rpm). The concentration of methanol in the headspace of the serum vials was measured using gas chromatography (Section 2.7.1). After depletion of methanol, samples were resupplied with 250 µM of methanol. After six days, samples were harvested from the enrichment for DNA extraction (Time point 1). 1 ml of standard dNMS was supplied to the serum vials on day seven and the experiment continued. After 17 days 200 µmol of ^{13}C had been consumed by all test groups. Soil was collected for DNA extraction (Time point 2). This is expected to be equivalent to the incorporation of 50 µmol of carbon per gram of soil (Chen and Murrell, 2010). DNA was extracted from all harvested soil samples (Section 2.4.1).

16S rRNA genes were amplified by PCR (Section 2.5.2), using DNA extracted from all test groups from the methanol enrichment series. The 16S rRNA gene profile of each sample was then analysed by 16S rRNA gene DGGE profiling. Bands of interest were picked from the DGGE gel, amplified by PCR and sent for Sanger sequencing.

DNA was ultracentrifuged and fractionated in order to separate the ^{13}C and ^{12}C labelled DNA according to the established protocol (Neufeld *et al.,* 2007c). Briefly, a cesium chloride (CsCl) solution of 7.163 M with a density of 1.88-1.89 g ml^{-1} was prepared. The amount of DNA and gradient buffer required to achieve the desired density of 1.725 g ml^{-1} when combined with the CsCl was calculated, and the corresponding volumes of each added to 4.8 ml of CsCl for each sample. The refractive index of the solution was measured using a refractometer (Reichert Analytical Instruments, NY, USA) calibrated with nuclease-free water. The desired refractive index nD-TC value was 1.4038. The density and nD-TC values were adjusted through the addition of gradient buffer and CsCl. The mixtures were loaded into pollyallomer quickseal centrifuge tubes, which were then heat-sealed and loaded into a VTI 65.2 rotor. Tubes were loaded and weighed to be balanced to within 10 mg. The rotor was loaded into a Beckman Optima XL-100K ultracentrifuge and run at 44,100x g for a minimum of 38 hours at 20°C. Deceleration was set to no brakes to prevent the gradient being disturbed. For each T2 sample processed, 4ug of DNA was loaded for ultracentrifugation. DNA from each replicate

within a test group from T1 sample was pooled to provide sufficient DNA for ultracentrifugation and fractionation. The amount of DNA loaded for T1 samples for ultracentrifugation varied from 0.5-2ug. Representative ^{13}C and ^{12}C samples for each environment and time point were processed through ultracentrifugation and fractionation.

The ultracentrifuged samples were processed through gradient fractionation. This process involves running autoclaved ddH_2O through a tube using a peristaltic pump calibrated to run at ~425 µL per minute. A 0.6mm needle was attached to the tube using a connector. This needle was inserted into the top of ultrancentrifuge tubes secured into a clamp stamp. The underneath of each tube was also pierced with a sterile needle. After activating the pump, the CsCl:DNA mixture was collected in 1.5 ml microcentrifuge tubes. The tube was changed every minute, collecting the sample across 12 1.5 ml microcentrifuge tubes. The refractive index of 40 µL of each fraction was measured using a refractometer.

DNA was precipitated in each fraction with 20 µg of linear polyacrylamide and 900 µL polyethylene glycol-NaCl solution (30% (w/v) polyethylene glycol, 1.6M NaCl solution). Tubes were inverted to mix, and incubated at room temperature overnight. The tubes were centrifuged at 14,000x g for 30 min. The supernatant was discarded, and the pelleted DNA washed with 400 µL 70 % (v/v) ethanol. The tubes were centrifuged at 14,000x g for 10 min, and the ethanol aspirated. The DNA pellets were air-dried for 10 min before being resuspended in 36 µL of nuclease-free water.

The concentration of DNA in each fraction was measured through fluorescence (Qubit, Invitrogen, UK). 16S rRNA genes were amplified using PCR and primers for DGGE (Section 2.5.2 and 2.5.11) from all of the fractions from each processed test group (unplanted soil, pea, and wheat rhizosphere soil), and time point (T1=seven days, T2=twenty days). A series of 16S rRNA gene DGGE profiles (Section 2.5.11) were produced using these 16S rRNA gene PCR products to compare the diversity of the bacterial communities between the time points and between the different environments.

16S rRNA genes were amplified using PCR (Section 2.5.2) from the heavy and light DNA fractions of each ^{13}C methanol and ^{12}C methanol enriched test group. These PCR products were purified and sent for sequencing by Molecular Research LP (USA). DNA from the heavy fractions of the T2 samples was pooled, quantified and sent for metagenome sequencing by the Centre for Genomic Research at the University of Liverpool (Section 2.5.12).

2.7.3 Identification of active methylotrophs in Church Farm soil using RNA stable isotope probing (RNA-SIP) and ^{13}C methanol

An enrichment was established with 10 g of Church Farm soil and 200 ml of autoclaved ddH_2O. The concentration of ^{12}C methanol supplied to two test groups was 2.5 µM and 250 µM. An additional test group was supplied with ^{13}C methanol at the concentration of 2.5 µM. A no substrate control was also established. Samples of soil were taken from each enrichment at three time points (six, twelve and twenty-four hours). RNA was extracted from the harvested soil samples using the Griffiths technique (Section 2.4.1). Superscript III reverse transcriptase was used to yield cDNA (Section 2.5.4). This cDNA was then used for 16S rRNA gene amplification and 16S rRNA gene DGGE profiling of each sample (Section 2.5.11).

RNA from the third time point for the 2.5 µM supplied test groups was prepared for ultracentrifugation and fractionation. Samples were prepared and processed according to established protocol (Whiteley *et al.*, 2007) in order to separate the ^{12}C and ^{13}C labeled RNA. For each sample, 4.5 ml of Cesium trifluoroacetate (CsTFA) (GE Healthcare Life Sciences, IL, USA) was combined with 197.5 µL formamide and 850 µL gradient buffer. The refractive index of the solution was measured as above (Section 2.8.2). The desired refractive index nD-TC value was 1.3725. 300-400 ng of RNA from each sample was loaded for ultracentrifugation. Following the addition of RNA to the mixture, the nD-TC value was adjusted through the addition of gradient buffer and CsTFA. The mixtures were loaded into centrifuge tubes and subsequently loaded into a rotor and ultracentrifuge as above (Section 2.8.2). The ultracentrifuge was run for 38,000x g for a minimum of 64 hours at 20°C. Deceleration was set to “no brakes” to prevent the gradient being disturbed.

The samples were processed through gradient fractionation as above (Section 2.8.2). The tube for the peristaltic pump was cleaned by running 100% ethanol through the tube prior to the nuclease free water. RNA was precipitated in all fractions with 0.1 x volume sodium acetate (3 M, pH 5.2), 20 µg glycogen, and 2 volumes of cold 96% (v/v) ethanol. Samples were incubated for 1 hour at room temperature or overnight at -20°C. Precipitated RNA was pelleted by centrifugation at 18,000x g for 30 min at 4°C. Pellets were washed with 150 µl of ice-cold 70% (v/v) ethanol, and centrifuged at 18,000x g for 15 min at 4°C. Pelleted RNA was air-dried for 5 min, and resuspended in 16 µl of nuclease free water. Superscript III reverse transcriptase was used to yield cDNA from each fraction (Section 2.5.4). This cDNA was then used for PCR amplification of 16S rRNA genes and 16S rRNA gene DGGE profiling (Section 2.5.11).

2.8 Identification of exudate utilising bacteria in the rhizosphere of pea and wheat plants using DNA and RNA stable isotope probing with $^{13}CO_2$

2.8.1 DNA-Stable isotope probing (DNA-SIP) of the rhizosphere of pea and wheat plants with $^{13}CO_2$ under short day length growth conditions

Pea and wheat plants were grown in Church Farm soil (collected in 2014) under short day growth conditions (8:16) for a total of 28 days. Unplanted controls were maintained in parallel to the growing plants. The plants and unplanted controls were in triplicate. 16 days after planting, one pea plant, one wheat plant, and one unplanted control were transferred to clear acrylic tubes of 4.75 L volume. The acrylic tubes were flushed with carbon dioxide depleted air, sealed with plastic lids, and $^{13}CO_2$ injected to a final concentration of 1000ppmv. This test group was pulsed for twelve days. The concentration of CO_2 in the tubes was monitored using gas chromatography (Section 2.7.2) in order to calculate the decline in CO_2 concentration over time. The concentration of CO_2 was maintained through the injection of $^{13}CO_2$ into the sealed acrylic tubes, and kept below 1000 ppmv to prevent harm to plants. At the end of each 8 hour light period the acrylic tubes were opened. Before the start of each light period, the tubes were flushed with carbon dioxide depleted air, resealed, and injected with $^{13}CO_2$. 22 days after planting another of each test group was transferred to acrylic tubes, and pulsed for six

consecutive days. The remaining plants and the unplanted control were left to grow in standard growth room conditions. After 28 days of growth, the pea plants, wheat plants, and unplanted controls were harvested (Section 2.3.2). The rhizosphere soil was collected for DNA extraction (Section 2.4.1). 4 μg of DNA for each sample was processed via ultracentrifugation and fractionation (Section 2.8.2).

2.8.2 DNA-Stable isotope probing (DNA-SIP) of the rhizosphere of pea plants with $^{13}CO_2$ under long day length growth conditions

Pea plants were grown in Church Farm soil collected in 2015. The pea plants were grown under long day growth conditions (16:8) for 16 days and medium day growth conditions for 12 days. 16 days from planting, eight pea plants and eight unplanted controls were transferred to acrylic tubes of 4.75 L volume. Remaining plants and unplanted control were left to grow under standard growth room conditions. All pea plants and unplanted controls were transferred to medium day light conditions (12:12). The acrylic tubes were flushed as above (Section 2.8.3.1). In duplicate, pea plants and unplanted controls were injected with either $^{13}CO_2$ or $^{12}CO_2$ to a final concentration of either 350 ppmv or 1000 ppmv. The concentration of CO_2 in the tubes was monitored and maintained as above (Section 2.8.2). The tubes were pulsed with CO_2 for 12 consecutive days. After 12 days the plants were harvested (Section 2.8.3.1) and DNA extracted from the rhizosphere soil (Section 2.4.1). 4 μg of DNA for each sample was processed via ultracentrifugation and fractionation (Section 2.8.2).

2.8.3 DNA and RNA-Stable isotope probing (DNA-SIP and RNA-SIP) of the rhizosphere of pea and wheat plants with $^{13}CO_2$ under medium day length growth conditions

Pea plants and wheat were grown in Church Farm soil collected in 2016. The plants were grown under medium day growth conditions (12:12) for 34 days. 22 days from planting, six pea plants, six wheat plants, and six unplanted controls were transferred to acrylic tubes of 4.75 L volume. Remaining plants and unplanted control were left to grow under standard growth room conditions. All test groups were in triplicate. The acrylic tubes were flushed with the carbon dioxide depleted air, sealed with plastic lids, and plants and unplanted controls injected with either $^{13}CO_2$ or $^{12}CO_2$ to a final concentration of 350 ppmv. The concentration of CO_2 in the tubes was maintained and monitored as

before (Section 2.8.2). The CO_2 concentration was monitored to ensure it did not exceed 400 ppmv. The tubes were pulsed for 12 consecutive days.

After 4 days of supplying CO_2 to the plants, the pulsing schedule of the wheat plants was altered to ensure the CO_2 concentration did not exceed 400 ppmv. The wheat plants were flushed with carbon dioxide depleted air every four hours, the tubes resealed, and the CO_2 reinjected to the concentration of 350 ppmv.

After 12 days the plants and unplanted control were harvested (Section 2.8.3.1) for RNA and DNA extraction (Section 2.4.1.) from the roots and rhizosphere soil. DNA from each rhizosphere sample was processed individually. RNA from the rhizosphere soil and DNA and RNA of the root samples was pooled prior to processing. 4 µg of DNA and 400 ng of RNA was processed for each sample via ultracentrifugation and fractionation (Section 2.8.2 and 2.8.3).

Chapter 3: Isolation, characterisation and genome sequencing and analysis of methanol-utilising methylotrophs

The ubiquitous nature of methanol in the soil environment results in the equally ubiquitous occurrence of methanol-utilising methylotrophic bacteria. There are multiple sources of methanol in the soil environment, with the primary source being the demethylation of pectin in plants through the action of pectin methyl esterase enzymes (80 – 250 Tg yr^{-1}) (Galbally *et al.*, 2002; Heikes 2002). An additional source of methanol in the terrestrial environment include the release of methoxy groups from the decomposition of lignin, pectin and additional compounds in decaying plant tissues (12-23 Tg yr^{-1}) (Heikes 2002; Millet *et al.*, 2008). Moreover, methylotrophs have been detected in the rhizospheres of several plant species (Madhaiyan *et al.*, 2010; Schreiner *et al.*, 2010; Madhaiyan *et al.*, 2013; Doronina *et al.*, 2015; Poroshina *et al.*, 2015). Due to the incomplete understanding of the methanol cycle with regards to the soil environment, the isolation and subsequent characterisation of methylotrophs is important to furthering our understanding of methanol utilisation in this environment. The culturing of isolated methylotrophs also helps to improve our understanding of the physiological capabilities of these organisms and methylotrophy in general. Further to this, genome sequencing of isolated methanol-utilising methylotrophs has led to characterisation of alternate metabolic pathways involved in C1 metabolism and obligate and facultative methylotrophy (Mustakhimov *et al.*, 2013; Kalyuhznaya *et al.,* 2009; Beck *et al.,* 2011; Anthony 1983). The primary aim of this work was to isolate methylotrophs that were either novel, or could be shown to be relevant to methanol oxidation in the Church Farm (CF) soil and the rhizospheres of plants grown in this soil (Chapter 4, 5, 6).

3.1 Sampling Site

As previously described (Chapter 2) the main collection site for all experiments was the CF site in Bawburgh (Norfolk, United Kingdom) (52.6276 N 1.1786 E). This is a John Innes Foundation owned farm, which is mostly used for the growth and characterisation of novel wheat cultivars. However, a small portion was left as unmaintained former grassland (Figure 3.1), and this is the section that soil was collected from. The soil at this site was previously analysed (Tkacz, 2013), and it was shown to be poor with regards to nutrients (NO_3^- 3.49 mg/kg, PO_4^{3+} 120.5 mg/kg, K^+ 168.2 mg/kg, Mg^{2+} 33.55 mg/kg). The

pH of the soil was shown to be neutral and with an amount of organic matter that is typical for grassland soil (2.92%).

Figure 3.1 The location of the soil collection site at CF, Bawburgh

3.2 Enrichment and isolation of methanol-utilising methylotrophs

dNMS and modified variants of dNMS were used throughout the enrichments as opposed to NMS and related media. dNMS was chosen as NMS and AMS consistently selected strongly for strains of *Hyphomicrobium* when used in preliminary studies.

3.2.1 Enrichment and isolation of methylotrophs using soil from CF using dNMS modified with lanthanides

Nine strains of bacteria were isolated using this enrichment regime (Section 2.2.3.1). 16S rRNA gene PCR amplification and sequencing was performed to provide phylogenetic information for each strain (Table 3.1).

Table 3.1 Organisms isolated from CF soil using dNMS supplemented with lanthanides

Isolate	16S rRNA gene closest match in NCBI nt database	Identity (%)	Growth on Methanol	Successful PCR amplification of functional genes		
				mxaF	*xoxF5*	*xoxF3*
MM1	[1]*Variovorax paradoxus* S110	99	+	-	+	-
CF2	[2]*Burkholderia terricola*	99	+	-	+	-
CF3	[3]*Hyphomicrobium denitrificans*	100	+	+	+	-
CF4	*Hyphomicrobium denitrificans*	100	+	+	+	-
CF5	*Hyphomicrobium denitrificans*	100	+	+	+	-
CF6	[4]*Dyadobacter fermentans*	99	-	-	-	-
CF7	[5]*Acinetobacter albensis*	98	-	-	-	-
CF8	[6]*Caulobacter flavus*	98	-	-	-	-
CF9	[7]*Flavobacterium breve*	98	-	-	-	-

([1]Satola *et al*., 2013; [2]Verlag, 2002; [3]Urakami *et al.,* 1995; [4]Chelius and Triplett 2000; [5]Krizova *et al.,* 2015; [6]Wei *et al.,* 2015; [7]Vandamme *et al.,* 1994)

Isolates CF6-9 did not grow on methanol as a sole carbon source. They also did not produce a positive PCR product with any of the *mxaF* or *xoxF* primer sets. These strains were therefore excluded from further analysis. Strains of *Flavobacterium* have previously been shown to grow in co-culture with methylotrophic bacteria as a result of cross feeding as opposed to utilising methanol as a carbon source (Kalyuzhnaya *et al.*, 2005; Hernandez *et al.*, 2015). Therefore it is possible these strains were enriched through cross feeding on the metabolic by-products of the methylotrophic bacteria present in the enrichment series. It is also possible that these strains were using the agar as a carbon source, indicating the necessity to test for growth on methanol in both liquid and solid media.

Burkholderia terricola CF2 had high (99 %) 16S rRNA gene identity to several species of *Burkholderia*. In order to assign this strain to a specific species of *Burkholderia,* its growth was tested on multiple carbon sources (Goris *et al.*, 2002, 2004). Based on the ability of this strain to grow on citrate, sucrose and lactose as carbon sources, CF2 was tentatively assigned to the species *Burkholderia terricola* (Goris *et al.*, 2002). Due to the high similarity of the *xoxF5* and 16S rRNA gene sequences of this strain to several known species of *Burkholderia* and the absence of the genus from the 16S rRNA gene profile of the CF soil, this strain was not characterised further. Additionally, based on the identical 16S rRNA gene sequence of the three *Hyphomicrobium* isolates to the species *Hyphomicrobium denitrificans* and the absence of this genus from the results of methanol SIP experiment (Chapter 5), these strains were also not characterised further.

Members of the genus *Variovorax* have previously been shown to be capable of methanol oxidation (Anesti *et al.*, 2005), with several genomes containing *xoxF5* methanol dehydrogenases. *Varivorax paradoxus* MM3 showed very high similarity to *Variovorax paradoxus* S110 at the 16S rRNA gene level (Satola *et al.*, 2013). *Varivorax paradoxus* consistently appeared in the 16S rRNA gene profile of the CF soil (Chapter 5) and as a member of the exudate utilisers in the pea rhizosphere (Chapter 6). *Varivorax paradoxus* MM3 was therefore characterised further (Section 3.5).

3.3 Characterisation of *Variovorax paradoxus* MM1

Due to the apparent relevance of the genus *Variovorax* to methanol oxidation in the rhizosphere environment (Chapter 5 and 6), the strain of *Variovorax paradoxus* isolated

from the CF soil (Section 3.2.1) was sent for genome sequencing. This genome adds to the growing list of *Variovorax paradoxus* genomes which are publicly available (16 at time of writing). The genome of *Variovorax paradoxus* MM1 has a genome size of 7.1 Mb with GC 67.2 Mol%. The other *Variovorax paradoxus* genomes vary in size between 6.5 – 9.6 Mb with GC 66.5 -69.2 Mol%. The genus *Variovorax*, and specifically the species *Varivorax paradoxus*, has been shown to be metabolically versatile (Kim *et al.*, 2006; Miwa *et al.*, 2008; Im *et al.*, 2010; Satola *et al.*, 2013; Brandt *et al.*, 2014). Strains have been isolated from varied environments, including marine and terrestrial, as well as pristine and contaminated (Anesti *et al.*, 2005; Kim *et al.*, 2006; Yoon *et al.*, 2006b; Miwa *et al.*, 2008; Im *et al.*, 2010; Jin *et al.*, 2012; Schreiter *et al.*, 2014). The organism and its diverse metabolism makes it an ideal study system for the degradation of several compounds (Satola *et al.*, 2013; Brandt *et al.*, 2014). Plant growth promoting traits have also been shown to be present in the species (Han *et al.,* 2011; Satola *et al.,* 2013).

3.3.1 General metabolic pathways

Variovorax paradoxus MM1 represents a facultative methylotroph, as it is able to utilise C1 and multicarbon compounds for growth and energy (Anthony 1983). *Variovorax paradoxus* MM1, and additional methylotrophic strains of *Variovorax paradoxus*, can be further classified as a less restricted facultative methylotroph due to the broad range of substrates on which they are able to grow.

Similar to the genomes of other sequenced *Variovorax paradoxus* species (Satola *et al.*, 2013; Brandt *et al.*, 2014), the genome of *Variovorax paradoxus* MM1 contains genes which encode for a complete TCA cycle. The genome also contains all of the genes required for assimilation of formaldehyde through the serine cycle. All of the genes comprising the complete Calvin-Benson-Bassham cycle for the assimilation of carbon from carbon dioxide are also present within the genome. The genome also contains genes that encode for the glyoxylate shunt, possessing both an isocitrate lyase and a malate synthase. In addition to its role in two-carbon assimilation this, or the alternative EMC pathway, is essential for the regeneration of glyoxylate in methylotrophs that utilise the serine cycle (Korotkova *et al.*, 2002; Chistoserdova *et al.*, 2009; Peyraud *et al.*, 2011; Keltjens *et al.*, 2014).

The genome of MM1 contains one copy of a *xoxF* methanol dehydrogenase gene. The sequence of this gene was aligned at the amino acid level with a database of *xoxF* sequences to identify the clade of this methanol dehydrogenase. The sequence clustered with *xoxF5*, the most genetically diverse and phylogenetically distributed of the five clades of *xoxF* methanol dehydrogenase (Keltjens *et al.*, 2014). The strain with the highest identity to MM1 at the 16S rRNA gene level, *Variovorax paradoxus* S110, possesses a *xoxF3* and a *xoxF5* gene. Due to the draft nature of the genome of MM1, which does not contain a *xoxF3*, DNA extracted from MM1 was used as template in a PCR assay to confirm the absence of a *xoxF3* gene in this organism and no product was obtained. Therefore, it is presumed that this strain of *Variovorax paradoxus* only has a *xoxF5*. The *xoxF5* sequence has high identity (96-99%) to *xoxF5* genes encoded in the genomes of five other strains of *Varivorax* (Figure 3.2). The genetic region upstream and downstream of the *xoxF5* methanol dehydrogenase encoding gene is conserved between the genomes of *Variovorax paradoxus* MM1, S110 and B4 (Figure 3.3). This region includes accessory genes known or predicted to play a role in methanol dehydrogenase formation (Keltjens *et al.*, 2014). In addition, the genes that encode ribulose-1,5-bisphosphate, the key enzyme of the CBB pathway, are upstream of the *xoxF5* gene. However, the question of whether expression of this enzyme could be linked to the expression of *xoxF5*, and the relative contribution of the serine cycle and CBB cycle to the growth of MM1 when grown on methanol as a carbon source would be need to be validated through further physiological characterisation of this strain.

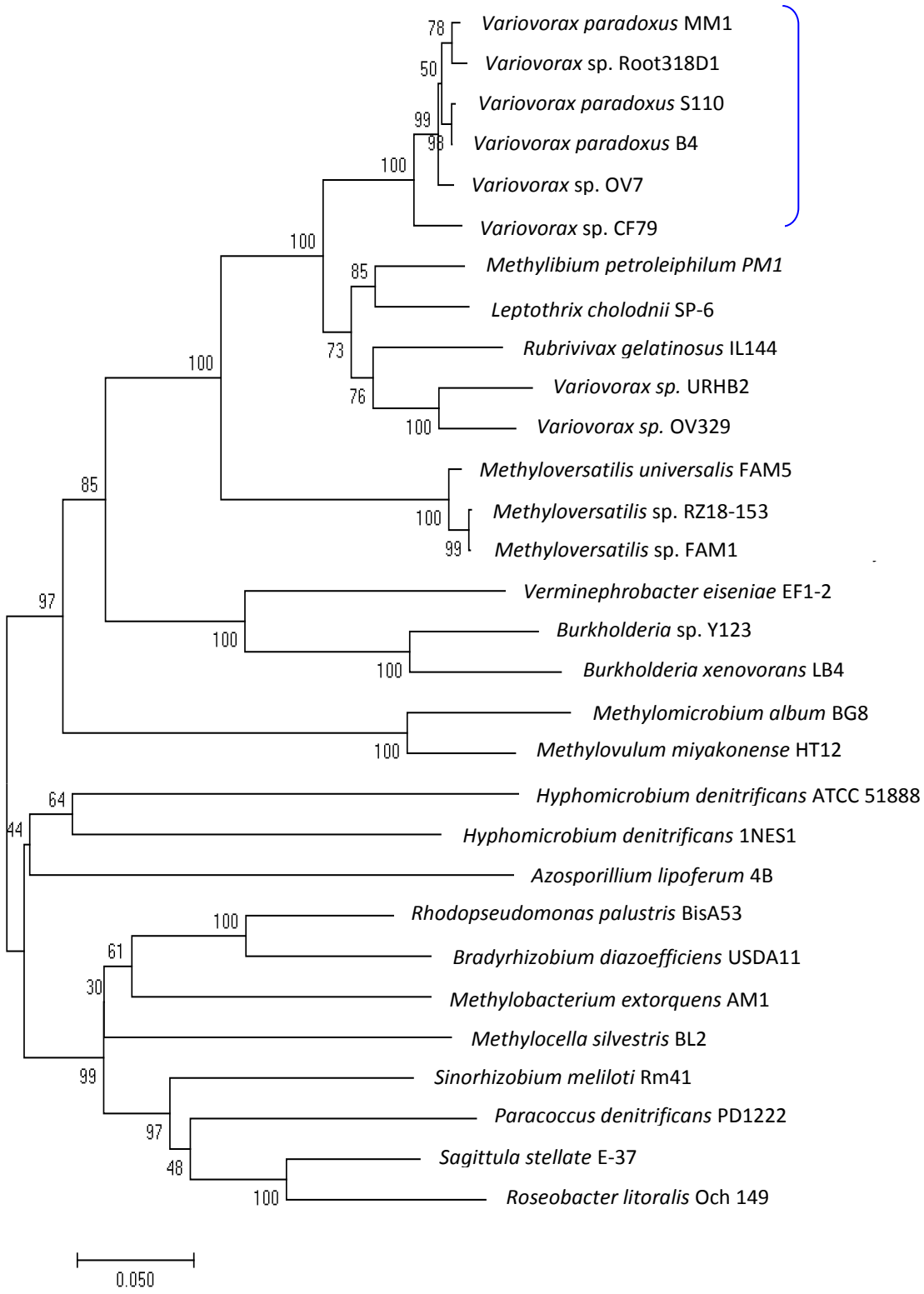


Figure 3.2 Phylogenetic analysis of *xoxF5* gene sequences from *Variovorax paradoxus* MM1 aligned with additional *xoxF5* genes, aligned at the deduced amino acid level, with the phylogenetic tree constructed from nucleotide sequences. The blue bracket marks the *Variovorax paradoxus xoxF5* sequences. The evolutionary history was inferred using the Neighbour-Joining method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position.

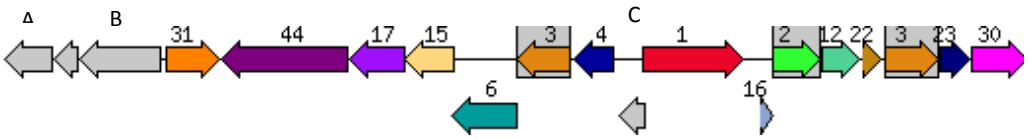


Figure 3.3 Gene cluster surrounding the region surrounding the *xoxF5* methanol dehydrogenase gene of *Variovorax paradoxus* MM1. A-B, ribulose bisphosphate carboxylase small subunit and large subunit; 31, LysR family transcriptional regulator; 44, Outer membrane receptor protein; 17, tricarboxylate transport protein; 15, multridrug transport system; 6, hypothetical protein; 3, hypothetical protein; 4, ATP binding protein; C, hypothetical protein; 1, methanol dehydrogenase *xoxF5;* 16, Cytochrome c55; 2, hypothetical protein; 12, hypothetical protein; 23, hypothetical protein; 30, *moxR.*

The genome of MM1 contains genes encoding enzymes of two formaldehyde oxidation pathways. It possesses the glutathione-dependent formaldehyde oxidation pathway (Wilson *et al.*, 2008). Initially, a glutathione formaldehyde activating enzyme (Gfa) converts formaldehyde to hydroxymethyl-glutathione. A glutathione dependent formaldehyde dehydrogenase (GSH-FALDH) then oxidises this to S-formyl GSH, which is then converted to formate by a formyl-glutathione hydrolase. Further analysis of the genome showed that it contained the genes required for the tetrahydrofolate (H4F) linked pathway of formaldehyde assimilation (Vorholt, 2002). The reaction between H4F and formaldehyde produces methylene-H4F, which can be inserted into the serine cycle for assimilation or oxidised further to formate. Genes required for the dissimilation of formaldehyde from methylene-H4F are present. FolD, a bifunctional enzyme capable of methylene-H4F dehydrogenase and methenyl-H4F cyclohydrolase activity would convert the methyl-H4F to 10-formyltetrahydrofolate. This would then be converted to formate and tetrahydrofolate by the enzyme 10-formly-H4F hydrolase (Chistoserdova *et al.*, 2009; Keltjens *et al.*, 2014). The genome also contains genes encoding for three formate dehydrogenases, FDH1, FDH2 and FDH3.

3.3.2 Further metabolic traits

The genome of *Variovorax paradoxus* MM1 contains genes encoding for an assimilatory nitrate reductase (NasAB) and two dissimilatory nitrite reductases (NirBD). In addition to this, the genome contains genes that encode for a 2-nitropropane dioxygenase, an enzyme that converts 2-nitropropane to acetone and nitrite. The genome also contains

genes encoding two nitrilases, converting nitriles to a carboxylate and ammonia (Howden *et al.*, 2009). Both of these enzymes would require experimental validation to determine their functionality, but would expand the metabolic capability of the strain with regards to nitrogen. The genome of *Variovorax paradoxus* MM1 was also predicted to contain two inactive prophages.

As previously mentioned, *Variovorax* is considered an important genus for the degradation of natural and polluting aromatic compounds. Analysis of the genome of MM1 showed it to contain genes encoding for the degradation of aromatic compounds to acetyl-CoA and succinyl CoA, allowing for subsequent utilisation by central metabolic pathways (Satola *et al.*, 2013; Liang *et al.*, 2014). The degradation pathways present are for nitrobenzene and naphthalene as are the pathways for the subsequent utilisation of catechol and 3-oxoadipate. These pathways would require experimental testing to confirm their functionality, however other closely related strains of *Varivorax* have been implicated in the degradation these compounds (Brandt *et al.*, 2014; Liang *et al.*, 2014; Posman *et al.*, 2016).

3.4 Enrichment and isolation of methylotrophs using soil from CF using dNMS and sloppy agar

Of 14 potential isolates from this enrichment regime (Section 2.2.3.2), two showed growth on methanol as a sole carbon source. The two successful cultures were plated onto modified dNMS plates and R2A plates in order to confirm purity. No colonies developed on the R2A plates, but colonies did develop on the modified dNMS plates. Individual colonies were used in colony PCR for the amplification of the 16S rRNA gene and for screening with the *xoxF* and *mxaF* primers, with the results shown in Table 3.2. Based on the low 16S rRNA gene similarity to their closest relatives within their respective genera (Figure 3.4, Table 3.2), these two strains were characterised further (See Section 3.3).

Table 3.2 Identity and basic characterisation of organisms gained using 0.2% agar dNMS with lanthanides

Isolate	Closest Blast Match on NCBI nt database	Identity %	PCR amplification of functional genes		
			mxaF	*xoxF3*	*xoxF4*
MM2	*Methylovorus glucosetrophus* SIP3-4	96	+	-	+
MM3	*Methylobacillus flagellatus* KT	96	-	-	+

3.4.1 General characteristics of *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3

Based on the generally accepted criteria for the designation of novel species, *Methylovorus* sp. MM2 and *Methylobacillus* sp. MM3 were proposed to represent two novel species, *Methylovorus methylotrophus* sp. nov (Type strain MM2) (me.thy.lo.tro'phus. N.L. n. *methylum* the methyl radical; Gr. n. *trophos*, feeder, one who feeds; N.L. masc. adj. *methylotrophus*, methyl radical-consuming) and *Methylobacillus denitrificans* sp. nov. (Type strain MM3) (de.ni.tri'fi.cans. N.L. v. *denitrifico*, to denitrify; N.L. part. adj. *denitrificans*, denitrifying). The two isolates were characterised to further support their designation as novel species and to identify differences between them and other members of their respective genera.

Methylovorus methylotrophus MM2 and *Methylobacillus denitrificans* MM3 are both Gram negative motile rods. *Methylovorus methylotrophus* MM2 is 0.3-0.4 by 1.5-1.6 µM in size. Colonies are 1-3 mm, white and translucent, with an entire and circular surface and convex elevation after growth on modified dNMS plates with methanol as the sole carbon source for four days. *Methylobacillus denitrificans* MM3 is 0.4-0.6 by 1.4-1.6 µM in size. Colonies are 1-4 mm, translucent and cream in colour with an entire and circular surface and convex elevation after growth on modified dNMS plates with methanol as the sole carbon source for four days.

The growth characteristics of both species are detailed in Table 3.3 and 3.4.

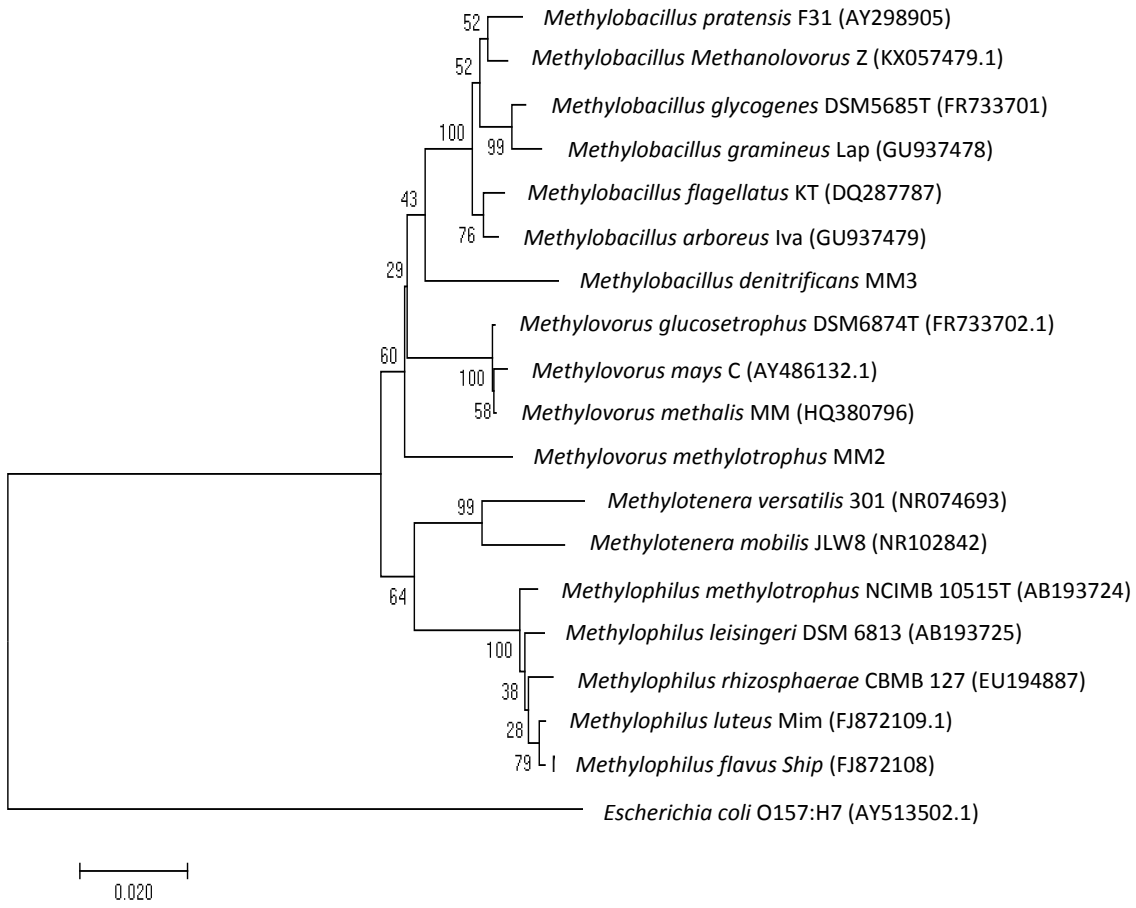


Figure 3.4 Phylogenetic analysis of 16S rRNA gene sequences from isolates *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3 and other cultivated representatives of the *Methylophilaceae*. The evolutionary history was inferred using the Neighbour-Joining method (Saitou *et al.*, 1987). The scale bar represents nucleotide substitution per position. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein 2009). There were a total of 1376 nucleotides in the final dataset.

Table 3.3 General characteristics of Methylovorus methylotrophus MM2 and Methylobacillus denitrificans MM3

	Methylovorus methylotrophus MM2	*Methylobacillus denitrificans* MM3
Temperature growth range (°C)	4-35	4-37
Temperature optimum (°C)	20-25	35-37
pH growth range	4.5-9	5-9.5
pH optimum	6-7	6-7
NaCl growth range (%)	0-0.5	0-1
NaCl optimum (%)	0	0
Nitrogen sources utilised	Nitrate, urea, ammonia	Nitrate, urea, ammonia
Nitrate reduction	-	+
Carbon sources utilised	Methanol	Methanol, methylamine, dimethylamine, trimethylamine
Catalase	+	+
Oxidase	+	+
IAA production with tryptophan supplied	+	+
Starch hydrolysis	-	-
Gelatin hydrolysis	-	-
Vitamin B12 auxotrophy	-	+
Siderophore production	+	+
Polyhydroxybutarate production	+	+

Table 3.4 Antibiotic resistance and susceptibility of *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3

	Methylovorus methylotrophus MM2	*Methylobacillus denitrificans* MM3
Anitiobtic susceptibility ($\mu g\ ml^{-1}$)	Gentamycin (10) Neomycin (30) Streptomycin (10) Nalidixic acid (30) Novobiocin (5) Kanamycin (30) Tetracycline (10)	Gentamycin (10) Neomycin (30) Ampicillin (100) Lincomycin (2) Chloramphenicol (10) Tetracycline (10)
Antibiotic resistance ($\mu g\ ml^{-1}$)	Ampicillin (100) Lincomycin (2) Chloramphenicol (10)	Streptomycin (10) Nalidixic acid (30) Novobiocin (5) Kanamycin (30).

3.4.2 Analysis of methylotroph genomes

As *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3 represented novel species within their respective genera, their genomes were sequenced as described in Chapter 2. These genomes contribute to our understanding of both genera, as there are only two genomes that are publicly available for either genus. Genome statistics are summarised in Table 3.5.

Table 3.5 General genome features of *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3

Genome data	*Methylovorus methylotrophus* MM2	*Methylobacillus denitrificans* MM3
Number of contigs	25	67
Genome size (bp)	2,425,793	2,958,606
GC content (%)	46.8	57.6
Number of Coding Sequences (CDS)	2291	2897
tRNAs	46	50
All rRNAs	3	5

3.4.3 Carbon utilisation

3.4.3.1 Central Metabolism

Both strains possess an incomplete tricarboxylic acid (TCA) cycle, similar to the other members of the *Methylophilaceae* (Chistoserdova *et al.*, 2007; Hendrickson *et al.*, 2010; Lapidus *et al.*, 2011a; Vorobev *et al.*, 2013). The TCA cycle was incomplete in both genomes due to the absence of a-ketoglutarate dehydrogenase, malate dehydrogenase and the alpha subunit of succinate dehydrogenase. These are the same enzymatic lesions as in the TCA cycle of *Methylobacillus flagellatus* KT, *Methylobacillus glycogenes,*

Methylovorus sp. MP688 and *Methylovorus glucosetrophus* SIP3-4 (Chistoserdova *et al.*, 2007; Hendrickson *et al.*, 2010; Lapidus *et al.*, 2011a).

Both strains possessed the KDPG aldolase variant of the ribulose monophosphate (RUMP) pathways for assimilation and dissimilation of formaldehyde (Anthony 1983). It is therefore possible that both of these species are capable of oxidising formaldehyde through the cyclic oxidative pathway via 6-phosphogluconate dehydrogenase, which has been proposed to be the main formaldehyde oxidation pathway in methylotrophs that possess the RUMP cycle. (Anthony 1983; Chistoserdova *et al.,* 2015). *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3 both possess all the genes for the tetrahydromethanopterin (H4MPT) pathway for the oxidation of formaldehyde to formate, which is subsequently oxidised to carbon dioxide (Vorholt *et al.*, 1999; Chistoserdova *et al.*, 2000). The H4MPT pathway has been shown to be non-essential for growth on methanol in members of the *Methylophilaceae* who possess the cyclic oxidation pathway, but it has been proposed to play a role in mitigating stress from a build-up of formaldehyde (Chistoserdova *et al.*, 2000).

Both genomes contain genes encoding formate dehydrogenases. The genome *Methylovorus methylotrophus* MM2 contained genes encoding FDH2 and FDH4 (Chistoserdova *et al.*, 2004, 2007; Lapidus *et al.*, 2011a). The genome of *Methylobacillus denitrificans* MM3 contained genes encoding formate dehydrogenases FDH1, FDH2 and FDH3 (Laukel *et al.*, 2003; Chistoserdova *et al.*, 2004). This varies from the complement of formate dehydrogenases found in *Methylobacillus glycogenes* (FDH2) and *Methylobacillus flagellatus* KT (FDH1, FDH2 and FDH4) (Chistoserdova *et al.*, 2007; Hendrickson *et al.*, 2010).

3.4.3.2 C1 metabolism

Blast searches of the genomes of the two strains revealed that the genome of *Methylovorus methylotrophus* MM2 contained one set of the classical methanol dehydrogenase encoding genes *mxaFI. Methylobacillus denitrificans* MM3 did not possess a copy of *mxaFI*.

Clustered with the *mxaFI* genes are the accessory genes *mxaJRSACKLD*. An alignment at the amino acid level of the *mxaF* sequence to a database of *mxaF* sequences showed a

high level of similarity to the *mxaF* methanol dehydrogenase gene possessed by *Methylovorus glucosetrophus* SIP3-4 (Figure 3.5 and Table 3.6).

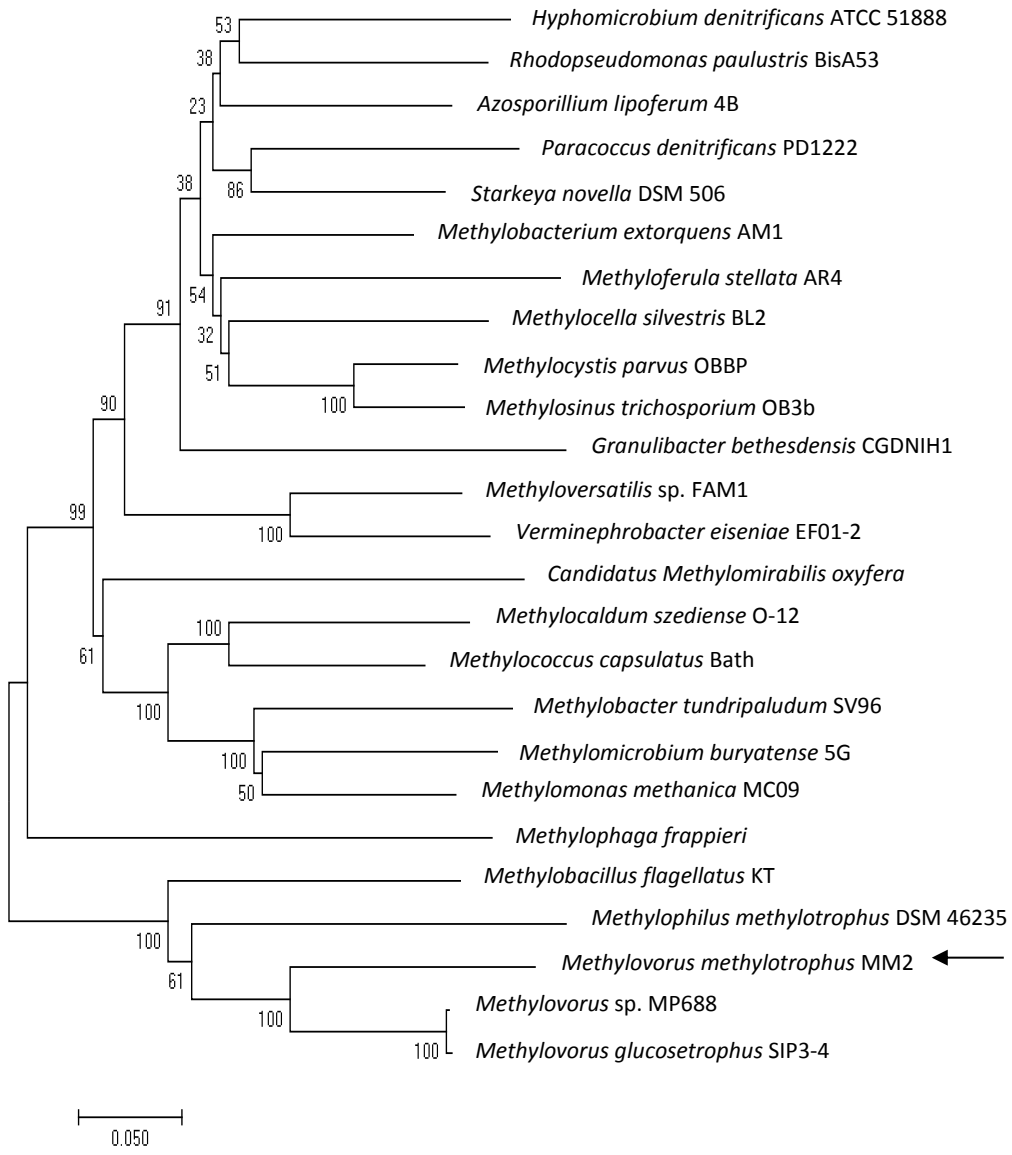


Figure 3.5 Phylogenetic analysis of the *mxaF* gene sequence from *Methylovorus methylotrophus* MM2 (designated by the black arrow) and other representative sequences aligned at the deduced amino acid level, with the phylogenetic tree constructed from nucleotide sequences. The evolutionary history was inferred using the Neighbour-Joining method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position.

Blast searches for the alternate methanol dehydrogenase encoding gene *xoxF* revealed that both isolates contain three copies of *xoxF*. These gene sequences were aligned at the amino acid level with a database of *xoxF* sequences, and clustered with the *xoxF4*

clade of methanol dehydrogenase (Figure 3.6). *xoxF4* methanol dehydrogenase genes are only found in members of the *Methylophilaceae* (Keltjens *et al.*, 2014).

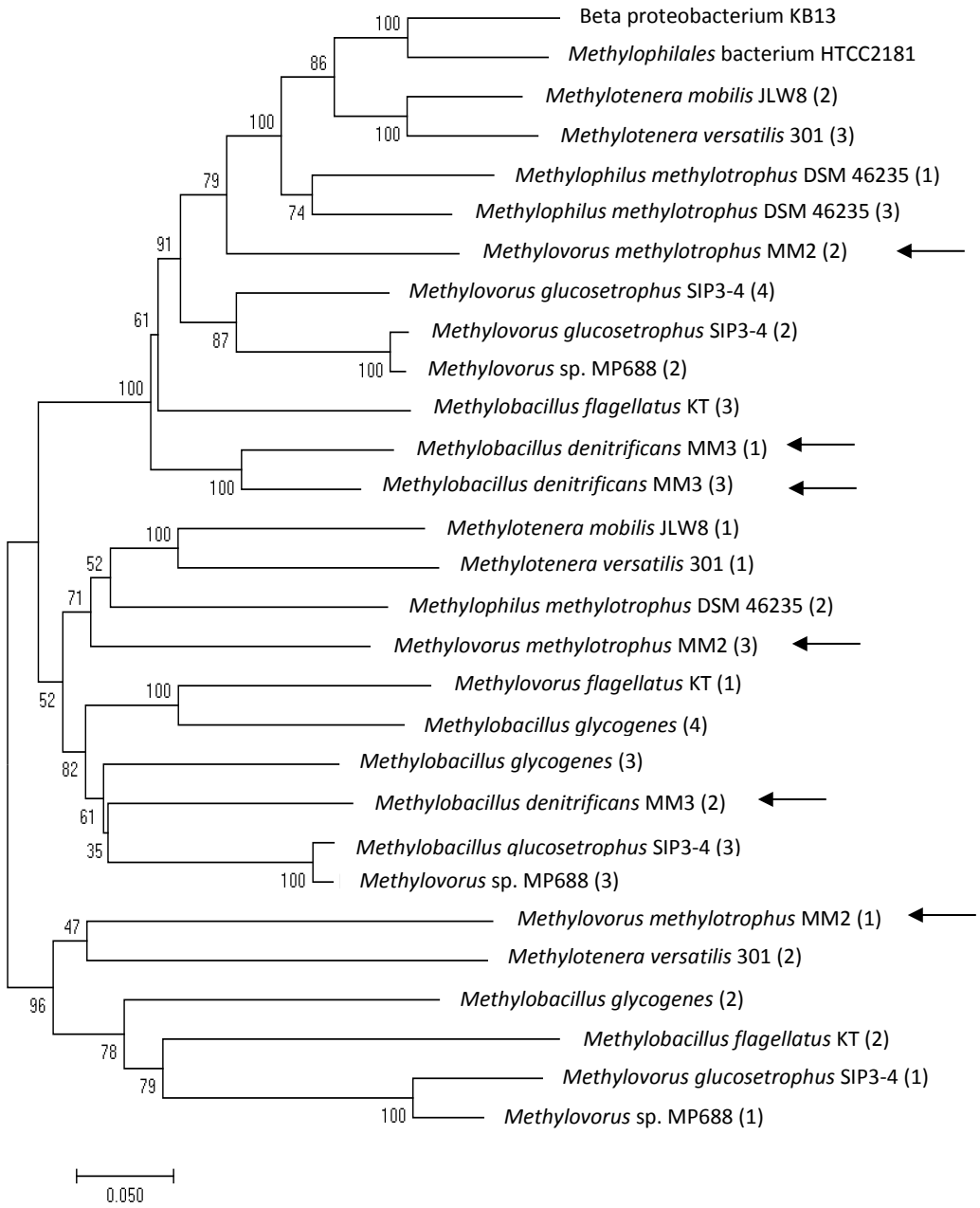


Figure 3.6 Phylogenetic analysis of *xoxF4* gene sequences from *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3 (designated with black arrows) aligned with databases of their respective *xoxF* clade aligned at the deduced amino acid level, with the phylogenetic tree constructed from nucleotide sequences. The evolutionary history was inferred using the Neighbour-Joining method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position.

The highest identity matches according to Blast further confirms their high similarity to methanol dehydrogenase sequences from the same genera (Table 3.6).

Table 3.6 Phylogeny of methanol dehydrogenase genes in the genome of *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3

Species	Gene	Closest Blastp Match	Identity %
Methylovorus methylotrophus MM2	*mxaF*	*Methylovorus glucosetrophus* SIP3-4	94
	xoxF4	*Methylovorus* sp. MP688	76
	xoxF4	*Methylovorus glucosetrophus* SIP3-4	92
	xoxF4	*Methylovorus glucosetrophus* SIP3-4	87
Methylobacillus denitrificans MM3	*xoxF4*	*Methylobacillus flagellatus* KT	87
	xoxF4	*Methylotenera mobilis*	84
	xoxF4	*Methylovorus glucosetrophus* SIP3-4	90

The number and clade of methanol dehydrogenase genes contained in the genomes of both *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3 are divergent from the other genome-sequenced members of their respective genera (Table 3.7).

Table 3.7 Complement of methanol dehydrogenase genes possessed by genome sequenced *Methylobacillus* spp. and *Methylovorus* spp.

Species	Copy number of methanol dehydrogenase genes		
	xoxF3	*xoxF4*	*mxaF*
Methylobacillus flagellatus KT	1	3	1
Methylobacillus glycogenes	1	3	1
Methylobacillus denitrificans MM3	0	3	0
Methylovorus sp. MP688	0	3	1
Methylovorus glucosetrophus SIP 3-4	0	4	1
Methylovorus methylotrophus MM2	0	3	1

Methylovorus methylotrophus MM2 contains one less *xoxF4* methanol dehydrogenase gene than *Methylovorus glucosetrophus* SIP 3-4, but has the same contingent of methanol dehydrogenases as *Methylovorus* sp. MP688. The genome of *Methylobacillus denitrificans* MM3 does not contain an *mxaF* or *xoxF3* gene, unlike the other two sequenced *Methylobacillus* genomes. *mxaF* has also been confirmed to be possessed by the five other species within the genus *Methylobacillus* (Doronina *et al.*, 2004; Chistoserdova *et al.*, 2007; Gogleva *et al.*, 2011; Madhaiyan *et al.*, 2013). The absence of a classical methanol dehydrogenase suggested that methanol oxidation in *Methylobacillus denitrificans* MM3 would be lanthanide dependent, as the strain only possesses the lanthanide dependent XoxF methanol dehydrogenases (Farhan Ul-Haque *et al.*, 2015; Chu and Lidstrom, 2016; Vu *et al.*, 2016). To assess the impact of lanthanides on the growth of *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3 on methanol as a sole carbon source, they were grown on dNMS with 20 mM

methanol and supplemented with either 5 µM lanthanum, 5 µM cerium or no lanthanides. The impact of the supply of lanthanides on the growth of *Methylobacillus denitrificans* MM3 is shown in Figure 3.7.

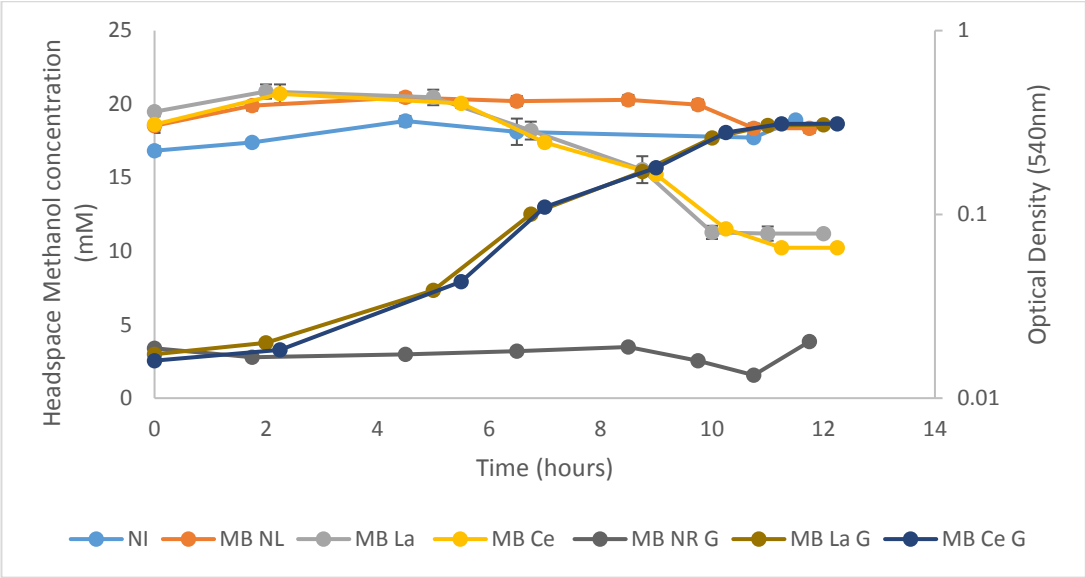


Figure 3.7 Growth of *Methylobacillus denitrificans* MM3 on methanol, with the aqueous concentration of methanol inferred from the concentration in the headspace relative to a series of standards. Cultures were supplied with 5 µM Cerium, 5 µM Lanthanum or no lanthanides to the culture. Error bars represent variation between three replicates. NI represents no inoculum controls. NL designates no supply of lanthanide. G designates OD of growing *Methylobacillus denitrificans* MM3.

Methylobacillus denitrificans MM3 exhibited lanthanide dependent growth, with no measurable oxidation of methanol or growth occurring over 48 hours in the absence of lanthanum or cerium. There was no difference between the growth of the strain when grown with lanthanum or with cerium, consistent with other studies that indicate that the first four elements of the lanthanide series can all support the lanthanide dependent methanol oxidation of the *xoxF* methanol dehydrogenase enzymes (Vu *et al.*, 2016). *Methylovorus methylotrophus* MM2 did not exhibit lanthanide dependent growth and this was expected due to its possession of both an *mxaF* methanol dehydrogenase in addition to the *xoxF* methanol dehydrogenases. Although it is possible that the supply of lanthanides affected the transcription of these genes, it had no measurable impact on growth. However, *Methylovorus methylotrophus* MM2 did grow in an aggregated manner when grown with a supply of 5 µM lanthanum or cerium. This growth response is typically seen when the cells are stressed, however why this would occur in this

instance is unknown. This response has been observed in other methylotrophic bacteria when the concentration of lanthanum supplied exceeded 50 µM (Fitriyanto *et al.*, 2011), and it is therefore possible that different species vary in the extent to which they are able to tolerate lanthanides (Hu *et al.*, 2004; Oliveira *et al.*, 2015).

There is a degree of synteny surrounding the methanol dehydrogenase genes in the genomes of *Methylovorus methylotrophus* MM2, *Methylobacillus denitrificans* MM3 and methanol dehydrogenase genes from other genome-sequenced members of the *Methylophilaceae* (Figure 3.8B). The region upstream (5') of the *mxaF* gene of *Methylovorus methylotrophus* MM2 has a histidine kinase and a DNA binding response regulator, LuxR family protein. Upstream of this is another DNA binding response regulator and histidine kinase transcribed in the opposite direction. This gene order is present in the genomes of *Methylovorus glucosetrophus* SIP3-4 and *Methylovorus* MP688, but is absent in the other *Methylophilaceae* genomes. In the genome of MM2 there is a SAM-dependent methyltransferase between the two DNA binding response regulators, but this is the only difference in this region. Downstream of the *mxaF* gene are genes encoding a cytochrome (*mxaG*), the small subunit of the methanol dehydrogenase (*mxaI*) and methanol dehydrogenase associated accessory genes (*mxaRSACKLD*) (Keltjens *et al.*, 2014). This is a region conserved in all genome sequenced members of the *Methylophilaceae* possessing an *mxaF*.

Directly upstream of *xoxF4*, designated *xoxF4*-2 in MM2 and *xoxF4*-3 in MM3 (Figure 3.8C), there is a gene encoding a proline imminopeptidase. Directly downstream of the *xoxF* gene there are genes encoding two cytochromes (*xoxG*), an NADH dehydrogenase, a transmembrane protein, an ATPase and a thiol peroxidase. This region (highlighted in blue) is conserved throughout the *Methylophilaceae*, with every genome sequenced member of the family possessing one *xoxF4* with this gene order in the region surrounding the *xoxF4* gene. It is of interest that the signal sequence that is proposed to direct XoxF to the periplasm (Nakagawa *et al.*, 2012) ends with an alanine, which is recognized by proline imminopeptidases (Gilbert *et al.,* 1994). This alanine has also been noted as being present in the proposed signal peptide sequence of a XoxF possessed by *Methylobacterium extorquens* AM1 (Nakagawa *et al.*, 2012). The possible role of this proline imminopeptidase in signal sequence cleavage would need to be confirmed

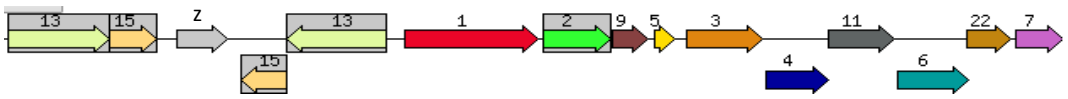
experimentally. The cAMP binding protein directly upstream of the proline imminopeptidase in the genome of *Methylovorus methylotrophus* MM2 is shared by additional *Methylophilaceae* genomes. The region further upstream of the cAMP binding protein, highlighted in red, is identical in gene order to that of a *xoxF4* possessed by *Methylobacillus flagellatus* KT.

The region surrounding MM2 *xoxF4*-3 and MM3 *xoxF4*-2 (Figure 3.8D) is also conserved within the *Methylophilaceae* (highlighted in green), with all of the genome sequenced species of the *Methylotenera*, *Methylovorus* and *Methylobacillus* containing genes coding for an acetoin catabolism regulatory protein directly upstream of the *xoxF4* gene.

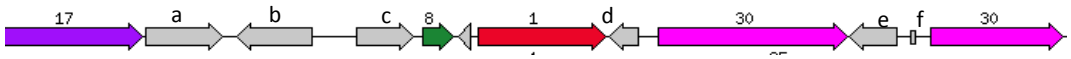
The third *xoxF4* contained by the two species (Figure 3.8 B, E) have no similarity in the genes upstream and downstream of the methanol dehydrogenase encoding gene. This lack of synteny appears to be a common trait in all genome-sequenced members of the *Methylophilaceae* that possess a third *xoxF4* methanol dehydrogenase.

The third *xoxF4* gene of *Methylobacillus denitrificans* MM3 (Figure 3.8E) is directly downstream of the nitrous oxide reductase genes and upstream of the respiratory nitrate reductase genes in the genome, in addition to a series of nitrite/nitrate transporters. *xoxF* previously had a proposed interaction with denitrification (Kalyuhznaya *et al.*, 2009; Mustakhimov *et al.*, 2013), with a suggested role of enhancing the rate of denitrification, so it is possible this gene order is a reflection of this interaction.

A. *Methylovorus methylotrophus* MM2 mxaF

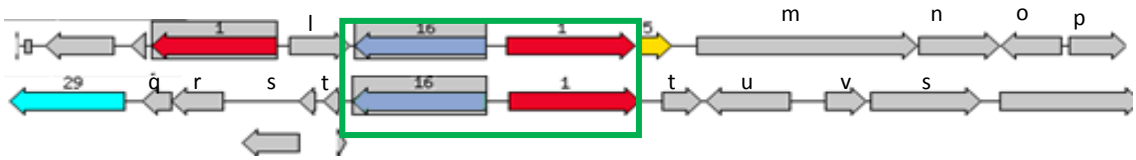


B. *Methylovorus methylotrophus* MM2 *xoxF4*-1



C. *Methylovorus methylotrophus* MM2 *xoxF4*-2 and *Methylobacillus denitrificans* MM3 *xoxF4*-3

D. *Methylovorus methylotrophus* MM2 *xoxF4*-3 and *Methylobacillus denitrificans* MM3 *xoxF4*-2



E. *Methylobacillus denitrificans* MM3 *xoxF4*-1

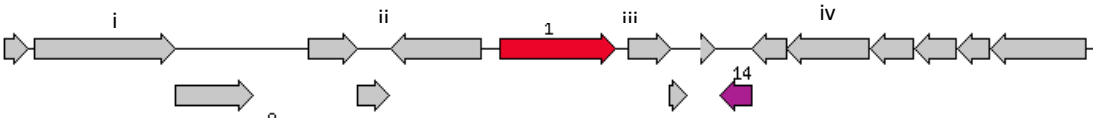


Figure 3.8 Gene clusters surrounding the methanol dehydrogenase genes in the genomes of *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3. *xoxF* numbers based on order in the assembled genome.

A *Methylovorus methylotrophus* MM2 *mxaF.* 13, histidine kinase; 15, LuxR DNA binding response regulator; z, Sam-dependent methyltransferase; 1, methanol dehydrogenase large subunit (*mxaF*); 2, *mxaG*; 9, *mxaI;* 5, *mxaR;* 3, *mxaS,;* 4, *mxaA;* 11, *mxaC;* 6, *mxaK;* 22, *mxaL.*

B *Methylovorus methylotrophus MM2 xoxF4*-1. 17, sensory box; a, diuguanylate cyclase; b, xenobiotic reductase; c, decarboxylase; 8, cytochrome cL (*xoxG*); d, hypothetical protein; 1, methanol dehydrogenase; e, hypothetical protein; 30, diguanylate cyclase; f, uracil gylosylase; g, tRNA-pseudo-GCA.

C *Methylovorus methylotrophus* MM2 *xoxF4*-2 and *Methylobacillus denitrificans* MM3 *xoxF4*-3. 33, rhodanese; 28, ferrichrome iron receptor; 25, iron uptake factor PiuC; 19, cAMP binding proteins; 15, proline imminopeptidase; 1, methanol dehydrogenase; 2, extracellular solute binding protein; 5, Cytochrome Cl; 21, NADH dehydrogenase; 26, transmembrane protein; 29,

ATPase; 32, Thiol peroxidase; h, DNA primase; i, RNA polymerase sigma factor; j, tRNA-Met-CAT; 3, cytochrome cL (*xoxG*). Blue and red boxes designate regions of conserved gene order

D *Methylovorus methylotrophus* MM2 *xoxF4*-3 and *Methylobacillus denitrificans* MM3 *xoxF4*-2. 1, glucose dehydrogenase; l, hypothetical protein; 16, acetoin catabolism regulatory protein; 1, methanol dehydrogenase; 5, cytochrome cL (*xoxG*); m, metal resistance protein CzcA; n, metal efflux protein; o, transcriptional regulator; p, luciferase like monoxygenase; q, Ferrichrome iron receptor; r, Channel protein MotA; s, TonB ferrichrome receptor; t, hypothetical protein; u, cyclopropane fatty acyl phospholipid cyclase; v, alkyl hydroperoxide protein. The green box designates a region of conserved gene order.

E *Methylobacillus denitrificans* MM3 *xoxF4*-1. i, Nitrous oxide reductase; ii, transcriptional regulator; 1, methanol dehydrogenase; iii, hypothetical protein; 14, cytochrome cL (*xoxG*); iv, Respiratory nitrate reductase.

3.4.3.3 Methylamine utilisation

Both genomes were screened for genes encoding enzymes involved in the utilisation of methylamine as a carbon or nitrogen source. The ability to metabolise of methylamine is widespread throughout the *Methylophilaceae* (Chistoserdov *et al.*, 1994; Chistoserdova *et al.*, 2007; Hendrickson *et al.*, 2010; Doronina *et al.*, 2011, 2016; Vorobev *et al.*, 2013). There are two pathways for methylamine utilisation. One pathway involves a methylamine dehydrogenase, which performs direct oxidation of the methylamine to formaldehyde and ammonia (Anthony 1983). This enzyme is encoded by the genes *mauABCDE* (Slotboom *et al.*, 1995). The indirect pathway for formaldehyde utilisation involves the transfer of the methyl group of the methylamine to a glutamate by the enzyme Y-glutamylmethylamide synthetase (*gmaS*). This product is then converted to N-methylglutamate by NMG synthase (*mgsABC*), regenerating glutamate and also producing ammonia as a by-product (Chen *et al.*, 2010; Latypova *et al.*, 2010). NMG is then converted to tetrahydrofolate-bound formaldehyde by NMG dehydrogenase (*mgdABCD*) (Chen *et al.*, 2010; Latypova *et al.*, 2010). All genes required for both the direct (*mauABCDE*) and indirect pathways (*gmas, mgsABC, mgdABCD*) are found in the genomes of *Methylobacillus flagellatus* KT and *Methylobacillus glycogenes*, whereas the genomes of *Methylovorus glucosetrophus* SIP3-4 and *Methylovorus* sp. MP688 both contain only the genes encoding the indirect pathway (Chistoserdova *et al.*, 2007; Lapidus *et al.*, 2011a).

Blast searches for genes involved in the methylamine utilisation indicated the absence of the direct methylamine utilisation pathway in the genomes of *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3. However, the genome of

Methylobacillus denitrificans MM3 was shown to encode the complete pathway for indirect methylamine utilisation. The genome of *Methylobacillus denitrificans* MM3 also contained genes that encode dimethylamine dehydrogenase and trimethylamine dehydrogenase enzymes, commensurate with the ability of *Methylobacillus denitrificans* MM3 to grow on both dimethylamine and trimethylamine as sole carbon and nitrogen sources. Trimethylamine dehydrogenase catalyses the conversion of trimethylamine to dimethylamine and formaldehyde. Dimethylamine dehydrogenase subsequently converts the dimethylamine to monomethylamine and formaldehyde, which then feed into the *gmaS* and formaldehyde utilisation pathways (Anthony, 1983). Growth on di- and trimethylamine has not been tested in the other species of *Methylobacillus*.

3.4.5 Nitrogen cycling-related genes

The genome of *Methylovorus methylotrophus* MM2 contains genes that encode assimilatory nitrate reductase (*nasAB*) and dissimilatory nitrite reductase (*nirBD*). *Methylobacillus denitrificans* MM3 has genes that encode the dissimilatory nitrate reduction pathway (*narGHI* and *nirBD*), in addition to the assimilatory nitrate reductase (*nasAB*). *Methylobacillus denitrificans* MM3 also contains all genes required for the complete denitrification pathway (*narGHI, nirK, nirS, norBC, nosZ*). This therefore represents the first *Methylobacillus* genome to contain genes encoding for the complete denitrification pathway. The reduction of nitrate to nitrite by *Methylobacillus denitrificans* MM3 was confirmed using the Greiss reagent assay and growth was shown to occur under anaerobic conditions with nitrate. The production of nitric oxide and nitrous oxide was confirmed to occur under anaerobic conditions using gas chromatography by Alexander Goodchild (University of East Anglia).

3.4.6 Additional genome features

The genomes of *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3 contain two inactive prophages. *Methylovorus methylotrophus* MM2 was also predicted to contain an intact prophage with a genome 46.4 kb in size. The most common bacteriophage sequences were from the sequenced bacteriophage *Mesorhizobium* phage vB MloP Lo5R7ANS, a dsDNA virus in the *Podoviridae*. Whether this phage is active would need to be confirmed with experimental validation, with the

attempted induction of the phage. Were this phage active, characterisation of its host range within the genus *Methylovorus* and family *Methylophilaceae* might prove valuable in furthering our understanding of the impact of bacteriophage on methylotrophic bacteria, an area that has received little research attention.

3.4.7 Comparison to the closest related species

To further support the classification of *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3 as novel species, the genomes sequences of both organisms were compared with genomes of members of the genera *Methylovorus*, *Methylobacillus* and *Methylotenera*. In-silico DNA:DNA hybridisation (DDA) was performed using the online program "Genome to Genome Distance Calculator" (server 2.1) (GGDC). The GGDC utilises three distinct algorithms to assess the similarity of genome, weighting either genome size and the length of regions with high similarity (Formula 1) or the length and number of regions with high similarity (Formula 2) as more important. Formula 3 is a combination of 1 and 2, scoring distance using both the number of similar regions and genome length (Meier-Kolthoff *et al.*, 2013). Further to this, the second formula is recommended as the most reliable for genomes which are not complete and to compare genomes which are variable in length, as it does not consider genome length. Average Nucleotide Identity (ANI) and Average Amino acid Identity (AAI) calculations were also performed to further assess the designation of species.

The results for in-silico DNA:DNA hybridisation for both *Methylovorus methylotrophus* MM2 (Table 3.8) and *Methylobacillus denitrificans* MM3 (Table 3.9) show low DDH similarity scores to all of the available genomes. The score for members of the same species is 70% (Goris *et al.*, 2007; von Jan, *et al.*, 2010), so this provides further support for the designation of both isolates as novel species. Due to the low score of the ANI calculations, AAI was performed and yielded low scores for both *Methylovorus methylotrophus* MM2 (71.25%) and *Methylobacillus denitrificans* MM3 (64%) when compared to genome sequenced members of their respective genera.

Table 3.8 *in silico* DDH scores for the genome of *Methylovorus methylotrophus* MM2 in comparison with other genome sequences of members of the *Methylophilaceae*.

Reference genome	Formula one	Formula two	Formula three
Methylovorus glucosetrophus SIP3-4	13.7	18.6	14.0
Methylovorus sp. MP688	13.8	18.5	14.0
Methylobacillus flagellatus KT	13.2	18.3	13.5
Methylobacillus glycogenes	13.2	18.6	13.5
Methylotenera mobilis	13.0	21	13.4
Methylotenera versatilis	13.0	19.8	13.3

Table 3.9 *in silico* DDH scores for the genome of *Methylobacillus denitrificans* MM3 in comparison with other genome sequences of members of the *Methylophilaceae*.

Reference genome	Formula one	Formula two	Formula three
Methylovorus glucosetrophus SIP3-4	13.8	19.5	14.0
Methylovorus sp. MP688	13.8	19.1	14.0
Methylobacillus flagellatus KT	13.6	18.9	13.9
Methylobacillus glycogenes	13.3	18.1	13.6
Methylotenera mobilis	12.8	18.0	13.1
Methylotenera versatilis	12.6	36.7	13.0

In addition to the above description of support from analysis of the genomes, there are also several major physiological characteristics that indicate both strains of methylotrophic bacteria represent novel species within their respective genera (Table 3.10 and 3.11).

Table 3.10 Major characteristics of the species within the genus *Methylobacillus*

Species	*Methylobacillus denitrificans* MM3	*Methylobacillus arboreus* (Gogleva *et al.*, 2011)	*Methylobacillus pratensis* (Doronina *et al.*, 2004)	*Methylobacillus gramineus* (Gogleva *et al.*, 2011)	*Methylobacillus flagellatus* (Kaparullina *et al.*, 2017)	*Methylobacillus glycogenes* (Kaparullina *et al.*, 2017)	*Methylobacillus methanolivorans* (Kaparullina *et al.*, 2017)
Optimum temp	35-37	19-24	25-30	19-24	42	30-33	29-35
Optimum pH	6-7	7.9-8.5	6.5-7.5	7.2-7.8	7.2-7.3	6-8	6.5-7.5
Highest NaCl conc tolerated (%)	1	3	2	2	3	2	0.5
Nitrate reduction	+	-	+	-	+	+	+
Growth on Methylamine	+	-	+	-	+	+	v
GC content %	57.6	54.0	61.5	50.5	53.5	53.2	51.0

v designates variable presence

Table 3.11 Major characteristics of the species within the genus *Methylovorus*

Species	*Methylovorus methylotrophus* MM2	*Methylovorus menthalis* (Doronina *et al.*, 2011)	*Methylovorus mays* (Doronina *et al.*, 2011)	*Methylovorus glucosetrophus* (Doronina *et al.*, 2016)
Optimum temp	20-25	24-26	35-40	35-37
Optimum pH	6.0-7.0	8.5-9.0	7.0-7.5	7.0-7.2
Highest NaCl conc tolerated (%)	0.5	2	3	N
Nitrate reduction	-	+	+	N
Utilisation of Methylamine	-	-	-	v
GC content %	46.8	54.5	57.2	55.8

v designates variable presence

3.5 Enrichment and isolation of methylotrophs using soil from CF using repeated pulsing of methanol

Two novel isolates were identified through this isolation series, (Section 2.3.3.3) *Methylophilus flavus* CF1 and *Methylobacterium pseudosasae* CF4. Both of these genera were highly enriched in the ^{13}C labelled DNA of $^{13}CH_3OH$ enrichments (Chapter 5). A strain indistinguishable from *Methylobacillus denitrificans* MM3 was also obtained from this enrichment, and was also enriched in the ^{13}C labelled DNA (Chapter 5).

3.6 Enrichment and isolation of methylotrophs using water from Norfolk Broads and soil from Marburg Forest and Strumpshaw Landfill

Eleven strains were isolated from the three different environments using this enrichment regime (Section 2.3.3.4) (Table 3.12).

Table 3.12 Identity of organisms isolated from a range of environmental samples

Isolate	Closest Blast Match (NCBI nt database)	Identity (%)	Environment
LF1	[1]*Methyloversatilis discipulorum*	100	Landfill
LF3	[2]*Hydrogenophaga pseudoflava*	99	Landfill
LF4	[3]*Oharaeibacter diazotrophicus*	99	Landfill
LF6	[4]*Starkeya koreensis*	99	Landfill
LF	[5]*Azohydromonas australica*	100	Landfill
BR2	*Methylobacterium extorquens*	100	Broads
BR13	[4]*Starkeya koreensis*	99	Broads
BR10	[6]*Methylophilus TWE2*	99	Broads
BR11	[7]*Methylophilus leisingeri*	99	Broads
BR14	[8]*Burkholderia sartisoli*	98	Broads
F1	[9]*Burkholderia unamae*	98	Forest

([1]Smalley *et al.,* 2015; [2]Willems *et al.,* 1989; [3]Haoxin *et al.,* 2017.; [4]Im *et al.,* 2005; [5]Xie and Yokota 2005; [6]Xia *et al.,* 2015; [7]Madhaiyan *et al.,* 2009; [8]Vanlaere *et al.,* 2008; [9]Paredes-Valdez 2004)

Isolate LF represents the first member of the genus *Azohydromonas* to be confirmed to grow on methanol (Palleroni and Palleroni 1978; Xie and Yokota 2005). The genome of the type strain for the genus, *Azohydromonas lata* (Xie and Yokota 2005; Palleroni and Palleronit 1978), contains one *xoxF5* gene and the species most closely related to isolate LF, *Azohydromonas australica*, contains a *xoxF5* and two *xoxF3* genes. Both of these strains were previously reported as being incapable of growing on methanol (Xie and Yokota 2005; Palleroni and Palleroni 1978), perhaps since lanthanides were not supplied in the growth medium. Isolate LF3 represents the second member of the *Hydrogenophaga* to be confirmed to be capable of growth on methanol as a sole carbon source (Eyice and Schäfer, 2015). Although previously shown to be enriched in methanol fed bioreactors (Ginige *et al.*, 2004; Osaka *et al.*, 2006), the isolated members of this genus were considered incapable of growth solely on methanol as a carbon source (Willems *et al.*, 1989; Ginige *et al.*, 2004).

Isolate LF4 represents the second isolated methanol-utilizing representative of the genus *Oharaeibacter*. When LF4 was screened using PCR it yielded a *xoxF1* and a *xoxF5* PCR product, the former of which represents the first of this clade of methanol dehydrogenase from this genus (Figure 3.9).

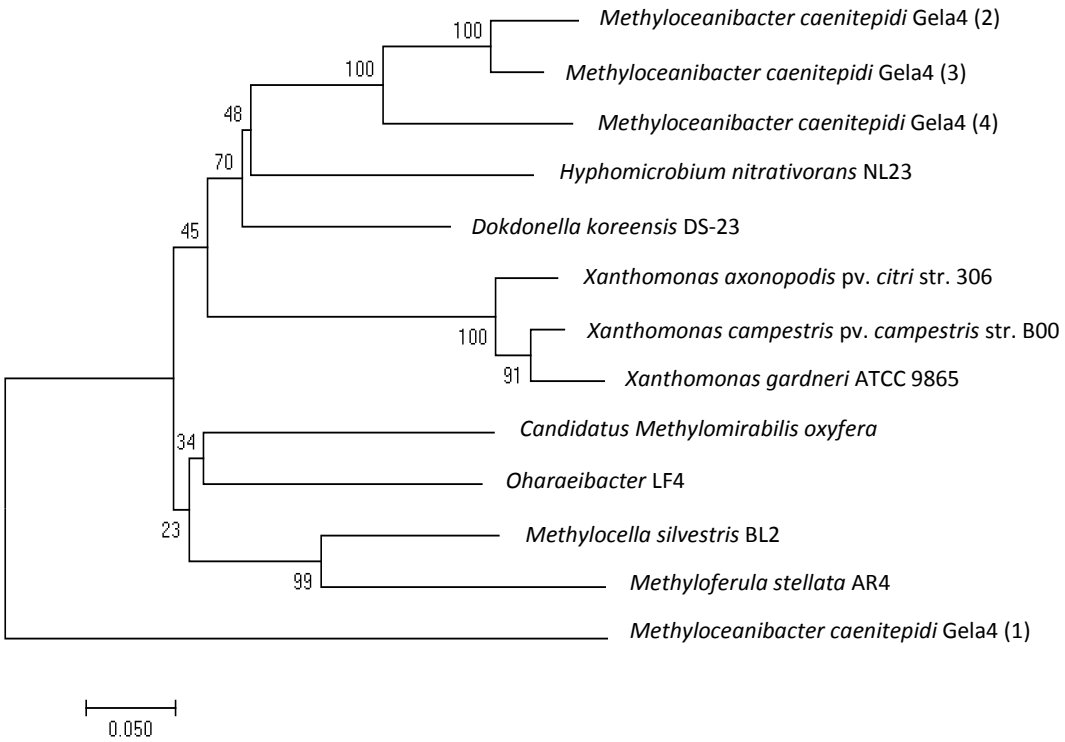


Figure 3.9 Phylogenetic analysis of the *xoxF1* gene sequence from *Oharaeibacter* LF4, constructed from nucleotide sequences aligned at the deduced amino acid level. The evolutionary history was inferred using the Neighbour-Joining method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position**.**

3.7 Discussion

3.7.1 Enrichment and isolation of methylotrophs

Using a varied range of approaches, several strains of methylotrophic bacteria were isolated across a range of different environments. A supply of lanthanides to media and the use of a dilute version of NMS facilitated the isolation of a greater diversity of methylotrophs, but also captured strains isolated using more traditional methods (*Burkholderia, Starkeya, Methylophilaceae, and Hyphomicrobium*). This further shows the need for varied approaches in order to maximise the diversity of strains isolated from environmental samples.

These isolations were important not only for the characterisation of the individual strains, but also in connection with subsequent culture independent work (Chapter 4).

The confirmation of methanol oxidation as present within a genus where it has never been reported, or has been reported as absent, was beneficial to identify putative methanol oxidisers in sequenced 16S rRNA gene amplicons (Chapters 5 and 6). Furthermore, the isolation of novel methylotrophs and the amplification and sequencing of their methanol dehydrogenase genes was instrumental in the expansion of the database of these genes. The expanded methanol dehydrogenase gene database improved the analysis of methanol dehydrogenase gene sequences obtained by PCR from DNA extracted from environmental samples (Chapter 4).

3.7.2 Analysis of the genome of *Variovorax paradoxus* MM1

The genome of *Variovorax paradoxus* MM1 was sequenced, enabling insight into its metabolic capabilities. Genes of interest involved in the metabolism of methanol were identified. Additional genes of interest included genes encoding enzymes that degrade aromatic compounds. These metabolic pathways would need testing to assess if they are functional in this organism, but it is interesting to observe the potential metabolism possessed by this methylotrophic organism.

3.7.3 Characterisation of *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3

Enrichment of soil from CF in Bawburgh with methanol using a semi-solid medium enabled isolation of two novel methylotrophs from the family *Methylophilaceae*. There is a large degree of support for the classification of the two methylotrophs, currently named *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3, as novel species within their respective genera. Both have 96% sequence identity at the 16S rRNA gene to the closest related species. Both *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3 vary in their physiological traits with regards to the other species. *Methylobacillus denitrificans* MM3 represents the only characterised member of the *Methylobacillus* to lack an MxaFI methanol dehydrogenase, therefore conferring lanthanide dependence to this strain's ability to oxidise methanol. It also lacks a *xoxF3* methanol dehydrogenase, an FDH4 formate dehydrogenase or a methylamine dehydrogenase. The genome of MM2 does however contain genes that encode for a dimethylamine dehydrogenase, a trimethylamine dehydrogenase, an FDH3 formate dehydrogenase and a complete denitrification pathway, all of which are absent in the other two *Methylobacillus* genomes. *Methylovorus methylotrophus* MM2 does

not possess a methylamine utilisation pathway, commensurate with the fact it cannot use methylamine as a nitrogen or carbon source. It is also incapable of growth on fructose or glucose as a sole carbon source unlike the most closely related species. Furthermore, following genome sequencing and subsequent DDH and AAI comparison to other species within the *Methylophilaceae*, both scored below the thresholds for belonging to an existing species.

Chapter 4: Characterisation of the diversity of methylotrophic bacteria in environmental samples

4.1 Introduction

Methylotrophic bacteria are present across a range of environments, including more extreme environments with regards to physical parameters such a pH and temperature (Hutchens *et al.*, 2003; Han *et al.*, 2009; Kolb, 2009; Antony *et al.*, 2010; Chistoserdova, 2011a; Kolb *et al.*, 2013). Within specific environments, certain genera are consistently detected e.g. *Methylobacterium* in the phyllosphere of several plant species and *Hyphomicrobium* in soils (Knief *et al.*, 2008, 2012; Delmotte *et al.*, 2009; Kolb, 2009; Stacheter *et al.*, 2013). Additional genera are consistently detected at low abundance, but are consistently favoured by conventional enrichment strategies e.g. *Methylophilaceae* in soils and *Methylophaga* in marine environments (Eyice 2015a; Eyice *et al.*, 2015b; Grob *et al.*, 2015). The favouring of certain methylotrophs in enrichments and the inability to isolate all methanol oxidising bacteria from environmental samples means that cultivation independent approaches must be utilised to characterise the diversity of methylotrophic bacteria.

One approach to characterise the diversity of methylotrophic bacteria within an environment is to use functional gene probes to amplify genes that encode enzymes involved in the oxidation of methanol. As previously described (Chapter One), there are multiple types of methanol dehydrogenase. The focus of the cultivation independent research with regards to primer design and the sequencing of these functional genes has focused on those possessed by Gram-negative methylotrophic bacteria, specifically *mxaF* and *xoxF*. An improved understanding of the role of the XoxF methanol dehydrogenases in the oxidation of methanol has led to an appreciation of the potential for methanol oxidation in species where this trait was previously considered absent, weak or variable (Fitriyanto *et al.,* 2011; Bosch *et al.,* 2009; Haoxin *et al.,* 2017.).

Prior to this work, there were no primer sequences available for the amplification of the *mdh2* gene from environmental or isolate DNA. This gene is much more restricted in its phylogenetic distribution than *mxaF* and *xoxF*, as it has only been detected and characterised in two genera from the Betaproteobacteria (Kalyuzhnaya *et al.*, 2008; Lu *et al.*, 2011; Lu *et al.,* 2012). Mdh2 has been shown to be a functional methanol

dehydrogenase enzyme, capable of oxidizing methanol and ethanol, with upregulation of the transcription of the *mdh2* encoding gene occurring in the presence of both substrates (Lu *et al.*, 2012). In spite of its reduced phylogenetic distribution, as a confirmed methanol dehydrogenase it was important to consider with regards to characterising the diversity of methylotrophs.

4.2 Design of *mdh2* primers

4.2.1 Design of *mdh2* primers

Primers were designed using nucleotide sequences of *mdh2* genes shown to encode functional methanol dehydrogenases. These sequences were aligned at the amino acid level to identify conserved regions. Alignments of *mdh2* sequences with *xoxF5* and *mxaF* sequences were used to avoid the selection of a region common to all PQQ alcohol dehydrogenases (Kalyuzhnaya *et al.*, 2008).

4.2.2 PCR amplification of *mdh2* genes from isolate DNA

The amplification of the *mdh2* gene was optimised using DNA extracted from *Methylibium* sp. ROOT1272 and *Methyloversatilis discipulorum* LF1 (The latter isolated and detailed in Chapter 3). The annealing temperature was optimised using genomic DNA templates. DNA from *Methylobacillus denitrificans* MM3 and *Hyphomicrobium denitrificans* (which contain *mxaF*, *xoxF4* and *xoxF5*) were used as negative controls to test for amplification of additional PQQ alcohol dehydrogenases. The amplified PCR products were aligned with methanol dehydrogenase sequences to confirm the classification of the amplified products as *mdh2* sequences. The products were subsequently aligned with representative *mdh2* sequences to produce phylogenetic trees.

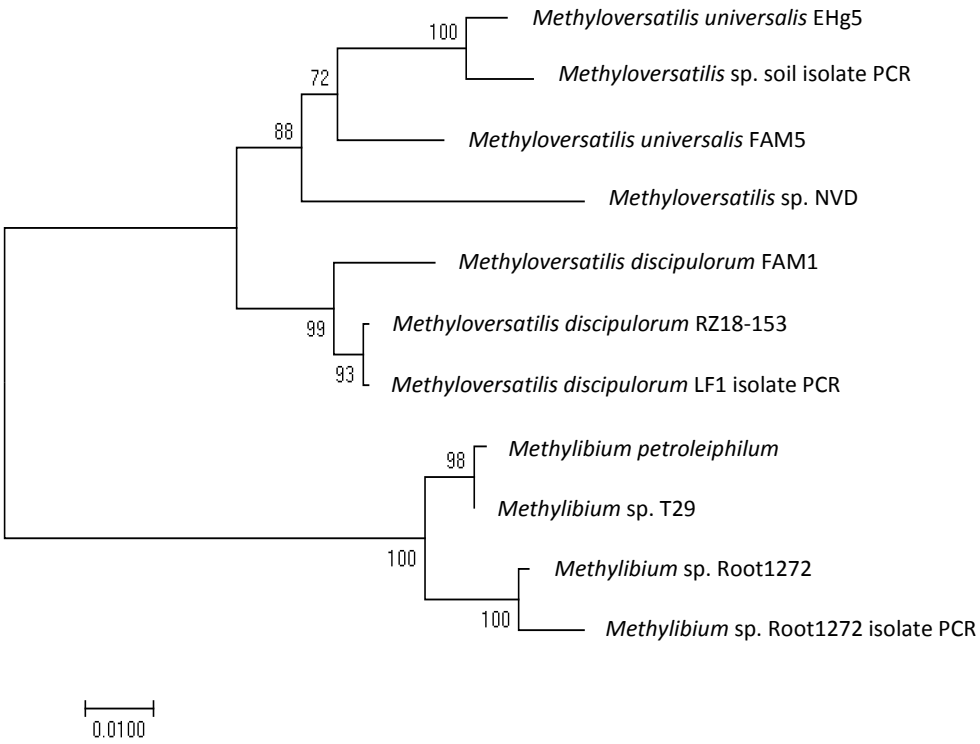


Figure 4.1 Phylogenetic analysis of the *mdh2* gene from isolates *Methylibium* sp. Root1272, *Methyloversatilis* sp. soil and *Methyloversatilis discipulorum* LF1, together with other representative sequences. The phylogenetic tree was constructed from nucleotide sequences aligned at the deduced amino acid level. The evolutionary history was inferred using the Neighbour-Joining method with a bootstrap value of 500. The scale bar represents nucleotide substitutions per position. Isolate PCR designates amplicons produced using DNA extracted from isolates.

4.2.3 PCR amplification of *mdh2* genes from DNA extracted from environmental samples

To test the *mdh2* primers as an assay for the diversity of *mdh2* sequences in the environment, DNA extracted from a range of environments was used as template for the PCR reactions. These environments include landfill soil, Norfolk Broads water from Hickling, Norfolk Broads sediment from Hickling, CF soil, methanol-enriched CF soil and methanol-enriched rhizosphere soils. Additional DNA used as templates in the PCR was rhizosphere soil collected from pea plants (pea rhizosphere soil) and from wheat plants (wheat rhizosphere soil) that were grown in the CF soil for four weeks. DNA extracted from roots that were collected from four week old pea (pea roots) and wheat (wheat roots) plants and washed with PBS was also used as a template. Products of ~500bp were used to produce clone libraries (2.5.5). Where additional bands were obtained, gel extraction was performed to extract the band of interest.

Amplicons of the correct size were obtained from DNA extracted from methanol-enriched pea rhizosphere soil, Norfolk Broads water and landfill soil. These amplicons were used to produce clone libraries. Twenty clones produced using DNA from the Norfolk Broads water and landfill soil were then screened by RFLP (2.5.7). RFLP profiling indicated that the diversity of *mdh2* sequences in these two environments was low and that they were dominated by one phylotype in all of the environments. Products representative of each profile were purified and sequenced, revealing that the dominant *mdh2* sequence for each environment had high similarity to *mdh2* sequences from members of the genus *Methyloversatilis* (Figure 4.2). The remaining profiles were found to result from non-specific amplification, with none of the sequences showing high identity with any clade of PQQ alcohol dehydrogenase. Three clones from the clone library produced using DNA from the methanol enriched pea rhizosphere soil were sent for sequencing. Sequencing showed these three clones to be identical to each other and to also have high identity to *Methyloversatilis*.

The primers are capable of amplifying *mdh2* sequences belonging to a member of the genus *Methylibium*, which share only 80% identity with *mdh2* sequences from *Methyloversatilis* strains. This suggests that primer bias is not solely responsible for the amplification of only *Methyloversatilis*-like *mdh2* sequences from the environment. Instead, it would suggest that the gene is not very diverse in the environments screened. Whether there is more diversity of this gene in other environments would require a more extensive screening effort in the future.

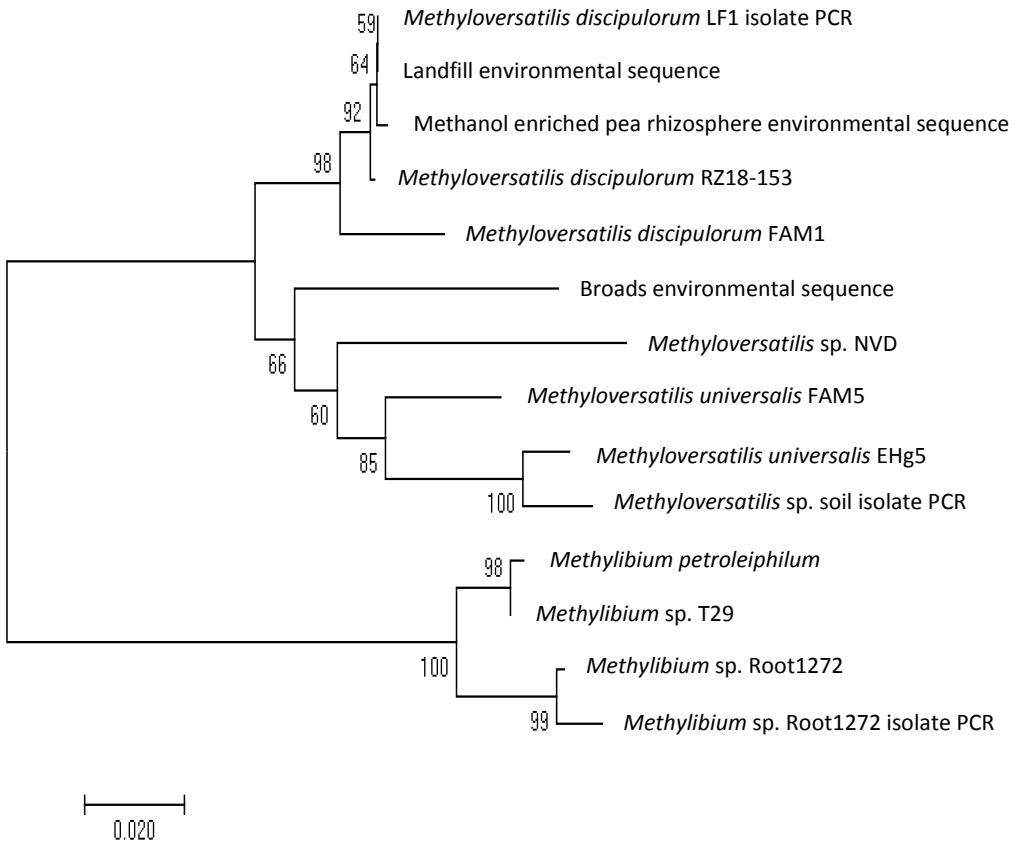


Figure 4.2 Phylogenetic analysis of the *mdh2* gene sequences retrieved by PCR from environmental samples, together with other representative *mdh2* sequences. The phylogenetic tree was constructed from nucleotide sequences aligned at the deduced amino acid level. The evolutionary history was inferred using the Neighbour-Joining method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position. Isolate PCR designates amplicons produced using DNA extracted from isolates. Environmental sequence designates amplicons products from DNA extracted from environmental samples.

4.3 PCR amplification of *mxaF* and *xoxF*1-5 genes using DNA extracted from environmental samples

Primers for *xoxF* genes were developed for, and have been mainly applied to, the marine environment (Taubert *et al.,* 2015). The potential for their use in terrestrial environments was therefore assessed using soil samples from a range of environments.

4.3.1 CF soil, pea rhizosphere soil and wheat rhizosphere soil and soil enriched with methanol and CF soil cDNA

mxaF, xoxF1, *xoxF2*, *xoxF3* and *xoxF5* were amplified from DNA extracted from the CF soil. DNA extracted from methanol-enriched CF soil (Chapters 2 and 5) was also used as template, yielding an additional *xoxF4* product. DNA extracted from additional

environments was screened with the *xoxF* primers (Table 4.1). Additional environments screened included DNA extracted from pea roots, wheat roots, pea rhizosphere soil and wheat rhizosphere soil. cDNA was also generated from RNA extracted from the CF soil and this was used as an additional template for screening the *xoxF* primers. In addition to DNA extracted from the CF soil and the related rhizosphere environments, DNA extracted from different environments was used as a template, including Norfolk Broads water, Norfolk Broads sediment, landfill soil and permafrost soil at 5 cm and 30 cm depth. The clades of successfully amplified methanol dehydrogenase gene varied when using cDNA and DNA from the CF soil, with the cDNA yielding only *xoxF3* and *xoxF5*. The landfill and permafrost soil DNA yielded PCR products for every *xoxF* gene. *mxaF* and *xoxF5* were successfully amplified from DNA from all of the screened environments.

Table 4.1 PCR amplification of *xoxF* genes from DNA extracted from a range of environmental samples

Gene	Norfolk Broads		CF soil		Pea rhizosphere	Wheat rhizosphere	Methanol-enriched CF	Methanol-enriched Pea rhizosphere	Landfill soil	Permafrost soil
	Sediment	Water	DNA	cDNA						
xoxF1	×	✓	✓	×	✓	✓	✓	✓	✓	✓
xoxF2	×	×	✓	×	✓	✓	✓	✓	✓	✓
xoxF3	✓	×	✓	✓	✓	✓	✓	✓	✓	✓
xoxF4	×	✓	×	×	×	×	✓	✓	✓	✓
xoxF5	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
mxaF	✓	✓	✓	×	✓	✓	✓	✓	✓	✓
mdh2	×	✓	×	×	×	×	×	✓	✓	×

4.4 Diversity of methanol dehydrogenase sequences amplified from CF DNA

4.4.1 mxaF profile of CF soil and pea rhizosphere soil

After quality control (Chapter 2) the number of sequences from the CF soil (2014) and the pea rhizosphere soil (2015) was reduced to 2,870 and 3,073 respectively. The number of the OTUs produced from the sequenced *mxaF* amplicons in both environments was low, with four OTUs produced from both environments. Three OTUs from both environments showed high similarity to the *mxaF* gene sequences of species of *Hyphomicrobium*. *Hyphomicrobium* was present at 4.5 - 6 % of the 16S rRNA gene profile in the CF soil and pea rhizosphere communities (Supplementary Table 1). Of the genera predicted to contain the *mxaF* gene, it is the most abundant within these environments and therefore the prominence of this genus within the *mxaF* profiles is not unexpected. The remaining diversity was represented by less than 1% of the *mxaF* sequences. These sequences had high identity to the *mxaF* gene sequences of *Methylobacterium* and members of the family *Methylocystaceae*. *Methylobacterium* and *Methylocystaceae* were both less abundant than *Hyphomicrobium* in the 16S rRNA gene profile in the CF soil, present at 1 % and 0.3 %, respectively, potentially explaining their lower abundance in the *mxaF* profile relative to the *Hyphomicrobium*.

4.4.2 *xoxF5* profile of CF soil, pea rhizosphere soil and wheat rhizosphere soil

The *xoxF5* amplicons from CF soil, pea rhizosphere and wheat rhizosphere contained 1,249, 1,117 and 3,109 reads respectively following quality control. After clustering the sequences to OTUs, 13 OTUs could be identified in the CF *xoxF5* amplicon, 14 OTUs in the wheat rhizosphere and 19 OTUs from the pea rhizosphere. The majority of OTUs detected from all three environments showed high identity to *xoxF5* sequences from the members of the classes Alphaproteobacteria and Betaproteobacteria.

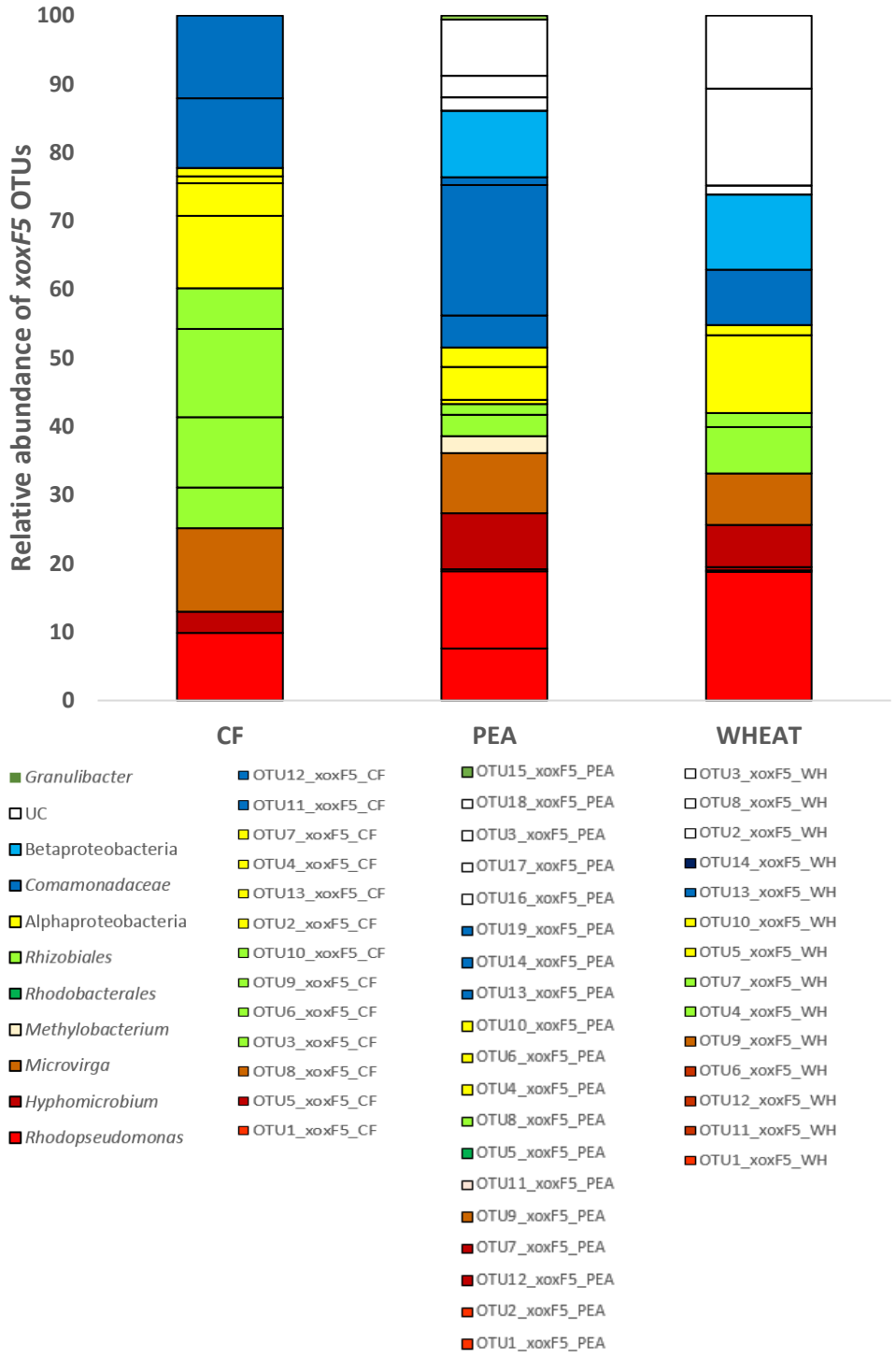


Figure 4.3 Relative abundance of *xoxF5* OTUs (at the highest level of phylogenetic resolution) in the CF soil, pea rhizosphere soil and wheat rhizosphere soil. Sequences were obtained by 454 sequencing.

OTUs from each of the *xoxF5* profiles showed high identity to *xoxF5*s from the genus *Hyphomicrobium* (OTU5_*xoxF5*_CF, OTU8_*xoxF5*_CF, OTU7_*xoxF5*_PEA, OTU12_*xoxF5*_PEA, OTU11_*xoxF5*_WH OTU12_*xoxF5*_WH, OTU26_*xoxF5*_WH) (Figure 4.3). An OTU with high identity to *Microvirga xoxF5* sequences was also identified in the *xoxF5* profiles of the different environments (OTU8_*xoxF5*_CF, OTU9_*xoxF5*_PEA, OTU9_*xoxF5*_WH). *Microvirga* is a genus within the *Methylobacteraceae*, with its closest phylogenetic relative being *Methylobacterium*. All three environments also had OTUs with high identity to *Rhodopseudomonas xoxF5* sequences, with both the pea and wheat rhizosphere environments having a higher relative abundance of the *Rhodopseudomonas* related OTUs than the CF soil (OTU1_*xoxF5*_CF, OTU1_*xoxF5*_PEA, OTU2_*xoxF5*_PEA, OTU1_*xoxF5*_WH). Members of the genus *Rhodopseudomonas* can grow on methanol as a sole carbon source and have a varied metabolic capability, growing as chemotrophs and phototrophs, as well as autotrophically and heterotrophically (Larimer *et al.,* 2004; Douthit and Pfenning 1981; Siefert and Pfennig 1979; Quayle and Pfennig 1975). OTUs with high identity to *xoxF5* sequences from members of the *Commamonadaceae* were also detected in the three environments (OTU11_*xoxF5*_CF, OTU12_*xoxF5*_CF, OTU13_*xoxF5*_PEA, OTU14_*xoxF5*_PEA, OTU19_*xoxF5*_PEA, OTU13_*xoxF5*_WH). The pea rhizosphere *xoxF5* profile had a higher relative abundance of *Commamonadaceae* related OTUs relative to the CF soil, whereas these were less abundant in the wheat *xoxF5* profile. OTUS that could not be assigned to a higher resolution than Betaproteobacteria were also more abundant in the *xoxF5* profile of the wheat rhizosphere soil and pea rhizosphere soil than in the CF soil. The pea rhizosphere *xoxF5* profile also contained OTUs with high identity to *xoxF5* sequences from the genera *Methylobacterium* (OTU11_*xoxF5*_PEA) and *Granulibacter* (OTU15_*xoxF5*_PEA). *Granulibacter* acetic acid bacterium have been isolated from plants, soil and water and is linked to infection of granulomas (Greenberg *et al.,* 2007; Falcone *et al.,* 2016; Greenberg *et al.,* 2006).

4.4.3 *xoxF1* and *xoxF2* profile of CF soil

The sequencing of the *xoxF1* amplicon produced 4,446 sequences after quality control. These sequences formed seven OTUs (Figure 4.4). The OTUs were divided between three genera within the order *Rhizobiales* and a genus within the order *Xanthomonadales* (Figure 4.5).

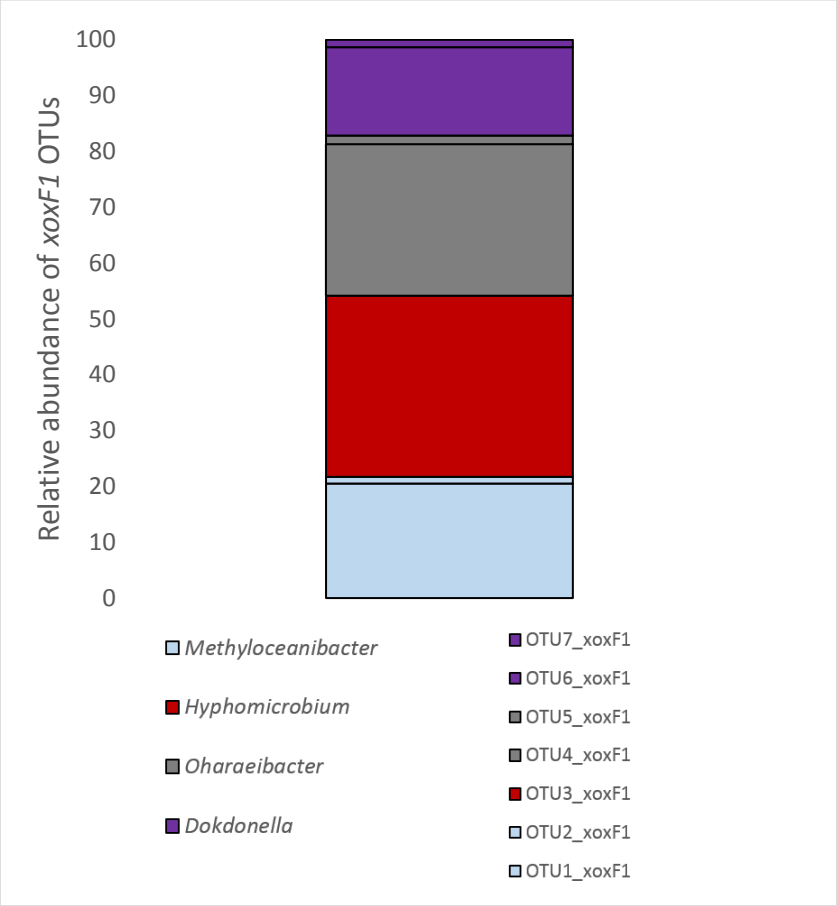


Figure 4.4 Relative abundance of *xoxF1* OTUs (at the genus level) of the CF soil. Sequences were obtained by 454 sequencing.

Thirty percent of sequences were assigned to an OTU (OTU3_*xoxF1*) with high identity to the *xoxF1* sequence of *Hyphomicrobium nitrativorans* (Figure 4.5), further demonstrating the relevance of this genus to methanol oxidation within the CF soil. The two additional members of the *Rhizobiales* detected in the *xoxF1* sequences are the *Oharaeibacter* (OTU4_*xoxF1* and OTU5_*xoxF1*) and *Methyloceanibacter* (OTU1_*xoxF1* and OTU2_*xoxF1*). Members of the genus *Oharaiebacter* have been isolated from the rhizosphere of a rice plant (Haoxin *et al.,* 2017) and from landfill soil (Chapter 3). Whilst

both of these environments have elevated methane concentrations, *xoxF1* sequences that cluster with that of *Oharaiebacter* LF4 are also present and abundant in the CF soil.

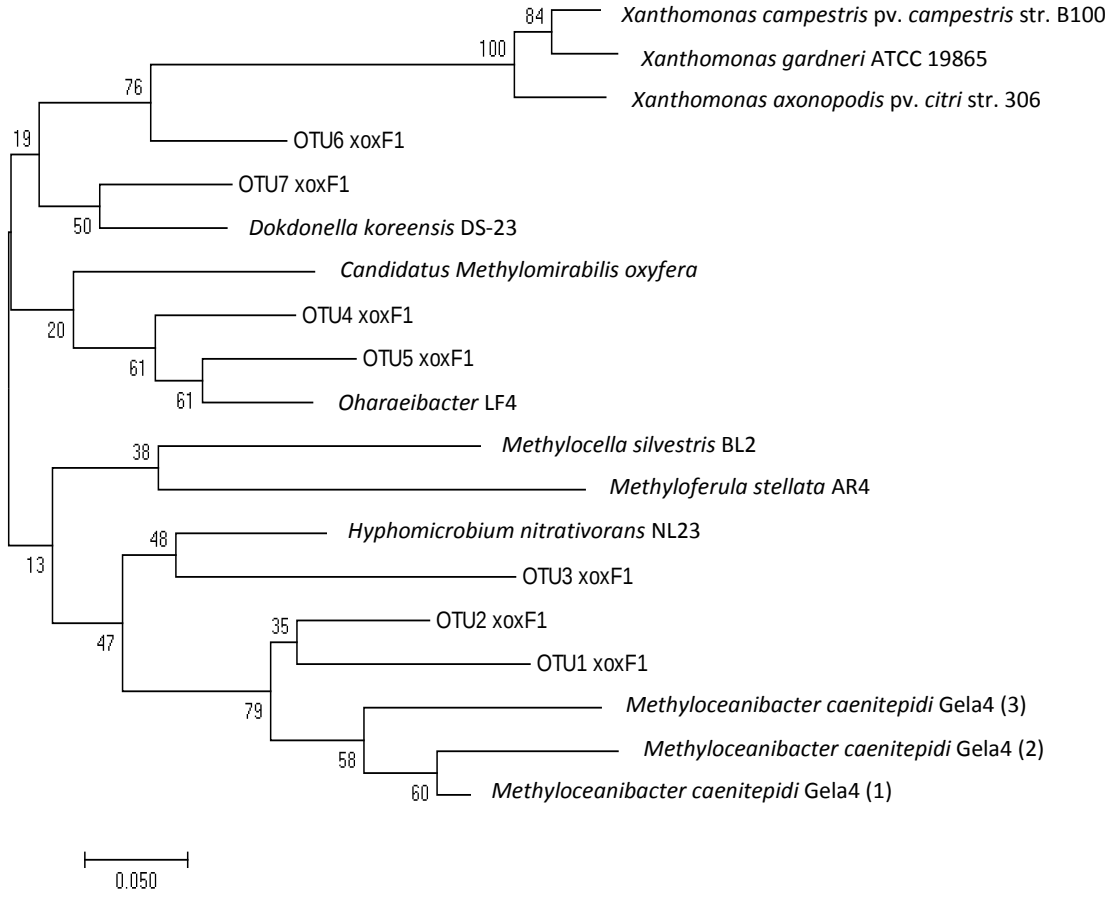


Figure 4.5 Phylogenetic analysis of the *xoxF1* gene sequences amplified from DNA extracted from CF soil. Sequences were analysed with a database of *xoxF1* sequences. The phylogenetic tree was constructed from nucleotide sequences aligned at the deduced amino acid level. The evolutionary history was inferred using the Neighbour-Joining method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position.

Methyloceanibacter has previously only been detected within the marine environment. However, characterisation of the isolated strains has shown that some do not require high salinity to grow (Vekeman 2016a; Vos *et al.,* 2016; Vekeman *et al.,* 2016b; Takeuchi *et al.,* 2014) and it has been shown there is variability in the metabolic capabilities of the different strains (Vekeman *et al.*, 2016a; Vos *et al.*, 2016). It is therefore possible that members of this genus occupy a niche within the terrestrial environment in addition to those that have been detected in the marine environment, as has been shown to occur with other genera (Dixon *et al.*, 2013; Chistoserdova, 2015). A fifth of the *xoxF1*

sequences cluster with references sequences belonging to members of the *Xanthomonadales*. Members of the genus *Dokdonella* have previously been isolated from the soil environment and in association with plants (Yoon *et al.*, 2006a; Ten *et al.*, 2009). However methylotrophy has not been confirmed as a trait within this genus. Therefore, it is possible that this gene is not functional with regards to methanol oxidation within these organisms. It is also of note that all of the genera with which the *xoxF1* sequences cluster can be classified as facultative i.e. capable of growth on methanol in addition to a range of additional carbon sources.

Only 144 *xoxF2* sequences were obtained from the 454 sequencing following quality control (reduced from 247). The reason for the low sequence number was probably the result of complications with the sequencing and this specific primer sequence (Dowd, Molecular Research LP, personal communication). The number of OTUs produced though 454 sequencing of the *xoxF2* methanol dehydrogenase amplicon obtained was low. There was one OTU at the 70% identity threshold and three OTUs at 80%, dominated by one OTU that represented 96 sequences. The most prominent OTU at 80% identity and the sole OTU produced at 70% identity were identical in sequence to a previously sequenced clone of the same PCR amplicon (Section 4.3). Therefore the clone sequence was used for further phylogenetic analysis due to its increased length of 500 bp compared to 200 bp.

The cloned *xoxF2* sequence clustered with the reference sequence of BAC10-4 (Figure 4.6). BAC10-4 is a fosmid constructed using DNA from the sediment of Lake Washington, containing a *xoxF* in addition to several additional methylotrophy-linked genes (Kalyuzhnaya *et al.*, 2005). Additional reference *xoxF2* sequences were identified using NCBI Blastp. The sequences that have the highest identity to the main *xoxF2* OTU, 82-84%, belong to binned genomes from metagenome datasets belonging to members of the Phyla *Candidatus* Rokubacteria and Gemmatimonadetes. It has been proposed that both of these phyla have major roles in the nitrogen and sulphur cycles and are metabolically versatile (Bernard *et al.*, 2007; Debruyn *et al.*, 2011; Butterfield *et al.*, 2016; Hug *et al.*, 2016). Further to this, their PQQ alcohol dehydrogenases were expressed in soil, suggesting that these enzymes may be functional in these specific bacteria (Butterfield *et al.*, 2016). 80 % identity was also observed with the soil *xoxF2*

OTU and the PQQ alcohol dehydrogenase sequences encoded within the genomes of strains of the squid endosymbionts *Candidatus Entotheonella* (Sennett *et al.*, 2008; Wilson *et al.*, 2014; Liu *et al.*, 2016). This additional *Candidatus* genus has been detected in the marine environment, with genomes constructed following metagenomic sequencing of DNA extracted from the Chinese and Japanese seas. These organisms are also proposed to be metabolically versatile (Sennett *et al.*, 2008; Wilson *et al.*, 2014; Liu *et al.*, 2016).

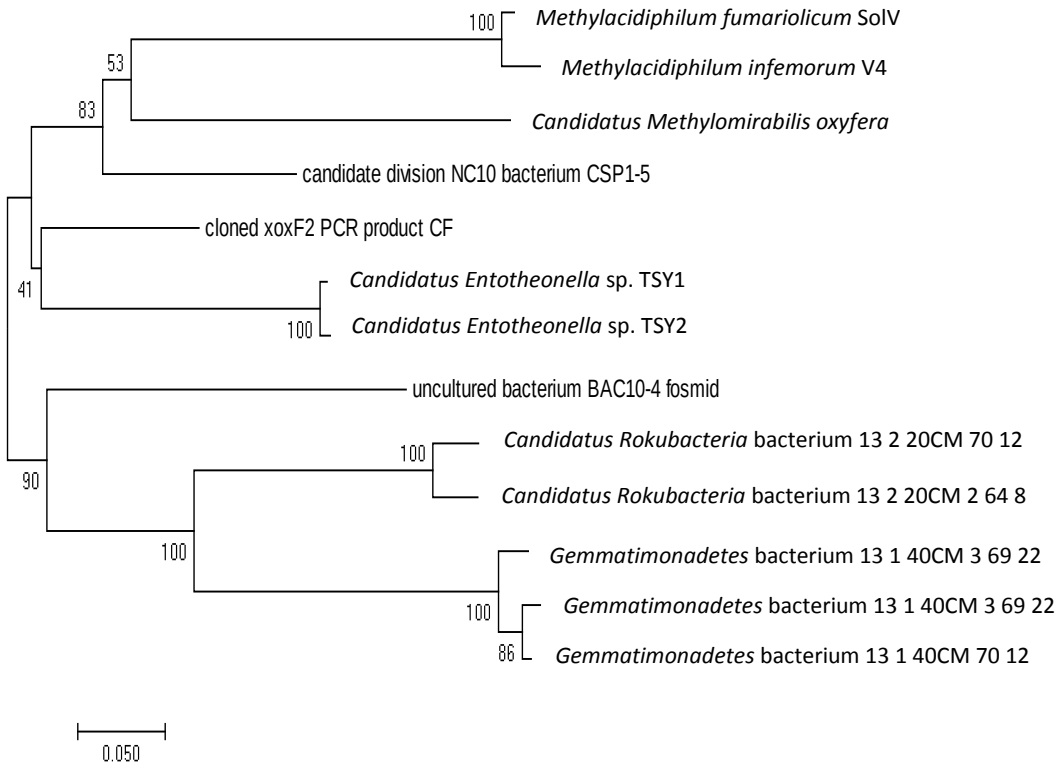


Figure 4.6 Phylogenetic analysis of the *xoxF2* gene sequences amplified from DNA extracted from CF soil. Sequences were analysed with a database of *xoxF2* sequences. The phylogenetic tree was constructed from nucleotide sequences aligned at the deduced amino acid level. The evolutionary history was inferred using the Neighbour-Joining method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position.

4.4.3 *xoxF3* profile of CF soil

The two dominant RFLP profiles, accounting for 53/100 of the clones, were *xoxF2* sequences and these were therefore excluded from further analysis. The sequence of the dominant *xoxF3* RFLP profile (OTU1_*xoxF3*), accounting for 26 of the remaining 47

clones, was most closely related to the *xoxF3* methanol dehydrogenase gene of *Methylobacterium nodulans* (Figure 4.7 and 4.8). However, *xoxF3* is not as widespread throughout the genus *Methylobacterium* as *xoxF5* and *mxaF*. Another RFLP profile revealed clones (OTU4_*xoxF3* and OTU5_*xoxF3*) with high identity to *xoxF3* of species within the genus *Azosporillium*. The genus, also within the Alphaproteobacteria, contains species that are typically plant associated and nitrogen fixing (Lu *et al.*, 2006; Chung *et al.*, 2012; Moghaddam *et al.*, 2012). Until recently there were no described species within the genus capable of the oxidation of methanol. However, a characterised and genome sequenced species of the genus, *Azosporillium thiophilum*, has now been shown to contain *mxaF* and *xoxF3* methanol dehydrogenase genes and grow on methanol (Orlova *et al.*, 2016). Phylogenetic analysis of the two *xoxF* gene sequences of *Methylosinus trichosporium* OB3b clusters one of these genes outside of the *xoxF3* methanol dehydrogenase clade. This *xoxF* gene has therefore been considered a different subtype of *xoxF* (Keltjens *et al.*, 2014). As this clade is closest to the *xoxF3* clade, it will therefore be referred to as *xoxF3b* for further discussion. The diversity of the *xoxF3* clones (OTU2_*xoxF3* and OTU3_*xoxF3*) that clustered with the *xoxF3b* subtype were included in the diversity profiling for this gene, as the subtype designation could be the result of only one candidate sequence being considered in the classification of the clades.

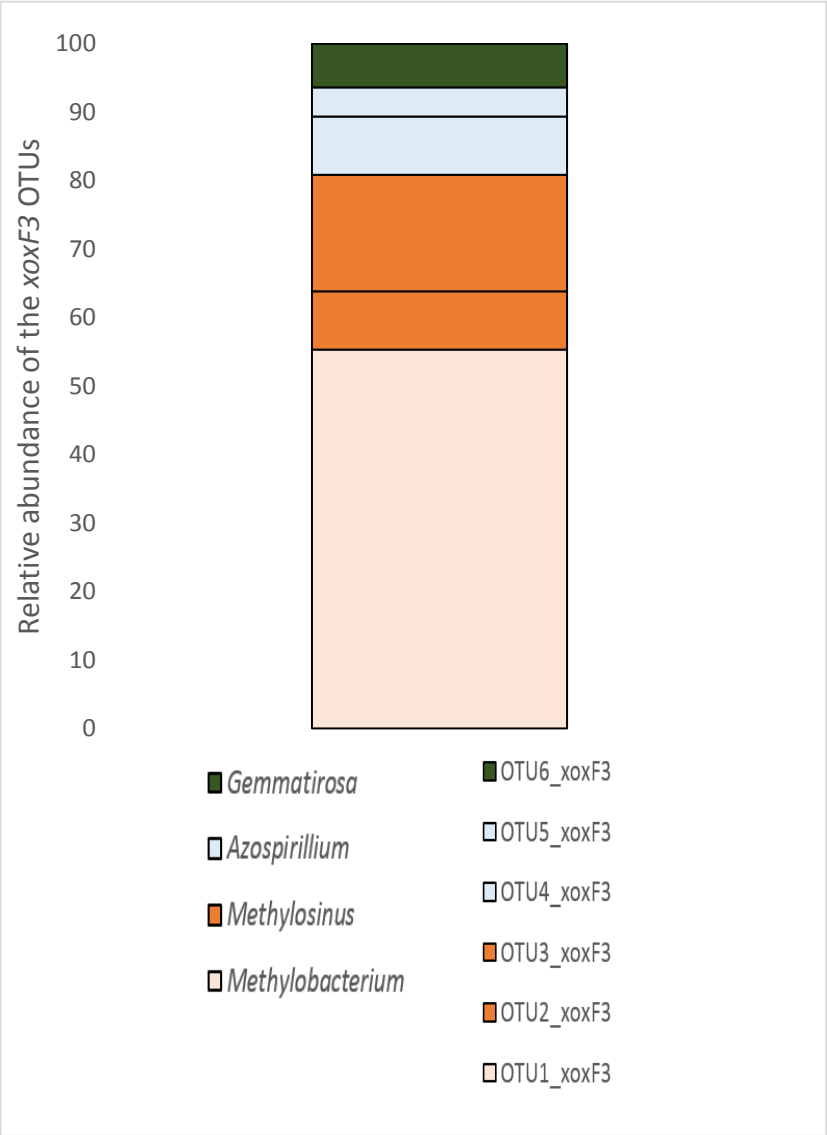


Figure 4.7 *xoxF3* profiles from bacteria (at the genus level) of the CF soil. Sequences were obtained by Sanger sequencing.

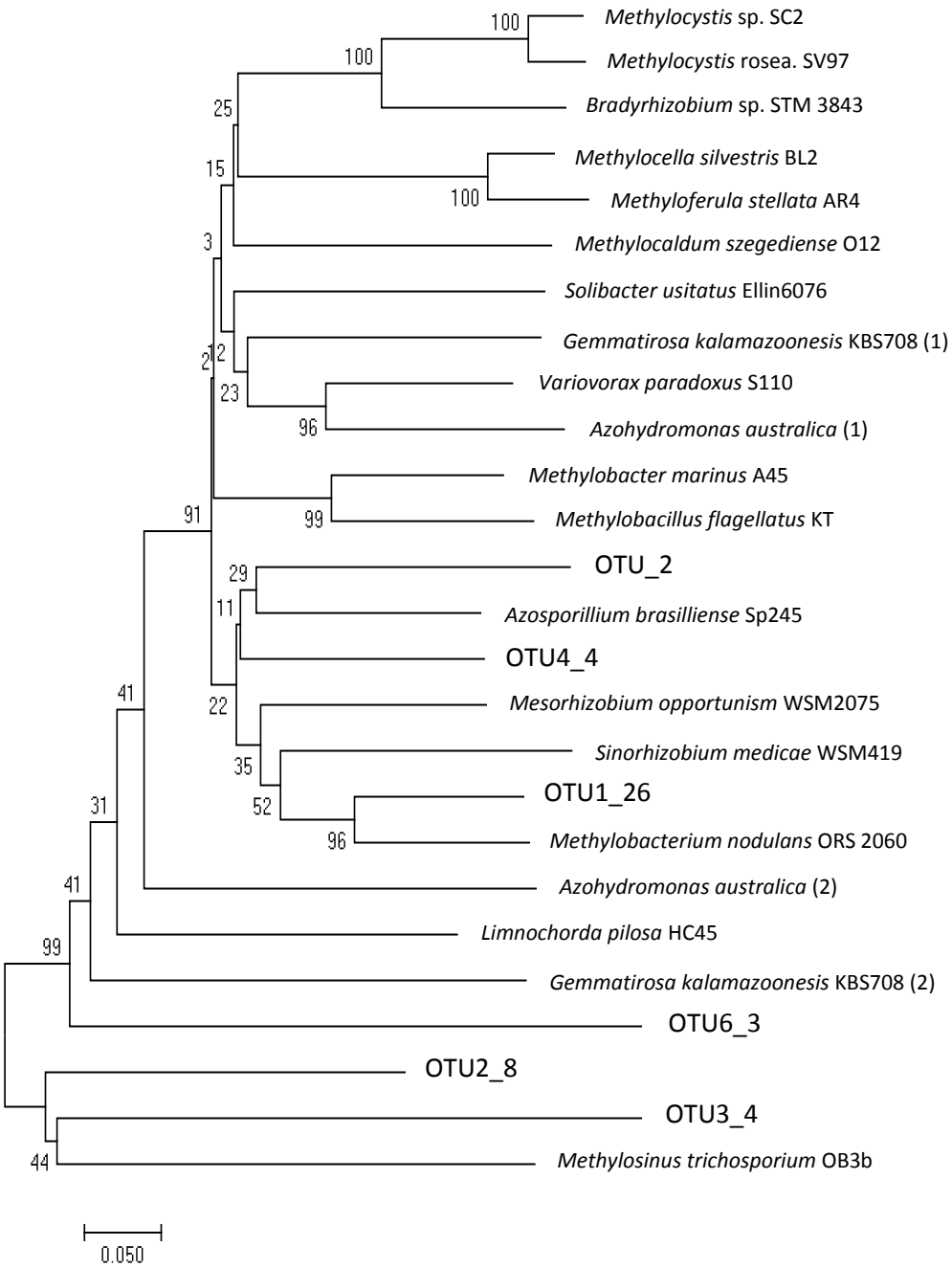


Figure 4.8 Phylogenetic analysis of the *xoxF3* gene sequences amplified from DNA extracted from CF soil. Sequences were analysed with a database of *xoxF3* sequences. The phylogenetic tree was constructed from nucleotide sequences aligned at the deduced amino acid level. The evolutionary history was inferred using the Neighbour-Joining method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position. The number (OTU_n) designates the number of clones that share the RFLP profile of the sequenced clone.

4.5 Quantification of *mxaF* and *xoxF5* gene abundance through qPCR

The sequencing results of the *xoxF*, *mdh2* and *mxaF* genes amplified from DNA extracted from the CF soil (4.2 and 4.5) indicated that the most relevant methanol dehydrogenase genes for considering the diversity of methylotrophs in this environment were *xoxF*5 and *mxaF*. Measuring the abundance of genes involved in methanol oxidation will allow detection of differences between environments. Therefore qPCR assays were developed for the amplification of both of these genes. The existing primer sets for PCR amplification of *mxaF* and *xoxF* were used in the qPCR for both of these genes and the reactions were optimised as described in Chapter 2.

4.5.1 Quantification of *mxaF* and *xoxF5* gene abundance in environmental samples through qPCR

The *mxaF* and *xoxF5* qPCR assays were used to quantify the abundance of *mxaF* and *xoxF5* genes present within DNA extracted from the CF soil, pea rhizosphere soil, wheat rhizosphere soil and pea and wheat roots (Section 4.3.2). The abundance of the methanol dehydrogenase genes was normalised to 16S rRNA gene copies. Three biological replicates from each environment, each with three technical replicates, were tested using this assay.

The melt curve for the methanol dehydrogenase gene qPCR assay using DNA extracted from pea roots and wheat roots had two peaks. This additional peak was not present in the other environmental samples tested, implying that it was not a result of an overlong elongation time or primer dimers. Therefore, it is possible that this peak was produced as a result of the amplification of an additional gene for which the primers were sufficiently cross specific. As this additional peak only occurred for the samples that were extracted from plant roots it is tempting to speculate that this additional product was amplified from plant DNA. The variation between replicates was very high when DNA extracted from the pea roots and wheat roots was used as template, so these environmental samples were excluded from further analysis.

The qPCR assays showed that within the soil environments tested, after normalising for the abundance of 16S rRNA genes, *xoxF5* was present at greater abundance then *mxaF* genes (Figure 4.9). *xoxF5* was present at a copy number of 0.1-0.2 per 16S rRNA gene copy relative to a copy number of 0.003-0.006 per 16S rRNA gene copy for *mxaF*. There

was no significant difference in the abundance of the *xoxF5* and *mxaF* copy number normalised by 16S rRNA gene abundance between the CF soil and the rhizosphere soils in spite of a shift in the *xoxF5* diversity profiles between the environments. However, the standard error in the abundance of these genes did increase in the rhizosphere samples relative to the CF soil.

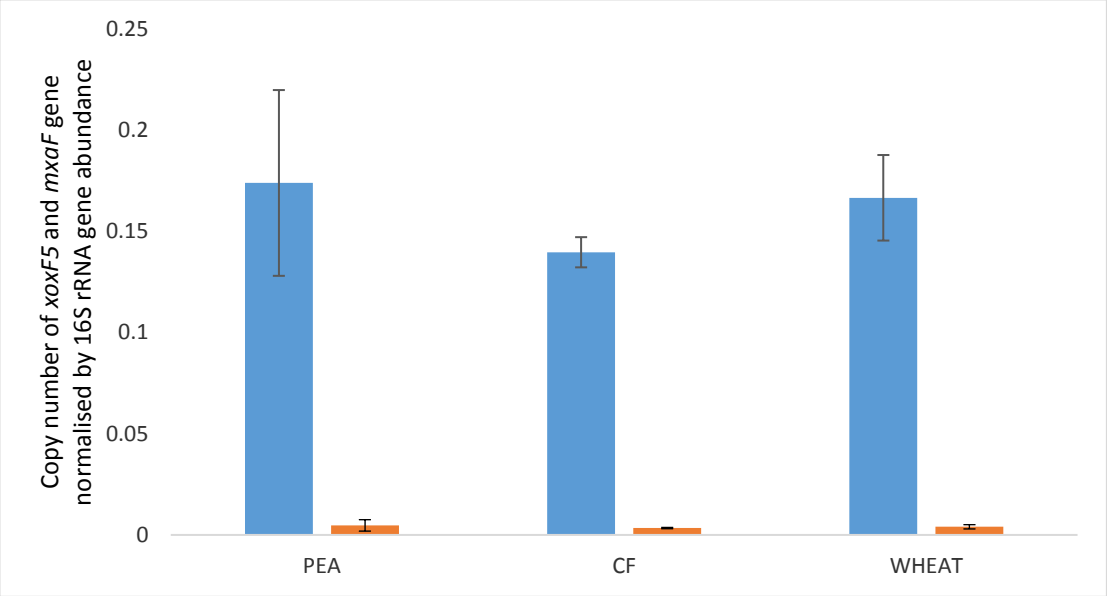


Figure 4.9 qPCR assay of xoxF5 (blue) and mxaF (orange) in DNA extracted from pea rhizosphere soil (PEA), CF soil (CF) and wheat rhizosphere soil (WHEAT). The abundance of both methanol dehydrogenase genes was normalised to the abundance of 16S rRNA genes.

4.6 Discussion

4.6.1 Amplification of the *xoxF* genes in DNA extracted from environmental samples

It is possible to reliably and consistently amplify *xoxF* genes from DNA extracted from environmental samples in which these genes are present. The ability to amplify the *xoxF* genes enhances our ability to characterise the diversity of methylotrophic bacteria within an environment. The relevance of the *xoxF* methanol dehydrogenase genes to methanol oxidation in both marine and terrestrial environments is being increasingly shown (Taubert *et al.*, 2015; Howat 2016; Ramachandran and Walsh 2015; Knief *et al.,* 2012; Delmotte *et al.,* 2009), with this work providing an indication as to the diversity of methylotrophic bacteria that was previously undetected through the sequencing of the *mxaF* methanol dehydrogenase encoding genes alone. This work also provides the first

confirmation of the capacity of the *xoxF*1-3 primer sets to amplify these clades of methanol dehydrogenase gene from environmental samples. In addition to the greater detection of diversity, the sequencing of *xoxF* genes enables the detection of a shift in the diversity of methylotrophic bacteria between the soil and rhizosphere environments. This shift in diversity was not detected by the sequencing of *mxaF* in the CF soil and pea rhizosphere soil, and further shows the importance of being able to characterise these additional genes.

The extent of the cross-specificity of the *xoxF* primers raises the issue of whether a universal primer set for the amplification of the *xoxF* genes could be developed. As the *xoxF5* primer set amplifies genes from every clade of *xoxF* and *mxaF* genes it is clear it would be possible to design a primer set to intentionally amplify these genes. However, a universal primer set would preferentially amplify the most abundant *xoxF* genes and not capture representative sequence diversity of the less abundant *xoxF* genes. Therefore, although a universal *xoxF* primer set could exist, the specific primers to use would depend on the question the research was attempting to answer. Although the existing *xoxF* primers are all cross-specific they are useful in assessing the diversity of the individual clades. In the absence of markedly improved results through further next generation sequencing techniques, the construction of clone libraries with subsequent RFLP analysis may be a better strategy to obtain for *xoxF2* and *xoxF3* sequences from DNA of environmental samples. This is based on the low number and diversity of *xoxF2* sequences and low quality of *xoxF3* sequences that were produced by 454 sequencing of these amplicons.

4.6.2 Characterisation of the diversity of methylotrophic bacteria in environmental samples

Several *xoxF5* OTUs increased in relative abundance in the plant associated soils relative to the bulk soil. These OTUs included those with high identity to *Methylobacterium*, *Microvirga*, *Commamonadaceae* and *Rhodopseudomonas*. These phylogenetic groups are found across a range of environments, including in association with plants (Sy *et al.*, 2001; Schmalenberger *et al.*, 2007; Knief *et al.*, 2008, 2012; Caputo *et al.*, 2016; Safronova *et al.*, 2017) . Due to these phylogenetic groups possessing highly varied metabolisms it is not possible to determine the reason for the change in abundance of

these OTUs within the *xoxF5* profiles following the growth of plants for four weeks. It is also interesting to note the increase in their relative abundance in the *xoxF5* profile when considering their overall relative abundance in the 16S rRNA gene profile does not alter to a large extent following growth of the plant (Chapter 5).

A portion of the diversity of *xoxF* sequences showed high identity to reference sequences from organisms where methylotrophy has either not been tested, or has been shown to be absent. For example, no species of *Microvirga* have been reported to utilise methanol as a carbon source, with an ability to utilise C1 compounds used as a delineating trait between *Microvirga* and *Methylobacterium* (Ardley *et al.*, 2012; Caputo *et al.*, 2016; Safronova *et al.*, 2017). This reinforces the need for the retesting of these organisms for growth on methanol with the addition of lanthanides. There is also a need to sequence the genomes of more methylotrophic bacteria. This would allow identification of methylotrophy-related genes in genera where methylotrophy is not typical and the methanol dehydrogenase gene may have not been identified (Boden *et al.*, 2008; Madhaiyan *et al.*, 2010; Eyice *et al.,* 2015a). The genome sequencing of these organisms would allow the expansion of the sequence databases in addition to potentially identifying novel pathways in methylotrophy.

A large amount of the *xoxF* sequence diversity captured from the CF soil could not be classified to a low phylogenetic level. The sequence with the highest identity to the *xoxF2* OTU was 84 % and the sequence identity of some of the *xoxF3* clones and *xoxF5* OTUs showed less than 70 % identity to reference sequences. Unclassifiable *xoxF* sequences have also been detected in the marine environment (Taubert *et al.,* 2015). This indicates the high levels of diversity of methylotrophic bacteria that remain to be characterised, as the ability to classify sequences depends on the availability of reference sequences of sufficiently high identity for phylogeny to be inferred. It is interesting to note that of the clades of methanol dehydrogenase, *xoxF2* is represented the most by phyla that are *Candidatus* and is absent in all sequenced members of the proteobacteria, the most studied phylum with regards to methylotrophy. Relating phylogeny to a gene sequence is also complicated by horizontal gene transfer. For instance, the *xoxF3* encoded within the genome of *Mesorhizobium opportunism* is a result of the integration of a plasmid into its chromosome (Reeve *et al.*, 2013). This

plasmid is found in other species of *Mesorhizobium* (Nandasena *et al.*, 2009; Reeve *et al.*, 2013). *Azosporillium brasilliense* and *Microvirga ossetica* both possess a *xoxF5* gene located on a plasmid, and *Methylobacterium nodulans* contains *mxaFI* genes on a plasmid (Sy *et al.*, 2001). Furthermore, the presence of multiple *xoxF* genes of the same clade within a genome that are divergent from each other further suggests a role for horizontal gene transfer (Taubert *et al.,* 2015). Therefore, this is something that needs to be considered when attempting to classify sequences from environmental samples.

A reduced number of clades were successfully amplified from the cDNA produced from RNA extracted from the CF soil relative to the DNA. This indicates that of the methanol dehydrogenase genes detected in this environment, not all of them are actively transcribed. *xoxF3* and *xoxF5* may represent the most actively transcribed methanol dehydrogenase genes within the CF soil environment. With the exception of *Hyphomicrobium*, genera containing *xoxF1* and *xoxF2* are not abundant within the CF soil, so it is also possible that these *xoxF* genes are expressed but the abundance of the *xoxF* transcripts is too low to detect within this environment. It is worthwhile noting that *xoxF3* methanol dehydrogenase genes have not been confirmed to code for functional methanol dehydrogenases. Every *xoxF3* possessing organism in which growth on methanol has been confirmed also possesses an additional methanol dehydrogenase-encoding gene and organisms that only possess *xoxF3* have either not been tested for growth on methanol or were shown not to grow (Pankratov *et al.*, 2008; Nandasena *et al.*, 2009; Reeve *et al.*, 2013).

4.6.3 Amplification of the *mdh2* genes in DNA extracted from environmental samples

The diversity of *mdh2* was shown to be low in the environmental samples. In spite of the low diversity of this gene, the ability to detect and sequence an additional methanol dehydrogenase gene from an environmental sample develops our ability to characterise the diversity of methylotrophic bacteria. Further characterisation of methylotrophs will be needed to determine the diversity of the methanol dehydrogenase genes that are not PQQ-dehydrogenases, including those located within the Gram positive bacteria, that have thus far been overlooked (Van Ophem *et al.*, 1993; Kolb *et al.*, 2013; Stacheter *et al.*, 2013; Wu *et al.*, 2016).

4.6.3 Optimisation of the quantification of the *xoxF* and *mxaF* genes in DNA extracted from environmental samples

The ability to quantify the methanol dehydrogenase genes within an environment is useful to assess the relative abundance of these genes between clade and between different environmental samples. *xoxF5* being more abundant in the CF soil than *mxaF* could be explained by both the higher copy number of *xoxF5* within several genomes and the broader phylogenetic distribution of the *xoxF* genes relative to *mxaF* (Chistoserdova, 2011a; Keltjens *et al.*, 2014). The cross specificity of the *xoxF5* primers does not account for the difference in abundance of the *xoxF5* and *mxaF* genes in the CF soil. Even using the highest reported extent of cross specificity of the *xoxF5* primers and reducing the normalised copy number of *xoxF5* by 10% (Taubert *et al.,* 2015) the difference in copy number between the two methanol dehydrogenase genes is still over an order of magnitude. This further shows the importance in sequencing the *xoxF* genes when attempting to characterise the diversity of methylotrophic bacteria within an environmental sample.

Chapter 5: Identification of active methylotrophs in the Church Farm soil through stable isotope probing with ^{13}C methanol

5.1 Introduction

Stable Isotope Probing (SIP), as described in the Introduction, is a powerful technique in microbial ecology that allows us to link processes to defined members of the population through the metabolism of a substrate enriched with a stable isotope (Dumont *et al.*, 2005). The usefulness of this technique, especially DNA-SIP and RNA-SIP using a ^{13}C label, is reflected in the rapid expansion of its use following its inception and the range of processes that it has been used to characterise (Coyotzi *et al.*, 2016). It is able to relate metabolic processes to a specific phylogenetic group e.g. *Beijerinckiaceae* and methane oxidation (Radajewski *et al.*, 2002). Early SIP work investigated the identity of methanotrophic and methylotrophic bacteria within different environments (Morris *et al.*, 2002; Radajewski *et al.*, 2002; Lueders *et al.*, 2003). These experiments tended to use high concentrations of labelled substrate and long incubation times, that can result in microbial activities not being representative of the *in situ* conditions within an environment (Neufeld et al., 2007a) . The extent to which enrichment occurs depends on the substrate used, the duration of the experiment and the concentration of the substrate supplied relative to the ambient concentration. However, this needs to be balanced against acquiring sufficient amounts of labelling (Lueders *et al.*, 2004). The duration of the experiment is important to consider due to cross feeding, whereby the products of metabolism are utilised by additional organisms as this results in secondary labelling and has been shown to occur in several SIP studies (Morris *et al.*, 2002; Lueders *et al.*, 2003; Pankratov *et al.*, 2008; Hernandez *et al.*, 2015). RNA SIP is a more sensitive technique than DNA SIP (Manefield *et al.*, 2002), enabling identification of labelling after shorter amounts of time, as it does not require replication for incorporation of label.

The identification of active methanol-utilising methylotrophs in the Church Farm (CF) soil, pea rhizosphere soil and wheat rhizosphere soil is complicated by many factors. These include species that possess *xoxF* methanol dehydrogenase genes where function has not been shown, species in which *mxaF* has been shown to be non-functional and the utilisation of alternate carbon sources by facultative methylotrophs (Kalyuzhnaya *et al.*, 2008; Keltjens *et al.*, 2014). Therefore, a DNA SIP experiment was performed with ^{13}C methanol using the CF soil and pea rhizosphere soil and wheat rhizosphere soils

collected from pea and wheat plants that were grown in CF soil for four weeks. This was done to identify the active methylotrophic bacteria of these three environments and to identify changes in the diversity of the active methylotrophs following the growth of the plant.

Research has shown that a supply of 0.25-1 µM of lanthanum or cerium to cultures of species of methylotrophic bacteria was sufficient to abolish the expression of the methanol dehydrogenase encoding gene *mxaF* (Chu and Lidstrom, 2016; Vu *et al.*, 2016) and enhance the expression of the alternate methanol dehydrogenase encoding gene, *xoxF* (Chu and Lidstrom, 2016; Vu *et al.*, 2016). A direct supply of lanthanides at 5 µM has been shown to cause a change in the rate of methanol oxidation in marine water samples (Howat, 2016), implying that lanthanides are limiting in that environment. The total measured concentration of lanthanides in soil in the UK is in the range of 0.0003-3µM (Ramos *et al.*, 2016). However, this issue is complicated by the difficulty in measuring the biologically available concentration of lanthanides in the soil and the fact that the system by which methylotrophs detect and take up lanthanides is unknown.

5.2 Analysis of the 16S rRNA gene profiles of Church Farm, pea rhizosphere and wheat rhizosphere soils

16S rRNA genes were amplified from DNA extracted from the Church Farm (CF) soil and from pea rhizosphere soil and wheat rhizosphere soil. These 16S rRNA gene amplicons were sequenced by Illumina to characterise the general bacterial communities of the three environments. This enabled identification of genera that either contain species shown to be capable of methanol oxidation (confirmed as methylotrophs), or contain species that possess *xoxF* methanol dehydrogenase genes (proposed to be methylotrophs).

5.2.1 Identification of methylotrophic genera present in the CF soil community

The 16S rRNA gene profile of the CF soil was shown to contain 34 proposed and confirmed methylotrophs (Supplementary Table 1). The diversity of methylotrophs present in the rhizospheres of pea and wheat plants was represented by 35 genera. Methylotrophic genera comprised a similar percentage of the total community within the three environments of the bulk soil, pea rhizosphere and wheat rhizosphere (15.1 %, 15.4 %, 14.0 %) in spite of a difference in diversity between the environments.

A threshold of 1.5 fold was used to identify methylotrophs that differed in abundance at the species level. Of 80 species putatively identified as methylotrophic, 25 species were present at higher abundance in the pea rhizosphere (20 confirmed and five proposed), and 18 species were present at higher abundance in the wheat rhizosphere (13 confirmed and five proposed). In addition to this higher abundance, over 50 species of methylotroph (51 and 58 respectively) were absent from the rhizosphere soils. This shift in abundance could indicate selection for specific methylotrophic species within these rhizosphere environments.

5.2.2 Methylotrophic genera enriched in the rhizosphere relative to the CF soil community

Methylotrophic genera present at higher relative abundance in pea and wheat rhizosphere soil relative to the bulk soil included *Azosporillium*, *Bradyrhizobium*, *Hyphomicrobium*, *Methylobacterium*, *Variovorax*, *Verminephrobacter* and *Verrucomicrobia*. The increased abundance of *Variovorax* in the pea rhizosphere relative to the bulk soil was previously shown through a metatranscriptomics study (Turner *et al.*, 2013), but this study did not observe enrichment of this genus in the wheat rhizosphere, which was also examined. Several of the detected *xoxF* containing organisms were tested for the ability to oxidise methanol in the absence of lanthanides. This includes *Verminephrobacter* (Pinel *et al.*, 2008), *Meganema* (Thomsen *et al.*, 2006; McIlroy *et al.*, 2015) and *Leptothrix* (Nakatsu *et al.*, 2006).

Genera present at higher abundance only in the pea rhizosphere relative to the bulk soil included *Methylotenera* and *Methylophilus*, also previously detected as enriched in the pea rhizosphere by Turner et al. (2013). *Methylosinus*, *Meganema*, *Oharaeibacter* and *Sphingomonas* (Described in Chapter 3 and 4) were also at higher abundance. *Cupriavidus* was also present at higher abundance, a genus that has an NAD dependent methanol dehydrogenase in addition to the alternate PQQ methanol dehydrogenase gene *xoxF* (Wu *et al.*, 2016).

Genera present at higher relative abundance in the wheat rhizosphere than in the bulk soil were *Methyloceanibacter* and *Methyloversatilis*, previously described in Chapter 4. Additional genera included *Xanthobacter*, a genus that contains methylotrophic

autotrophic bacteria, and *Leptothrix*, a genus that contains several species of *xoxF5* containing organisms (Meijer *et al.*, 1990) .

5.3 Identification of active methylotrophs in the Church Farm soil through DNA stable isotope probing with ^{13}C methanol

5.3.1 Set-up of the methanol SIP experiment

DNA stable isotope probing experiments were established with ^{13}C methanol and soil from CF, pea rhizosphere soil and wheat rhizosphere soil. Briefly, 120 ml serum vials were established in triplicate for each test group (CF soil, pea rhizosphere soil and wheat rhizosphere soil) with 2 g of soil and 40 ml of autoclaved RO water. ^{13}C labelled methanol was added to give a concentration of 250 µM. Parallel enrichments were established with ^{12}C methanol. Samples were incubated at 30 °C with shaking at 120 rpm. Headspace methanol was measured using gas chromatography (2.7.1) and samples were resupplied with methanol following depletion. Vials were opened and vented to prevent them becoming anoxic. Figure 5.1 shows the depletion of methanol in the methanol incubated CF soil, pea rhizosphere soil and wheat rhizosphere soil. The wheat and CF soil test groups showed an initial lag phase before methanol oxidation started. There was no lag phase for the pea rhizosphere samples. After six days of incubation the oxidation of methanol ceased in all replicates in all of the test groups and this was therefore chosen to represent time point one (T1) in the experiment. As the CF soil has been shown to be nutrient poor (Tkacz, 2013), it was predicted the methylotrophs could have become nutrient limited and therefore 1 ml of dNMS was supplied to the enrichments. The consumption of methanol resumed in all test groups following the supply of dNMS. All samples were harvested upon the estimated incorporation of 50 µmol (methanol) g^{-1}, with the final time point (T2) being between 15 - 17 days. DNA was extracted from the samples and used as template in a 16S rRNA gene PCR for denaturing gradient gel electrophoresis (DGGE) profiling.

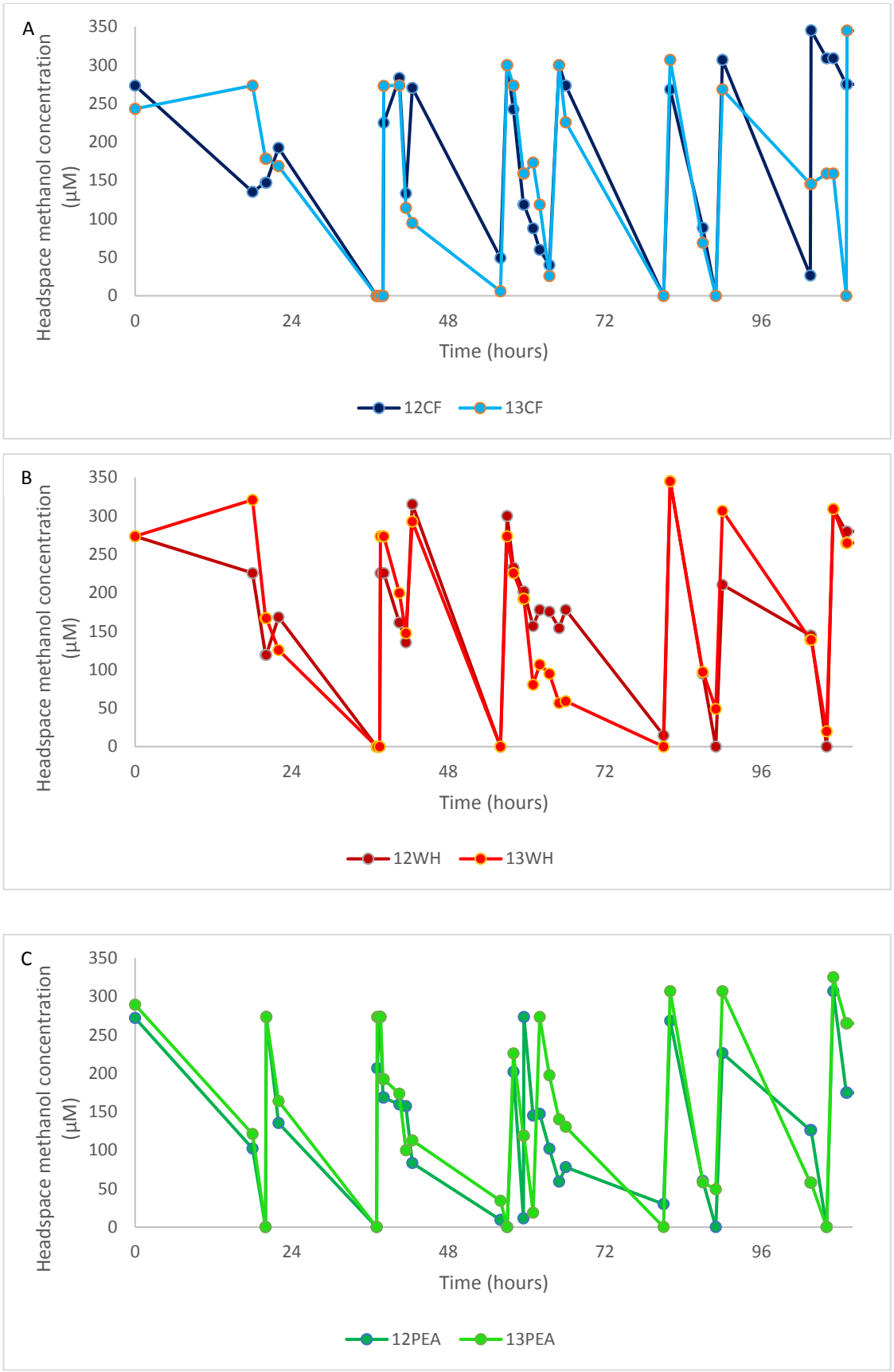


Figure 5.1 GC measurements of the concentration of methanol in methanol enriched A. Church Farm soil (CF) B. wheat rhizosphere soil (WH) and C. pea rhizosphere soil (PEA) from T0 to T1.

5.3.2 16S rRNA gene profiling of the methanol enriched samples through DGGE

The 16S rRNA gene profiling through DGGE of the unfractionated T1 and T2 samples from each test group (Figure 5.2-5.5) showed differences in the 16S rRNA gene

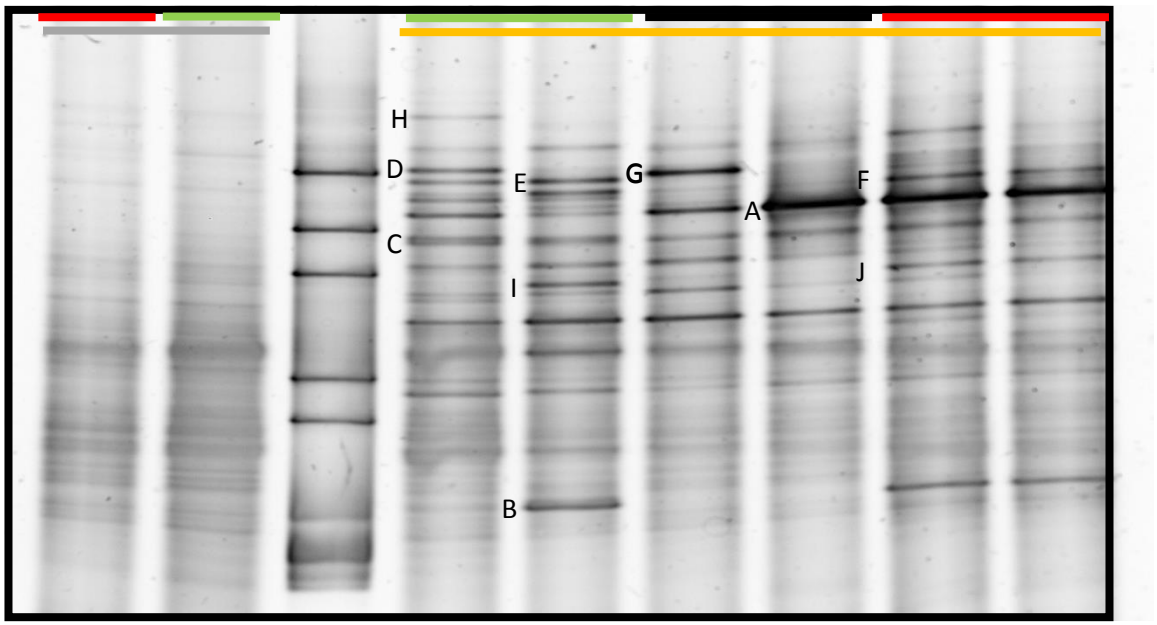


Figure 5.2 DGGE profiles of 16S rRNA genes amplified from unfractionated DNA extracted from T1 methanol-enriched (Orange) and non-enriched (Grey) pea (green) and wheat (red) rhizosphere soil and CF (CF) soil (black). Bands marked with letters correspond to sequenced bands, detailed in Table 5.1. The figure shows two representative samples out of 6 (selected to illustrate the diversity) from each soil type (incubations in triplicate for ^{12}C and ^{13}C-methanol enrichments).

profiles between the different test groups. Therefore DNA of ^{13}C and ^{12}C representatives of each profile were processed through ultracentrifugation and fractionation. Bands of interest were picked for re-amplification. The closest relatives of the sequenced bands (Figure 5.2 and Table 5.1) revealed the presence of members of the *Methylophilaceae* in all test groups.

Methylophilus was the genus most represented in the DGGE profiles. The pea rhizosphere 16S rRNA gene profiles contained the greatest number of unique bands and these showed high sequence identity to *Methylobacillus* and *Methylotenera*. One band present at greater intensity in the pea rhizosphere and the wheat rhizosphere 16S rRNA DGGE profiles showed high sequence identity to *Methylobacterium*. No variation within the test groups existed uniquely between the ^{12}C and ^{13}C test groups indicating that differences in the unfractionated profiles within a test group were not a result of the

isotope utilised in these enrichments. There was variation within the pea rhizosphere soil and CF soil test groups, with two distinct 16S rRNA gene profiles for each of these environments, designated A and B. The main difference within the pea test group was the differential presence of two bands, that both showed high identity to species of *Methylotenera*. In the CF DGGE profiles one band was present in some profiles at greater intensity, which showed high identity to *Methylobacillus* species.

Table 5.1 Identity of bands picked from 16S rRNA gene DGGE profiles of methanol enriched CF soil, pea rhizosphere soil and wheat rhizosphere soil

Sequence	Highest match using NCBI Blast	Percentage identity
A	*Methylophilus methylotrophus*	100
B	*Methylobacterium aminovorans*	100
C	*Methylobacillus flagellatus*	98
D	*Methylobacillus flagellatus*	97
E	*Methylobacillus methanolivorans*	98
F	*Pseudomonas spp.*	98
G	*Methylobacillus flagellatus*	99
H	*Methylotenera mobilis*	97
I	*Methylotenera mobilis*	99
J	*Methylophilus methylotrophus*	100

The program GelCompar II was used to further analyse the 16S rRNA DGGE gel profiling of the methanol enriched soil communities (Figure 5.3-5.5). A ranked Pearson correlation of the 16S rRNA DGGE profiles based on band intensity showed that, based on DGGE band position and intensity, the samples clustered according to the test groups (Figure 5.6). The T1 CF samples also clustered according to test group but the T2 CF samples showed more variation, with two of the profiles clustering outside of their test group. For each test group the samples from the same time point clustered together. This supports the observation that there is a change in the DGGE profiles of the

environmental samples from time point one to time point two (Figure 5.3-5.5). The distinct profiles (designated A and B) within the CF and pea rhizosphere communities also still cluster together in spite of the variable presence of specific bands. DNA from all test groups was processed through ultracentrifugation and fractionation to separate the ^{13}C and ^{12}C DNA. The DNA in each fraction was quantified (Chapter 2). All ^{13}C test groups produced a second peak of DNA concentration in fractions where ^{13}C labelled DNA (1.725 g ml^{-1}) would be expected (Figure 5.7).

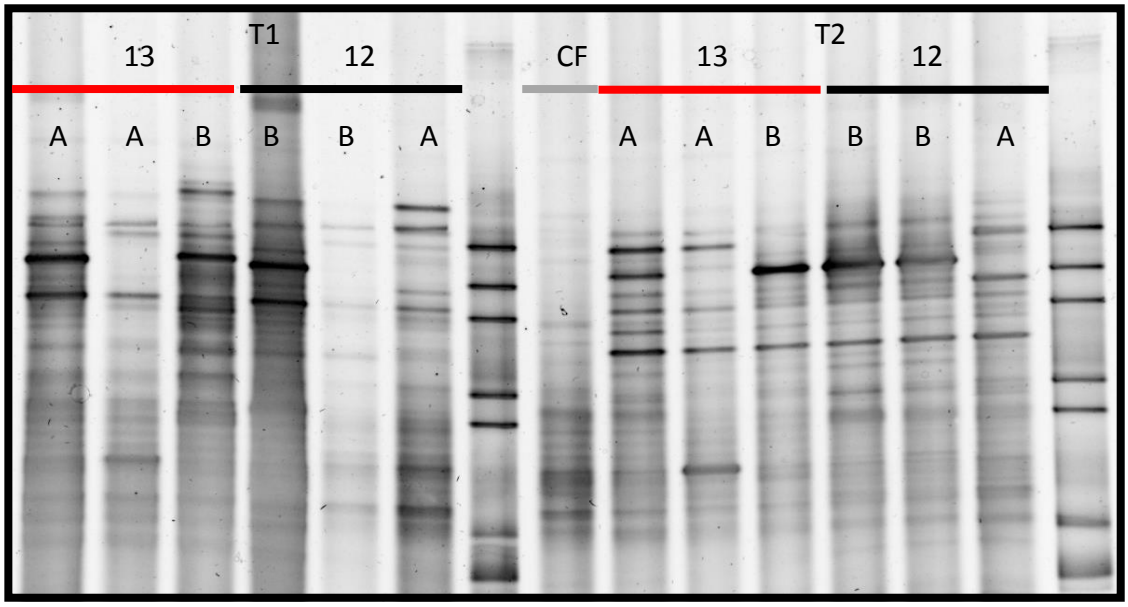


Figure 5.3 DGGE profile of 16S rRNA genes amplified from unfractionated DNA from methanol-enriched (T1 and T2) and non-enriched (T0) unplanted Church Farm soil. A and B designate profile type. CF designates T0 CF soil community. Red designates ^{13}C, black designates ^{12}C and grey represents T0.

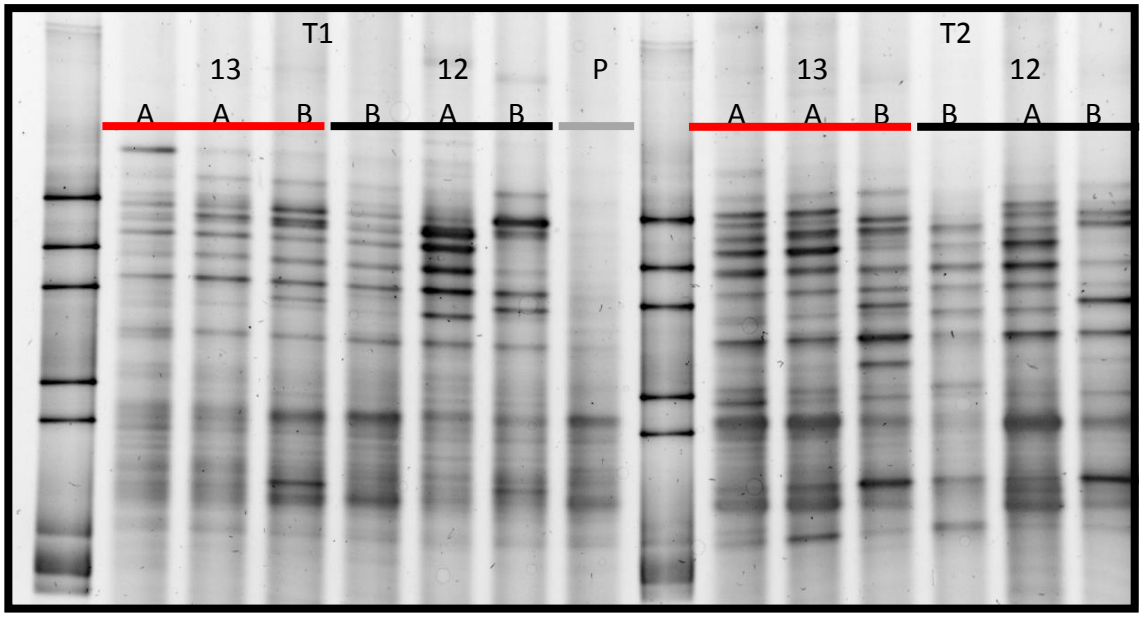


Figure 5.4 DGGE profile of 16S rRNA genes amplified from unfractionated DNA from methanol-enriched (T1 and T2) and non-enriched (T0) pea rhizosphere soil. A and B designate profile type. P designates T0 pea rhizosphere community. Red designates ^{13}C, black designates ^{12}C and grey represents T0.

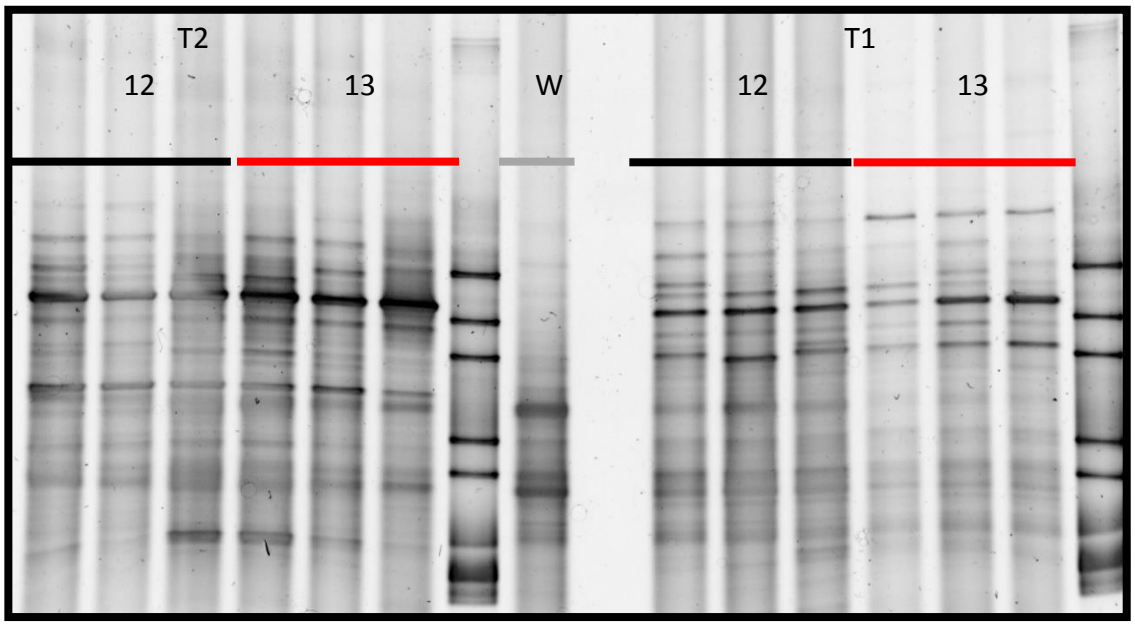


Figure 5.5 DGGE profile of 16S rRNA genes amplified from unfractionated DNA from methanol-enriched (T1 and T2) and non-enriched (T0) wheat rhizosphere soil. W designates T0 wheat rhizosphere community.

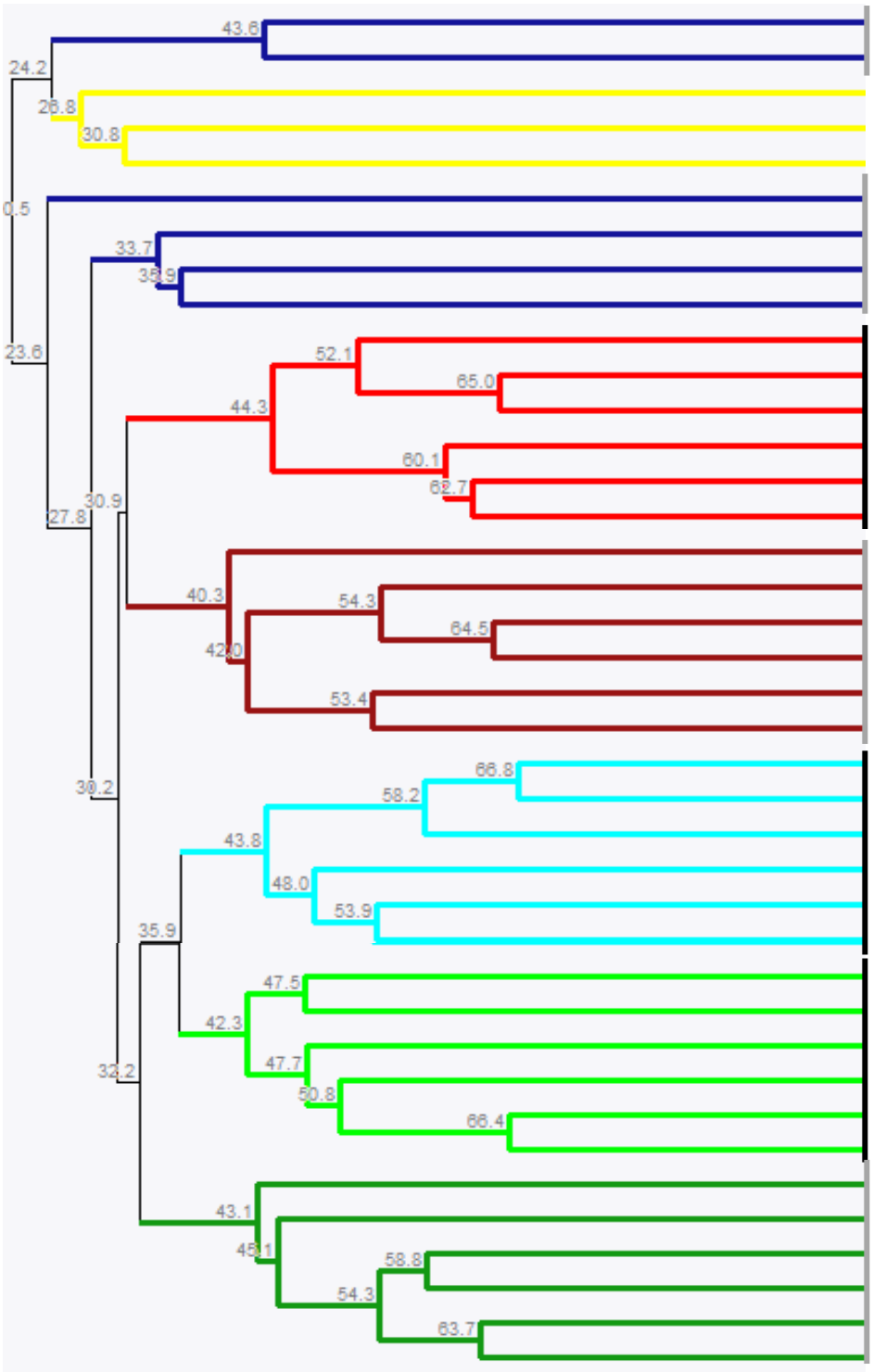


Figure 5.6 Dendogram showing a ranked-Pearson coefficient of 16S rRNA DGGE profiles of methanol enriched and unenriched CF soil, pea rhizosphere soil and wheat rhizosphere soil. Red designates wheat rhizosphere, green designates pea rhizosphere and blue designates CF. Black designates T1. Grey designates T2. Yellow designates T0.

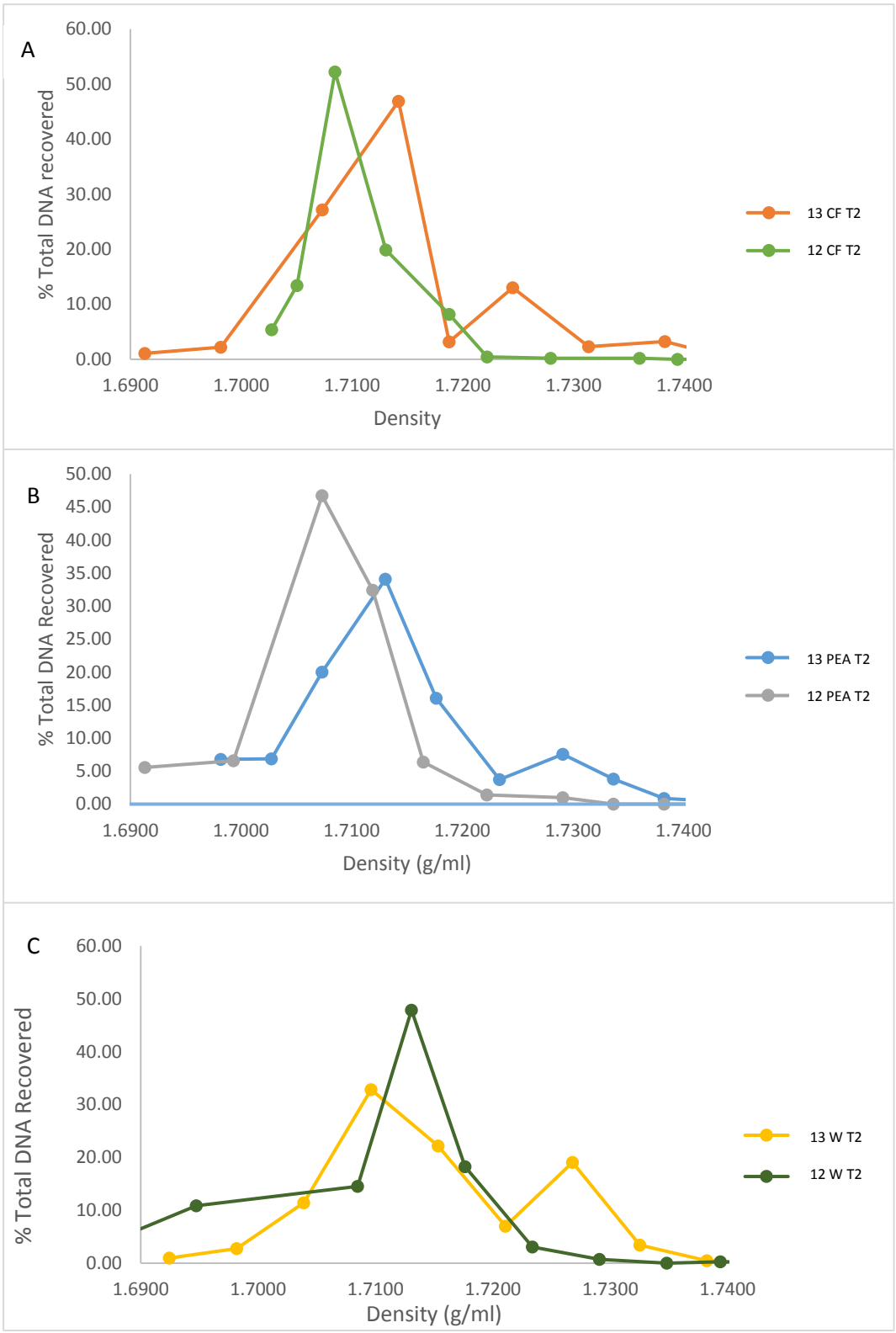


Figure 5.7 Percentage of DNA recovered from fractionated DNA from ^{13}C and ^{12}C methanol enriched environmental samples. A. Unplanted CF soil, B. pea rhizosphere soil, C. wheat rhizosphere soil.

Each test group was further analysed following fractionation and 16S rRNA gene DGGE profiling. The presence of specific bands in the heavy fractions of the ^{13}C test groups that are not enriched in the heavy fractions of the ^{12}C test group further support that ^{13}C labelled DNA was successfully obtained. The differences between the two distinct profiles within the CF soil and pea rhizosphere soil test groups persisted following fractionation. However, given the bands in the pea 16S rRNA gene profiles represented species of the same genera, these samples were pooled for further molecular analysis.

5.3.3 Sequencing of the 16S rRNA gene from the heavy and light fractions of the methanol enriched test groups

The 16S rRNA genes amplicons amplified from the heavy and light fractions of the T1 and T2 ^{12}C and ^{13}C samples from each environment were sent for Illumina sequencing. 16S rRNA gene sequencing confirmed that there were distinct differences in the community of active methylotrophs between the environments. It also showed a shift in the labelled community between time point one and time point two for each environmental sample. Genera were classified as ^{13}C labelled if they were present at ten-fold greater relative abundance in the heavy fraction compared to the light fraction of the ^{13}C-methanol-enriched samples and this was not observed between the ^{12}C heavy and light fractions.

5.3.3.1 Genera enriched in the methanol enriched samples at time point one

The heavy fraction of the pea rhizosphere contained four labelled genera that have been shown to be methylotrophs (Figure 5.8). These genera include *Methylophilus*, *Methylobacillus*, *Methylotenera* and *Methylobacterium*. *Desulfococcus* was also enriched in the heavy fraction, possibly as a result of cross feeding (Antony *et al.*, 2010; Dumont *et al.*, 2011), as the genome sequenced species of *Desulfococcus* do not possess methanol dehydrogenase genes and there is no indication in previous characterisations that they are capable of methanol utilisation (Imhoff-Stuckle *et al.*, 1983; Bridge *et al.*, 1999; Kleindienst *et al.*, 2014; Dörries *et al.*, 2016). The heavy fraction of the T1 methanol-enriched wheat rhizosphere contained the same methylotrophic genera, but with a higher relative abundance of *Methylophilus* (Figure 5.9). Additional groups labelled in the heavy fraction that were present at low abundance were the genus *Stigmatella* and members of the phylum *Lentisphaerae*. Based on their low abundance in the heavy fraction, the genomes of the sequenced strains lacking genes encoding

methanol dehydrogenases and previous characterisation of the two groups it is presumed they are labelled due to cross-feeding (Sutherland, 1978; Cho *et al.*, 2004; Choi *et al.*, 2013; Sood *et al.*, 2015). Fewer genera were labelled in the T1 heavy fraction of the CF samples than in the pea rhizosphere and wheat rhizosphere samples (Figure 5.10). The heavy fraction is represented by the genus *Methylophilus*, present at 90% abundance of the heavy fraction, and *Methylotenera*.

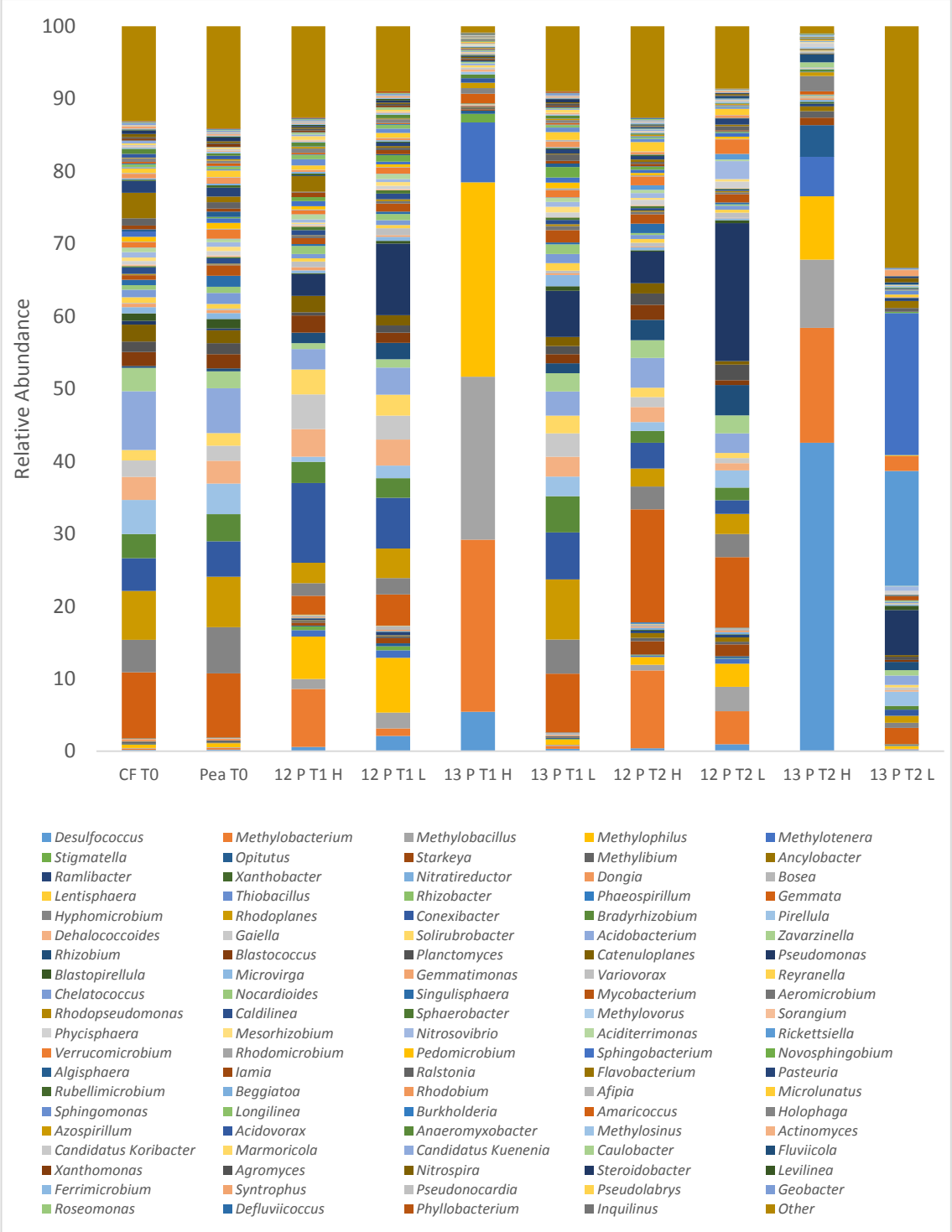


Figure 5.8 16S rRNA gene sequence profiles produced through amplicon sequencing of DNA extracted from T0 CF and pea rhizosphere soil and the heavy and light fractions of ^{13}C and ^{12}C methanol enriched pea rhizosphere soil.

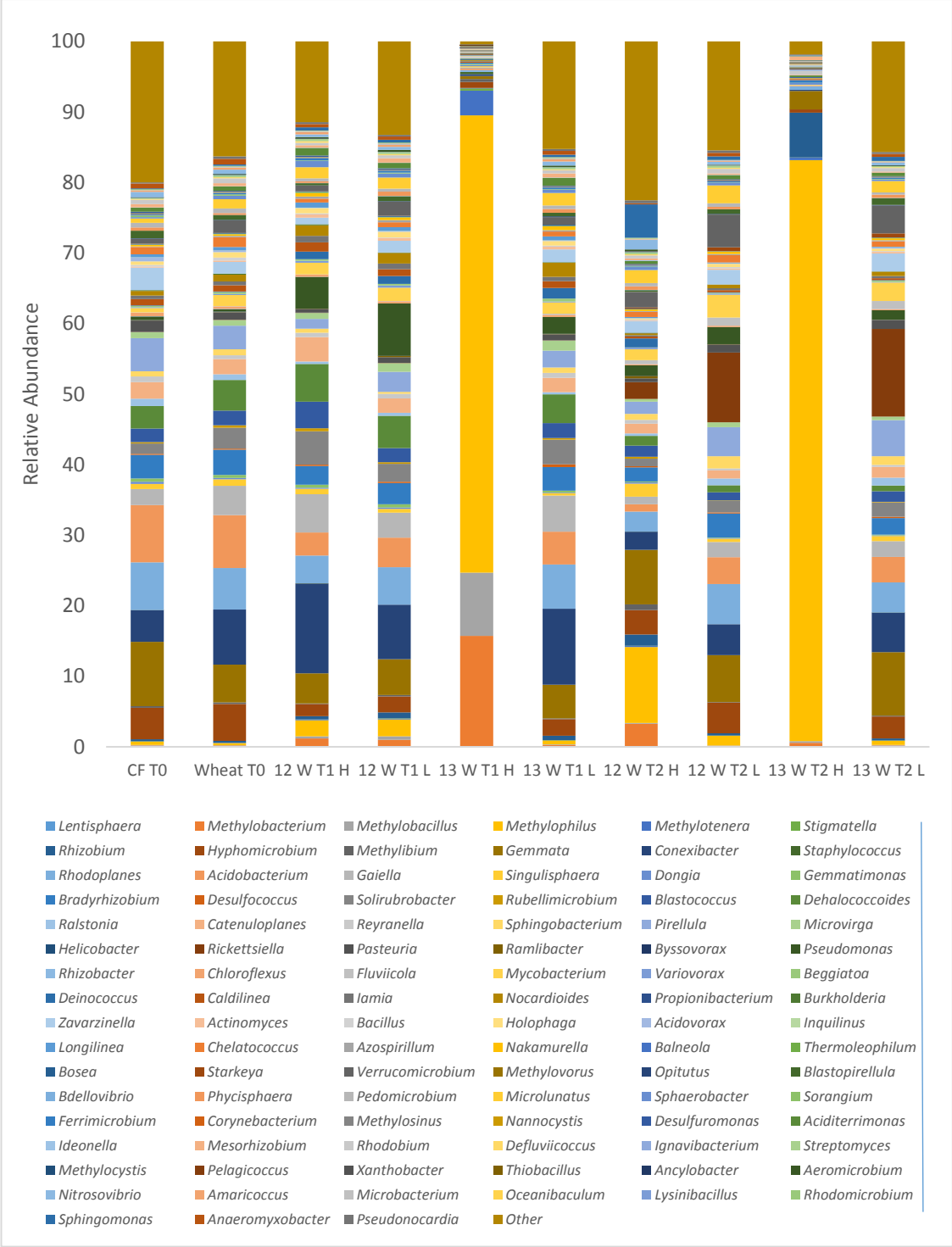


Figure 5.10 16S rRNA gene sequence profiles produced through amplicon sequencing of DNA extracted from T0 CF soil and wheat rhizosphere and the heavy and light fractions of ^{13}C and ^{12}C methanol enriched wheat rhizosphere soil.

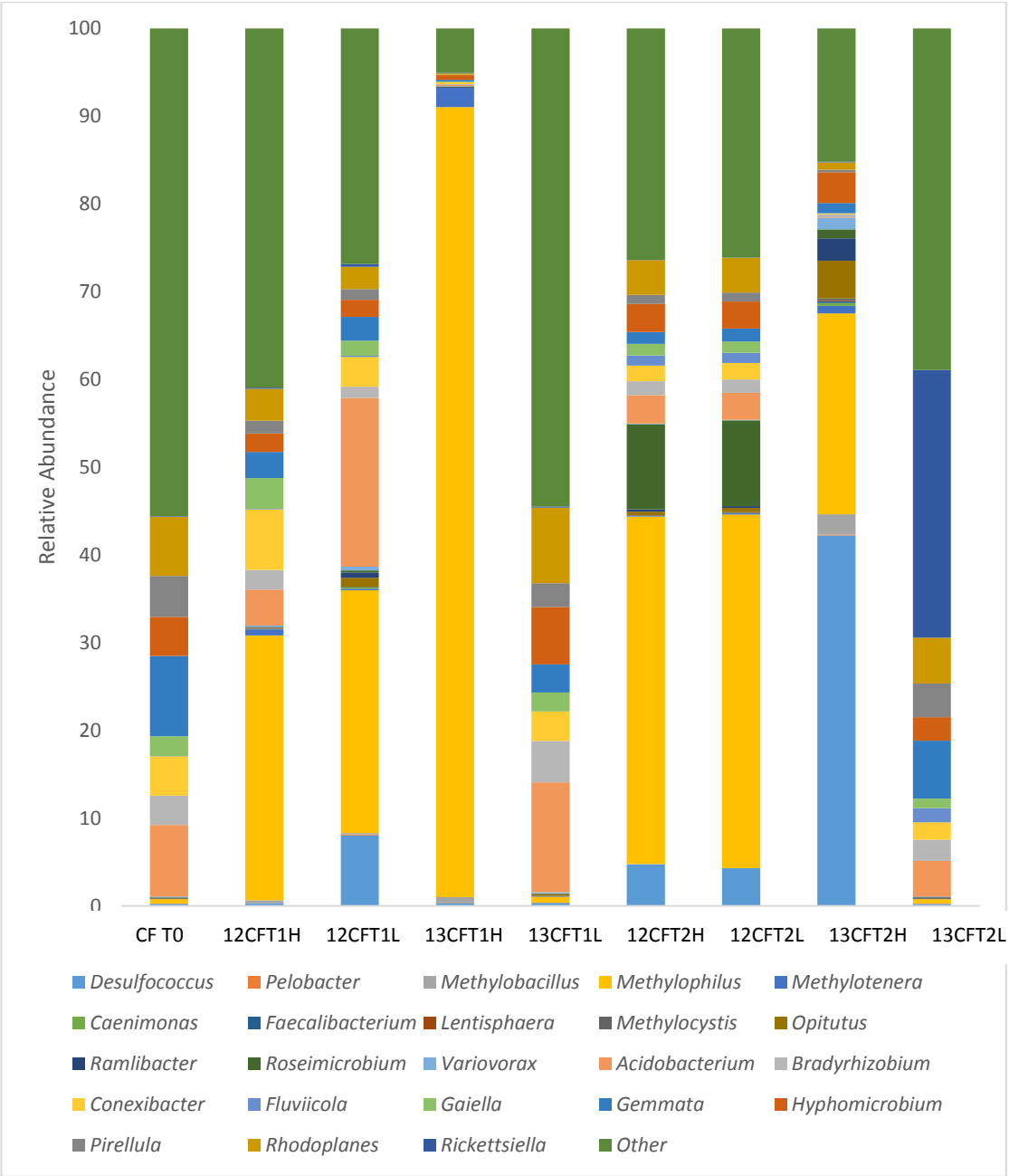


Figure 5.9 16S rRNA gene sequence profiles produced through amplicon sequencing of DNA extracted from T0 CF soil and the heavy and light fractions of ^{13}C and ^{12}C methanol enriched unplanted CF soil.

5.3.3.2 Genera enriched in the methanol enriched samples at time point two

The number of labelled genera in the T2 wheat rhizosphere samples was lower than the T1, with only *Methylophilus* and *Methylotenera* enriched in the heavy fraction. The relative abundance of *Methylotenera* decreased tenfold from T1 to 0.34 %, whereas the abundance of *Methylophilus* increased from 64 % to 82 %. It is interesting to note that there appears to be no labelling of "cross-feeding bacteria" in the heavy fraction of the wheat rhizosphere. The diversity of the T2 pea rhizosphere samples was increased in comparison to T1, with 13 additional genera enriched in the heavy fraction. However, of this diversity, only *Starkeya* and *Opitutus* were present at over 1% abundance. *Opitutus* is presumed to be a cross feeder due to the previously described reasons (5.3.3.1). Of the labelled genera, all except *Desulfococcus* decreased in abundance, with *Desulfococcus* increasing from 5% of the heavy fraction to 24%. The length of the incubation and the decrease in abundance of the genuine methylotrophs supports cross-feeding being the reason for this increase in abundance. The labelled community of the T2 ^{13}C methanol enriched CF soil increased in number to thirteen. Amongst these genera are genuine methylotrophs, *Methylobacillus*, *Methylocystis* and *Methylotenera*, that are collectively present at 5% relative abundance. Additional genera enriched in the heavy fraction were *Opititus* and *Ramlibacter*. *Desulfococcus* was also enriched in the heavy fraction, present at 42% relative abundance.

5.3.4 Analysis of the Metagenomes produced from the T2 ^{13}C heavy fractions

DNA from the T2 test groups was sent for shotgun metagenome sequencing. Replicates for each T2 test group were pooled. Sequencing was performed using paired-end sequencing (2 x 150 bp) on an Illumina Hiseq 4000. Assembly and bioinformatic analyses of the metagenomes was subsequently performed by Dr. Jennifer Pratscher. Short sequences and sequences of poor quality were excluded from the files using the program Trimmomatic (Bolger *et al.*, 2014). The metagenome sequences were assembled into contigs using the program Megahit (Li *et al.*, 2015) and annotated using myRast. The quality of these metagenome assemblies was then assessed using Quast (Table 5.2)

Table 5.2 Quast analysis of metagenomes

	Metagenome		
	Pea	CF	Wheat
# contigs (>= 0 bp)	1151414	1251579	981758
# contigs (>= 1000 bp)	195697	192658	106074
Total length (>= 0 bp)	934363676	922084398	616537133
Total length (>= 1000 bp)	456772046	392066999	186491273
# contigs	576782	616682	446171
Largest contig	720645	641982	87667
Total length	717825918	682211092	415542434
GC (%)	63.9	64.58	65.92
N50	1397	1168	916
NG50	116134	35098	9029
N75	788	734	656
NG75	73356	20181	6593
L50	112046	145393	128331
LG50	48	112	652
L75	288162	333682	264310
LG75	103	308	1309

Metaphlan (Segata *et al.*, 2012) was used to analyse the phylogenetic composition of the metagenomes sequenced from the heavy fractions of the T2 unplanted CF soil, wheat rhizosphere and pea rhizosphere soil (Figure 5.11). Metaphlan assigns phylogeny to reads by comparing contigs to a catalogue of reference sequences from the IMG database. Abundance is then estimated by normalising read based counts by the average genome size of each clade (Segata *et al.*, 2012).

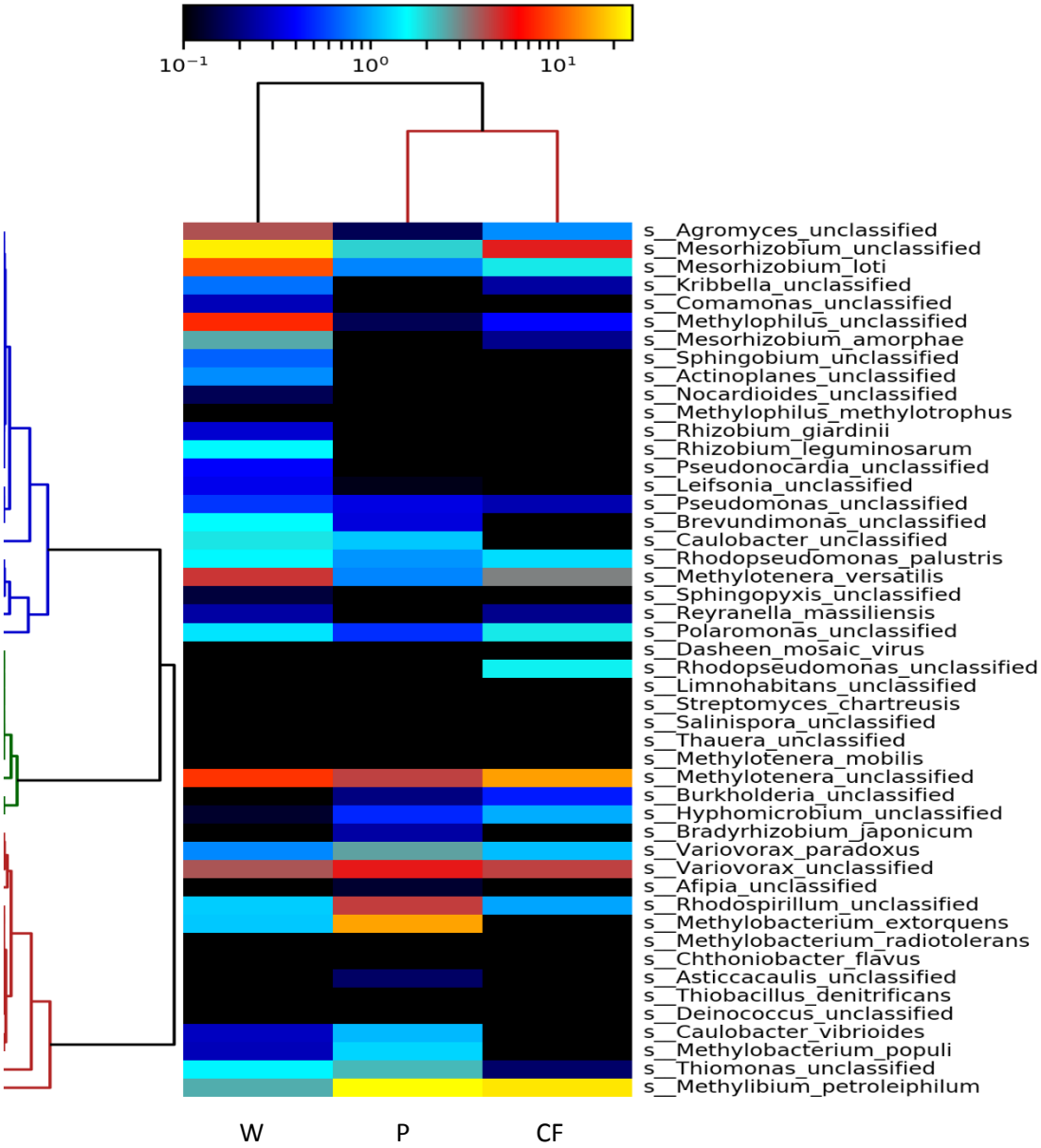


Figure 5.11 Metaphlan phylogenetic analysis of metagenomes constructed from DNA from the heavy fraction of ^{13}C-methanol enriched CF soil, wheat rhizosphere soil and pea rhizosphere soil at T2

The Metaphlan analysis showed that there were differences between the ^{13}C-labelled communities of the unplanted soil, pea rhizosphere and wheat rhizosphere. Bacteria unique to the ^{13}C labelled community of the wheat rhizosphere were *Agromyces*, *Comamonas*, *Sphingobium*, *Actinoplanes* and *Rhizobium*. *Methylophilus* was also more abundant in the wheat rhizosphere, than in the pea rhizosphere and unplanted samples. The abundance of *Methylophilus* in the wheat rhizosphere is consistent with the 16S rRNA gene profile of the T2 community. *Bradyrhizobium* was exclusively present in the

^{13}C labelled community of the pea rhizosphere. *Varivorax* was present in the heavy fractions of all three environments, but this genus was most abundant in the pea rhizosphere. Genera present at a higher abundance in the plant associated rhizosphere samples relative to the CF samples include *Caulobacter* and *Methylobacterium*. This is interesting as *Methylobacterium* is one of the key delineating genera between the plant associated environments and the unplanted soil in the 16S rRNA gene profile at T1. This presence of *Methylobacterium* persists in the metagenomes in spite of it being absent in the 16S rRNA gene profile of the T2 wheat heavy fraction. There were no genera unique to the ^{13}C labelled community of the CF soil. *Mesorhizobium* was abundant in the heavy fractions of the wheat and unplanted environments. *Methylotenera* and *Rhodopseudomonas* were present in all three environments, with higher presence in the wheat rhizosphere and unplanted soil sample. *Mesorhizobium* has not been shown to contain species capable of growth on methanol, but several *Mesorhizobium* genomes contain *xoxF* genes and there are species of *Mesorhizobium* that grow on methylamine and therefore have the metabolic pathways for the incorporation of the carbon from a C1 compound into cellular biomass (Wischer *et al.*, 2014). *Burkholderia* and *Hyphomicrobium* were both present in the heavy fraction of the pea rhizosphere and unplanted sample and *Methylibium* was also much more abundant in these two environments relative to the wheat rhizosphere.

5.3.5 Analysis and description of binned genomes

The metagenome sequences were assembled into contigs using the program Megahit (Li *et al.*, 2015). The bioinformatics program Metabat was used for binning of the sequenced metagenomes into genomic bins (Kang *et al.*, 2015). The completeness, contamination and heterogeneity of these genomes was then assessed using the program CheckM (Parks *et al.*, 2015). The binning was performed using two algorithms, “verysensitive” and “superspecific”. The “verysensitive” algorithm provides greater sensitivity for binning with a simple community. “superspecific” is the most specific algorithm. Both of these algorithms do not recruit contigs by abundance correlation. These two algorithms yielded different results, producing genomes with a varying degree of completeness and contamination. Genomes with a completeness score above 45% were assessed further, with details of their assembly below (Table 5.3). Where

genomes produced with the different algorithms were identified as highly similar these were analysed together, producing a range in genome characteristics.

Seven of the eighteen binned genomes with levels of completeness over 45 were identified as members of the order *Methylophilales*, with some genomes only being classified to the level of order. This is not unexpected given the presence of this order in the 16S rRNA gene sequence profiles and Metaphlan analysis of the metagenomes. None of the binned genomes contained 16S rRNA gene sequences. The majority of the genomes possessed methanol dehydrogenase gene sequences that were aligned with a database of methanol dehydrogenase sequences for assignment to a clade.

Eight of the ten genomes belonging to potential and confirmed methylotrophic organisms possessed methanol dehydrogenase encoding genes. The genome identified as a *Rubrivivax* contained a *xoxF5* methanol dehydrogenase gene. Using NCBI Blastp this gene was shown to have high identity with methanol dehydrogenase genes from species of *Rhizobacter* and *Methylibium*. A Neighbour joining tree produced from an alignment of the *xoxF* methanol dehydrogenase genes (Figure 5.12) showed that this gene clustered with the methanol dehydrogenase gene sequences from other members of the order *Burkholderiales*. This includes methanol dehydrogenase gene sequences from *Methylibium*, *Rubrivivax, Variovorax* and additional members of the *Commamonadaceae*.

Table 5.3 Details of genomes binned using the program Metabat

Marker lineage	Strain Designation	Genome size	Contigs	GC	Completeness	Contamination	Strain heterogeneity	MDH genes	Clade
Rubrivivax	2631	6.37	48-49	67.7	97.66	0.93	0	1	*xoxF5*
Bdellovibrio	7093	2.5	323-325	46.6	78.29	1.98	33.33	0	
Archaea (UID2)	0043	3.67	692	70.2	93.33	3.88	75	0	
Methylophilales	7798	1.94-2.03	396-408	51.8-52	74.52-76.94	11-13.22	35.14-36.17	0	
Methylophilaceae	1577	1.41-1.78	262-431	44.7-45	61.13-69.43	4.2-17.51	54.41-73.33	0	
Methylotenera	0503	1.28-1.81	174-227	45.2-45.7	62.07-67.24	1.72	0	1	*xoxF3*
Methylophilales	0201	2.0-2.6	320-410	57.7-58	69.18-84.84	4.61-5.06	60-73.33	1	*xoxF4*
Methylophilales	1312	1.23-1.3	250-255	46.6-46.9	45.32-47.45	0.88-1.09	66.67-75	1	*xoxF4*
Methylobacterium	1848	7.88-8.66	632-804	69	92.84-95.82	64.37-73.08	4.49-82.46	3	*mxaF*
									mxaF
									xoxF5
Methylophilales	2829	1.34-1.81	192-252	46.9-47.1	62.17-80.19	1.18-1.98	50-66.67	4	*mxaF*
									xoxF4
									xoxF4
									xoxF4
Methylotenera	5900	1.71	423	44.8	69.32	15.38	55.74	1	*xoxF4*
Methylobacterium	0020	3.97	640	68.9	71.85	1.73	87.5	1	*mxaF*
Deltaproteobacteria	68	6.06	448	66.1	92.69	5.04	18.75	0	
Verrucomicrobia	76	7.35	1446	62	71.82	35.1	11.24	0	
Verrucomicrobia	53	6.11	1036	56.1	80.66	4.94	27.27	0	
Verrucomicrobia	71	6.35	1078	56.4	84.04	5.61	25	0	
Verrucomicrobia	119	6.25	1326	62.7	69.26	15.88	14.29	0	
Verrucomicrobia	101	6.63	872	61.2	85.08	10.47	0	0	

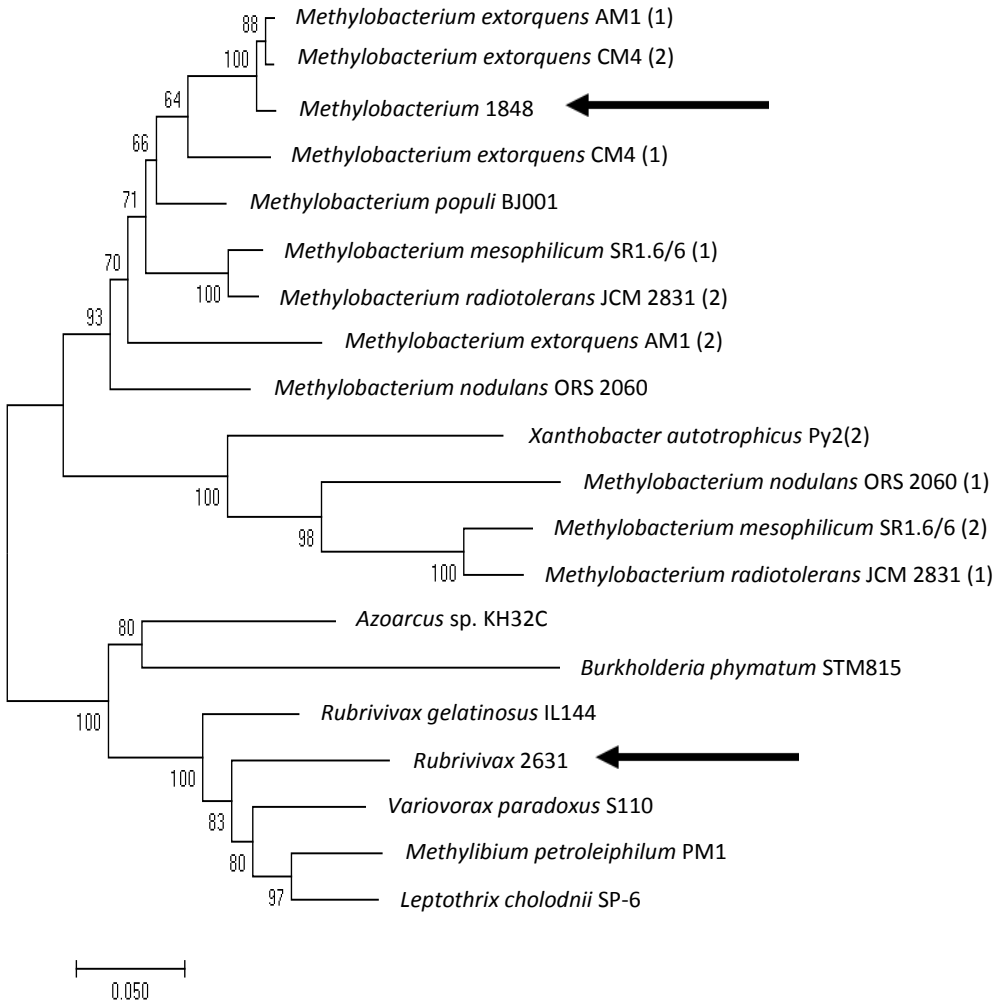


Figure 5.12 Phylogenetic analysis of the *xoxF5* genes from the genome of *Rubriviax* 2631 and *Methylobacterium* 1848 (indicated with a black arrow). Sequences were analysed with a database of *xoxF5* sequences. The phylogenetic tree was constructed from nucleotide sequences aligned at the deduced amino acid level. The evolutionary history was inferred using the Neighbour-Joining method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position.

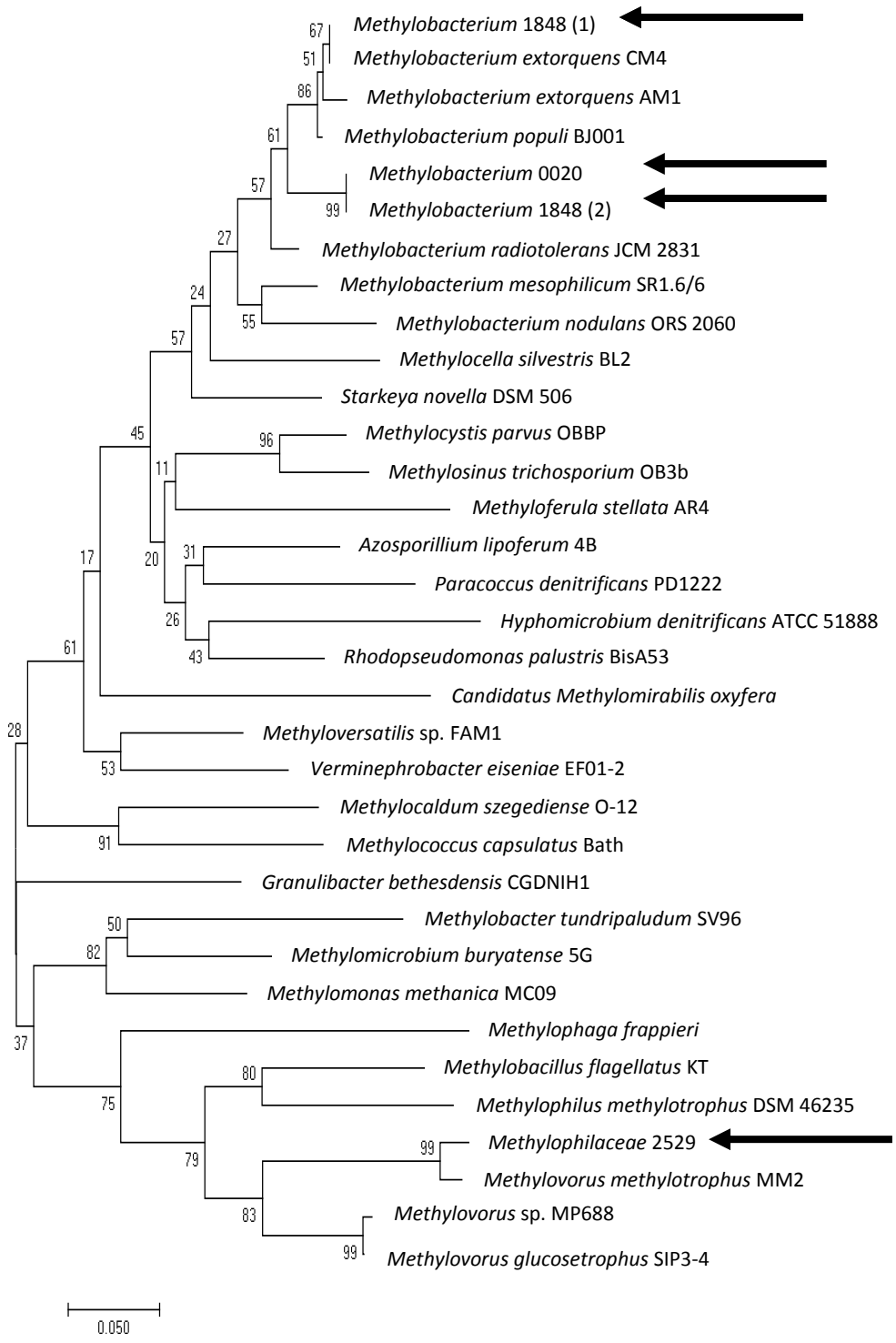


Figure 5.13 Phylogenetic analysis of the *mxaF* genes from binned *Methylobacterium* genomes 0020 and. *Methylobacterium* 1848 contained two *mxaF* genes, that were designated 1 and 2 (indicated with a black arrow). Sequences were analysed with a database of *mxaF* sequences. The phylogenetic tree was constructed from nucleotide sequences aligned at the deduced amino acid level. The evolutionary history was inferred using the Neighbour-Joining method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position.

Two genomes produced from the binning of the metagenomic sequence datasets were classified as *Methylobacterium*. One genome, 0020, was only produced with the "superspecific" algorithm. The "verysensitive" algorithm assigned contigs comprising 0020 to the other *Methylobacterium* genome, 1848. This results in genome 1848 containing two *mxaF* methanol dehydrogenase genes, which has not previously been shown to occur in the genomes of other methylotrophs (Chistoserdova 2009; Keltjens 2014). Conversely the 0020 genome contains one *mxaF* methanol dehydrogenase gene but no *xoxF* gene. No currently genome sequenced methylotroph possesses an *mxaF* methanol dehydrogenase gene in the absence of *xoxF* (Keltjens 2014; Taubert *et al.*, 2015). Given the completeness of the genome (71%), the *xoxF* gene was most likely not captured with this sequencing and binning. The methanol dehydrogenase genes of the binned *Methylobacterium* genomes show high identity to that of *Methylobacterium extorquens* AM1 and *Methylobacterium populi* (Figure 5.13).

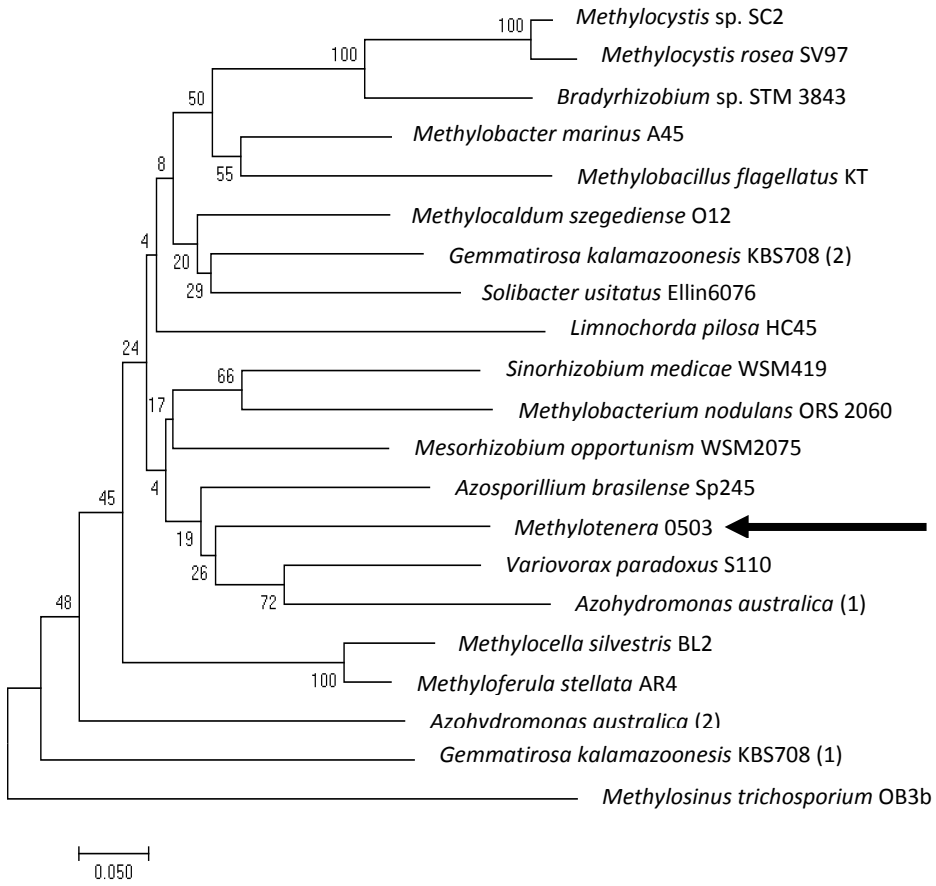


Figure 5.14 Phylogenetic analysis of the *xoxF3* gene from the genome of *Methylotenera* 0503 (indicated with a black arrow). Sequences were analysed with a database of *xoxF3* sequences. The phylogenetic tree was constructed from nucleotide sequences aligned at the deduced amino acid level. The evolutionary history was inferred using the Neighbour-Joining method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position.

Two of the *Methylophilaceae* genomes (7798 and 1577) did not possess methanol dehydrogenase genes in spite of relatively high levels of completeness. It is difficult to further assign these genomes to a higher level of phylogenetic revolution than that of the family level. The genome designated *Methylophilales* 0503 was divergent from the other genomes of this order as it only possessed a *xoxF3*. *xoxF3* is a clade of methanol dehydrogenase gene possessed by some species of *Methylobacillus* (Keltjens *et al.*, 2014), but the *xoxF3* of *Methylotenera* 0503 clustered with the *xoxF3* gene sequences of members of the *Commamonadaceae* (Figure 5.14). The genome of *Methylotenera* 0503 scored low for contamination (1.72 %). The *xoxF3* sequence of this binned genome could indicate there is a greater diversity to this clade than currently characterised. These genomes are also atypical of the *Methylophilaceae* due to the absence of a *xoxF4*

methanol dehydrogenase. However, as mentioned above, these genomes are not complete.

Some of the genome bins showed high levels of similarity to the genomes of two of the isolates described in Chapter 3. The methanol dehydrogenase gene sequences of three of the *Methylophilaceae* genomes (2829 and 0201) showed high identity to those of *Methylobacillus denitrificans* MM3 and *Methylovorus methylotrophus* MM2 (Figure 5.15). The methanol dehydrogenase gene of genome 0201 showed high identity to one of the *xoxF4* methanol dehydrogenase genes of *Methylobacillus denitrificans MM3* (97 %) at the amino acid level (Auch, *et al.*, 2010; Meier-Kolthoff *et al.*, 2013). Genome to genome distance calculator analysis of this genome indicates that it does not belong to the same species as MM2 (2.25 % probability), but this score could alter if the genome were more complete. The methanol dehydrogenase genes of genomes 2829 both show high identity to those of *Methylovorus methylotrophus* MM2. The genome of 2829 contains four methanol dehydrogenase genes that show 99-100% identity at the amino acid level to the methanol dehydrogenase genes of *Methylovorus methylotrophus* MM2. The GGDC showed that genome 2829 had a high probability of belonging to the same species as *Methylovorus methylotrophus* MM2, with the second formula of the GGDC showing sufficiently high identity for genome 2829 to be classified as the same subspecies (76-82 % identity) (Auch *et al.*, 2010; Meier-Kolthoff *et al.*, 2013). Given the differences between *Methylovorus methylotrophus* MM2 and other species of the same genera, this could account for the assignment of 2829 to the family level. 5900 is an additional genome that shows high identity to the *Methylophilaceae* and was classified to the genus *Methylotenera*. This genome also possesses a *xoxF4* methanol dehydrogenase. However, as opposed to the previously described genomes, the methanol dehydrogenase gene does not show high identity to those of either *Methylovorus methylotrophus* MM2 or *Methylobacillus denitrificans* MM3.

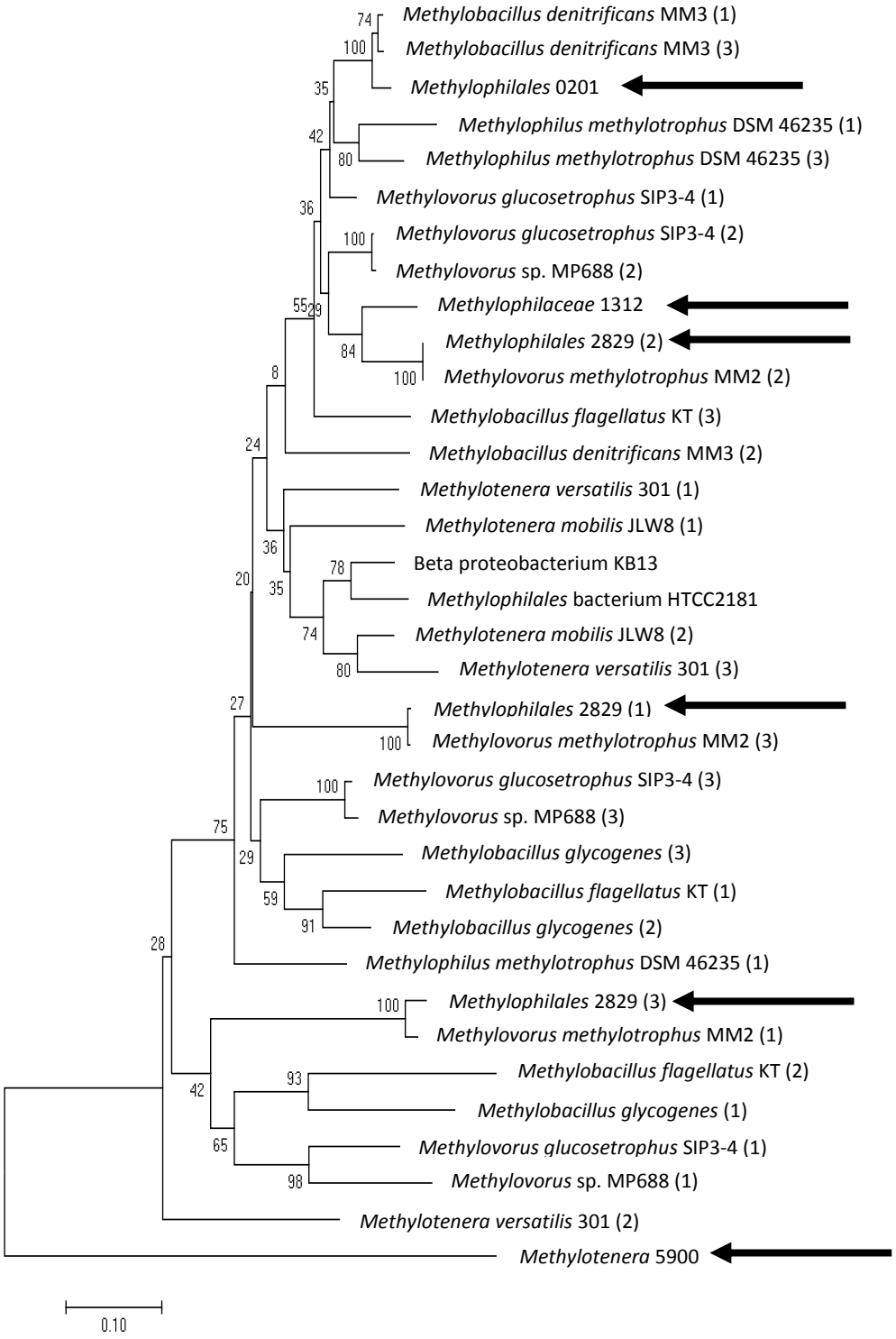


Figure 5.15 Phylogenetic analysis of the *xoxF4* gene from the binned *Methylophilales* genomes, *Methylophilales* 0201, *Methylophilaceae* 1312, *Methylophilales* 2829 (indicated with a black arrow). Multiple copies of *xoxF4* were designatednumbers. Gene Sequences were analysed with a database of *xoxF4* sequences. The phylogenetic tree was constructed from nucleotide sequences aligned at the deduced amino acid level. The evolutionary history was inferred using the Neighbour-Joining method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position.

In addition to the genomes that could be assigned to methylotrophic genera, additional binned genomes were produced. These included eight *Verrucomibiales* genomes, a *Bdellovibrio* genome and a genome assigned to the *Deltaprotobacteria*. The Phylum *Verrucomicrobia* does contain methanotrophic genera, however it also contains non-methylotrophic species (Chin *et al.*, 2001; Op den Camp *et al.*, 2009; Anvar *et al.*, 2014; Kotak *et al.*, 2015). Based on the observed enrichment of *Opitutus* in the 16S rRNA gene profiles these binned *Verrucomicroba* genomes could represent additional non-methylotrophic *Verrucomicrobia*. This possibility is supported by the absence of a methanol dehydrogenase gene in any of the 70-85% complete genomes. *Bdellovibrio* are predatory bacteria that prey upon gram negative bacteria (Feng *et al.*, 2017). Predation upon ^{13}C labelled bacteria would have resulted in the labelling of the DNA of these organisms akin to the labelling of the DNA of predatory nematodes in a methanol SIP study performed with forest soil (Lueders *et al.*, 2003). The Deltaproteobacteria could not be classified to a higher phylogenetic resolution but is also most likely enriched through cross-feeding, especially when considering the enrichment of *Desulfococccus* in the heavy DNA fraction of the pea and unplanted methanol enriched samples.

5.4 Identification of active methylotrophs in the Church Farm soil through RNA stable isotope probing with ^{13}C methanol

RNA SIP has a higher sensitivity than DNA-SIP as it does not require replication to occur following the supply of a ^{13}C labelled substrate. It is also possible to achieve labelling of RNA with a lower concentration of labelled substrate (Manefield *et al.*, 2002; Whiteley *et al.*, 2006). An RNA-SIP experiment was performed using soil from the Church Farm and ^{13}C labelled methanol. This was performed to attempt to identify the methylotrophic community of the CF soil active at an ambient concentration of methanol as opposed to an elevated concertation.

Briefly, 10 g of CF soil and 200 ml of autoclaved ddH_2O were combined in conical flasks (2 L). Test groups comprise soil supplied with methanol to a final concentration of 2.5 µM and 250 µM. Samples of soil were taken from each test group at three time points (six, twelve and twenty-five hours). RNA was extracted from the harvested soil samples using the Griffiths technique (Section 2.4.1) with subsequent DNase treatment. Superscript III reverse transcriptase was used to yield cDNA (Section 2.5.4). This cDNA

was used as template for 16S rRNA gene amplification and 16S rRNA gene DGGE profiling of each sample (Section 2.5.11).

16S rRNA gene DGGE profiling of the unfractionated test groups did not show a change in the active community profile in the cDNA synthesised from RNA extracted from the 2.5 µM enriched community relative to the unenriched sample. However, enrichment was apparent in the 16S rRNA gene profile of the 250 µM enriched sample (Figure 5.16).

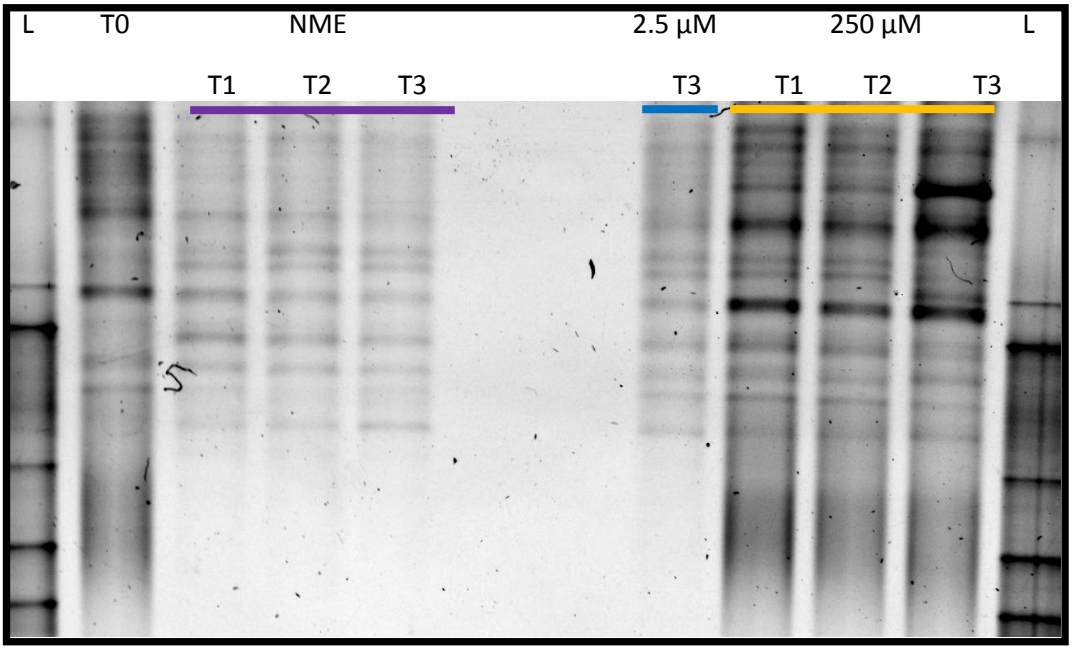


Figure 5.16 DGGE profile of 16S rRNA genes amplified from cDNA produced from RNA extracted from methanol-enriched (T1, T2 and T3) and non-enriched (T0) CF soil. NME designates non-methanol enriched soil. Purple designates non-enriched, blue designates 2.5 µM and orange designates 250 µM.

It is possible that enrichment occurred in the 2.5 µM test group, but the enrichment was occluded by the total community profile. RNA from the third time point of all test groups except the 250 µM enrichment was ultracentrifuged and fractionated according to established protocols (Whiteley *et al.*, 2007). Following fractionation, the RNA in all of the fractions was precipitated and reverse transcribed to enable 16S rRNA gene amplification through PCR and subsequent profiling through DGGE. The 16S rRNA gene profiles of the three processed test groups showed no difference between the

unenriched samples and the methanol supplemented test groups. No unique bands were present in the heavy fraction of the ^{13}C test groups relative to the other test groups.

It is possible enrichment of the methylotrophs active at an ambient concentration had occurred but that DGGE profiling was not sufficiently sensitive to be able to detect this enrichment. However, based on the 16S rRNA gene DGGE profiles, the labelling of the RNA of the methylotrophs that are active at this concentration of methanol, which is typical of in-situ concentrations (Conrad *et al.*, 2005), was not sufficient to enable detection of enrichment or for the separation of ^{13}C and ^{12}C labelled RNA. This means the methylotrophic community of the CF soil active at an ambient concentration of methanol could not be identified using this experimental setup.

5.5 Enrichment of Church Farm soil by supplementation with methanol and lanthanides

To assess the impact of the supply of lanthanides on the methylotrophic community of the CF soil and the oxidation rate of methanol, enrichments were performed with the addition of lanthanides to the soil (2.8.1). Given the diversity of methylotrophic bacteria that possess *xoxF* methanol dehydrogenase genes and the potential for lanthanides to be at a limiting concentration in certain soils, a pattern of increased methanol oxidation could be expected in certain terrestrial environments as observed in some marine environments (Howat, 2016).

120 ml serum vials were established with 5 g of soil in 5 ml of 1 % dNMS. These vials were established in triplicate with a final concentration of 3 mM methanol. The test groups were supplemented with either 5 µM lanthanum, 5 µM cerium or were not amended with lanthanides.

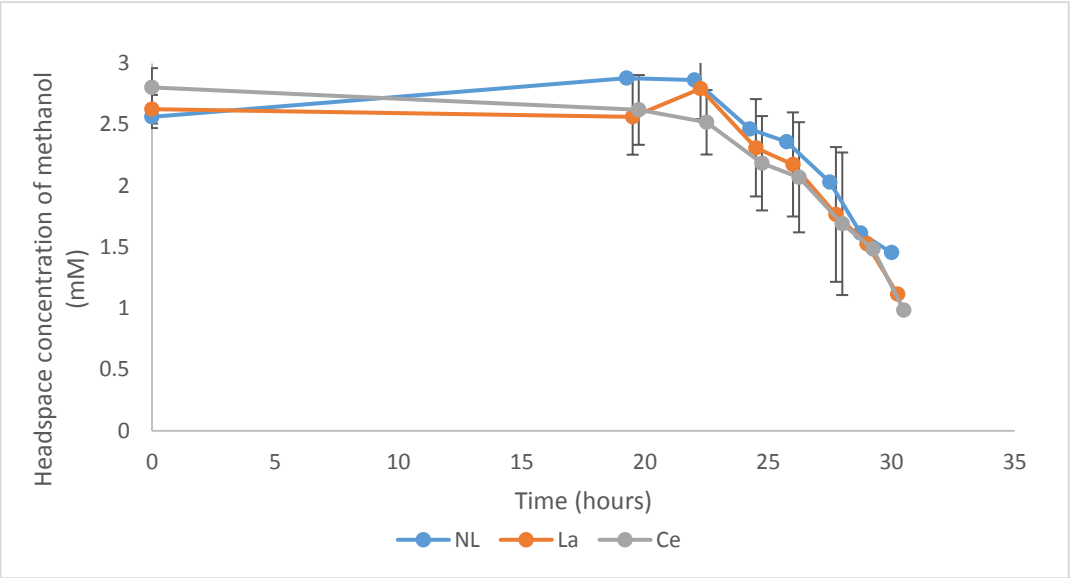


Figure 5.17 GC measurements of methanol concentration in the headspace of Church Farm soil enriched with methanol and lanthanides or without lanthanides. NL – No lanthanide supplemented, La – Lanthanum, Ce - Cerium

All samples had a lag phase of 22 hours. Following this initial lag phase the different test groups exhibited similar oxidation rates, with no significant difference between the test groups (Figure 5.17). Following consumption of the methanol all samples were harvested for DNA extraction. The DNA extracted from the test groups was then used as template in a PCR to amplify the 16S rRNA gene with DGGE specific primers. Figure 5.18 shows the total community profiles assessed through 16S rRNA gene DGGE profiling.

There are no bands unique to the community profile of any of the test groups, indicating that there is no change in the communities resulting from the supply of lanthanides (Figure 5.18). The absence of any clear difference in the 16S rRNA gene DGGE profiles and in the oxidation profile of the methanol in all test groups indicates that the supplementation of lanthanides to the soil samples had no significant impact on the methylotrophic community present over the period tested.

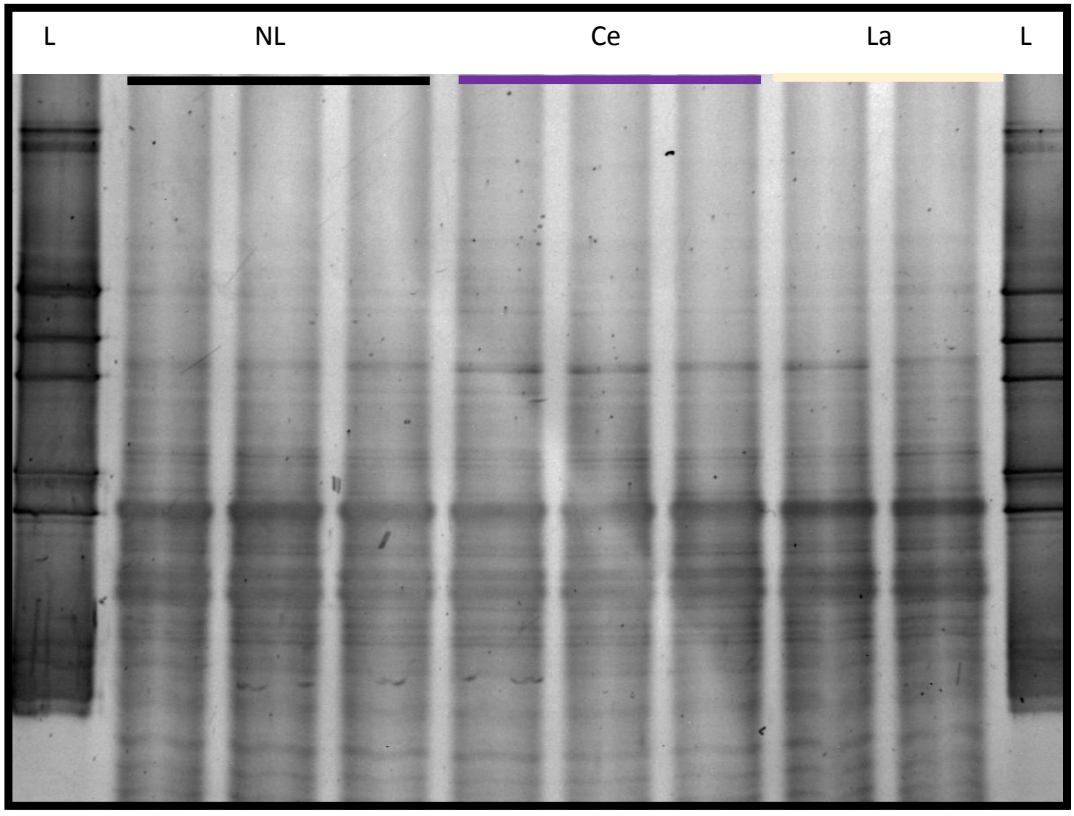


Figure 5.18 DGGE profile of 16S rRNA genes amplified from DNA extracted from methanol-enriched CF soil supplemented with lanthanum (La)(Pink), cerium (Ce)(Purple) or no lanthanides (NL)(Black).

5.6 Discussion

5.6.1 Characterisation of the active methylotrophs in the pea rhizosphere, wheat rhizosphere and CF soil through 16S rRNA gene sequencing and DGGE profiling

The methanol SIP experiment was successful in labelling the DNA of the active methylotrophs with ^{13}C, as shown by a second peak of DNA in the ^{13}C fractions, unique bands in the 16S rRNA gene DGGE profiles of the heavy fractions of the ^{13}C test groups and a shift in the 16S rRNA gene profiles of the ^{13}C test groups. There are differences in the 16S rRNA gene profiles between the different environments at both T1 and T2, indicating an impact of the plants on the methylotrophs within the soil. This difference is consistent with both the DGGE profiling and Illumina sequencing of the 16S rRNA gene of the different fractions. However, it was not possible to identify the precise reasons for these differences, with multiple possible causes following growth of the plant (Kuzyakov, 2002; Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012; Oliveira *et al.*, 2015). *Methylobacterium*, one of the clear differentiating genera between the rhizosphere-associated samples and the CF bulk soil, has been shown to be ubiquitously in association with plants in a range of studies (Knief *et al.*, 2008, 2010; Iguchi *et al.*, 2015). However, the majority of these studies have shown this genus to be enriched in the phyllosphere, with few studies showing increased presence in the rhizosphere relative to the bulk soil following growth of the plant (Sy *et al.*, 2001; Schreiner *et al.*, 2010; Minami *et al.*, 2016). The relative abundance of *Methylobacterium* increased between the T0 bulk soil community and the T0 pea rhizosphere community, but only from 0.17 % to 0.26 %. The wheat rhizosphere does not reveal an increase in the relative abundance of the genus. The labelling of *Methylobacterium* in rhizosphere samples could be due to the genus being more active in the plant associated soils than in the bulk soil.

It is interesting to note the higher diversity of the *Methylophilaceae* within the rhizosphere associated samples than the CF bulk soil. The family *Methylophilaceae*, previously described in Chapters 3 and 4, is comprised of four genera, *Methylobacillus, Methylophilus, Methylovorus* and *Methylotenera*. These genera have been studied in detail (Kalyuhznaya *et al.*, 2009; Lapidus *et al.*, 2011b; Vorobev *et al.*, 2013; Beck *et al.*, 2014). Several species from these genera have been isolated from the soil environment or in association with plants (Doronina *et al.*, 2004, 2011; Madhaiyan *et al.*, 2009;

Gogleva *et al.*, 2011; Madhaiyan *et al.*, 2013). Key differences between the genera include the metabolic capacity for denitrification (Kalyuhznaya *et al.*, 2009; Beck *et al.*, 2011; Mustakhimov *et al.*, 2013) and the absence of the classical methanol dehydrogenase gene in some species of *Methylotenera* and *Methylobacillus* (Lapidus *et al.*, 2011b; Keltjens *et al.*, 2014).

Cross feeding occurred in this enrichment, with several genera present at greater relative abundance in the T2 samples relative to the T1 time points. The proposed cross feeding bacteria are represented by a diverse array of genera, from four different classes of bacteria. Isolates of *Ramlibacter* have been shown to be aerobic heterotrophs, isolated from a range of environments including soils. Although the *Commamomadaceae* contains confirmed and proposed methylotrophic genera, there are no indications that species of *Ramlibacter* are capable of methanol oxidation (Heulin *et al.*, 2003; An *et al.*, 2012; Lee *et al.*, 2014). *Stigmatella* is a myxobacterium, with representatives isolated from several plant associated samples (Sutherland, 1978). *Lentisphara, Pelobacter, Opitutus* and *Desulfococcus* are genera that are typically associated with anaerobic terrestrial environments (Chin *et al.*, 2001; Cho *et al.*, 2004; Choi *et al.*, 2013; Kleindienst *et al.*, 2014; Kotak *et al.*, 2015). However, members of the *Lentisphaera, Opitutus* and *Desulfococcus* have both been shown to be present in aerobic environments under microaerophillic conditions (Bridge *et al.*, 1999; Choi *et al.*, 2013; Dörries *et al.*, 2016; El Khalloufi *et al.*, 2016). Shaking and opening of the serum vials was done to prevent any shift to anaerobic conditions. However, the enrichment of these genera indicates that either there were anaerobic conditions during the enrichment or there is a greater metabolic capacity within these genera than previously indicated. The specific compounds used by these genera to acquire the ^{13}C label is unknown. The metabolic capabilities of the different groups indicate that they could have used many ^{13}C compounds potentially produced by the methylotrophs. This includes carbon dioxide, compounds exuded by the methylotrophs or the cellular components of lysed methylotrophs (Pankratov *et al.*, 2008; Noar *et al.*, 2009; Dumont *et al.*, 2011; Hart *et al.*, 2013).

5.6.2 Characterisation of the active methylotrophs in the pea rhizosphere, wheat rhizosphere and CF soil through metagenomic sequencing

Analysis of the metagenomes produced using the heavy fraction DNA from the T2 communities supported the notion that there were differences between the methylotrophic communities of the CF soil and the rhizosphere environments. However, the Metaphlan results diverged from the results of the 16S rRNA gene sequencing. Key differences included the detection of additional genera at high abundance, such as *Methylibium* and *Mesorhizobium*. These differences suggest that the active methylotrophic communities of the pea rhizosphere and wheat rhizosphere were distinct from each other in addition to the CF soil. There were also differences detected in the enriched genera of the family *Methylophilaceae* relative to the 16S rRNA gene sequencing profile, with *Methylobacillus* being absent and *Methylotenera* being more enriched. This difference in profiles is potentially the result of members of this family being assigned to a different genera. The higher abundance of genera within the *Commamonadaceae* is another large divergence from the ^{13}C labelled 16S rRNA gene profiles in which this group is largely absent. It is also interesting to note in all environments that the genus *Desulfococcus* was absent in the metagenomes, in spite of the relatively high presence in the T2 16S rRNA gene profiles. The reasoning for the absence of *Desulfococcus* is harder to suggest. Again it is possible that this group was reassigned to a different genus within the Deltaproteobacteria or was not classified with a high phylogenetic resolution.

These differences are potentially the result the primers used to amplify the 16S rRNA genes possessing a bias, resulting in specific groups being discriminated against during the amplification of this gene, resulting in their absence or depletion within specific environmental samples (Bergmann *et al.*, 2012). Furthermore, the programs used to assign phylogeny to the metagenomes will have different pipelines and reference sequences resulting in a different output from 16S rRNA gene analysis alone. Therefore certain groups will be under- or over-represented in the 16S rRNA gene profile of the heavy fraction, but present in the sequenced metagenomes. This variation in community profile as a result of the different sequencing approach used shows that this is an important factor to consider in the design of stable isotope probing experiments.

An array of binned genomes were produced from the sequencing of the metagenomes. Amongst the diversity captured were two *Methylobacterium* genomes and several genomes that were assigned to the *Methylophilales*. This reinforces the importance of these two groups in this particular enrichment series and enables further assessment of this diversity. One of the genome bins exhibited high identity to *Methylovorus methylotrophus* MM2, supporting the potential relevance of this species to methanol oxidation in the soil environment. This is interesting to note given the divergence of this species in comparison to other species of *Methylovorus*. An additional *Methylophilales* genome was shown to possess a *xoxF3* methanol dehydrogenase gene that was divergent from the methanol dehydrogenase gene of this clade found within the *Methylophilaceae* (Chistoserdova *et al.*, 2007; Keltjens *et al.*, 2014), reflecting the remaining diversity within this family that remains to be cultivated (Kalyuhznaya *et al.*, 2009; Lapidus *et al.*, 2011b; Beck *et al.*, 2014). An additional binned genome was assigned to the family *Comamonadaceae*. This family possesses genera that possess *xoxF* methanol dehydrogenase genes and some have been shown to be capable of methanol oxidation (Satola *et al.*, 2013; Keltjens *et al.*, 2014; Eyice *et al*., 2015a). Clearly this family may be highly relevant to methanol oxidation in a variety of natural environments.

5.6.3 Insufficient labelling of RNA with ^{13}C following enrichment with an ambient concentration of methanol

The RNA-SIP experiment performed with CF soil at a typical environmental concentration of methanol failed to yield sufficient labelled RNA. A long-term enrichment of a soil sample with continual spiking of a low concentration of methanol, or a continuous supply of methanol to the soil samples, could potentially result in the successful labelling of the RNA and DNA of the methanol utilising methylotrophs in the soil. However, this approach would have the limitations of being an artificial setup and a long term incubation experiment. It is also possible that this experimental design would result in the build-up of methanol that would be unmonitored in the absence of a sufficiently sensitive assay technique such as PTR-MS (Abanda-Nkpwatt *et al.*, 2006).

5.6.4 Enrichment of the CF soil with methanol and lanthanides

There are multiple possible reasons for the lack of an impact on the oxidation of methanol by the Church Farm soil. Without further characterisation of the systems

involved in the regulation of methanol dehydrogenase gene expression, these reasons remain speculative. However, it is tempting to suggest that no change occurred in the community profile or rate of methanol oxidation following the supply of lanthanides because lanthanides are already present at a non-limiting concentration (Keltjens *et al.*, 2014). It was not possible to measure the concentrations of lanthanides in the CF soil. However, soils across the United Kingdom are shown to have a range of lanthanides that could be non-limiting (Ramos *et al.*, 2016). If the acquisition system is able to overcome the low availability of the lanthanides then it is possible that the results of this enrichment would be replicated with additional soils. This hypothesis could be tested through the use of a type soil with a lower concentration of lanthanides (Ramos *et al.*, 2016).

Chapter 6: Identification of active exudate utilisers in the pea rhizosphere and wheat rhizosphere through DNA stable isotope probing with $^{13}CO_2$

6.1 Introduction

Plants have a profound impact on the microbial communities present within soil (Haichar *et al.*, 2008; Ofek *et al.*, 2013). This is due to the amount of carbon released to the soil by the plant. This carbon takes the form of exuded compounds including organic acids, sugars and alcohols, mucilage and sloughed off cells (Dennis *et al.*, 2010; Cébron *et al.*, 2011). The available carbon pool in the soil is also increased by the plant through the breakdown of soil organic matter and release of organic acids to degrade SOM (Kuzyakov, 2002; Haichar *et al.*, 2008). The exudates released by a plant typically vary across the growth stages (Houlden *et al.*, 2008) and this variation in exudation across the life stages of the plant impacts on the microbial community of the rhizosphere (Houlden *et al.*, 2008; Haichar *et al.*, 2012). Characterisation of the rhizosphere communities of several plant species has consistently shown that they are dominated by Proteobacteria, Actinobacteria and Bacteroidetes (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012; Ai *et al.*, 2015). However, changes in relative abundance of bacteria in the soil following the growth of a plant could be due to multiple reasons, with some bacteria directly utilising carbon released by the plant, whereas others are enriched due to the enhanced breakdown of soil organic matter (Bernard *et al.*, 2007; Ai *et al.*, 2015).

Experiments described in this chapter assessed whether an increase in the relative abundance of methylotrophic bacteria and other bacteria in the rhizosphere of a cereal and a legume was due to exudate utilisation as opposed to the priming effect. This was tested using stable isotope probing by supplying wheat and pea plants with $^{13}CO_2$. This type of stable isotope probing experiment differs from SIP experiments in which the label supplied is the only major source of carbon available to the microbial community, as additional non-labelled carbon will be available. This can result in the dilution of the ^{13}C label of the organisms utilising the labelled substrate.

6.2 Experimental design of preliminary rhizosphere SIP experiment

A preliminary rhizosphere SIP experiment was performed to inform the selection of specific parameters for further experiments. This experiment was performed using $^{13}CO_2$ supplied to actively growing pea plants, wheat plants and unplanted controls at a concentration of 1000 ppmv. Seeds were germinated in petri dishes supplied with autoclaved RO water for three days. After three days the germinated seeds were transferred to pots of CF soil. Plants were grown under short day (8:16 hour) growth conditions. 16 days after planting, one pea plant, one wheat plant, and one unplanted control were transferred to acrylic tubes (Section 2.9) (Figure 6.1) for incubation with labelled CO_2.

Figure 6.1 Pea and wheat plants contained in acrylic tubing

The acrylic tubes were flushed with carbon dioxide-free air, sealed with plastic lids and $^{13}CO_2$ was injected to a final concentration of 1000 ppmv. This test group was pulsed for twelve days, with the concentration of CO_2 in the tubes monitored using gas chromatography (Section 2.7.2). The concentration of CO_2 was maintained through the injection of $^{13}CO_2$ when the concentration reached 700 ppmv. The concentration of CO_2 was kept below 1000 ppmv to prevent harm to plants. Tubes were opened at the end of

each light period and flushed with CO_2-free air before the start of the subsequent light cycle. This experiment was repeated as before, except that plants were grown for 22 days before incubation with labelled CO_2 for six days. The remaining plants and the unplanted control were grown in standard growth room conditions (termed open). After growth with labelled CO_2 (28 days total growth), all test groups were harvested (Section 2.3.2). The rhizosphere soil was collected, DNA was extracted and 4 µg of DNA for each sample was processed via ultracentrifugation and fractionation (Section 2.8.2).

The DNA retrieved was used as template for PCR amplification of 16S rRNA genes and PCR products were used for 16S rRNA gene profiling by DGGE. The DGGE profiles indicated that there had been sufficient labelling of the DNA of the exudate-utilising bacteria in the pea and wheat rhizosphere of plants supplied with $^{13}CO_2$ for 12 days. This was indicated through the presence of exclusive or more intense bands present in the heavy fraction of the ^{13}C test groups relative to the ^{13}C light fraction and the heavy fraction of the open test groups. However, there was no indication of labelling of the exudate utilising bacteria in the six day pulsed test groups. It is possible that six days allowed insufficient uptake of ^{13}C by the plant for subsequent exudation and assimilation by the rhizosphere community. Sequencing of the heavy and light fractions of the ^{13}C 12 day pulsed test groups of the pea and wheat rhizosphere further indicated that there was labelling of specific groups in the heavy fraction of both of these test groups (Table 6.1-6.2).

Table 6.1 OTUs over-represented in the H compared to L fraction based on the relative abundance 16S rRNA genes between the heavy fraction and light fraction of the pea rhizosphere at the family level

	Relative abundance in the 16S rRNA gene profile		
	^{13}C Pea Heavy	^{13}C Pea Light	Relative abundance $^{13}H/^{13}L$
Geobacteraceae	26.83	0.10	261
Comamonadaceae	15.15	5.81	2
Pseudomonadaceae	9.36	1.59	5
Rhodocyclaceae	7.52	1.64	4
Aeromonadaceae	3.02	0.15	19
Desulfobulbaceae	2.82	0.05	54
Veillonellaceae	0.15	0.05	2

Table 6.2 OTUs over-represented in the H compared to L fraction based on the relative abundance 16S rRNA genes between the heavy fraction and light fraction of the wheat rhizosphere at the family level

	Relative abundance in the 16S rRNA gene profile		Relative abundance
	^{13}C Wheat Heavy	^{13}C Wheat Light	^{13}H/^{13}L
Comamonadaceae	13.89	4.25	3
Rhodocyclaceae	6.84	0.56	12
Pseudomonadaceae	6.34	0.34	18
Oxalobacteraceae	3.45	1.72	2
Nostocaceae	2.58	0.04	60
Rhodospirillaceae	1.27	0.17	7
Paenibacillaceae	1.27	0.60	2
Geodermatophilaceae	1.12	0.34	3
Aeromonadaceae	1.06	0.25	4
Intrasporangiaceae	0.76	0.26	2
Clostridiaceae	0.56	0.26	2
Iamiaceae	0.46	0.21	2
Myxococcaceae	0.25	0.04	5
Nannocystineae	0.25	0.04	5
Isosphaeraceae	0.15	0.04	3
Candidatus Chloracidobacterium	0.10	0.04	2
Sphingobacteriaceae	0.10	0.04	2

Due to the absence of a sequenced ^{12}C control it was not possible to reliably analyse these communities further with regards to exudate utilisation. However, based on the results of this experiment, 12 days was selected as the length of pulsing to be used in further rhizosphere SIP experiments.

6.3 Experimental design of first rhizosphere SIP experiment

A rhizosphere SIP experiment was performed using $^{13}CO_2$ supplied to actively growing pea plants and unplanted controls. Two concentrations of carbon dioxide were supplied to the test groups, ambient (350 ppmv) and an elevated concentration (1000 ppmv). The experimental design was as above (Section 6.2), except the plants were grown under a long day growth cycle for the first 16 days (16:8 hours of light). The plants were switched to a medium day growth cycle (12:12) for the duration of the pulsing. All test groups were performed in duplicate. In addition to the ^{13}C and open test groups, an additional test group was pulsed with ^{12}C carbon dioxide. At the end of twelve days of pulsing,

samples were collected from all test groups for DNA extraction, processing and analysis as above (Section 6.2).

The 16S rRNA gene DGGE profiles from these different test groups indicated that labelling of the exudate-utilising community in the ^{13}C test groups was successful (Figure 6.2). This was identified through the presence of exclusive and more intense bands in the ^{13}C heavy fraction of the pea test group relative to the ^{13}C light fractions of the pea rhizosphere and the heavy fractions of the 350 ppmv and ^{12}C test groups. The extent of the labelling appeared to be greater in the 1000ppmv supplied test group compared to the 350ppmv supplied test groups. Bands that were present in both of these test groups were present at greater intensity in the 1000ppmv heavy fraction profiles. Bands were picked, amplified through PCR and sent for sequencing in order to identify the enriched bands.

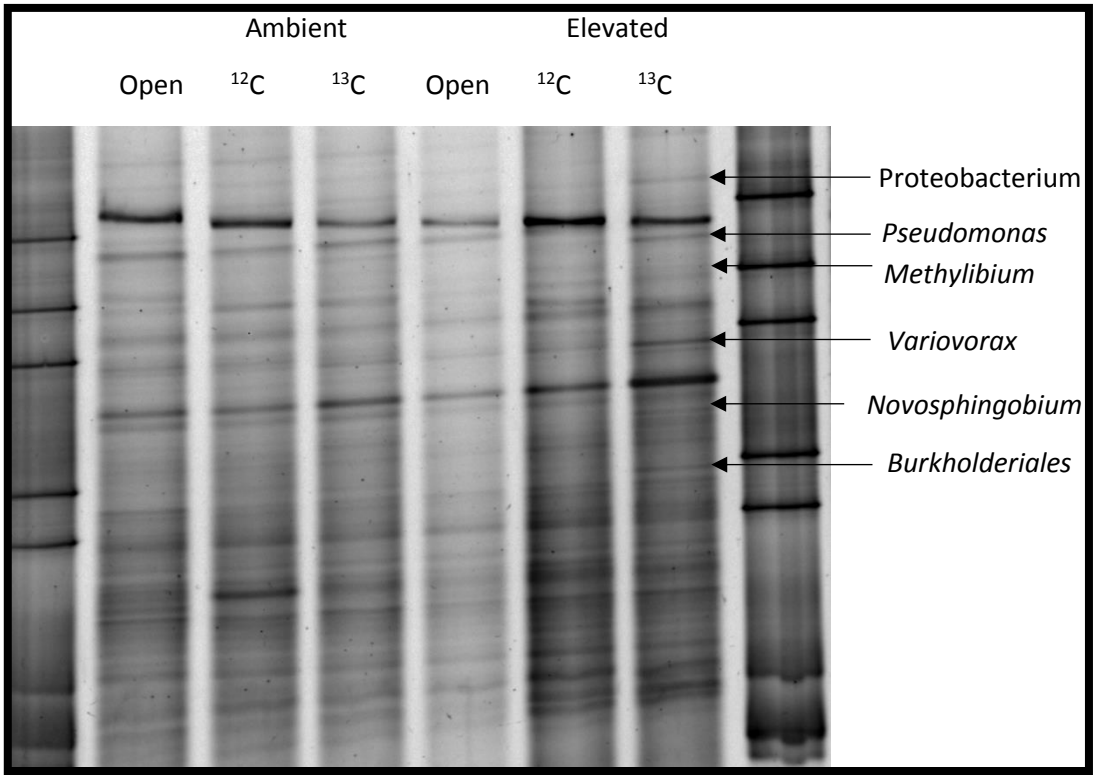


Figure 6.2 16S rRNA gene DGGE profile produced using the pooled heavy DNA fraction of each pea test group. Open represents profiles with DNA from rhizosphere soil of pea plants grown without pulsing of CO_2.

The 16S rRNA gene sequences retrieved from the selected bands were assigned to a broad range of Gram negative genera, including *Methylibium*, *Novosphingobium* and

Variovorax, that have shown to possess plant growth promoting traits (Nakatsu *et al.*, 2006; Smit *et al.*, 2012; Satola *et al.*, 2013).

The heavy fractions and a pooled light fraction for each test group was sent for 454 sequencing. Analysis of the sequenced 16S rRNA gene amplicons further supported the success of the SIP experiment, with clear differences being observed between the heavy fraction of the ^{13}C heavy fraction and the other test groups. A series of criteria was applied to the sequenced amplicons in order to identify genera that could be classified as labelled. Criteria applied to the 16S rRNA gene sequencing results from the DNA of the heavy fraction of the pea rhizosphere were:

1. For each OTU, relative abundance in the ^{13}C heavy fraction was more than twice the relative abundance in the ^{13}C light fraction
2. Reads are more than two times more abundant in the sequencing results of the ^{13}C heavy fraction compared to the sequencing results of the ^{12}C heavy fraction
3. Reads are less than two times more abundant in the sequencing results of the ^{12}C heavy fraction compared to the sequencing results of the ^{12}C light fraction

An additional criterion to control for autotrophs directly labelled from the $^{13}CO_2$ was applied. This criterion was that reads are less than two times more abundant in the sequencing results of the unplanted ^{13}C heavy fraction compared to the sequencing results of the ^{13}C light fraction. These criteria were applied to the 16S rRNA gene sequencing results to control for genera that appeared labelled in the ^{13}C heavy fraction as a result of GC content, incomplete separation of labelled and unlabelled DNA and autotrophic growth on $^{13}CO_2$.

The ten most enriched OTUs for this rhizosphere SIP experiment are summarised in Figure 3. It is interesting to note the genera that are common between the 16S rRNA gene DGGE profiles and those in the sequenced 16S rRNA gene amplicons. These genera include *Novosphingobium* and *Varivorax*, both present in the ^{13}C heavy fraction of the 350 ppmv supplied test groups. However, analysis of the sequence data indicated that *Methylibium* and *Pseudomonas* were not enriched in the heavy fraction. This difference in community profile captured by 454 sequencing indicates the value of utilising more than one profiling technique and using a high resolution approach. Furthermore,

sequencing was also able to capture labelled genera that were not apparent as enriched bands in the 16S rRNA gene DGGE profile, indicating the value of next generation sequencing in characterising the ^{13}C heavy fraction, identifying the more lightly labelled and less abundant members of the exudate utilising community (Prosser *et al.*, 2006). Sequencing of the ^{13}C heavy fraction focuses in on the heavy fraction, meaning the same number of reads are applied to a small subset. Therefore, OTUs are detected that are not detected in the ^{12}C light.

In total, 48 genera were detected as labelled in the 350 ppmv supplied pea rhizosphere ^{13}C heavy fraction and 46 genera were detected as labelled in the 1000 ppmv supplied test group. The diversity of the exudate-utilising genera can be broadly categorised into three groups, comprising Actinobacteria, including the antibiotic producing *Actinomycetes*, facultative methylotrophic bacteria and other heterotrophic genera.

6.3.1 Methylotrophs ^{13}C labelled in the ^{13}C heavy fraction of the 350 ppmv test group

Of the labelled taxa identified in the 350 ppmv pea rhizosphere test group, *Sphingomonas*, *Paracoccus*, *Variovorax* and *Flavobacterium* contain methylotrophic species (Table 6.3). These genera contain species of facultative methylotrophs, as previously described (Chapter 3 and 4). Some species of *Ramlibacter* also contain *xoxF* methanol dehydrogenase encoding genes and may be capable of metabolising methanol within the soil environment. This family has been shown to be potentially relevant to methanol oxidation in the CF soil previously in this work (Chapter 4 and Chapter 5). *Flavobacterium, Variovorax* and *Sphingomonas* also contain species shown to possess cellulase activity (Lee *et al.*, 2006; Haichar *et al.*, 2007; Boersma *et al.*, 2010). *Variovorax* has been detected in the rhizospheres of several plant species, including pea, lettuce and ginseng (Kim *et al.*, 2006; Im *et al.*, 2010; Turner, 2013). Both *Flavobacterium* and *Sphingomonas* are atypical methylotrophic genera, with methylotrophy present in the minority of isolated species from both genera, and the enzymatic systems for subsequent C1 metabolism have yet to be fully elaborated (Boden *et al.*, 2008; Munusamy Madhaiyan *et al.*, 2010). It is interesting to note that no genome sequenced members of the *Sphingomonadaceae* possess a PQQ methanol dehydrogenase.

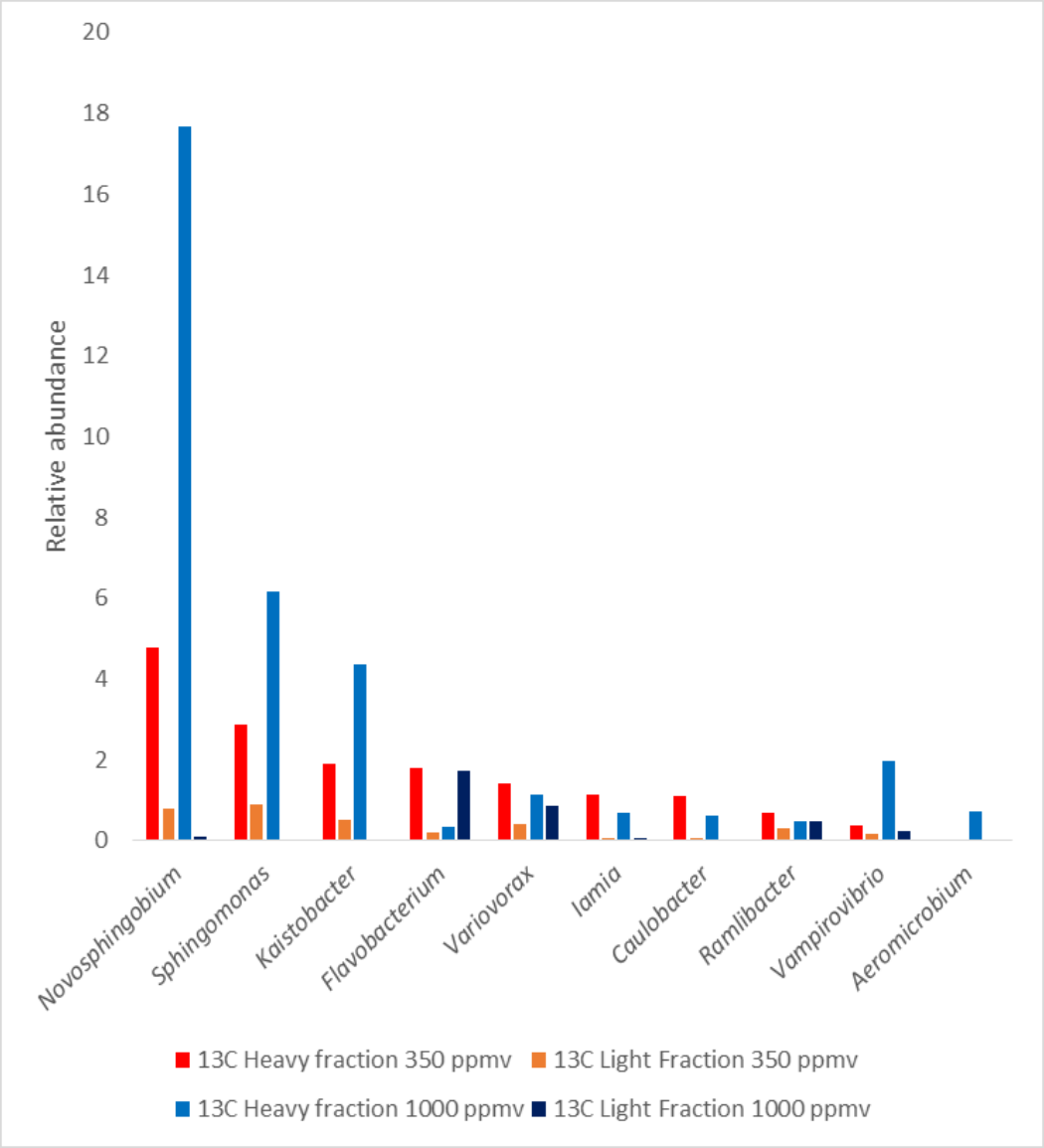


Figure 6.3 The relative abundance of the ten most abundant OTUs over-represented in the ^{13}C heavy fraction compared to ^{13}C light fraction based on their relative abundance in the 16S rRNA gene profile of the the rhizosphere of the 350 ppmv and 1000 ppmv $^{13}CO_2$ supplied pea plants

Table 6.3 20 most abundant OTUs over-represented in the ^{13}H compared to ^{13}L fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the pea rhizosphere 350 ppmv test group

	Relative abundance in the 16S rRNA gene profile (%)				Relative abundance
Genus	^{13}C Heavy fraction	^{13}C Light Fraction	^{12}C Heavy Fraction	^{12}C Light Fraction	^{13}H/^{13}L
Novosphingobium	4.79	0.79	0.53	1.76	6
Sphingomonas	2.87	0.89	1.03	1.35	3
Kaistobacter	1.91	0.50	0.46	0.49	3
Flavobacterium	1.80	0.19	0.91	3.71	9
Variovorax	1.40	0.41	0.61	1.09	3
Iamia	1.14	0.06	0.38	0.26	17
Caulobacter	1.10	0.06	0.11	0.15	17
Ramlibacter	0.67	0.32	0.19	0.30	2
Nakamurella	0.64	0.19	0.11	0.11	3
Marmoricola	0.62	0.29	0.11	0.22	2
Mitsuaria	0.40	0.06	ND	ND	6
Vampirovibrio	0.36	0.16	0.08	1.54	2
Clostridium	0.24	ND	0.08	0.19	NA
Paracoccus	0.18	0.03	ND	0.04	5
Actinomadura	0.16	ND	ND	0.08	NA
Actinoplanes	0.14	ND	ND	ND	NA
Candidatus Kuenenia	0.14	ND	ND	ND	NA
Deinococcus	0.12	0.03	ND	0.08	3
Phytophthora	0.10	ND	ND	0.08	NA
Nocardiopsis	0.10	ND	ND	ND	NA
Desulfobacca	0.09	ND	ND	0.08	NA

6.3.2 Methylotrophs ^{13}C labelled in the ^{13}C heavy fraction of the 1000 ppmv test group

The methylotrophic genera labelled in the 1000 ppmv supplied pea rhizosphere test group varied from those in the 350 ppmv supplied test group (Table 6.4). Of the confirmed methylotrophic genera, *Sphingomonas*, *Methylocapsa* and *Methylotenera* were shown to belong to the exudate utilising portion of the rhizosphere. It is interesting to note that *Sphingomonas* was present to a greater extent in the ^{13}C heavy fraction within the pea rhizosphere in a concentration of carbon dioxide above 350 ppmv. *Methylocapasa*, a genus of facultative methanotrophs (Dunfield 2010), has also previously been shown to be plant associated (Chen *et al.*, 2008b; Andreote *et al.*, 2009; Iguchi *et al.*, 2015). *Methylotenera*, described in Chapters 3 and 5, is a genus of facultative and obligate methylotrophs (Bosch *et al.*, 2009). The *Methylophilaceae* was

previously shown to increase in relative abundance following growth of both cereal and legume crops in soil from the Church Farm (Turner et al. 2013). However, in this experiment the members of the *Methylophilaceae* were only detected as labelled within the exudate utilising portion of the rhizosphere in the above 350 ppmv test group. It is worth noting that in the Turner 2013 study, the samples were sequenced to a greater depth (Turner, 2013) (100,000 reads compared to 3,000 reads per sample) and this could account for the lack of detection of the family in this experiment.

Additional *xoxF*-containing genera include *Dokdonella*, *Leptothrix*, *Polaromonas* and *Rubrivivax. Dokdonella* has not been confirmed to be capable of methanol oxidation but contains species that possess *xoxF* methanol dehydrogenase genes and has been shown to be associated with the rhizosphere and roots of maize plants (Haichar *et al.*, 2008; Dohrmann *et al.*, 2013). The latter three genera are have been detected in the rhizospheres of ryegrass, poplar trees and rice plants (Ramana *et al.*, 2006; Cébron *et al.*, 2011; Brown *et al.*, 2012) and are members of the family *Comamonadaceae*, previously been shown to be relevant to methanol oxidation within the CF soil (Chapter 5). Furthermore, the *xoxF* methanol dehydrogenase of *Leptothrix* has been detected as expressed in the soil environment, indicating that the gene may be functional (Knief *et al.*, 2012).

Table 6.4 20 most abundant OTUs over-represented in the ^{13}H compared to ^{13}L fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the pea rhizosphere 1000 ppmv test group

	Relative abundance in the 16S rRNA gene profile (%)				Relative abundance ^{13}H/^{13}L
	^{13}C Heavy fraction	^{13}C Light Fraction	^{12}C Heavy Fraction	^{12}C Light Fraction	
Novosphingobium	17.68	0.08	2.42	1.76	225
Sphingomonas	6.16	ND	1.34	2.71	NA
Kaistobacter	4.37	0.04	1.38	0.84	111
Vampirovibrio	1.98	0.23	ND	0.49	8
Aeromicrobium	0.71	ND	ND	ND	NA
Methylocapsa	0.50	0.04	0.20	0.11	12
Leptothrix	0.37	0.04	ND	0.07	9
Duganella	0.34	0.04	0.03	0.14	8
Massilia	0.30	ND	0.07	0.11	NA
Rhodoferax	0.22	ND	ND	0.04	NA
Sphingopyxis	0.18	ND	ND	0.11	NA
Polaromonas	0.16	0.08	ND	ND	2
Actinomyces	0.15	ND	0.03	0.04	NA
Actinoplanes	0.10	ND	ND	0.18	NA
Labilithrix	0.10	ND	ND	ND	NA
Rhodopila	0.10	ND	ND	ND	NA
Inquilinus	0.08	ND	0.03	0.04	NA
Candidatus Koribacter	0.07	ND	ND	ND	NA
Kaistia	0.06	ND	ND	ND	NA
Actinomycetospora	0.06	ND	ND	ND	NA
Rubrivivax	0.06	ND	ND	ND	NA

6.3.3 ^{13}C labelling of additional bacteria within the 350 ppmv and 1000 ppmv test groups

The exudate-utilising portion of the rhizosphere community also included further diversity. Amongst the heterotrophic bacteria ^{13}C labelled within the rhizosphere environment were additional nitrogen fixing members of the *Sphingomonadaceae* (White *et al.*, 1996; Videira *et al.*, 2009; Lin *et al.*, 2014). *Sphingomonas, Kaistobacter* and *Novosphingobium* were labelled in both the 350 ppmv and elevated test groups, with *Novosphingobium* the most abundant genus within the ^{13}C labelled exudate utilisers. The utilisation of exudates by members of the *Sphingomonadaceae* was also shown to occur in a stable isotope probing study studying the rhizosphere of rice plants (Hernández *et al.*, 2015). *Caulobacter*, *Achromobacter* and *Mitsuaria* were also identified as exudate utilisers. *Achromobacter* was previously isolated from the CF soil and was indicated to be actively selected by plants grown in the soil (Tkacz *et al.*, 2015). *Mistsuaria* has been used previously as a biocontrol agent due to the ability of some species to suppress phyopathogens (Rong *et al.*, 2012).

In addition to *Kaistobacter* and *Novosphingobium*, *Sphingopyxis* was also present within the exudate utilisers in the elevated CO_2 supplied test group. *Achromobacter* was not present within the elevated exudate utilising community, but several heterotrophic genera were, including *Massilia*, *Duganella* and *Stenotrophonomas*. *Massilia* was also shown to be enriched following growth of *Arabidopsis* in soil from the Church Farm (Tkacz *et al.*, 2015). Both test groups also saw the ^{13}C labelling of genera typically associated with infection in humans (*Clostrium*, *Stenotrophonomas* and *Inquilinus*) in addition to species that have been detected in the soil, as well as genera known to contain plant pathogens (*Ralstonia*) (Aliye et al. 2008; Berg et al. 2013 and references therein).

6.4 Design of the second rhizosphere SIP experiment

A second rhizosphere SIP experiment was performed using $^{13}CO_2$ supplied to pea plants, wheat plants and unplanted controls. Carbon dioxide was supplied to the plant at 350 ppmv concentration. The experimental design was as above (Section 6.3), except that plants were grown under a medium day growth cycle (12:12) for the duration of the experiment and the plants were grown open for 30 days and then for a further 12 days supplied with $^{12}CO_2$ or $^{13}CO_2$. All test groups were performed in triplicate, with ^{12}C, ^{13}C and open test groups. At the end of twelve days of CO_2 pulsing, rhizosphere samples and root samples were collected from all test groups, snap-frozen in liquid nitrogen and stored at -80 °C for molecular analysis. DNA and RNA was extracted from all of the test groups through established protocols (Section 2.4). DNA from the test groups was processed and analysed as above. RNA was also processed according to established protocols (Manefield *et al.*, 2002; Whiteley *et al.*, 2007). DNA from the plant roots and the RNA from the replicates within a test groups were pooled prior to ultracentrifugation and processing. RNA was reverse transcribed from processed test groups to produce cDNA.

16S rRNA gene profiling by DGGE indicated that the labelling of nucleic acids of the exudate utilisers in the root and rhizosphere environments supplied with $^{13}CO_2$ was successful. However, the variation between the ^{13}C heavy fraction and ^{13}C light fractions, and the heavy fractions of the additional test groups is present to a larger extent through bands of greater intensity as opposed to exclusive bands. Furthermore, the variation between the ^{13}C heavy fractions and the other test groups is less than that in the previous rhizosphere SIP experiment (Section 6.2 and 6.3). The DNA and cDNA heavy and light fractions of all processed test groups was used as template in the PCR amplification of the 16S rRNA gene, and these PCR products were purified and sent for Illumina sequencing.

6.4.1 ^{13}C labelling of methylotrophic genera within all test groups

Methylotrophic genera were shown to be ^{13}C labelled within the exudate utilising portions of all test groups. There were some genera shared between the plant species and between the DNA and cDNA profiles. However, there were also several genera exclusively ^{13}C labelled in only one test group. *Xanthomonas* represents the most

abundant genus amongst the exudate utilisers in the 16S rRNA gene profile of the wheat rhizosphere (Summaried in Figure 6.4 and Table 6.5). *Xanthomonas* is a genus containing plant pathogens that have been shown to possess *xoxF1* methanol dehydrogenase genes (Studholme *et al.*, 2011). The presence of plant pathogens within the exudate utilising portion of the rhizosphere has been observed in a previous study (Haichar *et al.*, 2008). *Gemmobacter* is another methylotrophic genus utilising plant carbon in the wheat rhizosphere. The first methylotrophic species of this genus, using methylamine as a sole carbon source, was isolated from Movile Cave (Wischer et al. 2014). The methylotrophic genera ^{13}C labelled within the cDNA profile of the wheat rhizosphere are present to a lesser extent than those in the DNA community (Table 6.6). Amongst this diversity is *Sphingomonas*, *Methylobacillus*, *Starkeya* and *Methylobacterium*. It is interesting to observe the presence of *Methylobacillus* as ^{13}C labelled because all extant species of *Methylobacillus* are obligate methylotrophs (Chapter 4). This suggests the activity of the genus within this environment is the result of the metabolism solely of C1 compounds. However, a broader metabolism possessed by uncultivated members of *Methylobacillus* could be present. *Methylobacterium* was also present within the active exudate-utilising community, showing the relevance of this genus following the growth of a plant within the CF soil and its increase in abundance during the growth of a cereal (Schreiter *et al.*, 2014).

The proportion of methylotrophs within the exudate-utilising members of the wheat root increased relative to that of the rhizosphere. The diversity of methylotrophic genera is also greater in the wheat root than in the wheat rhizosphere community (Summarised in Figure 6.5 and Table 6.7). *Methylocapsa* and *Beijerinkia* are both from the family *Beijerinkacaea*. The genus *Beijerinkia* has a varied metabolism. Some species are heterotrophs and one species can grow on methanol as a sole carbon source (Dedysh *et al.*, 2005). *Gemmobacter* is present within the wheat root exudate utilisers, having also appeared within the wheat rhizosphere. *Xanthobacter* and *Dokdonella* present in the exudate-utilising portion of the wheat root community in a previous rhizosphere SIP study (Haichar *et al.*, 2008).

Methylophaga was abundant within the ^{13}C labelled exudate utilising portion of the wheat root community (as determined from DNA extraction), that was unexpected due

to the low relative abundance of this genus within the 16S rRNA gene profiles of the CF soil (Supplementary Table 1, (Tkacz et al. 2015; Turner et al. 2013) and the fact that it is a genus associated with the marine environment. *Methylophaga* is a key player in C1 metabolism in the marine environment (Neufeld *et al.*, 2008; Grob *et al.*, 2015) and several species have been isolated from seawater (Doronina et al. 2003; Doronina et al. 2003; Janvier et al. 1985). However, there have been studies that show that *Methylophaga* proliferate in the soil and produce plant hormones (Bal *et al.*, 2013; El Khalloufi *et al.*, 2016). Being detected in both the ^{13}C labelled DNA and RNA communities of the wheat root and the DNA community of the pea root would suggest that there are *Methylophaga* capable of growing and thriving within association with plants. However, the relative abundance of *Methylophaga* is much lower in the ^{13}C labelled heavy fraction of the RNA than the DNA. Based on the greater stability of DNA relative to RNA this could indicate that the *Methylophaga* was more active before the final days of pulsing of $^{13}CO_2$.

The wheat root cDNA profile (Table 6.8) also contains other methylotrophic genera, *Methylophilus* from the family *Methylophilaceae and* members of the *Comamonadaceae*. *Comamonas* represented the most abundant proposed methylotroph within the active exudate-utilising bacteria. *Methylobacterium* was also present within active methylotrophs of the wheat root community utilising plant carbon.

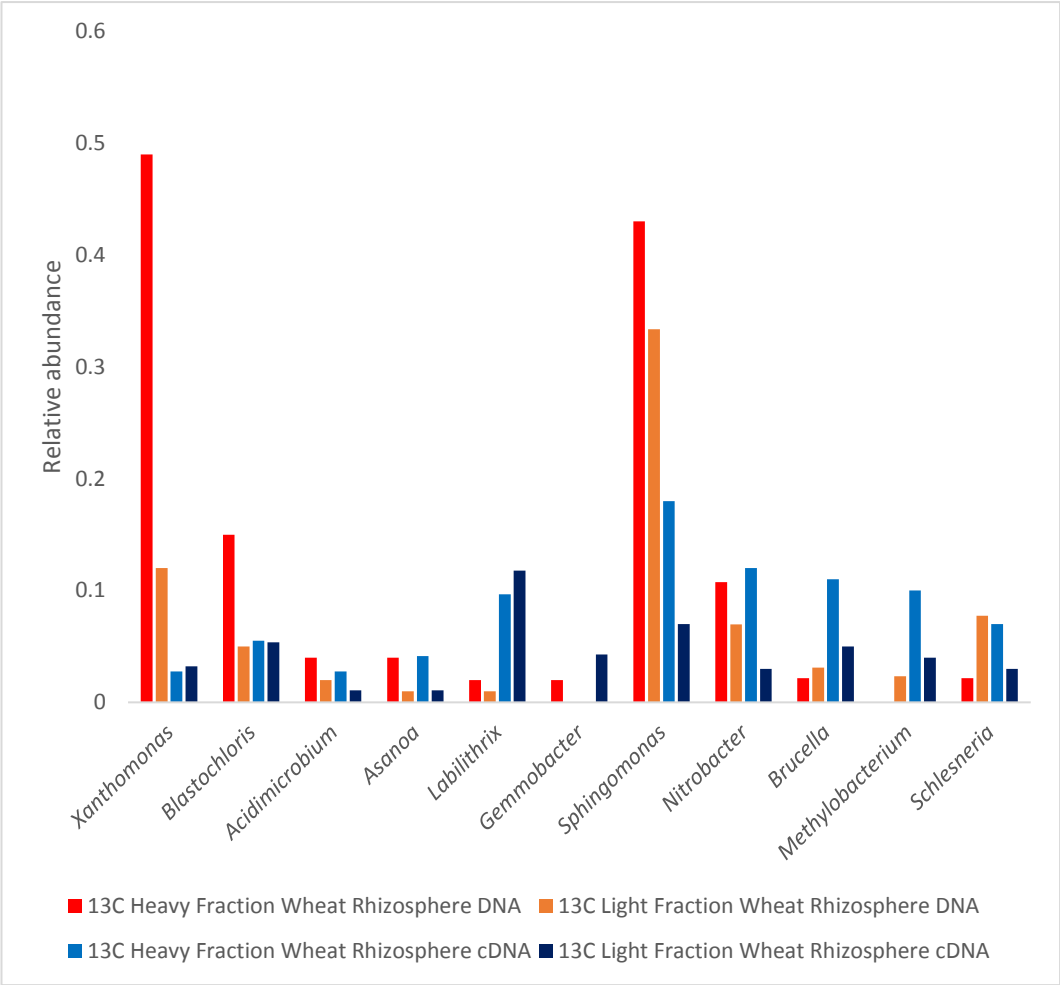


Figure 6.4 The relative abundance of the ten most abundant OTUs over-represented in the ^{13}C heavy fraction compared to ^{13}C light fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the DNA and cDNA from the rhizosphere of the $^{13}CO_2$ supplied wheat plants

Table 6.5 20 most abundant OTUs over-represented in the ^{13}H compared to ^{13}L fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the wheat rhizosphere DNA community

	Relative abundance in the 16S rRNA gene profile (%)				Relative abundance ^{13}H/^{13}L
	^{13}C Heavy fraction	^{13}C Light Fraction	^{12}C Heavy Fraction	^{12}C Light Fraction	
Xanthomonas	0.49	0.12	0.10	0.07	4
Blastochloris	0.15	0.05	0.058	0.03	3
Acidimicrobium	0.04	0.02	0.01	0.02	3
Asanoa	0.04	0.01	ND	ND	5
Labilithrix	0.02	0.01	ND	0.01	2
Gemmobacter	0.02	ND	ND	ND	NA
Simkania	0.02	0.01	ND	0.01	2
Desulfococcus	0.02	ND	ND	0.03	NA
Nostoc	0.02	0.01	ND	0.01	2
Corallococcus	0.02	0.01	ND	ND	2
Solibacillus	0.02	ND	ND	0.01	NA
Hippea	0.02	0.01	ND	0.01	2
Thermanaeromonas	0.02	ND	ND	0.01	NA
Erythrobacter	0.02	ND	ND	ND	NA
Thiodictyon	0.02	ND	ND	ND	NA
Nitrincola	0.02	ND	ND	ND	NA
Oscillochloris	0.02	ND	ND	ND	NA
Solimonas	0.02	ND	ND	0.01	NA
Ammoniphilus	0.02	ND	ND	0.03	NA
Algisphaera	0.02	0.01	ND	0.02	2

Table 6.6 20 most abundant OTUs over-represented in the ^{13}H compared to ^{13}L fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the wheat rhizosphere cDNA community

	Relative abundance in the 16S rRNA gene profile (%)				Relative abundance ^{13}H/^{13}L
	^{13}C Heavy fraction	^{13}C Light Fraction	^{12}C Heavy Fraction	^{12}C Light Fraction	
Sphingomonas	0.18	0.07	0.05	0.14	2
Nitrobacter	0.12	0.03	0.03	0.03	3
Brucella	0.11	0.05	0.03	0.02	2
Methylobacterium	0.10	0.04	0.03	0.03	2
Schlesneria	0.07	0.03	0.03	0.02	2
Agrobacterium	0.07	0.02	0.02	0.04	3
Nordella	0.06	0.01	0.01	0.04	5
Streptosporangium	0.04	ND	0.01	0.01	NA
Alloactinosynnema	0.04	0.01	ND	0.01	3
Candidatus Entotheonella	0.03	0.01	0.01	0.02	2
Thermomicrobium	0.03	0.01	ND	0.01	2
Paracraurococcus	0.03	ND	0.01	0.06	NA
Amphiplicatus	0.03	0.01	ND	0.01	2
Phaeospirillum	0.03	ND	ND	0.01	NA
Hyalangium	0.03	0.01	0.01	0.04	2
Alicyclobacillus	0.03	ND	ND	ND	NA
Thermanaerothrix	0.03	0.01	ND	0.01	2
Ferruginibacter	0.03	0.01	ND	0.01	2
Tepidamorphus	0.03	ND	ND	0.01	NA
Thermovum	0.03	ND	ND	ND	NA

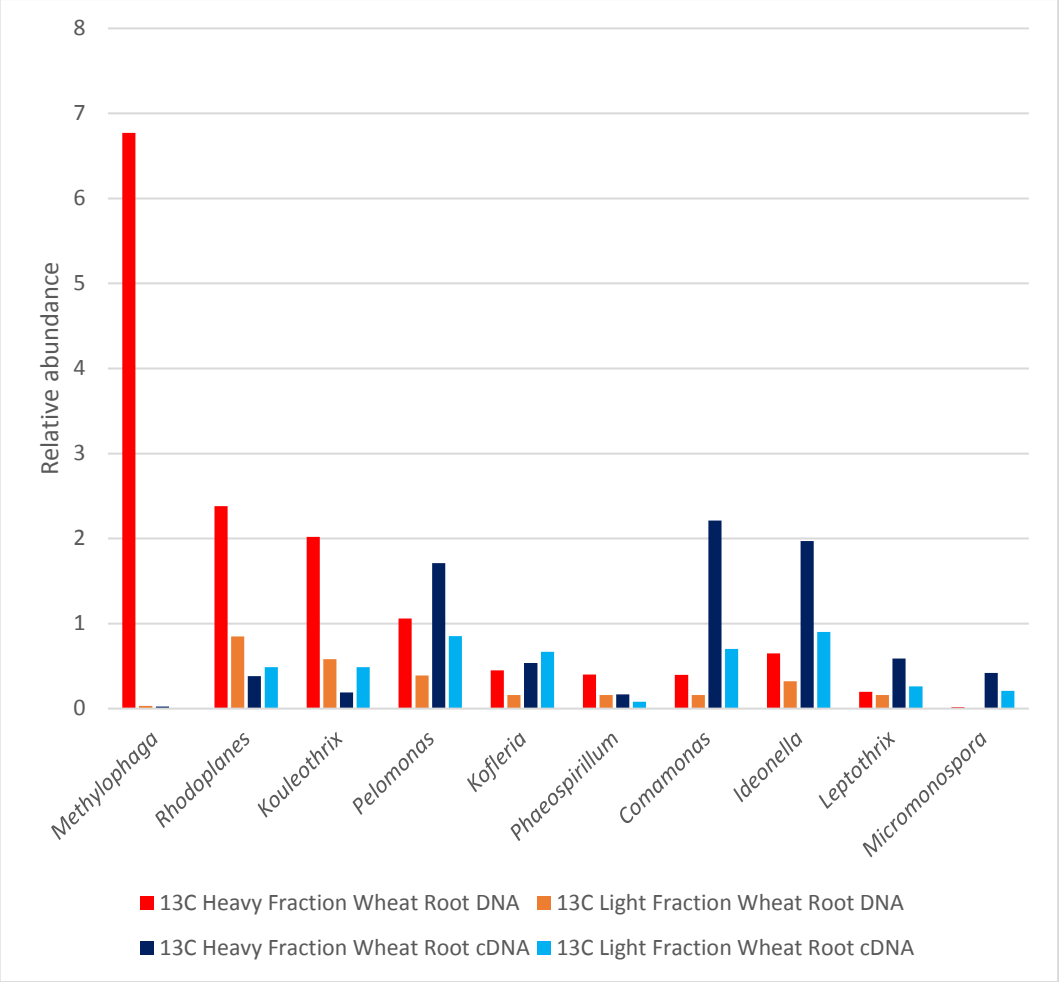


Figure 6.5 The relative abundance of the ten most abundant OTUs over-represented in the ^{13}C heavy fraction compared to ^{13}C light fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the DNA and cDNA from the root of the $^{13}CO_2$ supplied wheat plants

Table 6.7 20 most abundant OTUs over-represented in the ^{13}H compared to ^{13}L fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the wheat root DNA community

	Relative abundance in the 16S rRNA gene profile (%)				Relative abundance ^{13}H/^{13}L
	^{13}C Heavy fraction	^{13}C Light Fraction	^{12}C Heavy Fraction	^{12}C Light Fraction	
Methylophaga	6.77	0.03	ND	0.02	210
Rhodoplanes	2.38	0.85	1.06	0.68	2
Kouleothrix	2.02	0.58	0.44	0.53	3
Pelomonas	1.06	0.39	0.40	0.37	2
Kofleria	0.45	0.16	0.22	0.19	2
Phaeospirillum	0.40	0.16	0.13	0.11	2
Microlunatus	0.23	0.11	0.04	0.09	2
Acinetobacter	0.22	ND	ND	ND	NA
Herbaspirillum	0.20	0.05	0.09	0.05	3
Labilithrix	0.18	0.02	ND	0.02	8
Roseateles	0.11	0.03	ND	0.01	3
Beijerinckia	0.11	0.05	0.04	0.03	2
Rheinheimera	0.11	ND	ND	ND	NA
Dokdonella	0.11	0.02	ND	0.01	5
Rubellimicrobium	0.11	0.05	ND	0.03	2
Aquamicrobium	0.11	0.04	ND	0.02	2
Rickettsiella	0.07	ND	ND	0.04	NA
Alsobacter	0.07	0.03	ND	ND	2
Dichotomicrobium	0.07	0.03	ND	ND	2
Salinibacterium	0.05	0.01	ND	ND	5

Table 6.8 20 most abundant OTUs over-represented in the ^{13}H compared to ^{13}L fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the wheat root cDNA community

	Relative abundance in the 16S rRNA gene profile				Relative abundance ^{13}H/^{13}L
	^{13}C Heavy fraction	^{13}C Light Fraction	^{12}C Heavy Fraction	^{12}C Light Fraction	
Comamonas	2.21	0.70	0.19	0.3	3
Ideonella	1.97	0.90	0.32	0.53	2
Pelomonas	1.71	0.85	0.32	0.49	2
Leptothrix	0.59	0.26	0.11	0.35	2
Micromonospora	0.42	0.21	0.12	0.16	2
Saccharothrix	0.36	0.11	0.05	0.03	3
Virgisporangium	0.29	0.09	0.09	0.09	3
Microbacterium	0.22	0.09	0.11	0.18	2
Herpetosiphon	0.19	0.09	0.02	0.06	2
Acinetobacter	0.13	ND	ND	ND	NA
Methylophilus	0.12	0.05	0.02	0.09	2
Curtobacterium	0.09	0.04	ND	ND	2
Paracoccus	0.09	0.01	ND	ND	9
Dyella	0.08	ND	0.01	0.03	NA
Rubrivivax	0.08	ND	ND	0.01	NA
Methylobacterium	0.06	0.01	ND	0.02	6
Verminephrobacter	0.06	0.03	0.01	0.02	2
Woodsholea	0.04	0.02	0.01	0.01	2
Blastochloris	0.04	0.02	0.01	0.02	2
Methylophaga	0.02	ND	ND	ND	NA
Dokdonella	0.02	0.01	ND	0.01	2

Within the exudate utilisers in the pea rhizosphere (Summarised in Figure 6.6) are the genera *Polaromonas*, *Dokdonella* and *Methyloceanibacter* (Table 6.9). The number of methylotrophic genera classified as labelled in the active exudate-utilising portion of the cDNA increased in the pea rhizosphere relative to the wheat rhizosphere but represented a lower relative abundance of the total community (Table 6.10). Amongst this diversity were the genera *Comamonas* and *Polaromonas* (*Comamomadaceae*) and the diazotrophic genus *Oharaiebacter*. Also ^{13}C labelled is the genus *Sphingomonas*, that was shown to be present in the exudate utilisers of the pea rhizosphere in the previous DNA SIP experiment (Section 6.3).

Pseudomonas was the most abundant genus in the ^{13}C labelled pea root DNA-derived community. *Pseudomonas* has a broad metabolic diversity, with the genus containing species known to be commensal, pathogenic or beneficial to the host plant, in addition to producing siderophores, plant hormones and antifungal compounds (Lugtenberg et al. 2001 and references therein). *Pseudomonas* previously contained a high number of methylotrophic bacteria, but the majority of these were transferred to alternate genera (Pacheco *et al.*, 2003). A minority of methylotrophs remains within the genus *Pseudomonas*, but the genome sequenced species do not possess the *mxaF* or *xoxF* methanol dehydrogenases. These species have been shown to possess an alcohol dehydrogenase that is lanthanide dependent and has low levels of activity towards methanol (Wehrmann *et al.*, 2017). Several studies characterising the rhizosphere and root communities identified *Pseudomonas* as present, including in the rhizosphere of *Arabidopsis* and pea plants (Bulgarelli et al. 2012; Lundberg et al. 2012; Turner et al. 2013). Furthermore, the formate dehydrogenase of *Pseudomonas* has been shown to be upregulated upon exposure of *Pseudomonas* strains to plant exudates (Mark *et al.*, 2005).

The exudate utilisers within the pea root community (Summarised in Figure 6.7 and Table 6.11) include *Methylophaga*, indicating the labelled carbon compounds being utilised by this genus are similar between the roots communities given the limited metabolisms characterised within this genus (Grob *et al.*, 2015). The remaining methylotrophs were *Methyloceanibacter, Meganema*, *Solibacter* and *Azohydromonas*. *Meganema* and *Solibacter* represent only putative methylotrophic genera, but

Azohydromonas was confirmed to contain methylotrophs in this work (Chapter 3). *Azohydromonas* is also represented within the active exudate utilisers of the cDNA profile, representing a genus capable of both methylotrophy and nitrogen fixation (Xie *et al.*, 2005). Also within the cDNA profile (Table 6.12) are the genera *Leptothrix* and M*ethylocapsa.*

There were more methylotrophic genera in the root environments of both wheat and pea plants than in the rhizosphere environments. It is interesting to observe members of the *Methylophilaceae*, *Methylobacterium* and the *Comamomadaceae* as exudate utilisers here as well as active methanol utilisers in the methanol SIP experiment (Chapter 5).

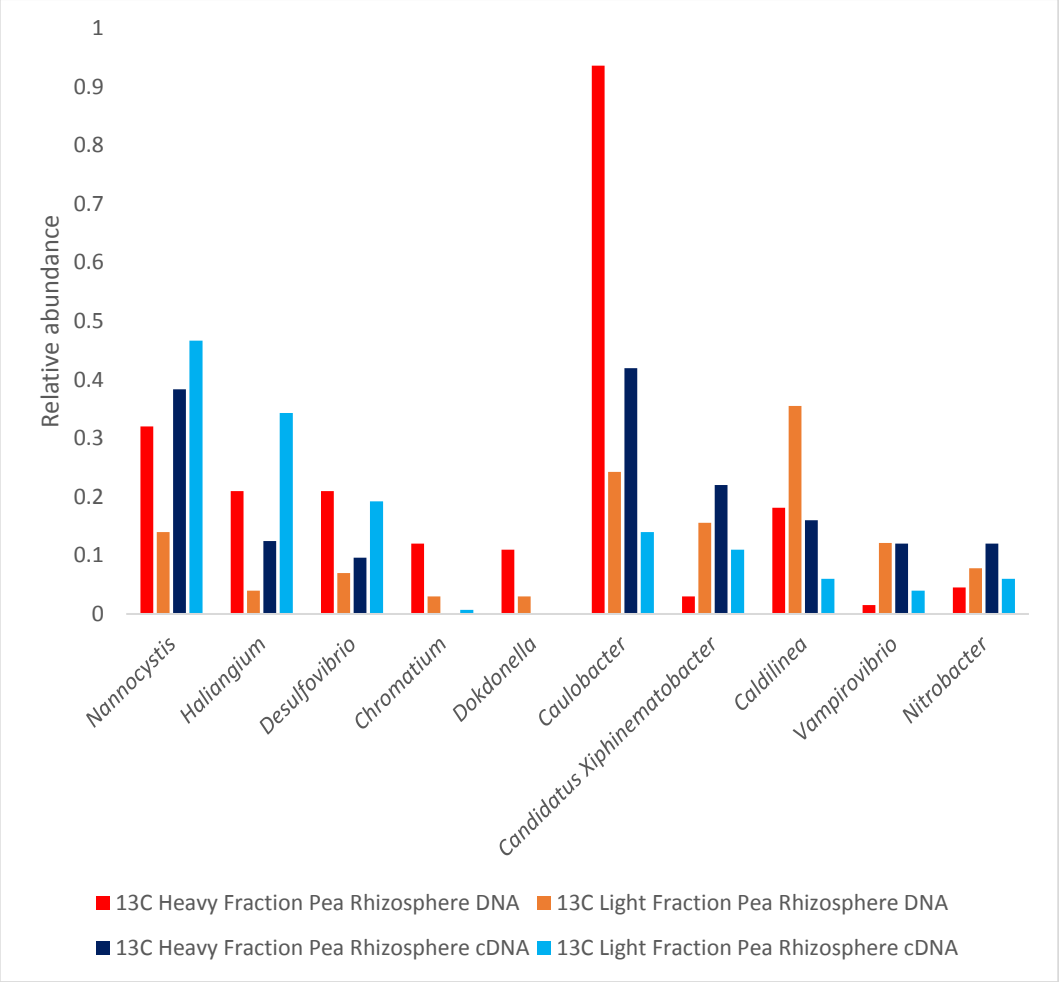


Figure 6.6 The relative abundance of the ten most abundant OTUs over-represented in the ^{13}C heavy fraction compared to ^{13}C light fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the DNA and cDNA from the rhizosphere of the $^{13}CO_2$ supplied pea plants

Table 6.9 20 most abundant OTUs over-represented in the ^{13}H compared to ^{13}L fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the pea rhizosphere DNA community

	Relative abundance in the 16S rRNA gene profile (%)				Relative abundance ^{13}H/^{13}L
	^{13}C Heavy fraction	^{13}C Light Fraction	^{12}C Heavy Fraction	^{12}C Light Fraction	
Nannocystis	0.32	0.14	0.16	0.31	2
Haliangium	0.21	0.04	0.10	0.13	4
Desulfovibrio	0.21	0.07	0.06	0.09	3
Chromatium	0.12	0.03	0.03	0.05	3
Dokdonella	0.11	0.03	0.04	0.05	4
Thermodesulfobacterium	0.09	0.04	0.01	0.05	2
Methyloceanibacter	0.09	0.02	0.03	0.03	5
Salicola	0.06	0.01	0.01	0.06	6
Thermomicrobium	0.06	0.03	0.01	0.09	2
Sphingobium	0.06	0.01	0.03	0.03	6
Cystobacter	0.05	ND	0.01	0.02	NA
Acidimicrobium	0.05	0.01	0.01	0.01	5
Frigoribacterium	0.05	0.01	0.01	0.02	5
Sterolibacterium	0.05	0.02	ND	ND	2
Actinomyces	0.03	0.01	ND	0.02	3
Geobacillus	0.03	0.01	ND	ND	3
Algisphaera	0.03	0.01	0.01	0.03	3
Candidatus Kuenenia	0.03	ND	ND	0.01	NA
Amycolatopsis	0.02	ND	ND	0.01	NA
Phaselicystis	0.02	ND	ND	0.02	NA

Table 6.10 20 most abundant OTUs over-represented in the ^{13}H compared to ^{13}L fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the pea rhizosphere cDNA community

	Relative abundance in the 16S rRNA gene profile (%)				Relative abundance ^{13}H/^{13}L
	^{13}C Heavy fraction	^{13}C Light Fraction	^{12}C Heavy Fraction	^{12}C Light Fraction	
Caulobacter	0.42	0.14	0.13	0.12	2
Candidatus Xiphinematobacter	0.22	0.11	0.07	0.15	2
Caldilinea	0.16	0.06	0.08	0.05	2
Vampirovibrio	0.12	0.04	0.03	0.03	3
Nitrobacter	0.12	0.06	0.04	0.05	2
Actinophytocola	0.09	0.03	0.01	0.03	2
Actinospica	0.08	0.01	0.01	0.02	5
Elusimicrobium	0.07	0.01	0.01	0.01	4
Rufibacter	0.07	0.01	ND	ND	9
Novosphingobium	0.05	0.02	0.01	0.05	2
Carnobacterium	0.04	ND	ND	0.02	NA
Pedobacter	0.04	0.01	0.01	0.02	2
Polaromonas	0.03	ND	ND	ND	NA
Alloactinosynnema	0.03	0.01	ND	ND	4
Dethiobacter	0.03	0.01	0.01	0.01	2
Comamonas	0.02	0.01	ND	0.02	2
Simkania	0.02	ND	ND	ND	NA
Lacibacterium	0.02	ND	ND	ND	NA
Sulfitobacter	0.02	0.01	ND	0.02	2
Natronocella	0.02	0.01	ND	0.01	2

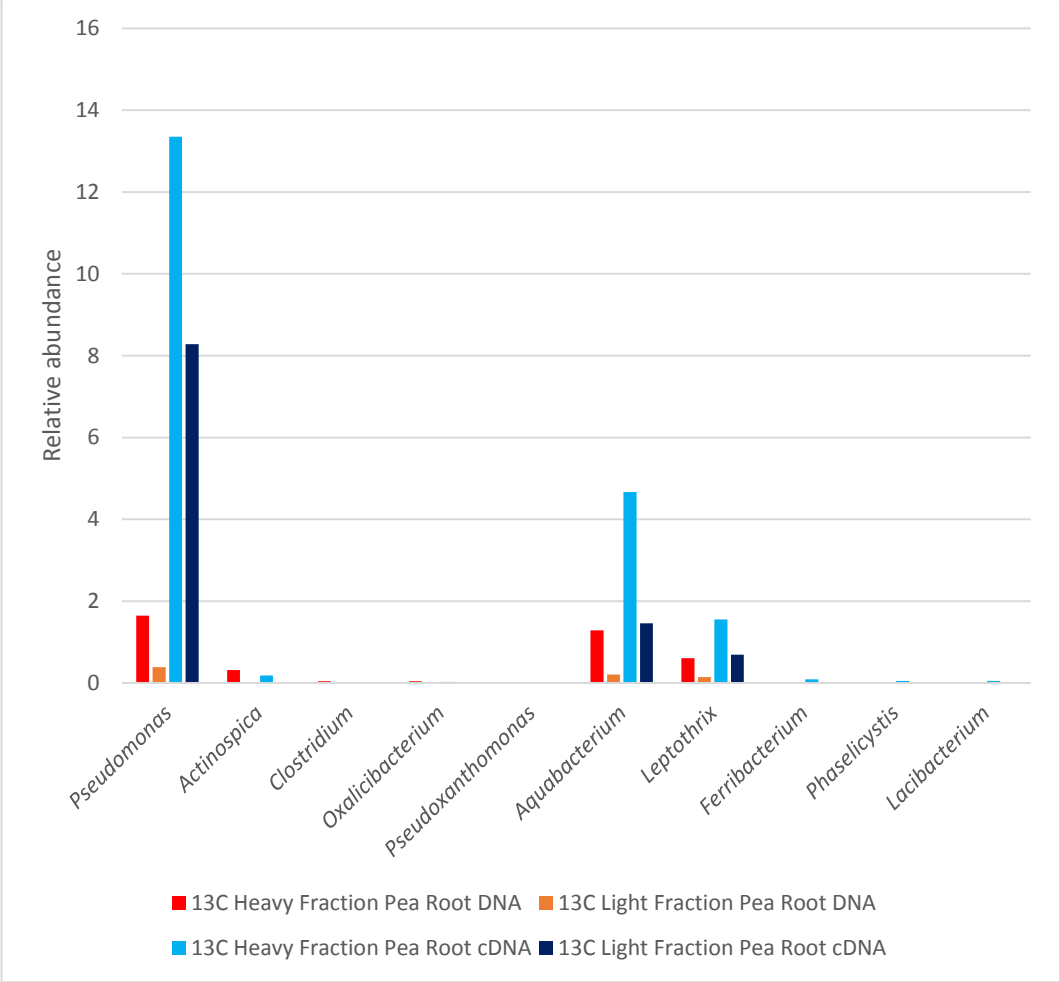


Figure 6.7 The relative abundance of the ten most abundant OTUs over-represented in the ^{13}C heavy fraction compared to ^{13}C light fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the DNA and cDNA from the roots of the $^{13}CO_2$ supplied pea plants

Table 6.11 20 most abundant OTUs over-represented in the ^{13}H compared to ^{13}L fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the pea root DNA community

	Relative abundance in the 16S rRNA gene profile (%)				Relative abundance ^{13}H/^{13}L
	^{13}C Heavy fraction	^{13}C Light Fraction	^{12}C Heavy Fraction	^{12}C Light Fraction	
Pseudomonas	1.65	0.39	0.54	0.96	4
Actinospica	0.32	0.01	ND	ND	26
Clostridium	0.04	0.01	ND	0.02	6
Oxalicibacterium	0.04	0.01	0.02	0.01	6
Pseudoxanthomonas	0.02	0.01	ND	ND	4
Ferrovum	0.02	0.01	ND	0.01	4
Arthrobacter	0.02	0.01	ND	0.02	2
Luteibacter	0.02	0.01	ND	ND	4
Marmoricola	0.02	0.01	ND	0.01	2
Daeguia	0.02	ND	ND	0.01	NA
Phaselicystis	0.02	ND	ND	ND	NA
Geothrix	0.02	ND	ND	ND	NA
Acidimicrobium	0.02	0.01	ND	ND	2
Alsobacter	0.02	ND	ND	ND	NA
Azohydromonas	0.02	ND	ND	ND	NA
Catellatospora	0.02	0.01	ND	0.02	2
Sinorhizobium	0.02	0.01	ND	ND	2
Candidatus Xiphinematobacter	0.01	ND	ND	ND	NA
Simkania	0.01	ND	ND	ND	NA
Parastreptomyces	0.01	ND	ND	0.01	NA

Table 6.12 20 most abundant OTUs over-represented in the ^{13}H compared to ^{13}L fraction based on their relative abundance in the 16S rRNA gene profile of the heavy fraction and light fraction of the pea root cDNA community

	Relative abundance in the 16S rRNA gene profile (%)				Relative abundance ^{13}H/^{13}L
	^{13}C Heavy fraction	^{13}C Light Fraction	^{12}C Heavy Fraction	^{12}C Light Fraction	
Aquabacterium	4.67	1.46	1.02	1.48	3
Leptothrix	1.55	0.69	0.33	0.63	2
Ferribacterium	0.09	0.01	ND	0.01	13
Phaselicystis	0.05	0.01	ND	0.01	7
Lacibacterium	0.05	0.01	0.01	0.01	3
Alcaligenes	0.05	0.01	ND	0.02	3
Azohydromonas	0.02	ND	0.01	0.01	NA
Cyanobacterium	0.02	0.01	0.01	0.01	3
Oxalicibacterium	0.02	0.01	ND	ND	3
Edaphobacter	0.02	ND	0.01	0.01	NA
Enterobacter	0.02	ND	ND	0.01	NA
Cellulomonas	0.02	ND	ND	0.01	NA
Pseudoxanthomonas	0.01	ND	ND	0.01	NA
Methylocapsa	0.01	ND	ND	ND	NA
Porphyrobacter	0.01	ND	ND	0.01	NA
Nitrobacter	0.01	ND	ND	0.01	NA
Thermodesulfobacterium	0.01	ND	ND	ND	NA
Geopsychrobacter	0.01	ND	ND	0.01	NA
Nocardia	0.01	ND	ND	0.01	NA

6.4.2 ^{13}C labelling of additional bacteria within the plant associated environments

The specific ^{13}C labelled genera tended to vary between test groups, as was seen with the methylotrophic genera. This includes plant associated heterotrophs and Actinobacteria, including several members of the Actinomycetes (Table 6.5-12). ^{13}C labelling of the heterotrophs *Myxococcaceae* occurred to a greater extent in the wheat and pea test groups in this experiment compared to the 350 ppmv and elevated test groups of the previous experiment.

Labelling of the bacterial nematode symbiont *Xiphinematobacter* (Vandekerckhove *et al.*, 2002) suggests that not only the bacterial community was labelled with ^{13}C through the utilisation of the exudates released from the plants, as the labelling of this genus would indicate that nematodes were also utilising ^{13}C labelled exudate from the plants. However, as this experiment focused on the characterisation of methylotrophs within the rhizosphere environment, the enrichment of eukaryotes would only be relevant to the species of eukaryotic methylotrophs shown to be capable of the utilisation of methanol, that previous work has shown to not be a major group in the CF soil or greatly affected by the growth of pea and wheat plants (Turner et al. 2013; Tkacz et al. 2015).

Cyanobacteria were also ^{13}C labelled in the rhizospheres of both the pea and wheat plants. This labelling was ruled out as being a result of the labelling of autotrophs within the soil through the ^{13}C labelling with the pulsed CO_2, due to the low levels of observed ^{13}C labelling in the unplanted soil test groups. The presence of Cyanobacteria in the rhizosphere has previously been observed (Prasanna *et al.*, 2009; Ahmed *et al.*, 2014). Cyanobacteria have been shown to be capable of nitrogen fixation and to benefit plant growth through inoculation experiments (Radha *et al.*, 2009; Prasanna *et al.*, 2013).

6.5 Discussion

6.5.1 350 ppmv and 1000 ppmv supplied rhizosphere SIP experiment

The differences in the rhizosphere communities of the 350 ppmv and 1000 ppmv pulsed test groups are not unexpected, as the supply of an elevated concentration of carbon dioxide to a growing plant has been shown to impact on the rhizosphere community (Drigo *et al.*, 2010, 2013). The shift in the rhizosphere community could be the result of higher levels of carbon being available to the plant (Bazzaz, 1990; Cheng *et al.*, 1998). Furthermore, the level of ^{13}C in the carbon pool of the plant will have been higher due

to higher uptake rates of carbon dioxide at 1000 ppmv relative to the 350 ppmv concentration of the CO_2. This is potentially the reason for the higher percentage of the heavy fraction qualifying as labelled in the 1000 ppmv supplied test group. However, given the presence of genera detected as ^{13}C labelled in the 350 ppmv test group that were absent in the elevated test group, this would indicate that the exudation profile and recruitment of bacteria from the soil changed. A 350 ppmv concentration of carbon dioxide was therefore used in the next rhizosphere SIP experiment to produce the least artificial labelled community. In addition to the general bacterial community shifting, the specific members of functional groups within the rhizosphere were also different, with recruitment of different methylotrophs in the exudate utilising portion of the rhizosphere community. This specific impact could potentially also be the result of enhanced growth of the pea plant due to the higher concentration of carbon dioxide resulting in higher amounts of restructuring of the plant cell walls resulting in enhanced methanol formation (Stulen *et al.*, 1993; Galbally *et al.*, 2002).

6.5.2 Comparison of the two rhizosphere SIP experiments

There is a stark difference in the relative abundance of the community that is classified as exudate utilising between the rhizosphere SIP experiments. The relative abundance of the community that is labelled in the wheat root and pea root are more comparable, with a much higher percentage of the community ^{13}C labelled. The low percentage of the second rhizosphere SIP experiment communities that is identified as exudate utilising could be a result of the additional supply of carbon to the rhizosphere occupying organisms through the increased breakdown of SOM. This would result in a supply of carbon that is not ^{13}C labelled, thereby reducing the percentage of labile carbon in the soil that is ^{13}C labelled. Members of the same genus that are utilising carbon from the plant exudates in addition to SOM would result in the presence of their 16S rRNA gene sequences in both the heavy and light fractions of the rhizosphere and reduce the levels of enrichment with ^{13}C (Haichar *et al.*, 2008). Alternatively, it is possible that high levels of cross-feeding occurred, resulting in a more even distribution of the ^{13}C label, causing lower levels of ^{13}C labelling detected in the primary utilisers. It was also proposed that a high level of community complexity results in higher levels of contamination of the ^{13}C heavy fraction with ^{12}C labelled DNA that would reduce the levels of labelling in the heavy fraction (Rangel-Castro *et al.*, 2005). As the plants used in these experiments

varied in age and growth stage the types of compound and the volume of carbon exudates released will have shifted (Houlden *et al.*, 2008; Haichar *et al.*, 2012). It is also possible that cellulolytic bacteria and other slow growing bacteria were not labelled due to utilising plant material that is more recalcitrant or tissues that are not as rapidly replaced and therefore remained ^{12}C labelled (Prosser *et al.*, 2006; Neufeld *et al*., 2007a).

6.5.3 Comparison of the RNA and DNA rhizosphere SIP experiments

It is interesting to note that although there are some ^{13}C labelled groups present in the DNA and cDNA profiles of the environments, there are also differences between these profiles. Given the differences in timespan required for successful labelling of the different nucleic acids it is not unexpected for there to be differences between these two profiles. The carbon compounds released by the plant into the soil will alter over the growth of the plant (Houlden *et al.*, 2008; Haichar *et al.*, 2012). The community present in the ^{13}C profile of the DNA may be divergent from the labelled RNA community, as the labelled DNA community results from cumulative exudate-uptake from the start of the experiment, whereas the labelled RNA represents a snapshot of the active community currently utilising carbon from the plant. Therefore genera identified in the DNA ^{13}C labelled community that are absent or less abundant in the RNA ^{13}C labelled community would be expected to be less active in the rhizosphere and utilising less plant derived carbon. Bacteria being more abundant in the RNA ^{13}C labelled community, e.g. *Aquabacterium* in the pea rhizosphere, would indicate these are genera that are more recently utilising ^{13}C labelled exudates and have not replicated sufficiently for incorporation into their DNA.

6.5.4 Methylotrophs identified as exudate utilisers

Methylotrophs were ^{13}C labelled in the exudate utilising rhizosphere and root communities of both plant species. Interestingly, some of the active methanol utilisers identified in the methanol SIP experiment were also identified as exudate utilisers in these environments. This is in spite of the artificially high concentration of methanol used in the methanol SIP experiment, implying that these genera are capable of utilizing methanol at a wide range of concentrations. Exudate-utilizing members of specific functional groups were not universally present across test groups, e.g. the

methylotrophic genus *Hyphomicrobium*. The reason for this differential enrichment of methylotrophic bacteria indicates that that there is selection for different genera in response to exudates released by the different species of plant. In this study, several facultative methylotrophs were ^{13}C labelled, implying that these bacteria could be metabolising methanol in addition to other carbon compounds released from the roots. However, plants have also been shown to impact other factors in the soil, such as the availability of micronutrients, soil structure and the pH and redox potential of the soil (Haichar et al. 2008; Turner et al. 2013; Philippot et al. 2009), that could also play a role in the selection of methylotrophic genera between the plant species.

6.5.5 Diversity of non-methylotrophs identified in the exudate utilising portion of the rhizosphere community

Members of the *Sphingomonadaceae* and *Comamonadaceae* were consistently detected in the CF soil and plant associated environments throughout this experiment and in previous studies (Hernández et al. 2015; Bulgarelli et al. 2012). Members of the *Commamonadaceae* have been shown to enhance the cycling of sulphur in soil (Schmalenberger *et al.*, 2007). Also within the exudate utilisers are Deltaproteobacteria that can be bacteriovorous (Lueders *et al.*, 2006; Sood *et al.*, 2015) and could therefore be labelled due to cross-feeding from the primary exudate utilisers. However, it is not possible to infer the metabolism of this phylogenetic group within this environment and it is also possible they were directly utilising carbon directly from the plant. ^{13}C labelling of the *Myxococcaceae* may be beneficial for the host plant due to suppression of fungal or bacterial pathogens within the rhizosphere soil, with some members of the *Myxococcaceae* used as biocontrol agents to support the growth of plants (Garcia *et al.*, 2009; Sood *et al.*, 2015). However, it is also possible that the group is labelled by their predation of other exudate-labelled microbial groups within the rhizosphere.

Cyanobacteria were also amongst the ^{13}C labelled bacteria within the exudate-utilising portion of the plant associated community and can enhance plant growth (Prasanna *et al.*, 2013). Some ^{13}C labelled genera are plant and human pathogens. The former is not unexpected, as strains of bacteria that are pathogenic for plants will seek to exploit the resources of the plant (Schreiner *et al.*, 2010; Berendsen *et al.*, 2012). The presence of genera with species shown to be pathogenic for humans within the exudate utilisers

indicates that either there is uncultivated diversity within these phylogenetic groups, or that the pathogens were able to survive in the soil (Berg *et al.*, 2013).

Additionally ^{13}C labelled were Actinobacteria, including the *Actinomycetes*, proposed to have a role as plant growth promoting bacteria through suppression of plant pathogens (Butler *et al.*, 2005; Badji *et al.*, 2006) in addition to the production of plant hormones and siderophores (Tokala *et al.*, 2002; Khamna *et al.*, 2009; van der Meij *et al.*, 2017). Further research could entail the sterilisation of the rhizoplane of the plant prior to extraction of DNA and RNA in order to assess the presence and diversity of endophytic bacteria that are capable of producing antimicrobial and antifungal compounds. Several Actinobacteria, including members of the Actinomycetes, produce antimicrobial or antifungal agents and have been shown to be rhizosphere associated or endophytic (van der Meij et al. 2017; and references therein). The labelling of this phylogenetic group has been shown to occur in previous $^{13}CO_2$ rhizosphere SIP studies characterising the exudate utilising bacteria in the rhizospheres of oil seed rape, wheat, maize and *Medicago truncatula* (Haichar *et al.*, 2008; Ai *et al.*, 2015). Plant associated soils and the roots of plants have been proposed to be an important site for the acquisition of novel antibiotic producing bacteria, due to the close association that the Actinomycetes have previously been shown to form with plants (Seipke et al. 2012; van der Meij et al. 2017; and references therein). The identification of members of this group within the exudate utilisers of the pea rhizosphere supports claims that they are enriched in the rhizosphere of different plant species, and are not restricted to the rhizospheres of cereals where this has previously been shown to occur (Bernard *et al.*, 2007; Haichar *et al.*, 2008; Li *et al.*, 2014)

6.5.6 Identification of the exudate utilising bacteria through stable isotope probing

To summarise, through pulsing $^{13}CO_2$ at the 350 ppmv concentration of carbon dioxide it is possible to label the exudate utilising portion of the rhizosphere and root community of pea and wheat plants. Genera that contain species capable of methylotrophy were identified as present within the exudate utilising community of the pea and wheat rhizospheres. However, it is not possible to assign active metabolism to the ^{13}C labelled genera as traits that are found within particular species of a genus cannot be presumed to be ubiquitous to every member of the genus, and the presence of a trait does not

confirm its activity, only that the metabolic potential is present. Therefore, although these experiments identified methylotrophic genera as utilising carbon directly from the wheat and pea plants there is a need for further characterisation as to why different genera are recruited by the pea and wheat plants. Further identification of activity could be gained by additional analysis through proteomics and metabolomics.

Chapter 7: Discussion

7.1 Isolation of and characterisation of novel methylotrophs

Chapter 3 described isolation attempts from CF soil and other environments. Isolates included a strain of *Azohydromonas*, a genus not known to grow on methanol, and a strain of *Oharaeibacter*, whose *xoxF1* sequence was fundamental to the expansion of the *xoxF* sequence database. Also isolated during this work were strains of *Variovorax* and *Methylobacterium* that were identified as exudate utilising and methanol utilising respectively in the SIP experiments (Chapter 5 and 6). The genome sequence of *Variovorax paradoxus* MM1 provides further insight into the metabolic capabilities of this versatile species. Two other isolates from the CF soil represent novel species within the family *Methylophilaceae*. The genomes of these two isolates were screened and their physiological capabilities assessed, revealing both *Methylovorus methylotrophus* MM2 and *Methylobacillus denitrificans* MM3 as highly divergent from members of their respective genera. *Methylobacillus denitrificans* MM3 was confirmed to be the first denitrifying species of *Methylobacillus*.

There is a need for more expansive enrichment regimes, as cultivation dependent work enhances our ability to perform further cultivation independent assessments of methylotrophic diversity in the environment. There are innumerable alterations to enrichment strategies that could enhance the diversity of methylotrophs isolated. These approaches could include the supply of substrates in addition to methanol, such as dimethylsulfide. Members of the *Methylophilaceae* have been linked to the oxidation of DMS in the environment and therefore this enrichment strategy could enrich for methylotrophs capable of utilising both methanol and DMS (Eyice *et al.*, 2015a). Additionally, further enrichments could be performed with variable oxygen concentrations, that has been shown to impact on the relative competitive ability of members of the *Methylophilaceae* (Hernandez *et al.*, 2015). Enrichments performed under anaerobic or microaerophilic conditions could also enrich for denitrifying methylotrophs, as occurred with the isolation of *Methylobacillus denitrificans* MM3 using media with 0.2 % agar.

There are studies that show variation between the impacts of the specific lanthanides on the growth of methylotrophs. This has been shown in *Methylomicrobium buryatense*, with copper partially attenuating the suppression of *mxaF* gene expression in the presence of cerium but not in the presence of lanthanum (Chu and Lidstrom, 2016). The lanthanides with an atomic mass greater than that of neodymium have also been shown to have a variable impact on the growth of methylotrophs, with the growth of *Methylacidiphilum fumarilolicum* SolV and an *mxaF* mutant of *Methylobacterium extorquens* on methanol enabled by the supply of samarium, but with a lower growth rate than with lanthanum, cerium, neodymium and praseodymium (Pol *et al.*, 2013; Vu *et al.*, 2016). It may be worthwhile performing enrichments using members of the lanthanide series in combination and individually to further investigate whether this has an impact on the specific methylotrophs that are isolated. Conversely, given the absence of a lanthanide requirement for several of the methanol dehydrogenases (NAD(P) dependent, Mdh2 and MxaF), further enrichments performed without the additional supplementation of lanthanides may assist in selection for methylotrophs that possess the less characterised methanol dehydrogenases. Selection for the gram positive methylotrophs could be enhanced through the use of a selective medium or a heat treatment, that Gram positive bacteria have been shown to be more resistant to (Jay 1986).

7.2 Assessment of the diversity of methanol dehydrogenase genes in the CF soil

Chapter 4 detailed the characterisation of methylotrophs present in the CF soil by sequencing PQQ methanol dehydrogenase encoding genes. Sequencing of the *xoxF* genes captured a greater diversity of methylotrophs within the CF soil than sequencing of the *mxaF* gene. It also enabled detection of a shift in community between the CF soil and rhizosphere soils. Sequencing of the *xoxF* genes also made apparent the need for previously characterised *xoxF* containing organisms to be re-tested for their ability to grow on methanol. The dependence of the methanol oxidising activity of the XoxF enzyme was previously unknown that means that species would have been tested for the ability to grow on methanol in the absence of lanthanides. This may have resulted in species being classified as non-methylotrophic. This retesting of the ability to oxidise methanol when supplied with REEs would enhance the identification of functional

methanol dehydrogenase genes in the environment and potentially expand the phylogenetic diversity where methanol oxidation is confirmed to occur. Clarification of the additional proposed roles of *xoxF,* enhancing the rate of denitrification and improving stress tolerances (Mustakhimov *et al.*, 2013; Firsova *et al.*, 2015), in both methylotrophic and non-methylotrophic organisms would also be valuable. The experimental approach through which these additional proposed functions could be assessed would entail the culturing of the wild type and *xoxF* deletion mutants under stressful (e.g. high temperature or high salinity) or anaerobic conditions and "standard" conditions (e.g. aerobic and non-stressful). Transcriptomic and proteomic analysis of these organisms under these conditions could reveal the transcriptomic changes underpinning the change in phenotype observed in these mutants. This may then enable the identification of the specific genes that the XoxF methanol dehydrogenase is interacting with.

The *mdh2* profile in two environments was dominated by one single phylotype. However, in spite of this low diversity it is still useful to be able to assess the diversity of *mdh2* in environmental samples given its confirmed function as a methanol dehydrogenase (Kalyuzhnaya et al. 2008). It would be interesting to screen further environments to establish whether there is a type of environment in which *mdh2* is more often detected, e.g. marine or terrestrial, and whether there are certain environments in which there are a greater level of diversity of this gene present. It is also interesting to observe that *mdh2* genes are still only found in the two genera they were identified in nearly ten years ago in spite of the expansion of the number of available genomes.

Attempts to characterise methylotrophs, both in this work and previous research, have focused on the gram negative methylotrophs that possess PQQ methanol dehydrogenases (Anthony 1983; Chistoserdova 2011a; Taubert et al. 2015; Keltjens et al. 2014 and references therein). This is overlooking some of the diversity of methylotrophs within the natural environment. This includes the unknown enzyme system for methanol oxidation in the methylotrophic species of *Sphingomonas* (Boden *et al.*, 2008), the NAD-dependent methanol dehydrogenase possessed by a species of *Cuprivavidus* and the methanol dehydrogenase genes that are encoded by gram positive

bacteria e.g. *Bacillus* and *Mycobacterium* (Vries et al. 1992; Arfman et al. 1992; Kato et al. 1988). The design of PCR primers to amplify these additional methanol dehydrogenase encoding genes would be a step towards expanding our ability to describe methylotrophs as a functional group.

7.3 Enrichment of methylotrophs from CF and rhizosphere soils

Chapter 5 detailed a DNA-SIP experiment that identified the active methylotrophs in CF soil and pea and wheat rhizosphere soils through the supply of ^{13}C labelled methanol. This revealed a greater diversity of members of the *Methylophilaceae* and the presence of the genus *Methylobacterium* in the plant associated soils. Differences in the labelled community were identified through 16S rRNA gene DGGE profiling, 16S rRNA gene sequencing and metagenome sequencing. This difference in profile shows the value of using multiple approaches when performing a DNA-SIP experiment in order to most effectively identify the ^{13}C labelled community. The metagenomes produced from the ^{13}C labelled DNA from the methanol SIP were binned into genomes. Some of the binned genomes were identified as *Methylobacterium*, *Methylophilaceae* and *Comamonadaceae*. These three phylogenetic groups were also detected as exudate utilisers in the rhizosphere SIP and representatives of these phylogenetic groups were also isolated from the CF soil (Chapter 3 and 6). Furthermore, *Methylobacterium* and *Comamonadaceae* were identified in the *xoxF* profile of the CF soil and pea and wheat rhizosphere soils (Chapter 5). The presence of genera that were ^{13}C labelled and identified as putative cross feeders, e.g. *Desulfocapsa*, reinforces the issues of SIP experiments that are performed for a long time or with a high concentration of labelled substrate. An RNA-SIP experiment using an environmentally relevant concentration of methanol was performed but this did not achieve sufficient labelling. This experiment could be repeated with a more sensitive assay for methanol, such as PTR-MS, to enable measurement of the depletion of this substrate at an environmentally relevant concentration. The ability to measure the methanol at this concentration would allow for the substrate to be supplied until sufficient labelling is achieved without concerns over an increase in the concentration of methanol. A setup that supplies the methanol at a constant concentration, as used in Lueders et al. 2004, could be used to ensure the concentration did not exceed a certain level. Several time points would need to be

harvested across this experiment to enable analysis of the methylotrophic community that is ^{13}C labelled with the shortest time.

An enrichment was performed with CF soil with the supplementation of lanthanides in addition to methanol. No differences were detected in the rate of methanol oxidation or in the methylotrophic communities between the lanthanide supplied test groups and the non-supplemented test group. There are multiple possibilities for why this occurred, and these could be elucidated through the measurement of lanthanides by ICP-MS (Ramos *et al.*, 2016), to ascertain whether they are at a concentration that is limiting in the CF soil. Given the mechanism by which methylotrophs sense and acquire lanthanides is unknown it is difficult to identify the bioavailability of these compounds in the soil, but it might be worth repeating this enrichment with soils with a lower total concentration of lanthanides (Ramos *et al.*, 2016). Given the plant growth promoting impacts of lanthanides and the accumulation of lanthanides by plants (Oliveira et al. 2015; Hu et al. 2004 and references therein), an additional experiment would be to supplement the soil with lanthanides prior to plant growth and then use this soil in an enrichment similar to the methanol SIP in chapter 5. This experiment would assess if the presence of the plant combined with the enhanced lanthanide concentration impacts on the methylotrophic community. Given the presence of lanthanides in several fertilisers (Kanazawa *et al.*, 2006), and the purposeful addition of lanthanides to agricultural soils in China (Pang et al. 2001 and references therein), it would be worthwhile characterising this interaction. Further assessment of the differences between the plant associated soils and the CF soil could be achieved through the supply of ^{14}C methanol and the concentration of $^{14}CO_2$ produced used as a proxy for methanol oxidation (Stacheter *et al.*, 2013). V_{max} and K_m could be calculated from the oxidation of a range of methanol concentrations. The V_{max} and K_m would provide further information on how the presence of a plant impacts methylotrophs present in the Church Farm soil.

7.4 Identification of active exudate utilisers in the plant associated communities of pea and wheat plants

Chapter Six described rhizosphere SIP experiment that labelled the DNA and RNA of the exudate utilising bacteria in the rhizosphere communities of pea and wheat plants through the supply of $^{13}CO_2$. Within the exudate utilising community of the pea and

wheat plants were methylotrophic bacteria, including putative methylotrophs. The majority of those enriched were facultative methylotrophs, e.g. *Variovorax, Methylobacterium* and *Methylocapsa*, with few obligate methylotrophs, e.g *Methylobacillus*, enriched through exudate utilisation. Also in the labelled community were antibiotic producing Actinomycetes and heterotrophic bacteria, e.g. *Sphingomonas*, *Leptothrix, Pelomonas and Comamonas*, that possess plant growth promoting traits (Schmalenberger *et al.*, 2007; Videira *et al.*, 2009). Several members of the *Comamonadaceae* were identified in most test groups in both experiments and the *Sphingomonadaceae* were heavily enriched in the first rhizosphere SIP experiment. Labelling of bacteria was greater in the root relative to the rhizosphere, with the exudate utilising community of the wheat roots and pea roots dominated by *Methylophaga* and *Pseudomonas* respectively. Further work in clarifying the enrichment of methylotrophs in this environment would be to sequence the methanol dehydrogenase genes from the ^{13}C labelled DNA.

The exudate utilisers within the plant associated environments could also be further assessed through the production of metagenomes and metatranscriptomes from the ^{13}C labelled DNA and RNA. However, given the low yields of nucleic acids in the heavy fractions, this would necessitate multiple replicates or MDA to produce a sufficient yield (Neufeld *et al*., 2007a; Chen and Murrell, 2010; Grob *et al.*, 2015). Proteomics could be applied in combination with the sequenced metagenomes and metatranscriptomes to provide further information on the specific metabolic processes being performed by the exudate utilising bacteria. It would also be interesting to grow the plants using soil collected from different seasons to assess the impact of seasonality, as this has been shown to impact on the microbial community and respiration rate in soils (Cheng *et al.*, 1998; Smalla *et al.*, 2001; Leake *et al.*, 2006; Ai *et al.*, 2015).

Further experiments using this technique could be improved by supplying the $^{13}CO_2$ label in an agricultural setting as this would reduce the artificial nature of the experiment. This alteration would introduce further difficulties to the experimental design but would provide results with more applicability. Complications would develop from the lack of access to a gas chromatograph, necessitating the collection of gas samples at several time points across the course of a day for analysis at a later time point to calculate the

rate of CO_2 uptake by the plants. The experimental design would also require an additional test group that has the same level of rain protection as the ^{13}C and ^{12}C test groups as this might impact on the microbial community of the soil. However, with this additional test group the experimental set up used could be broadly the same as the rhizosphere SIP experiments in chapter 6. The applicability of the data could also be enhanced by performing the labelling with plants at different growth stages up until the harvesting of the plant. This would necessitate the design of a larger vessel to contain a larger plant.

Accurate quantification of the release of methanol from plant roots across the life cycle of a plant would provide valuable data. Measurements of methanol released from plant roots have been infrequent in the literature and have been performed using PTR-MS (Steeghs et al. 2004; Abanda-Nkpwatt et al. 2006; Tsumaru et al. 2015). These measurements have typically occurred under gnotobiotic conditions. The release of methanol from plant roots is worth further quantification as it has important implications for methylotrophs in the soil. Therefore, the release of methanol by a range of plant species could be quantified initially in gnotobiotic roots using PTR-MS, with further experiments including roots inoculated with methylotrophic and non-methylotrophic bacteria. It is possible that the amount of methanol will change under gnotobiotic conditions relative to colonised test groups as has been shown with other plant exudates (Turner, 2013).

7.5 Conclusion

Cultivation dependent and independent work performed during the course of this PhD has provided a further insight into the phylogenetic and metabolic diversity of methylotrophs and their relationship with plants. This includes the isolation of novel methylotrophs, including two novel species belonging to the *Methylophilaceae,* and the testing, design and application of primers for the amplification and quantification of PQQ methanol dehydrogenase gene clades. Active and exudate utilising methylotrophs were identified using two SIP-based approaches. A methanol SIP experiment identified members of the *Methylophilaceae* and *Comamonadaceae* as key methylotrophs within the CF soil, with *Methylobacterium* enriched in the plant associated soils. The rhizosphere SIP experiments confirmed some of these methylotrophic genera as present

in the CF soil utilise carbon directly from the plant in the rhizosphere of pea and wheat plants.

List of abbreviations

AMS	ammonium mineral salts
AAI	avergae amino acid identity
ANI	average nucleotide identity
Blast	Basic Local Alignment Search Tool
bp	base pairs
BSA	bovine serum albumin
CF	Church Farm
CTAB	cetyl trimethylammonium bromide
dAMS	dilute ammonium mineral salts
dANMS	dilute ammonium nitrate mineral salts
DDH	DNA-DNA hybridisation
ddH2O	Double distilled water
DEPC	diethyl pyrocarbonate
DGGE	denaturing gradient gel electrophoreiss
DMS	dimethylsulfide
DNA	deoxyribonucleic acid
dNMS	dilute nitrate mineral salts
DTT	dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FID	flame ionisation detector
FAD	flavin-adenine dinucleotide
FDH	formate dehydrogenase
FGH	S-formylglutathione hydrolase
GC	gas chromatography
Gfa	glutathione-formaldehyde activating enzyme
GMA	gammaglutamylmethylamide
GSH	glutathione
H_4F	tetrahydrofolate
H_4MPT	tetrahydromethanopterin
ml	millilitre
mM	millimolar
NADH/NAD^+	nicotinamide adenine dinucleotide
NADPH/$NADP^+$	nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
nD-TC	average density
ng	nanogram
NMG	N-methylglutamate
NMS	nitrate mineral salts
OD	optical density
OTUs	operational taxonomic unit

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PQQ	pyrroloquinoline quinone
pMMO	particulate methane monoxygenase
ppmv	parts per million by volume
RFLP	restriction fragment length polymorphism
RO	reverse osmosis
SOB	super optimal broth
SDS	sodium dodecyl sulphate
sMMO	soluble methane monoyxgenase
SOM	soil organic matter
TAE	tris acetate EDTA
TCA	tricarboxylic acid
TE	tris EDTA
TEMED	tetramethylethylenediamine
TMA	trimethylamine
T-RFLP	terminal restriction fragment length polymorphism
µg	microgram
µM	micromolar
UMS	urea mineral salts
UP	unplanted
v/v	volume to volume
w/v	weight to volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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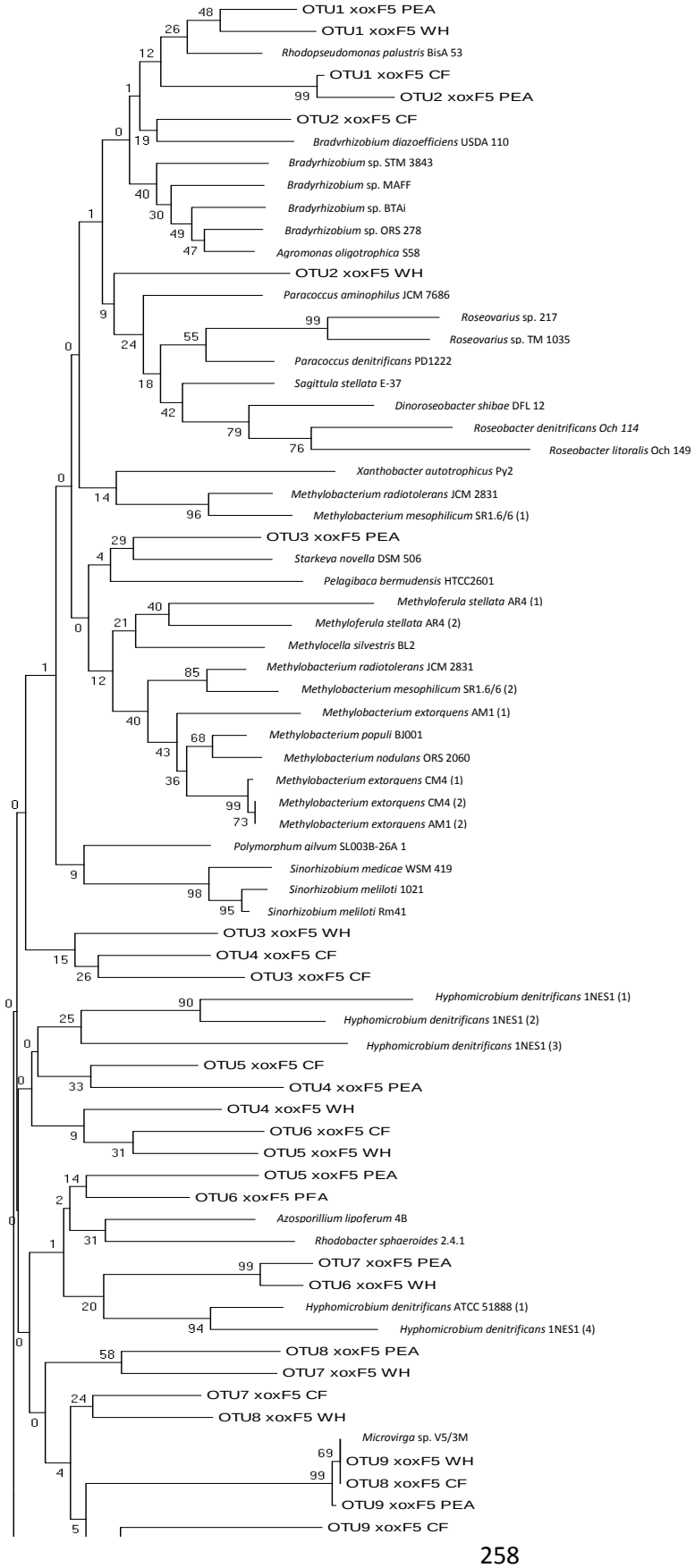
Appendices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Methylobacterium extorquens AM1 (1)
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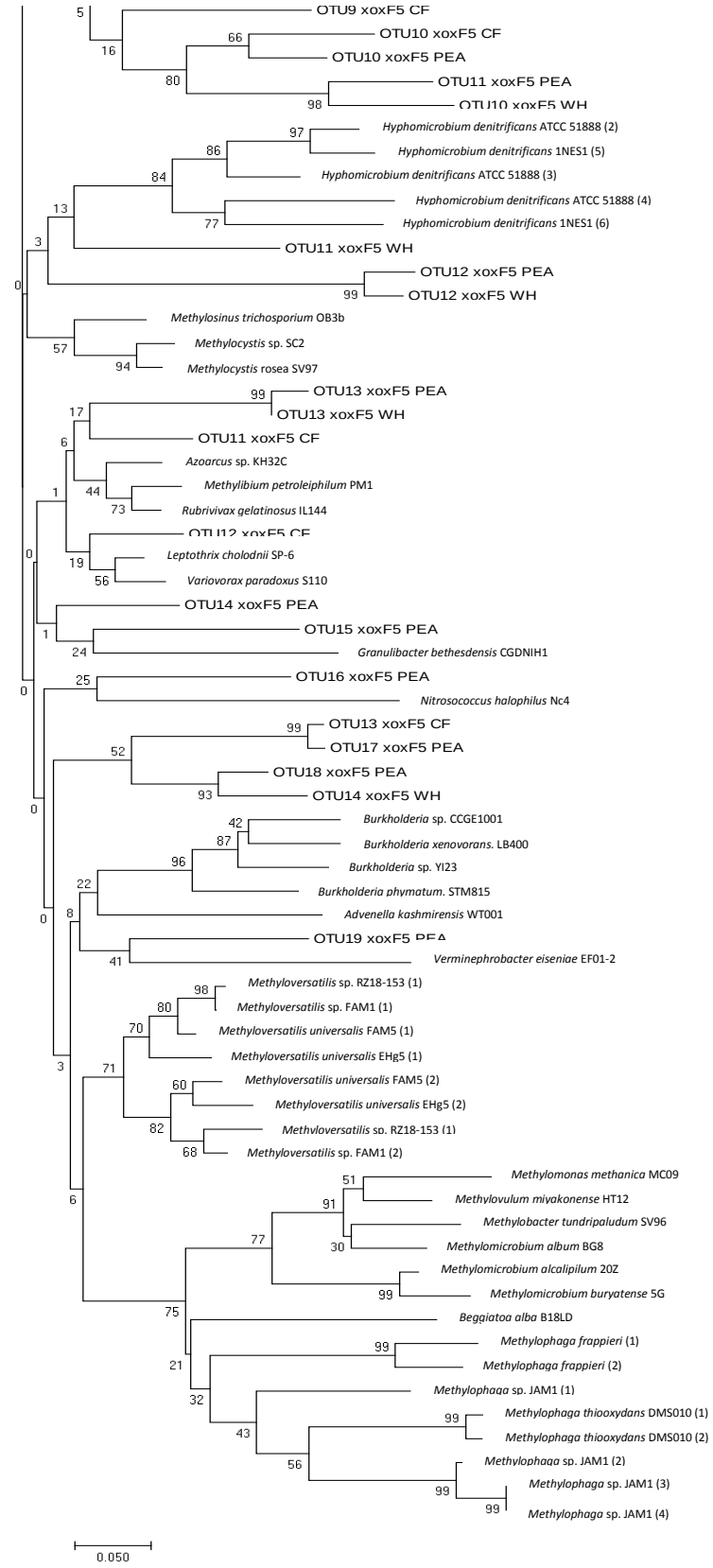
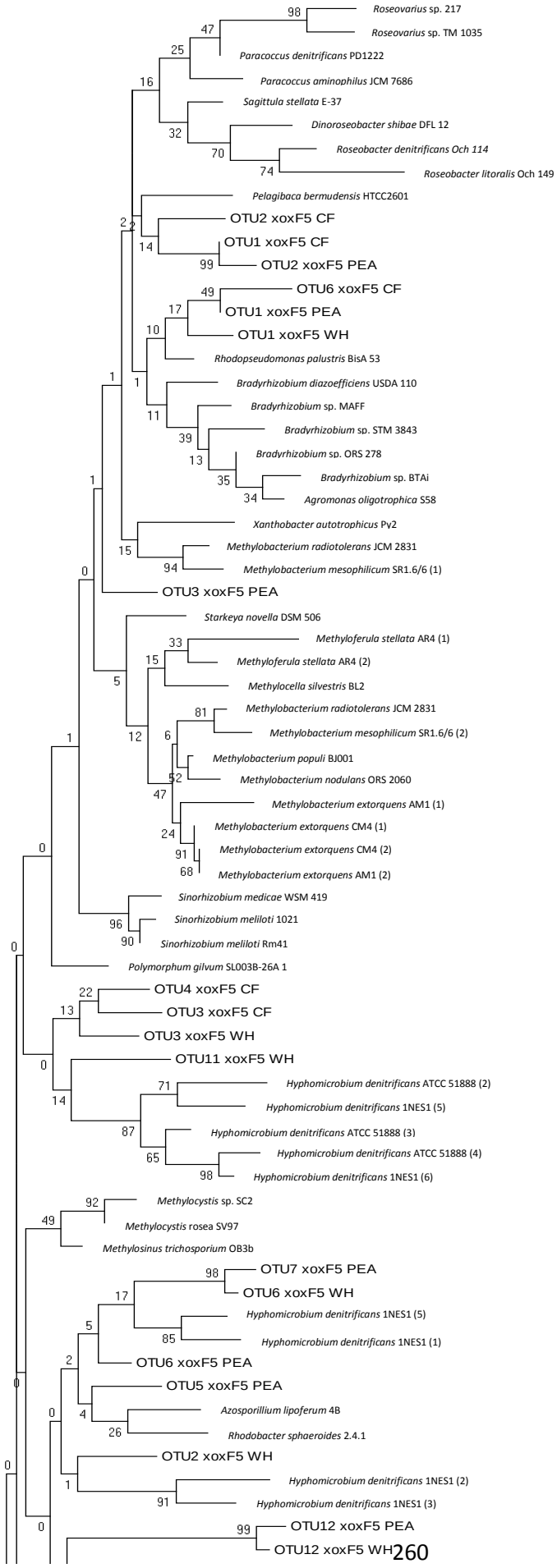


Figure A.1 Phylogenetic analysis of the *xoxF5* gene sequences amplified from DNA extracted from CF soil, pea rhizosphere soil and wheat rhizosphere soil. The phylogenetic tree was constructed from nucleotide sequences aligned at the deduced amino acid level. The evolutionary history was inferred using the maximum likelihood method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position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Roseobacter denitrificans Och 114
Roseobacter litoralis Och 149
Pelagibaca bermudensis HTCC2601
OTU2 xoxF5 CF
OTU1 xoxF5 CF
OTU2 xoxF5 PEA
OTU6 xoxF5 CF
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Rhodopseudomonas palustris BisA 53
Bradyrhizobium diazoefficiens USDA 110
Bradyrhizobium sp. MAFF
Bradyrhizobium sp. STM 3843
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Bradyrhizobium sp. BTAi
Agromonas oligotrophica S58
Xanthobacter autotrophicus Py2
Methylobacterium radiotolerans JCM 2831
Methylobacterium mesophilicum SR1.6/6 (1)
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Starkeya novella DSM 506
Methyloferula stellata AR4 (1)
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Methylocella silvestris BL2
Methylobacterium radiotolerans JCM 2831
Methylobacterium mesophilicum SR1.6/6 (2)
Methylobacterium populi BJ001
Methylobacterium nodulans ORS 2060
Methylobacterium extorquens AM1 (1)
Methylobacterium extorquens CM4 (1)
Methylobacterium extorquens CM4 (2)
Methylobacterium extorquens AM1 (2)
Sinorhizobium medicae WSM 419
Sinorhizobium meliloti 1021
Sinorhizobium meliloti Rm41
Polymorphum gilvum SL003B-26A 1
OTU4 xoxF5 CF
OTU3 xoxF5 CF
OTU3 xoxF5 WH
OTU11 xoxF5 WH
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Hyphomicrobium denitrificans 1NES1 (5)
Hyphomicrobium denitrificans ATCC 51888 (3)
Hyphomicrobium denitrificans ATCC 51888 (4)
Hyphomicrobium denitrificans 1NES1 (6)
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Methylosinus trichosporium OB3b
OTU7 xoxF5 PEA
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Hyphomicrobium denitrificans 1NES1 (1)
OTU6 xoxF5 PEA
OTU5 xoxF5 PEA
Azosporillium lipoferum 4B
Rhodobacter sphaeroides 2.4.1
OTU2 xoxF5 WH
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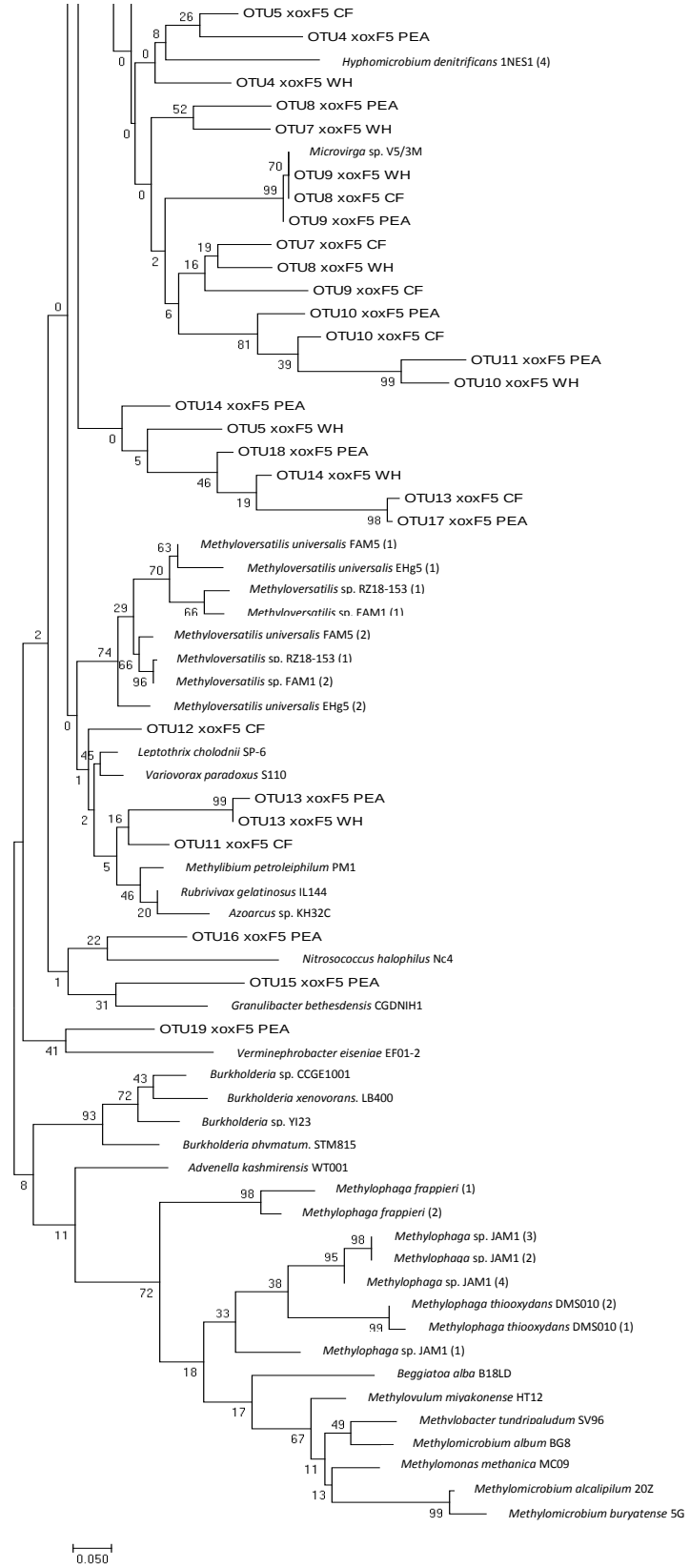

Figure A.2 Phylogenetic analysis of the *xoxF5* gene sequences amplified from DNA extracted from CF soil, pea rhizosphere soil and wheat rhizosphere soil. The phylogenetic tree was constructed from nucleotide sequences aligned at the deduced amino acid level. The evolutionary history was inferred using the maximum likelihood method with a bootstrap value of 500. The scale bar represents nucleotide substitution per position.

Supplementary table 1. 16S rRNA gene relative abundance of proposed and confirmed methylotrophic species in the CF soil, pea rhizosphere soil and wheat rhizosphere soil

Species	Proposed/ Confirmed	Church Farm Soil	Pea Rhizosphere	Wheat Rhizosphere
Acidovorax facilis	P	0.000	0.003	0.000
Acidovorax spp.	P	0.529	0.462	0.237
Ancylobacter sp.	C	0.008	0.009	0.010
Azospirillum brasilense	P	0.000	0.000	0.005
Azospirillum sp.	C	0.028	0.051	0.034
Azospirillum spp.	C	0.065	0.151	0.143
Beggiatoa spp.	C	0.187	0.217	0.250
Beijerinckia mobilis	C	0.003	0.003	0.003
Beijerinckia spp.	C	0.008	0.003	0.000
Bradyrhizobium canariense	P	0.011	0.027	0.021
Bradyrhizobium elkanii	P	0.263	0.290	0.344
Bradyrhizobium liaoningense	P	0.105	0.103	0.086
Bradyrhizobium sp.	P	0.074	0.085	0.065
Bradyrhizobium spp.	P	2.884	3.245	3.070
Burkholderia spp.	C	0.110	0.103	0.136
Cupriavidus necator	C	0.000	0.003	0.000
Flavobacterium aquatile	P	0.011	0.006	0.000
Flavobacterium columnare	P	0.107	0.027	0.018
Flavobacterium fluvii	P	0.011	0.006	0.000
Flavobacterium hauense	P	0.008	0.000	0.003
Flavobacterium sp.	P	0.834	0.118	0.120
Flavobacterium spp.	P	0.662	0.438	0.498

Flavobacterium succinicans	P	1.827	0.163	0.149
Flavobacterium swingsii	P	0.011	0.003	0.000
Flavobacterium tegetincola	P	0.008	0.000	0.000
Flavobacterium xanthum	P	0.023	0.003	0.008
Gemmatimonas spp.	P	0.469	0.420	0.425
Hyphomicrobium spp.	C	4.309	6.162	5.066
Hyphomicrobium sulfonivorans	C	0.003	0.009	0.000
Hyphomicrobium zavarzinii	C	0.017	0.048	0.031
Leptothrix sp.	P	0.037	0.048	0.057
Leptothrix spp.	P	0.037	0.024	0.016
Meganema perideroedes	P	0.003	0.006	0.000
Mesorhizobium loti	P	0.045	0.048	0.055
Methylibium petroleiphilum	C	0.028	0.015	0.013
Methylibium spp.	C	0.204	0.169	0.224
Methylobacillus flagellatus	C	0.008	0.000	0.000
Methylobacillus spp.	C	0.082	0.088	0.047
Methylobacterium adhaesivum	C	0.000	0.000	0.003
Methylobacterium aminovorans	C	0.054	0.072	0.034
Methylobacterium chloromethanicum	C	0.000	0.003	0.003
Methylobacterium extorquens	C	0.062	0.103	0.065

Methylobacterium hispanicum	C	0.000	0.003	0.005
Methylobacterium isbiliense	C	0.000	0.000	0.003
Methylobacterium jeotgali	C	0.006	0.006	0.013
Methylobacterium organophilum	C	0.006	0.009	0.005
Methylobacterium rhodinum	C	0.008	0.015	0.005
Methylobacterium sp.	C	0.023	0.024	0.021
Methylobacterium spp.	C	0.006	0.000	0.000
Methylobacterium suomiense	C	0.000	0.000	0.003
Methylobacterium zatmanii	C	0.003	0.009	0.008
Methylocapsa spp.	C	0.062	0.048	0.044
Methyloceanibacter caenitepidi	C	0.008	0.006	0.013
Methylocella spp.	C	0.076	0.088	0.068
Methylocystis spp.	C	0.023	0.012	0.026
Methyloligella solikamskensis	C	0.003	0.000	0.003
Methylophilus sp.	C	0.017	0.033	0.005
Methylophilus spp.	C	0.484	0.525	0.305
Methylosinus sp.	C	0.006	0.021	0.005
Methylosinus spp.	C	0.144	0.085	0.081
Methylotenera sp.	C	0.003	0.006	0.000
Methylotenera spp.	C	0.020	0.015	0.016
Methyloversatilis spp.	C	0.000	0.000	0.003
Oharaeibacter spp.	P	0.000	0.003	0.000

Pseudomonas sp.	C	0.011	0.003	0.010
Pseudomonas umsongensis	P	0.003	0.000	0.003
Rhodopseudomonas palustris	C	0.017	0.018	0.010
Rhodopseudomonas sp.	C	0.008	0.003	0.005
Rhodopseudomonas spp.	C	0.107	0.112	0.109
Roseomonas spp.	C	0.040	0.091	0.076
Sphingomonas sp.	C	0.025	0.072	0.031
Starkeya sp.	C	0.017	0.015	0.010
Subaequorebacter tamlense	P	0.003	0.000	0.003
Variovorax paradoxus	C	0.023	0.027	0.021
Variovorax sp.	C	0.059	0.148	0.122
Variovorax spp.	C	0.020	0.030	0.023
Verminephrobacter spp.	P	0.000	0.012	0.003
Verrucomicrobium spp.	C	0.715	1.259	1.730
Xanthobacter sp.	C	0.000	0.000	0.003
Xanthomonas albilineans	P	0.003	0.003	0.000