Characterisation of novel methylotrophs and the role of *xoxF* in coastal marine environments

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Abstract

Methanol is one of the most abundant volatile organic gases in the atmosphere, and whilst much is known about the sources of methanol, much less is known about the sinks. Methylotrophs are able to use one carbon compounds, such as methanol, as their sole source of carbon and energy. Seawater enrichments with methanol gave rise to the isolation of a novel species of the methylotroph *Methylophaga*. Some methylotrophs require a rare earth element (REE) when using the alternative methanol dehydrogenase (MDH) XoxF for growth on methanol. Addition of REEs to methanol seawater enrichments, using coastal waters from the south coast of the United Kingdom, showed REE stimulated methanol oxidation, whilst amplicon sequencing of the *xoxF5* gene revealed relative increases in unknown sequences. Isolation from enrichments containing lanthanum allowed the cultivation of a new member of the *Roseobacter* clade, strain La 6. A mutant in the only MDH gene in the genome and complementation and enzyme assays of this strain revealed the essential nature of *xoxF* during growth on methanol and ethanol. Genome sequencing revealed that stain La 6 has the largest genome of all *Roseobacters*, at 6.79 Mbp. This facultative methylotroph is metabolically very versatile, growing on some alkanes and aromatic compounds but it was also able to degrade and synthesise DMSP. Multilocus sequence analysis suggests that whilst it shares the core genes with subgroup 1 of the *Roseobacters*, it shares very little of its pangenome, suggesting unique genetic adaptations. Given this data, the new strain is proposed to be a new genus in the *Roseobacter* clade. Attempts to express different *xoxF* sequences in the *xoxF* mutant of La 6 revealed no phenotype, suggesting there may be as yet unidentified regulatory or accessory mechanisms involved during growth on methanol in this bacterium.

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Chapter 1

Introduction

1 Introduction

1.1 The importance of methylotrophy

Methylotrophs are organisms which are able to use reduced organic compounds with no carbon-carbon bonds, such as methane, methanol, or methylamine, as their sole source of carbon and energy (Anthony 1982; Chistoserdova 2011a). They are a very diverse group of organisms and are found in almost every environment. Reasons for the research into the genetics, metabolism and diversity of methylotrophs are twofold: to understand their role in the context of climate change and to develop novel systems for methanol-based biotechnology.

The ability of methylotrophs to use one-carbon compounds means that they are not only central to the global cycling of many atmospheric gases important in climate regulation, but also intrinsic to the cycling of carbon, sulfur and nitrogen (Kelly & Murrell 1999; Naqvi et al. 2005; Sharma et al. 2007; Trotsenko & Murrell 2008; Singh et al. 2010; Boden, Murrell, et al. 2011). Methanol is the second most abundant organic gas in the atmosphere, after methane, and is a biogeochemically active compound, significantly influencing the concentration of other atmospheric gases (Jacob et al. 2005; Heikes 2002). Although it is such an abundant gas, the few estimations of the global methanol budget are conflicting, partly due to the limited knowledge of the sources and sinks of methanol. The metabolism of methanol by methylotrophs is the only known biological sink of methanol. Therefore research into which microbes are involved in this process is necessary to determine the full impact of methylotrophs in the carbon cycle, and to be able to develop a more accurate account of the global methanol budget.

Moreover, the global methanol demand reached 87 billion litres in 2015, with thousands of products being synthesised from this (Methanol Institute, http://www.methanol.org/the-methanol-industry). Due to their methanol metabolising capabilities, in recent years methylotrophs have been genetically engineered to produce methanol-derived products such as amino acids, polyhydroxyalkanoates, single cell protein, insecticides, green fluorescent protein (GFP) and even human growth hormones (Ochsner et al. 2014 and references therein; Güneş et al. 2015). Therefore the isolation of new methylotrophs and the further study of their genetics, metabolism and diversity may contribute towards cheaper and more widely available products.

1.2 Aerobic methylotrophy

1.2.1 An overview of the history and phylogeny of methylotrophs

The first methylotroph, named *Bacillus methylicus*, was isolated in 1892 and was able to grow on methanol, formaldehyde and methylamine (and other multi-carbon compounds) (Loew 1892). Given the physiological description of this strain, it is highly likely that it was in fact the well studied model methylotroph *Methylobacterium extorquens*. Although some other methylotrophic bacteria were discovered after that, the next big discovery came from the isolation of the first methanotroph (organisms that grow on methane as sole carbon and energy source) in 1906 (Söhngen 1906). Methanotrophs oxidise methane to methanol using a methane monooxygenase, which can be either a membrane bound particulate monooxygenase (pMMO) or a soluble, cytoplasmic monooxygenase (sMMO) (Trotsenko & Murrell 2008; Murrell & Smith 2010). Since the isolation and characterisation of one hundred methanotrophs in 1970 by Whittenbury, research into methylotrophy has rapidly expanded (Whittenbury et al. 1970). To date, there are over 200 described species of methylotrophs belonging to the *Alpha-*, Beta-, and *Gammaproteobacteria, Verrucomicrobia, Cytophagales, Bacteroidetes, Firmicutes,* and *Actinobacteria* (Kolb & Stacheter 2013; Madhaiyan et al. 2010), although this number is likely much higher by now.

It has long become clear that methylotrophs are incredibly diverse in both the environments they inhabit and in their genetics (Chistoserdova et al. 2009; Anthony 1982). Whilst methanotrophs tend to be obligate, methanol utlisers are mostly facultative in nature, growing on a range of multi-carbon compounds as well as C1 compounds (Trotsenko & Murrell 2008; Kolb 2009). Through various isolation techniques and due to advances in meta-sequencing technologies, methylotrophs have been found in many environments. They are ubiquitous in the soil, on plants and in the oceans, but also in many more extreme environments such rice paddies, deserts, soda lakes, Antarctic soil, biological soil crusts, acidic volcanic mudpots and hydrothermal vents (Kolb 2009; Kolb & Stacheter 2013; Iguchi et al. 2015; Neufeld, Boden, Helene Moussard, et al. 2008; Sowell et al. 2011; Angel & Conrad 2009; Oyaizu-Masuchi &

Komagata 1988; Tambekar & Pawar 2013; Antony et al. 2012; Yergeau et al. 2009; Csotonyi et al. 2010; Pol et al. 2014; Duperron et al. 2007).

Research on methylotrophs has typically focused on a few specific species or genera, due to ease of cultivation in the laboratory, rapid growth and relatively simple systems for genetic manipulation (Chistoserdova et al. 2009). For example, *Methylomonas methanica, Methylosinus trichsporium*, and *Methylococcus capsulatus* have been intensively studied for decades as they each represent the three classic classes of obligate methanotrophs (type I, II and X, respectively). Type I methanotrophs are *Gammaproteobacteria* that contain bundles of disc-shaped vesicles as their internal cell membrane, whilst type II are *Alphaproteobacteria* and have paired peripheral membranes, both of which are used for assimilation of carbon (Trotsenko & Murrell 2008; Murrell et al. 2000). Class X methanotrophs are strains that have physiological properties of both Type I and II methanotrophs, but develop Type I intracytoplasmic membranes (Hanson & Hanson 1996). Moreover, type I methanotrophs contain the ribulose monophosphate (RuMP) pathway for formaldehyde, type II methanotrophs utilise the serine pathway, and type X methanotrophs can contain both. However, in the past few years, genome sequencing and advanced proteomics has shown these classifications to be often over simplistic (Chistoserdova 2011a).

Many methylotrophs are unable to grow on methane, but can grow on other C1 substrates such methanol or methylamine, much like the first isolated methylotroph *Bacillus methylicus,* now known as the model methylotroph *Methylobacterium extorquens*. This strain is an incredibly important facultative methylotroph, as *Methylobacterium* are consistently found to inhabit plants, soil, lake sediments, air and even humans (Green 2006; Anesti et al. 2004). Importantly, it is one of the most dominant groups found on plants, including the phyllosphere (Knief et al. 2008; Knief et al. 2010), rhizosphere (Omer et al. 2004; Egamberdieva et al. 2015) and endosphere (Lacava et al. 2004). Plants are responsible for releasing large amounts of methanol into the atmosphere due to the degradation of methyl ester groups by pectin methyl esterases (Fall & Benson 1996; MacDonald & Fall 1993; Finlay 2007). *Methylobacterium* sp. utilise much of the methanol released by the plants through the stomata, thereby reducing the overall amount of methanol emitted to the atmosphere (Abanda-Nkpwatt et al. 2006).

1.2.2 Metabolism of aerobic methylotrophs

The oxidation of methanol to formaldehyde is the first step in the metabolism of methanol for methylotrophs. In most methylotrophs, formaldehyde is the key intermediate that can either be further directed into the assimilatory pathways (the ribulose monophosphate (RuMP) pathway, serine pathway or via Calvin-Benson-Basham (CBB) cycle) or it can be dissimilated to carbon dioxide (CO_2) to generate reducing power and energy. Methylotrophs have various methods of metabolising the toxic formaldehyde, often with multiple methods in the same bacterium, and as such has been termed 'modular' in nature (Chistoserdova 2011a). Given the abundance of research and possible variations of these modules, it is beyond the scope of this thesis to discuss all scenarios, but most of them are depicted simply in Figure 1.1. However, a few of the most important and frequently used are described here briefly.

One important step is to bind the formaldehyde to tetrahydrofolate (H_4), forming the product methylene-H_4, or to tetrahydromethanopterin (H_4MPT), which forms the product methylene-H_4MPT. For bacteria that convert it to methylene-H_4, this can then be metabolised by the serine cycle for cell carbon, or oxidised to formate and further dissimilated (Chistoserdova 2011a and references therein). For bacteria using methylene-H_4MPT, this can then only be oxidised to formate. Other mechanisms employ an NAD-linked formaldehyde dehydrogenase (FaDH) to directly detoxify the formaldehyde straight to formate, whilst some bacteria such as *Paracoccus* and *Rhodobacter* employ a glutathione-dependent formaldehyde oxidation pathway that generates formate (not shown in Figure 1.1, but shown later in Figure 5.13) (Barber & Donohue 1998; Ras et al. 1995).

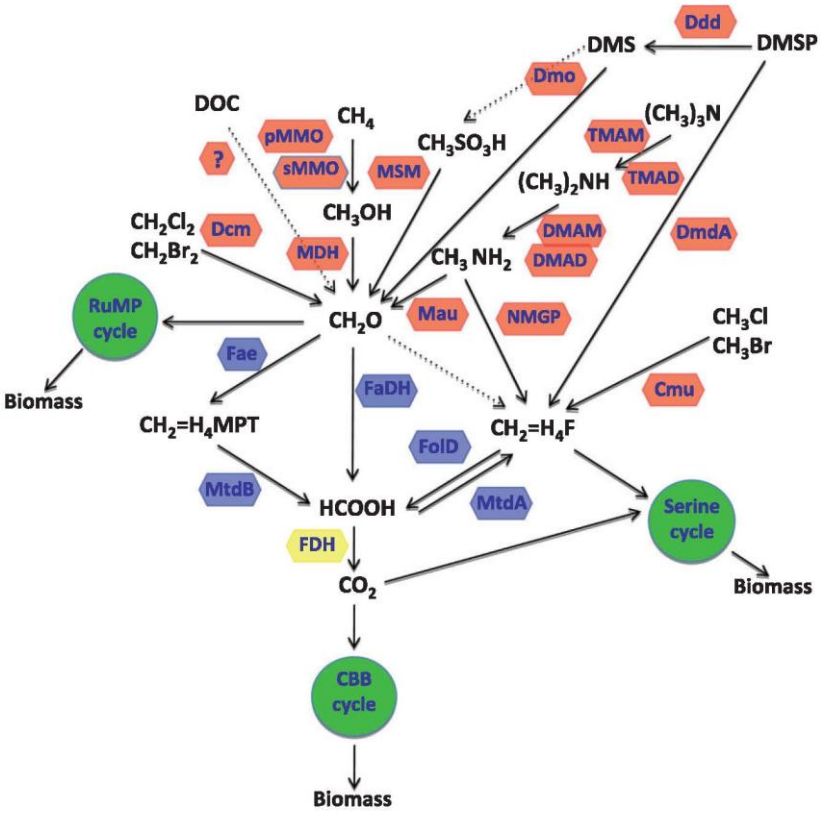

Figure 1.1 Simplified diagram showing the published methylotrophic modules involved in the degradation of different C1 compounds (taken from Chistoserdova (2011a)). Primary oxidation is shown in red, formaldehyde handling (methyl-H_4) modules are in blue, formate dehydrogenase is in yellow and carbon assimilation modules are shown in green. Dashed lines indicate non-enzymatic reactions, unknown, or both. Abbreviations (alphabetical order): Cmu, chloromethane methyltransferase; Dcm, dichloromethane dehalogenase; Ddd, DMSP lyase; DMAD, DMA dehydrogenase; DMAM, DMA monooxygenase; DmdA, DMSP demethylase, Dmo, DMS monooxygenase; FaDH, NAD-linked formaldehyde dehydrogenase; Fae, formaldehyde activating enzyme; FolD, bifunctional methylene-H_4F dehydrogenase– methenyl-H_4F cyclohydrolase; Mau, methylamine dehydrogenase; MDH, methanol dehydrogenase; MSM, methane sulfonate monooxygenase; MtdA, methylene-H_4F dehydrogenase; MtdB, methylene H_4 handling enzyme; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase; TMAD, TMA dehydrogenase; TMAM, TMA monooxygenase.

1.3 The oxidation of methanol to formaldehyde

1.3.1 Methanol dehydrogenases of methylotrophs

The catalysis of methanol to formaldehyde requires a methanol dehydrogenase (MDH). There are three main types of MDH found throughout methylotrophic organisms, the FAD-containing alcohol oxidase in yeasts, the NAD(P)+ dependent MDH in Gram positive bacteria, and a pyrroloquinoline quinone (PQQ)-dependant MDH in Gram negative organisms. *Pichia*, *Candida* and *Torulopsis* are the three genera of

methylotrophic yeasts, with *Pichia pastoris* being one of the most widely studied and utilised for heterologous expression of protein due to its rapid growth to high cell densities on methanol (van der Klei et al. 2006; Cereghino & Cregg 2000; Ahmad et al. 2014). This enzyme is an octameric acohol oxidase which contains one non-covalently linked FAD cofactor per subunit, is located in peroxisomes due to the toxic hydrogen peroxide (and formaldehyde) that is produced (Yurimoto et al. 2011; Sahm & Wagner 1973)

Gram positive bacteria utilise a cytoplasmic NAD(P)+ dependent MDH. The well studied methylotroph *Bacillus methanolicus* C1 contains a magnesium dependent, decemeric MDH which is a member of the type III alcohol dehydrogenase family (Müller et al. 2014). Other genera such as *Amycolatopsis methanolica*, *Mycobacterium gastri MB19*, *Rhodococcus rhodochrous* LMD 89.129 and *Rhodococcus erythropolis* DSM 1069 use N,N9-dimethyl-4-nitrosoaniline (DMNA)-dependent nicotinoprotein methanol:DMNA oxidoreductase (MDO for the oxidation of methanol (Van Ophem et al. 1993).

Gram negative methylotrophs almost always employ a periplasmic pyrroloquinoline quinone (PQQ)-dependent MDH (Anthony 1982; Duine et al. 1986). The first MDH discovered was in the model methylotroph *M. extorquens* by Anthony in 1964 (Anthony & Zatman 1964a; Anthony & Zatman 1964b), and has since been shown to be encoded in the genomes of most bacterial methylotrophs (Chistoserdova 2011a). Assays using purified enzyme shows that it has a broad substrate range, including primary alcohols, formaldehyde, ethanol and some aldehydes, to a lesser extent (Schmidt 2010 and references therein). The canonical MDH encoded by genes *mxaF* and *mxaI* is an $a_2\beta_2$ tetramer and has been known to be widespread in bacteria that grow on methanol for many decades. It was shown initially examined at the genetic level in *Methylobacterium extorquens* AM1 (Anderson et al. 1990). A homolog of *mxaF*, *mdh2*, is also known to encode an MDH in some organisms such as *Burkholderiaceae* and *Rhodocyclales* but is much less widespread in the environment (Kalyuzhnaya et al. 2008; Chistoserdova 2011a) .

1.3.2 The canonical methanol dehydrogenase, MxaFI

The PQQ-dependent MDH encoded by the genes *mxaFI* has now been researched for over fifty years. The soluble enzyme is located in the periplasm, along with an associated cytochrome c_L, encoded by *mxaG*, and a typical class I, cytochrome (c_H). The cytochrome c_L is a specific electron acceptor for MDH (Quilter & Jones 1984; Anthony 1986; Anthony 1992; Frank et al. 1993). The *mxaFI* and *mxaG* are all typically found together in a large gene cluster in organisms containing this system, along with other *mxa* accessory genes (Lidstrom et al. 1994; Amaratunga et al. 1997). Elsewhere in the genome are the seven genes, *pqqDGCBA* and *pqqEF* required for the 5-step PQQ biosynthesis (Morris et al. 1994).

At the centre of each large subunit (MxaF) contains one molecule of PQQ bound tightly but non-covalently at the centre of the protein, and one tightly bound divalent calcium ion, see Figure 1.2. This calcium ion is coordinated to both the PQQ and to different residues in the active site (Adachi et al. 1990; Blake et al. 1994; Richardson & Anthony 1992; White et al. 1993). The structure of the protein is very stable due to ion pair interactions between the large and small subunits. One of the distinctive characteristics of MDH proteins is the presence of the disulfide bridge between two cysteine residues Cys^{103}-Cys^{104}, of which the function is still not completely understood. However the PQQ is held very closely between these residues and a tryptophan residue inside the large subunit, which is folded into eight β-sheets arranged together in a propeller-like fashion (Ghosh et al. 1995; Anthony & Williams 2003; Williams et al. 2005).

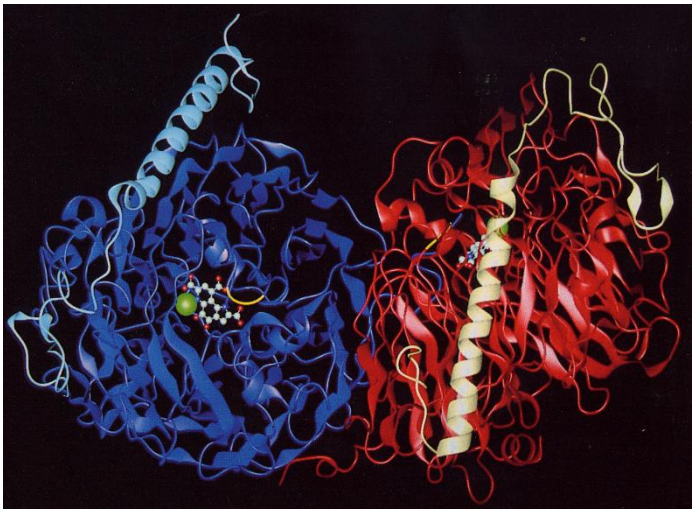

Figure 1.2 Crystal structure of methanol dehydrogenase, MxaFI, from *Methylobacterium extorquens*. Figure taken from Ghosh et al. (1995). The large, α subunits encoded by *mxaF* are seen in dark blue and red, whilst the smaller β subunits encoded by *mxaI* are seen in light blue and pale yellow. The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

The mechanism of the MDH proceeds as follows: the PQQ is reduced by methanol, formaldehyde is released (the product), and two electrons are transferred to the specific cytochrome $c_{L,}$ (and releasing two protons in the cytoplasm. These electrons are then passed to the typical cytochrome c_H, which are then transferred again onto an oxygen molecule at a terminal oxidase, combining with the two released protons at the cytoplasmic side of the membrane (Richardson & Anthony 1992; Toyama et al. 2003; Frank et al. 1993; Anthony 1986)

1.3.3 XoxF as a rare earth element-dependent methanol dehydrogenase

In the past 17 years, a whole new set of genes have been implicated in methanol metabolism. A protein with high similarity to the canonical MxaF subunit of *M. extorquens* was first discovered when studying proteins related to C1 metabolism (Chistoserdova & Lidstrom 1997). Sequencing of *M. extorquens* AM1 genome then later revealed that there were two more possible MDHs (other than MxaFI), termed *xoxF1* and *xoxF2*. XoxF1 of *M. extorquens* had a 50% amino acid similarity to MxaF, whilst XoxF1 and XoxF2 had 90% similarity to each other (Vuilleumier et al. 2009).

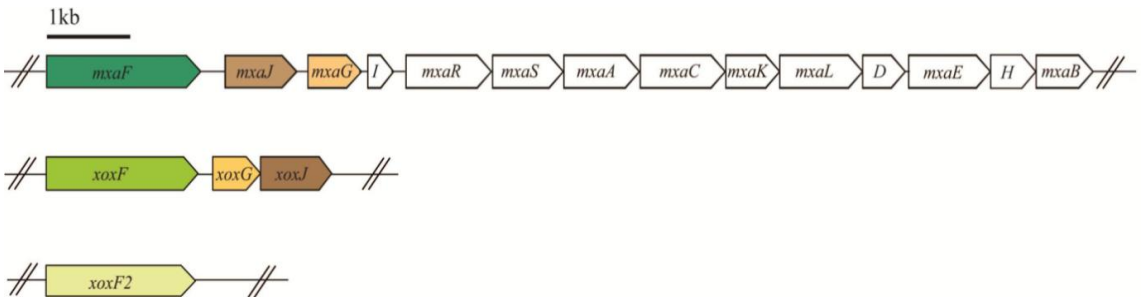

Figure 1.3 Comparison of the *mxa* and *xox* operons in *M. extorquens* AM1. Figure taken from Schmidt (2010). *MxaF* and *mxaI* encode for the large and small MDH subunits, respectively. *MxaG* encodes the associated cytochrome. *MxaJ* encodes for a periplasmic protein of unknown function. *MxaAKL* are required for insertion of calcium into the active site, *mxaD* is thought to be involved in stimulation of the interaction between MDH and the cytochrome cL, *mxaB* encodes a response regulator of *mxaF* transcription. It is unknown what the functions of the other *mxa* genes are. *xoxF1* and *xoxF2* are homologs of *mxaF*, whilst *xoxG* and *xoxJ* are homologs of *mxaG* and *mxaJ*, respectively.

The *xoxFJG* operon is present in all known *mxaFI*-containing methylotrophs to date (Chistoserdova 2011a), and is predicted to encode a methanol dehydrogenase, cyctochrome c and an unknown protein, respectively (see Figure 1.3). Although there are many more genes encoding accessory and regulatory proteins for the classic MDH, this module is very similar to the *mxaFJG* module which makes up part of the active MDH. However, although shown to be a functional MDH in many strains, many non-methylotrophs also have this *xoxFJG* module present in their genomes, such as *Rhizobiales* and *Burkholderiales* (Chistoserdova 2011a), and so there is still speculation as to the role of XoxF.

Both XoxF proteins of *M. extorquens* share many characteristics with the classic MDH of *M. extorquens* AM1, such as the conserved residues specific for PQQ and Ca^{2+} binding of MDH. Also like the classic MDH, XoxF proteins have a proposed active site base Asp^{303}, a $Cys^{103/104}$ disulphide bridge, a predicted signal peptide for periplasmic localisation and most have been found to be ammonium dependent (Schmidt et al. 2010; Anthony & Williams 2003; Nakagawa et al. 2012). These findings strongly supported the role of XoxF as an alternative MDH in *M extorquens* AM1. The native form of XoxF was under debate as some research suggested that the enzyme was a homodimer (Hibi et al. 2011; Nakagawa et al. 2012), whilst other research indicated a monomeric structure (Schmidt et al, 2010), unlike the classic MDH which is a heterotetramer ($\alpha_2\beta_2$).

Initial experiments done by Chistoserdova & Lidstrom (1997) suggested XoxF was not a MDH, since a *xoxF1* deletion mutant showed no growth deficiency with methanol as a sole source of carbon and energy. However Schmidt et al (2010) contradicted this by showing a 30% decrease in specific growth rate of the same mutant, and showed that it was less competitive than the wild-type strain during colonisation of the phyllosphere. They also showed a high affinity of purified XoxF1 for methanol (K_m = 11μM), although the specific activity was over 10 times lower (V_{max} = 0.015 U mg^{-1}) than that of MxaFI (V_{max} = 0.8 U mg^{-1}). Skovran et al. (2011) examined this further and made a *xoxF12* double mutant and showed that when both genes were absent, *M. extorquens* AM1 was unable to grow on methanol in liquid and solid media, and that this was due to the loss of gene expression from the *mxa* promoter. This loss of gene expression was due to a decrease in expression of the two-component regulatory systems *mxcQE* and *mxbDM* which regulates the *mxa* region. This led the authors to speculate that the role of XoxF was an environmental sensor which exerted its mechanism of action through the *mxcQE* and *mxbDM* systems. They also showed that the growth of a *xoxG* single and *xoxF2 xoxG* double deletion strain was similar to the wild type, suggesting that only the large subunit, XoxF, is required for growth on methanol (Skovran et al. 2011).

Research on the function of XoxF really started to become clear when work on *Methylobacterium radiotolerans* reported the induction of XoxF activity in this methylotroph by the rare earth element (REE) La^{3+} (Hibi et al. 2011), and soon XoxF1 was shown to be a La^{3+} dependent MDH in *M. extorquens* AM1 (Nakagawa et al. 2012). An *mxaF* deletion mutant in this strain was also unable to grow on methanol with Ca^{2+}, but its growth was restored upon La^{3+} addition, thereby supporting the role of XoxF as a La^{3+}-dependent MDH. XoxF purified from *M. extorquens* AM1 cells grown on methanol and La^{3+} exhibited a specific activity of 10 U mg^{-1} protein and contained 0.91 atoms of La^{3+} per dimer (Nakagawa et al. 2012). Studies that had previously shown that purified XoxF had very low specific activity (Schmidt et al, 2010) had obtained the enzyme from cells grown in minimal media lacking La^{3+}, and so it seemed likely that the lack of activity was due to the conditions not being optimum for a fully functional XoxF.

Another huge advance in the XoxF-REE story emerged when Pol et al. (2014) isolated an extremely acidophilic methanotroph, *Methylacidiphilum fumariolicum* SolV, from a

volcanic mudpot. This strain could only grow on methane when provided with the metal-rich mudpot water it was isolated from. Pol et al found that it was dependent on the REEs in the water, and these metals were the cofactors in the only MDH in the genome, XoxF. This XoxF had a high specific activity of 4µmol min^{-1} mg^{-1} protein and the highest known affinity of any MDH for methanol at 0.8µM when using La^{3+} as its cofactor. The *M. fumariolicum* SolV strain also took up much more Ce^{3+} from the medium than was required for maximum cell growth, suggesting the possibility of a storage mechanism. The first crystal structure was obtained for the XoxF of this strain, revealing the cerium metal coordinated where the calcium ion is in the MxaFI MDH (seen in Figure 1.4). This structure revealed that two of the amino acid residues that coordinate the calcium ion in XoxF were different to MxaFI, along with an additional residue, which was proposed to be specific to accommodating the larger REE metal.

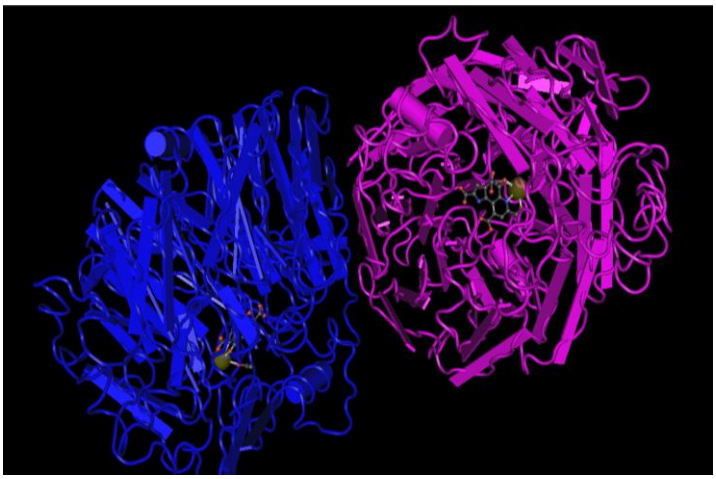

Figure 1.4 Crystal structure of methanol dehydrogenase, XoxF, from *Methylacidiphilum fumariolicum* SolV. Figure taken from Pol et al. (2014). The blue and purple are the XoxF subunits (homolog to the α subunit in MxaF). The cerium atom is shown in green, next to the PQQ prosthetic group.

Further evidence that supported the role of XoxF as a MDH came from the ability of some methylotrophs to metabolise methanol when there are no *mxaFI* genes present in their genome. *Methylotenera mobilis* JLW8, for example, has two *xoxF* genes and no *mxaF*-encoded MDH and has been shown to be one of the major species that oxidises methanol in freshwater lake sediment (Bosch et al. 2009). Research on *M. mobilis* has shown that there was a 150-fold higher abundance of XoxF1 peptides in methanol grown cells compared to methylamine grown cells (Beck et al. 2011). Moreover Mustakhimov et al. (2013) showed that mutant strains in *xoxF1* and *xoxF2* of *M.*

mobilis were able to grow on methanol but that a mutant in both *xoxF1* and *xoxF2* was unable to grow. Interestingly, the single mutant in *xoxF2* was able to grow better than the wild type on methanol. This unexpected phenotype has not been addressed further, but one possible explanation is that *xoxF2* functions as a regulator in methanol metabolism, rather than directly as an MDH. Although *M. mobilis* is able to grow on methanol, it does so very poorly and so proves difficult in measuring growth rates and MDH activity.

The photosynthetic methylotroph *Rhodobacter sphaeroides* has one *xoxF*5 and no *mxaF* in its genome but is able to grow on methanol during photosynthetic conditions. With well established genetic systems, it was therefore a good model for studying the role of XoxF. Wilson et al. (2008) constructed a *xoxF* deletion mutant (the only MDH present in the genome) and showed that it was unable to use methanol as a photosynthetic carbon source and was unable to perform methanol-dependent oxygen uptake. This strongly indicated the role of XoxF as the MDH responsible for methylotrophy in *R. sphaeroides*.

Recently there has been an explosion of research into the role of XoxF. Chistoserdova (2011a) performed a phylogenetic analysis on all *xoxF* sequences in sequenced genomes and revealed that *xoxF* sequences clustered into five different clades (1-5), and that *mxaF* seemed to cluster between two of the clades (see Figure 1.5). This clustering of *mxaF* within the *xoxF* clades suggests that *xoxF* may even be the ancestral MDH form, and that *mxaF* was the result of a secondary evolutionary event (Keltjens et al. 2014).

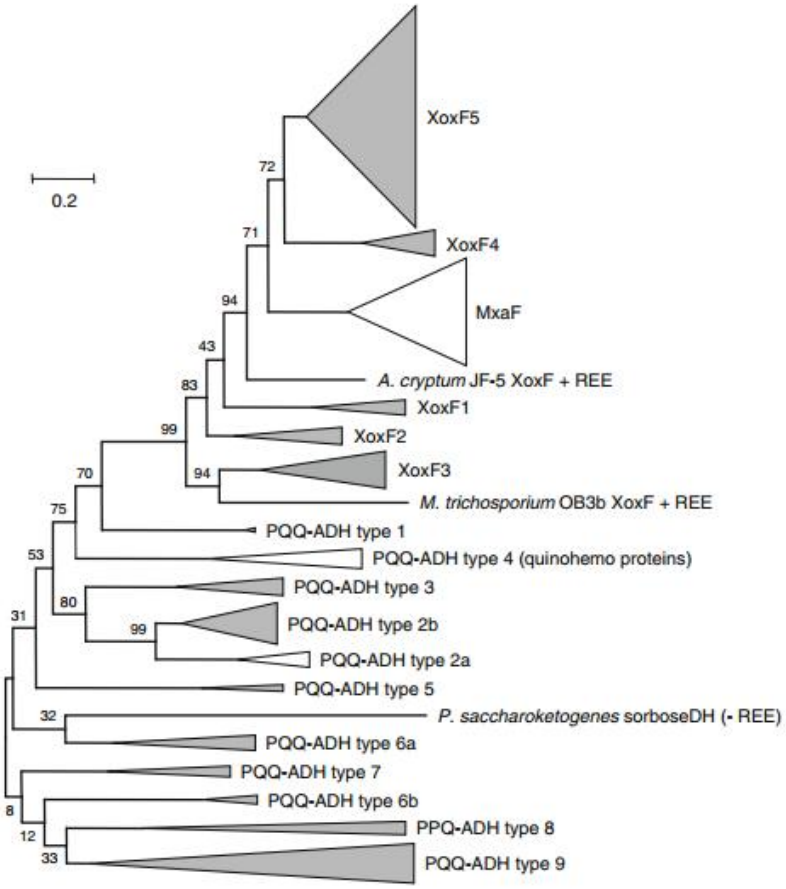

Figure 1.5 Phylogenetic tree of types I and II alcohol dehydrogenase quinoproteins. Figure taken from Keltjens et al. (2014), which was based on work done by Chistoserdova et al. (2009). The tree shows the relationship of the different *xoxF* sequences to the *mxaF* gene and other PQQ-dependent alcohol dehydrogenases.

Work done on *Methylomicrobium buryatense* showed that the addition of lanthanides increased *xoxF* expression, whilst reducing *mxaF* expression, and that this was regulated, in part, by the response regulator MxaB (Chu et al. 2016). Very shortly after, using mutants in both *xoxF* and *mxaF* and transcriptional reporter fusion strains, Vu et al. (2016) showed that expression of *mxaF* is repressed and *xoxF1* up-regulated at concentrations of above 100 nM lanthanum, with the strain preferentially utilising the XoxF MDHs when possible.

Much of the research on XoxF has been conducted using pure cultures or cell extracts, and so very little is known of the true role of XoxF in the environment. XoxF was not detected in 2D protein gels in methanol grown *M. extorquens* in minimal media (Laukel et al. 2003) and was found in numbers 100-fold less abundant than MxaF (Bosch et al.

2008). However it has been detected in the phyllosphere of soybean, clover and *Arabidopsis*, and was even the only MDH present detected in *Arabidopsis thaliana*, with no MxaF being detected (Delmotte et al. 2009). Recent environmental research suggests, however, that XoxF may also have a role in the metabolism of other C1 compounds. For example, XoxF expression was highly induced in the marine strain *Methylophaga* sp. DMS010 during growth on DMS compared to growth on methanol, whilst MxaF was found in cells grown under both conditions (Schäfer 2007). Moreover a proteomic analysis of *M. mobilis* JLW8 grown on methylamine revealed high amounts of XoxF and XoxG peptides (Bosch et al. 2009), which was subsequently confirmed by Beck et al, 2011. This supports the suggestion by Skovran et al, 2011 that XoxF acts as an environmental signal and that it might not only detect methanol, but a range of C1 compounds.

With regard to the complexities of alternative MDH systems, there are some non-methylotrophs such as *Bradyrhizobium japonicum, Dinoroseobacter shibae* DFL12 and *Sinorhizobium meliloti* 1021 which contain *xoxF* but do not as yet have a characterised function (Mühlencoert & Müller 2002). It is therefore still very difficult to assign a universal function for of XoxF, but its presence in every known *mxaF*-containing methylotroph across a range of environments suggests a key role in C1 metabolism in the environment.

1.4 Methanol in the environment

1.4.1 The global methanol budget

Methanol is a oxygenated volatile organic compound (OVOC) and is ubiquitous in the atmosphere, being the second most abundant organic gas in the atmosphere, after methane (Lewis et al. 2005). It is a biogeochemically active compound, significantly influencing the concentration of other atmospheric gases (Jacob et al. 2005; Heikes 2002). In the presence of nitrogen oxides (NOx), methanol oxidation enhances global tropospheric O_3 (Ebojie et al. 2016), whilst methanol also acts as a source of formaldehyde and hydrogen radicals through the reaction with hydroxyl radicals

(Heikes et al, 2002). Moreover, methanol photochemistry in clouds can produce formic acid, leading to increased acidity of rainwater (Heikes et al, 2002).

Due to the fairly long residence time of methanol (19 days (Bey et al. 2001)) it is fairly difficult to attribute which sources contribute to atmospheric standing concentrations of methanol, and from where. Coupling this to the difficulty of accurately measuring atmospheric concentrations of methanol means that estimations of the global methanol budget and its sources and sinks vary vastly (Sargeant 2013; Dixon et al. 2013). For example, it is widely known that plants are the largest contributor of methanol to the atmosphere (MacDonald & Fall 1993), with estimations of the global emissions due to plant growth varying between 50-280 Tg $year^{-1}$ (Sargeant 2013). Other forms of methanol production into the atmosphere are plant decay, anthropogenic emissions, biomass burning and atmospheric production (Read et al. 2012; Heikes 2002). Sources and sinks of methanol can be seen in Figure 1.6.

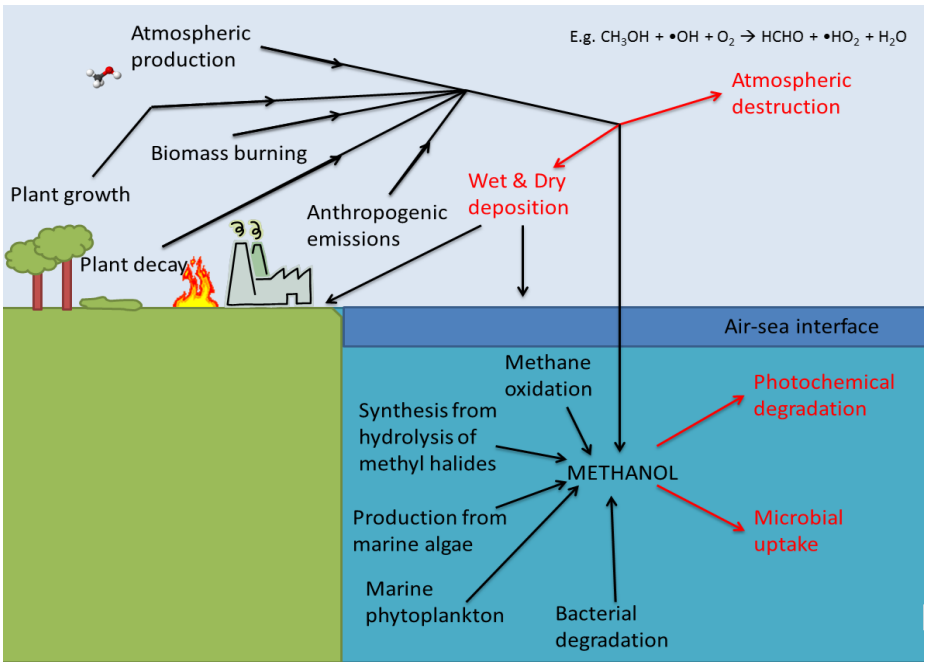

Figure 1.6 Sources and sinks of methanol in the environment. Figure taken from Sargeant (2013). Potential sources of methanol are indicated by black arrows whilst potential sinks of methanol are red arrows.

1.4.2 Methanol in the marine environment

Although estimations of the terrestrial and anthropogenic contribution to the global methanol budget vary widely, the overall finding is that they are net source of methanol to the atmosphere. However this is not true for the estimations of the marine contribution to the global budget. There are such large differences between data sets that some data suggest that the marine environment is a major net source of methanol at around 80 Tg $year^{-1}$ (Heikes 2002; Read et al. 2012), whilst other measurements suggest it is a sink (Heikes 2002; Millet et al. 2008). One of the main reasons why there are such large uncertainties about whether the marine environment is a source or sink of methanol is due to the analytical difficulty in measuring methanol concentrations in seawater (Dixon et al. 2011), and therefore in calculating the flux between the air and ocean. Thus the extent to which microbes are involved in the production and consumption of methanol in the marine environment is still to be fully understood.

However, various *in situ* measurements of methanol in the marine environment place concentrations of up to 420 nM (J. L. Dixon et al. 2011; Joanna L Dixon et al. 2011; Joanna L. Dixon et al. 2013; Beale et al. 2011; Read et al. 2012; Williams et al. 2004; Kameyama et al. 2010), indicating a high available carbon source for marine methylotrophs. For a long time the source of methanol was a topic of uncertainty, with Dixon et al. (2011) suggesting there may still be an unidentified *in situ* marine source in open ocean waters. Furthering this, Beale et al. (2015) studied the marine methanol concentrations, air methanol concentrations and methanol loss rates in shelf waters in UK shelf waters (station L4, Plymouth). They found that the highest concentrations of methanol were in the top 5 m sea surface layers, whilst the sea surface layer was under-saturated compared to the overlaying atmospheric methanol concentration. They also measured microbial methanol losses of 5.3 (±3.4) nmol/l/h. Using these parameters (and more), they calculated that the air–sea flux of methanol was only 2–20% of the total microbial oxidation, implying that (i) the atmosphere was not likely to be a dominant source of methanol to L4 surface waters and (ii) that there must be *in-situ* production of methanol to sustain the loss rates.

1.4.3 Methanol production by microbes

Previous research had suggested there may indeed be *in situ* biological sources of methanol, although none of this was from direct evidence. Measurements of the concentration of methanol in water surrounding intact macroalgae revealed increased concentrations compared to ambient seawater (Nightingale 1991). Reimer (1998) also showed that micromolar concentrations of methanol were produced in cultures of various phytoplankton, however no direct evidence was shown that these were in fact the cause of the methanol. It has now been revealed that various types of phytoplankton do produce methanol in cell culture (Mincer & Aicher 2016). All phytoplankton tested (*Synechococcus* spp. 8102 and 8103, *Trichodesmium erythraeum*, *Prochlorococcus marinus*, *Phaeodactylum tricornutum*, *Emiliania huxleyi*, *Rhodomonas salina*, and *Nannochloropsis oculata*) produced methanol between 0.8–13.7 micromolar in culture, although it varied greatly between species. Isotope ratio measurements of the phytoplankton in ^{13}C- labelling experiments with bicarbonate revealed that methanol is indeed produced from phytoplankton, labelled *de novo* from algal biomass. With methanol production being as much as 0.3% of the total cellular carbon, they estimated that (using only the lowest producing strain) phytoplankton could be the largest source of methanol emitted per year, exceeding that of plant emissions.

1.5 Methylotrophy in the marine environment

Given the recent findings that potentially massive amounts of methanol are released by phytoplankton into the marine environment, it is therefore hardly surprising that marine methylotrophs are ubiquitous. Much of the knowledge on marine methylotrophs has been based on the isolation and characterisation of novel isolates. However, since the development (and subsequent modification) of a PCR primer pair specific to the *mxaF* gene, (McDonald & Murrell 1997; Neufeld et al. 2007), the identification of methylotrophs in the environment became much easier. For example, using these primers, Dixon et al. (2013) found sequences relating to *Methylophaga* sp., *Burkholderiales* sp., *Methylococcaceae* sp., *Ancylobacter aquaticus*, *Paracoccus denitrificans*, *Methylophilus methylotrophus*, *Methylobacterium oryzae*, *Hyphomicrobium sp*. and *Methylosulfonomonas methylovora* in open Atlantic waters.

1.5.1 *Methylophaga* spp.

Methylophaga are members of the *Piscirickettsiaceae* family in the *Gammaproteobacteria* and are routinely isolated from marine enrichments (Janvier et al. 1985b; Kim et al. 2007) The first isolated strain was *Methylophaga* was originally named *Methylomonas*, and it was only the isolation and naming of *Methylophaga marina*, that *Methylomonas thalassica* was amended to *Methylophaga* (Janvier et al. 1985b). There are now ten officially described species, although only *M. marina*, *M. thalassica* and *M. aminisulfidivorans* were isolated from marine environments. Although *Methylophaga* strains are readily isolated from methanol seawater enrichments, they are numerically rare in the marine environment (Janvier et al. 2003), putting into question their ecological importance in marine methylotrophy.

However DNA stable-isotope probing (DNA-SIP) experiments using ^{13}C-labelled methanol revealed the presence of *Methylophaga*-related *mxaF* and 16S rRNA gene sequences in the heavy fractions of the DNA, suggesting the metabolism of this substrate by members of the *Methylophaga* genus (Neufeld et al. 2007). This was the first detailed example of a cultivation-independent study of marine methylotrophs. A further DNA-SIP experiment using lower concentrations of methanol and multiple displacement amplification once again showed *Methylophaga*-like sequences, whilst sequences of the genus were also found in a methanol DNA-SIP experiment phytoplankton bloom in a temperate coastal environment (Neufeld, Chen, et al. 2008; Neufeld, Boden, Hélène Moussard, et al. 2008). Recently, a methanol DNA-SIP experiment, using water from the coast of Plymouth, was combined with metagenomics analysis of the heavy DNA and metaproteomics to characterize an uncultivated *Methylophaga* that actively incorporated ^{13}C-labelled methanol into its biomass. Metagenomics allowed the construction of almost a complete genome of this uncultivated *Methylophaga*, whilst metaproteomics revealed which pathways the strain was utilising during growth on methanol (Grob et al. 2015).

1.5.2 SAR11 and OM43 clade

The SAR11 clade are members of the *Alphaproteobacteria* and are one of the most abundant, free living bacteria in the ocean, comprising up to 50% of all heterotrophic

bacteria (Giovannoni 1990; Morris et al. 2002). These bacteria are adapted to nutrient poor waters, such as the open ocean, but are found throughout the marine system (Gilbert et al. 2009; Sowell et al. 2011; Giovannoni 1990; Morris et al. 2002). These strains are capable of oxidising a range of C1 substrates such as methanol, formaldehyde and methylamine to produce energy (Tripp 2013). Work conducted by Sun et al. (2011) using ^{14}C-labelled methanol showed that strain HTCC1062 (*Candidatus* Pelagibacter ubique) seemed to use methanol as a supplementary energy source, rather than as a carbon source, only incorporating between 2-6% of the carbon from methanol into cell biomass. Sequencing the genome of a similar strain revealed an gene encoding an iron-containing alcohol dehydrogenase (Fe-ADH, PF00465) protein, likely involved in the metabolism of methanol in this strain.

Methylophilales bacterium HTCC2181 is a representative of a cluster of another one of the most abundant marine methylotrophs, the OM43 clade (of the *Betaproteobacteria*), which is a strain that contains only a XoxF-like MDH (Giovannoni et al. 2008). Moreover, high expression of XoxF-like proteins were also found to be highly expressed in the metaproteome of coastal oceanic microbial plankton (Sowell et al. 2011). Research on the growth of strain HTCC2181 showed that although unable to grow on methanol as sole source of carbon and energy, growth was enhanced when methanol was added to the culture media, suggesting that this strain may use methanol as an energy source, much like with SAR11 (Halsey et al. 2012). Moreover, bacteria of the OM43 clade have been found to be a dominant group of organisms during a diatom bloom (Morris et al. 2006). Given the recent research showing large amounts of methanol production by phytoplankton, it is highly possible that these strains were utilising the methanol during this bloom (Mincer & Aicher 2016).

1.5.3 Marine *Roseobacter* clade

The *Roseobacter* clade is also one of the most abundant groups of marine bacteria, often comprising over 20% of the total bacterial community in coastal environments. This group is significant as many members are involved in the global carbon and sulfur cycle (Pradella et al. 2010; Wagner-Döbler & Biebl 2006; Buchan et al. 2005). Importantly, many strains are found to be commonly associated with phytoplankton (Gonzalez et al. 2000; Grossart et al. 2005; Amin et al. 2012; Amin et al. 2015). Given that very

recently, phytoplankton have been found to release large amounts of methanol (Mincer & Aicher 2016), it is highly likely that many of these close associations may be due to the opportunistic methylotrophic nature of the *Roseobacter* clade. It is therefore very important that the methylotrophic capacity of the marine *Roseobacter* is re-examined. One potential example of this may be the strain *Marinovum algicola*, which was isolated from the dinoflagellate *Prorocentrum limais*, and is able to grow on methanol (Martens et al. 2006; Pradella et al. 2010).

Although it has not been tested for growth on methanol, analysis of the genome of *Sedimentitalea nanhaiensis* reveals a single *xoxF* gene, suggesting it may be methylotroph (Sun et al. 2010; Breider et al. 2014). *Methyloceanibacter caenitepidi* is a fairly newly isolated methylotroph from marine sediments near a hydrothermal vent (Takeuchi et al. 2014). This strain is particularly interesting as not only does it contain an *mxaF* gene, it also encodes four copies of the *xoxF1* gene. The *xoxF1* gene seems to be the least prevalent and diverse throughout methylotroph genomes (see Figure 1.5), and to my knowledge, this is the only strain that contains multiple copies. Moreover, environmental sequences highly related to *M. caenitepidi* (98-99% identity) have been found in various marine sediments worldwide, suggesting it may play a significant role in the metabolism of methanol in the marine environment.

Furthermore, amplicon sequencing of *xoxF5* genes amplified from four different coastal sites revealed high relative abundances of *Rhodobacteraeceae* genera such as *Sagittula* (a known marine methylotroph), but also of many unclassified *Rhodobacteraceae* sequences. This work, conducted by Taubert et al (2015, see Figure 1.7) may support the hypothesis that many members of the *Roseobacter* clade are capable of methylotrophy *in situ.*

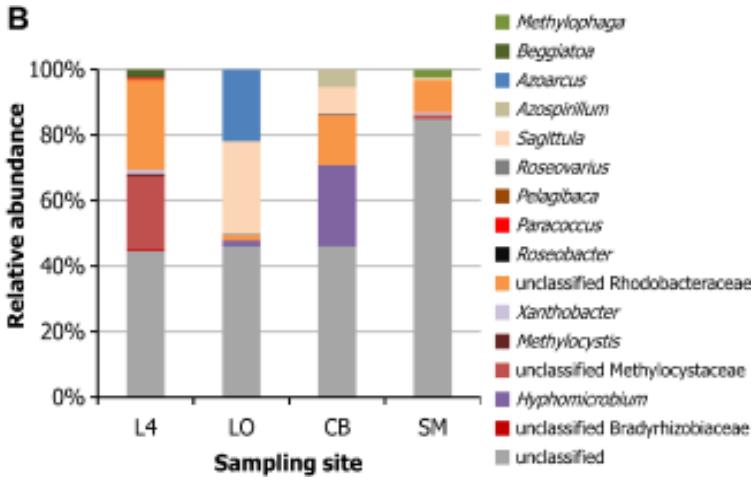

Figure 1.7 Relative abundance of *xoxF5* sequences from four coastal marine sites. Figure taken from Taubert et al. (2015). Sequences were retrieved by 454 amplicon sequencing. The 'unclassified' category contains all sequences that were unclassified at family level. Data were derived from samples collected at the Western Channel Observatory Station L4 (L4), Stiffkey Salt Marsh (SM), Cromer Beach (CB) and offshore of Lowestoft (LO).

1.6 Aims and objectives

The aims of the work described here were:

1. To isolate and characterise novel methylotrophs from the marine environment using physiological characterisation, genetic manipulation and genome sequencing;
    - Chapter 3 and 5 both detail the isolation of two new species and one novel genus from surface seawater using modified isolation procedures.
    - The strains are physiologically characterised with respect to their closest relatives.
    - The genomes of all three strains are sequenced and compared against their closest members, whilst the new genus is further analysed using comparative genomics.
    - The role of the *xoxF* gene in the new genus is characterised using single allelic disruption, complementation and growth studies on various carbon sources (Chapter 5).
2. To investigate the effect of rare earth elements on the microbial oxidation of methanol in enrichments, and to identify which members of the microbial community are involved in this process;
    - Chapter 4 addresses this by monitoring headspace methanol in seawater enrichments with added rare earth elements.
    - It shows the use of DGGE and amplicon sequencing on DNA extracted from enrichments that contained varying concentrations of methanol to profile the bacterial community.
3. To investigate the function of the *xoxF* gene from characterised methylotrophs and non-methylotrophs using expression systems in heterologous hosts.
    - Chapter 6 details the use of both *E. coli* and a novel strain carrying a mutated *xoxF* as hosts for expressing five different *xoxF* sequences.
    - Various methods are employed to optimise expression and activity of the XoxF proteins.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Chemicals and reagents

Analytical grade reagents and chemicals used in this research were from Sigma-Aldrich Corporation (St Louis, USA), Fisher Scientific (Loughborough, UK), or Melford Laboratories Ltd (Ipswich, UK). Molecular biology grade reagents were obtained from Promega UK (Southampton, UK), Bioline Reagents Ltd. (London, UK) and Fermentas Molecular Biology Tools (Leon-Rot, Germany).

2.2 Bacterial strains, plasmids and primers

Table 2.1 List of organisms and plasmids used in this study

Strains/Plasmids	Description/genotype	Reference/source
Strains		
Escherichia coli TOP10	F– *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 *ΔlacX74 recA1 araD139* Δ(*ara leu*) *7697 galU galK rpsL* (StrR) *endA1 nupG*	Invitrogen
Escherichia coli BL21(DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm]* Δ*hsdS*	Invitrogen
E. coli p672*xoxF*	*E. coli* TOP10 carrying p672*xoxF* vector	This study
E. coli pET16	*E. coli* BL21 carrying pET16 vector	This study
E. coli pETSAG	*E. coli* BL21 carrying *xoxF* from *Sagittula stellata* E-37 on plasmid pETSAG vector	This study
E. coli pETMSV	*E. coli* BL21 carrying *xoxF* from *Methylocella silvestris* on plasmid pETMSV vector	This study
E. coli pETRDN	*E. coli* BL21 carrying *xoxF* from *Roseobacter denitrificans* OCh 114 on plasmid pETRDN vector	This study
Methylophaga marina	Wild-type strain	(Janvier et al. 1985a)
Rhodobacter sphaeroides Ga	Wild-type strain	(Wilson et al. 2008)
Roseobacter denitrificans Och 114	Wild-type strain	(Shiba et al. 1991)
Sagittula stellata E-37	Wild-type strain	(Gonzalez et al. 1997)
Methylobacterium extorquens AM1	Wild-type strain	Lidstrom lab collection
Methylocella silvestris BL2	Wild-type strain	Warwick culture collection
Methylophaga AH1 L4	Wild-type strain	This study
La 6	Wild-type strain	This study
La 6^{Rif}	Wild-type strain, Rif^R	This study
La 6 $XoxF^-$	La 6^{Rif} *XoxF::*p672*xoxF*, Km^R	This study

XoxF$^-$ p509LA6	La 6 XoxF$^-$ complemented with the wild-type *xoxF* gene on plasmid p509LA6, Gm^R	This study
XoxF$^-$ p509MEX	La 6 XoxF$^-$ carrying *xoxF* from *Methylobacterium extorquens* AM1 on plasmid p509MEX, Gm^R	This study
XoxF$^-$ p509MPH	La 6 XoxF$^-$ carrying *xoxF* from *Methylophilales bacterium* HTCC2181 on plasmid p509MPH, Gm^R	This study
XoxF$^-$ p509SAG	La 6 XoxF$^-$ carrying *xoxF* from *Sagittula stellata* E-37 on plasmid p509SAG Gm^R	This study
XoxF$^-$ p509MSV	La 6 XoxF$^-$ carrying *xoxF* from *Methylocella silvestris* on plasmid p509MSV, Gm^R	This study
XoxF$^-$ p509RDN	La 6 XoxF$^-$ carrying *xoxF* from *Roseobacter denitrificans* OCh 114 on plasmid p509RDN, Gm^R	This study
Plasmids		
pGEM-T	Ap^R TA cloning vector	Promega
pRK2013	Km^R, RK2 vector, self transmissible, helper plasmid	(Figurski & Helinski 1979)
pK19*mob*	Km^R, RP4-mob, mobilizable cloning vector	(Schafer et al. 1994)
p672*xoxF*	pK19*mob* containing a 672bp internal fragment of *xoxF* from La 6	Invitrogen
pET21	Ap^R expression vector with T7 promoter	This study
pETSAG	pET21 containing *xoxF* from *Sagittula stellata* E-37	This study
pETMSV	pET21 containing *xoxF* from *Methylocella silvestris* BL2	This study
pETRDN	pET21 containing *xoxF* from *Roseobacter denitrificans* OCh 114	This study
pUCMPH	Synthesised pUC57 containing *xoxF* from *Methylophilales bacterium* HTCC2181	This study; Genscript
pLMB509	Gm^R expression vector with inducible taurine promoter (tauAP); *gfp* excised	(Tett et al. 2012)
p509LA6	pLMB509 containing *xoxF* from strain La 6	This study
p509MEX	pLMB509 containing *xoxF* from *Methylobacterium extorquens* AM1	This study
p509MPH	pLMB509 containing *xoxF* from *Methylophilales bacterium* HTCC2181	This study
p509SAG	pLMB509 containing *xoxF* from *Sagittula stellata* E-37	This study
p509MSV	pLMB509 containing *xoxF* from *Methylocella silvestris* BL2	This study
p509RDN	pLMB509 containing *xoxF* from *Roseobacter denitrificans* OCh 114	This study

Table 2.2 PCR primers used in this study

Name	Target gene	Sequence (5'-3')	Reference
27F	16S rRNA	AGAGTTTGATCMTGGCTCAG	(Lane 1991)
1492R		TACGGYTACCTTGTTAGGACTT	
341-GC		CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGCCTACGGGAGGCAGCAG	(Muyzer et al. 1993)
518R		CCAGCAGCCGCGGTAAT	
27Fmod		AGRGTTTGATCMTGGCTCAG	
519Rrmodbio		GTNTTACNGCGGCKGCTG	
1003F	*mxaF*	GCGGCACCAACTGGGGCTGGT	(Neufeld et al. 2007)
1555R		CATGAABGGCTCCCARTCCAT	
xoxF1F	*xoxF1*	TAYGCCGAYGGCAAGSTGST	(Taubert et al. 2015)
xoxF1R		CCGTCRTARTCCCAYTGRTCGAA	
xoxF2F	*xoxF2*	GGCYTAYCAGATGACBCCNTGG	
xoxF2R		GCCTTRAACCAKCCRTCCA	
xoxF3F	*xoxF3*	GGHGAGWCCATSACVATGGC	
xoxF3R		TCCATSGTKCCGTAGAA	
xoxF4F	*xoxF4*	TTYCCHAAYAACGTNTAYGC	
xoxF4R		GGRTTRCCHGTHCCGTAGTA	
xoxF5F	*xoxF5*	GAYGAVTGGGAYTWYGACGG	
xoxF5F		GGYTCVTARTCCATRCA	
mauAF1	*mauA*	ARKCYTGYGABTAYTGGCG	(Neufeld et al. 2007)
mauAR1		GARAYVGTGCARTGRTARGTC	
557F	*gmaS*	GARGAYGCSAACGGYCAGTT	(Chen 2012)
1332R		GTAMTCSAYCCAYTCCATG	
Euk1A	18S rRNA	CTGGTTGATCCTGCCAG	(Díez et al. 2001)
Euk516r-GC		ACCAGACTTGCCCTCCCGCCCGGGGCGCGC CCCGGGCGGGGCGGGGGCACGGGGGG	
pLMB509F	Inside MCS	CAAAAAGCGGGGCGACATAA	Robert Green,
pLMB509R	of pLMB509	TTGACACCAGACAAGTTGGT	Unpublished

Sequences underlined are the added GC-rich sequence.

2.3 Cultivation and maintenance of strains

Solutions and growth media were prepared in Milli-Q water and sterilised by autoclaving at 15psi for 15 minutes at 121˚C. Solutions sensitive to autoclaving, were sterilised using 0.2µM pore-size sterile filter units (Sartorius Minisart, Göttingen, Germany) and were added to cooled autoclaved media. Other solvents were used on occasion, such as methanol or ethanol. Solid media were prepared by the addition of 1.5

% (w/v) Bacto Agar (Difco) before autoclaving. Bacterial strains and plasmids used in this study are shown in Table 2.1, and PCR primers relevant to this section are in Table 2.2.

2.3.1 Antibiotics

Antibiotics were filter sterilized and added aseptically to cooled, autoclaved growth medium at these concentrations: ampicillin 100 µg ml^{-1}; kanamycin, 25 µg ml^{-1}; gentamicin 5 µg ml^{-1}; tetracycline 10 µg ml^{-1}, rifampicin 10 µg ml^{-1}, unless otherwise indicated.

2.3.2 Escherichia coli

All *E. coli* strains were grown in Luria-Bertani (LB) medium (Sambrook and Russell, 2001) and incubated at 37˚C, unless otherwise indicated. 1.5% (w/v) Bacto Agar (Difco) was added before autoclaving to make LB agar plates. Liquid cultures were shaken at 200 rpm. Strains were stored at -80˚C after the addition of 25% (v/v) glycerol and flash freezing in liquid nitrogen.

2.3.3 Preparation and transformation of chemically competent *E.coli*

SOB medium:

The following were dissolved in 900 ml deionised water: yeast extract, 5 g; tryptone, 20 g; NaCl, 0.5 g. KCl solution (10 ml of 250 mM) was added, the pH adjusted to 7.0 with 5 M NaOH, the volume made up to 1 litre with water, and the solution sterilised by autoclaving. Before use, sterile $MgCl_2$ solution (2 M) was added to 10 mM.

SOC medium:

Filter sterilised glucose solution (1 M) was added to SOB medium to a final concentration of 20 mM.

CCMB80 buffer:

The following were dissolved in 800 ml deionised water: KOAc solution pH 7.0 (10 ml of a 1 M stock); $CaCl_2.2H_2O$, 11.8 g; $MnCl_2.4H_2O$, 4 g; $MgCl_2.6H_2O$, 2 g; glycerol, 100

ml. The solution was adjusted to pH 6.4 with 10% (v/v) HCl, made up to 1 litre with water and sterilised by filtration.

Chemically competent *E. coli* cells were prepared by a modified variant of the Hanahan protocol (Hanahan et al. 1991) using CCMB80 buffer. A 1 ml seed stock of *E. coli* cells was inoculated into 250 ml of SOB medium and grown to an OD_{600} of 0.3. The cell culture was centrifuged (3000 g at 4 °C for 10 minutes) and the supernatant discarded. The pellet was gently re-suspended in 80 ml ice cold CCMB80 buffer and stored for 20 minutes on ice. The cells were centrifuged again, the supernatant discarded and the pellet re-suspended in 10 ml ice cold CCMB80 buffer. After 10 minutes incubation on ice, the OD_{600} of the cell suspension was adjusted to an OD_{600} of 1.5 using ice cold CCMB80 buffer. 50 µl aliquots were flash frozen in liquid nitrogen and stored at -80 °C.

2.3.4 *Methylophaga* spp

Marine Ammonium Mineral Salts (MAMS) was used for the growth of both *Methylophaga AH1* and *M. marina*, and was prepared according to Goodwin (et al. 2001) as shown below.

MAMS:

The following were dissolved in 900 ml deionised water: 20 g NaCl, 10 ml $(NH_4)_2SO_4$ solution (10 g/100 ml), 10 ml $CaCl_2.2H_2O$ solution (2 g/100 ml), 10 ml MS solution (per 100 ml: 10 g $MgSO_4.7H_2O$, 20 mg $FeSO_4.7H_2O$, 0.2 g $Na_2MoO_4.2H_2O$), 30 µl $Na_2WO_4 \cdot 2H_2O$, 1 ml SL10 trace element solution (Widdel et al. 1983) and 0.5 µl Na_3VO_4 + Na_2SeO_3 solution (10 µg/ml). The volume was adjusted to 990 ml and autoclaved. Phosphate solution containing 3.6g KH_2PO_4 and 23.4g K_2HPO_4 (anhydrous) per 100 ml was autoclaved separately and added at 10 ml per l. 1 ml of filter sterilised vitamin solution as prepared by Kanagawa (et al. 1982) was also added.

Methylophaga AH1 and *M. marina* were routinely cultivated and maintained in 120 ml serum vials containing 30 ml MAMS medium and 20 mM methanol, then sealed with grey butyl rubber seals. Other carbon sources were added at varying concentrations as detailed in Chapter 3. Vials were inoculated with a single colony or with a 3% (minimum) inoculum from a previously grown culture. Vials were incubated at 25°C,

shaking at 150 rpm for 3 days or until growth was observed. Growth was monitored by measuring the OD_{540} using a UV-1800 Shizmadzu (Milton Keynes) spectrophotometer. $(NH_4)_2SO4$ was omitted from the MAMS medium when testing for the ability to use 20 mM monomethylamine as a nitrogen source. Agar plates were incubated in gas-tight chambers with 100 µl volatilised methanol which was replenished every few days.

2.3.5 *Oceanicola* strain La 6

Marine Basal Medium (MBM) was used for the growth of strain La 6 in minimal medium, with the addition of lanthanum chloride heptahydrate and cerium chloride heptahydrate to a final concentration of 5 µM, as shown below. Unless otherwise stated, 5 mM succinate was used as the sole carbon source. Marine Broth 2216 (MB) was used for growth in a rich medium, and was prepared according to the manufacturer's instructions (BD Biosciences). Other media specific to experiments are detailed in the relevant sections.

MBM:

The following were dissolved in 700 ml deionised water and autoclaved: 20 g Sea Salts (Sigma-Aldrich), 250 ml Basal Media (per 525 ml: 150 ml 1 M Tris-HCl pH 7.5 solution, 87 mg K_2HPO, 1.5 g NH_4Cl). Separately autoclaved and added was 25 mg FeEDTA sodium salt in 50 ml. 1 ml of filter sterilised vitamin solution as described by Kanagawa et al (1982) was added, as well as 200 µl of $LaCl_3.7H_2O$ (37.14 mg /10 ml) and $CeCl.7H_2O$ (37.26 mg/10 ml) solutions, unless otherwise stated.

Strain La 6 was maintained in 30 ml MBM in 120 ml serum vials with either 5 mM methanol or 5 mM succinate as the sole carbon source. Cultures were incubated at 25 ˚C, with shaking at 150 rpm. NH_4Cl was omitted from the MBM recipe when testing substrates as nitrogen sources. Vitamin B12 was omitted when testing for vitamin B12 requirement. La 6 was also grown in 1 l conical Quickfit flasks, containing 400 ml of either MBM or Marine Broth, fitted with SubaSeal (Sigma-Aldrich) stoppers and incubated at 25 °C, with shaking at 150 rpm.

2.3.6 Conjugation of strain La 6

Plasmids were transferred by tri-parental mating from *E. coli* to strain La 6^{Rif} using the helper plasmid pRK2013 (Figurski & Helinski 1979). Briefly, 0.5 ml of overnight cultures (containing no antibiotic) of each of the *E. coli* pRK2013 and *E. coli* containing the desired plasmid were centrifuged together (6,000 g x 3 min, 21 °C) and the supernatant discarded. A volume of (MB-grown) overnight culture of strain La 6^{Rif} was added, centrifuged again and the resultant pellet re-suspended in 100 µl residual media to give a final cell number of roughly 1:1:2 of *E.coli* pRK2013, *E. coli* containing the desired plasmid and La 6^{Rif}, respectively. The cell suspension was placed on to a 0.2 µm pore-size nitrocellulose filter (Millipore, Billerica, MA, USA) which was on top of a MB plate and incubated overnight at 30 °C. The cells were washed off the filter with 1 ml MB media and 50 µl plated onto MB media containing Rif and other selective antibiotics. A streak-plate of the same suspension was also made. Plates were incubated for 3-4 days until colonies formed.

2.4 Bacterial purity checks and microscopy

Cell cultures were regularly examined under 1,000 x magnification in phase-contrast to assess morphology and purity using a Zeiss Axioskop 50 microscope, 130 VA Type B, and documented using the AxioCam camera system and Axiovison Rel 4.8 software (all supplied by Carl Zeiss Ltd, Cambridge UK). On occasion, further purity checks were performed by amplifying the 16S rRNA gene by PCR from single colonies and subsequent DNA sequencing. Strains were also diluted and plated onto either MB or R2A agar and incubated at 25 ˚C for 4 days to check for contaminants.

2.5 Extraction of nucleic acids

2.5.1 Environmental DNA extraction

DNA from seawater and seawater enrichments was extracted by the initial filtration of seawater through 0.22 µm Sterivex polyethersulfone filters (Millipore). Environmental DNA was extracted from Sterivex filters. 1.6 ml SET buffer (0.75 M sucrose, 40 mM EDTA, 50 mM Tris-HCl pH 9) and 0.2 ml 10% (w/v) SDS were added into the Sterivex and incubated at 55°C for 2 h, rotating. Lysates were withdrawn with a syringe, and

another 1 ml of SET buffer and 150 µl of SDS solution added for another 30 min. Both lysates were then combined, and two extractions using 2 ml phenol : chloroform : isoamyl alcohol (25:24:1) and one with 2 ml chloroform : isoamyl alcohol (24:1) were performed. 25 µg glycogen (Roche, Basel, Switzerland), 1 ml of 7.5 M ammonium acetate and 8 ml of pure ethanol was then added to the aqueous phase, and DNA left to precipitate overnight at −20°C. Samples were then centrifuged (17, 000 g x 20 min x 4 °C). The pellet was washed twice in 1 ml 70% (v/v) ethanol, dried and re-suspended in 600 µl (or more) Tris 5 mM pH 8.5 buffer.

2.5.2 Genomic DNA extraction

High molecular mass DNA was extracted for genome sequencing using the CTAB (cetyltrimethylammonium bromide) method of Doyle & Doyle (1987). 50 ml cell culture was centrifuged (10,000 g x 10 min, 21 °C) in a 50 ml conical tube and the pellet re-suspended in 5 ml resuspension buffer (20 mM Tris, 2 mM EDTA, pH 8), 60 µl lysozyme (100 mg ml-1, Sigma) and incubated for 1 h at 37 °C. 375 µl Proteinase K (10 mg ml^{-1}) (Melford Laboratories) and 7 µl RNase A was added and incubated for a further 15 min at 37 °C. 780 µl N-laurylsarcosine (10% w/v in re-suspension buffer) was added and gently rotated and incubated at 60 °C for 1 h. 1,012 µl of 5 M NaCl and 803 µl of warm CTAB (10% v/v in 0.7 M NaCl) were added and incubated for 15 min at 60 °C. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed rigorously and incubated for 10 min at 60 °C. The mixture was shaken vigorously again, centrifuged (8,000 g x 5 min) and the supernatant transferred to a new tube. This was repeated twice, with 5 min incubation at room temperature each time, and then again using chloroform/isoamyl alcohol (24:1). 2 volumes of ice-cold ethanol were added to the aqueous phase to precipitate the nucleic acids, incubated for 60 min (or overnight) at -20 °C and then centrifuged (17, 000 g x 20 min x 4 °C). The pellet was washed twice in 1 ml 70% (v/v) ethanol, dried and re-suspended in 600 µl (or more) Tris 5 mM pH 8.5 buffer.

2.5.3 Small scale plasmid extraction

Plasmids were extracted using the GeneJET kit (Fermentas) according to the manufacturer's instructions, using 2-5 ml of overnight *E. coli* or strain La 6 (MB grown) cultures.

2.6 Nucleic acid manipulation techniques

2.6.1 Quantification of DNA

DNA amount and quality was measured on a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). DNA concentration was also estimated by comparing a known quantity of DNA (1kb ladder, Fermentas) on an agarose gel to a known volume of extracted DNA. On occasion, DNA concentration was quantified on a Qubit 2.0 (Invitrogen).

2.6.2 Polymerase Chain Reaction (PCR)

PCR reactions were set up in 50 µl volumes in a Tetrad (Bio-Rad) thermal cycler. A typical reaction would contain 1 x master mix (2x Master Mix, NEB), 0.08% BSA, 0.4 µM forward and reverse primer and 10-40 ng DNA. If colony PCR was being performed then 2.5% (v/v) DMSO was added. General PCR cycling conditions were: primary denaturation at 95°C, 5 min (10 min for colony PCR); 30 cycles of denaturation at 95°C, 30 s; annealing at 55 °C (variable on primer Tm), 30 s; elongation at 72 °C, 1 min/kb; final elongation at 72 °C, 7 min. Template-free reactions were performed as negative controls.

2.6.3 Restriction digests

Restriction digestion of DNA was performed with enzymes from Invitrogen or Fermentas according to the manufacturers' recommendations.

2.6.4 DNA purification

DNA from PCR and restriction digests was purified using NucleoSpin Gel and PCR clean up (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. DNA excised from agarose gels was also purified using the recommended instructions.

2.6.5 Agarose gel electrophoresis

DNA fragments were separated on 1% (w/v) agarose gels containing ethidium bromide (0.5 µg ml^{-1}) in 1 x TBE buffer. GeneRuler 1kb DNA (Fermentas) ladder was used for estimation DNA fragment size.

2.6.6 DNA ligations and cloning of PCR products

Ligations were typically carried out in 10 µl reactions containing a total of 100 ng DNA, with efforts made to have equimolar concentrations of plasmid to insert DNA. PCR fragments were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) according to the manufacturers' instructions. If the plasmid was previously digested with restriction enzymes, it was treated with FastAP Thermosensitive Alkaline Phosphatase (Fermentas) according to the manufacturers' recommendations, prior to the ligation reaction. Reactions were carried out at 20 °C for 1 hour or overnight at 4 °C. 2 µl of ligation reactions were transformed into Top10 *E.coli* cells using the heat shock method. Inserts of clones were sequenced using primers M13F/M13R (as described in Table 2.2 for pGEM-T or the appropriate primers for other vectors).

2.6.7 DNA sequencing and assembly

PCR purified products (20-100 ng) were sequenced using the Sanger sequencing method at Source BioScience (Cambridge, UK). DNA sequences were analysed manually using Chromas (Technelysium Pty Ltd) and aligned using MEGA5 (Tamura et al. 2011).

16S rRNA gene and *xoxF*5 PCR amplicons were sent for 454 pyrosequencing at MR DNA (Texas, U.S.A).

DNA extracted from *Methylophaga AH1* and *Methylophaga marina* was sequenced by MR DNA using Illumina kits on a MiSeq machine with a paired end approach. Raw sequences were checked for quality control using FastQC on BaseSpace. Assembly was done using Spades v.3.8 (Bankevich et al. 2012), whilst ORF-calling and annotation was done using the RAST server (Aziz et al. 2008).

The genome of strain La 6 was sequenced by collaborators John Vollmers and Anne Kaster, (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany) as follows:
Standard and Mate-Pair sequencing libraries were produced using Illumina kits and run on a Miseq machine using V3 chemistry with a paired end approach and 301 cycles per read. Reads were adapter-clipped and quality trimmed using Trimmomatic (Bolger et al. 2014). Mate-pair reads were additionally clipped, sorted and re-orientated using NxTrim (O'Connell et al. 2015). Potential PhiX and vector contamination was filtered out using fastq_screen (http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/), while low complexity reads (consisting entirely of only one base type or direct short oligonucleotide repeats) were removed using prinseq (Schmieder & Edwards 2011). Potential overlapping paired-end reads were merged using FLASH (Magoč & Salzberg 2011). Assembly was done using Spades v.3.8. ORF-calling and annotation was done using the PROKKA pipeline v.1.12 (Seemann 2014).

2.6.8 Multi-Locus Sequence Analysis

For the Multi Locus Sequence Analysis (MLSA), the unique core-genome of 210 gene products with a combined length of 89169 amino acid residues was determined using the bidirectional BLAST approach implemented in proteinortho5 (Lechner et al. 2011), excluding all genes with duplicates in any comparison genome. After alignment with muscle (Edgar 2004), the gene products were concatenated and un-alignable regions were filtered out using gblocks (Castresana 2000), leaving 54554 aligned amino acid residues for phylogenetic analysis. Clustering was performed using the Neighbour Joining algorithm with 1000 bootstrap permutations.

2.6.9 Gene content analysis

A binary matrix was constructed, representing the presence or absence of orthologeous group identified by the bidirectional BLAST approach as described for the MLSA. In order to prevent artefacts caused by fragmented or falsely predicted genes, all singletons were excluded from the analyses (requiring each considered orthologeous group to be present in at least two different genomes). This resulting binary matrix was converted into a distance matrix and clustered using the Neighbor Joining algorithm and 1000 bootstrap permutations.

2.6.10 Denaturing gradient gel electrophoresis

16S rRNA and 18S rRNA amplicons amplified by primers containing GC-rich regions (see Table 2.2) were separated by denaturing gradient gel electrophoresis (DGGE) on a 10 % (w/v) polyacrylamide gel with a 30-70 % (w/v) linear denaturant gradient or 6 % (w/v) with a 20-45 % (w/v) linear denaturant gradient for 16S rRNA and 18S rRNA amplicons, respectively. Each 14 ml gel was prepared as below and run using the DCode^{TM} Universal Mutation Detection System (Bio-Rad).

	18S rRNA gel 6 % acrylamide		16S rRNA gel 10 % acrylamide		Top up gel
	20 %	45 %	30 %	70 %	
40 % (w/v) acrylamide/bis (37.5:1)* (ml)	2.1	2.1	3.5	3.5	0.75
50 x TAE (ml)	0.28	0.28	0.28	0.28	0.1
Formamide (ml)	1.12	2.52	1.68	3.92	-
Urea (g)	1.17	2.65	1.76	4.12	-
Milli-Q water (ml)	to 14	to 14	to 14	to 14	4.1
10 % (w/v) Ammonium persulfate (µl)	126	126	126	126	50
TEMED (N,N,N',N'-tetramethylethane-1,2-diamine) (µl)	12.6	12.6	12.6	12.6	5

* premixed solution, Amresco, Solon, OH, USA

9 sequences of varying length were synthesised by PCR, mixed in equimolar concentrations and used as a molecular ladder alongside samples. Electrophoresis was performed at 75 V for 16 h at 60 °C in a running buffer of 1 x Tris-acetate-EDTA (TAE) solution. Gels were stained for 1 h in 300 ml 1 x TAE containing 3 µl SYBR®

Gold Nucleic Acid Gel Stain (Invitrogen), washed three times in Milli-Q water and imaged using a BioRad GelDoc imaging system.

2.7 Harvesting cells and preparation of cell free extracts

Cells (*E. coli* or strain La 6, for example) were harvested by centrifugation (6,000 g x 20 min, 4 °C), washed once in growth media and centrifuged again. Pellets were either frozen at -20 °C immediately for later use or gently re-suspended in as small a volume as possible of ice cold 40 mM phosphate buffer, pH 7.5 and 1 mM benzamidine chloride was then added. Cells were passed through a French pressure cell (American Instrument Company, Silver Spring, MD) three times at 110 MPa and maintained on ice. Cells were centrifuged (10, 000 g x 15 min, 4 °C) to separate the soluble and insoluble fractions. The supernatant was removed and either used immediately as the soluble extract or flash frozen in liquid nitrogen and stored at -80 °C. The pellet was washed twice and re-suspended in a small volume of 40 mM phosphate buffer, pH 7.5 and used as the insoluble/membrane fraction.

When working with small volume cultures (e.g. 5 ml), sonication was used for cell lysis. Cells were sonicated (3 × 15 s) using an ultrasonic processor VC50 sonicator (Jencons).

2.8 Protein analysis

2.8.1 Quantification

Total protein was quantified using the Qubit 2.0 according to the manufacturer's recommendations.

2.8.2 SDS-PAGE

Polypeptides were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5 % (w/v) resolving gel and a 4% (w/v) stacking gel. The gels were prepared as seen below and run using an X-cell SureLock apparatus (Novex).

	12.5 % resolving gel	4 % stacking gel
Milli-Q water	5.41 ml	3.17 ml
40 % (w/v) acrylamide/bis (37.5:1)*	3.125 ml	0.5 ml
3 M Tris-HCl pH 8.8	1.25 ml	-
0.5 M Tris-HCl pH 6.8	-	1.25 ml
10 % (w/v) SDS	100 µl	50 µl
10 % (w/v) Ammonium persulfate	75 µl	25 µl
TEMED (N,N,N',N'-tetramethylethane-1,2-diamine)	5 µl	5 µl

* premixed solution

Polypeptides were analysed using either whole cells, soluble extract or insoluble extract. After protein quantification, samples were boiled in a microcentrifuge tube for 8 minutes in SDS-PAGE sampling buffer (64 mM Tris, 5 % (v/v) β-mercaptoethanol, 10 % (v/v) glycerol, 2 % (w/v) SDS and 0.0025 % (w/v) bromophenol blue. Samples were placed immediately on ice for 3 minutes, centrifuged (17, 000 g x 2 min, 4 ˚C) and the supernatant loaded on to the gel. Approximately 20-25 µg protein was loaded per lane. The amount of protein loaded from the insoluble fraction was estimated by using the quantified amount in the soluble fraction and the very rough assumption that 20 % of total cell protein is either membrane bound or insoluble. PageRuler Unstained or PageRuler Plus Prestained protein ladders (Fermentas) were used as molecular markers. Polypeptides were run through the stacking gel at 90V and at 160V through the resolving gel in a running buffer containing Tris base, 3 g l^{-1}; glycine, 14.4 g l^{-1} and SDS, 1 g l^{-1}. Gels were stained using Coomassie stain (0.1 % (w/v) Coomassie brilliant blue R-250, 40 % (v/v) methanol, 10 % (v/v) acetic acid and 50 % (v/v) water) and destained in 40 % (v/v) methanol and 10 % (v/v) acetic acid.

2.8.3 Peptide Mass Fingerprinting

Bands of interest were excised and sent for analysis at the Proteomics Facility at the John Innes Centre. Samples were digested with trypsin and analysed by peptide mass fingerprinting using the Bruker Autoflex Speed Maldi-TOF/TOF. Polypeptides were identified using databases provided of the derived amino acid sequences from the whole genome sequence of each bacterial strain.

2.9 NAD(P)-independent alcohol dehydrogenase assay

Methanol dehydrogenase-like activity was assayed using the artificial electron acceptor phenazine methosulfate (PMS) coupled to the reduction of dichlorophenolindophenol (DCPIP) as described by Anthony & Zatman (1964). Unless otherwise stated, reactions (1ml) contained Tris buffer (pH9, 100mM), PMS (1mM), DCPIP (0.08mM), NH_4Cl (15mM), protein, and substrate (typically 10mM). Reactions were initiated by the addition of ammonium and followed spectrophotometrically at 600nm, using water as a blank. Reactions lacking protein, ammonium or substrate were also performed as controls. Significant transient activity occurred without substrate and was not subtracted from the substrate-induced activity (Day & Anthony 1990). Cell extract was kept on ice at all times and used for no longer than 3 hours. Activity was calculated using ε_{600} (molar extinction coefficient at 600nm) = 1.91 x 10^4 M^{-1} cm^{-1} for DCPIP (Basford & Huennekens 1955).

2.10 Measurements of substrates

2.10.1 Headspace methanol

Headspace methanol was measured by gas chromatography (GC) on an Agilent 7890A instrument, using a flame ionisation detector, a Porapak Q column (30 m x 0.530 mm, 40 μM film) and nitrogen carrier gas. The following settings were used:

Injector temperature:	300 °C
Detector temperature:	300°C
Column temperature:	115 °C
Injection volume	100 μl

The run time was adjusted to 7 minutes, with the retention time of methanol at 2.9 minutes. Standards were prepared in sterile water in the same volume and vials as the relevant experiment. The detection limit for methanol was around 0.5 mM.

2.10.2 Methanol in enrichment medium

For concentrations of ≤ 500 μM, methanol was measured using an method using alcohol oxidase (Sy et al. 2001), modified here for marine enrichment samples. Briefly, 600 μl sample was centrifuged (17, 000 g x 2 min, room temperature) and 500 μl of the supernatant transferred to a 2 ml microcentrifuge tube. 900 μl of Tris buffer (0.1 M Tris-HCl, pH 7.5) and 20 μl alcohol oxidase solution (10U/ml alcohol oxidase in 0.1 M phosphate buffer, pH 7.5) were added, vortexed and incubated for 1 h at 37 °C. 200 μl acetyl acetone solution (per 100 ml: 15.4 g $NH_4CH_3CO_2$, 200 μl acetyl acetone, 300 μl acetic acid) was added, vortexed and incubated for 20 min at 60°C. The reaction mixture was then measured spectrophotometrically at room temperature at 412 nm, with the reaction mixture containing Milli-Q water in place of sample as the blank. Standards were prepared with methanol dissolved in Milli-Q water at concentrations between 10-500 μM and treated in the same way as samples. The detection limit for methanol was around 5 μM. When performing the reaction in non-marine samples, the Tris buffer was replaced with 0.1 M potassium phosphate buffer, pH 7.5, which is more sensitive.

2.10.3 Headspace dimethylsulfide

Dimethylsulfide (DMS) in the headspace was measured using an Agilent 7890A instrument, using a flame photometric detector (GC 2010; Shimadzu, Milton Keynes, UK), CP-Sil 5CB column (30 m x 0.53 mm x 40 μm); Varian Inc., Oxford, UK) and a Gerstel multipurpose sampler (Anatune, Cambridge, UK) and nitrogen carrier gas (60 ml/min). The following settings were used:

Injector temperature	250°C
Detector temperature	200 °C
Column temperature:	175 °C
Injection volume	50 μl
Split ratio	2:1

The run time was adjusted to 6 minutes, with the retention time of DMS at 5.1 minutes. A calibration curve was made using standards made by lysing dimethylsulfoniopropionate to headspace DMS using 10 M NaOH.

2.10.4 Total dimethylsulfoniopropionate

Total dimethylsulfoniopropionate (DMSP) (in the medium and headspace combined) was quantified by lysing 300 µl culture with 100 µl of 10 M NaOH, left overnight in the dark and then the subsequent headspace DMS quantified by GC (as above).

2.10.5 Indole acetic acid in culture media

Aliquots of cell cultures grown in MB supplemented with 2.5 mM tryptophan were centrifuged (17, 000 g x 3 min, room temp) and the supernatant used for measuring indole-3-acetic-acid (IAA) using the method of Pilet & Chollet (1970). An equal volume of Salkowski reagent (12g l^{-1} $FeCl_3$ in 7.9 M H_2SO_4) was added to the supernatant and incubated at room temperature in the dark for 30 min. Controls included inoculated cultures without tryptophan and un-inoculated cultures with tryptophan. The mixture was then examined spectrophotometrically at 530 nm and the assay was calibrated by generating a standard curve for samples in MB (supernatant after centrifugation) containing 0-30 µg IAA.

2.10.6 Thiosulfate in culture media

Aliquots of cell cultures grown on (10 mM) acetate or succinate in MBM supplemented with 10 mM thiosulfate were centrifuged (17, 000 g x 3 min, room temp) and the thiosulfate in the medium was measured using the method described by González et al. (2003). 20 µl of Ellman's reagent (0.5 g l^{-1} 5,5'-dithiobis-2-nitrobenzoic acid in 50 mM potassium phosphate buffer, pH 7) was added to 350 µl of the supernatant and incubated at 20 ˚C in the dark for 30 min. Controls included inoculated cultures without thiosulfate and un-inoculated cultures with thiosulfate. Samples were made up to 925 µl with MBM and the thiosulfate then determined spectrophotometrically at 412 nm. The assay was calibrated by generating a standard curve of samples containing 0-15 mM thiosulfate.

2.11 Physiological characterisation

2.11.1 Gram stain, catalase and oxidase test

Gram staining was performed as described by (Gerhardt et al. 1994). The catalase test was performed by the addition of 3 % (v/v) hydrogen peroxide to colonies after 2 days of growth on MB plates. A single colony was tested on a few drops of 1 % N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride (Kovac's oxidase reagent) on filter paper for the oxidase test.

2.11.2 Carbon source utilisation

Utilisation of different carbon sources was tested by monitoring cell density increases in OD_{540} in duplicate compared to controls with no inoculum at 25 ˚C, shaking at 150 r.p.m. Cultures were grown for a minimum of 8 days. Substrate concentrations are detailed in the relevant chapter.

2.11.3 Temperature, pH and salinity ranges

Growth at different temperatures, pH and salinity were monitored by increases in OD_{540} in triplicate compared to controls with no inoculum for 8 days. Temperatures of 4, 8, 10, 15, 20, 25, 30, 37, 40, 42 and 45 ˚C in MB were tested. Growth at different pH was tested in MB at every pH increase of 0.5 between 3.5-10, using 10 % (v/v) HCl and 10 M NaOH to adjust the pH. Each pH medium was used to blank its respective test condition. Growth at different NaCl concentrations was tested using an artificial salt water (ASW) media, as described by (Cho & Giovannoni 2003) and shown below.

ASW medium:

Per litre of media (pH 8):

1·0 g $MgCl_2$ $6H_2O$

5·0 g $MgSO_4$ $7H_2O$

0·7 g KCl

0·15 g $CaCl_2$ $2H_2O$

0·5 g NH_4Cl

0·1 g KBr

0·27 g KH_2PO_4
0·04 g $SrCl_2$ $6H_2O$
0·025 g H_3BO_3
5·0 g peptone
1·0 g yeast extract

Concentrations of NaCl were added at 1, 2, 2.5, 3, 3.5, 4% (w/v), and then at each 1% increase to 10%, then 12, 15 and 20 % NaCl.

2.11.4 Sensitivity to antibiotics

Sensitivity to antibiotics was tested by two methods: inoculating in 10 ml MB media and monitoring for cell growth by OD_{540} and by using the disc method. Overnight cultures (100 µl) were spread on MB plates; discs were impregnated with antibiotics and placed onto the plate surfaces and incubated at 30 ˚C for 5 days. The following antibiotics were tested at 20, 50 and 100 µg/ml: chloramphenicol, nalidixic acid, kanamycin, tetracycline, streptomycin, ampicillin, puromycin, erythromycin, vancomycin, rifampicin, gentamycin, and cyclohexamide.

2.11.5 Dimethylsulfide production

Production of dimethylsulfide (DMS) in the headspace was tested by inoculating strain La 6 into 300 µl MBM containing 5 mM succinate in gas tight 2 ml vials, incubated overnight at 30 ˚C and assayed for DMS by GC (see in section 2.10 Measurements of substrates). Un-inoculated media containing succinate served as controls. After DMS quantification, cell cultures were centrifuged (4 min x 17, 000 g at 4 ˚C), resuspended and sonicated using an ultrasonic processor VC50 sonicator (Jencons) for 10 sec x 3 in 50 mM phosphate buffer, pH 7.5. Samples were centrifuged again and the protein in the supernatant measured. DMSP production was expressed as pmol min^{-1} μg^{-1} protein.

2.11.6 Dimethylsulfoniopropionate degradation

Degradation of dimethylsulfoniopropionate (DMSP) was examined by the same method as for DMS production, but with the addition of 1 mM DMSP to the medium. DMSP was quantified by lysis to DMS, as described in section 2.10 (Measurements of

substrates). Protein was quantified as described for DMS production and also expressed as DMS produced in pmol ug protein^{-1} min^{-1}.

2.11.7 Dimethylsulfoniopropionate production

Production of dimethylsulfoniopropionate (DMSP) was examined by the same method as for DMS production except that DMSP was then quantified by lysis to DMS, as described in section 2.10 (Measurements of substrates). Protein was quantified as described for DMS production and was expressed as DMSP in pmol ug protein^{-1} min^{-1}.

2.11.8 Carbohydrate oxidation and fermentation

The ability to metabolise glucose and lactose by fermentation or oxidation was tested using Hugh and Leifson's OF basal medium (Hugh & Leifson 1953), with replacement of the NaCl with Sea Salts (Sigma-Aldrich). The following were dissolved in 1 litre deionised water: 2 g peptone, 20 g Sea Salts, 0.03 g bromthymol blue, 3 g agar, 0.3 g K_2HPO. The solution was adjusted to pH 7.1 prior to autoclaving. A 10% (w/v) glucose or lactose solution was filter sterilised and added to a final 1% (w/v) concentration.

Overnight cultures were stabbed 2 cm below the surface of 5 ml agar in test tubes (13 x 100 mm). 1 cm of mineral oil was overlaid for testing for fermentation. Tubes were incubated for 3 days at 30 ˚C and checked for colour changes compared to an un-inoculated control and a glucose fermenting strain (*E. coli*).

2.11.9 Gelatin hydrolysis

Gelatinase activity was tested by stab inoculating an overnight culture into a medium containing gelatin. The following were dissolved in 1 l deionised water: 20 g Sea Salts, 5 g peptone, 3 g beef extract, 120 g gelatin. The pH was adjusted to 6.8. For each test, 3 ml was dispensed into test tubes (13 x 100 mm), autoclaved and allowed to cool in an upright position. Overnight cultures were stabbed 2 cm below the surface and incubated for 5 days at 30˚C. Cultures tested positive for gelatinase activity if the medium remained liquid after chilling on ice for 1 h, compared to the un-inoculated control.

2.11.10 Cellulase, xylanase and amylase activity

Cellulase, xylanase and amylase activities were all investigated by monitoring for clearing zones around 5 µl of overnight culture which was spotted onto the surfaces of agar containing the relevant substrate. All plates were incubated for 7 days at 30 ˚C. Water-inoculated and no carbon source plates served as negative controls. Cellulose degradation was tested as described by Kauri & Kushner (1985); MBM plates contained both 10 mM succinate and 0.5 % Avicel (microcrystalline cellulose, type PH-105; FMC Corporation) or only Avicel as the carbon source. Half of the plates then had roughly 3 mm of MBM agar (no carbon source) poured on top. *Sagittula stellata* E-37 was used as a positive control.The same method was performed for xylanase activity, using 0.5% birchwood xylan (Sigma), but with no overlaid agar plates. Amylase activity was assayed by streaking a colony onto MB agar plates containing 0.5 % soluble starch (Sigma-Aldrich), incubating as previously mentioned and flooding the plate with Gram's iodine solution.

2.11.11 Motility

Three different types of motility were tested, using varying agar concentrations. 25 ml MB plates were made containing 0.3 % (w/v) agar for swarming motility, 0.5 % for swimming motility and 1 % (w/v) agar for twitching motility. 5 µl of overnight culture was placed on top of the agar of swarm plates, the same volume stab inoculated inside the centre of the agar of swim plates, and a colony stabbed to the bottom of the 'twitching plates'. Plates were incubated at 30˚C for 48 h and checked for motility rings compared to the water-inoculated controls. *Ruegeria pomeroyii* DSS-3 was used as a positive control for swimming motility. Cells were also examined under 1000 x magnification using phase-contrast for motility in MB and MBM media.

2.11.12 Bacteriochlorophyll a and pigment production

The production of bacteriochlorophyll a or other pigments was investigated using the method of (Shiba et al. 1991). Briefly, triplicate 20 ml MBM cultures in 120 ml serum vials containing 2 mM succinate were incubated in either a 12 hour light/dark cycle or in the dark. Cultures were incubated at room temperature, shaking 150 rpm for 7 days. 15 ml cell culture was centrifuged (5,000 x g, 15 min) and 1 ml of an acetone-methanol

(7:2 v/v) mixture added. The samples were centrifuged again and the absorption spectrum of the supernatant measured between 600-100 nm, with bacteriochlorophyll a pigment expected around 770 nm (Shiba et al. 1991). Negative controls with no inoculum and positive controls with *Roseobacter denitrificans* Och114 were performed.

2.11.13 Nitrate and nitrite reduction

The ability to reduce nitrate or nitrite was tested using nitrate/nitrite broth supplemented with Sea Salts. The following were dissolved in 1 l deionised water: 5 g peptone, 3 g beef extract, 20 g Sea Salts, 1 g KNO_3 or KNO_2 and the pH was adjusted to pH 7. For each test, 6 ml was dispensed into test tubes (13 x 100 mm), a Durham tube added and autoclaved. 1 ml of overnight culture was inoculated, fitted with SubaSeal stoppers and incubated for 3 days at 30 ˚C. 1 ml aliquots were centrifuged (17, 000 g x 3 min, room temp) and 100 µl of the supernatant added to 100 µl of Greiss' Reagent (Sigma) to assess for reduction of nitrate to nitrite. Zinc dust was then added to reduce any nitrate to nitrite for further confirmation. Non-inoculated and *E.coli* inoculated cultures were tested alongside as controls.

2.12 Cellular fatty acid analysis

100 mg wet biomass of an exponentially growing culture in MB was analysed by DSMZ using the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.).

Chapter 3

Isolation and characterisation of *Methylophaga AH1* strain L4

3 Isolation, and characterisation of *Methylophaga AH1* strain L4

3.1 Introduction

Previous research has shown that methanol in the oceans can reach up to concentrations of 420 nM (Joanna L Dixon et al. 2011; J. L. Dixon et al. 2011; Beale et al. 2013; Dixon et al. 2013; Beale et al. 2011; Read et al. 2012; Williams et al. 2004 and Kameyama et al. 2010). There are large uncertainties as to whether the ocean is a source or sink of methanol, however current research suggests there may still be an unidentified *in situ* marine source in open ocean waters (Joanna L Dixon et al. 2011). Thus the extent to which microbes are involved in the production and consumption of methanol in the marine environment is vital to further understand the global cycling of methanol.

Huge seasonal variablities in the structures of microbial communities have been found to occur at Plymouth, station L4, and community structure could be correlated with environmental parameters such as temperature and nutrient concentration (Mary et al. 2006; Gilbert et al. 2009). Moreover active marine methylotrophs are associated with phytoplankton blooms in the English Channel (Neufeld, Boden, Helene Moussard, et al. 2008), whilst as yet uncultivated *Methylophaga* have been shown to be present during methanol and methylamine DNA-SIP experiments using seawater from the same location (Neufeld et al. 2007; Neufeld, Chen, et al. 2008; Grob et al. 2015).

Therefore there was the potential for the isolation of a novel methylotroph through enrichment experiments. The primary aim of this work was to isolate novel methylotrophs from the coastal waters off Plymouth, and to characterise them with respect to their metabolic and genetic capabilities, as described in Objective 1 of this thesis. A novel species of *Methylophaga* was isolated, physiologically characterised and the genome sequenced (along with another already isolated strain) in order to compare against its closest relatives.

3.1.1 Sampling site

Station L4 is located approximately 10 nautical miles south-west of Plymouth (Figure 3.1) and is used by the Western Channel Observatory (WCO) in research as it represents a typical coastal environment, influenced by tidal, estuarine and human activities. It has

one of the longest sampling programmes in the world for zooplankton and phytoplankton, with weekly sampling dating back to 1988. Many environmental parameters are also monitored weekly by scientists at Plymouth Marine Laboratory (PML), including suspended particulate matter, chlorophyll, alkalinity, nutrients, temperature, and dissolved oxygen. These data provide us with a wealth of knowledge of this marine environment. Moreover, *in situ* measurements performed by Beale et al (2015) at station L4 showed that there is a standing concentration of 16-78 nM methanol, suggesting there is a substantial source of carbon available for the growth of methylotrophs.

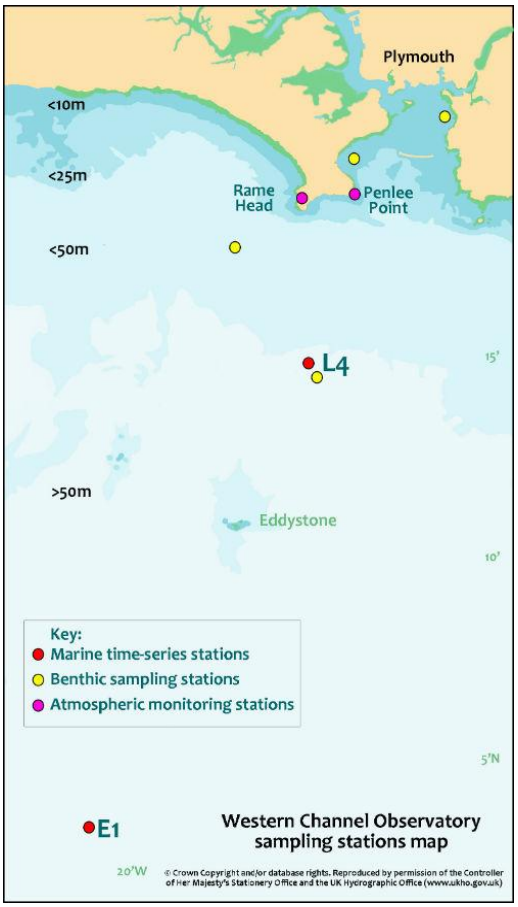

Figure 3.1 Map of the coast of Plymouth showing sampling stations of the Western Channel Observatory, including station L4. Image taken from the Western Channel Observatory website: http://www.westernchannelobservatory.org.uk/

3.2 Isolation and preliminary characterisation of a new *Methylophaga* sp.

3.2.1 Isolation

Strain L4 was isolated from surface water from the Western Channel Observatory (WCO) station L4 (50°15.0'N; 4°13.0'W) on 20th December 2012, kindly provided by Jo Dixon of Plymouth Marine Laboratory (PML).

Samples were collected in sterile carboys and transported within 3-4 hours of its collection to the laboratory (overnight transportation at room temperature). 50 ml enrichment cultures were established with addition of 1% (v/v) MAMS media and 5 mM methanol. Enrichments were incubated at 25°C in a shaking incubator (50 r.p.m.) for 5 days, serial dilutions were then plated onto MAMS medium and incubated with headspace methanol in a gas tight chamber for 8 days. Colonies were re-streaked to purify and growth on methanol was confirmed by inoculation into liquid MAMS with 5 mM methanol, to rule out possible growth on the agar or trace organic compounds within the agar. Strains that exhibited growth at an $OD_{540} \leq 0.05$ more than non-inoculated controls were investigated further.

Several strains of *Methylophaga* were isolated growing on methanol, as identified by 16S rRNA gene sequencing of strains from the enrichment. All 8 *Methylophaga* strains had ≥ 97% identity to a previously isolated and characterised *Methylophaga* species, whilst 2 strains had 99% identity to *Dyadobacter tibetensis*. To determine which isolates were of particular interest, all isolates were further tested for characteristics of classic methylotrophs. *Methylophaga* species are well characterised methylotrophs that are routinely isolated from methanol enrichments, however there are currently no strains of *Dyadobacter* that have been described as methylotrophs. As many methylotrophs are also able to grow using methylamine as sole carbon and energy source, the ability of the isolates to grow on 5 mM methanol and methylamine was examined in 20 ml cultures in 120 ml serum vials. They were all also screened for the key functional genes involved during growth on methanol and methylamine using colony PCR (using primers listed in Table 2.2 in Methods and Materials chapter); *xoxF* and *mxaF* for methanol metabolism and *gmaS* and *mauA* for methylamine metabolism. Table 3.1 summarises the data from

sequencing of the isolates 16S rRNA genes from isolates, the growth experiments and PCR assays.

Table 3.1 Phylogeny and basic characterisation of isolates from Plymouth L4.

Strain	**Phylogeny**[1]	**Identity**[2] **(%)**	**Growth on carbon sources**		**Presence of functional genes**[3]			
			MeOH	MMA	*mxaF*	*xoxF*	*gmaS*	*mauA*
1	*Dyadobacter tibetensis*	99	-	-	-	-	-	-
2	*Methylophaga thiooxydans*	99	+	+	+	+	+	+
4	*Methylophaga thiooxydans*	99	+	+	+	+	+	+
5	*Methylophaga thiooxydans*	99	+	+	+	+	+	+
6	*Methylophaga thiooxydans*	99	+	+	+	+	+	+
7	*Dyadobacter tibetensis*	99	-	-	-	-	-	-
8	*Methylophaga thiooxydans*	99	+	+	+	+	+	+
AH1 L4	*Methylophaga sulfidovorans*	97	+	-	+	+	+	-
10	*Methylophaga sulfidovarans*	97	+	-	+	+	+	-
11	*Methylophaga nitratireducenticrescens*	99	+	+	+	+	+	+

Abbreviations: MeOH, methanol; MMA, methylamine. Carbon sources were supplied at 5 mM.
[1]Organisms shown are type strains.
[2]Identity refers to 16S rRNA gene sequence identity over ≤700 bp.
[3]Presence or absence was based on the amplification and sequencing of a PCR product on a single colony

Isolates 1 and 7 did not grow on methanol or methylamine and had no functional genes involved in methylotrophy and so were therefore considered to be false positives and investigated no further.

Isolates 2, 4, 5, 6 and 8 are all related to *Methylophaga thiooxydans* (99% identity), were able to grow on methanol and methylamine, and have both sets of genes present involved in methanol and methylamine metabolism. *Methylophaga thiooxydans* is known to have these characteristics (Boden et al. 2010; Boden, Ferriera, et al. 2011) and because the isolates were very likely the same strain that had been isolated multiple times (all shared 99.9% 16S rRNA identity to each other), they were considered to be very closely related strains of *M. thiooxydans* DMSO10, and not examined further.

Isolate 11 had 99% identity to *M. nitratireducenticrescens* at the 16S rRNA level, was able to grow on methylamine and also had both sets of genes involved in methanol and methylamine metabolism. However *M. nitratireducenticrescens* did not grow on methylamine (Villeneuve et al. 2012) and analysis of its genome reveals that it did not have *mauA* or *gmaS* sequences. Isolate 11 may therefore be a new strain of *Methylophaga thiooxydans*.

Isolate 9 (*AH1* L4) had the lowest identity to the 16S rRNA genes out of all isolated strains, and was most closely related to *Methylophaga sulfidovorans* RB-1 at the 16S rRNA sequence level (97% identity). *M. sulfidovorans* was able to grow on methylamine, whilst isolate 9 could not. They both contain *gmaS* but no *mauA*, and both contain the MDH genes *mxaF* and *xoxF*.

The generally recommended and accepted criteria for delineating bacterial species is having a 16S rRNA gene sequence dissimilarity greater than 3 % (or having a DNA–DNA relatedness of less than 70 % as measured by hybridization (Erko & Ebers 2006). Due to the low 16S rRNA identity with its closest relative and its inability to grow on methylamine (discussed in more depth later), it is therefore proposed that the strain represents a novel species, *Methylophaga AH1* sp. nov. (type strain L4^{T}). *Methylophaga* AH1 falls within the *Methylophaga* genus at the phylogenetic level, see Figure 3.2.

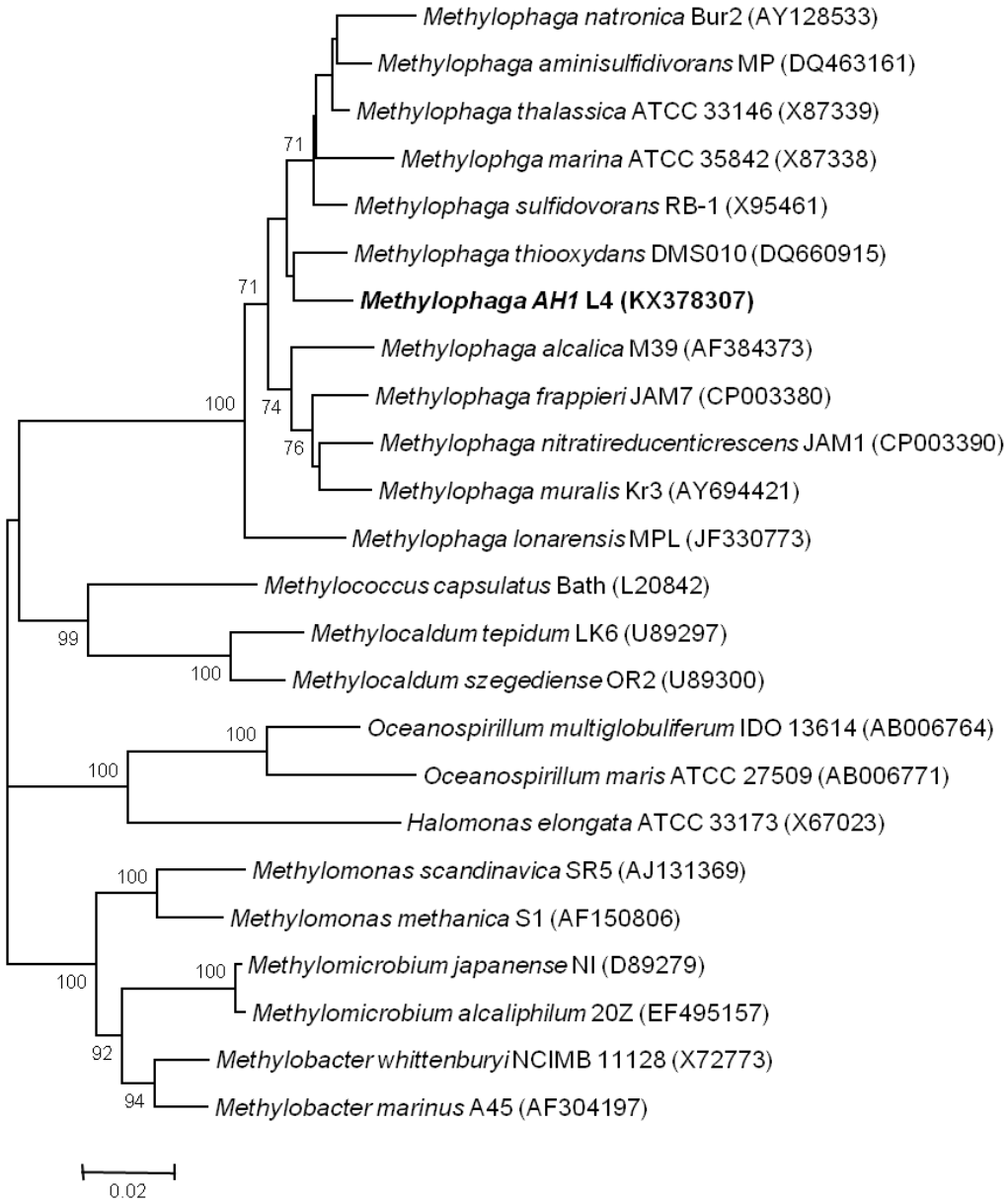

Figure 3.2 Phylogenetic analysis of 16S rRNA gene sequences of species of the genus *Methylophaga*. Evolutionary distance among 16S rRNA gene sequences of species *AH1* (bold) and all type strains of the species within the genus *Methylophaga* is illustrated by an unrooted neighbour-joining phylogenetic tree. The tree was inferred from a matrix of pairwise distance using aligned sequences containing minimum 1,400 bp positions using MEGA6. The numbers at the branches indicate the percentage of 1,000 bootstrap resamplings. Bootstrap values greater than 70% are shown. Numbers in parentheses are GenBank accession numbers. 16S rRNA sequences from representatives from the Gammaproteobacteria are also shown. The scale bar indicates nucleotide substitutions per position. A phylogenetic tree constructed using the maximum-liklihood method showed a similar topology.

3.2.2 Cell and colony morphology

Methylophaga AH1 is a Gram-negative, motile rod, 1.5-2 µm long and 0.4-0.8 µm wide in minimal media (Figure 3.3). Colonies are very pale cream, and 0.5-1 mm in diameter, uniformly circular, convex and slightly translucent after growth on MAMS minimal media at 25 °C for 4 days.

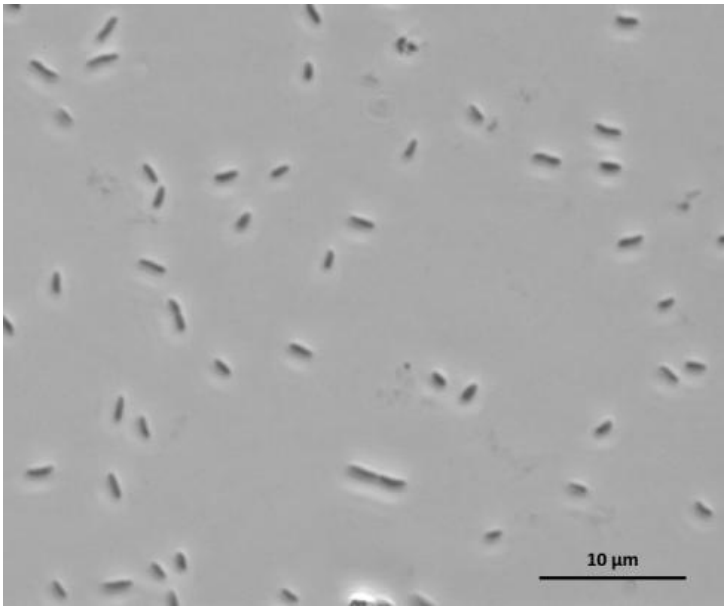

Figure 3.3 Micrograph of *Methylophaga AH1* under 1000 x magnification.

3.3 Substrate utilisation profile of *Methylophaga AH1* strain L4

3.3.1 Use of MMA as a nitrogen source, not a carbon source

Justification for the designation of strain L4 to a new species, *Methylophaga AH1*, was based on the fact that it does not grow on MMA since many of the *Methylophaga* strains do. There are two possible pathways for the oxidation of MMA by Gram negative bacteria; the direct conversion of MMA into formaldehyde (releasing NH_4^+) by a methylamine dehydrogenase (MaDH) (Anthony 1982) or by an indirect pathway in which MMA is converted to tetrahydrofolate-bound formaldehyde via gammaglutamylmethylamide (GMA) and N-methylglutamate (NMG) (Latypova et al. 2010; Chistoserdova 2011b); see Figure 3.4. In this pathway, the methyl group of MMA is transferred to glutamate by the enzyme GMA synthetase (*gmaS*), producing GMA.

This is then converted into NMG by NMG synthase, and then lastly into tetrahydrofolate-bound formaldehyde by NMG dehydrogenase.

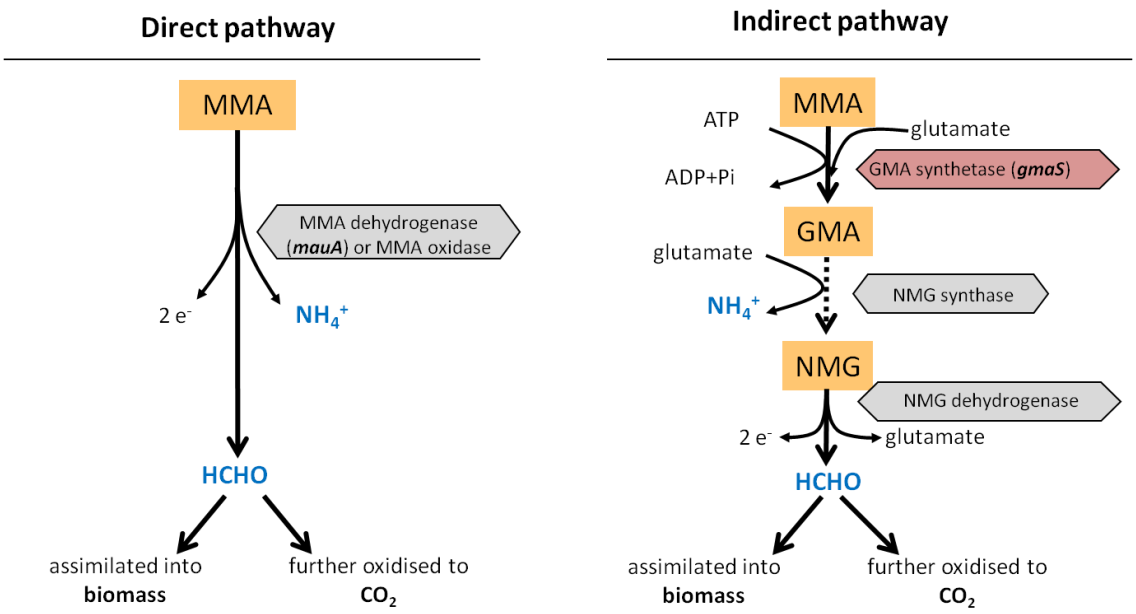

Figure 3.4 Schematic of the direct (left) and indirect (right) pathways used by Gram negative methylotrophs for the oxidation of MMA. MMA, monomethylamine; GMA, gamma-glutamylmethylamide; NMG, N-methylglutamate. Figure taken from Wischer, 2014.

Work conducted by Chen et al. (2010) on the indirect pathway showed that there are many non-methylotrophic bacteria which use this pathway for the metabolism of MMA as a nitrogen source. As shown in Table 3.1, colony PCR specific for *gmaS* and *mauA* indicated that *Methylophaga AH1* does have *gmaS* but not *mauA* and that it did not use MMA as a sole carbon source. Therefore the genomes of all available *Methylophaga* species were screened by BLAST (blastn and tblastn) for both genes, and then compared to whether they were able to grow on MMA as a carbon source (from the literature). Table 3.2 summaries the BLAST and growth data.

Table 3.2 Methylamine metabolism and functional gene markers in *Methylophaga* spp.

Strain	Growth on MMA	*gmaS*	*mauA*
M. AH1	-	+	-
M. sulfidovorans	+	+	-
M. marina	+	+	+
M. thiooxidans	+	+	+
M. aminisulfidivorans	+	+	-
M. lonarensis	-	+	+
M. frappieri	-	-	-
M. nitratireducenticrescens	-	-	-
M. alcalica	+	N/A	N/A
M. muralis	+	N/A	N/A
M. natronica	+	N/A	N/A
M. thalassica	+	+*	-*

Summary of the ability of all type strains of each species within the *Methylophaga* genus to grow on methylamine (MMA) as sole carbon source, and the presence or absence of the functional genes for the direct pathway using methylamine dehydrogenase (*mauA*) and the indirect pathway using gammaglutamylmethylamide synthetase (*gmaS*) of MMA metabolism. Functional gene results are based on BLAST results of the genome sequences. *Results are based on enzyme assays results from Janvier et al. (1985). N/A indicates that no genome sequence is available and no PCR or assay has been performed.

Although there are unfortunately some data missing from the literature, based on current available data, there are no other species of *Methylophaga* which cannot grow on methylamine as a carbon source and which do not have *mauA* but do contain *gmaS.* Based on this analysis it seems that is no obvious way of predicting whether any strain is unable to grow on MMA based solely on the presence or absence of *mauA* and *gmaS; M. sulfidovorans*, *M. aminisulfidivorans* and *M. thalassica* contain only *gmaS*, as does *Methylophaga AH1*, but are all able to grow on it, whilst *M. lonarensis* has both present and is unable to grow on MMA.

As noted earlier, however, some methylotrophs that have *gmaS* are able to use MMA as a nitrogen source, whilst growing on an alternative carbon source. Therefore the ability of *Methylophaga AH1* to use MMA as a nitrogen source was assessed. Triplicate cultures of 20 ml MAMS in 120 ml serum vials containing either methanol and MMA or methanol and NH_4Cl (standard MBM medium) were monitored for growth spectrophometically. Inoculated vials containing methanol only (no nitrogen source),

MMA only (no additional nitrogen source) and MMA and NH_4Cl served as controls for growth on contaminating nitrogen, growth on MMA as sole carbon and nitrogen source, and growth on MMA as sole carbon source but not as a nitrogen source, respectively. *M. marina* was used as a positive control for strains able to use MMA as a nitrogen source. Growth data are summarised in Figure 3.5.

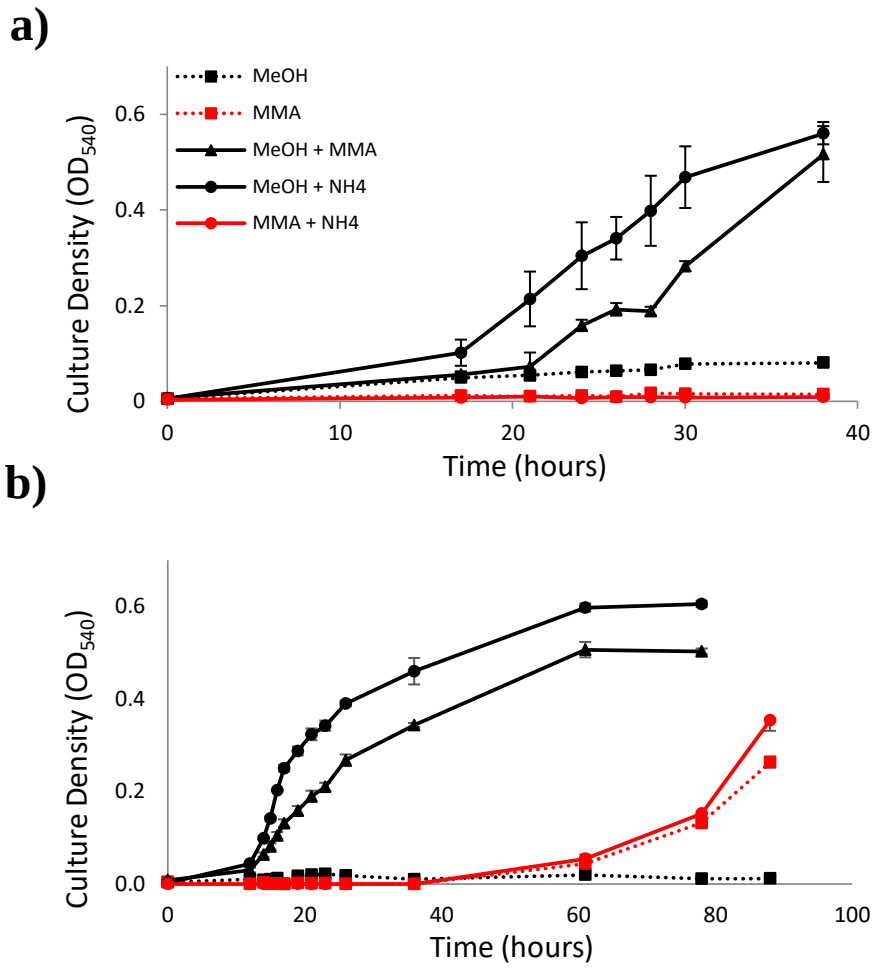

Figure 3.5 Growth of *Methylophaga AH1* (a) and *Methylophaga marina* (b) on methanol or methylamine as sole source of carbon and/or nitrogen. Abbreviations in legend: MeOH, methanol; MMA, methylamine; NH4, ammonia added as NH_4Cl. The legend applies to both graphs. *Methylophaga AH1* did not grow under any of the conditions tested after an additional 60 hours of monitoring (data not shown). Error bars show standard error of triplicate cultures.

Methylophaga AH1 grew on methanol with MMA supplied as a nitrogen source, but was unable to grow with it as sole carbon source (either as sole carbon and nitrogen, or as just sole carbon source with NH_4Cl as a nitrogen source). *Methylophaga marina* is an example of one of the species containing only *gmaS* but is able to grow on MMA as

sole carbon source and nitrogen source (De Zwart et al. 1996). It is therefore fairly surprising that *M. AH1*is unable to also do the same.

3.3.2 Growth on other carbon compounds

In order to assess if *Methylophaga AH1* was substantially different from its closest relative, *M. sulfidovorans*, and other characterised *Methylophaga* spp. to classify it as a new species, its ability to grow on other carbon sources was examined. Growth was evaluated in 25 ml cultures in 120 ml serum vials in triplicate, using methanol-grown cells as starter inoculum. Growth data are summarised in Table 3.3 alongside data from all other validly published *Methylophaga* type strains as a comparison.

Table 3.3 Growth of all *Methylophaga* spp. on a range of carbon compounds.

Concentration (mM) **Strain**	**MMA (10)**	**DMA (10)**	**TMA (10)**	**DMS (2)**	**DMSO (20)**	**Fructose (5)**	**Formate (5)**
M. AH1	-	-	-	-	-	-	-
M. sulfidovorans	+	+	-	+	-	-	-
M. marina	+	+	-	-	N/A	+	-
M. thiooxydans	+	+	+	+	-	+	-
M. aminisulfidivorans	+	+	+	+	+	+	N/A
M. lonarensis	-	-	-	-	N/A	-	N/A
M.frappieri	-	N/A	N/A	N/A	N/A	-	N/A
M.nitratireducenticrescens	-	N/A	N/A	N/A	N/A	-	N/A
M. alcalica	+	-	-	-	-	-	N/A
M. muralis	+	-	+	-	-	+	-
M. natronica	+	+	-	-	N/A	+	
M. thalassica	+	+	+	-	-	+	-

Numbers in brackets are the substrate concentrations used to test *M. AH1*. Data are taken from previously published work: *M.sulfidovorans* (De Zwart et al. 1996), *M. marina* (Janvier et al. 1985b; Li et al. 2007)*; M. thiooxydans* (Boden et al. 2010), *M. aminisulfidivorans* (Kim et al. 2007), *M. lonarensis* (Antony et al. 2012), *M. frappieri* (Villeneuve et al. 2012), *M.nitratireducenticrescens* (Villeneuve et al. 2012), *M. alcalica* (N. V. Doronina et al. 2003), *M. muralis* (Doronina et al. 2005), *M. natronica* (N. Doronina et al. 2003), *M. thalassica* (Janvier et al. 1985b). All strains are can grow on methanol (data not shown).

M. AH1 was unable to grown on any carbon sources tested (except methanol) whereas *M. sulfidovorans* is able to grow on MMA, DMA and DMS as a sole carbon source. Based on these data, *M. AH1* seems to be an obligate methylotroph and is more similar

in its metabolic capabilities to *M. lonarensis*, *M. frappieri* and *M. nitratireducenticrescens* than *M. sulfidovorans*, although some data are lacking in previously published work. This suggests that *M. AH1* is indeed a new species.

3.4 Effect of p-nitrophenylhydrazine during growth on methanol

Inhibition of bacterial oxidation of methanol by both p-nitrophenylhydrazine (p-NPH) and cyclopropanol has been reported in the literature (Anthony & Zatman 1964b; Mincey et al. 1981). These compounds were of interest as it may be possible to use these as a control to show directly the role of MDH in the oxidation of methanol. Moreover if one of these was found to inhibit the canonical MDH (encoded by *mxaF*) but not *xoxF*, this might also be used to show the extent to which *xoxF* plays a role as an active MDH. P-NHP has been shown to cause 100% inhibition of methanol oxidation by whole cells at 10μM and at 50% with 1μM (Anthony & Zatman 1964b) whilst cyclopropanol has been shown to inhibit methanol oxidation on cell extracts (Mincey et al. 1981).

Therefore p-NPH was used to assess inhibition of the growth of *Methylophaga AH1*, as *Methylophaga AH1* contains both forms of MDH. The effect on the growth of *Methylophaga* AH1 on the addition of the metal lanthanum was also examined, as recent research has shown the role of rare earth elements (REEs) in the catalytic site of methylotrophs utilising the MDH XoxF (Keltjens et al. 2014; Farhan Ul-Haque et al. 2015; Vu et al. 2016). Growth of *M. AH1* was evaluated in 30ml MAMS cultures in triplicate containing methanol (50mM) with either 5 μM lanthanum, 20 μM p-NPH, or both. Cultures containing only *M. AH1* and methanol served as controls. Growth data are summarised in Figure 3.6.

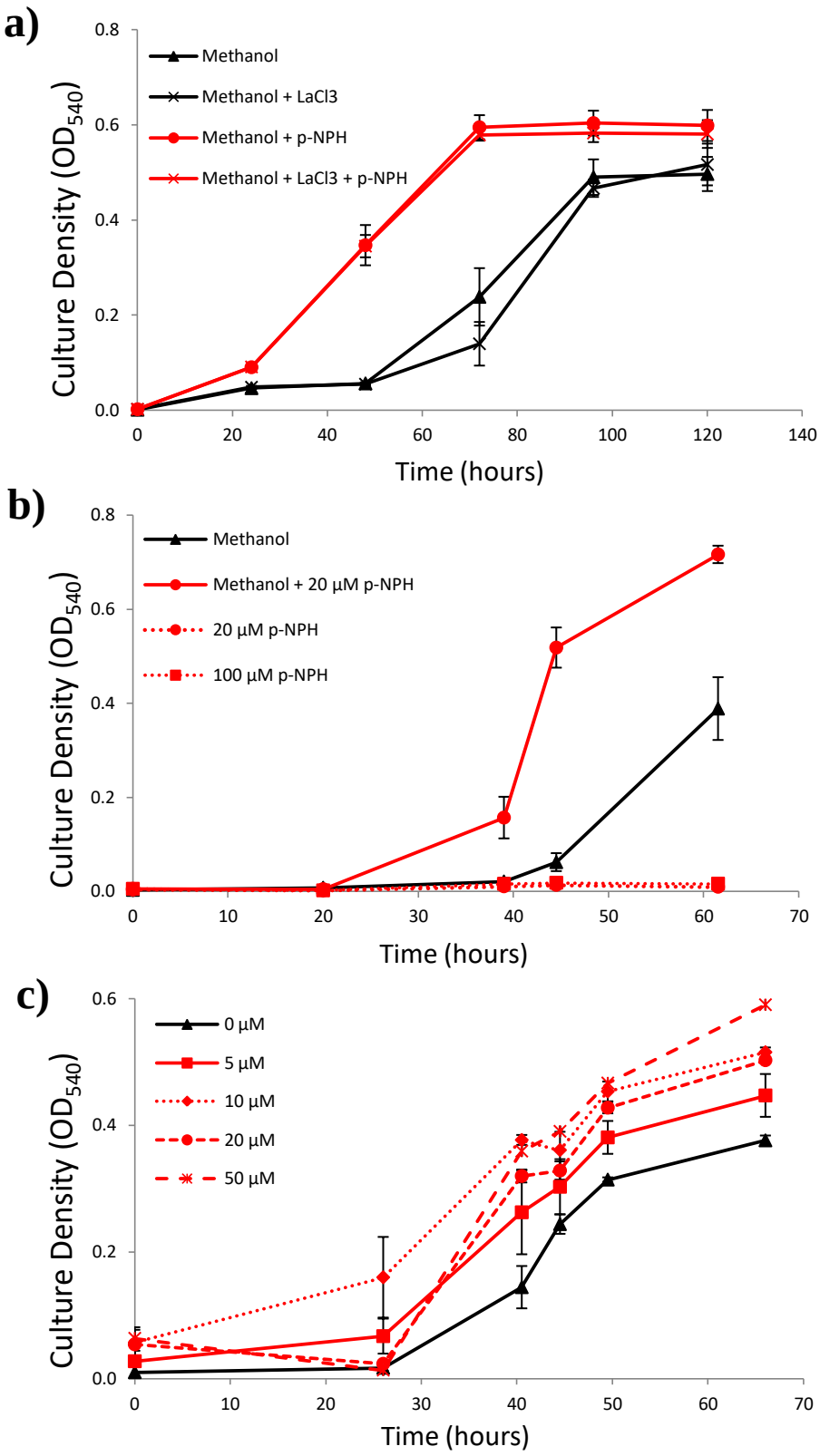

Figure 3.6 The effect of p-nitrophenylhydrazine (p-NPH) on the growth of *Methylophaga AH1* during growth on methanol. a) Preliminary tests on the effect of the addition of 20 µM p-NPH and 5 µM lanthanum on *AH1*. b) The effect of two different concentrations of p-NPH on the growth of *AH1* in the presence and absence of methanol. c) The effect of increasing concentrations of p-NPH on *AH1*. Unless otherwise shown, the concentration of p-NPH is 20 µM, methanol is 50 mM and lanthanum chloride ($LaCl_3$) is 5 µM. Error bars show the standard error of triplicate cultures.

Surprisingly, initial tests on *Methylophaga AH1* showed that the strain was able to grow better when p-NPH was present, whilst the addition of lanthanum had no effect (Figure 3.6a). Tests to see if p-NPH was serving as a carbon source for *M. AH1* revealed that it was stimulating growth on methanol, rather than acting as a carbon source (Figure 3.6b). Further experiments using increasing concentrations of p-NPH confirmed the previous results (Figure 3.6c), showing an increase in growth with increasing p-NPH concentrations. Given that previous research showed inhibition of methanol oxidation, these data are rather surprising. Further research would need to be conducted in order to assess the molecular mechanism of how p-NPH is causing this stimulation.

3.5 Genome analysis of *Methylophaga AH1* strain L4 and *Methylophaga marina*

Given the previously discussed data suggesting that *M. AH1* is a new species of *Methylophaga*, strain L4 was sent for genome sequencing. *M. marina* was also sent for genome sequencing, as although it has been considered a type strain for many years, little is known about its genetics. Therefore both genomes were analysed and discussed here. Genome statistics are summarised in Table 3.4.

Table 3.4 General genome statistics of *Methylophaga AH1* strain L4 and *Methylophaga marina*

Genome data	***Methylophaga AH1* strain L4**	***Methylophaga marina***
Number of contigs	4	8
Genome size (bp)	2,874,120	3,045,419
Smallest contig (bp)	5,389	782
Largest contig (bp)	1,295,104	765,050
Average contig size (bp)	718,530	380,677
Median contig size (bp)	850,760	432,709
N50	850,760	761,829
L50	2	2
GC content (%)	42.3	43.8
Number of genes	2,824	2,985
Number of Coding Sequences (CDS)	2,779	2,936
Number of hypothetical proteins (%)	580 (20)	585 (20)
tRNAs	39	37
rRNAs	6	12

3.5.1 Overview of the general metabolic pathways in *M. AH1* and *M. marina*

Local nucleotide database files of the genome sequences of *M. AH1* and *M. marina* were created using BioEdit software. BLAST searches against these databases and use of the KEGG (Kyoto Encyclopedia of Genes and Genomes) recruitment plots created by the RAST server provided a framework for establishing the potential metabolic pathways. The work is based solely on genetic inference and is not supported by experimental evidence.

Both genomes had an incomplete tricarboxylic acid cycle (TCA) pathway, missing 6-phosphofructokinase, like all other *Methylophaga* species. They were also missing the 2-oxoglutarate dehydrogenase complex, also like the other *Methylophaga* members. They contained all genes of the pentose phosphate pathway, Entner-Doudoroff and Ribulose Monophosphate (RuMP) pathways. They also contained all genes required for ammonia assimilation (GOGAT). They both contained all genes required for both the tetrahydromethanopterin (H_4MPT) and tetrahydrofolate (H_4F) pathways for formaldehyde handling.

3.5.2 Methylotrophy gene clusters in *M. AH1* and *M. marina*

BLAST searches of the genomes of both *Methylophaga* species revealed they both contained the full *mxaFI* gene cluster, in the classic gene order as seen in other *Methylophaga* genomes, seen in Figure 3.7 (Grob et al. 2015). Searches for the *xoxF* gene also revealed that both genomes encoded a total of five *xoxF5* genes, with almost identical genetic organisation to each other. Three *xoxF* genes are found together in one cluster, along with the genes encoding for PQQ synthesis (see Figure 3.8). Another is separate but is next to the associated *xoxFJ*, whilst the last is alone, with no methylotrophy-associated genes nearby. The one difference between the genetic organisation between the genomes is the presence of a DNA-binding response regulator, LuxR family protein between two *xoxF* genes. To my knowledge, this is the highest number of *xoxF* genes found in the genome of any bacteria, further stressing the importance of the role of *xoxF*.

Lastly, searches for methylamine related genes revealed the gene cluster encoding for the full N-methylglutamate pathway (NMG) for methylamine metabolism in both

genomes, whilst *M. marina* also contained the full cluster encoding for methylamine dehydrogenase, as expected (see section 2.3).

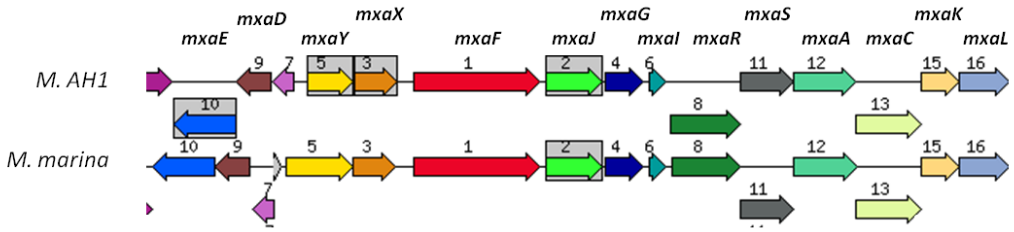

Figure 3.7 Gene cluster surrounding the predicted methanol dehydrogenase genes *mxaFI* of *Methylophaga AH1* and *Methylophaga marina*. *mxaF* and *mxaI* correspond to the large and small subunit of the MDH; *mxaG* encodes the associated cytochrome; *mxaJ* is a gene of unknown function; *mxaDE* and *mxaYX* have regulatory roles in transcription, *mxaRSACKL* are required for maturation and activation of MDH.

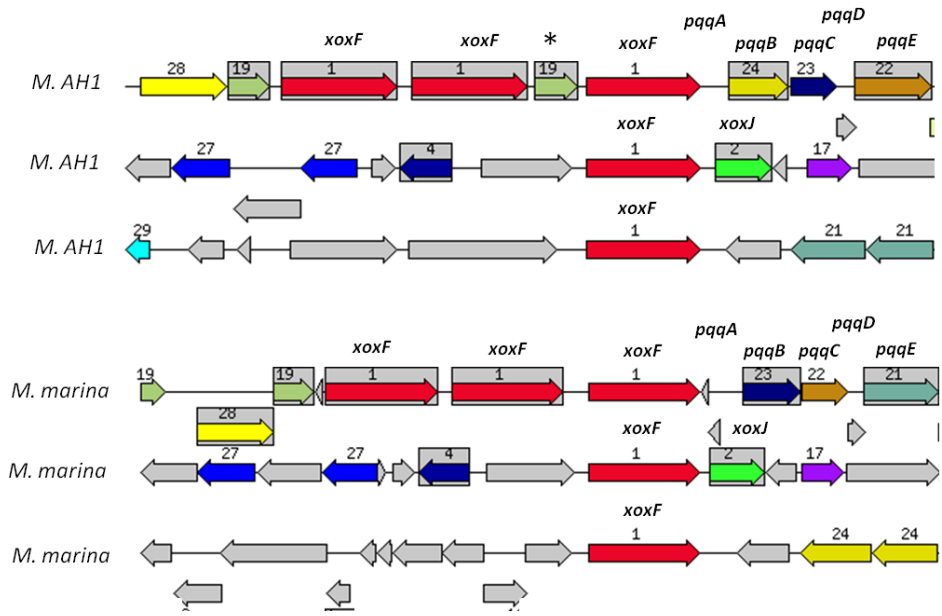

Figure 3.8 Gene clusters surrounding the predicted methanol dehydrogenase genes, *xoxF*, of *Methylophaga AH1* and *Methylophaga marina*. *mxaJ* is a gene of unknown function; *pqqABCDE* gene cluster encodes for proteins involved in PQQ biosynthesis. *encodes for a DNA-binding response regulator, LuxR family protein.

3.5.3 Comparative genomics with *Methylophaga AH1*

As *M. AH1* was proposed to be a new species of *Methylophaga* based on physiological tests and functional PCR screens, the genome was compared to all other sequenced *Methylophaga* genomes using the online tool 'Genome-to-Genome-Distance Calculator' to see how related they were based on *in silico* DNA-DNA hybridisation (DDH).

According to the tool, *M. AH1* shared a maximum of 18% DDH similarity across all *Methylophaga* genomes (Table 3.5). Genomes considered to be within the same species have more than 70% DDH, and so these data suggest that *M. AH1* is indeed a new species. Moreover, calculations of the average nucleotide identity, ANI, (another tool used to delineate species) between *M. AH1* and other *Methylophaga* species also supported this finding, with all between 75% and 79% identity (calculated on the Kostas lab using the algorithm developed by Goris et al. 2007).

Table 3.5 Digital DDH similarities between *Methylophaga AH1* strain L4 and other *Methylophaga* species, calculated *in silico* with the GGDC server version 2.0 (Meier-Kolthoff et al. 2013)

Reference species	Formula 1	Formula 2	Formula 3
M. aminisulfidivorans	22.5	18	20.8
M. lonarensis	13.3	17.7	13.6
M. marina	22.5	18	20.7
M. frappieri	13.4	17.8	13.7
M.nitratireducenticrescens	13.6	18	13.9
M. thiooxidans	15.5	17.2	15.4

Formula 2 is recommended, particularly for draft genomes. The distance formulas are explained in Auch et al. (2010).

3.6 Discussion

Methanol enrichments using seawater from station L4, Plymouth gave rise to the isolation of a new methylotroph *Methylophag*a, strain L4. The 16S rRNA gene of this strain had 97% identity to the 16S rRNA of *M. sulfidovorans* RB-1, indicating it was likely a new species. Initial PCR analyses suggested this strain contained the *gmaS* gene for methylamine metabolism and not *mauA*, like *M. sulfidovorans*. However, unlike *M. sulfidovorans*, growth tests using methylamine as sole source of carbon and energy revealed that strain L4 was unable to grow on methylamine and could only utilise it as a nitrogen source, again suggesting it was a new species. Moreover, strain L4 was unable to grow on any other carbon compounds tests, indicating it is an obligate methylotroph,

more similar to *M. lonarensis*, *M. frappieri* and *M. nitratireducenticrescens.* The strain is therefore proposed to be a novel species, named *Methylophaga AH1* strain L4.

Genome sequencing of strain L4 allowed the comparison to other *Methylophaga* genomes using *in silico* DNA-DNA hybridisation (DDH), and confirmed the designation of the strain as a new species given the low DDH. Genome sequencing of *M. AH1* and *M. marina* also revealed full metabolic pathways required for aerobic methylotrophic lifestyles. The both contained the full *mxaFI* and PQQ genes required for growth on methanol. Moreover they both contained five *xoxF5* genes, arranged in similar clusters, which is the highest number of *xoxF* genes seen in the genome of any bacteria.

Isolation and characterisation of this new species of *Methylophaga* supports some previous research. Firstly, that isolation series for new methylotrophs using traditional methods from well studied sites can still yield new isolates, and so no sampling site should ever be considered exhausted. Secondly, *Methylophaga* are consistently found to be active methylotrophs in coastal waters, as seen in previous culture-independent studies (Neufeld et al. 2007; Neufeld, Chen, et al. 2008; Grob et al. 2015).

Of particular interest here is the stimulatory effect that p-nitrophenylhydrazine appears to have on *M. AH1* during growth on methanol, which is in complete contrast to previous research (Anthony & Zatman 1964b). Aside from this work and that done by Anthony & Zatman, very little has been done in the way of p-NPH on MDH. Given the potential industrial applications of this compound on methylotrophs if this is a stimulant for growth on methanol, further work on the mechanism of action on the MDH, and the effects on other strains should be conducted. For example, it may be that this strain is stimulated by p-NPH via another mechanism (i.e. central metabolism), other than through directly with an MDH. *Methylophaga AH1* needs to be further characterised in order to find more carbon compounds it is able to grow on. This may then allow the direct comparison between the inhibitory effect of p-NPH on methanol and other carbon compounds.

Chapter 4

Addition of rare earth elements to methanol seawater enrichments

4 Addition of rare earth elements to methanol seawater enrichments

4.1 Introduction

Recent research has revealed the importance of rare earth elements (REEs) such as cerium and lanthanum during the growth of XoxF-utilising methylotrophs (Keltjens et al. 2014; Farhan Ul-Haque et al. 2015; Vu et al. 2016). Not only have lanthanum and cerium been shown to be at the catalytic site of XoxF, but they are also involved in the up-regulation of the expression of *xoxF* and down-regulation of the expression of the *mxaFI* genes encoding the classic MDH (Nakagawa et al. 2012; Pol et al. 2014; Bogart et al. 2015; Wu et al. 2015; Keltjens et al. 2014; Farhan Ul-Haque et al. 2015).

REEs are highly insoluble and are rarely found in pure form (Hu et al. 2004), and due to the relative difficulty in quantifying REEs, measurements during biological sampling is not commonplace. Studies have shown that concentrations can range from the high nM in estuarine and coastal environments (Elderfield et al. 1990; Hatje et al. 2014a) to pM concentrations in open oceans (Garcia-Solsona et al. 2014; Greaves et al. 1991). However it is not known how much of this is bioavailable. In contrast, the *xoxF* gene has been shown to be present in the genomes of a broad range of bacteria and is widely distributed throughout marine environments (Taubert et al. 2015) However all studies on the marine environment and rare earth elements have been conducted on pure isolates *in vitro*; there has been no research as to what role REEs have on the methylotrophic communities as a whole.

Therefore the aim of this work was to investigate what effect, if any, the addition of REEs had on the microbial oxidation of methanol in seawater enrichments, and if this had any impact on the overall microbial community. As previously detailed in Objective 2, the effect of rare earth elements was examined by investigating the impact of the addition of the metals to seawater methanol enrichments from various sampling sites and monitoring methanol depletion. The impact of this on the bacterial community was examined using DNA profiling and amplicon sequencing, whilst the artificial concentrations of methanol used in the enrichments is also addressed.

4.2 REE enrichments with high methanol concentrations

4.2.1 Preliminary station L4 REE methanol enrichments show increased methanol oxidation

The effect of the addition of REEs to seawater enrichments containing methanol was examined initially using surface seawater from station L4, Plymouth. The first set of enrichments were established in 250 ml conical Quickfit flasks fitted with SubaSeal stoppers, containing 50 ml seawater, 1% (v/v) MAMS medium and 5 mM methanol. Duplicate flasks had either 50 nM lanthanum, cerium, both metals or no metals added (as chloride heptahydrate salts). Control flasks with both metals but no methanol were also set up (no MeOH). Headspace methanol concentration was quantified periodically by GC (as described in Materials and Methods) as a measure of bacterial methanol oxidation.

A second, more in depth look at the effect of REEs was established in the same way but with triplicate flasks, whilst 50 ml seawater was also filtered through Sterivex filters on day 0 (T0), 1 ml samples taken from each flask at day 9 (T1) when measurements of methanol showed substantial decreases. The Sterivex filters and samples were frozen immediately at -20 ˚C for later use. GC data are summarised in Figure 4.1.

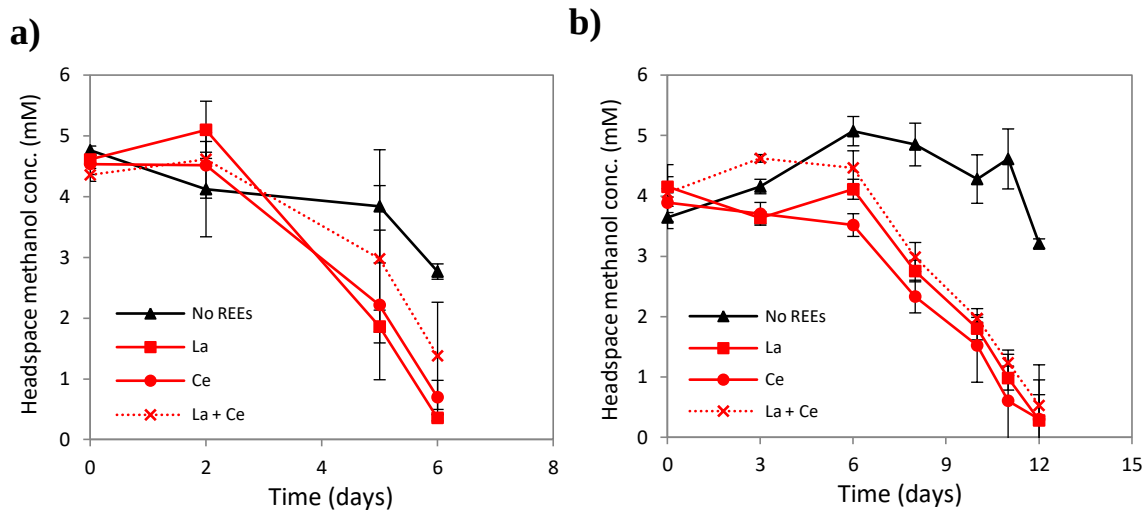

Figure 4.1 Headspace methanol depletion of REE methanol seawater enrichments using samples from station L4, Plymouth. a) Preliminary experiment with duplicate vials; error bars indicate the range. b) Second experiment with triplicate vials; error bars indicate the standard error. REEs were added in the form of chloride heptahydrate salts. All enrichments contained methanol (shown) except control vials containing both REEs but no methanol, which were only measured on the GC on the first and last days (not shown).

Preliminary methanol enrichments containing REEs showed an increase in methanol headspace depletion compared to the enrichments with no added REEs (Figure 4.1a). However as cultures were only in duplicate and the enrichment was too short to draw significant conclusions, a second enrichment experiment was established (Figure 4.1b). This confirmed results from the first experiment, with a significant increase in methanol depletion in all cultures containing added REEs ($p \leq .05$) compared to those without, suggesting that the bacterial oxidation of methanol is stimulated by REEs. It also suggested that the concentration of REEs at station L4 water sampled at that time was lower than those required for the maximum growth of methylotrophs on methanol.

4.2.2 Analysis by denaturing gradient gel electrophoresis (DGGE) profiling of the bacterial community of preliminary REE enrichments using station L4 water

To examine what effect the addition of REEs had on the bacterial community, 16S rRNA sequences were amplified by PCR using specific primers (in Table 2.2 in Materials and Methods) and profiled by denaturing gradient gel electrophoresis (DGGE), as shown in Figure 4.2.

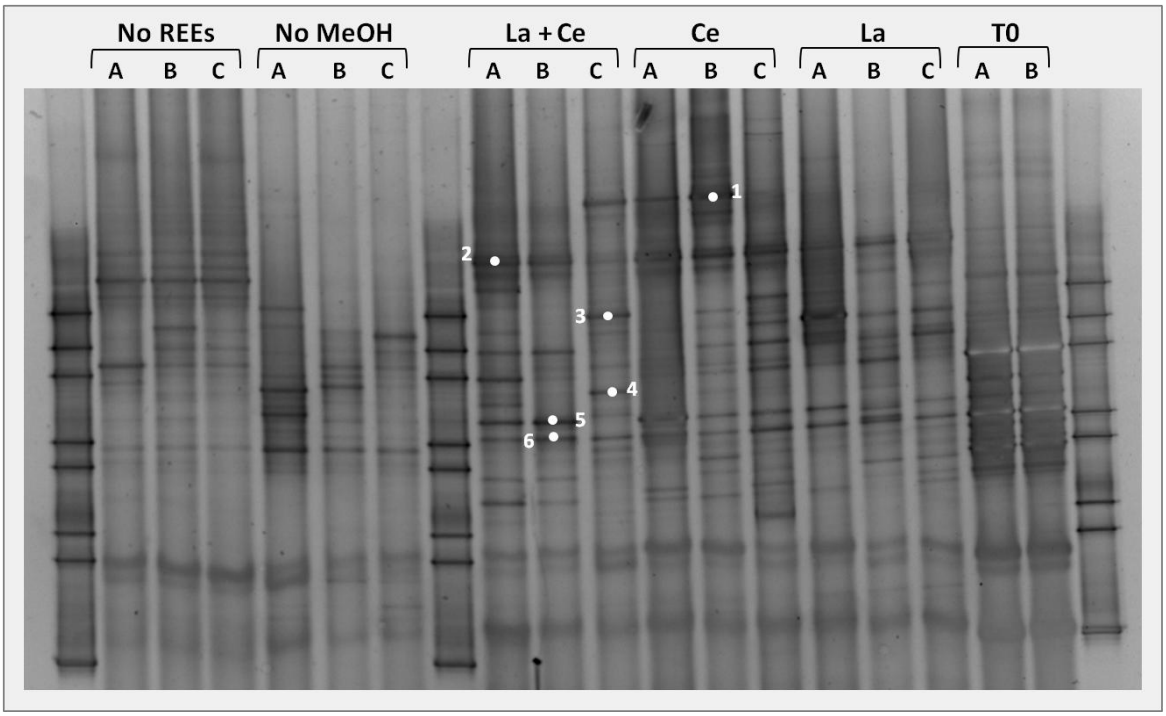

Figure 4.2 16S rRNA gene DGGE profile of the bacterial community of the second of the REE enrichments (shown in Figure 2.1b) using seawater from station L4, Plymouth. Each lane represents a single enrichment, with replicates labelled as either A, B, or C. All conditions contained methanol except control vials containing both REEs but no methanol (No MeOH). T0 lanes represent the bacterial profile of DNA extracted before the enrichments were set up. Numbered white dots represent bands that were picked and sequenced.

DGGE analysis of the REE enrichments revealed differences between the enrichments with no REEs added, the enrichments with methanol only, and the enrichments with either lanthanum, cerium or both REEs added. Although not in all replicates, bands were present in enrichment conditions with added cerium or lanthanum (or both) that were not in either the methanol-only conditions (No REEs) or in those with methanol but no added REEs (no MeOH). Some of these bands, labelled 1-6 in Figure 4.2, were picked, re-amplified by PCR and sequenced using Sanger sequencing. A summary of the 16S rRNA gene sequences obtained is presented in Table 4.1.

Table 4.1 Closest 16S rRNA gene relatives of sequences picked from the DGGE gel of preliminary REE enrichments from station L4

Band[1]	Phylogeny	Accession number	Identity[2] (%)
1	*Flavobacteriaceae* bacterium RC2-3 16S rRNA	JQ408440	94
2	*Scutiococilliatia* SL-220 18S rRNA	KC287215	99
3	*Methylophilaceae* bacterium strain AY117 16S rRNA	AB930174	100
4	*Tenacibaculum* sp. ODE7 16S rRNA	AB822595	98
5	*Alteromonas macleodii* strain CSB14KR 16S rRNA	KX380760	99
6	*Alteromonas confluentis* strain DSSK2-12 16S rRNA	NR_137375	99

[1]Band number corresponds to the numbers seen in Figure 4.2.

[2]Identity refers to 16S rRNA gene sequence identity using around ~170 bp of DNA sequence.

Sequencing of bands that appeared in the DGGE profile of the REE enrichments from station L4 revealed that a relative of the known methylotrophic family *Methylophilaceae* was enriched in some incubations containing cerium (Band 3, Table 4.1 and Figure 4.2), suggesting that this strain may require this element for growth on methanol.

Members from the family *Flavobacteriaceae* were also enriched in the cerium or lanthanum (or both) containing incubations compared to those without REEs. For example, band 4 was related to the 16S rRNA gene of a member of the *Tenacibaculum* genera, whilst band 1 was related to *Flavobacteriaceae* bacterium. There are known methylotrophs within the *Flavobacteriaceae*, such as some *Flavobacterium* species (Moosvi et al. 2005; Boden et al. 2008) however no members of the *Tenacibaculum* genera have been shown to be methylotrophs.

Sequences of DGGE bands 5 and 6 were most closely related to the 16S rRNA genes of members of the *Alteromonas* genera, also not known to be methylotrophs. One of the most prominently enriched bands in the conditions containing REEs (Band 2) was most closely related to the 18S rRNA gene sequence of the eukaryote *Scutiococilliatia* SL-220. The 16S rRNA primers used for DGGE do have some cross-specificity with parts of the 18S rRNA sequence and so may explain this anomaly. However it is worth noting that it is only heavily enriched in those incubations containing both methanol and REEs.

4.2.3 Analysis by 454 16S rRNA gene amplicon sequencing of the bacterial community of preliminary REE enrichments with station L4 water

To further examine the effect of REEs on the bacterial community, samples of each triplicate of the enrichments containing no REEs and the enrichments with both lanthanum and cerium (No MeOH and La + Ce, respectively) were combined and the 16S rRNA genes amplified using the primer set 27Fmod/519R modbio (Table 2.2 in Materials and Methods). The amplicons were then purified and sent for 454 sequencing at MR DNA (Texas). Data were analysed according to Dowd et al (2011) and DeSantis et al (2006). The data are summarised in Figure 4.3 and Figure 4.4.

Surprisingly, when analysing the 16S rRNA sequence data from the No REEs enrichments (Figure 4.3a), there did not seem to be any *bona fide* methylotrophs, with groups such as *Massilia*, *Thiobacillus* and *Polaribacter* being most dominant. However when there were REEs present (Figure 4.3b), sequences related to the 16S rRNA gene sequences of two known marine methylotrophs were present; *Sagittula* and *Methylotenera*. Sequences affiliated with *Alteromonas* and *Tenacibaculum* found in the DGGE profile (Figure 4.2 and Table 4.1) were also present in the La + Ce 16S rRNA gene amplicon sequencing, confirming the enrichment of these sequences compared to the No REEs enrichment.

An analysis of the two sets of 16S rRNA gene sequencing data at the class level (Figure 4.4) also showed an overall increase in *Alphaproteobacteria* and *Gammaproteobacteria* and a decrease in *Betaproteobacteria* when REEs were present. However as this data set does not show the individual replicate sequence data, nor sequence data from DNA obtained before the enrichments were established (i.e. environmental DNA) it is not possible to be confident of how accurately the combined data truly reflect each replicate. Therefore these data are simply presented to provide a preliminary analysis of the potential effects of the addition of REEs to methanol enrichments.

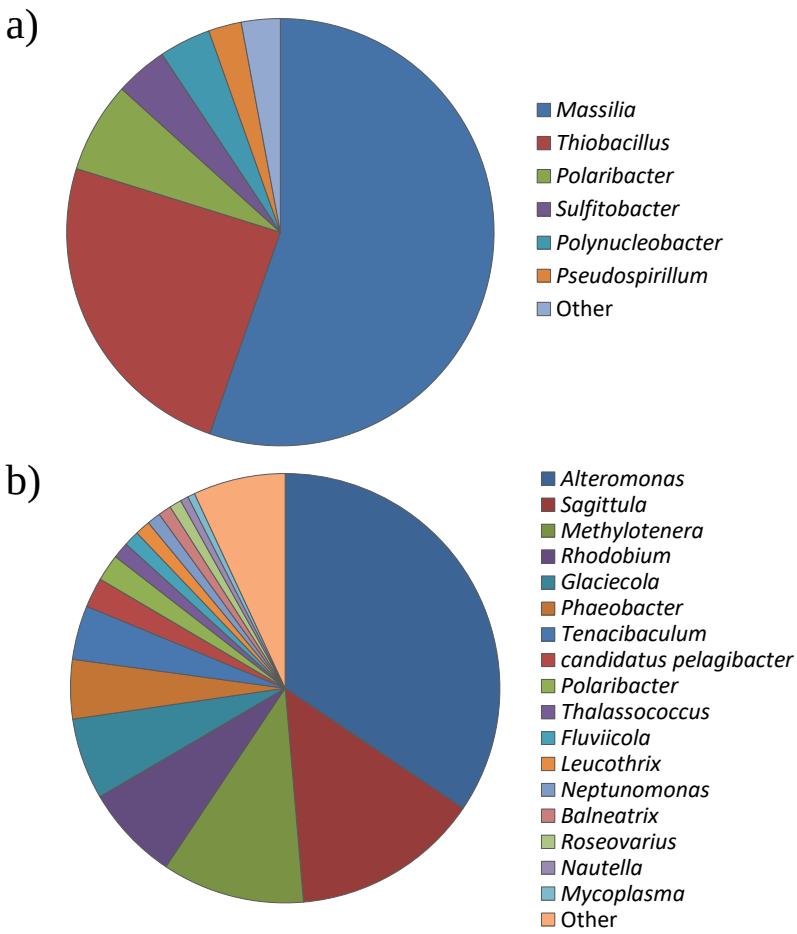

Figure 4.3 16S rRNA gene profiles at the genus level of the bacterial communities in the second of the REE methanol enrichments using seawater from station L4 (retrieved by 454 amplicon sequencing). a) Combined triplicates of No REEs enrichments. b) Combined triplicates of La + Ce enrichments. Only genera representing >0.5% of the community are shown.

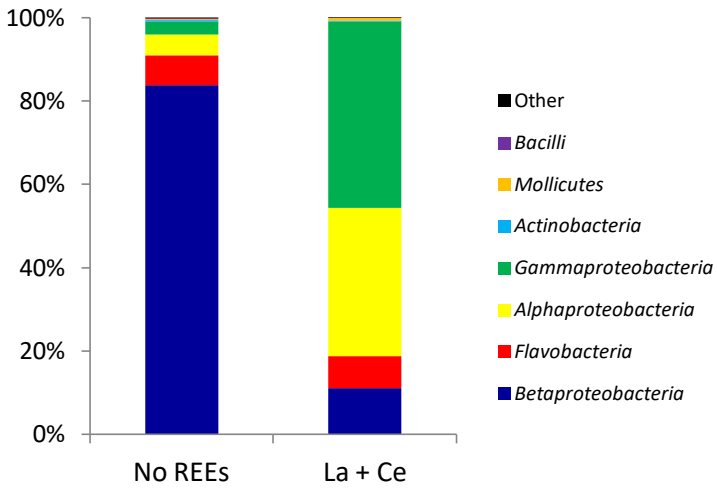

Figure 4.4 16S rRNA gene profiles at the class level of the bacterial communities in the second of the REE methanol enrichments using seawater from station L4 (retrieved by 454 amplicon sequencing).

4.2.4 REE enrichments from three different marine sites show different methanol oxidation profiles

Enrichments were set up using coastal seawater from three locations in order to examine if different types of seawater were affected by REEs differently. As mentioned in Chapter 3, station L4 represents a coastal site and is heavily influenced by the flow of the Tamar estuary. Station E1 is located around 40 miles off the coast of Plymouth (50˚ 02'N, 4˚ 22'W; depth 75 m) (Figure 3.1 in Chapter 3) and was chosen because although it is tidally influenced, it represents an open shelf site, well away from coastal freshwater influences (Smyth et al. 2010). Lastly, water collected from Cefas, Lowestoft is from the bottom of a water column near the coast, only a few meters deep and has been filtered through sediment before reaching the point of collection.

Enrichments were set up as before using 1% (v/v) MAMS medium and 5mM methanol, but with an increase in concentration of the REES to 5μM. Enrichments contained either no REEs, lanthanum, or cerium and were again monitored for headspace methanol depletion. Water was also filtered before enrichment to determine the initial bacterial community. Samples were sacrificed at the end of the experiment by filtration through Sterivex filters and the DNA was then extracted for later use.

GC data are summarised in Figure 4.5 and show that the addition of either lanthanum or cerium to seawater enrichments stimulates methanol oxidation in station E1 (compared to enrichments with no REEs), whilst there is no effect of the presence of REEs in the enrichments using water from station L4 or Lowestoft. Indeed, during the fastest rate of decrease in the E1 enrichments, those containing lanthanum are over 2.5 times the rate of those with no REEs, whilst those with cerium are over 1.5 times the rate (No REEs, 0.2 mM h^{-1}; La, 0.53 mM h^{-1}; Ce, 0.36 mM h^{-1}, Figure 4.6). The lack of difference between conditions in station L4 enrichments is in contradiction to the preliminary enrichments, suggesting that the water sampled at the two different times contained different REE concentrations. It also suggests that standing concentrations of REEs at station E1 may be lower than those at station L4 and Lowestoft.

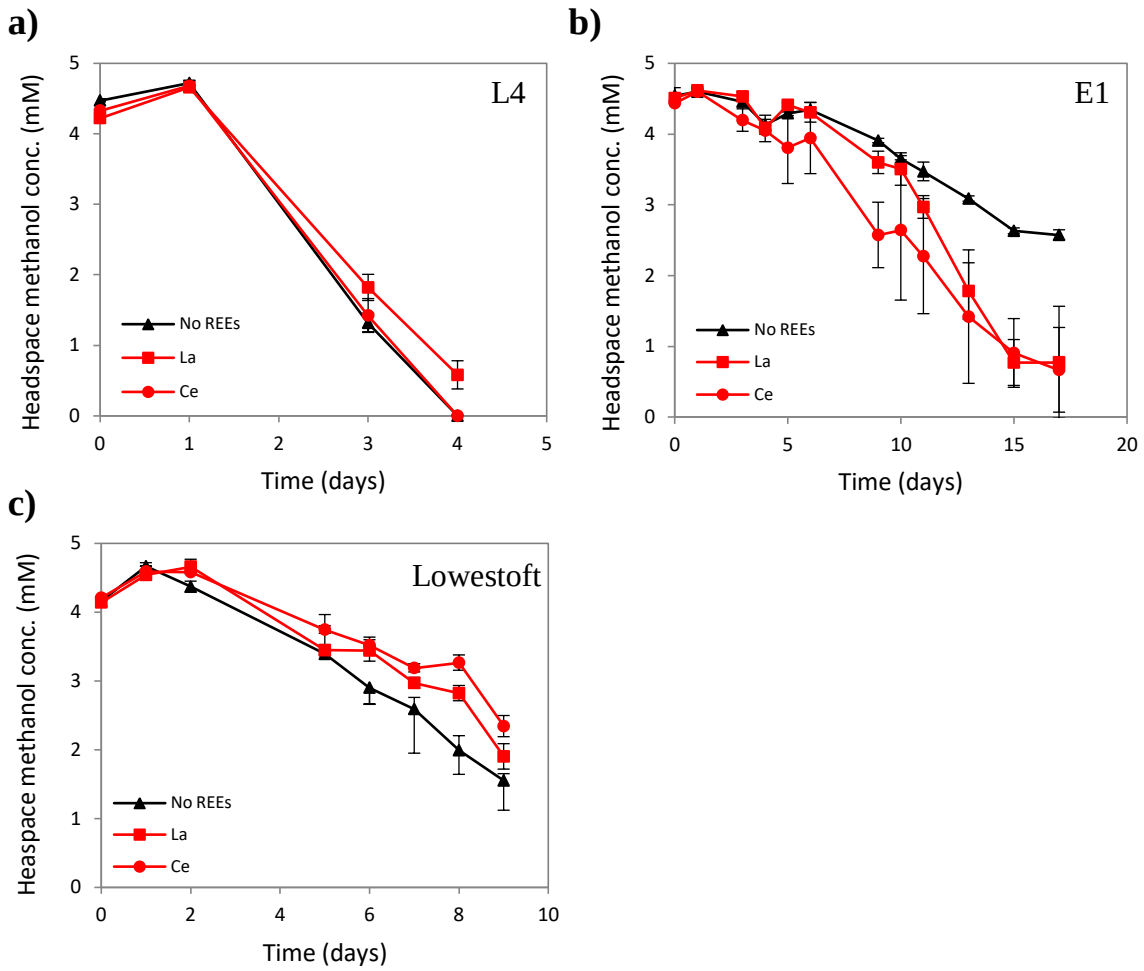

Figure 4.5 Headspace methanol depletion of REE enrichments using seawater from station L4 (a), station E1 (b) and Lowestoft (c). Error bars show standard error of triplicate vials, except for in Ce enrichment in b) due to one vial being discarded due to no activity, thus data represents duplicates and bars show the relative range.

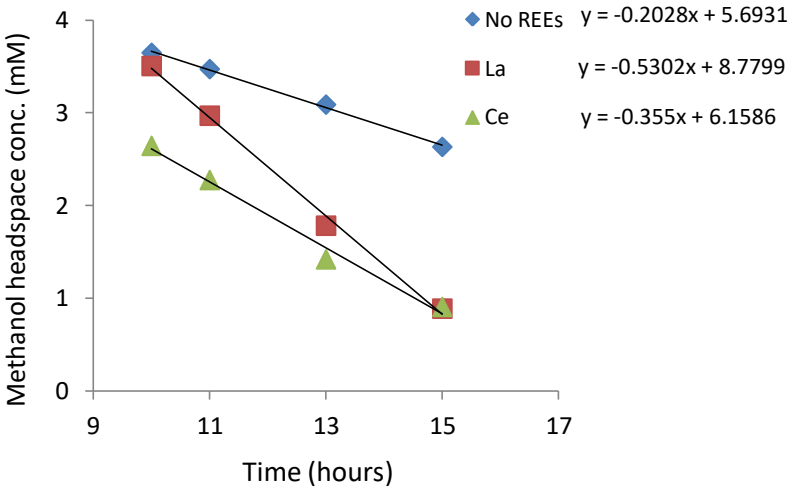

Figure 4.6 Calculated rates of methanol oxidation for station E1 REE enrichments, from Figure 4.5b.

4.2.5 16S rRNA gene denaturant gradient gel electrophoresis (DGGE) analysis of the bacterial community of REE enrichments from three different marine sites

16S rRNA gene sequences in all DNA samples were amplified by PCR and analysed by DGGE. DGGE profiles of all three enrichment experiments showed high variability within replicates, as well as between conditions; Figure 4.7 shows the DGGE profile of station E1 as an example. In the case of the E1 enrichments, this makes it very difficult to establish which, if any, 16S rRNA genes sequences represent those enriched due to the presence of REEs. This variability may be due to the 'bottle effect', which is a commonly found phenomenon whereby microorganisms are non-specifically affected by their confined environment (Hammes et al. 2010; Agis et al. 2007).

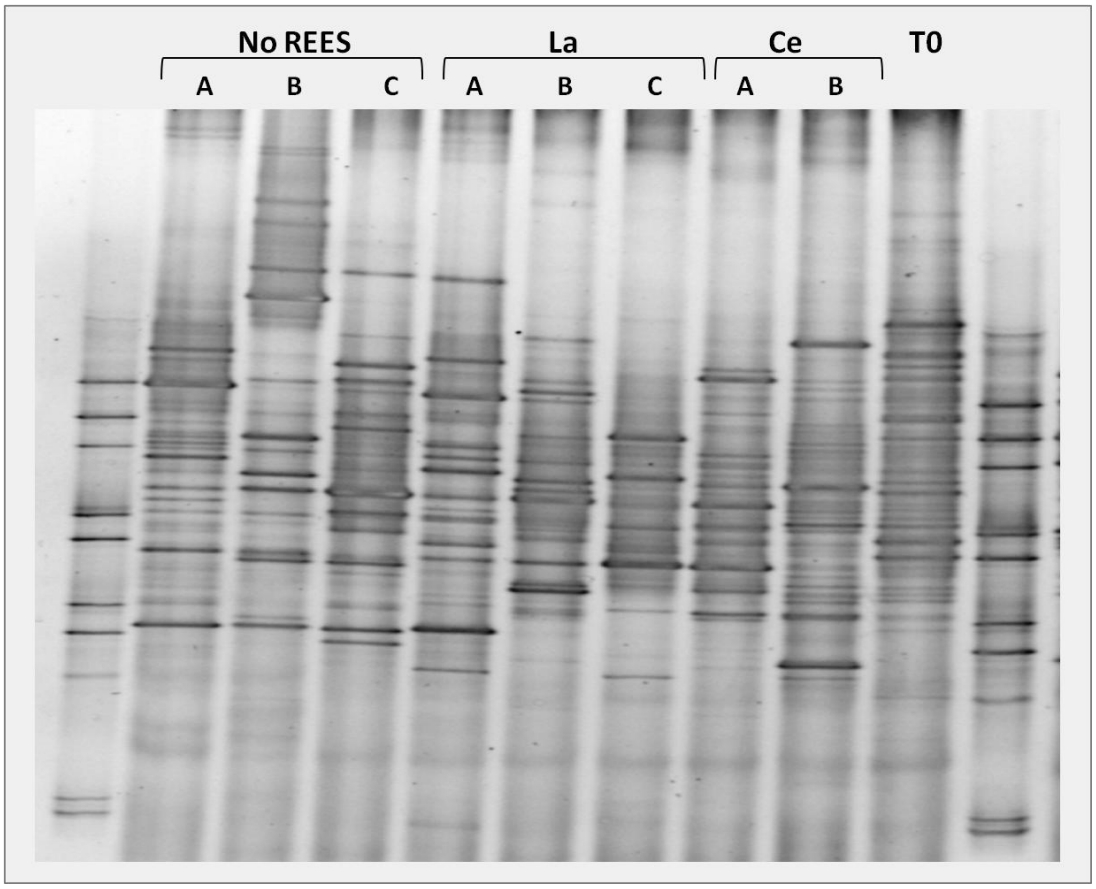

Figure 4.7 16S rRNA gene DGGE profile of the bacterial community of the REE enrichments using station E1 seawater. Each lane represents a single enrichment, with replicates labelled as either A, B, or C. T0 represents the bacterial profile from DNA extracted before the enrichments were set up.

4.2.6 Analysis of the bacterial community of station E1 and L4 REE enrichments by 454 16S rRNA gene amplicon sequencing

The 16S rRNA gene sequences were obtained by PCR from the DNA of each replicate and T0 from the station E1 enrichments, and from the pooled DNA of replicates from station L4 enrichments. These were purified and sent for 16S rRNA gene amplicon sequencing to profile the overall bacterial community. As REEs have been shown to be involved in the growth of methylotrophs using the XoxF MDH, the *xoxF5* gene was also amplified from pooled DNA of each treatment from both station E1 and L4 using *xoxF5* specific primers (Table 2.2 in Materials and Methods chapter) and sent for amplicon sequencing. Primers specific for *xoxF1*, *2*, *3* and *4* were also tested on the extracted DNA, but no amplicons could be amplified. *XoxF5* sequences received were analysed according to Taubert et al (2015) using the software packages MOTHUR (Schloss et al. 2009) and USEARCH (Edgar 2013) and phylogenetic trees constructed using MEGA (Tamura et al. 2011). The *xoxF* database was the same used in Taubert et al (2015) and included the top BLAST hits of the sequences with the highest OTUs.

Data are summarised in Figure 4.8 (station E1, 16S rRNA), Figure 4.9 (station E1, *xoxF5*), Figure 4.10 (station L4, 16S rRNA) and Figure 4.11 (station L4, *xoxF5*). Phylogenetic trees used to assign *xoxF5* sequences to groups are shown in Appendix Figure 10.1 (station E1, *xoxF5*) and Appendix Figure 10.2. (station L4, *xoxF5*). Sequence information is too small to be read in printed format but can be read in the available digital format.

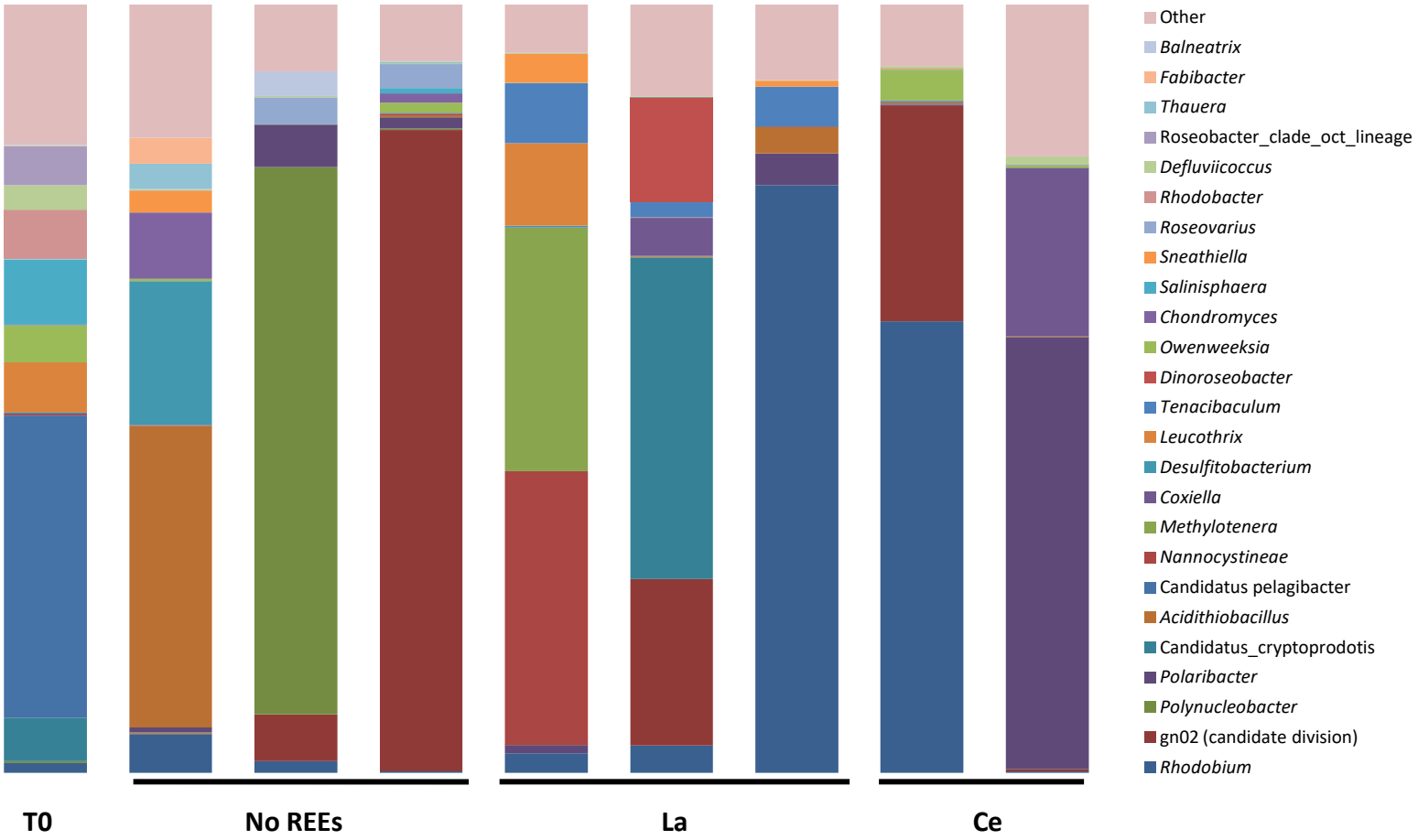

Figure 4.8 16S rRNA profiles of the bacterial communities (at the genus level) of station E1 REE methanol enrichments, as retrieved by 454 amplicon sequencing. Bars represent individual replicates. Only genera representing >3% in any one replicate are shown.

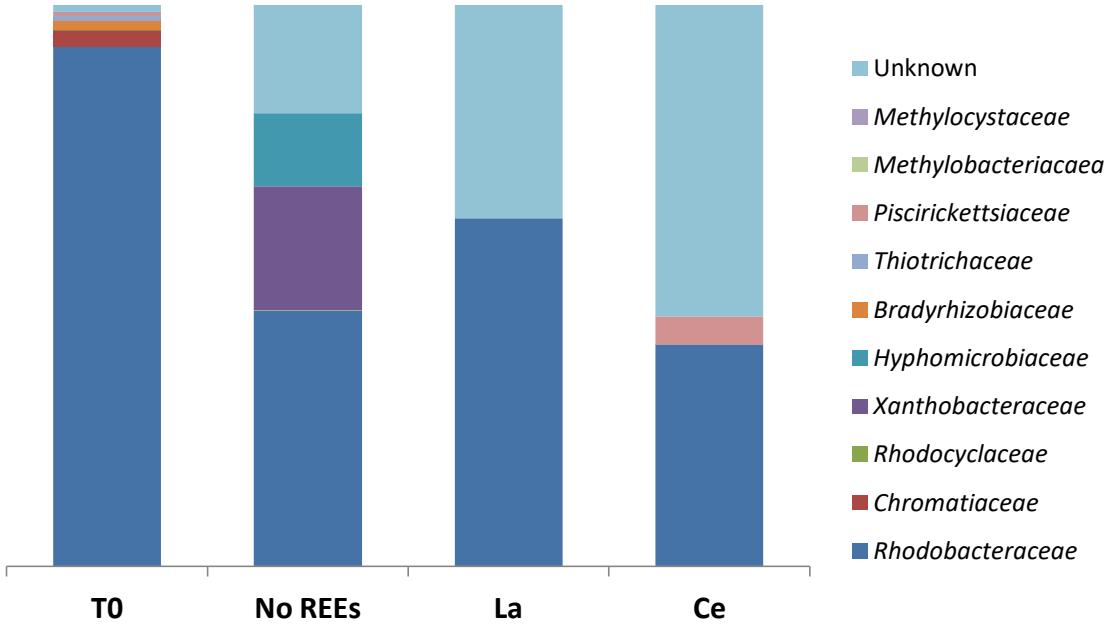

Figure 4.9 *xoxF5* profiles of the bacterial communities (at the family level) of station E1 REE methanol enrichments, as retrieved by 454 amplicon sequencing. Bars represent the *xoxF5* sequences pooled of replicates.

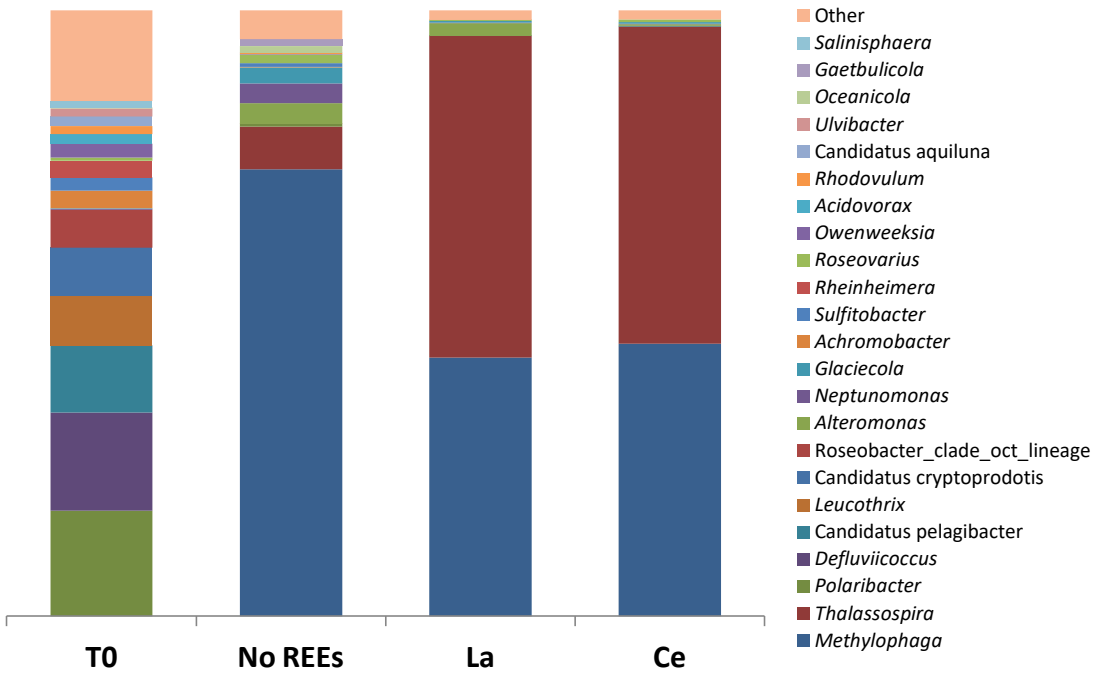

Figure 4.10 16S rRNA profiles of the bacterial communities (at the genus level) of station L4 REE methanol enrichments, as retrieved by 454 amplicon sequencing. Bars represent the 16S rRNA gene sequences of pooled replicates. Only genera representing >1% in any condition replicate are shown.

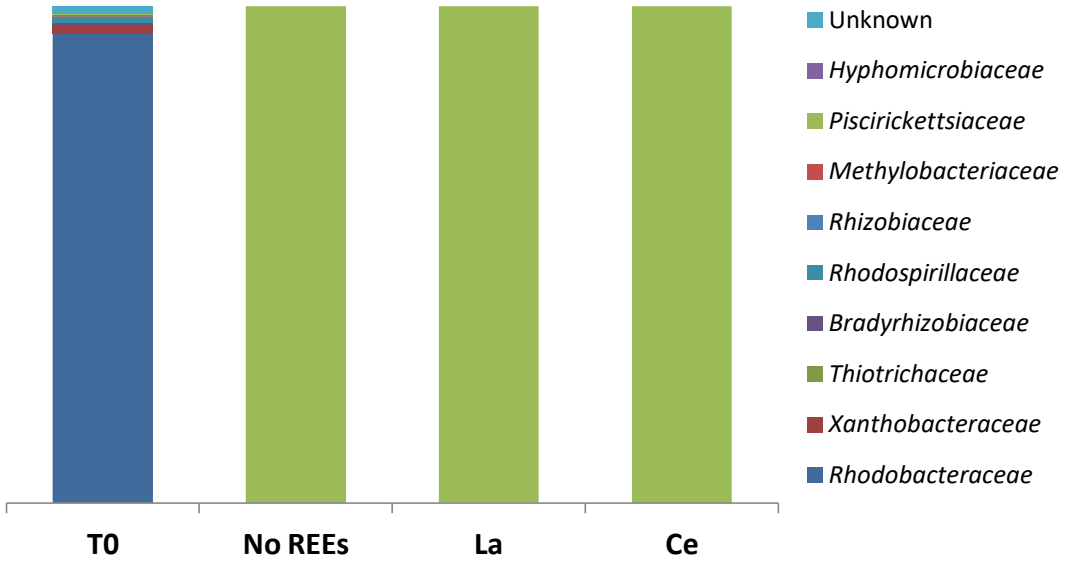

Figure 4.11 *xoxF5* profiles of the bacterial communities (at the family level) of station L4 REE methanol enrichments, as retrieved by 454 amplicon sequencing. Bars represent the *xoxF5* sequences of pooled replicates.

Analysis of 16S rRNA sequences retrieved from amplicon sequencing of station E1 enrichments reveals that, as seen in the DGGE profile, there was very high variability between replicates as well as between enrichments (Figure 4.8). It is therefore difficult to draw too many conclusions from this. However it is worth noting that in one each of a lanthanum and cerium replicates there is a high abundance of *Rhodobium*, seen previously in the preliminary La + Ce station L4 enrichment. Also present in one of the lanthanum enrichments is an abundance of *Methylotenera*, also observed in the preliminary La + Ce L4 enrichments.

When analysing the pooled *xoxF5* sequencing data from the same enrichments (Figure 4.9), there was a marked increase in *xoxF5* sequences from the lanthanum and cerium enrichments which do not closely affiliate with any sequences in the NCBI database, compared to the No REEs enrichments. In fact, these sequences could not be assigned to a family, and more than 95% of the sequences in the 'Unknown' family group (in all three enrichments) could not be assigned to a phylum either. This therefore suggests that the presence of REEs stimulates the growth of potentially novel bacteria containing *xoxF*.

Although there were no differences between the methanol oxidation rates of any of the station L4 REE enrichments, the 16S rRNA data derived from the pooled enrichments showed a relative increase in the genus *Thalassospira* in both REE containing enrichments compared to those without REEs (Figure 4.10). No extant member of this group has been tested for growth on methanol and so the type strain *Thalassospira lucentensis* QMT2 was ordered from DSMZ and tested for growth on (5mM) methanol as sole carbon source with lanthanum and cerium, and also in combination with succinate (as a co-substrate). This strain was unable to grow in either test conditions (data not shown). It is therefore difficult to deduce whether the 16S rRNA sequences represent those of a new species which is able to grow on methanol or if the REEs alone stimulate the growth of this bacterium.

Sequenced genomes of the genera *Thalassospira* do not contain any *xoxF* (or *mxaF*) and so it is not surprising that we do not see sequences annotated as *Thalassospira* in the *xoxF5* data sets (Figure 4.11). Also fairly unsurprising is the massive relative enrichment of the *Piscirickettsiaceae* in all three enrichments, as previous research has shown the enrichment of the genus *Methylophaga* from station L4 methanol enrichments, and in the isolation of *Methylophaga AH1* strain L4 in Chapter 3 (Grob et al. 2015; Neufeld et al. 2007).

Lastly, although the *xoxF5* primers were designed to be specific to the *xoxF5* clade, when all the sequences were aligned in a phylogenetic tree with a database containing all five clades, some sequences were found to cluster within the *xoxF1* clade. In fact one OTU from the cerium enrichment contained 1386 sequences, representing 26% of the total *xoxF* sequences (Appendix Figure 10.3). This OTU clustered closely with a *xoxF1* gene sequence from a relatively newly isolated facultative methylotroph, *Methyloceanibacter caenitepidi*, with 96% amino acid identity (or 94 % DNA identity) (Takeuchi et al. 2014). The 16S rRNA gene of this methylotroph has been found in numerous marine sediments and environments, suggesting that it is ubiquitous and may be important in the metabolism of methanol. The relative enrichment of *xoxF1* of sequences very similar to this methylotroph when provided with additional cerium may support this idea.

4.3 REE enrichments with low concentrations of methanol

4.3.1 REE enrichments using station E1 seawater and lower concentrations of methanol

As station E1 water showed REE stimulated methanol oxidation activity with 5 mM methanol, enrichments using lower concentrations of methanol and nutrients (MAMS) were established to investigate the enrichment of methylotrophs in conditions closer to those *in situ*. Lanthanum was chosen as it had yielded the fastest methanol oxidation rates. Triplicate enrichments were set up in 2 litre bottles containing 750 ml seawater and 0.1 % (v/v) MAMS. Lanthanum was added at 5 μM. Previous research using 100 μM methanol showed enrichment of methylotrophs (Grob et al. 2015) and so this concentration was chosen. A second set of enrichments with 1 μM was established, after which 1 μM methanol was added each day. Cultures were incubated at 25 ˚C, shaking at 150 rpm. Methanol in the enrichments was monitored using the alcohol oxidase assay until the 100 μM methanol enrichments had all of the methanol depleted (1 μM is below the limit of detection). Cultures were then sacrificed as described before. The different enrichment conditions are summarised in Table 4.2.

Table 4.2 Summary of low methanol E1 REE enrichment set up

Enrichment name (as in Figure 4.12)	**Enrichment supplements**
100 No REEs	100 μM methanol
100 La	100 μM methanol and lanthanum
3 No REEs	3 μM methanol (1 μM added daily for 3 days)
3 La	3 μM methanol and lanthanum
No MeOH	lanthanum
Control	Nothing

All enrichments contain 0.1% MAMS.

There was a lag phase of two days in which the enrichments containing 100 μM methanol concentration did not show any methanol depletion. The 100 μM was then completely depleted within the following day (between two measurements) so no unfortunately no rate could be calculated. It therefore took three days for the methanol in the enrichments containing 100 μM methanol to be depleted. Since 1 μM methanol was added daily to the lower methanol enrichments during this time, the cumulative concentration added to these was 3 μM after the three days.

4.3.2 Analysis by DGGE of the bacterial and eukaryotic community of station E1 low methanol enrichments

The 16S rRNA profile of the bacterial community in REE low methanol enrichments was analysed by DGGE (as described previously) and is shown in Figure 4.12.

Many members of the marine *Roseobacter* clade have been shown to be in commensal relationships with phytoplankton, invertebrates and vertebrates, and are most abundant during blooms of phytoplankton (Buchan et al. 2005; Buchan et al. 2014; Moran et al. 2007). Given that the 16S rRNA and *xoxF5* gene profiles of station E1 shows such a high proportion of members of this group (T0, Figure 4.8 and T0, Figure 4.9, respectively) it was possible that the eukaryotic population was also influenced by the REE enrichments. Therefore the 18S rRNA gene profile was also examined by amplifying the 18S rRNA gene sequence using EUKF and EUKR primers (Table 2.2 in Materials and Methods) and analysing the PCR product on a DGGE gel containing 20-45 % (w/v) denaturing conditions and 6% (w/v) polyacrylamide. The DGGE profile is shown in Figure 4.13.

The 16S rRNA DGGE profile of the low methanol E1 REE enrichments show no differences between the enrichments containing lanthanum to those without (100 μM or 3 μM conditions), indicating that at these concentrations, lanthanum has no visible affect on the bacterial population. There are a few bands which are more strongly enriched in the 100 μM methanol enrichments compared to the 3 μM, and some bands in the 3 μM that are not in the 100 μM enrichment. These bands are not present in the 'No MeOH' or 'Control' enrichments, suggesting that not only does the addition of methanol have an impact on the microbial community, but also that the concentration of methanol is important.

The 18S rRNA DGGE profile shows a few bands that are present in both the 100 μM and 3 μM methanol containing lanthanum enrichments that do not seem to be present in either of the methanol-only enrichments (Figure 4.13). This also does not appear in either the lanthanum only (No MeOH) or the control enrichments, suggesting there may be eukaryotes which are enriched when both methanol and lanthanum are provided. Whilst very faintly present in some non-lathanum containing enrichments, one band (band 1 Figure 4.13), also seems more enriched in all those containing lanthanum and methanol.

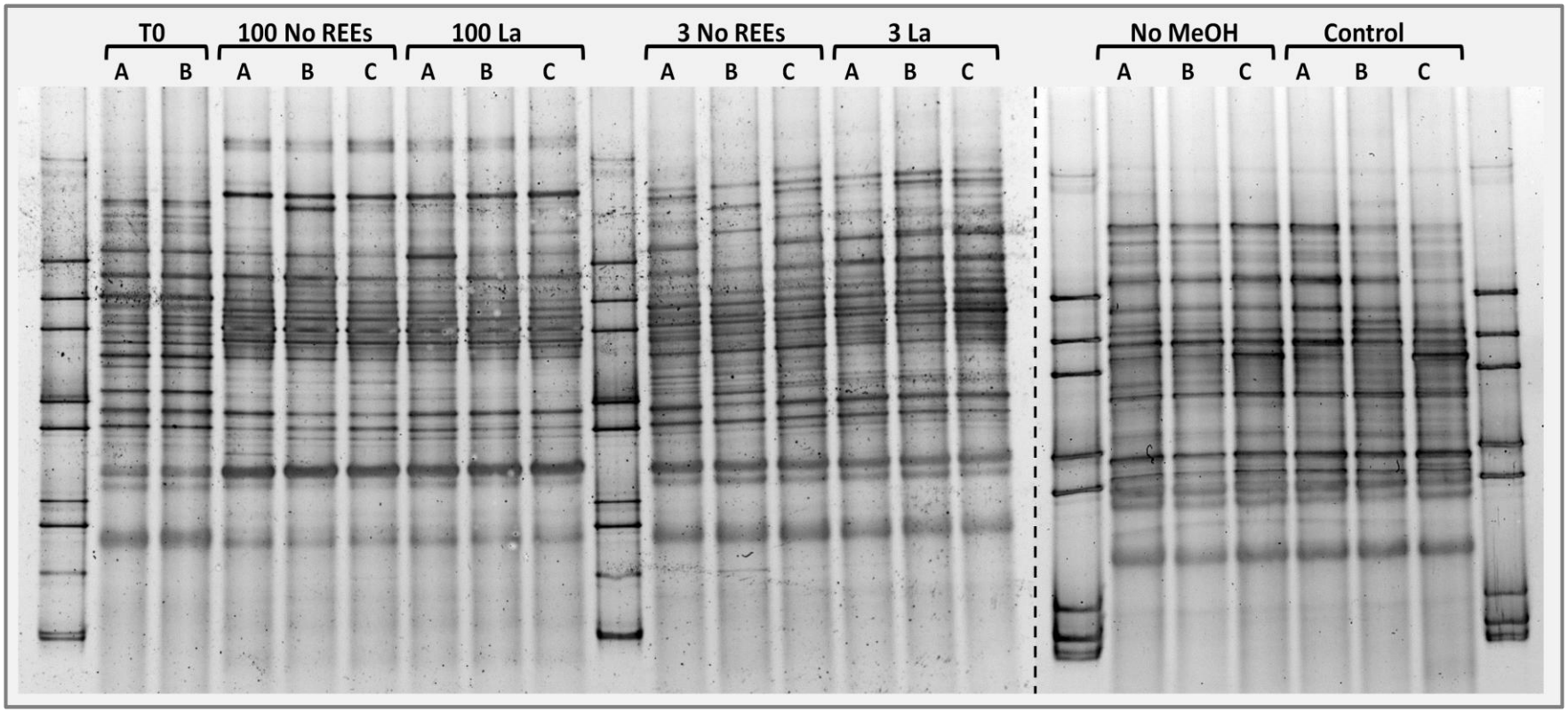

Figure 4.12 16S rRNA gene DGGE profile of the bacterial community of the low methanol station E1 REE enrichments.100 or 3 indicate the cumulative concentration of methanol in μM added to the enrichments. No MeOH enrichments contain only lanthanum. Control enrichments contain no methanol or lanthanum (only 0.1% v/v MAMS). The dotted line indicates the use of two different DGGE gels (left and right of it).

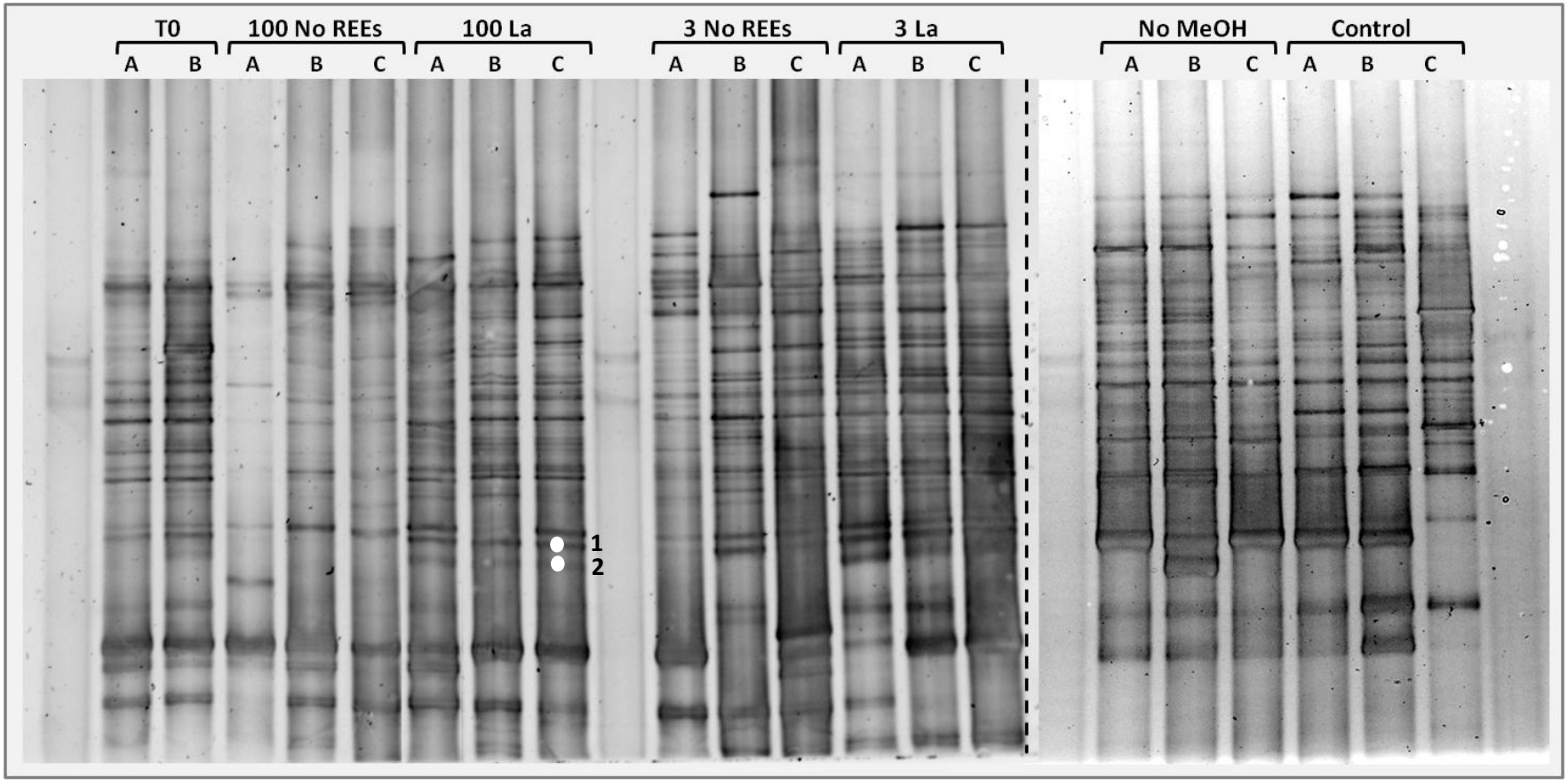

Figure 4.13 18S rRNA gene DGGE profile of the bacterial community of the low methanol station E1 REE enrichments. 100 or 3 indicate the cumulative concentration of methanol in μM added to the enrichments. No MeOH enrichments contain only lanthanum. Control enrichments contain no methanol or lanthanum (only 0.1% v/v MAMS). The dotted line indicates the use of two different DGGE gels (left and right of it). The white dots show bands of interest.

4.4 Discussion

4.4.1 REE enrichments with high concentrations of methanol

Methanol seawater enrichments with rare earth elements (REEs) were used to assess the impact of REEs on the rate of methanol oxidation and the subsequent change in the microbial community.

Preliminary enrichments using high methanol concentrations and water from the coastal station L4, Plymouth showed a significant increase in the rate of methanol oxidation when either lanthanum or cerium were added. 16S rRNA gene sequencing revealed increases in the relative abundance of known methylotrophs *Sagittula* and *Methylotenera* (at the genus level), and an increase in the *Alphaproteobacteria* and *Gammaproteobacteria* (at the class level) in the enrichments containing REEs.

Enrichments established using station L4 water collected at a different date showed no difference in methanol oxidation rates with the addition of REEs, as did enrichments using water from another coastal site, Lowestoft. Whilst a lot of the REEs are removed during the mixing process in estuaries, rivers and estuaries are a source of REE to the oceans (Elderfield et al. 1990). Measurements of REEs in the Tamar have shown that 3-4 fold changes in REE concentrations can occur over timescales of just a few days (Elderfield et al. 1990). Moreover the flow rate of the Tamar river also impacts station L4. Therefore the observation of an REE induced methanol oxidation profile in the first station L4 experiment but not in the second may be down to the changes in the flow of the Tamar estuary, and so it is possible that of the flow of Tamar was particularly low on the date of the first sampling and so concentrations of REEs were rate limiting. whilst the flow was high on the second sampling, thus providing a greater source of REEs to station L4, and therefore the seawater was saturated and addition of REEs had no impact.

However enrichments using water from station E1, Plymouth did show an increase in methanol oxidation upon the addition of lanthanum or cerium. Generally, rivers and estuaries have much higher concentrations of REEs than coastal and open oceans (Elderfield et al. 1990; Greaves et al. 1991; Hatje et al. 2014b; Garcia-Solsona et al. 2014), and given that the site represents an open sea shelf, it is possible that

concentrations of REEs were even lower than station L4 and so therefore showed a greater response to added REEs.

16S rRNA amplicon sequences from the E1 REE enrichments showed very high variability between both replicates and different conditions, therefore making it difficult to draw solid conclusions. Of note, however is the marked increase in *xoxF5* sequences in the lanthanum and cerium enrichments that could not be classified into any known class compared to the methanol only enrichments. This suggests there is much still to do in elucidating exactly what microorganisms the *xoxF* sequences are from and what role they have in methanol metabolism in the marine environment.

Such large variabilties between individual enrichments might be due to them having such a high concentration of methanol but also as the incubation period was for 16 days, allowing sufficient time for cross feeding and the 'bottle effect' to take hold. For example, with only three replicates available, it is highly possible that within the relatively small volume of 50 ml, clumps of different types of algae or other organic debris may be present in some but not other vials. Research has shown that a specific *Roseobacter* strain of the *Sulfitobacter* species is important for algal growth and survival in the marine environment (Amin et al. 2015). Therefore any minute difference in algae population between incubations might carry a large difference in initial *Roseobacter* populations. *Roseobacter* strains are heterotrophs, and so are likely to be able to rapidly take advantage of any exogenous carbon source (such as methanol) and outcompete surrounding other communities. To address these potential 'bottle effect' problems, much lower methanol concentrations, larger enrichment volumes and shorter incubation times were adopted in the 'low methanol enrichments'.

4.4.2 REE enrichments with low concentrations of methanol

The lower methanol enrichments using station E1 water showed very few differences in 16S rRNA gene profiles in the different enrichments when analysed by DGGE, suggesting that at these concentrations lanthanum has no visible effect on the bacterial population. There were, however two bands in the 18S rRNA DGGE profile of the 100 μM and 3 μM methanol and lanthanum enrichments that were not present in the methanol only enrichments. Very little work has been done on the role of marine eukaryotic methylotrophs and so it is very interesting to find that REEs may also play

an important role in their metabolism. Some strains of yeasts that have been shown to be methylotrophic have been found in different marine environments (Kutty & Philip 2008 and references therein), and so it is certainly not impossible that they could play a role in marine methanol turnover when rare earth elements are added. However methylotrophic yeasts do not use a XoxF or even an MDH, but rather an alcohol oxidase to metabolise methanol (Yurimoto et al. 2011). Until very recently, XoxF was thought to be the only alcohol-type oxidation system to use lanthanides as a cofactor. Just last year, however, an ethanol dehydrogenase (ExaF) was discovered to be responsible for the lanthanide-dependant metabolism of ethanol and other alcohols in *M. extorquens,* including methanol (Nathan M. Good et al. 2016). It may well be that we are on the cusp on the discovery of many more lanthanide-dependent enzymes, of which those used in eukaryotic methylotrophy may be included.

As such, it would be interesting to continue on the analysis of the low methanol enrichments, such as sequencing the 18S rRNA gene to see if there are any small but possibly significant differences in the community profiles. Moreover, sequencing of the 16S rRNA gene and direct comparison to the 18S rRNA sequences may reveal, if any, relationships between eukaryotic and prokaryotic communities, with and without rare earth elements. Moreover, if the switch between XoxF and MxaFI-mediated methylotrophy is regulated by lanthanide concentrations, as shown in recent work (Vu et al. 2016), then DNA-dependent techniques such DGGE and amplicon sequencing may not detect such subtle switches. Transcriptomics or proteomics could be used in a similar enrichment-style experiment to follow changes in expression rather than growth of bacteria, which would also detect methylotrophs which only metabolise methanol as an energy source, such as the SAR11 and OM43 clade.

Chapter 5

Characterisation of a XoxF utilising member of the *Roseobacter* clade

5 Characterisation of a XoxF utilising member of the *Roseobacter* clade

5.1 Introduction

As discussed in Chapter 3, a large part of understanding the role of methylotrophs in the marine environment has come from the isolation, characterisation and genetic analysis of novel methylotrophs. Due to the increasing evidence that rare earth elements (REEs) are directly involved in the metabolism of many methylotrophs, the addition of these metals to enrichment and isolation media is becoming standard practice. Moreover, as shown in Chapter 4, the effect of the addition of lanthanum and cerium to seawater enrichments stimulates the biological oxidation of methanol and causes the relative increase of *xoxF5*-containing bacteria. This work describes the isolation and physiological and genetic characterisation of a novel methylotroph isolated from a seawater enrichment containing methanol and lanthanum.

5.2 Isolation and preliminary characterisation of a novel *Roseobacter*

5.2.1 Isolation

Strain La 6 was isolated from surface sea water from the Western Channel Observatory station L4 (50°15.0'N; 4°13.0'W) on 9th October 2014 off the coast of Plymouth, UK. Samples were collected in sterile carboys and transported to the laboratory (overnight transportation at room temperature). 0.75 L of seawater was used for enrichments in 2 L gas tight bottles, with the addition of 0.1% (v/v) MAMS media, 5 mM methanol and 5 µM lanthanum or cerium. Enrichments were incubated at 25°C in a shaking incubator (50 r.p.m.) for 8 days, serial dilutions of this plated onto MBM minimal media containing lanthanum and incubated with headspace methanol in a gas tight chamber for 8 days. Colonies were re-streaked to purify and growth on methanol was confirmed by inoculation into liquid MAMS with methanol and lanthanum. Microscopy was performed to check for purity.

The 16S rRNA gene sequence of isolates was amplified by PCR and sequenced for identification. Isolate La 6, named after the metal it was isolated on, had 99% identity to the 16S rRNA gene sequence of *Oceanicola marinus* AZO-C (over 1350 bp). However as *O. marinus* itself does not cluster with the other members of the *Oceanicola* genus on a phylogenetic level, it seemed unlikely that *O. marinus* or La 6 were indeed true members of the *Oceanicola* genus (Figure 5.1). Moreover no extant *Oceanicola* species are able to grow on methanol or contain any MDH genes in their genome, suggesting again that La 6 may not be a member of this genus. Therefore strain La 6 was chosen for further analysis.

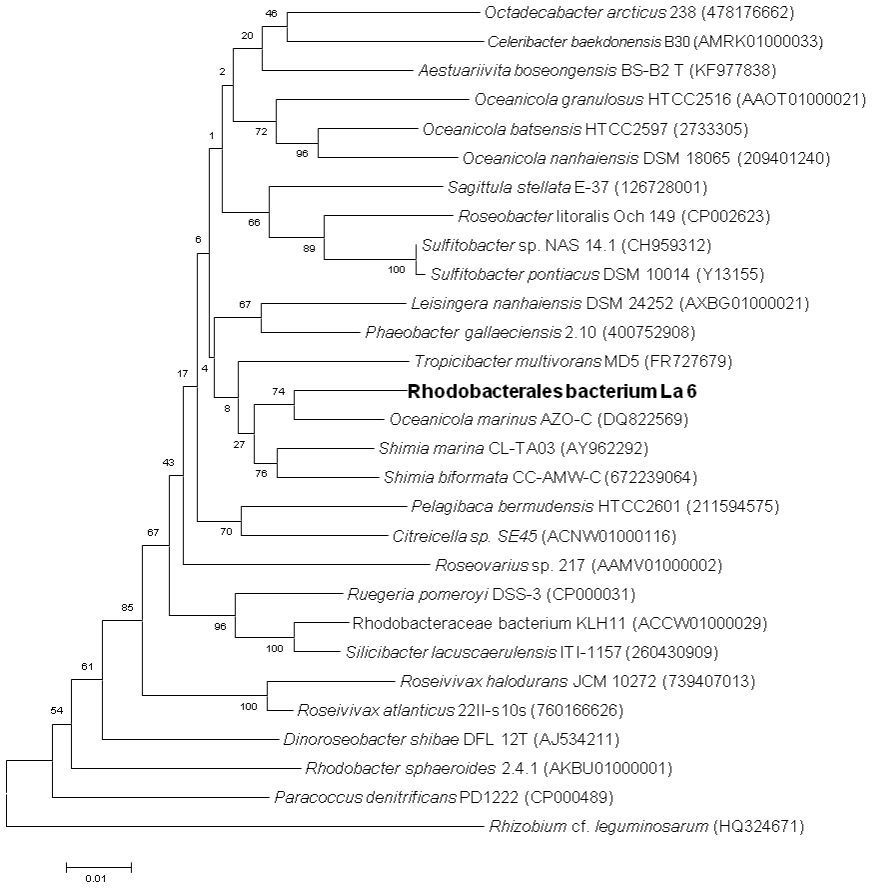

Figure 5.1 Phylogenetic analysis of 16S rRNA gene sequences of strain La 6 and members of the Roseobacter clade. Evolutionary distance among 16S rRNA gene sequences of Rhodobactereales bacterium La 6 (bold) and all type strains of the species within the genus *Oceanicola* and members *Roseobacter* clade is illustrated by an unrooted neighbour-joining phylogenetic tree. The tree was inferred from a matrix of pairwise distance using aligned sequences containing a minimum of 1400 bp positions using MEGA6. The numbers at the branches indicate the percentage of 1000 bootstrap resamplings. Numbers in parentheses are GenBank accession numbers. The scale bar indicates nucleotide substitutions per position. A phylogenetic tree constructed using the maximum-likelihood method showed a similar topology.

5.2.2 PCR and sequencing of functional genes

Isolate La 6 was further characterised by functional gene PCR screens in order to assess what methylotrophy-associated genes it contained. All methanol dehydrogenase (MDH) genes were tested (*mxaF, xoxF1, 2, 3, 4* and *5*) as well as the methylamine metabolism genes (*mauA* and *gmaS*). PCR amplicons obtained were purified and sent for Sanger sequencing. A summary of the results is shown in Table 5.1.

Table 5.1 Functional gene screen of strain La 6 and the phylogenetic affiliations of the translated amino acids sequences.

Functional gene	Presence	Closest relatives from GenBank	Amino acid identity (%)
mxaF	-	-	-
xoxF (clades 1-5)	*xoxF5*	*Loktanella* sp S4079	84
gmaS	+	*Pelagibaca bermudensis* HTCC2601T	86
mauA	-	-	-

Functional gene PCR revealed that strain La 6 did not contain the canonical MDH encoded by *mxaF*, but contained the alternative MDH *xoxF5*, which was most closely affiliated with *xoxF5* from *Loktanella* sp S4079. As most methylotrophs contain multiple copies of *xoxF*, the amplicon was cloned (as described in Materials and Methods) and sequenced, indicating that it was present in only one copy. La 6 also possessed one of the pathways for methylamine metabolism, *gmaS*, most closely related to the *gmaS* gene of *Pelagibaca bermudensis* HTCC2601T. It did not contain *mauA*, the gene encoding for a subunit of the other methylamine degrading enzyme, methylamine dehydrogenase.

5.2.3 Lanthanum stimulates growth on methanol and ethanol

Strain La 6 was isolated from an enrichment containing lanthanum, and since preliminary PCR data suggested that the strain contained only one MDH, encoded by *xoxF*, the effect of the addition of lanthanum to MBM medium whilst growing on methanol was investigated. Ethanol was also investigated in the same way, as MDHs are also capable of oxidising ethanol, as well as some other alcohols. Triplicate 120 ml serum vials containing MBM, 5 mM carbon source and either with the addition or without 5 µM lanthanum were inoculated with a single colony and monitored for growth by spectrophotometry. The headspace depletion of methanol was also monitored by GC. Non-inoculated and succinate containing vials served as negative and positive controls, respectively (data not shown). Growth and GC data are summarised in Figure 5.2.

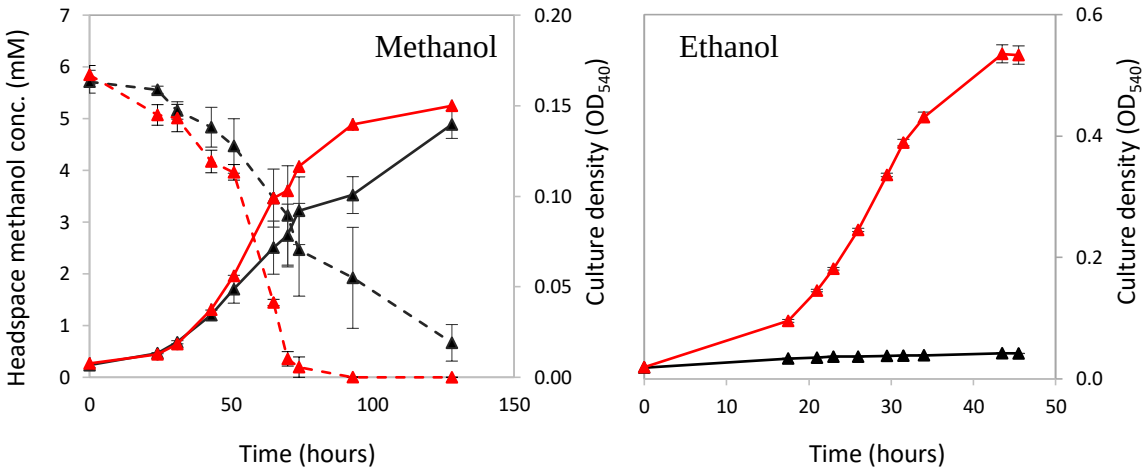

Figure 5.2 Effect of the presence (red) or absence (black) of lanthanum on the growth (solid lines) of strain La 6 on methanol (left) and ethanol (right). Dotted lines represent headspace methanol concentrations. Error bars are the standard error of three replicates.

The addition of lanthanum to the medium stimulated the growth of La 6 on methanol compared to the control. Interestingly there was a requirement for the metal when La 6 grew on ethanol, as the cultures containing no metal did not grow at all. Moreover, when aligned with the XoxF sequence of *M. fumariolicum* and other XoxF sequences shown to require lanthanum, the XoxF sequence of strain La 6 contains the characteristic amino acid residues required for coordination (Asp319) and accommodation (Thr278 and Gly190) of lanthanide atoms (Pol et al. 2014).

5.3 Genetics and growth characteristics of a *xoxF* mutant

5.3.1 Organisation of the methanol dehydrogenase gene, *xoxF*

Previous functional gene PCR screens of isolate La 6 revealed that it contained only one copy of the alternative methanol dehydrogenase (MDH), *xoxF5*, and that did not contain the canonical MDH encoded by *mxaF*. Genome sequencing confirmed this finding, and revealed *xoxF5* to be in a cluster with *xoxG* (encoding an associated cytochrome c used as an electron acceptor during methanol oxidation; Anthony 1992) and *xoxJ*, encoding a putative periplasmic binding protein (of which very little is known). Adjacent genes are similar to those found in the known methylotrophs *Rhodobacter sphaeroides* and *Paracoccus aminophilus* JCM7686 that employ the glutathione-dependent formaldehyde oxidation pathway (Wilson et al. 2008; Dziewit et al. 2015), both of which, like La 6, only contain *xoxF5* (Figure 5.3).

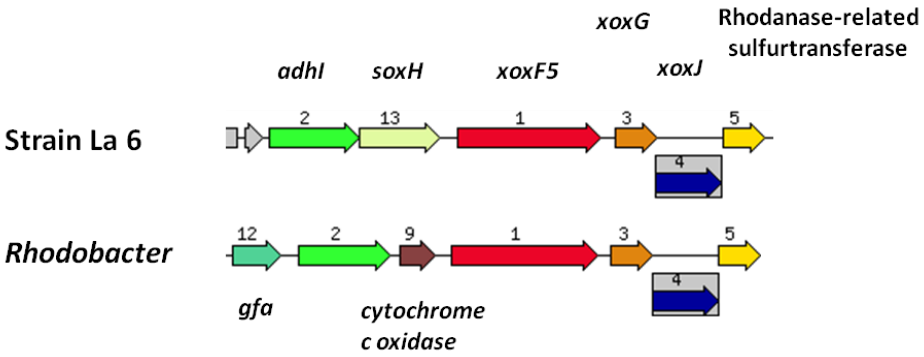

Figure 5.3 Gene cluster surrounding the predicted methanol dehydrogenase gene *xoxF5* (locus tag La619760) and comparison to the methylotroph *Rhodobacter sphaeroides* 241. Colour and numbers indicate predicted similar functions of genes between the two organisms. *adhI*, glutathione-dependent formaldehyde dehydrogenase; *soxH*, putative protein SoxH; *xoxF5*, methanol dehydrogenase; *xoxG*, cytochrome c-553i; *xoxJ*, hypothetical periplasmic binding protein; *gfa*, homologue of glutathione-formaldehyde activating enzyme.

At the time of the isolation and sequencing of La 6, the mutational analysis *Paracoccus aminophilus* JCM7686 had not been published, and so *R. sphaeroides* was the only strain similar to La 6 that had shown *xoxF5* was the sole MDH (Dziewit et al. 2015; Wilson et al. 2008). With so few methylotrophs containing only one MDH, there was little evidence of the direct role of the *xoxF5* gene in methanol metabolism. Therefore the role of the *xoxF5* gene of strain La 6 was investigated by the method of gene disruption.

5.3.2 Construction of a *xoxF* mutant, strain XoxF$^-$

A single allelic exchange method was used to generate an insertional mutation in the *xoxF5* gene. A 672bp internal fragment of the *xoxF* gene was amplified with primers La6delBamF and La6delPstR that incorporated *BamH*I and *Pst*I sites, respectively (Table 5.2). This was ligated into digested suicide vector pK19mob (Schafer et al. 1994) to form p672xoxF and was transformed into *E. coli* TOP10 cells. Confirmation of a p672xoxF positive transformant was checked by a plasmid miniprep, digestion with *BamH*I and *Pst*I and analysis of the cleaved 672 bp product and plasmid on an agarose gel. Plasmid p672xoxF was then conjugated from this strain into strain La 6^{Rif} in triparental matings with helper plasmid pRK2013 (Figurski & Helinski 1979), using the method described in Materials and Methods. Rif^R and Kan^R single cross over transformants were checked using colony PCR with primers CheckmutF and CheckmutR that amplified a 1580 bp region spanning from within the disrupted genomic *xoxF* gene to inside the kanamycin cassette of the incorporated p672xoxF plasmid. Figure 5.4 shows the agarose gel showing the correctly amplified product. The single cross over mutant strain was termed La 6 strain XoxF$^-$. Primers relating to this work can be found in Table 5.2.

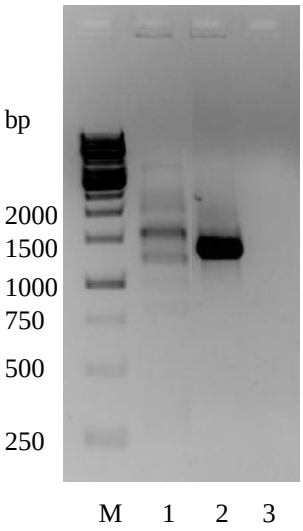

Figure 5.4 PCR primers CheckmutF and CheckmutR were used to confirm the single cross over event of plasmid p672*xoxF* into the genomic *xoxF gene*, creating the mutant strain *XoxF::*p672*xoxF*, termed La 6 XoxF$^-$. Lane 1: wild-type strain La 6; lane 2: *XoxF::*p672*xoxF*; lane 3: no template control.

5.3.3 Growth of strain XoxF⁻ on methanol and ethanol

The ability of La 6 strain XoxF⁻ to grow on methanol and ethanol was assessed by the same method described for the effect of lanthanum during growth on methanol and ethanol. Growth data are summarised in Figure 5.5.

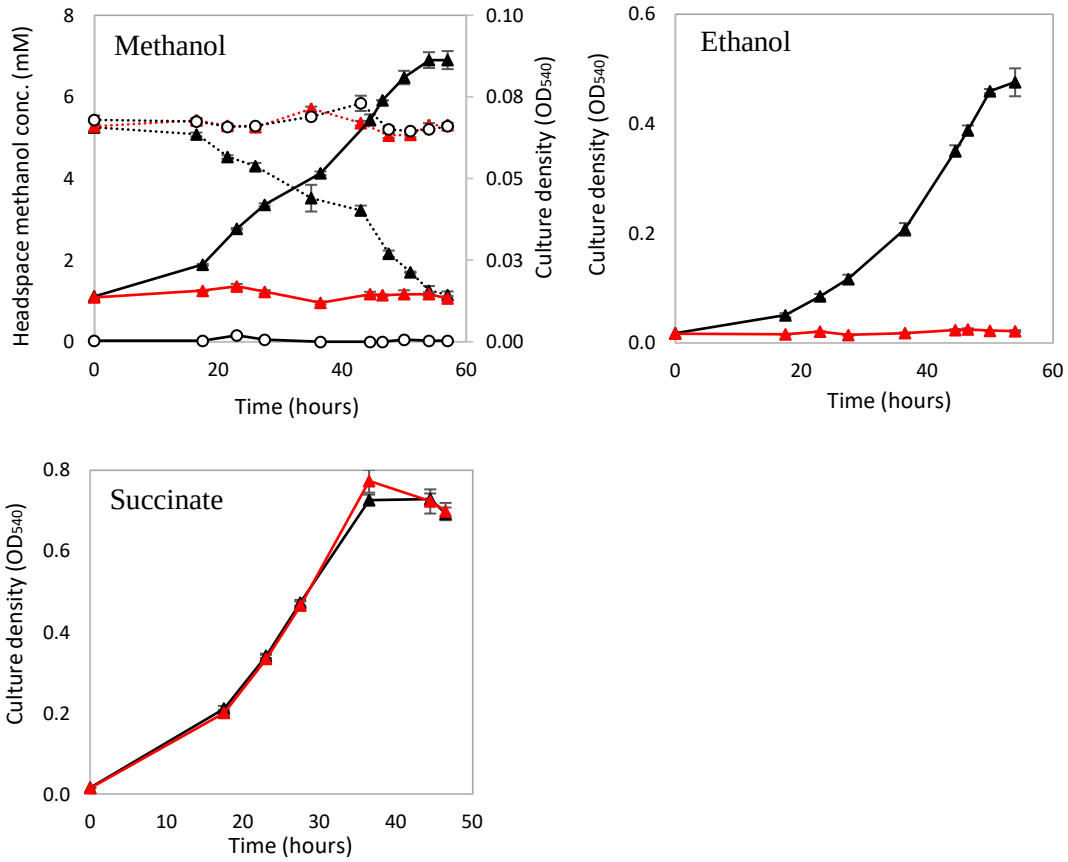

Figure 5.5 Growth of La 6 wild-type strain (black triangles), strain XoxF⁻ (red triangles) or no inoculum controls (white circles) on 5 mM methanol (top, left), ethanol (top, right) or succinate (bottom). Dashed lines represent methanol headspace concentrations. Error bars show standard error of three replicate cultures.

Mutation of the *xoxF* gene abolishes growth of strain La 6 XoxF⁻ on both methanol and ethanol, whilst it is still able to grow comparably to the wild-type on succinate. This suggests that *xoxF* is directly involved in the turnover of methanol and ethanol and that it is essential for methylotrophic survival and confirms the idea that *xoxF* functions as an MDH in this organism.

5.3.4 Complementation of XoxF$^-$

To show that the inability of strain XoxF$^-$ to grow on methanol and ethanol was directly due to the loss of a functional XoxF rather than due to a polar mutation, the strain was complemented with the wild-type *xoxF* on a taurine inducible plasmid (pLMB509). To construct the plasmid, the full *xoxF* sequence was amplified by PCR using primers La6xoxFNdeF and La6xoxFSacR that incorporated *NdeI* and *SacI* sites respectively. This was ligated into pGEM-T Easy vector and transformed into TOP10 *E.coli* cells, as described in Materials and Methods. The plasmid was extracted by miniprepping, digested with *NdeI* and *SacI* and the correct sized product extracted and purified from an agarose gel. The fragment was ligated into the *NdeI* and *SacI* digested broad host range vector, pLMB509 (Tett et al. 2012) and transformed into *E. coli* TOP10. Transformants containing the correct insert were screened using the primers used to originally amplify the *xoxF* gene. The insert was sequenced using PCR primers pLMB509F and pLMB509R. The confirmed vector was termed p509LA6. This vector was the conjugated into La 6^{Rif} in triparental matings using the method described in Materials and Methods. The strain was termed La 6 XoxF$^-$ p509LA6.

The ability of La 6 strain XoxF$^-$ p509LA6 to grow on methanol, ethanol and succinate was assessed. A single colony was inoculated into 10 ml MB media containing kanamycin (to maintain the insertional mutation), gentamycin and 10 mM taurine to induce *xoxF* expression. This was used as a 5% (v/v) inoculum into duplicate serum vials containing 120 ml MBM, kanamycin, gentamycin and taurine. Growth was then monitored by spectrophotometry. The growth data, which are summarised in Figure 5.6, show that complementation restores growth on methanol and ethanol similar to the wild-type. This again supports the idea that *xoxF* is the sole MDH in strain La 6.

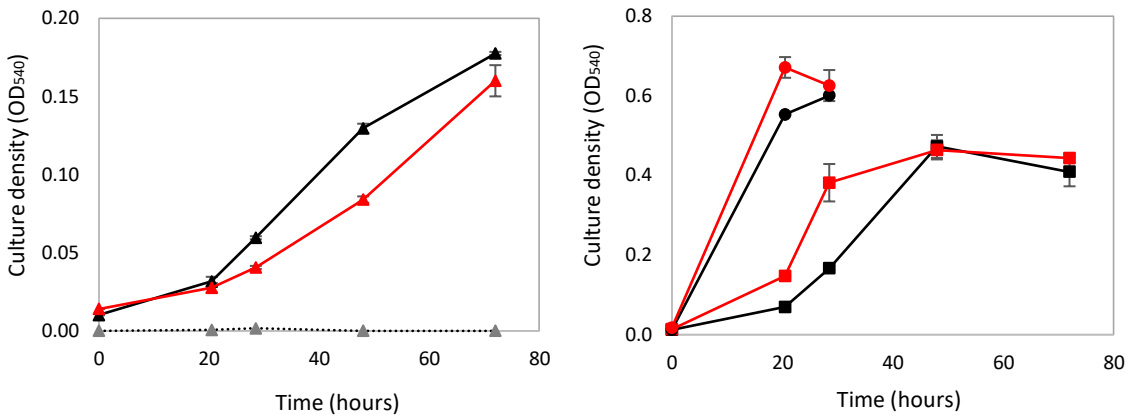

Figure 5.6 Growth of La 6 wild-type strain (black), complemented strain La 6 XoxF⁻ p509LA6 (red) and no-inoculum controls (grey) on 5 mM methanol (left), ethanol (right, squares) and succinate (right, circles). Error bars show the range of duplicate cultures.

5.3.5 Methanol dehydrogenase expression and activity in strain La 6 wildtype and XoxF⁻

To confirm the loss of XoxF expression in strain XoxF⁻, the strain was grown on 5 mM succinate or benzoate and the soluble fractions analysed by SDS-PAGE. These were directly compared to the soluble fractions of the wild type strain grown on 5 mM methanol, ethanol, succinate and benzoate, seen in Figure 5.7a. The presence of a band around 65 kDa in all wild-type conditions, and the lack of it in both XoxF⁻ conditions supports the previous growth data, in that strain La 6 requires the expression of the (predicted) 64.9 kDa XoxF protein to grow on methanol. It also suggests that the enzyme is constitutively expressed, as it is present during growth on all carbon compounds tested, not just methanol. Excision of the band indicated by the arrow and analysis by mass spectrometry (MS) confirmed the identity of XoxF (25% protein sequence coverage). Analysis of the mutant and the complemented strain XoxF⁻ P509LA6 grown on a mixture of 5 mM succinate and methanol also confirmed the presence of the over-expressed XoxF band in XoxF⁻ P509LA6, shown in Figure 5.7b.

The soluble fractions of cell extracts of the wild-type grown on methanol were assayed for methanol dehydrogenase activity (MDH) using the standard PMS/DCPIP linked assay as described in Materials and Methods. The optimum pH for this enzyme was determined to be pH 9.0, and ammonium was required for activity, so all assays were further conducted with these conditions. The wild-type strain grown on methanol had a

specific activity of 262 nmol min^{-1} mg^{-1} protein (± 6 s.e), whilst there was no activity in the $XoxF^-$ strain grown on the combined substrates of methanol and succinate, again confirming the role of XoxF in the metabolism of methanol.

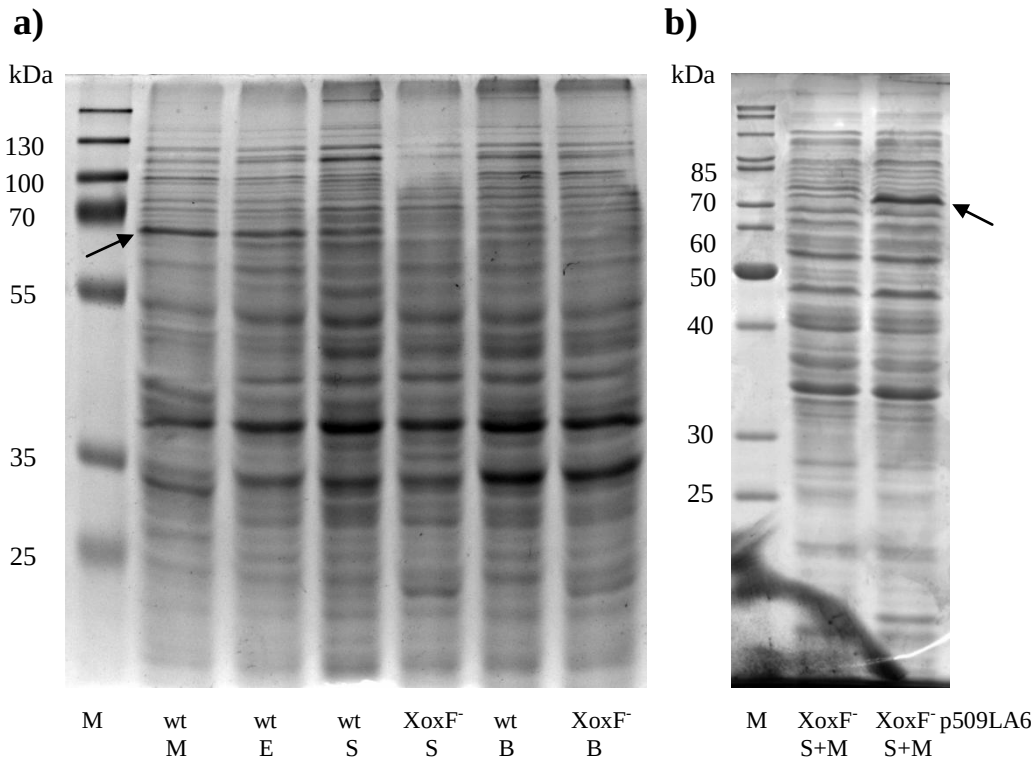

Figure 5.7 SDS-PAGE of strains $XoxF^-$ and $XoxF^-$ p509LA6 grown on different carbon sources. a) Strain $XoxF^-$ grown on succinate and benzoate, showing the missing XoxF band compared to the presence in the wild type grown on methanol, ethanol, succinate and benzoate. b) Strain $XoxF^-$ p509LA6 showing restored expression of XoxF when grown on succinate and methanol compared to the mutant $XoxF^-$. Abbreviations: M, methanol; E, ethanol; S, succinate; B, benzoate. The band corresponding to the XoxF polypeptide is indicated by the arrow.

Table 5.2 PCR primers used in the work described in this chapter. Restriction sites are underlined.

Primer name	**Sequence (5'-3')**	**Amplicon length (bp)**	**PCR conditions Ann temp (°C), cycles**
La6delBamF	GCGGATCCTTGGTGCCCAGGGCCGCC	672	62, 30
La6delPstR	GGCTGCAGTCGCACCTGACCGCCTA		
CheckmutF	CACCGTGGTGGCGCTGGATGC	1580	64, 35
CheckmutR	ACCCAAGCGGCCGGAGAACCT		
La6xoxFNdeF	GGATCCCATATGAAAAAGTTTGTCGCATGCCTG	1819	62, 30
La6xoxFSacR	CAGCGAGCTCTCAGTCGGGCAGCGCGAAGAC		

5.4 Physiological characterisation of strain La 6

Isolate La 6 is a Gram-negative, non motile, ovoid rod, 0.8-2.2 µm long and 0.5-1.2 µm wide in minimal media, shown in Figure 5.8. It is non motile when tested on swimming, swarming or twitching motility plates and in liquid medium. Colonies are very pale cream and 0.5-1.0 mm in diameter, uniformly circular, convex and opaque after growth on MBM minimal media at 25 °C for 6 days. Colonies are cream and 0.6-1.2 mm in diameter, uniformly circular, convex and opaque after growth on Marine Agar 2216 at 25 °C for 4 days.

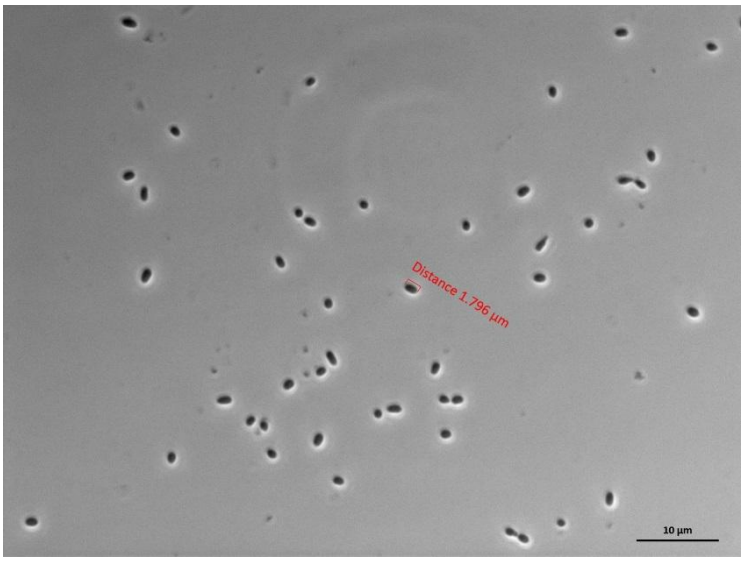

Figure 5.8 Phase contrast micrograph of La 6 grown on methanol in MBM medium. Bar indicates 10 µm.

5.4.1 Growth on carbon sources

The ability of strain La 6 to grow on a range of carbon compounds, including sugars, alcohols, alkanes, aromatic compounds and amino acids was tested as described in Materials and Methods. Compounds that the strain was able to grow on, concentrations used and maximum cell density (OD_{540}) are summarised in Table 5.3, whilst those it was unable to utilise are listed below that.

Table 5.3 Compounds utilised by strain La 6 as sole source of carbon and energy, presented in alphabetical order. Inoculum used was grown on succinate.

Carbon source	Concentration	OD_{540}	Carbon source	Concentration	OD_{540}
1-butanol	0.05 % (v/v)	0.82	Fructose	5 mM	1.20
1-propanol	0.05 % (v/v)	0.28*	Glucose	5 mM	1.20
2-propanol	0.05 % (v/v)	0.07*	Glycerol	5 mM	0.13
4-hydroxybenzoate	3.6 mM	0.20	Glycine betaine	10 mM	0.13
Acetate	5 mM	0.37	Malate	5 mM	0.71
Acetone	0.05 % (v/v)	0.13*	Mannitol	5 mM	0.76
Acrylic acid	10 mM	0.17	Mannose	5 mM	0.96
Alanine	0.2 % (w/v)	0.28	Methanol	5 mM	0.18
Arabinose	5 mM	0.50	Propanal	5 mM	0.30*
Arginine	0.2 % (w/v)	0.24	Propane	20 % (v/v)	0.05*
Benzoate	5 mM	1.00	Propionate	5 mM	0.42*
Butane	20 % (v/v)	0.17	Protocatechuate	5 mM	0.87
Catechol	5 mM	1.32	Pyruvate	5 mM	0.40
Citrate	5 mM	0.49	Ribose	5 mM	0.18
Ethanol	5 mM	0.54	Serine	0.1 % (w/v)	0.10
Formate	5 mM	0.08	Sorbitol	5 mM	0.82
Formate	20 mM	0.13	Succinate	5 mM	0.80

*Cell densities reached higher when-propane grown inoculum was used, described later in the chapter.

Strain La 6 was unable to utilise the following carbon compounds as sole source of carbon and energy, in alphabetical order: 2-butanol (0.05%), 3-hydroxybenzoate (3.6 mM), 4-chlorobenzoate (saturated solution), benzene (1 mM), cysteic acid (10 mM), dimethylamine (10 mM), dimethylsulfide (2 mM), dimethylsulfoniopropionate (2 mM), dimethylsulfoxide (0.5 mM), ethane (20 % v/v), glycine (5 mM), glyoxylate (5 mM), lactose (5 mM), methane (20 % v/v), methane sulfonic acid (20 mM), methionine (0.2%), monomethylamine (10 mM), naphthalene (saturated solution), p-cresol (1 mM), p-xylene (1 mM), phenol (0.04%), rhamnose (5 mM), sucrose (5 mM), taurine (10 mM), toluene (0.5 mM), trimethylamine (10 mM), trimethylamine N-oxide (30 mM), urea (5 mM), vanillate (saturated solution).

5.4.2 Antibiotic sensitivity

The strain is sensitive to gentamycin, kanamycin, ampicillin, puromycin, rifampicin, tetracycline, vancomycin, chloramphenicol, streptomycin and erythromycin using the filter disk method. It is slightly resistant to tetracycline at 20 µg and resistant to naladixic acid and cyclohexamide at 100 µg.

5.4.3 General physiology

The temperature range for growth was 4-45°C, with the optimum at 37°C. The pH range for growth was pH 4.5-9 (optimum pH 7.5) and the NaCl concentrations for growth were 0-15% w/v (optimum 3%), with no growth at 20%. Strain La 6 did not grow under anaerobic conditions and did not reduce nitrate or nitrite. It did not hydrolyse cellulose, gelatin or starch, or ferment glucose or lactose aerobically or anaerobically. The strain was negative for thiosulfate oxidation. It produced indole acetic acid when supplemented with tryptophan, but not without. The strain did not produce any acetone/methanol extractable pigments or bacteriochlorophyll a after growth in either a light/dark cycle or in the dark after 5 days at 22 °C, therefore suggesting growth of the isolate is exclusively non-photosynthetic chemoheterotrophic. La 6 required vitamin B12 for growth, and was oxidase and catalase positive.

5.4.4 Dimethylsulfonioproionate (DMSP) and dimethylsulfide (DMS) metabolism

Although strain La 6 was unable to grow on DMSP as sole source of carbon and energy, some members of the *Roseobacter* clade are able to metabolise DMSP by either demethylating it or cleaving it, releasing dimethylsulfide (DMS) into the atmosphere. DMS is a compound of high environmental interest as its oxidation products can act as cloud condensation nuclei, it can act as a chemo attractant for many marine animals and it is also a huge source of organic sulfur in the sulfur cycle (Schäfer et al. 2010; Curson et al. 2011; Moran et al. 2012, and references therein). When tested strain La 6 did indeed degrade DMSP, with DMS being produced at a rate of 72 pmol µg protein^{-1} min^{-1} (4.8 s.e.).

Not only can bacteria degrade DMSP, but ongoing research by Todd et al at UEA (data unpublished) has shown that some bacteria can also produce DMSP breaking the long standing dogma that only eukaryotes such as phytoplankton and angiosperm are able to synthesise DMSP. Indeed, when tested, La 6 could synthesise DMSP at a rate of 2.3 pmol μg protein^{-1} min^{-1} (0.15 s.e.).

Lastly, some *Roseobacter* strains are also able to produce DMS independently of DMSP via methylation of methane thiol (Carrión et al. 2015), however no DMS was detected when strain La 6 was tested for this trait.

5.4.5 Analysis of the growth on propane and potential secondary metabolites

Sequencing and analysis of the genome revealed two predicted methane/phenol/toluene hydroxylases, La638380 and La63840 within a cluster of other alkane metabolism genes as illustrated in Figure 5.9 and the annotations summarised in Table 5.4. Work conducted by Crombie (2011) showed that *Methylocella silvestris* is able to grow on propane, metabolising it using a propane monooxygenase, with the gene encoding for the alpha monooxygenase hydroxylase subunit designated Msilv1651. The relatively high amino acid identity (77%) that La63840 shares with *M. silvestris* gene 1651, and the strong similarity between the surrounding clusters in *M. silvestris* and La 6 suggested that La 6 may also encode a functional propane monooxygenase. The cluster surrounding La63840 of La 6 and closely related clusters in other bacteria is shown in Figure 5.9. Moreover, phylogenetic analysis of the amino acid sequence of La63840 with other short chain alkane and alkene and related monooxygenases revealed that it clearly clusters with other propane monooxygenases (Figure 5.10).

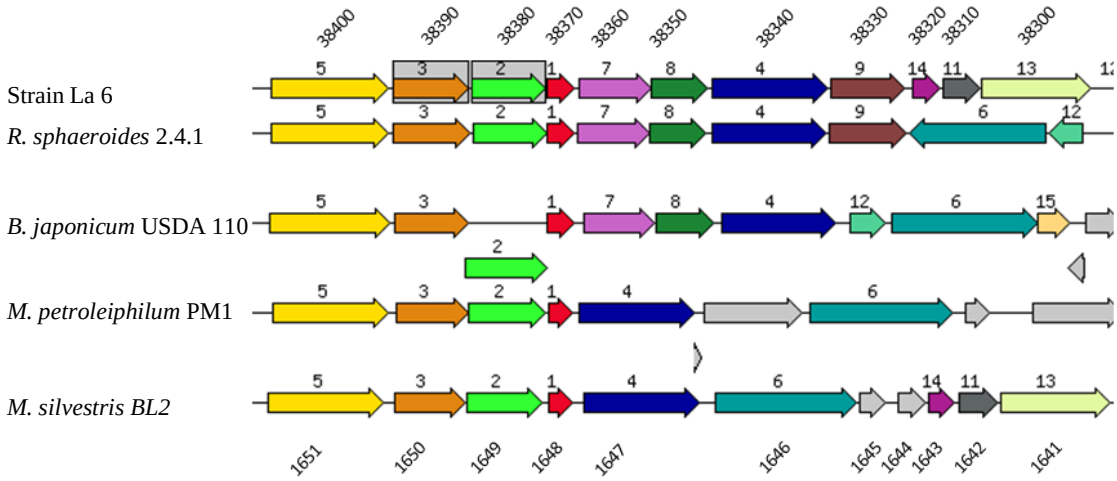

Figure 5.9 Gene cluster surrounding the predicted methane hydroxylase genes La638380 and La638400 and closely related clusters in other organisms. Colour and numbers indicate predicted similar functions between genes. Diagonal numbers above La 6 and underneath *M. silvestris* genes are their corresponding gene locus tags.

Table 5.4 Protein annotations of the gene cluster surrounding the predicted methane hydroxylase genes La638380 and La638400 in strain La 6 and their closest blastp hits and identities from the *Methylocella silvestris* BL2 genome. Gene number corresponds to the numbers above genes in Figure 5.9. Gene 6 not present in La 6 but within the *M. silvestris* cluster (locus 1646) is included for completeness.

Gene number in Figure 5.9	Locus tag (La 6)	Annotation	Closest blastp hit to *M.silvestris*, locus tag	Identity (%)
1	38370	MmoB/DmpM family protein	Phenol 2-monooxygenase, 1648	61
2	38380	Methane/Phenol/Toluene Hydroxylase	Methane/phenol/toluene hydroxylase, 1649	61
3	38390	2-polyprenylphenol hydroxylase	Oxidoreductase, 1650	53
4	38340	Chaperonin GroEL (HSP60 family)	60 kDa chaperonin, 1647	45
5	38400	Methane/Phenol/Toluene Hydroxylase	Methane monooxygenase, 1651	77
6	-	-	σ^{54} transcriptional regulator, 1646	-
7	38360	Putative metal-dependent hydrolase of the TIM-barrel fold protein	-	-
8	38350	putative metal-sulfur cluster	-	-
9	38330	Zn-dependent alcohol dehydrogenase	Zinc-dependent alcohol dehydrogenase, 1821	33
11	38310	Gluconate 2-dehydrogenase subunit 3	Uncharacterised, 1642	48
13	38300	Choline dehydrogenase	Gluconate 2-dehydrogenase, 1641	72
14	38320	Putative enzyme	Glyoxylase-like protein, 1643	60

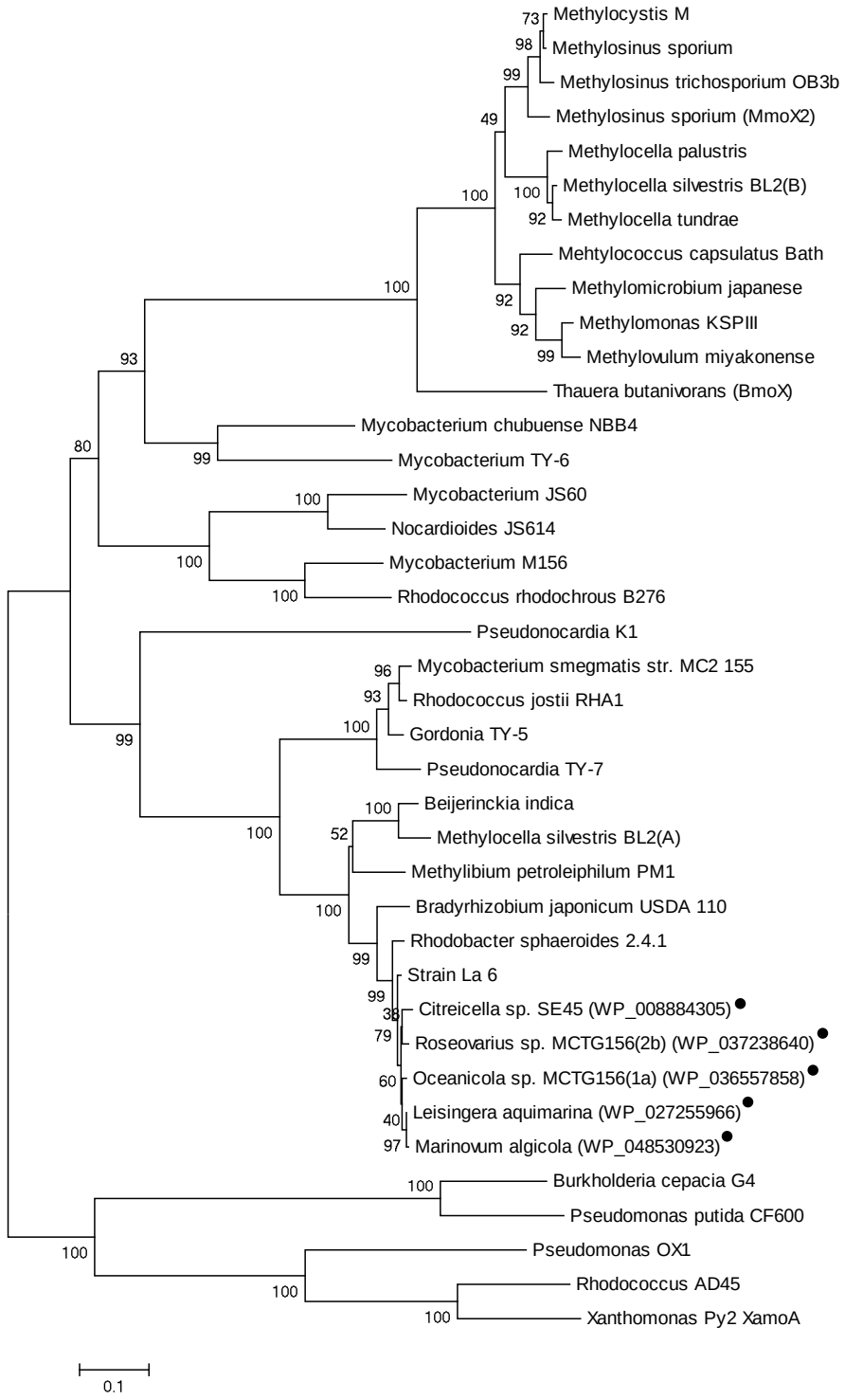

Figure 5.10 Phylogenetic analysis of the amino acid sequence of the alkane monooxygenase alpha subunit of strain La 6 and the corresponding polypeptides of other soluble di-iron monooxygenases (SDIMOs). The tree was constructed based on sequences from Crombie 2011; accession numbers of sequences not shown in the tree can be found within. The tree was constructed using the Neighbour joining method using MEGA6, with bootstrap values representing 1000 replications. Black dots indicate the closest relatives from a blastp search.

Tests of the ability of strain La 6 to grow on propane (20% v/v) revealed it was able to do so, although only to cell densities of around OD_{540} 0.05 after 8 days, as seen in Table 5.3 (or 0.100 after 15 days). Therefore strain La 6 was tested to see if it could grow on potential metabolites of propane. The oxidation of propane can be metabolised in two ways via a monooxygenase; oxidation of the terminal carbon atom to 1-propanol or oxidation of the sub-terminal carbon to 2-propanol. These are then metabolised further by different pathways, simplified in Figure 5.11.

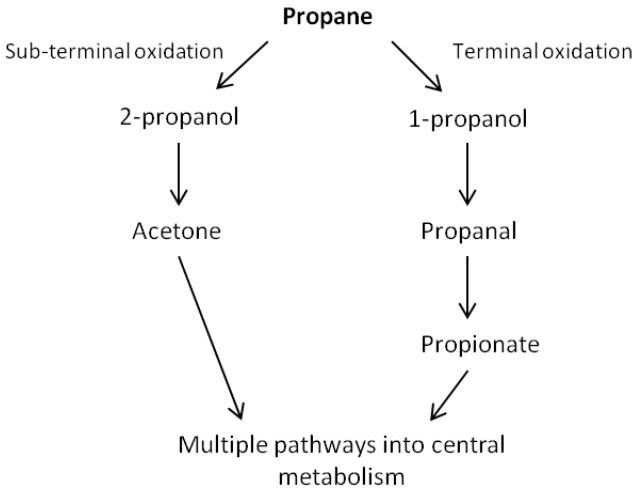

Figure 5.11 Summary of the products of bacterial metabolism of propane via the terminal or sub-terminal pathways, leading into central metabolism.

Therefore strain La 6 was tested for growth on the metabolites of both oxidation pathways. Crombie (2010) and Patel (et al. 2012) showed that cultures of *M. silvestris* grown on propane highly expressed not only the propane monooxygenase proteins, but also those that are involved in the metabolism of the sub-terminal oxidation products of propane metabolism. These same proteins were not detectable in succinate-grown cells. Since strain La 6 grew very poorly on propane, attempts were made to enhance growth by using inoculum that may already be expressing propane metabolising proteins. Comparison against cultures given succinate-grown inoculum and then growing these on potential propane metabolites may have revealed insights into which pathway La 6 used. Triplicate cultures were inoculated with either succinate or propane grown cells and monitored for growth. Growth data are summarised in Figure 5.12.

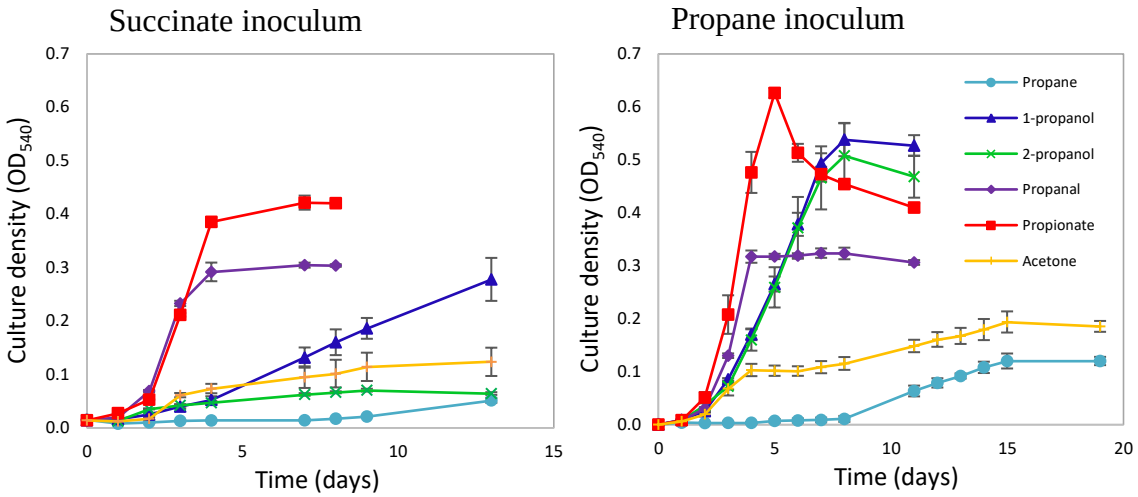

Figure 5.12 Growth of strain La 6 on propane and possible intermediates of propane metabolism using succinate (left) or propane (right) grown inoculum. 1-propanol, 2-propanol and acetone was added at 0.05% (v/v), propanal and propionate at 5 mM and propane at 20% (v/v). Error bars represent the standard error of three replicates.

Strain La 6 was able to grow well on propionate, propanal and 1-propanol from both succinate and propane inoculum, although growth on propionate and 1-propanol and are greatly stimulated when using cells pre-grown on propane, suggesting the use of the terminal oxidation pathway. However, although 2-propanol was not metabolised to a high degree using a succinate grown inoculum, La 6 was able to grow to a similar cell density on 2-propanol as 1-propanol using an inoculum of propane grown cells. This suggests that the sub-terminal oxidation pathway may be employed, although growth on acetone was only very slightly stimulated when propane grown inoculum was used, compared to succinate grown inoculum. Based on only these few data, it is difficult to draw conclusions as to which pathway strain La 6 uses during growth on propane. Much like the work done by Crombie (2011), analysis of the metabolites produced in the media during growth, expression studies and mutational analyses must be performed to fully understand the metabolism of propane in strain La 6.

5.4.6 Fatty acid analysis

One tool which is used widely used as a chemotaxonomic standard in taxonomy is fatty acid analysis (Mergaert et al. 2001; Da Costa et al. 2011). As strain La 6 may be a novel genus, the strain was sent for analysis. Table 5.5 summarises the results and compares La 6 against members of the *Oceanicola* species, the genus which it has the highest identity at the 16S rRNA gene level, and *Sedimentitalea nanhaiensis*, the bacterium most closely related to La 6 at the multi-locus sequence analysis (MLSA) level (see later for further discussion on MLSA). The presence of four different types of fatty acids in La 6 that are not present in any of the comparison organisms suggests that the strain is substantially different and may indeed be a new genus.

Table 5.5 Cellular fatty acid content of strain La 6, species of the *Oceanicola* genus and the closest relative based on MLSA, *Sedimentitalea nanhaiensis*. Values are percentages of total fatty acids. -, not detected.

Fatty acid	***O. marinus* AZO-C**	***O. batsensis* HTCC2597**	***O. granulosus* HTCC2516**	***O. nanhaiensis* SS011B1-20**	***S. nanhaiesensis* NH52F**	***La 6***
10:0	-	-	0.1	0.2	-	-
10:0 3-OH	0.1	0.4	1.5	1.0	3.9	-
12:0	-	2.0	-	0.1	-	-
12:0 3-OH	2.1	-	1.6	-	2.9	-
12:1ω11c	-	4.9	-	-	-	-
14:0	0.2	1.5	-	0.3	-	-
14:0 3-OH	-	-	-	0.9	-	-
Summed feature*	-	-	-	-	-	7.31
15:0	-	0.9	-	-	-	-
15 : 0 3-OH	-	-	-	-	-	0.87
Summed feature**	1	-	1.2	1.8	0.6	-
16:0	14.7	15.0	11.9	7.0	3	5.36
16 : 0 2-OH	-	-	-	-	-	6.19
16 : 1 2-OH	-	-	-	-	-	1.13
17:0	-	1.5	0.4	0.4	-	-
17:1ω8c	-	-	0.3	0.4	-	-
17:0 anteISO	-	-	0.2	-	-	-
17:0 cyclo	-	-	0.2	-	-	-
18:0	1.0	2.4	0.9	1.3	-	1.16
18:1ω7c	49.1	31.0	62.8	81.2	71.9	67.83
18:1 methyl	6.6	-	8.1	4.3	3	6.71
19:0 cyclo	24.6	40.4	10.8	1.1	-	3.44
Summed feature***	0.4					
20:2ω6,9c	0.2	-	-	-	-	-
Unknown 11.799	-	-	-	-	5.7	-

*14:0 3-OH/16 : 1 iso I

** 15:0 iso 2-OH / 16:1*ω*7*c*

***19:1*ω*6*c* / 19:0*ω*10*c* cyclo

5.5 Preliminary genome analyses

5.5.1 Overall genome features

The genome of strain La 6 was sequenced and assembled by collaborators John Vollmers and Anne Kaster at DSMZ (Germany) as described in the Materials and Methods. General genome statistics are summarised in Table 5.6. The majority of protein-coding genes were assigned a putative function, whilst one quarter of them were classified as 'hypothetical'. The genome was checked for completeness and contamination by the CheckM tool (Parks et al. 2015), indicating that it was 99.36% complete and had 0.85% contamination.

Table 5.6 General genome statistics of strain La 6.

Genome data	
Number of contigs	15
Genome size (bp)	6,789,082
Smallest contig (bp)	948
Largest contig (bp)	3,672,580
Average contig size (bp)	454,605
Median contig size (bp)	103, 981
N50	3,672,580
L50	1
GC content (%)	65.6
Number of genes	6, 554
Number of Coding Sequences (CDS)	6, 502
Number of hypothetical proteins (%)	1, 646 (25)
tRNAs	52
rRNAs	6

5.5.2 The general metabolic pathways in strain La 6

Local nucleotide database files of the genome sequence of strain La 6 were created using BioEdit software. BLAST searches against these databases and use of the KEGG (Kyoto Encyclopedia of Genes and Genomes) recruitment plots created by the RAST server provided a framework for establishing the potential metabolic pathways. Most of

the work is based solely on genetic inference and is not supported by experimental evidence; however it is discussed in more detail where experimental data has been conducted.

The genome had a complete tricarboxylic acid cycle (TCA) pathway and genes for the pentose phosphate pathway, Entner-Doudoroff and Embden-Meyerhof pathways. It also contained all genes required for ammonia assimilation (GOGAT).

5.5.3 Predicted methylotrophy pathway in strain La 6

As discussed in section 5.3, analysis of the genome of strain La 6 revealed the clustering of genes in involved in the glutathione-linked pathway of formaldehyde oxidation around *xoxF5*, suggesting this was the pathway employed during growth on methanol. BLAST searches of the genome for the tetrahydromethanopterin (H_4MPT) pathway employed by many methylotrophs for the oxidation of formaldehyde (such as the model methylotroph *Methylobacterium extorquens* AM1, Chistoserdova 2011) revealed that this pathway was not present, supporting the idea of the use of the glutathione-dependent pathway.

The role of this pathway was first shown in *Rhodobacter sphaeroides* strain Ga through deletion studies by Wilson et al (2008) (Figure 5.13). Briefly, the formaldehyde produced by XoxF is converted to hydroxymethyl-gluthathione (GS-CH_2OH) by a glutathione- formaldehyde activating enzyme (Gfa) or by a spontaneous reaction. This is then oxidised by a glutathione-dependent formaldehyde dehydrogenase, GSH-FDH (encoded by *adhI*), to S-formylGSH (GS-CHO), then converted to formate by S-formylGSH hydrolase (FGH). Lastly this is converted to CO_2 by a formate dehydrogenase (FDH).

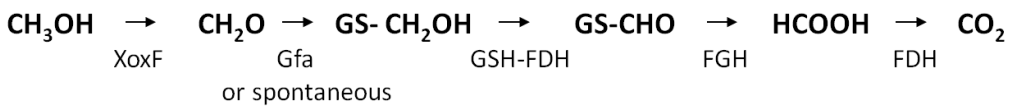

Figure 5.13 Pathway of methanol metabolism in Rhodobacter sphaeroides, as described by Wilson et al. (2008). XoxF, methanol dehydrogenase; Gfa, gluthathione-formaldehyde activating enzyme, GSH-FDH, glutathione-dependent formaldehyde dehydrogenase; FGH, *S*-formylglutathione hydrolase; FDH, formate dehydrogenase.

As seen in Figure 5.13, the cluster around *xoxF5* of strain La 6 does not encode the gene *gfa*, encoding for the glutathione-formaldehyde activating enzyme, like *R. sphaeroides*. BLAST searches of the genome showed seven potential gene sequences annotated as 'gfa-like' sequences that may perform this role. However none of them had an amino acid identity higher than 35%, suggesting that these may not be likely candidates. It was therefore possible that strain La 6 did not contain a gene responsible for converting the toxic formaldehyde to GS-CH_2OH, and relied solely on the spontaneous chemical reaction.

Research on the necessity of Gfa during growth on methanol is conflicting; deletion in *R. sphaeroides* did not affect the ability of the strain to grow on methanol, whilst purified Gfa from *Paracoccus deniftrificans* showed accelerated formation of S-hydroxymethylglutathione from formaldehyde and glutathione (Goenrich et al. 2002) (although recent work suggests it acts as a glutathione carrier, not as an enzyme, Hopkinson et al. 2015). Strain La 6 is unable to grow on methanol concentrations much higher than 10 mM (data not shown), with growth only ever reaching a maximum of around OD_{540} 0.180. It is tempting to speculate that the lack of a Gfa to detoxify the formaldehyde is the reason for the inability of strain La 6 to grow well on methanol; with XoxF rapidly converting the methanol to formaldehyde, and then relying on a spontaneous reaction to the GS-CH_2OH.

Further analysis of the genome revealed that it contains the tetrahydrofolate-linked (H_4F) pathway for metabolism, like *Paraccocus* and *Rhodobacter* strains (Figure 5.14). This pathway generates the key metabolite methylene-H_4T, which can either feed into the serine cycle for assimilation or serve as a further source of formate for generating energy. Current research would suggest that, in strain La 6, this pathway would rely on the spontaneous reaction between formaldehyde and H_4F. However, as suggested by Chistoserdova (2011), it may also be possible that FolD can function in the reductive direction and generate methylene-H_4 to provide carbon for assimilation into the serine pathway. Formate generated through the glutamate-linked pathway could be fed via the reversible enzyme formyl-H_4F ligase (FtfL) and methenyl-H_4F cyclohydrolase onto FolD. The genome of strain La 6 contained all genes encoding for three formate dehydrogenases (FDH); FDH1, 2, and 3.

As mentioned previously, strain La 6 contained all the genes for the serine pathway for assimilation. Methylotrophs utilizing the serine cycle require an additional pathway for regenerating glyoxylate; strain La 6 encodes all the genes for the ethylmalonyl-CoA pathway (EMCP) and does not contain isocitrate lyase. Lastly, strain La 6 also encodes for methyl-H_4F reductase (MetF) which oxidises methyl-H_4F originating from demethylation reactions such as in the metabolism of DMSP or chloromethane (Studer et al. 2001; Studer et al. 2002; Reisch et al. 2008; Curson et al. 2011). However, strain La 6 did not contain the *cmuAB* or *dmdA* (discussed in more detail later) genes that would suggest metabolism of these compounds.

The full predicted metabolic pathway for growth on methanol is summarised below in Figure 5.14.

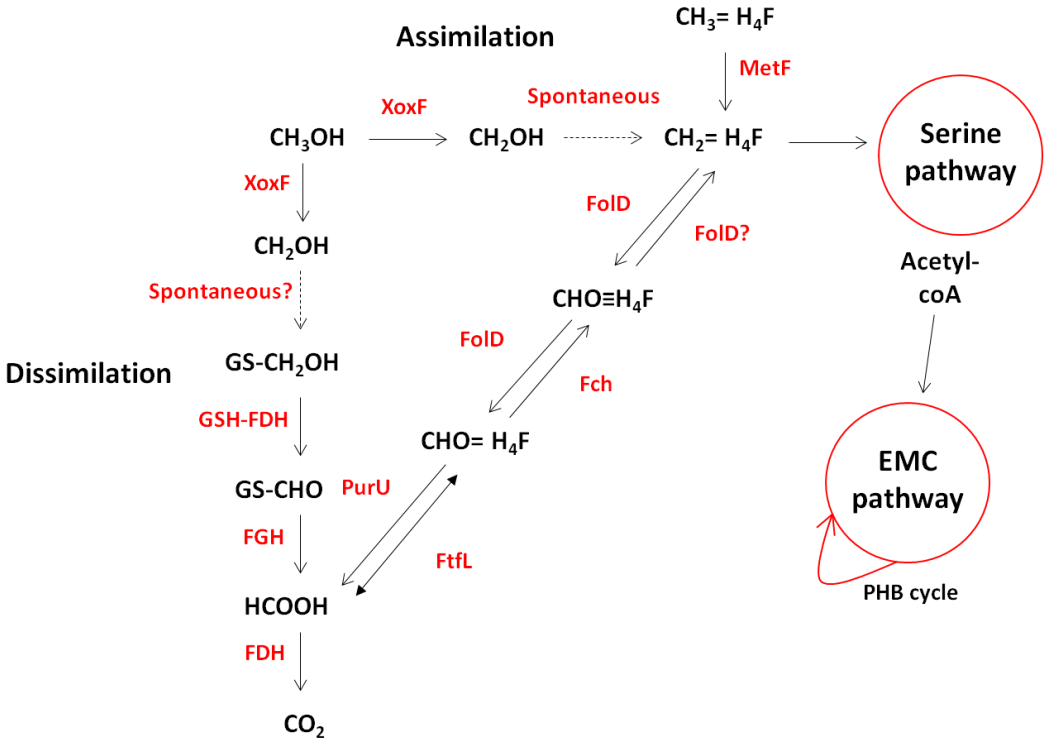

Figure 5.14 Predicted metabolic pathway of methanol metabolism in strain La 6 based on genome sequence analysis. Enzymes are shown in red whilst compounds or names of pathways are in black. Dashed arrows indicate reactions are non enzymatic (Spontaneous) or unknown (Spontaneous?). XoxF, methanol dehydrogenase; GSH-FDH, glutathione-dependent formaldehyde dehydrogenase; FGH, *S*-formylglutathione hydrolase; FDH, formate dehydrogenase; PurU, 10-formyl-H_4F hydrolase; FtfL, formyl-H_4F ligase; FolD, bifunctional methylene-H_4F dehydrogenase and methenyl-H_4F cyclohydrolase; Fch, methenyl-H_4F cyclohydrolase; MetF, methyl-H_4F reductase.

5.5.4 Other predicted metabolic pathways

Methylated amines

As discussed in section 5.2.2, strain La 6 contained the *gmaS* (but not *mauA*) for the metabolism of monomethylamine (MMA). However tests revealed that the strain was unable to grow on MMA as sole source of carbon and energy, but was able to utilise it as a nitrogen source when supplemented with succinate as an alternative carbon source. It was also unable to grow on dimethylamine (DMA), trimethylamine (TMA) or trmethylamine N-oxide (TMAO) as sole source of carbon and energy. Analysis of the genome revealed that it contained a gene with high sequence similarity (75%) to the trimethylamine monooxygenase gene (*tmm*) of *Methylocella silvestris* and to the TMAO demethylase (*tdm*, 71% similarity) of *Ruegeria pomeroyii* (Chen et al. 2011; Lidbury 2014), together in the same gene cluster. An adjacent gene only showed fairly low (48%) similarity to the putative gene involved in DMA metabolism. Many strains of the *Roseobacter* are able to utilise TMA as a nitrogen source and some use TMA and TMAO as a supplementary energy source (Lidbury et al. 2015). Although not tested here, strain La 6 may therefore also be able to use these as nitrogen or supplementary sources.

DMSP and DMS metabolism

The ability of strain La 6 to cleave DMSP to DMS (see 5.4.4) is likely due to the presence of the DMSP lyase gene *dddL* in the genome. There are various DMSP lyase proteins; they act on DMSP by cleaving DMSP into DMS and either acrylate (DddL, P, Q, W, Y) or 3-hydroxypropionate (3HP) (DddP) (Curson et al. 2011). Some strains are capable of then metabolising the acrylate to 3HP via the action of AcuNK. This 3HP is then available as sole source of carbon and energy for many strains. Other bacteria contain a method of demethylating DMSP via the demethylase DmdA, producing methylmercaptopropionate (MMPA), which is then also further metabolised as sole source of carbon and energy. A comprehensive review can be found in Curson et al. (2011).

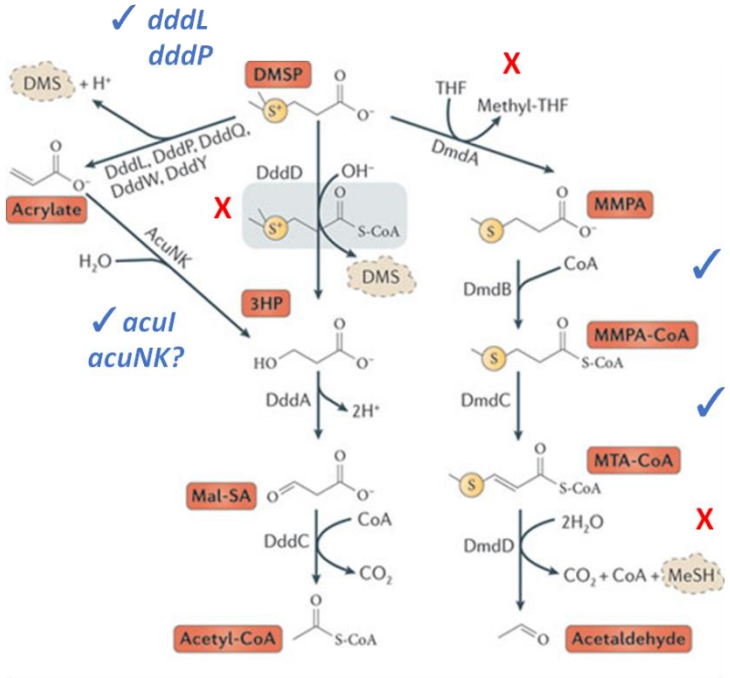

Figure 5.15 Biochemical pathways for dimethylsulfoniopropionate (DMSP) degradation in bacteria, modified from Curson et al (2011). Enzymes involved in demethylation and cleavage are shown next to the arrows. Blue ticks indicate presence of a specific gene in strain La 6 whilst red crosses indicate absence. DMS, dimethylsulfide; 3HP, 3-hydroxypropionate; Mal-SA, malonate semi-aldehyde; THF, tetrahydrofolate; MMPA, methyl- mercaptopropionate; MTA-CoA, methylthioacryloyl-CoA; MeSH, methanethiol.

Strain La 6 did not contain the gene for the demethylase, DmdA, in its genome and so it was therefore unable to grow on DMSP via the demthylation pathway. Although it contained downstream genes for this pathway, *dddB* and *dddC*, these genes are fairly widespread and found in genomes of bacteria that not metabolise DMSP at all. La 6 did contain genes that had some similarity to *acuN*, *acuK* and *acuI* (59%, 70% and 65% similarity, respectively) although these were not found clustered near any DMSP-related genes (as seen in *Halomonas* and *Alcaligenes,*, Curson et al. 2011) and may in fact encode for CaiB and CaiD, two enzymes that are involved in converting carnitine to γ-butyrobetaine in some bacteria. This may therefore explain why strain La 6 was unable to utilise DMSP as sole carbon source and can only cleave off DMS.

Lastly the production of DMSP by strain La 6 may be explained by the presence of a putative methyltransferase gene, termed *mmtB* by Jonathon Todd at UEA (in review). A mutant in the *mmtB* gene in strain *Labrenzia agreggata* IAM12614 was no longer able to synthesise DMSP, suggesting its direct role in DMSP production. The *mmtB* gene of strain La 6 had a 73% amino acid similarity to *mmtB* of *L. agreggata*, suggesting it did encode a functional MmtB.

Aromatic and phenolic compound degradation

Members of the *Roseobacter* clade are known for growing on various aromatic-related and phenolic compounds (Buchan 2001; Buchan et al. 2004; Alejandro-Marín et al. 2014). The ability of these organisms to degrade naturally occurring compounds produced from the decay of lignin but also of potentially harmful compounds from chemical contamination, such as from the degradation of polycyclic aromatic hydrocarbons (PAHs) make the *Roseobacter* clade an ecologically important group (Seo et al. 2009). Strain La 6 was able to grow on a range of tested compounds (see section 5.4.1), notably benzoate, 4-hydroxybenzoate, protocatechuate and catechol.

Analysis of the genome revealed gene clusters that may explain such capabilities, such as the *benABCD* cluster involved in benzoate metabolism, and the *pcaQDCHGB* cluster for protocatechuate metabolism (Buchan et al. 2004; Alejandro-Marín et al. 2014). Figure 5.16 summarises the genes found for some of the predicted pathways. The inability of strain La 6 to grow on 3-hydroxybenzoate supports the use of the *benABDC* system during growth on benzoate, as 3-hydroxybenzoate is one of the metabolites of benzoate degradation in the alternative pathway and so strain La 6 would have to metabolise the 3-hydroxybenzoate in order to grow on benzoate too.

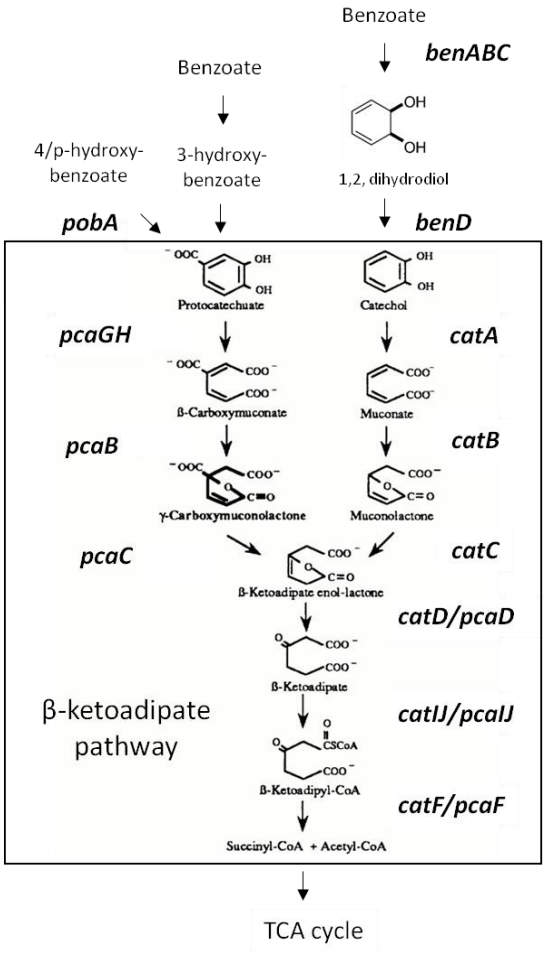

Figure 5.16 Genes identified in the genome of strain La 6 predicted to be involved in the degradation of some aromatic compounds. The β-ketoadipate pathway inside the box has been adapted from Harwood & Parales (1996) to show genes that encode for the enzymes at each step. Enzymes encoded by the gene shown: *pobA*, *p*-hydroxybenzoate hydroxylase; *pcaGH*, protocatechuate 3,4-dioxygenase; *pcaB*, β-carboxy-cis,cis-muconate lactonizing enzyme; *pcaC*, γ-carboxymuconolactone decarboxylase; *benABC*, benzoate dioxygenase; *benD*, benzoate dehydrogenase; *catA*, catechol 1,2-dioxygenase; *catB*, cis,cis-muconate lactonizing enzyme; *catC*, muconolactone isomerase; *catD*/*pcaD*, enol- lactone; *catIJ*/*pcaIJ*, β-ketoadipate:succinyl-coA transferase; *catF*/*pcaF*, β-ketoadipyl-coA thiolase; CMH, β-carboxymuconolactone hydrolase.

Sulfur oxidation

Whilst many *Roseobacters* are involved in the sulfur cycling via DMS and DMSP production, as previously mentioned, others have also be shown to oxidise inorganic sulfur compounds such as sulphite, sulphide and thiosulfate (Sorokin 1995; Gonzalez et al. 1999; Sass et al. 2010; Muthusamy et al. 2014). Although strain La 6 was unable to oxidise thiosulfate under the conditions tested (see Methods and Materials), it was not tested for the ability to oxidise sulphite or sulphide. However the strain did contain the full set of *sox* genes involved in the oxidation of thiosulfate, suggesting it may have the metabolic capacity to oxidise some types of inorganic sulfur. Some of the *sox* gene cluster can be seen in Figure 5.17.

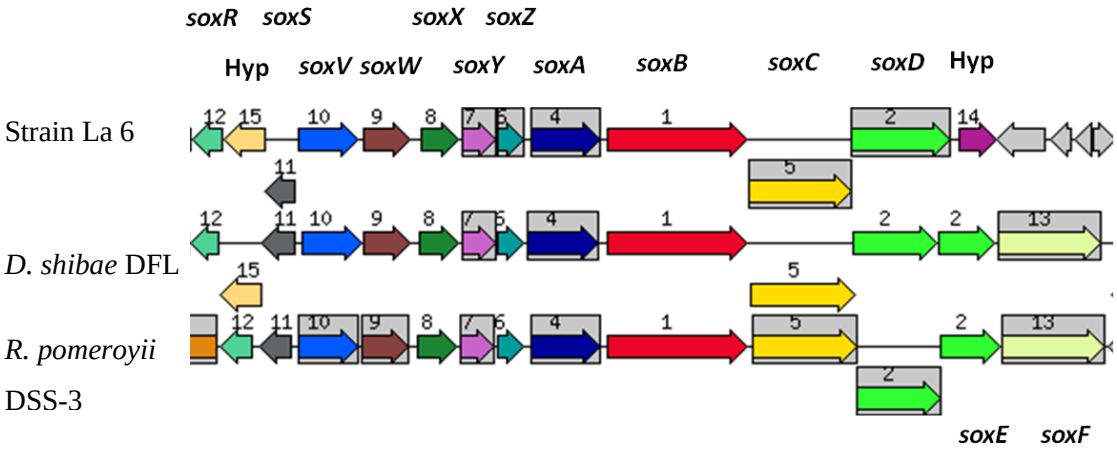

Figure 5.17 Gene organisation of the sulfur oxidation cluster, *sox,* in strain La 6 and related organisms. Colour and numbers indicate predicted similar functions of genes between the two organisms. *soxR*, regulatory protein; Hyp, hypothetical protein; *soxS*, regulatory protein, *soxW*, thioredoxin; *soxV*, sulfur oxidation V protein; *soxXYZAB* sulfur oxidation X, Y, Z, A and B proteins. *soxC*, molybdopterin C protein; *soxD*, sulfite dehydrogenase cytochrome subunit.

Carbon monoxide oxidation

The genomes of many members of the *Roseobacter* clade contain genes encoding for a carbon monoxide monoxygenase. *Ruegeria pomeroyii* DSS-3 oxidises carbon monoxide (Cunliffe 2011), and bacterial oxidation of CO is the primary CO sink in the marine environment (Zafiriou 2003). Therefore the presence of a CO cluster in the genome of strain La 6 may suggest it could also metabolise CO, although it does not contain the entire set of genes (missing *coxE* and *coxG*). Moreover it only contains form I of *coxL*, whilst those that do actively metabolise CO contain two copies, *coxL* form I and II. No physiological tests were done to test the organism. See Figure 5.18 for a schematic of the gene cluster.

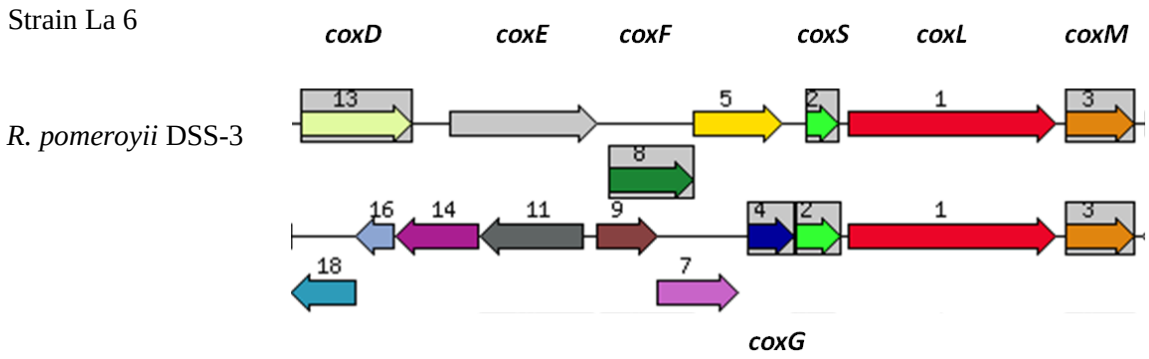

Figure 5.18 Gene organisation of the carbon monoxide (CM) oxidation cluster, *cox*, (form II) in strain La 6 and related organisms. Colour and numbers indicate predicted similar functions of genes between the two organisms. *coxD*, CM dehydrogenase D protein; ; *coxE*, CM dehydrogenase E protein; *coxF*, CM dehydrogenase F protein; *coxG*,; CM dehydrogenase G protein; ; *coxS*, CM dehydrogenase small subunit; *coxL*, CM dehydrogenase large subunit; *coxM*, CM dehydrogenase medium subunit.

5.6 Comparative genomics

5.6.1 Genome sizes of the *Roseobacter* clade

Roseobacters are known for having large genomes, versatile metabolic capabilities and fairly high G + C contents, all of which isolate La 6 is no exception (Luo & Moran 2014). However isolate La 6 has by far the largest genome of all sequenced members of the *Roseobacter* clade to date at 6.79 Mbp in size, compared to the next largest genome

of *Roseovarius indicus* DSM 26383 at 6.1 Mbp, as shown in Figure 5.19. Members of the *Oceanicola* genus have genome sizes between 3.8 to 4.86 Mbp, again suggesting that strain La 6 is a member of a novel genus.

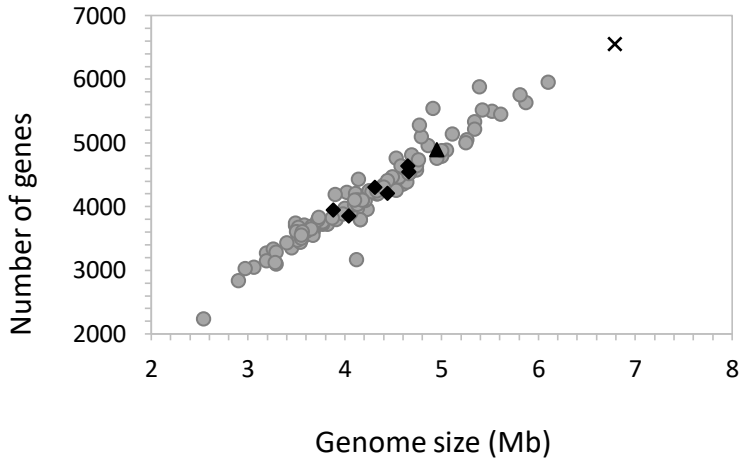

Figure 5.19 Relationship between genome size and number of genes in the genome of strain La 6 compared to the genomes of 114 members of the *Roseobacter* clade. The genome of strain La 6 is the represented by the cross, the triangle is *Sedimentitalea nanhaiensis* NH52F, diamonds are members of the *Oceanicola* genus and circles are all other members of the *Roseobacter* clade.

5.6.2 Multi-Locus Sequence Analysis (MLSA) and gene content analysis

The work in this section is credited to Dr John Vollmers, with permission, as he performed the bioinformatic analysis and created the following figures. It is presented here as it was undertaken due to the close collaboration between him and myself and is directly relevant to the work and enhances the understanding of strain La 6.

Multi-Locus Sequence Analysis was performed (as described in Methods and Materials) in order to examine the phylogenetic relationship between the core genome of strain La 6 and other sequenced *Roseobacter* genomes. Gene content analysis was performed and compared against the MLSA to investigate the similarities and differences in gene composition between comparison genomes, seen in Figure 5.20. Stain La 6 clusters coherently within Subgroup 1 of the *Roseobacter* group, which currently consists of the genera *Leisingera*, *Pseudophaeobacter*, *Phaeobacter, Ruegeria* and *Sedimentitalea*. However, at gene content level, this organism clusters distinctly apart from subgroup 1 and far more closely with *Oceanicola* and *Celeribacter* strains, indicating unique genetic adaptations.

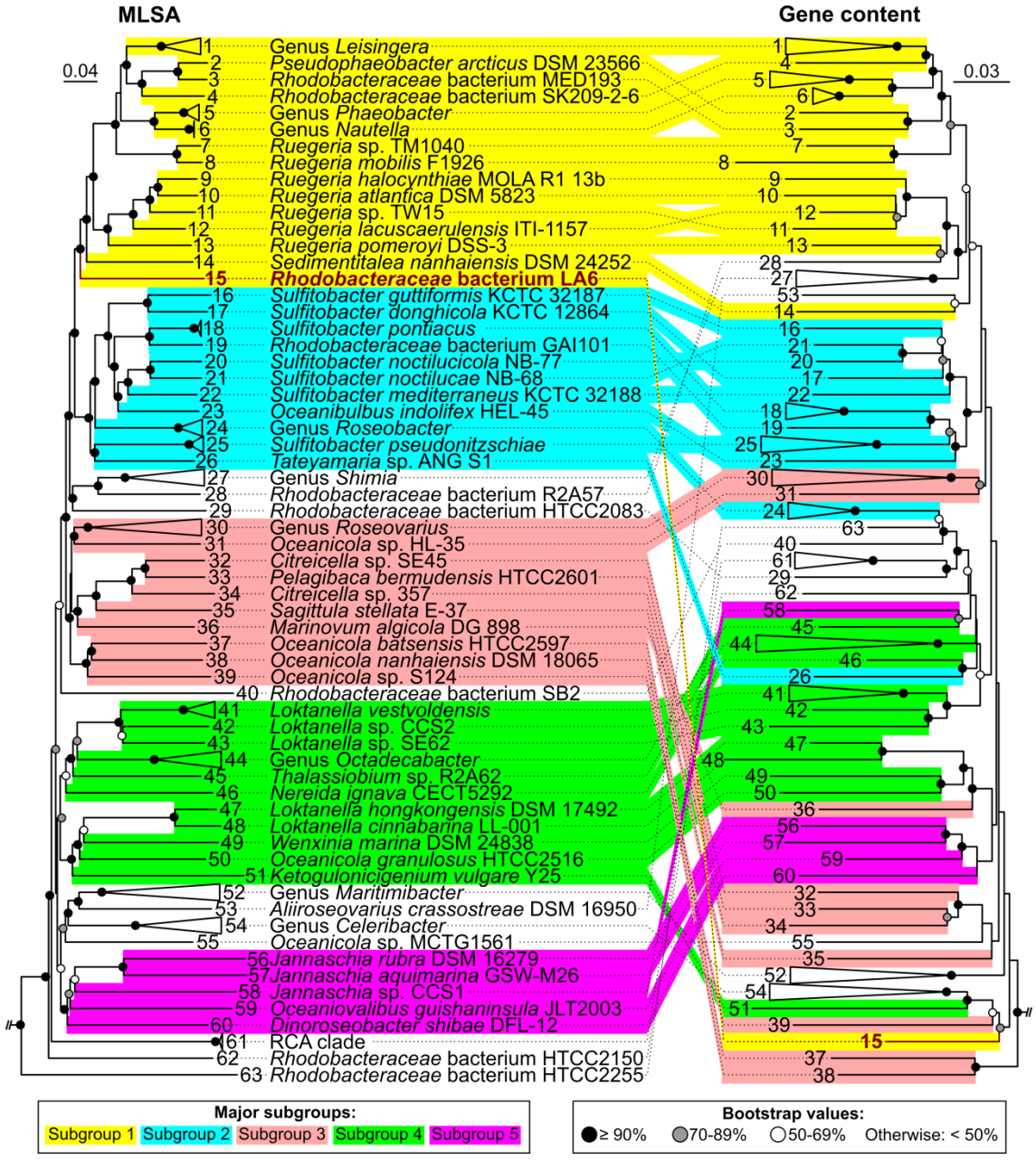

Figure 5.20 Clustering of *Roseobacter* group genomes showing the relationships between sequenced strains based on Multi Locus Sequence Analyses (MLSA) as well as gene content. MLSA (left) is based on concatenated aligned core-genome gene product sequences and illustrates phylogenetic relationships with high confidence. Coherent clusters corresponding to the 5 subgroups originally described by Newton et al (2010) are marked in colour. Corresponding leaves between the MLSA and gene content tree are indicated by identical numbering. For ease of viewing, genera and species consisting of multiple genomes which cluster coherently in the MLSA as well as the gene content tree are shown collapsed. Furthermore, the-outgroup (*Parvularcula bermudensis* HTCC2503) is not shown. In contrast, gene content clustering (right) is based on the presence and absence of orthologs shared between the comparison genomes. This illustrates similarities and differences in gene composition between comparison genomes, thereby reflecting adaptations to individual niches and lifestyles. Divergences between MLSA- and gene content-based clustering show that even closely related strains may possess strongly diverging gene compositions. Please refer to Appendices Figure 10.4 and Figure 10.5 for un-collapsed versions of these trees, including the out-group.

5.6.3 Analysis of the genome for plasmids

Genome sequencing and assembly of strain La 6 produced fifteen contigs of varying length (see Table 5.7). The *Roseobacter* clade are renowned for containing multiple plasmids (Pradella et al. 2010; Petersen et al. 2011; Beyersmann et al. 2013), and as the genome had not yet been closed, the genome was analysed for genes encoding for replication and partitioning proteins for both chromosome and plasmids.

Although contig 1 contained what looked like plasmid-related genes, it also contained all genes such as *dnaAN, gyrB, and recF* necessary for chromosomal replication and so was very likely part of the chromosome. Contig 2 contained no plasmid-related genes and is a particularly large contig and so was therefore also likely to be chromosomal. Contigs 10, 11, and 15 also did not contain any plasmid-related genes but were much smaller and so it is very difficult to speculate if they were of chromosomal or plasmid origin.

However many of the contigs had some form of replication-related genes (*repAB*C or *dnaA-like*) and some had partitioning genes *(parAB*) as shown in Table 5.7. It is highly likely that contigs 1b and 9 are plasmids as not only did they contain the genes required, but they could also be circularised. Although contig 13 did not contain any genes directly related to plasmid partitioning, it was 100% identical (at the nucleotide and amino acid level) to a plasmid pP73E from *Celeribacter indicus* P73 and could also be circularised.

To our knowledge, this is the first example of two strains from completely different genera in the *Roseobacter* clade containing naturally occurring identical plasmids, suggesting inter-genus plasmid exchange. In order to confirm that the sequence truly belonged to strain La 6 and was not from contamination, PCR analyses were conducted. Two sets of primers were designed that would specifically amplify chromosomal genes from La 6, two sets to amplify *C. indicus* P73 chromosomal genes, and two sets specific to the 7.18 Kbp plasmid. All primer sets were used on genomic DNA from both strains. All products were sequenced to confirm correct amplification. As shown in Figure 5.21, the plasmid was indeed confirmed to be present in the genome of strain La 6 (as well as *C. indicus* P73) and was not a product of contamination. Primers used in this section are shown in Table 5.8.

Table 5.7 Potential genes involved in replication and partitioning of plasmids in strain La 6.

Contig	Length (bp)	Replication proteins	Partitioning proteins	Other info
1	3,672,580	*dnaA*-like	*parA*	'Replication protein' next to *parA*
1b**	180,483	*repA, repC*	-	'Replication protein' between *repA* and *repC.* Recombinase next to *repA.*
2	1,788,622	-	-	
3*	114,798	*repA, B, C*	-	'ParB/RepB/Spo0J family partition protein' next to *repABC*. Type IV secretion system downstream.
4*	361,830	*repA, B, C*	*parA, B*	
5*	134,154	*repA,B, C*	-	Recombinase next to *repC.*
6*	118,200	*repA, B, Ci*; *repCii*	-	Type IV secretion system downstream of *repCi*
7*	103,981	*repC*	*parA, B*	Type IV secretion system downstream of *repC*
8*	102,248	*repB*	*parA, B*	
9**	78,561	*dnaA*-like	*parA, B*	
10	78,374	-	-	
11	28,172	-	-	
12*	18,951	*repA, B, C*	-	Recombinase next to *repABC.* ParB/RepB/Spo0J family partition protein downstream
13**	7,180	-	-	
15	948	-	-	

*Hypothesised to be a plasmid based on organisation of repetition/partition modules, based on Petersen et al. (2013).

**Hypothesised to be a plasmid based on organisation of repetition/partition modules (as above) and ability to circularise.

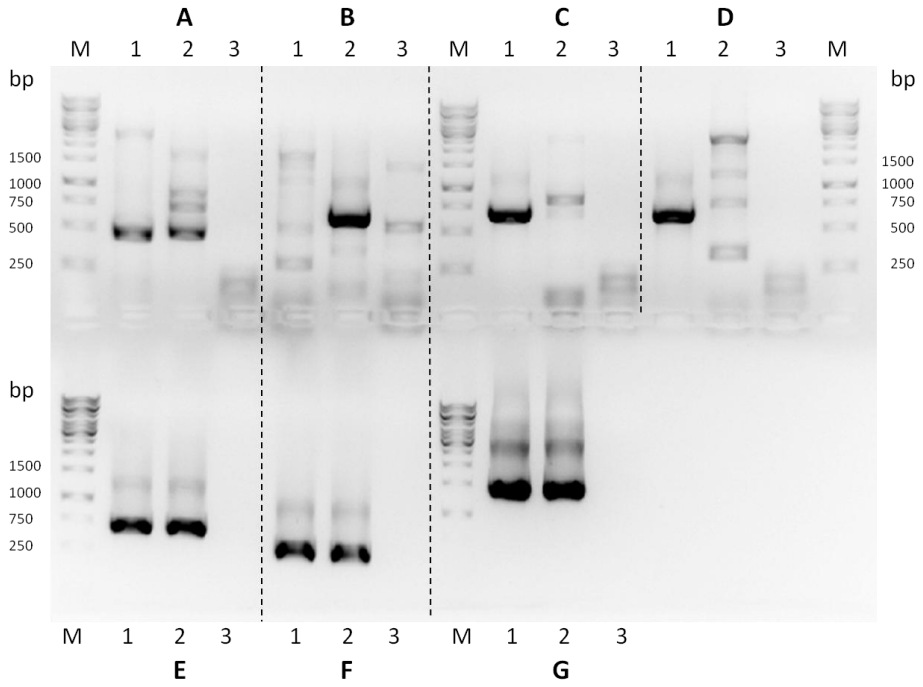

Figure 5.21 PCR confirmation of the presence of plasmid p73 in strain La 6, which is also found in *Celeribacter indicus* P73 using six primer sets, A – F, as shown in Table 5.8. Primer set G is 16S rRNA gene primer pair as a control reaction. Lanes: M, 1 kb ladder; 1, La 6 gDNA template; 2, *C. indicus* P73 gDNA template; 3, no template control. All PCR reactions had 50 cycles. The unexpected product in A1 was found, when sequenced, to be non-specific amplification from elsewhere in the genome.

Table 5.8 Primer pairs used in PCR reactions shown in Figure 5.21

	Primer pair	Target gene	Forward primer	Reverse primer	Amplicon (bp)
A	Cel_xanf/r	Xanthosine triphosphate pyrophosphatase P73_0217	ATTTCCCCGA AGGTCTCTGT	CTCGCCTCC CACAACAAG	503
B	Cel_ostf/r	Organic solvent tolerant proein OstA P73_2660	AGATCGACAC GGAACTCCAG	GATGCCGTAA TCGACGAGAT	638
C	La6_xoxf/r	Methanol dehydrogenase XoxF La619760	TCCGGGTCAT AGGAATACCA	AACACGCGCT ATTCGAAACT	668
D	La6_prmf/r	Propane monoxygenase PrmA La63840	GGTCGAATGG ATGAAGCTGT	CCACATCTCC GCATAGGATT	628
E	p73_mobf/r	Mobilisation protein P73_4824	TCTTGTTCCA GCTCCTTGGT	AAGGTCGAGG TTCTGGAAGG	698
F	P73_chromf/r	Chromate resistant protein P73_4820	TGAAATCCCC GTATCTGCTC	CGATCATGGT ATCGAACGTG	554

5.7 Discussion

5.7.1 Isolation and preliminary characterisation of a novel *Roseobacter*

The addition of lanthanum to methanol seawater enrichments from station L4, Plymouth, allowed the isolation of an *Oceanicola*-related isolate based on the 16S rRNA gene sequence. The ability to grow on methanol and the presence of a *xoxF* (and no *mxaF*) gene suggested this was not a member of this genus. Moreover, the addition of lanthanum to media stimulated the growth of the strain on methanol, whilst the metal was required for growth on ethanol. Given that lots of research has shown that XoxF requires lanthanides at its catalytic site, and that expression of the protein (in *M. extorquens* AM1) requires only 50-100 nM lanthanum, it is possible that strain La 6 also requires this metal for growth on methanol, but that it was able to scavenge metals from the glassware (Martinez-gomez 2015; Nathan M Good et al. 2016).

5.7.2 Methylotrophic growth and genetics of strain La 6

Sequencing of the genome revealed that the only MDH in the genome, *xoxF5*, was in a cluster with genes related to those used in the glutathione-dependent formaldehyde oxidation pathway. It also contained a gene, *soxH*, encoding a predicted sulfur oxidation protein, SoxH, that has as yet, not been linked to methylotrophy. A mutant strain of the *xoxF* gene (XoxF$^-$) was no longer able to grow on methanol or ethanol as sole source of carbon and energy or show MDH activity. Growth was restored on both compounds when complemented with the wild-type gene on a BHR plasmid, and MDH activity was also restored. SDS-PAGE and mass spec analysis suggested constitutive expression of XoxF on succinate and benzoate, and that strain XoxF$^-$ no longer expressed the band corresponding to XoxF. These data therefore strongly suggest that *xoxF* encodes a functional MDH, required for growth on methanol and ethanol in strain La 6. However, the genome did not encode for a glutathione-formaldehyde activating enzyme typically responsible for detoxifying the formaldehyde in organisms containing this pathway. It is tempting to speculate that this may be partly responsible for the limited growth of strain La 6 on higher concentrations of methanol.

5.7.3 Physiological characterisation of strain La 6 and genomic context

Strain La 6 is a non-motile, Gram negative ovoid rod. The temperature range for growth was 4-45°C, with the optimum at 37°C. The pH range for growth was pH 4.5-9 (optimum 7.5) and the NaCl concentrations for growth were 0-15% w/v (optimum 3%), with no growth at 20%. Strain La 6 did not grow under anaerobic conditions and did not reduce nitrate or nitrite. It produced indole acetic acid when supplemented with tryptophan, but not without. It did not produce any acetone/methanol extractable pigments or bacteriochlorophyll a, therefore suggesting growth of the isolate is exclusively non-photosynthetic chemoheterotrophic. La 6 required vitamin B12 for growth.

Like many other members of the *Roseobacter* clade, strain La 6 was able to grow on a range of carbon compounds, including some aromatic compounds such as protocatechuate, benzoate, catechol and 4-hydroxybenoate. Analysis of the genome revealed genes responsible for benzoate and aromatic compound degradation. Surprisingly, it was able to grow on propane and butane, whilst the genome contained a propane monooxygenase cluster that may be responsible for this metabolism. Most strains are not routinely tested for this ability and so it is unknown how many more members of the *Roseobacter* may be able to degrade these compounds.

It was unable to grow on DMSP as sole source of carbon and energy but could produce DMSP-dependent DMS, likely via a demethylase encoded by a *dddL* gene in the genome. It was also able to synthesis DMSP, possibly by a methyltransferase encoded by the gene *mmtB*. It could not oxidise thiosulfate but did contain the sulfur oxidation gene cluster, *sox*. It also contained the carbon monoxide monoxygenase cluster, *cox*, but was not tested for growth on CO. It contained the gene *gmaS* (but not *mauA*) for the metabolism of monomethylamine (MMA) but was only able to use it as a nitrogen source, not a carbon source. Strain La 6 contained four different types of fatty acids in La 6 that are not present in any closely related organisms.

5.7.4 Comparative genomics

The genome of strain La 6 is the largest to date of all sequenced members of the *Roseobacter* clade, at 6.79 Mbp and is far larger than any member of the *Oceanicola* species, supporting the idea that it is a member of a novel genus. Moreover, MLSA and gene content analyses conducted by John Vollmers and Anne Kaster (DSMZ) revealed that whilst it clustered in subgroup 1 of the *Roseobacter* clade based on core genome genes (MLSA), it clustered far away from these on gene content, indicating unique genetic adaptations and again suggesting strain La 6 represents a novel genus. Lastly analysis for plasmid partitioning and replication genes suggested that the genome contains multiple plasmids (at least three, but possibly ten) and that one of these is identical to a plasmid encoded by *Celeribacter indicus* P73.

To summarise, this chapter details the isolation and characterisation of a novel methylotroph, fulfilling Objective 1 of this thesis. The data shown strongly support the conclusion that strain La 6 is a member of a novel genus within the *Roseobacter* clade, and that it utilises the MDH XoxF for growth on methanol and ethanol. The description of the use of XoxF by this strain is important in revealing the potential of many of the other *Roseobacter* strains that contain just a single copy of *xoxF* and either have not been shown to grow on methanol or have had no genetic confirmation of its role. Furthermore, the vast differences seen between La 6 and its closest neighbours at the 16S rRNA, MLSA and gene content level clearly demonstrates the need for comparative genomics to be used as a tool for fully understanding relationships between strains.

Chapter 6

Expression of *xoxF* from different bacteria in heterologous hosts

6 Expression of *xoxF* from different bacteria in heterologous hosts

6.1 Introduction

In the last few years there has been an explosion of research into the role of *xoxF*, encoding for the alternative methanol dehydrogenase (MDH), during growth on methanol in methylotrophs. There are five different clades of *xoxF* gene (see Figure 1.5) of which clade 5 seem to be the most diverse and widespread among bacteria (Chistoserdova 2011b; Taubert et al. 2015). Much of the work has been conducted in the model methylotroph *Methylobacterium extorquens* AM1, with some excellent work showing that XoxF may acts as both a sensor for lanthanides for regulation of the canonical *mxaFI*-encoded MDH, and as a functional MDH (Skovran et al. 2011; Vu et al. 2016). The fact that many methylotrophs contain not only two different types of MDH (*xoxF* and *mxaF*) but that that there are often multiple copies of the *xoxF* genes has meant that the genetics required to study their role is much more complicated.

Moreover, there are many non-methylotrophs which also contain *xoxF* genes, confusing the story even more. XoxF was also highly expressed during the growth of *Methylophaga thiooxidans* DMS010 on dimethylsulfide, but not on methanol, again suggesting it may have alternative roles than just as an MDH (Schäfer 2007). One method often used to examine the function of a protein is the expression in a heterologous host that does not contain the gene of interest in its genome (Frommer & Ninnemann 1995). This method is particularly good to assess the function of proteins that do not seem to have an expected phenotypic role in organisms, such as the *xoxF* in non-methylotrophs. Schmidt (2010) attempted to express *xoxF* from *M. extorquens* AM1 in *E. coli* but was unable to get sufficient expression to perform any biochemical analyses. Apart from this, all other research has focused on expression of *xoxF* genes in either the wild type or mutant of the original strain.

This chapter reports the attempt to examine the role of different *xoxF* genes in heterologous hosts, with the aim to characterise their enzymatic properties. This involved the use of *E. coli* and subsequently strain La 6 XoxF$^-$ as hosts, and whilst unfortunately unsuccessful, the results are reported here for completion sake and to provide a comprehensive overview of a substantial proportion of the work undertaken

throughout this PhD. Moreover it may also provide a framework for further investigation into the role of *xoxF*.

6.2 Expression of three *xoxF5* genes in *E. coli*

6.2.1 Selection of *xoxF* sequences

Three different *xoxF* genes were chosen for expression; *xoxF5* from *Sagittula stellata*, E-37, *Methylocella silvestris* BL2 and *Roseobacter denitrificans* OCh 114. *XoxF5* was chosen as this is this most widespread and diverse of the five clades (Chistoserdova 2011a) but has also been found to be very diverse in different coastal marine waters (Taubert et al. 2015). The three strains were selected for numerous reasons; all three are type strains and so are important in representing their respective genera, whilst *M. silvestris* and *R. denitrificans* have closed genomes and have had extensive physiological and metabolic characterisation.

S. stellata was of particular interest as it is one of the few marine methylotrophs to contain only one copy of *xoxF* and no *mxaF*, and whilst the strain has been shown to use methanol as an energy source, the metabolism of methanol has not been directly linked to the *xoxF5* in its genome (Gonzalez et al. 1997). Moreover, *xoxF* sequences classified as *Sagittula* sequences were present in high relative abundance in *xoxF5* gene amplicon sequencing data from two coastal marine sites (Taubert et al. 2015). *R. denitrificans* was selected as this marine bacterium cannot grow on methanol as sole source of carbon and energy (Shiba 1991), nor could it use it as an energy source (like *S. stellata*) when supplemented with succinate as a carbon source when tested here (data not shown). Therefore it is unknown what role *xoxF* has in this organism. Lastly *M. silvestris* was chosen as it is an example of a methanotroph which contains not only the canonical *mxaFI,* but also two *xoxF5* genes and one *xoxF1* and *xoxF3* (Chen, Crombie, et al. 2010), and so is therefore impossible to speculate what role they have with the current published work on the strain. Interestingly, when *M. silvestris* was grown on methane, the selected *xoxF5* (and the other *xoxF5*) was detected in a proteomic analysis of the total protein content, but not when grown on propane, suggesting that it does have a role during growth on methanol (Patel et al. 2012).

The primary choice of host to over-express *xoxF* genes was *Rhodobacter sphaeroides* Ga *xoxF* deletion strain TP19 kindly provided by Timothy Donohue (University of Wisconsin-Madison), however we were unable to reproduce the methanol stimulated whole-cell O_2 uptake using oxygen electrode as shown previously (Wilson et al. 2008).

Although attempted by Schmidt, (2010) expression of the *xoxF5* genes was then attempted in *E. coli* BL21. Problems with the lack of expression in her work may have been linked to the absence of REEs in the medium used, as the link between REEs and XoxF activity only started emerging from the first publications by Fitriyanto et al and Hibi et al in 2011, and so REEs would not have been added to medium at that time. The presence of REEs may have a stabilising effect on the protein and so therefore, in these studies described below, lanthanum and cerium were added to all expression media and assays.

6.2.2 Expression of three *xoxF* genes in *E. coli*

Complete *xoxF5* sequences (including the stop codon) were amplified by PCR using primers described in Table 6.1 that incorporated restriction sites. Products were then cloned into pET16 using the method described for pLMB509 expression in Chapter 5. Transformants containing the correct size insert were screened using the primers used to originally PCR amplify the *xoxF* gene. The confirmed vectors were termed pETSAG, pETMSV and pETRDEN carrying the *S. stellata*, *M. silvestris* and *R. denitrificans* *xoxF5* genes, respectively. *E. coli* strains carrying these vectors were named according to these.

Single colonies of *E. coli* carrying pET21 with each *xoxF* gene were inoculated into 5 ml LB (containing 5 μM lanthanum and cerium), grown to an OD_{540} of 0.4 at 25 ˚C (shaking at 250 rpm) and induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 18 ˚C. Cell suspensions from 1 ml aliquots were sonicated, and the soluble and insoluble fractions boiled and run on an SDS gel (as described in Methods and Materials), shown in Figure 6.1.

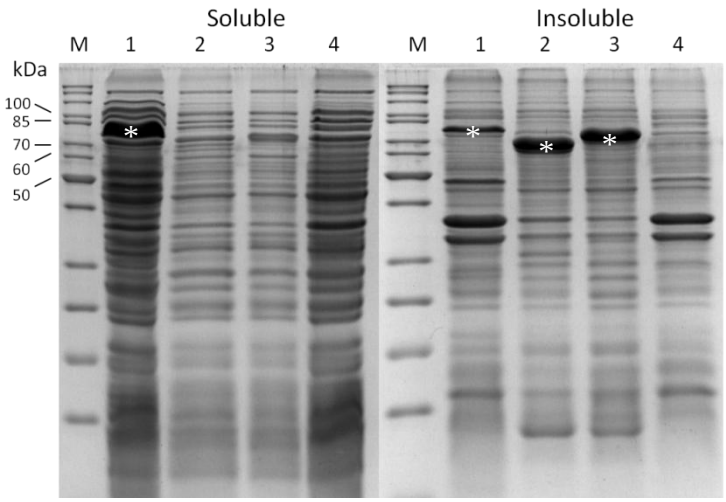

Figure 6.1 SDS PAGE of *E. coli xoxF* expression strains. M, PageRuler Unstained Protein Ladder; lane 1, *E. coli* pETSAG; lane 2, *E. coli* pETMSV; lane 3, *E. coli* pETRDN; lane 4, *E. coli* pET16. White stars show expected XoxF bands, confirmed by mass spec analysis.

Expression was seen for strains containing *xoxF*, although the protein bands were present in the insoluble fractions for *E. coli* strains carrying pETMSV and pETRDN, whilst it was in the soluble fraction for *E. coli* pETSAG. This suggested that the protein was being deposited into inclusion bodies by the cell, and examination under the microscope showing large refractive granules revealed this to be the case. The PSM/DCPIP-linked assay for MDH activity relies on functional, active protein in the soluble fraction, and so attempts were made to optimise the conditions.

6.2.3 Troubleshooting *E. coli* expression conditions

One leading cause of inclusion bodies is due to rapid levels of protein expression (Rosano & Ceccarelli 2014, and references therein), and so the experiment was repeated as before (5 ml, 25 ˚C, 0.1 mM IPTG), but using just *E. coli* pETMSV, and taking samples over a time course. Once cultures were induced, samples were taken at 0, 2, 7 and 22 hours and processed and analysed by SDS-PAGE again. The SDS-PAGE image can be seen in Figure 6.2, showing that even after only three hours there is a massive amount of protein present in the insoluble fraction, and none in the soluble fraction. The control *E. coli* pET16 showed no protein band at the same size (data not shown).

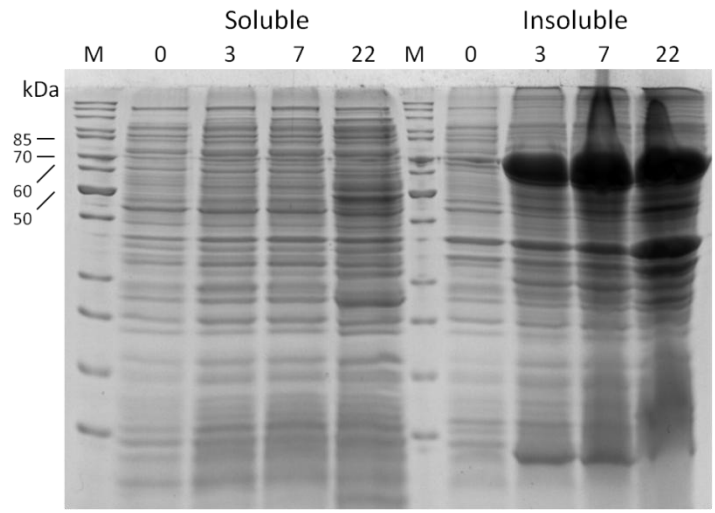

Figure 6.2 SDS PAGE of *E. coli* pETMSV expression strain. M, PageRuler Unstained Protein Ladder; Numbers indicate hours at which sample was taken after induction**.**

In order to slow down the expression and so thus allow proper protein folding, the temperature was decreased to 18 ˚C, and the IPTG concentration tested at two lower concentrations; 50 μM and 5 μM. The amount of oxygen available is also an important factor (Rosano & Ceccarelli 2014) and so two ratios of media to flask volumes were also tested, 5 ml in a 10 ml vial (as before) and 50 ml in a 250 ml flask. Samples were taken at the same time points as before. Unfortunately none of the tests significantly changed the expression profile compared to that seen in Figure 6.2 (data not shown), suggesting that the problem could be more of a genetic one. For example, *E. coli* does not contain any of the regulatory PPQ genes required for production of the PQQ cofactor, although Schmidt (2010) did indeed try adding PQQ to the media to alleviate this problem, with no success.

6.3 Expression of five *xoxF* genes in the methanol dehydrogenase mutant strain XoxF$^-$

Since most of the *xoxF*-only containing methylotrophs do not contain the accessory and regulatory *mxa* genes, lack of expression in *E. coli* was unlikely to be due to the absence of these. However there may be as yet unidentified regulatory or accessory genes in *xoxF*-containing strains that are important in XoxF function, and so strain XoxF$^-$ was selected as an alternative host. Strain La 6 did contain all PQQ synthesis genes, but did not contain the *mxa* genes. However, given that the strain had already been

complemented with its own wild-type *xoxF5* gene with vector pLMB509, the system was already established.

6.3.1 Selection of two more *xoxF* genes

Due to the expression problems in *E. coli*, another expression strain containing *xoxF5* from *M. extorquens* AM1 was made. The *xoxF5* from this strain has been shown to be functional as an MDH (termed *xoxF1*, Schmidt 2010; Nakagawa et al. 2012) and so this served as a theoretical positive control, i.e. as this protein functioned as an MDH, strain XoxF$^-$ should theoretically be complemented (or at least have MDH activity with the DCPIP assay), but those XoxF proteins which are not true MDH would not. A strain of La 6 XoxF$^-$ containing the *xoxF4* gene from the methylotroph *Methylophilales* bacterium HTCC2181 on pLMB509 was also made, as the protein sequence of this gene was one of the most highly expressed proteins in a coastal upwelling system, suggesting it plays a significant role in this environment (Sowell et al. 2011). The *xoxF4* gene is the only MDH present in the genome of this strain, and so the aim was to show that this gene was responsible for the methylotrophic lifestyle of this strain.

6.3.2 Construction of five *xoxF*-carrying strains in strain XoxF$^-$

To construct the expressions vectors, *xoxF* sequences were amplified using the same forward primers as those for amplification into the pET16 vector (except for *M. extorquens*) and reverse primers designed specifically for pLMB509 (see Table 6.1). The *xoxF4* of *Methylophilales* was synthesised in pUC57 as plasmid pUCMPH, digested and ligated immediately into pLMB509. Vectors were conjugated into XoxF$^-$ as previously described in Chapter 5. Vectors were termed p509SAG, p509MSV, p509RDN, p509MEX and p509MPH carrying the *S. stellata*, *M. silvestris*, *R. denitrificans*, *M. extorquens* and *Methylophilales xoxF* genes, respectively.

6.3.3 Expression of *xoxF* sequences using strain XoxF$^-$

Strains carrying the different *xoxF* genes were first tested to see if expression of the gene would complement strain XoxF$^-$ by restoring the ability to grow on methanol as

sole source of carbon and energy. These were established as described in Chapter 5 for strain XoxF$^-$ p509LA6. None of the strains were able to grow on methanol, whilst strain XoxF$^-$ p509LA6 was able to, indicating that these could not replace the wild-type gene during growth on methanol.

Although unable to support growth on methanol, the expressed XoxF proteins might still have functioned as an MDH in the mutant. Strains were initially grown in MBM containing succinate and methanol and induced, but no expression was seen in any strains, except for a small amount in XoxF$^-$ p509LA6 (data not shown). Therefore strains were inoculated into 400 ml MB medium containing 5 mM methanol and 5 μM lanthanum and cerium heptachloride salts, and induced overnight at 25 ˚C (shaking at 150 rpm).

Half of the culture was then processed as described previously in Chapter 5 (and Method and Materials) and assayed for MDH activity. The other half was concentrated by centrifugation (6,000 g x 5 min x room temperature), into triplicates of 10 ml MB media containing 5 mM methanol in 120 ml serum vials. The concentration of methanol in the headspace was assayed at 0, 24 and 48 hours using GC. The GC data are summarised in Figure 6.3.

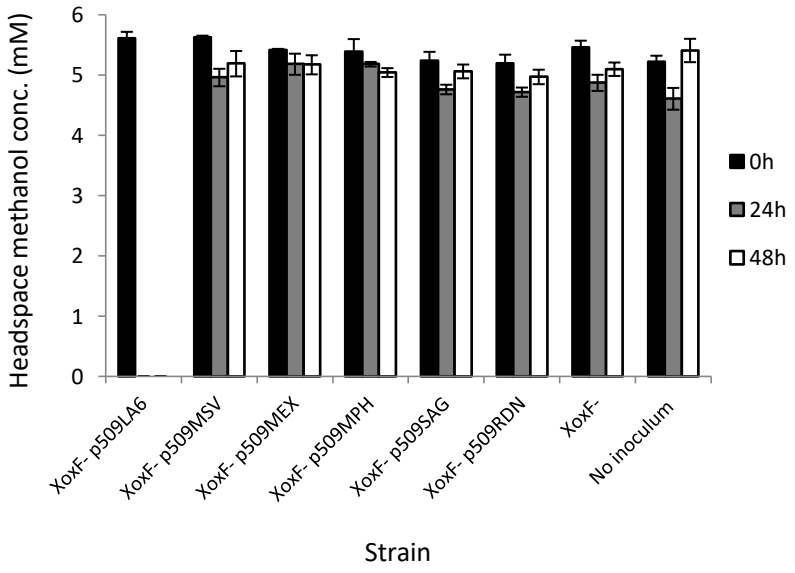

Figure 6.3 Depletion of headspace methanol in concentrated cultures of XoxF$^-$ expressing *xoxF* from various bacteria, grown on MB and 5 mM methanol. Measurements were taken at 0, 24 and 48 hours of incubation. Error bars represent the standard error of three replicate measurements.

None of the strains containing *xoxF* genes from other bacteria were able to deplete any methanol over the course of two days, whilst strain XoxF$^-$ p509LA6 expressing the wild-type *xoxF* used all the headspace methanol within the first twenty-four hours. When tested for MDH activity using the PMS/DCPIP-linked assay, again, only XoxF$^-$ p509LA6 showed methanol dependent oxidation (data not shown). The soluble and insoluble fractions were run on an SDS-PAGE gel to check for expression in all strains, shown in Figure 6.4. Expression was seen all strains, although at a much lower level compared to strain XoxF$^-$ p509LA6 and for some, mostly in the insoluble fraction. Bands were excised and analysed by mass spec analysis to confirm their identity.

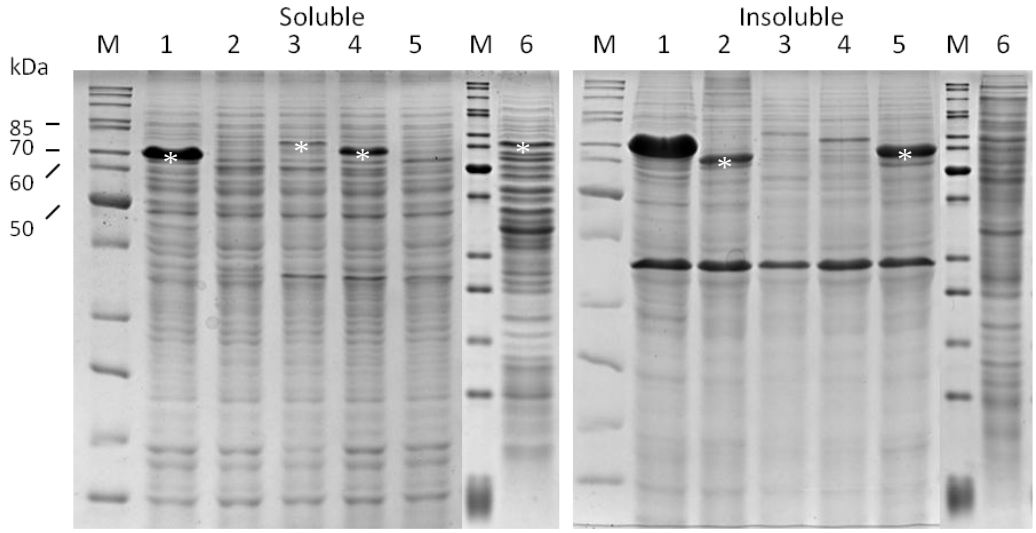

Figure 6.4 SDS-PAGE of La 6 XoxF$^-$ expressing *xoxF* genes from different bacteria. Lanes: M, PageRuler Unstained Protein Ladder; lane 1, XoxF$^-$ p509LA6; lane 2, XoxF$^-$ p509MEX; lane 3, XoxF$^-$ p509SAG; lane 4, XoxF$^-$ p509RDN; lane 5, XoxF$^-$ p509MSV; lane 6, XoxF$^-$ p509MPH. White stars show expected XoxF bands, confirmed by mass spec analysis.

6.4 Discussion

6.4.1 Expression of three *xoxF* sequences in *E. coli*

Heterologous expression of *xoxF* sequences from *S. stellata*, *R. denitrificans* and *M. silvestris* in *E. coli* was successful in that protein was produced in all strains, but the protein was consistently found to be in the insoluble fraction, due to formation of inclusion bodies during growth. It was hoped that the addition of REEs to the media

may prove to be sufficient for successful protein expression and stability. Modifications to the expression protocol, including decreases in induction temperature, lower IPTG concentration, time of sampling and different media to flask volume ratio all showed no changes in protein expression from the insoluble to the soluble fraction.

There are various reasons why *E. coli* may not be able to express these proteins well. Methanol dehydrogenases are periplasmic proteins, encoding a signal peptide which allows transport to the periplasm, where it is then cleaved off (Goodwin & Anthony 1995; Anderson et al. 1990; Fassel et al. 1992). The presence of this signal peptide may have caused localisation in the periplasm, and with such huge amounts being produced, this may have created a stress response from *E. coli* to dispose of the protein quickly. Moreover, as mentioned previously, *E. coli* does not contain any of the regulatory PPQ genes required for production of the PQQ cofactor, although Schmidt (2010) did indeed try adding PQQ to the media to alleviate this problem, without success.

6.4.2 Expression of five *xoxF* genes in the methanol dehydrogenase mutant strain XoxF$^-$

It was thought that expression problems may have been due to *E. coli* lacking as yet unidentified regulatory or accessory proteins that are present in methylotrophs. Since the La 6 strain XoxF$^-$ was readily available and theoretically contained all the required methylotrophy genes, this strain was chosen as an alternative heterologous host. Moreover, complementation with its wild-type *xoxF* using broad-host range expression vector pLMB509 revealed that this expression system was functional in this strain, and so XoxF$^-$ p509LA6 served as a positive control in experiments.

Tests in minimal media containing methanol as sole source of carbon and energy revealed that none of the five *xoxF* genes (the same previous three *xoxF* genes and one from *M. extorquens* and *Methylophilales* bacterium HTCC2181), except the wild-type, were able to complement the mutant strain XoxF$^-$ and restore growth on methanol. Tests for MDH activity in cultures grown in MB media and methanol also showed no activity, whilst concentrated samples of the same cultures were also unable to deplete (headspace) methanol over forty-eight hours. SDS-PAGE and mass spec analysis

revealed that bands corresponding to *XoxF* were expressed in the cultures, although at a reduced level compared to the wild-type.

It is difficult, therefore, to suggest why these proteins were unable to function as MDHs in strain XoxF$^-$. The *xoxF5* from *M. extorquens* is a functional MDH in its wild-type host, (Schmidt 2010; Nakagawa et al. 2012) and it is 73% identical (and 83% similar) to the wild-type XoxF protein (Table 6.2). It is therefore surprising that this was also unable to restore growth on methanol or show PMS/DCPIP-linked activity here. It is possible that either strain XoxF$^-$ managed to recognise the protein as foreign and dispose of it. When comparing the sequences of signal peptides alone, the identities are lot lower than compared to the overall peptide sequence (Table 6.2) and so perhaps the synthesised protein was not being recognised and exported to the periplasm, therefore not forming a functional MDH. Lastly, the presence of the *soxH* in the same gene cluster as *xoxF* of strain La 6 suggests it may be involved in methanol metabolism, and so this may also play an unknown role in methylotrophy which is not compatible with other XoxF proteins. However without further genetic analyses, it is not possible to speculate any further.

Whilst it is clear that, in the tested conditions, none of the over-expressing constructs are able to support methylotrophic growth in XoxF$^-$, it might be possible to transform a heterologous host to express these *xoxF* sequences (including La 6 *xoxF*). For example, *Methylobacterium extorquens* AM1 strain ES1100 is mutant in its *mxaF* and both *xoxF* genes (Vu et al. 2016). Since the construct p509MEX already carries one of its own wild-type *xoxF* sequences, this would be a good positive control to assess complementation of the constructs in this strain.

Table 6.1 PCR primers used to amplify *xoxF* sequences described in this chapter. Restriction sites are underlined.

Name	Vector	Target gene, locus tag	Sequence (5'-3')
Sagxoxf	pET16 and pLMB509	*S. stellata xoxF5*, SSE37_03200	GGATCCCATATGGCAAACAGCGATCTGATTGAGC
Sagxoxr	pET16		GGATCCCATATGGCAAACAGCGATCTGATTGAGC
Sag509r	pLM509		GACATATGTCAATCCGGCAGTGCGAACAC
Rdenxoxf	pET16 and pLMB509	*R. denitrificans xoxF5;* RD1_RS04090	GGATCCCATATGGCAAACAGCGATCTGATTGAGC
Rdenxoxr	pET16		GGATCCCTCGAGCCACCGCTTAGTTCGGCAGAGCG
Rden509r	pLMB509		GACATATGTTACTTACCGTGCAGAGCGAAAACG
Msilxoxf	pET16 and pLMB509	*M. silvestris xoxF5;* Msil_1587	GGATCCCATATGCGCAAAATCCTATTGATG
Msilxoxr	pET16		GCGAATTCAGGCTTACTTACCGTGCAGAGC
Msil509r	pLMB509		GACATATGTTACTTACCGTGCAGAGCGAAAACG
Am1xoxf	pLMB509	*M. extorquens xoxF5;* MEXAM1_RS08325	GCGAATTCAGGCTTACTTACCGTGCAGAGC
Am1xoxr	pLMB509		GACATATGTTAGTTGTTCGGCAGCGAGAAGAC

Table 6.2 DNA and protein relatedness of sequences used in this chapter to *xoxF* of strain La 6.

Host organism (*xoxF* clade)	Accession, locus tag	DNA identity (%)	Amino acid identity / similarity (%)	Signal peptide identity / similarity (%)
Sagittula stellata E-37 (5)	AAYA01000026, SSE37_03200	79	81 / 87	3 / 5
Roseobacter denitrificans OCh 114 (5)	110677421, RD1_RS04090	76	80 / 89	32 / 59
Methylocella silvestris BL2 (5)	CP001280, Msil_1587	75	73 / 83	27 / 59
Methylobacterium extorquens AM1 (5)	NC_012808, MEXAM1_RS08325	78	79 / 87	29 / 43
Methylophilales bacterium HTCC2181 (4)	NZ_AAUX01000001, MB2181_RS01880	51	51 / 62	14 / 28

*at the amino acid level.

Chapter 7

Summary and future prospects

7 Summary and future prospects

7.1.1 Isolation and characterisation of a novel *Methylophaha AH1* strain L4

Chapter 3 described the isolation and characterisation of a novel member of the genus *Methylophaga* from methanol enrichments from station L4, Plymouth. This strain was named *Methylophaga AH1*, strain L4. The genome of this strain contained only the *gmaS* methylamine utilisation pathway, and as such was unable grow on methylamine as sole source of carbon and energy, but could use it as a nitrogen source. It was unable to grow on any other carbon compounds tested and so is described as an obligate methylotroph. *In silico* DDH analyses with other members of the *Methylophaga* genus supported the designation of strain L4 as a new species.

Sequencing and analysis of the genomes of both *M. AH1* and *M. marina* revealed a very similar genetic organisation of their *xoxF* clusters, both containing five *xoxF* genes. To my knowledge, this is the highest number of *xoxF* sequences found in the genome of any bacteria. Whilst there has a been a small amount of research into activities of the MxaFI, G and J, of some *Methylophaga* strains, none has been done on the role or function of their XoxF (Heiber-langer et al. 1992; Kim et al. 2005; Kim et al. 2012). *Methylophaga* sequences have been found in numerous cultivation-independent methanol enrichment experiments and are readily isolated from marine environments and saline lakes. Moreover XoxF expression was highly induced in the marine strain *Methylophaga* sp. DMS010 during growth on DMS compared to growth on methanol, whilst MxaF was found in cells grown under both conditions (Schäfer 2007). It is therefore clear that *Methylophaga* are important methylotrophs, and that whilst XoxF may not play a direct role in methanol metabolism, the abundance of copies of the gene in the genomes and clear physiological role during growth during some conditions suggest there is much more of the story to unravel. Both *M AH1* and DMS010 strains would be useful to study if we are to determine what role *xoxF* plays in this genus of methylotroph.

7.1.2 Addition of rare earth elements to methanol seawater enrichments

Chapter 4 detailed the investigation of the effect of rare earth element (REE) addition to methanol seawater enrichments from different locations. Using initial crude, high methanol concentrations with water from station L4, Plymouth, the addition of REEs stimulated an increase in methanol oxidation. REE-stimulated methanol oxidation seemed to be site dependent, with coastal station L4 seeing variable profiles, whilst enrichments using water from a more 'open shelf' (station E1) also showed REE stimulation. This is likely due to differences in the standing concentrations of REEs in the seawater, affected by coastal and estuarine run-off, although concentrations in seawater enrichments used was unknown. Although replicates had large variabilties, the addition of REEs seemed to stimulate the relative enrichment of bacteria containing as yet unclassified *xoxF5* sequences, suggesting the importance of REEs in XoxF-mediated methylotrophy.

Attempts to examine the effects of REE addition at methanol concentrations closer to those *in situ* revealed no significant differences in the bacterial population. A possible explanation for this could be that REEs simply stimulate the oxidation of methanol in all methylotrophs, and so populations may not necessarily change. Also possible is that some XoxF-utilising methylotrophs may grow more slowly than their MxaFI-utilising counterparts, and so it could take longer to see any significant shift in population, especially when using a technique such as DGGE to examine these changes. One way of addressing this might be to compare the transcription of both *mxaF* and *xoxF* genes upon the addition of REE in marine methanol enrichments, which would provide a much more detailed analysis of the response of XoxF-utilising methylotrophs to REEs.

Moreover, very low concentration ^{14}C methanol labelling experiments, such as those used by Dixon & Nightingale (2012) could be applied in REE enrichments that may provide rates of methanol oxidation that could not be gained through methods used here. Such measurements are much more sensitive and can reveal rates within hours, whilst it can reveal those strains metabolising methanol as an energy source but not as a carbon source. For example, highly ecologically important strains such as the SAR11 and OM43 can account for a substantial proportion of bacterioplankton in both coastal and open ocean waters (Giovannoni 1990; Morris et al. 2002) and research has shown that *Methylophilales bacterium* HTCC2181 of the OM43 clade uses methanol only as a

supplementary energy source, whilst *Candidatus* Pelagibacter ubique of the SAR11 clade used it almost exclusively as an energy source too (Sun et al. 2011; Halsey et al. 2012). It is therefore very important that the impact of lanthanides on these strains in the environment are further examined, using methods such as these.

Based on the 18S rRNA DGGE analysis, there did seem to be a few bands enriched in the enrichments containing both lanthanum and methanol, although this analysis is merely a preliminary and crude look into the effects. As previously discussed, there are yeasts capable of methylotrophy, although they use an alcohol oxidase rather than a XoxF-mediated method of oxidation. Interestingly, a previous study showed that the addition of lanthanides to metal-deplete cultures of a freshwater algae alleviated the effects of metal deficiency (such as calcium and manganese), and so stimulating cellular growth and photosynthetic competence (Goecke et al. 2015). It is reasonable to postulate that such effects may also be seen in marine algae. As already discussed, many important bacterioplankton such as members of the *Roseobacter* clade are closely associated with algae, and so the direct stimulation of algae by lanthanides are likely to impact these bacterial populations too (Ramanan et al. 2016 and references therein). The effects of lanthanides on phytoplankton should also be investigated for the same reason.

However in order to do this, measurements of lanthanides must become more routine in order to determine their likely role in methylotrophy. For example, data from experiments such as the methanol and lanthanum enrichments conducted here could be supplemented with the standing concentrations of lanthanides. So far all studies on the measurement of lanthanides in rivers, estuaries, coastal waters and open ocean have been from a purely biogeochemical perspective, with little attention drawn to the biological role they play (Elderfield et al. 1990; Hatje et al. 2014a; Garcia-Solsona et al. 2014; Greaves et al. 1991). If they are not already established in the lab, methods used for quantification of lanthanides such as inductively coupled plasma mass spectrometry (ICPMS) can be labour intensive and make routine analysis complicated or expensive (Neal 2007). However given the sudden recent explosion of research into the impact of lanthanides on biological systems, it is likely (and hopeful) that more labs will invest in the technique or outsource for more routine analyses.

7.1.3 Characterisation of a XoxF utilising member of the *Roseobacter* clade

Chapter 5 described the isolation and characterisation of a novel member of the *Roseobacter* clade from a methanol enrichment using water from Plymouth, L4, with the addition of the REE, lanthanum. The growth of this strain was stimulated on methanol upon the addition of lanthanum, likely explaining why it was so readily isolated when the metal was added. It also showed a requirement for lanthanum when growing on methanol. A mutant in the *xoxF5* gene, the only MDH-encoding gene in the genome, abolished the strain's ability to grow on methanol and ethanol, and complementation with the wild-type gene restored this growth, demonstrating the role of XoxF during methylotrophic growth in this strain.

Analysis of the genome sequence suggested this strain used the glutathione-formaldehyde linked pathway for formaldehyde, although it seemed to be lacking the first gene in the detoxification pathway (*gfa*), suggesting that either it uses a different enzyme for this process or relies on the spontaneous reaction. Moreover, the presence of *soxH*, a gene encoding for a protein has an unknown role in sulfur oxidation, in the *xoxF* gene cluster is intriguing (Friedrich et al. 2000; Rother et al. 2001; Rother et al. 2005). Analyses of the genomes of other strains reveals genes annotated as *soxH* also near methanol dehydrogenases, associated cytochromes and genes of the glutathione-formaldehyde linked pathway (Figure 10.6 and Table 10.1 in Appendix). It is possible that this gene may be involved in either methanol or formaldehyde metabolism (or both) in many types of bacteria, and so further mutational analysis of *soxH* in strain La 6 may shed some light on the matter.

In order to determine if the *soxH* gene is involved in methanol metabolism in strain La 6, a mutant in this gene could be constructed. In parallel, XoxF$^-$ could be used to create another gene mutation, in the *soxH* gene (marker exchange). These single *soxH* and double *xoxF soxH* mutants could then be physiologically characterised with respect to methylotrophy and other *Roseobacter*-specific traits: do they have any phenotypic differences to the single *xoxF* strain and each other? Does complementing with the *xoxF* or *soxH* genes on their own restore growth on methanol, or do both need to be simultaneously expressed for growth? It could be possible that SoxH is involved in regulation of expression of either *xoxF* or other downstream methylotrophy genes. qRT-PCR could be performed on the wild-type La 6 of both *xoxF* and *soxH* genes during

growth on methanol and succinate to see if transcription of the two genes differ under the different conditions.

Further genomic analysis and subsequent physiological examination revealed strain La 6 to grow on a range of aromatic carbon compounds and some alkanes, cleave DMSP to DMS and synthesise DMSP. Comparative genomics using MLSA and gene content analysis revealed that whilst strain La 6 clusters with subgroup 1 of the Roseobacter clade based on core genes, it clusters far away on gene content, indicating unique genetic adaptations. Further analysis for potential plasmids suggested there are a number of plasmids in the genome (likely between three and ten), with one being identical to a plasmid found in another *Roseobacter, Celeribacter indicus* P73 (Lai et al. 2014; Cao et al. 2015). Interestingly this strain was also highly related to strain La 6 on gene content. Attempts to close the genome of strain La 6 would reveal the true number of plasmids in the genome and allow further genetic analysis on a strain that has been shown to be metabolically diverse, perhaps revealing more insights into the nature of the ecologically important group of bacteria, the *Roseobacter* clade.

7.1.4 Expression of *xoxF* from different bacteria in heterologous hosts

Chapter 6 described the investigation of the function of *xoxF* genes from different bacteria using two different heterologous hosts. The *xoxF5* from two methylotrophs, *Sagittula stellata* and *Methylocella silvestris*, and one non-methylotroph, *Roseobacter denitrificans*, were initially expressed in *E. coli* BL21, with the addition of REEs. Although expression occurred, the protein was consistently found in the insoluble fraction, even after changes in induction temperature, IPTG concentration, media to flask volume ratio and sampling time.

Given that the mutant strain XoxF$^-$ was readily available with a working expression system, this was the next obvious choice as host. There was also the addition of two further *xoxF* genes, *xoxF5* from *Methylobacterium extorquens* and *xoxF4* from *Methylophilales* bacterium HTCC2181 to the experiment. Although expression was slightly more successful in strain XoxF$^-$ (i.e. some protein was found in the soluble fraction), none of the strains could complement the mutant, show any MDH activity or deplete methanol in the headspace of concentrated cell cultures.

Given the limited data here, it is not possible to confidently suggest why none of the *xoxF* sequences showed any function at all in strain La 6. This be may interesting in itself, as mentioned earlier, it could suggest that there are as yet unidentified regulatory or accessory mechanisms involved in strain La 6 such as the *soxH* gene. However, a possible alternative heterologous host to express these *xoxF* sequences could be the *mxaF* and double *xoxF* mutant strain ES1100 of *Methylobacterium extorquens* AM1 (Vu et al. 2016). Any further information on the role of these *xoxF* genes that are so abundant and diverse throughout bacteria may further our understanding of not only the mechanisms within methylotrophy, but perhaps of the evolution of the sequences themselves.

7.1.5 Conclusion

In summary, the discovery of the novel and obligate methylotroph *Methylophaga AH1* strain L4 and the representative species of a novel genus in the *Roseobacter* clade, strain La 6, has provided further insights into the incredibly diverse mechanisms methylotrophs employ to grow on methanol. The knowledge that concentrations of REEs in the marine environment may influence which bacteria are able to metabolise methanol, and that some of those are completely uncharacterised provides an exciting platform for the future research into the environmental role of XoxF.

8 List of abbreviations

ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANI	average nucleotide identity
Ap^R	ampicillin resistance
ASW	artificial salt water
ATP	adenosine triphosphate
BHR	broad-host range
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
CBB	Calvin Benson Bassham cycle
CTAB	cetyl trimethylammonium bromide
Da	Dalton
DCPIP	2,6-dichlorophenolindophenol
DDH	DNA-DNA hybridisation
DGGE	denaturing gradient gel electrophoresis
DMA	dimethylamine
DMS	dimethylsulfide
DMSO	dimethylsulfoxide
DMSP	dimethylsulfoniopropinoate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylendiaminetetraacetic acid
EMC	ethylmalonyl-CoA
FID	flame ionisation detector
FAD	flavin-adenine dinucleotide
FDH	formate dehydrogenase
FGH	S-formylglutathione hydrolase
g	gram / acceleration due to gravity
GC	gas chromatography
Gfa	glutathione-formaldehyde activating enzyme
Gm^R	gentamicin resistance
GMA	gammaglutamylmethylamide
GS-CHO	S-formylglutathione
GSH	glutathione
$GS\text{-}CH_2OH$	hydroxymethyl-gluthathione
GSH-FDH	glutathione-dependent formaldehyde dehydrogenase
h	hour
H_4F	tetrahydrofolate
H_4MPT	tetrahydromethanopterin
ICL	isocitrate lyase
KDPG	2-keto-3deoxy-6-phosphogluconate
Km^R	kanamycin resistance
l	litre
M	molar
MaDH	methylamine dehydrogenase

MAMS	marine ammonium mineral salts
MB	marine broth
MBM	marine basal medium
MCS	multiple cloning site
MDH	methanol dehydrogenase
mg	milligram
min	minute
ml	millilitre
MLSA	multi locus sequence analysis
mM	millimolar
MMA	monomethylamine
mol	mole
mRNA	messenger RNA
MS	mass spectrometry
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NCBI	National Centre for Biotechnology Information
ng	nanogram
NMG	N-methylglutamate
OD_{540}	optical density at 540 nm
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PIPES	1,4-piperazinediethanesulfonic acid
PMS	phenazine methosulfate
p-NPH	p-nitrophenylhydrazine
PQQ	pyrroloquinoline quinone
pMMO	particulate methane monooxygenase
PrMO	propane monooxygenase
RBS	ribosomal binding site
REE	rare earth element
Rif^R	rifampicin resistance
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
RubisCO	ribulose 1,5-bisphosphate carboxylase-oxygenase
RuMP	ribulose monophosphate
s	seconds
SDIMO	soluble diiron monooxygenase
SDS	sodium dodecyl sulphate
sMMO	soluble methane monooxygenase
TAE	tris acetate EDTA
TCA	tricarboxylic acid
TE	tris EDTA
TEMED	*N*,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamine
TMA	trimethylamine
TMAO	trimethylamine N-oxide
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid

v/v	volume to volume
w/v	weight to volume

9 References

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10 Appendices

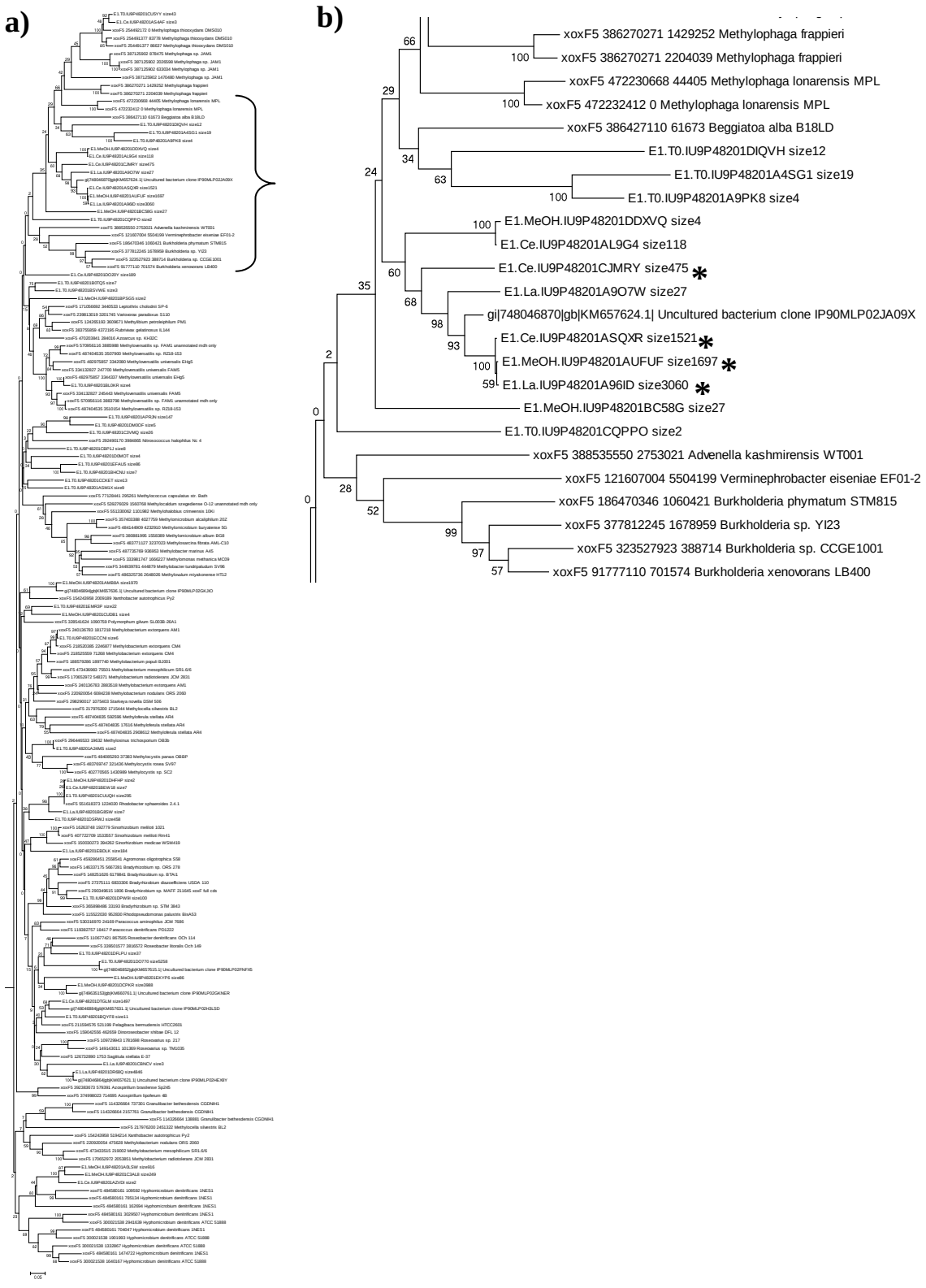

Figure 10.1 Neighbour-joining phylogenetic tree of amino acid deduced *xoxF5* sequences from station E1 REE enrichments. a) All sequences. b) An enlargement of the indicated section on a) where most of the sequences classified as 'Unknown' cluster on the tree, shown with *. The numbers at the branches indicate the percentage of 1,000 bootstrap resamplings. The scale bar indicates nucleotide substitutions per position. A phylogenetic tree constructed using the maximum-liklihood method showed a similar topology.

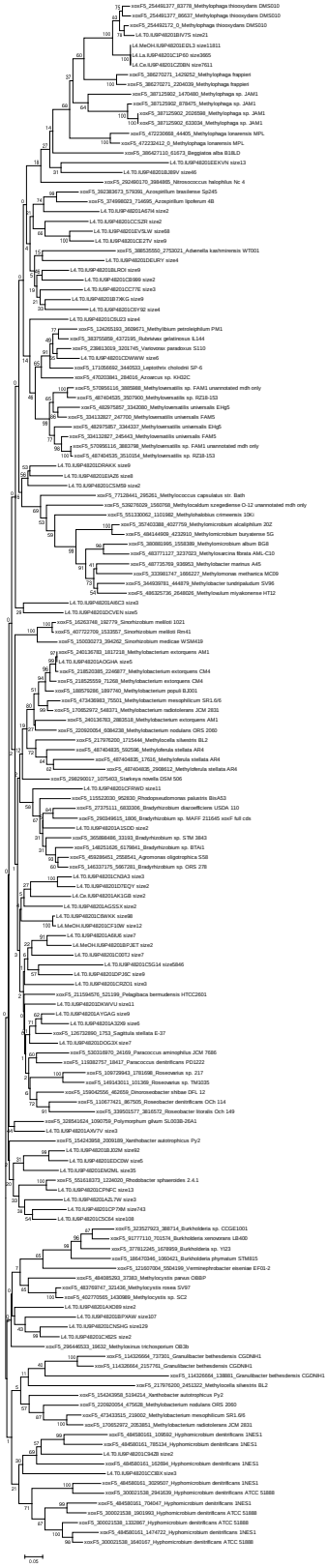

Figure 10.2 Neighbour-joining phylogenetic tree of amino acid deduced *xoxF5* sequences from station L4 REE enrichments. The numbers at the branches indicate the percentage of 1000 bootstrap resamplings. The scale bar indicates nucleotide substitutions per position. A phylogenetic tree constructed using the maximum-liklihood method showed a similar topology.

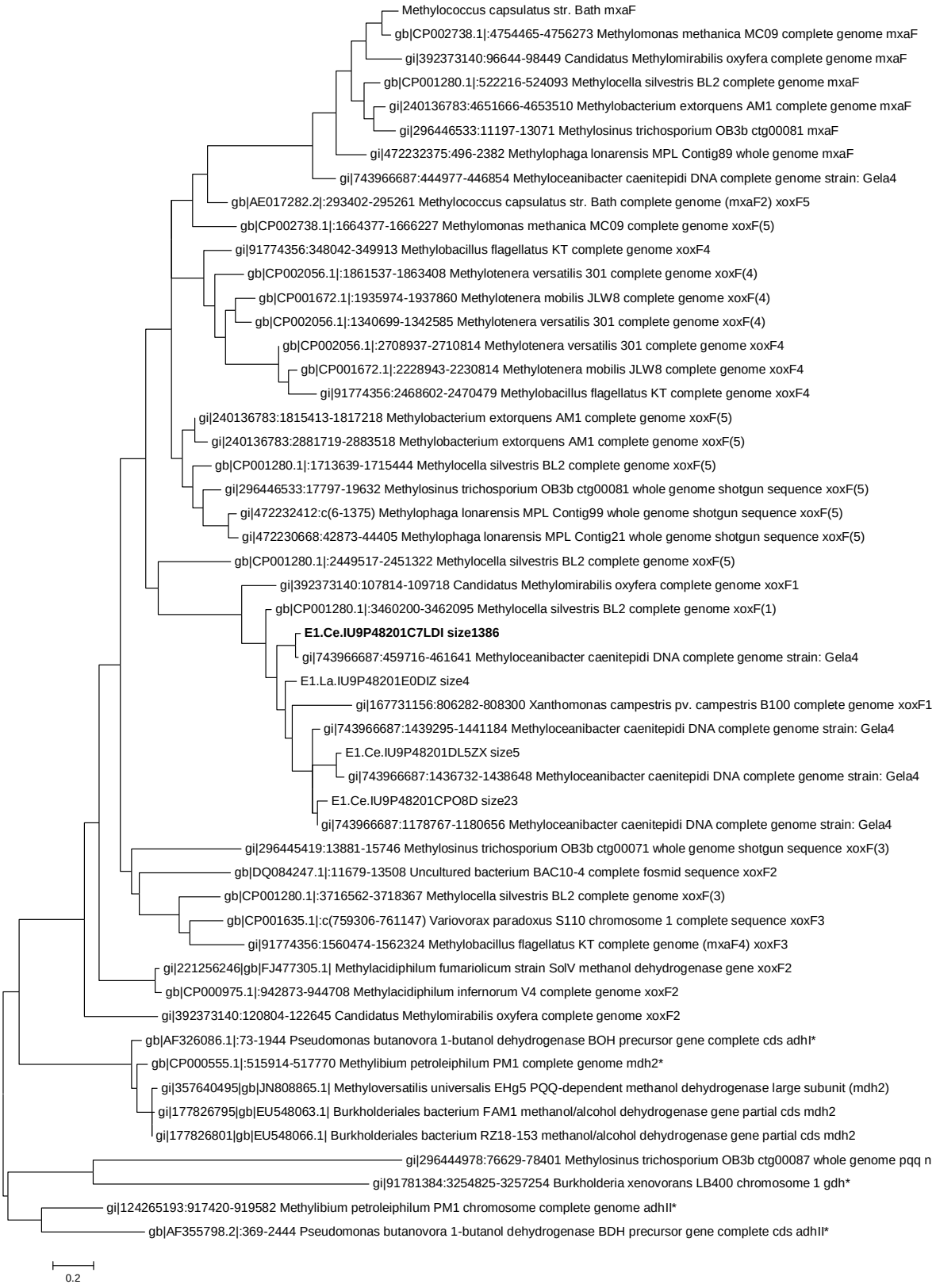

Figure 10.3 Neighbour-joining phylogenetic tree of amino acid deduced *xoxF1* sequences from the *xoxF5* gene amplicon sequencing of station E1 REE methanol enrichments. The numbers at the branches indicate the percentage of 1000 bootstrap resamplings. The scale bar indicates nucleotide substitutions per position. A phylogenetic tree constructed using the maximum-liklihood method showed a similar topology.

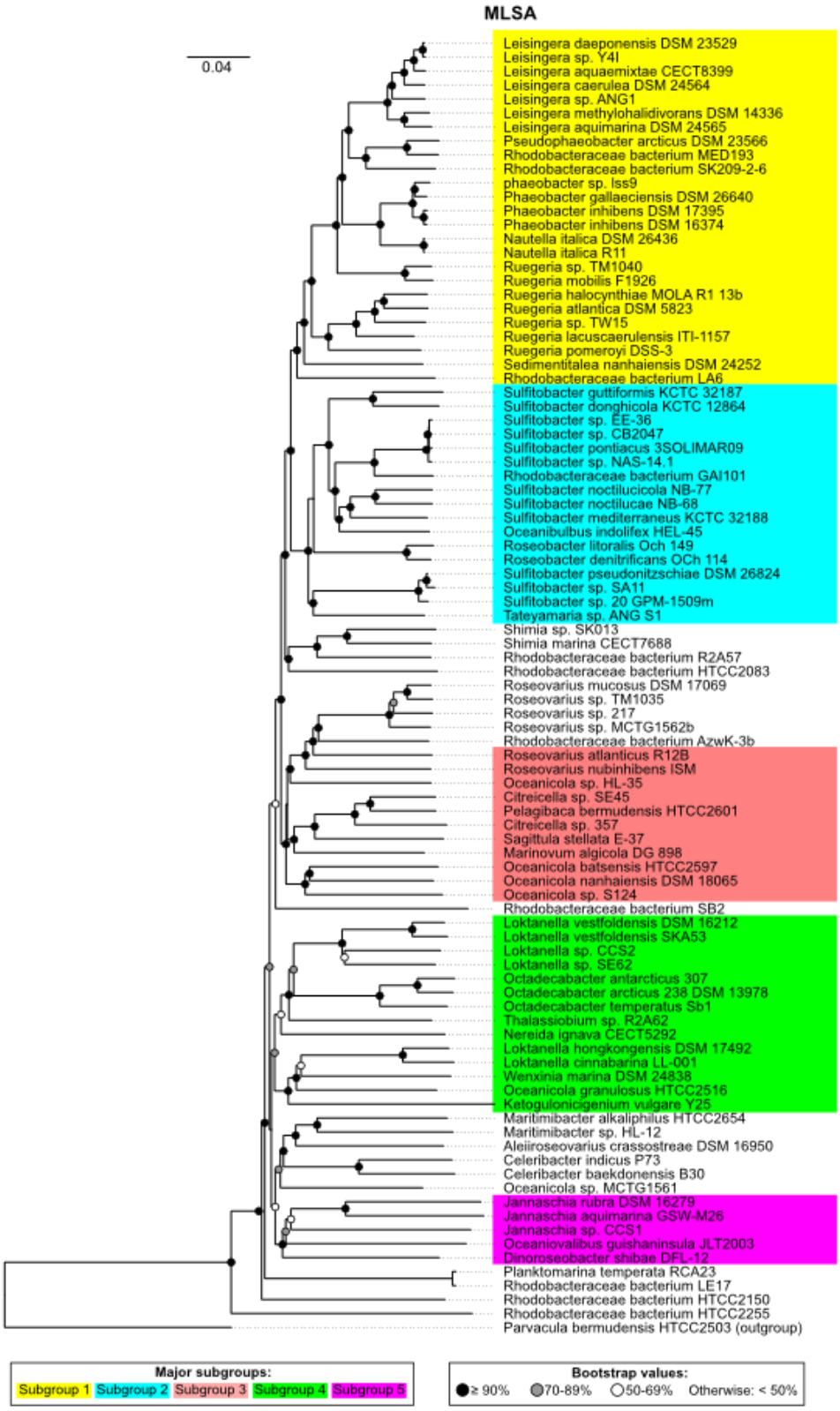

Figure 10.4 Clustering of *Roseobacter* group genomes showing the relationships between sequenced strains based on multi locus sequence analysis (MLSA). Analysis is based on concatenated aligned core-genome gene product sequences and illustrates phylogenetic relationships with high confidence. Coherent clusters corresponding to the 5 subgroups originally described by Newton et al (2010) are marked in colour. The outgroup *Parvularcula bermudensis* HTCC2503 is shown.

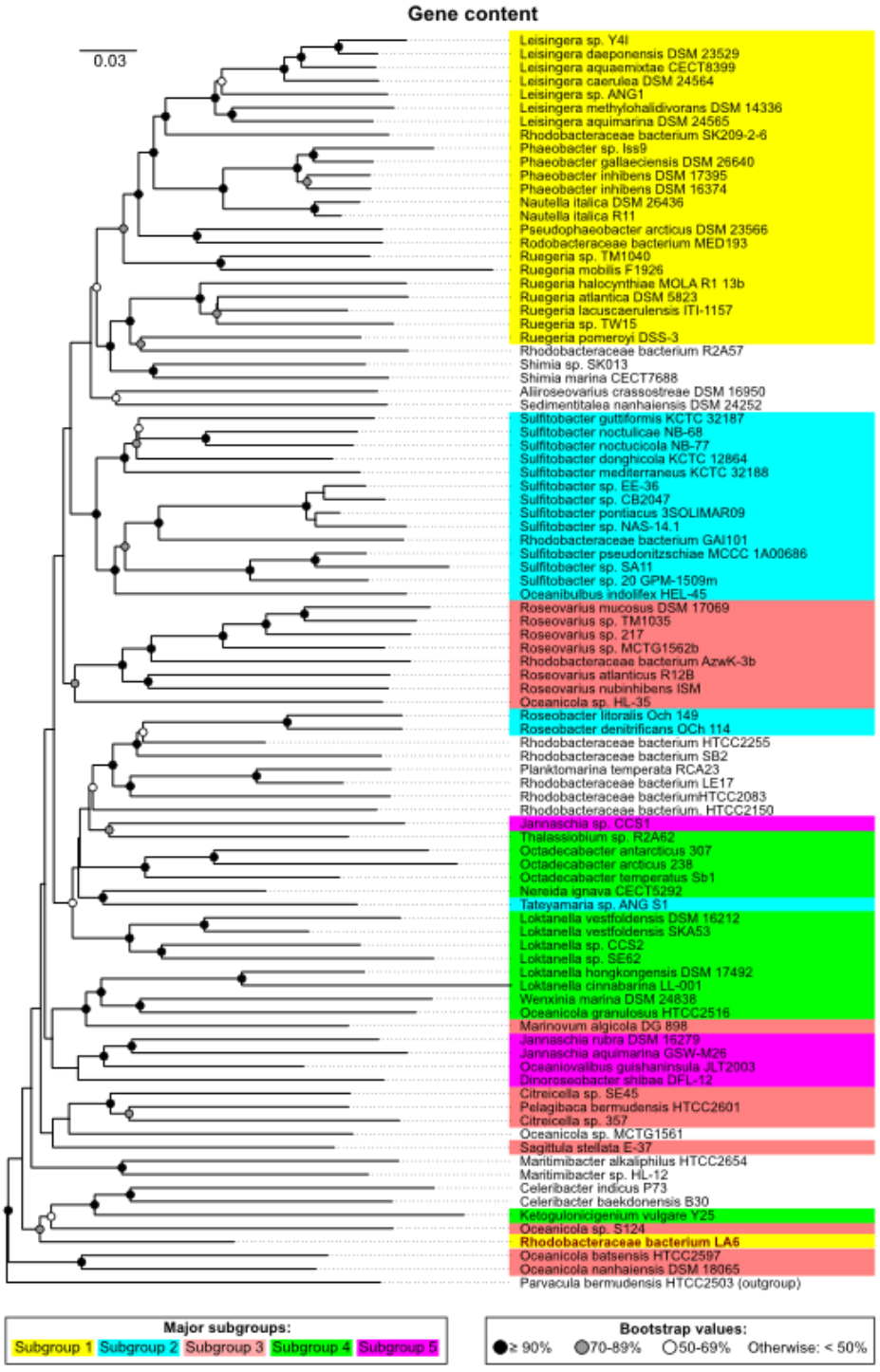

Figure 10.5 Clustering of *Roseobacter* group genomes showing the relationships between sequenced strains based on gene content. Gene content clustering is based on the presence and absence of orthologs shared between the comparison genomes. This illustrates similarities and differences in gene composition between comparison genomes, thereby reflecting adaptations to individual niches and lifestyles. Coherent clusters corresponding to the 5 subgroups originally described by Newton et al (2010) are marked in colour. The outgroup *Parvularcula bermudensis* HTCC2503 is shown.

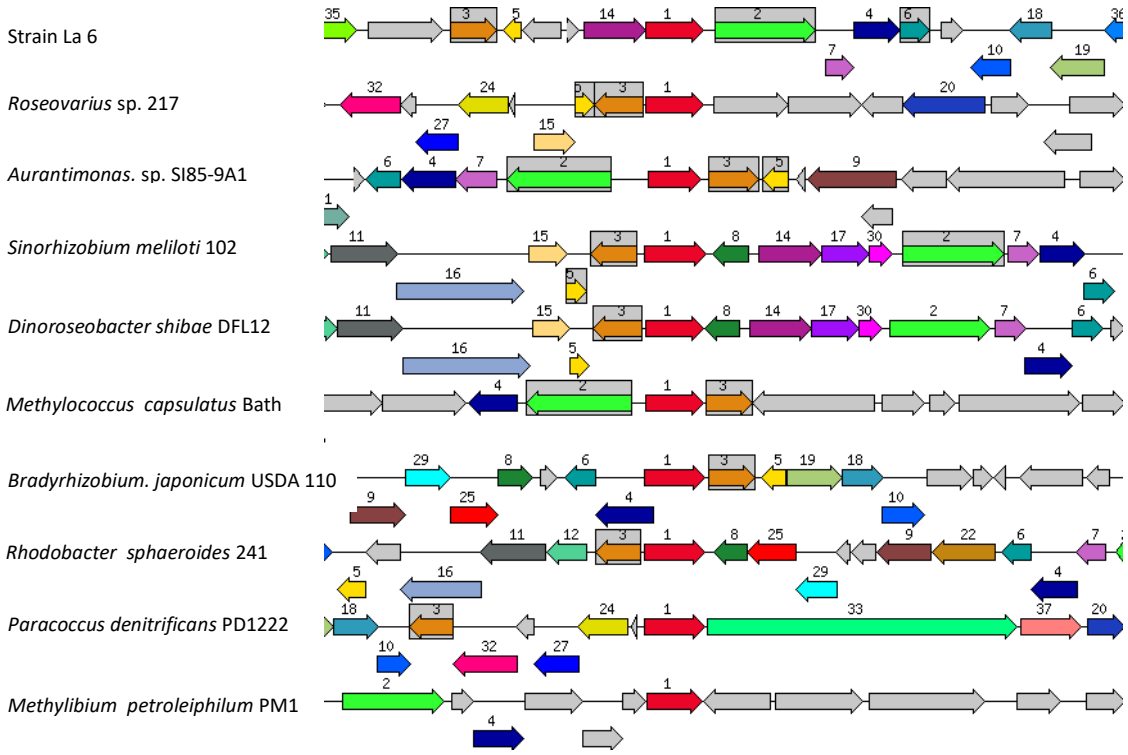

Figure 10.6 Gene clusters surrounding the *soxH* gene in the genomes of different bacteria.

Number	Annotation	Number	Annotation
1	*soxH*	8	hypothetical
2	*xoxF*	14	*adhI* (Gfa)
3	hypothetical	17	*fghA* (FGH)
4	*xoxJ*	24	Coenzyme PQQ biosynthesis B
5	hypothetical	27	Coenzyme PQQ biosynthesis C
6	Rhodenase-related sulfurtransferase	30	cytochrome oxidase
7	*xoxG*	32	Coenzyme PQQ biosynthesis E

Table 10.1 Annotations of genes of interest surrounding *soxH* in various bacteria; numbers correspond to those shown in Figure 10.6.