Molecular Ecology Studies of Methanotrophs in a Freshwater Lake Sediment

Thesis by

Andria M. Costello

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Abstract

Major advances have been made recently in the application of molecular biological techniques to environmental settings. The methane oxidizing bacteria, methanotrophs, are ubiquitous in nature and grow on methane as their sole source of carbon and energy. They have important roles in both the global carbon cycle and the degradation of hazardous compounds. Methanotrophs are capable of degrading a number of halogenated compounds including the common groundwater contaminant, trichloroethylene (TCE). In spite of their environmental importance, genetic tools for the methanotrophs have not been widely developed or applied for studying natural populations of these organisms in situ.

In this thesis, genes for the particulate methane monooxygenase (pMMO) were cloned from pure cultures of methanotrophs. Using these data and others, robust molecular tools were developed for use in the methanotrophs. These include PCR primers and oligonucleotide probes designed for the 16S rRNA and *pmoA* genes in methanotrophs. The tools were utilized in this study to examine the diversity of methanotrophs in the sediments of Lake Washington, a freshwater lake habitat. The data obtained suggest that the methanotrophs detectable using the newly developed genetic tools demonstrate a diversity as broad as the known methanotrophs from all mesophilic environments. These results are in contrast to other environments, such as peat and marine environments, that appear to be dominated by a limited diversity of methanotrophs.

The capacity of the Lake Washington methanotrophic populations for TCE degradation under conditions that mimic intrinsic and enhanced bioremediation protocols

was investigated. The changes in the populations were followed by observing methane and TCE oxidation rates in addition to hybridization with methanotroph specific oligonucleotide probes. The data obtained suggest that the soluble methane monooxygenase (sMMO) is not expressed under methane enrichment conditions typical of enhanced bioremediation protocols. Thus, in situ bioremediation protocols involving methanotrophs will probably involve degradation by the pMMO and not the sMMO, as is currently believed. These results have strong implications for modeling solvent bioremediation by methanotrophs and must be addressed in future designs.

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Chapter 1
Introduction to the Molecular Ecology of Methanotrophs

As we prepare to move into the 21st century, enormous environmental challenges exist that remain to be solved. Daily reports can be found outlining the problems that are prevalent in the pollution of our air, water, and land. As environmental engineers, our jobs are to identify the problems, analyze them, and provide solutions that lead to better living conditions for all. In these efforts, we are often aided by advances in technological understanding. One of the areas of importance to Environmental Engineering that has benefited recently by such an advance is microbial ecology. The advances in this field have come in the form of tools that allow us to examine natural microbial populations with greater depth and precision than ever before. Using these tools, we are able to make more accurate predictions regarding the activities of in situ microbial populations both for assessment and monitoring purposes.

1.1 Molecular Microbial Ecology

Traditionally, the most frequently employed methods of quantifying microbial cells in environmental samples have been viable plate counts and the most probable number (MPN) approach, both of which require growth under laboratory conditions. However, it has now been shown that these techniques select for a minor subset of the existing microorganisms and are inadequate for examining diversity in natural populations (1). It is well known that numbers from microscopic cell counts exceed the numbers of culturable cells by many orders of magnitude. Estimates are that only 0.01-1% of the microbial communities in soil and aquatic environments are routinely cultured in the laboratory (1,2,16). This phenomenon is often referred to as the "great plate count

anomaly" (30). Because of the problems associated with identifying cells from natural environments, the extent of microbial diversity is still unknown (1).

Recently, great strides have been made in the field of molecular microbial ecology in response to the problems associated with identifying cells in the environment. Major advances have occurred in the modification of nucleic-acid-based molecular biological techniques and their application to environmental settings. Advances include developments in colony hybridization, ribosomal RNA (rRNA) techniques, the polymerase chain reaction (PCR), oligonucleotide gene probing and messenger RNA (mRNA) assays. These techniques have been adapted from the lab to environmental samples and can now be carried out for a variety of natural habitats. The use of these techniques allows researchers to investigate natural microbial communities without the bias of culturing.

One of the most promising techniques for characterizing environmental samples without culturing is studying rRNA molecules amplified by PCR. Ribosomal RNAs are biological molecules that are functionally constant, universally distributed, and moderately well conserved across broad phylogenetic distances (20). By using PCR, rRNA gene fragments can be amplified from mixed DNA samples, the fragments can be sequenced, and the identity of organisms in the diverse microbial communities determined (Figure 1.1). This method eliminates the need for culturing and thus alleviates the bias associated with traditional methods.

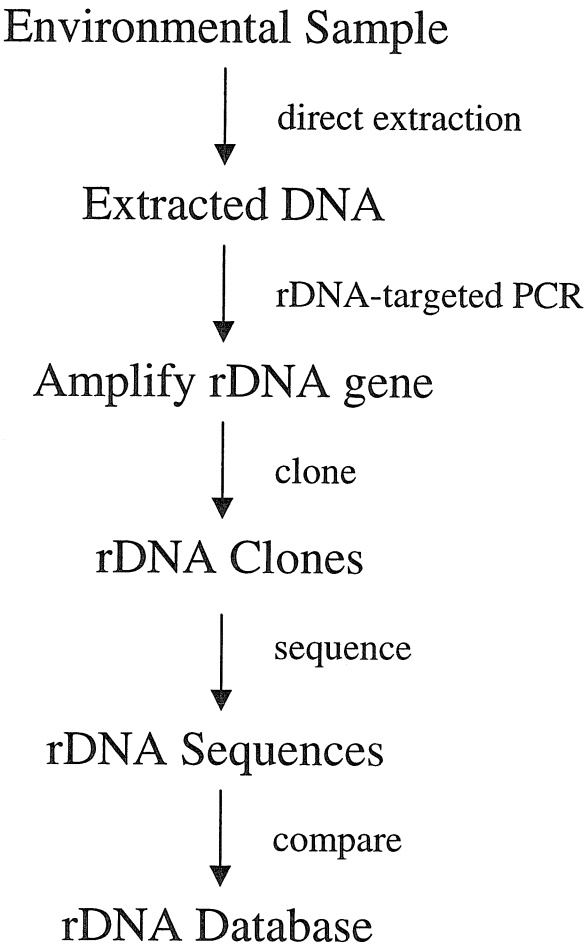


Figure 1.1: Pathway for obtaining and studying rRNA genes from environmental samples. Adapted from (1).

1.2 Bioremediation

Recently, nucleic-acid-based methods have been used to monitor the performance of in situ bioremediation and to diagnose the potential for successful bioremediation before deploying a field system (7). In bioremediation, microorganisms and microbial processes are employed to remove environmental pollutants. It is rapidly becoming a more widely utilized method in the United States for the cleanup of hazardous waste and is expected to become a $500 million-per-year industry by the year 2000 (19). For bioremediation to be effective, it is necessary to have a means of identifying and quantifying both the organisms that are responsible for contaminant removal and their activities (32). Nucleic-acid-based methods are valuable diagnostic tools for these purposes and can help to demonstrate that contaminant loss in the field corresponds to biological removal processes and is not simply due to the contaminant being transferred from one location to another (7). Applying new molecular biological methods to bioremediation can provide important information that cannot be obtained by other microbiological analyses. This information is critical for addressing the prolonged regulatory issues regarding the feasibility of implementing bioremediation for removal of environmental pollutants.

In situ bioremediation can be carried out in two ways: enhanced and intrinsic. Enhanced, or accelerated, bioremediation involves the addition of substances such as nutrients and electron acceptors into the contaminated area to stimulate the growth of indigenous microorganisms able to degrade the contaminants. Enhanced bioremediation systems are often chosen when an accelerated biodegradation rate is desired to reduce the duration of cleanup (19). In contrast, intrinsic bioremediation allows degradation of

contaminants to proceed without any stimulation of the microorganisms present. Intrinsic bioremediation is desirable when the rate of degradation is already significant or when the contaminant can be contained for the duration of breakdown. Even though enhanced bioremediation should be faster than intrinsic bioremediation, it also involves more costly methods and greater human and environmental exposure to contaminants (19).

1.3 Trichloroethylene

Trichloroethylene (TCE) is a synthetic chlorinated hydrocarbon manufactured as an organic solvent with resistance to fire. It has been widely utilized in degreasing metals, dry cleaning, and the manufacturing of plastics (11,15). TCE is a suspected carcinogen and has been shown to damage the central nervous system, heart, liver, and kidneys (29). Because of its widespread use and improper disposal, TCE has been classified as a priority pollutant by the United States Environmental Protection Agency (USEPA). In addition, the Safe Drinking Water Act has established a drinking water standard for TCE of 5 μg/l or approximately 38 nM.

TCE has proven to be a recalcitrant environmental contaminant and is currently the most prevalent contaminant found at all National Priorities List (Superfund) sites (www.epa.gov/superfund/sites/index.htm#nplsites). The remediation of soil and groundwater at locations like Superfund sites often involves the use of traditional pump and treat technology. However, it is difficult to remove hydrophobic organic contaminants, such as TCE, from the subsurface using this method (3). Predictions estimate that pumping may take hundreds of years to lower concentrations of pollutants by a factor of 100 even under the best conditions (3,34). Because of this grim outlook,

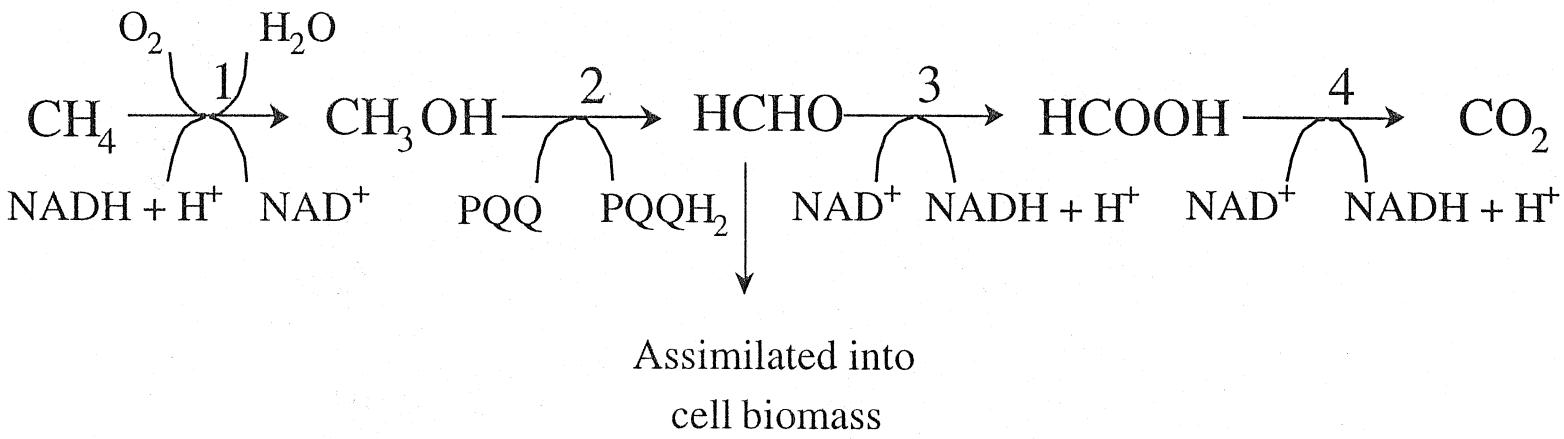
researchers have begun to address alternate methods of remediating contaminated sites, including sites where TCE is present. Bioremediation is one alternative approach to pump and treat technology that has been proposed for site remediation of TCE.

1.4 Methanotrophs

The methane oxidizing bacteria (methanotrophs) are a group of organisms that have been shown to degrade TCE. Methanotrophs include gram negative rods, vibrios, and cocci that grow on methane as their sole source of carbon and energy. They can be isolated from a wide variety of environments and are believed to be ubiquitous in nature (13). Increasing attention has recently been focused on the ecological implications of methane oxidation and the role of methanotrophs in the global methane budget (17,24,25). While the main global sink for methane is chemical oxidation by $^{\cdot}OH$ radicals in the troposphere, biological oxidation has been estimated to be responsible for up to 10% of the global removal of methane (26).

By oxidizing methane to formaldehyde, methanotrophs are able to obtain energy by generating $PQQH_2$ which is a respiratory chain intermediate. The formaldehyde can be further oxidized to CO_2, yielding reducing equivalents in the form of NADH. Alternately, this formaldehyde can be assimilated into cell biomass. These processes are shown in Figure 1.2.

The first step in the methane oxidation pathway is the conversion of methane into methanol by the enzyme, methane monooxygenase (MMO). Two forms of the MMO exist, a membrane-bound or particulate form (pMMO) and a cytoplasmic or soluble form (sMMO) (13). All known methanotrophs contain pMMO, and, in addition, a few also



1. Methane Monooxygenase
2. Methanol Dehydrogenase
3. Formaldehyde Dehydrogenase
4. Formate Dehydrogenase

Figure1.2: Pathway of methane oxidation.

contain sMMO. Differences exist between the two types of enzymes with respect to structure, sensitivity to inhibitors, and substrate specificity (9,21,22). Both enzymes are complex systems that are distinct from each other, with environmental growth conditions responsible for selection of the type of MMO present in the cell (10).

The expression and activity of the MMO in cells that contain genes for both sMMO and pMMO have been shown to depend on the concentration of copper available during growth (23,31). sMMO activity is observed at a low [Cu]/[biomass] ratio (less than about 10^6 atoms/cell) and has three components: a hydroxylase, a reductase, and a regulator (10). The soluble MMO has been studied in detail and both biochemical and genetic information are available. The sMMO has a high K_m (the apparent whole cell half-saturation constant) for methane and contains iron in its active site. The sMMO cooxidizes a wide range of substrates including a variety of chlorinated alkanes, alkenes, and aromatic compounds (13).

Unlike the sMMO, the pMMO is found in all known methanotrophs. The activity of the pMMO is observed at a high [Cu]/[biomass] ratio (greater than 10^6 atoms/cell) and it has been shown that this enzyme has an obligate requirement for copper (21,35). The pMMO has proven to be more difficult to isolate and characterize than the sMMO and at the time this project started, very little was known about it. In the past few years, however, advances have been made and active preparations of the enzyme have been isolated (21,35). It is now known that the pMMO consists of three major polypeptides with molecular masses of approximately 47,000, 27,000, and 25,000 Da. Genetic information for the pMMO has also become available (14,28). In contrast to the sMMO,

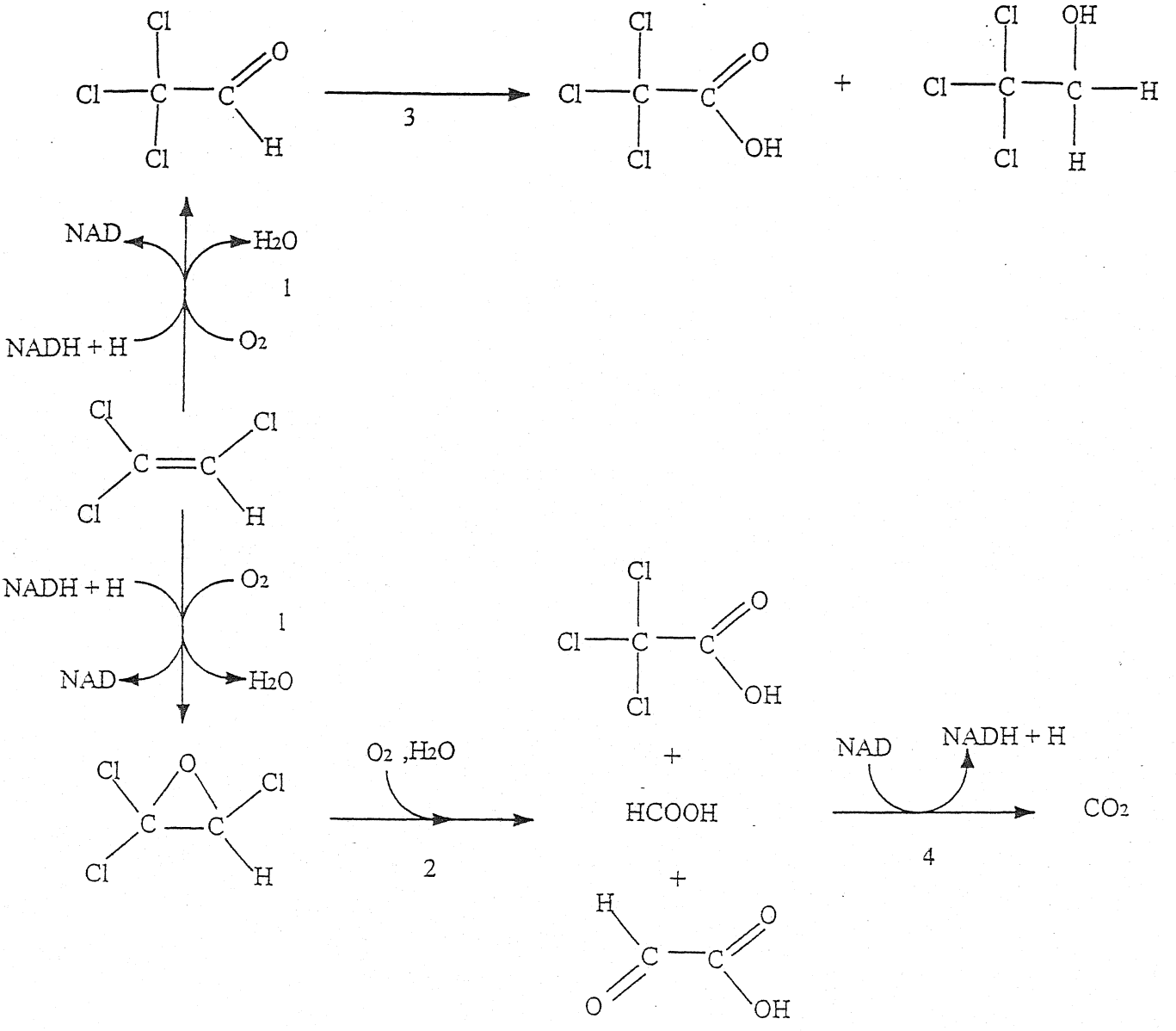
the particulate enzyme has a low K_m for methane and a more narrow range of substrates, although it does cooxidize short-chain halogenated alkanes and alkenes, such as TCE.

Methanotrophs have been classified on the basis of chemotaxonomic studies and 16S ribosomal RNA (rRNA) phylogenetic analyses into eight recognized genera: *Methylobacter*, *Methylomonas*, *Methylomicrobium*, *Methylococcus*, *Methylosinus*, *Methylocystis*, *Methylocaldum*, and *Methylosphaera* (4-6). These recognized genera can be further divided into subgroups that each have distinct characteristics dealing with methane oxidation kinetics, carbon assimilation pathways, and ability to fix N_2. These subgroups and their properties are outlined in Table 1.1. The subgroups fall into two major phylogenetic classes, the alpha- and gamma-proteobacteria. The alpha-proteobacteria are also known as Type II methanotrophs and are characterized by having intracytoplasmic membranes in rings around the periphery of the cell. Type I methanotrophs, the gamma-proteobacteria, have stacks of membranes throughout the cytoplasm.

The distinctions between the methanotrophic subgroups and between the sMMO and pMMO become important when studying the biodegradative capacities of methanotrophs in situ. Methanotrophs are capable of degrading TCE (see Figure 1.3) in a cometabolic reaction where the secondary substrate (TCE) provides no energy benefit to the cells and could not be used as a primary substrate for growth (27). Therefore, the cells have an obligate requirement for a primary substrate, such as methane for the methanotrophs. The degradation of TCE by methanotrophs has been studied using both pure and mixed cultures (8,12,18,33). It has been shown that both types of MMO are able to degrade TCE. In most contaminated systems, sMMO expression is desirable because it

Genera	*Methylococcus*	*Methylosinus*	*Methylobacter, Methylomonas, Methylomicrobium, Methylosphaera*	*Methylocystis*	*Methylocaldum*
Type Classification	I	II	I	II	I
Phylogenetic Position	γ-proteobacteria	α-proteobacteria	γ-proteobacteria	α-proteobacteria	γ-proteobacteria
pMMO (K_m= 2-4 μM)	yes	yes	yes	yes	yes
sMMO (K_m= 30-90 μM)	yes	yes	no[a]	no[a]	no
Oxidize aromatics (under low copper)	yes	yes	yes	no	no
Fix N_2	yes	yes	no	yes	no
RuMP cycle (high carbon conversion efficiency)	yes	no	yes	no	yes

Table 1.1: Characteristics of the methanotroph subgroups. a) One *Methylomonas* and one *Methylocystis* have been found to contain sMMO.



1. Methane Monooxygenase
2. Abiotic hydrolysis and oxidation
3. Heterotrophic biological transformations
4. Formate dehydrogenase

Figure 1.3: TCE degradation by methanotrophs.

has a much higher rate of TCE degradation than the pMMO and would be useful for remediating high concentrations of TCE. Alternately, the pMMO has a lower rate of TCE degradation but a higher affinity for TCE. Thus, it would be desirable to use the pMMO for TCE clean-up at sites with low concentrations of TCE. To use methanotrophs effectively for in situ bioremediation, it is necessary to examine the factors that affect expression of the two MMO's under different conditions.

1.5 Significance of This Work

To date, no one has investigated the difference in the degradation of TCE under intrinsic versus enhanced bioremediation conditions using in situ environmental samples. In addition, robust molecular tools that would allow monitoring of methanotrophs at the microbial level have yet to be employed to examine methanotrophs and their role in TCE remediation. These data are important in order to understand how methanotrophs and TCE interact under specific environmental conditions. Without this information, the evaluation of a bioremediation project involving methanotrophs and TCE cannot be carried out with any degree of certainty.

In this study, molecular tools for the methanotrophs have been developed and used to examine natural communities of methanotrophs and their responses to situations that mimic conditions of intrinsic and enhanced bioremediation protocols. In addition, the use of a chamber system that allows for the incubation and manipulation of sediment samples while preserving the vertical stratification profiles was investigated. This study addresses major issues surrounding the use, monitoring, and optimization of natural methanotrophic populations for TCE bioremediation. It is the first step in developing well

defined systems to study the metabolic activities of organisms in situ. Furthermore, this study provides a new approach for obtaining the kinds of information about environmental microbial populations that are necessary to develop predictive models enabling us to make more effective decisions about bioremediation.

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Chapter 2
Genes for the Particulate Methane Monooxygenase in Methanotrophs

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Stolyar, S., A. M. Costello, T. L. Peeples, and M. E. Lidstrom. 1999. Role of multiple gene copies in particulate methane monooxygenase activity in the methane oxidizing bacterium *Methylococcus capsulatus* Bath. In Press for Microbiology.

2.1 Abstract

DNA from the methanotroph *Methylococcus capsulatus* Bath was screened with a 0.9 kb *Pst*I fragment corresponding to an internal portion of the *pmoB* gene. A 2.8 kb *Kpn*I fragment was identified that hybridized to the probe. This fragment was cloned and sequenced. The sequence revealed that the fragment contained the 5' end of *pmoB* and extended 1.8 kb upstream. A search of both protein and DNA databases revealed significant identity to only one entry, *amoA*, a gene for a subunit of the ammonia monooxygenase in nitrifying bacteria. The translated amino acids of the new open reading frame showed 46.7% identity to those of *amoA* and this gene is proposed to be designated *pmoA*. Upstream of the newly designated *pmoA* gene, an additional open reading frame of 0.8 kb was identified. A region near the N-terminus of this open reading frame revealed an almost perfect match to an amino acid sequence that had been determined by Edman degradation for the 23 kDa subunit of the pMMO. This open reading frame was designated *pmoC*. In addition to the 2.8 kb *Kpn*I fragment, two additional *Eco*RI fragments were identified that hybridized to the 0.9 kb *Pst*I probe. These two fragments were sequenced and were determined to contain two distinct copies of *pmoB*. A portion of the *pmoA* and *pmoB* genes in another methanotroph, *Methylomicrobium album* BG8, was also sequenced. These results provide the first genetic information regarding the genes for the particulate methane monooxygenase in methanotrophs.

2.2 Introduction

As described in Chapter 1, two types of MMO are known, a cytoplasmic or soluble form (sMMO) and a membrane-bound or particulate form (pMMO). The sMMO has been purified from several methanotrophs and has been characterized in detail (2,7,8,16). In addition, the genes for the subunits of sMMO have been cloned and sequenced from *Methylococcus capsulatus* Bath, *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M (2,14,25).

The pMMO is less well studied than the sMMO and at the time this project initiated, it had never been reproducibly obtained in active form (17,24). Recently, advances have been made and active preparations of the enzyme have been isolated (18,30). It is now known that the pMMO consists of three major polypeptides with molecular masses of 47 kDa, 27 kDa and 25 kDa. The 27 kDa polypeptide has been shown to bind acetylene and both the 27 and 47 kDa polypeptides appear as major components in the particulate fractions of cells that have pMMO activity (3-5). However, there is still much that is unknown about the structure and genetics of the pMMO. It is important to obtain a better understanding of the pMMO because of the role of methanotrophs in the global methane cycle and in the bioremediation of hazardous wastes. Additionally, because the pMMO is the only diagnostic enzyme found in all methanotrophs, it is important to obtain sequence information so that a gene probe can be developed to assess natural populations of methanotrophs.

In the recent past, studies of another enzyme, the ammonia monooxygenase (AMO) have provided information relevant to the pMMO. The AMO shares many characteristics with the pMMO and is thought to be a homologous enzyme (9). Both

enzymes have similar substrate ranges, are inhibited by acetylene, are highly unstable after lysis and are thought to contain copper (1,6,11,15,27). Like the pMMO, the AMO contains a polypeptide of approximately 43 kDa that copurifies with an acetylene-binding polypeptide of approximately 27 kDa (11,15). Although little was known about the genetics of the pMMO prior to this study, the gene encoding the acetylene-binding subunit of the AMO (*amoA*) had been cloned and sequenced from the nitrifier *Nitrosomonas europaea* (15). The sequence predicts the actual size of AmoA to be 32 kDa. A second gene encoding a 43 kDa AMO subunit was discovered downstream of *amoA* and was labeled *amoB*. In these same studies, it was shown that *N. europaea* contains two gene copies of *amoA* and *amoB*.

In the study presented here, we have cloned and sequenced genes for the 3 subunits of the pMMO in the methanotroph *Methylococcus capsulatus* Bath. We have also sequenced two copies of the genes for one of the subunits. In addition, preliminary work was done with another methanotroph, *Methylomicrobium album* BG8, to sequence the genes from two of the pMMO subunits.

2.3 Materials and Methods

2.3.1 Bacterial Strains, Plasmids and Growth Conditions

Escherichia coli strains DH5α, InvαF'(Invitrogen) and S17-1 were used in this study (23)(see Table 2.1). They were grown in Luria-Bertani (LB) medium in the presence of appropriate antibiotics as described by Sambrook *et al.* (20). Methanotrophs were grown on nitrate mineral salts medium in batch culture with copper added as $CuSO_4 \cdot 5H_2O$ (28). The cells were shaken at 200 rpm under a methane-air headspace

Strain or Plasmid	Relevant trait(s)	Source or reference
E. coli DH5α		Bethesda Research Labs, Inc.
E. coli InvαF'		Invitrogen, San Diego, CA
E. coli S17-1	Tc^r	(23)
pUC19	*lacZ*	(29)
pCR2.1	Ap^rKm^r*lacZ*α	Invitrogen, San Diego, CA
***Methylococcus capsulatus* Bath**		
pAMC100	pUC19 with 2.9 kb *Kpn*I insert	This study (22)
pAMC101	pUC19 with 1.8 kb *Kpn*I/*Pst*I insert	This study (22)
pAMC102	pUC19 with 2.5 kb *Eco*RI insert	This study (22)
pAMC103	pUC19 with 1.0 kb *Eco*RI insert	This study (26)
***Methylomicrobium album* BG8**		
pAMC105	pCR2.1 with 1.8 kb insert	This study (9)
pAMC108	pUC19 with 1.8 kb *Mun*I insert	This study

Table 2.1: Bacterial strains and plasmids used in this study.

(approximately 1:1 [vol/vol]) or grown on agar plates with the same medium. *Methylomicrobium album* BG8 was grown at 30°C and *Methylococcus capsulatus* Bath was grown at 42°C.

2.3.2 DNA Purification

Chromosomal DNA was isolated from the methanotrophs by a modified version of the Marmur technique (13). Cells were first pelleted by centrifugation at 5,000 rpm. The cells were then washed once with saline-EDTA (0.15 M NaCl, 0.1 M EDTA [pH 8.0]) and resuspended in 10 ml of saline-EDTA to which 10 mg of lysozyme per ml was added. The suspensions were frozen at -80°C. Before thawing, 10 ml of Tris-SDS (0.1M Tris, 1% [wt/vol] SDS, 0.1 M NaCl [pH 9.0]) with 1 mg of proteinase K per ml was added and the solution was placed in a 50°C water bath for 30 min or until the solution became translucent. The DNA was extracted with a phenol-saline-EDTA solution and then precipitated with isopropanol and resuspended in water.

2.3.3 DNA Manipulations

Plasmid isolation, transformation of *E. coli*, restriction enzyme digestion, ligation, blunting ends with T4 DNA polymerase, or filling in ends with Klenow were carried out as described by Sambrook *et al.* (20).

2.3.4 Cloning of *pmo* Genes from Methanotrophs

Fractions of the restriction endonuclease-digested *Methylococcus capsulatus* Bath chromosome that hybridized with the 0.9 kb *Pst*I probe were isolated from agarose gels (20). Each fraction was ligated with the vector pUC19 to generate partial clone libraries. These were used to transform *E. coli* and approximately 1000 colonies from each library were screened by hybridization to the 0.9 kb *Pst*I probe. Libraries constructed with

chromosomal DNA digested with *Kpn*I (2 to 3 kb fraction) and with *Eco*RI (0.8 to 1 kb and 2.0 to 3.0 kb fractions) resulted in colonies that hybridized to the probe. Plasmids were isolated from hybridizing colonies and sequenced on both strands.

A 1.9 kb PCR product containing partial *pmoA* and *pmoB* genes from *Methylomicrobium album* BG8 was obtained (9). *Mun*I-digested fractions of *Methylomicrobium album* BG8 that hybridized to the PCR product were isolated from agarose gels and ligated into pUC19. Approximately 1000 colonies were screened from the clone library by hybridization to the 1.9 kb PCR product. One clone from a *Mun*I fraction (1.6 to 1.9 kb) hybridized to the probe. The hybridizing plasmid was isolated and sequenced on both strands.

2.3.5 DNA Sequencing and Homology Searches

DNA sequencing was carried out from both strands by the Caltech Sequencing Facility using an Applied Biosystems automated sequencer. The Wisconsin Genetics Computer Group (GCG) program was used to search for nucleotide and polypeptide sequences with similarity to the cloned pieces of DNA.

2.3.6 Nucleotide Sequence Accession Numbers

The GenBank accession number for the *Methylococcus capsulatus* Bath fragment containing copy 1 of *pmoCAB* is L40804 and the accession number for copy 2 of *pmoB* is U94337. The DNA fragment from *Methylomicrobium album* BG8 was not submitted.

2.4 Results

2.4.1 Cloning and Sequencing of *pmoA*, *pmoB* and *pmoC* from *Methylococcus capsulatus* Bath

Prior to this study, Andrei Chistoserdov in our laboratory was able to clone a 0.9 kb *Pst*I fragment containing part of *pmoB* from *Methylococcus capsulatus* Bath (22). This fragment contains the 5' two-thirds of *pmoB*. Using the cloned 0.9 kb *Pst*I fragment as a probe, restriction digested DNA from *Methylococcus capsulatus* Bath was examined. Two *Kpn*I fragments of 2.9 and 4.0 kb were identified that hybridized to the 0.9 kb piece of *pmoB*. Attempts were made to clone both fragments but only the 2.9 kb piece was obtained (pAMC100). Restriction mapping showed that this fragment overlapped at the 5' end of *pmoB* and extended 1.9 kb upstream (Figure 2.1). The 2.9 kb *Kpn*I fragment was sequenced on both strands. An open reading frame of 248 amino acids was identified and a search of both protein and DNA databases revealed significant identity only to the sequence of *amoA*. The translated amino acids of this open reading frame showed 46.7% identity to those of *amoA* and this gene is proposed to be designated *pmoA*. The hydropathy plot of PmoA predicts four membrane-spanning regions, with no classical leader sequence at the N-terminus. This structure is similar to that predicted for AmoA, except that the third predicted membrane-spanning region is approximately twice as long in PmoA as it is in AmoA (15).

A 1.8 kb *Kpn*I-*Pst*I fragment containing most of *pmo*A and 1.0 kb of the upstream region was subcloned into pUC19 to generate pAMC101 (Figure 2.1). Analysis of sequence information from this region revealed an additional open reading frame upstream of *pmoA*. After this sequence was obtained, the N-terminal sequence of the 23

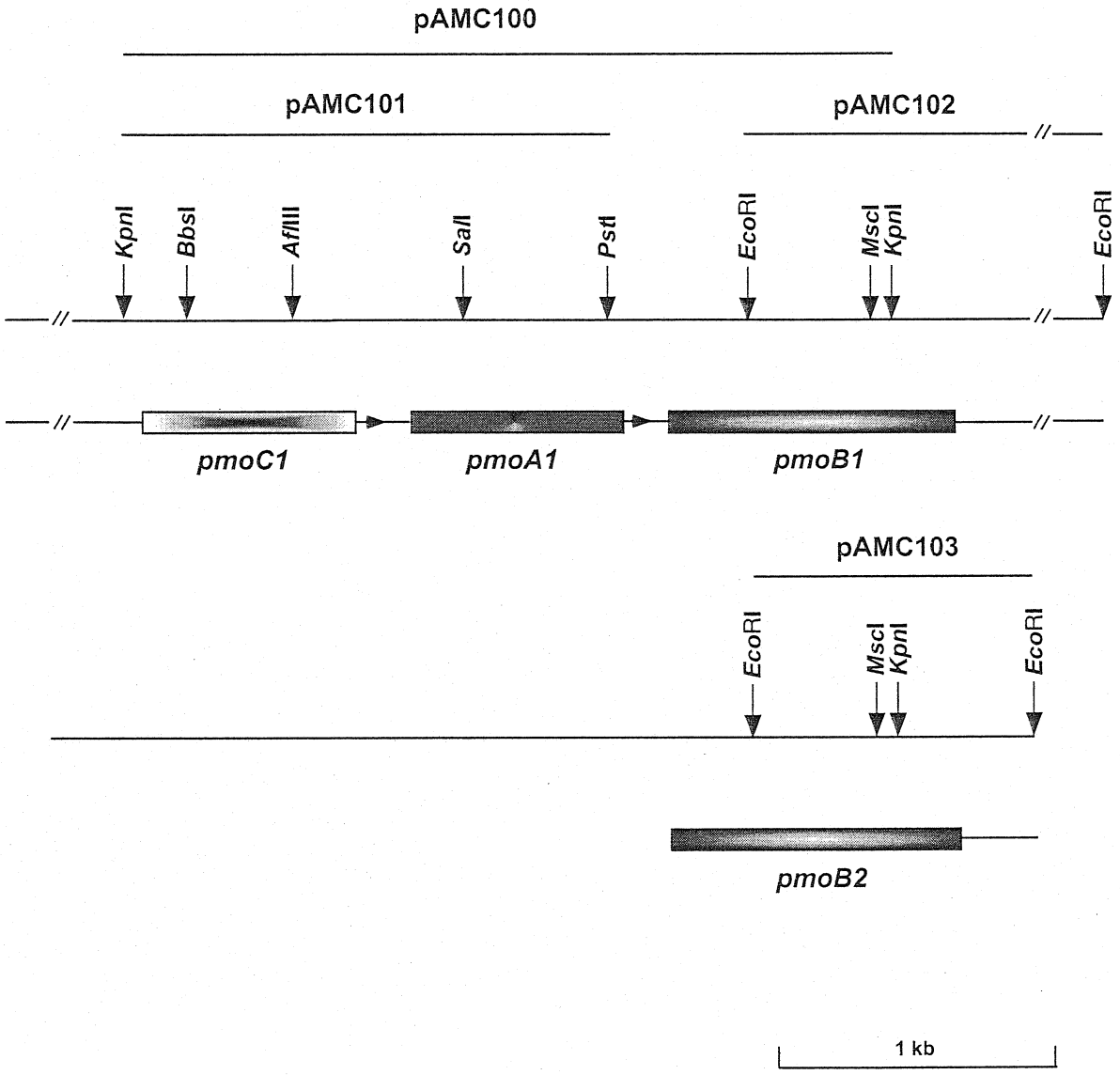

Figure 2.1: *pmo* Genes in *Methylococcus capsulatus* Bath.

kDa pMMO subunit became available (18). This amino acid sequence revealed an almost perfect match to the predicted amino acid sequence for the gene upstream of *pmoA*, starting 10 residues after the first methionine. This open reading frame was designated *pmoC* and is predicted to encode a polypeptide of 260 amino acids with a molecular mass of 29 kDa, assuming it starts at the position determined by Edman degradation. A hydropathy analysis of PmoC predicts a minimum of 3 and a maximum of 6 membrane-spanning regions with no classical leader sequence at the N-terminus. At the time *pmoC* was sequenced, a search of both protein and DNA databases revealed no significant identity with any genes. More recently, sequences have become available for the *amoC* genes from ammonia-oxidizing bacteria that show significant identity with *pmoC* at the amino acid level (12,21).

Restriction digested DNA from *Methylococcus capsulatus* Bath was examined further to identify fragments that might contain the 3' region of *pmoB*. In the *Eco*RI digest, two fragments were identified that hybridized with the *Pst*I probe but did not hybridize with an oligonucleotide probe designed from the first seven amino acids of *pmoB*, suggesting that the fragments did not contain the 5' end of *pmoB*. Jeremy Semrau in the laboratory had previously shown that two copies of *pmo* genes were present in this methanotroph, suggesting that these two fragments might represent a part of each *pmo* copy. The two *Eco*RI fragments (2.5 kb and 1.0 kb) were cloned and sequenced (pAMC102 and pAMC103, respectively). The sequence of the 2.5 kb fragment matched an overlapping 300 bp portion of the 0.9 kb *Pst*I fragment exactly, while that of the 1.0 kb *Eco*RI fragment diverged slightly. Therefore, it was assumed that the 2.5 kb *Eco*RI fragment was the one that overlapped the 0.9 kb *Pst*I fragment on the chromosome

(Figure 2.1). Analysis of the sequence of the 2.5 kb fragment revealed an open reading frame that matched the open reading frame of *pmoB* and terminated within the *Eco*RI fragment. The entire open reading frame contains 414 amino acids and is predicted to encode a polypeptide of 45,540 Da with a 32-residue leader sequence. The N-terminal amino acid of the isolated PmoB is the His at residue 33 of the open reading frame, immediately following the proposed leader sequence.

The 1.0 kb *Eco*RI fragment contains the 3' portion of the second copy of *pmoB* (Figure 2.1). The sequence showed high identity to the same region from the 2.5 kb *Eco*RI fragment and an open reading frame is present that shows high identity to PmoB. The predicted amino acid sequences from the 2.5 kb *Eco*RI fragment (PmoB1) and from the 1.0 kb *Eco*RI fragment (PmoB2) differ only at residue 385 where PmoB1 contains a serine instead of an arginine as in PmoB2. All attempts to clone the *Kpn*I and/or *Eco*RI fragments upstream of *pmoB2* were unsuccessful. Subsequent work from other members of the laboratory suggests these genes are toxic in *E. coli.*

The sequences of the two gene copies in the 2.5 kb and 1.0 kb *Eco*RI fragments begin to diverge 80 nucleotides downstream of the termination codon for *pmoB*, after which the identity drops to 35%. Immediately before this divergent point are conserved sequences that could form a double-hairpin structure. This structure is a candidate for a transcriptional terminator. Because of the similarity in the sequences of the two copies, in all cases they were sequenced at least twice from different DNA preparations, and in each case the sequences agreed exactly.

2.4.2 Cloning and Sequencing of *pmoA* and *pmoB* from *Methylomicrobium album* BG8

Using the conserved regions of the sequence of the *pmo* genes from *Methylococcus capsulatus* Bath in comparison with the sequence of the *amo* genes from *Nitrosomonas europaea*, broad specificity oligonucleotide primers were designed (9,15). These primers were used to perform polymerase chain reaction (PCR) amplification of partial regions of the *pmoA* and *pmoB* genes from the methanotroph *Methylomicrobium album* BG8 (9). A 1.8 kb PCR product from *Methylomicrobium album* BG8 was amplified, cloned and sequenced (pAMC105) (9). The PCR product contains the 3' portion of *pmoA* and the 5' portion of *pmoB* (Figure 2.2). Using the 1.8 kb PCR product as a probe, restriction digested DNA from *Methylomicrobium album* BG8 was examined. In *Mun*I digests, a 1.8 kb fragment was identified that hybridized to the PCR product probe. This fragment was isolated, cloned and sequenced (pAMC108). Analysis of sequence data revealed that the clone overlapped the 1.8 kb PCR product in 840 bp at the 3' end of *pmoB* (Figure 2.2). Analysis of the 0.9 kb sequence downstream of *pmoB* did not reveal any significant open reading frames. Attempts to clone the *pmoCA* region upstream of the PCR product were unsuccessful.

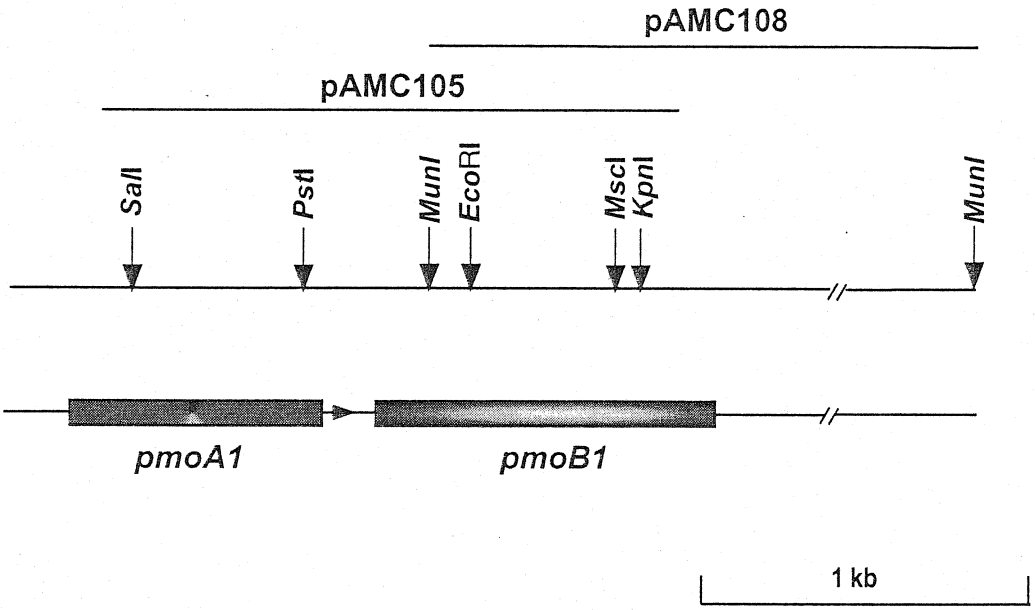

Figure 2.2: *pmo* Genes in *Methylomicrobium album* BG8.

2.5 Discussion

The pMMO is present in all known methanotrophs and is responsible for the conversion of methane to methanol. Although some biochemical and structural data are available concerning the pMMO, little is known about the genetics of the enzyme. In this study, we describe the first steps towards a genetic approach to studying the pMMO. We have cloned and sequenced genes from two methanotrophs, *Methylococcus capsulatus* Bath and *Methylomicrobium album* BG8 that encode polypeptides with high similarity to the subunits of the AMO. The available data suggest that these genes are subunits of the pMMO and that at least some of the genes exist in duplicate copies in the methanotrophs studied.

We have cloned and sequenced genes from *Methylococcus capsulatus* Bath for *pmoCAB* and for *pmoAB* from *Methylomicrobium album* BG8. We were able to obtain sequence for two different copies of the *pmoB* gene (*pmoB1* and *pmoB2*) in *Methylococcus capsulatus* Bath. These results are highly similar to results subsequently obtained for the *amo* genes in nitrifiers. It is now known that in that system, the *amo* genes are present in duplicate copies in *Nitrosomonas europaea* and that other nitrifiers have been found that contain three sets of *amo* genes (10,19,21).

Hybridization data from restriction digested DNA from a variety of methanotrophs has shown that the *pmoCAB* genes are most likely present in duplicate copies in the strains examined (22). The role of these multiple gene copies is currently not understood in either the methanotrophs or the nitrifiers. In this study, duplicate gene copies were only obtained for the *pmoB* gene from *Methylococcus capsulatus* Bath. It will be necessary to clone and sequence both sets of the *pmo* genes from different methanotrophs to elucidate the potential role(s) of the duplicated sets of genes. Select

methanotrophs, such as *Methylococcus capsulatus* Bath, contain both the pMMO and sMMO for methane oxidation. The physiological role of the pMMO is therefore likely to be different in these methanotrophs than in nitrifiers that possess only the AMO system for oxidation of ammonia.

While the role of the multiple genes is not yet clear, the data that we have collected thus far have enabled us to develop broad specificity oligonucleotide primers for the putative catalytic subunit of the pMMO, PmoA (9). Using these primers, the *pmoAB* genes from *Methylomicrobium album* BG8 were obtained and sequenced, providing additional *pmo* gene sequence information. More recently, the *pmoA* gene sequence has also been determined from a number of other methanotrophs, greatly expanding the available sequence database for the *pmo* genes (9). It is now possible to use this sequence information to obtain clones for the additional *pmo* genes, *pmoC* and *pmoB* in methanotrophs, work that is in progress by other researchers in the laboratory. In addition, because the pMMO is the diagnostic enzyme for all methanotrophs, as more *pmo* sequences become available it will be possible to create more specific oligonucleotide probes. These probes will enable us to study the diversity of natural populations of methanotrophs and their important roles in the global methane cycle, nutrient cycling and bioremediation of hazardous wastes.

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Chapter 3
Molecular Characterization of Functional and Phylogenetic Genes from Natural Populations of Methanotrophs in Lake Sediments

Data submitted to Applied and Environmental Microbiology

3.1 Abstract

The 16S rRNA and *pmoA* genes from natural populations of methane oxidizing bacteria (methanotrophs) were PCR amplified from total community DNA extracted from Lake Washington sediments encompassing the area of peak methane oxidation. Clone banks were constructed for each of the genes and approximately 200 clones were analyzed from each bank using restriction fragment length polymorphism (RFLP) and the tetrameric restriction enzymes *Msp*I, *Hae*III, and *Hha*I. The PCR products were grouped based on their respective RFLP patterns and representatives of each group were sequenced and analyzed. Studies of the 16S rRNA data obtained indicated that the existing primers did not uncover the total methanotrophic diversity present when compared with pure culture data from the same environment. New primers specific for methanotrophs were developed and used in constructing more complete clone banks. Phylogenetic analyses of both the 16S rRNA and *pmoA* gene sequences indicated that these new primers should detect these genes over the known diversity of methanotrophs. In addition to these findings, 16S rRNA data obtained in this study were combined with existing phylogenetic data to identify operational taxonomic units (OTU's) that can be used to identify methanotrophs at the genus level.

3.2 Introduction

Methanotrophs are a group of gram-negative bacteria capable of growth on methane as their sole source of carbon and energy. They are widespread in nature and have gained increased attention in the past two decades due to their potential role in the global methane cycle (10) and their ability to cometabolize a number of environmental contaminants (14). Methanotrophs include a total of 6 recognized genera (5,6) that fall into 2 major phylogenetic classes, the α-proteobacteria (also known as Type II methanotrophs) and the γ-proteobacteria (also known as Type I methanotrophs). In addition, two new genera of methanotrophs have recently been described. These new genera include a group of thermophilic methanotrophs and a psychrophilic methanotroph that both form distinct clusters in the γ-subgroup (3,4).

Traditionally, studies with natural populations of methanotrophs have focused on culture-based techniques (14) that may or may not represent the true diversity in nature (1). More recently, however, researchers have recognized the need for culture-independent analyses of natural methanotrophic populations and these types of analyses have been facilitated by recent advances in the molecular biology and molecular phylogeny of methanotrophs (15,22,26). To aid in these studies, PCR primers targeted to the 16S rRNA genes in methanotrophs have been developed (7,16). Additionally, preliminary work has been carried out to identify primers that detect *pmoA*, one of the genes for the diagnostic enzyme for methanotrophs, the particulate methane

monooxygenase (pMMO) (15). These primers also detect *amoA*, which encodes the analogous subunit of the ammonia monooxygenase in nitrifying bacteria (24).

Most studies to date involving non-culture based analyses of natural populations of methanotrophs have been focused on marine and peat bog environments (16,21,23). In these experiments, nucleic acid based techniques have been used to obtain information on methanotrophic 16S rRNA and *pmoA* genes. These studies have expanded the known sequence diversity for these genes and have suggested that these environments contain limited methanotroph diversity at the genus level. The environmental sequences that have been published from peat environments all cluster with the Type II methanotrophs (21,23), while the two published so far from marine and estuarine environments are both Type I strains (16,31).

Our laboratory is interested in investigating natural populations of methanotrophs in freshwater sediments. However, it is not yet clear whether the molecular tools currently available detect the full range of in situ methanotroph genera in these environments. Methanotrophs in freshwater sediments are important to the global methane cycle as these environments are predicted to produce an amount of methane equivalent to approximately 40-50% of the annual global atmospheric methane flux (10,17,29). However, most of this methane never reaches the atmosphere as it is consumed by methanotrophs (17). Some data suggest that freshwater environments may contain a greater methanotroph diversity than peat and marine environments since both pure culture isolation methods and phospholipid fatty acid (PLFA) analyses indicate a mixture of Type I and Type II strains (2,8).

Currently, no data are available characterizing in situ populations of methanotrophs in freshwater environments using 16S rRNA or *pmoA* genes, and it is not known whether the published methanotroph primers can effectively assess the in situ methanotroph diversity in these habitats. Therefore, the objective of this study was twofold: to develop a database of methanotroph 16S rRNA and *pmoA* sequences for a freshwater sediment and to use this information to develop robust molecular tools for studying in situ methanotrophs in freshwater habitats. The study site chosen was Lake Washington, which we have previously analyzed for methanotrophic activities in carbon and oxygen cycling (18,19).

3.3 Materials and Methods

3.3.1 Collection of Samples

Sediment was collected from a 62m deep station in Lake Washington, Seattle, Washington, USA, using a box core sampler that allows for the collection of relatively undisturbed sediment. Subsections of the box cores were taken and sectioned into 0.5 cm slices to a depth of 5 cm. Samples were kept on ice for approximately 1-2 hours and were then used or stored at -20°C.

3.3.2 DNA Extraction and Purification

DNA was extracted from sediment at the area of peak methane oxidation (Auman and Lidstrom, unpublished) using a protocol by Gray and Herwig (13). The amount of sediment used per extraction was 600 mg. Protocol modifications included replacing the Spin-Bind columns with Sephadex G-200 spin columns. The sephadex spin columns were constructed by filling a 1 ml syringe with glass wool and approximately 1-2 cm of

TE (Tris-EDTA)-saturated sephadex. After passage through the column, the DNA was further purified from residual humic acids by electrophoresis on a 1% agarose gel and purification with the Qiagen Gel Extraction kit (Qiagen, Inc.). DNA obtained after this treatment was used in PCR reactions.

3.3.3 PCR Amplification of 16S rRNA and *pmoA* Genes

The 16S rRNA genes were PCR amplified from total DNA extracted from sediment using the methanotroph phylogenetic group-specific primers Mb1007, Mc1005, Mm1007, and Ms1020 (16) in conjunction with the bacteria-specific primer f27. Furthermore, the 16S rRNA primers Mm835 (5'gctccacyactaagttc 3') and Type2b (5' cataccggrcatgtcaaaagc 3') were designed using new and existing sequences to specifically amplify genes from the genera *Methylomonas* and *Methylosinus/Methylocystis*, respectively (Table 3.1). These primers were also used in PCR reactions with the f27

Primer	Sequence (5'-3')	Target genus/gene	Reference
Mb1007r	cactctacgatctctcacag	*Methylobacter* *Methylomicrobium*	16
Mc1005r	ccgcatctctgcaggat	*Methylococcus*	16
Mm1007r	cactccgctatctctaacag	*Methylomonas*	16
Ms1020r	cccttgcggaaggaagtc	*Methylosinus*	16
Mm835	gctccacyactaagttc	*Methylomonas*	This study
Type2b	cataccggrcatgtcaaaagc	*Methylosinus/Methylocystis*	This study
A189gc	ggngactgggacttctgg	*pmoA*	15
mb661	ccggmgcaacgtcyttacc	*pmoA*	This study

Table 3.1: Methanotroph-specific primers used in this study.

primer. All reactions were carried out in a total volume of 30 µl using approximately 100 ng of sediment DNA, 10 pmol of each primer, 1.5 mM Mg^{2+}, Gibco buffer and Gibco

Taq polymerase. The reactions were carried out in a Perkin Elmer GeneAmp PCR System 9600 thermal cycler with 25 cycles of 92°C for 1 min, 55°C for 1.5 min (50°C for primer mm835) and 72°C for 1 min, with a final extension at 72°C for 5 min. In addition, amplifications were also carried out with primers specific for *pmoA*. For design of *pmoA* specific primers, *pmoA* and *amoA* sequences available from GenBank were aligned and the primer mb661 (5'ccggmgcaacgtcyttacc 3') was designed (Table 3.1). Primer mb661 was used in conjunction with the primer A189gc (15). Together, primers A189gc and mb661 amplify an approximately 470 bp internal piece of *pmoA* and give a strong signal with all methanotrophs tested. The primers produce no product with *Nitrosomonas europaea* DNA as tested in PCR reactions and show low identity to other *amoA* genes in the GenBank database.

3.3.4 Construction of Clone Banks and RFLP Analyses

All PCR products were checked for size and purity on 1% agarose gels (30). The PCR products were purified using the Qiagen PCR Purification Kit (Qiagen, Inc.) and ligated into the pCR2.1 vector supplied with the TA Cloning kit (Invitrogen) following manufacturer's instructions. Individual colonies containing inserts were suspended in 50 µl of water, boiled for 5 minutes, cell debris spun down and 1 µl of the supernatant used in PCR reactions to re-amplify the insert from the vector using the appropriate primers. The re-amplified product was used in restriction digests with tetrameric restriction enzymes. The 16S rRNA genes were digested with the enzymes *Msp*I, *Hha*I and *Hae*III. The *pmoA* genes were digested with *Hha*I and a combination of *Msp*I/*Hae*III. Digests were resolved on 3% NuSieve GTG agarose (FMC) gels and grouped manually based on the restriction patterns.

3.3.5 16S rRNA and *pmoA* Genes from Pure Cultures

Pure cultures requiring methane for growth were obtained from enrichment cultures using Lake Washington sediments (A. Auman, S. Stolyar, and M. Lidstrom, unpublished results). Chromosomal DNA was isolated from each strain using cells grown on agarose plates. Cells were washed from the agarose surface using 500 µl of TEN (50 M Tris EDTA + 150 mM NaCl) and the liquid was collected in 1.5 ml tubes. Tubes were spun for 5 min at 14,000 rpm and the supernatant was poured off. The pellet was resuspended by adding 500 µl TEN with 4 mg/ml lysozyme and incubated at 37°C for 1 hour. Next, 50 µl of 20% SDS was added to the tubes and the tubes were incubated in a 45-50°C water bath approximately 30 min. DNA was extracted using phenol and precipitated using ethanol by standard procedures (30). DNA from each of the isolates was used in PCR reactions as described above. The 16S rRNA genes were amplified using the bacteria specific primers f27 and 1492r (12). The *pmoA* genes from each of the isolates were amplified using the primer pair A189gc/mb661 as described above.

3.3.6 Data Analyses

Analyses and translation of DNA and DNA-derived polypeptide sequences were carried out using the Genetics Computer Group (GCG) programs (Wisconsin).

3.3.7 Phylogenetic Analyses

16S rRNA gene sequences were compared against the small subunit rRNA database of the Ribosomal Database Project (RDP) using the Similarity_Rank program (20). 16S rRNA sequences were aligned manually to the representative sequences of the nearest phylogenetic neighbors as defined by the RDP using the SeqApp program. Dendrograms were constructed using the programs DNADIST, DNAPARS, DNAML,

NEIGHBOR and SEQBOOT from the PHYLIP v3.5c package (11). Tree files generated by PHYLIP were analyzed using the program TreeView (27). The RDP program Check_Chimera was used to examine 16S rRNA gene sequences for chimeras. *pmoA* sequences were aligned manually with *pmoA* and *amoA* sequences obtained from the GenBank database. Dendograms were constructed using the programs PROTDIST, PROTPARS, NEIGHBOR, and SEQBOOT from PHYLIP v3.5c (11) and tree files were analyzed using TreeView (27).

3.3.8 DNA Sequencing

DNA sequencing of the 16S rRNA and *pmoA* genes was carried out from both strands using the ABI Prism BigDye terminators sequencing kit (Applied Biosystems). The sequences were analyzed by the University of Washington Center for AIDS Research DNA Sequencing Facility and the Department of Biochemistry Sequencing Facility using an Applied Biosystems Automated Sequencer.

3.3.9 Nucleotide Sequence Accession Numbers

The GenBank accession numbers for the Lake Washington 16S rRNA and *pmoA* gene sequences described in this study are AF150757–AF150803. The accession numbers for the pure culture methanotrophs are: *Methylosinus trichosporium* OB3b, AF150804; *Methylocystis parvus* OBBP, AF150805; *Methylomonas methanica* S1, AF150806; *Methylomonas rubra*, AF150807.

3.4 Results

3.4.1 RFLP Analysis of Known Methanotrophs

Tetrameric restriction enzymes have been shown to be useful tools for screening environmental clone banks by RFLP analysis (9,25,28,32,34). Common restriction fragments from such analyses that distinguish between taxonomic groups are known as operational taxonomic units (OTU's) (25). The identification of OTU's for methanotrophs would facilitate rapid screening of both isolates and environmental clones. Therefore, a number of representatives of methanotrophic 16S rRNA genes available from the GenBank database were examined by computer aided digestion with the tetrameric restriction enzymes *Msp*I, *Hha*I, and *Hae*III to determine whether OTU's could be identified. These enzymes were predicted to produce useful patterns from regions used previously for PCR analysis (16) and a comparative computer analysis indicated that each genus could be identified by a distinct set of patterns (Table 3.2). To test these predictions experimentally, the same PCR products were generated using DNA from representative strains and were digested by the three restriction enzymes. The RFLP patterns obtained for the strains tested all corresponded to the patterns predicted from the published sequences except for *Methylomonas methanica* S1, *Methylomonas rubra*, *Methylocystis parvus* OBBP and *Methylosinus trichosporium* OB3b. The discrepancies observed suggested the possibility of errors in the deposited sequences. The 16S rRNA genes from these cultures were resequenced and significant apparent errors were identified in the original sequences. The new sequences we obtained were 86.9-99% identical to the previously published sequences and matched the RFLP patterns observed from the digests with chromosomal DNA, suggesting that they are correct. The RFLP

Genus[1]	*Hha*I	*Msp*I	*Hae*III
***Methylomicrobium*:**			
Methylomicrobium album (X72777)[2]	75,126,160,170,478	11,33,**67**,109,**347**,442	34,53,59,66,66,85,99,129,418
Methylomicrobium agile (X72767)[2]	76,124,126,160,170,352	11,33,**67**,109,**347**,441	34,52,66,67,85,99,128,153,324
Env. Clone pAMC421	76,126,126,160,171,350	**67**,110,**348**,484	34,53,66,67,99,129,561
Env. Clone pAMC466	76,126,126,160,171,352	**67**,110,**348**,486	34,53,66,67,85,99,129,478
***Methylobacter*:**			
Methylobacter whittenburyi (X72773)[2]	75,126,126,160,170,353	11,33,110,**414**,442	34,53,66,66,85,99,129,478
Methylobacter luteus (X72772)[2]	75,126,160,170,479	11,33,110,**414**,442	34,53,66,66,85,99,129,478
Isolate LW1	75,126,160,170,478	11,32,110,**414**,442	34,53,59,66,85,98,195,419
Env. Clone pAMC405	76,126,160,171,477	32,110,**415**,453	34,59,67,99,119,129,129,374
Env. Clone pAMC415	76,126,160,171,478	11,32,110,**415**,443	34,53,59,66,67,85,99,129,419
Env. Clone pAMC417	76,126,160,172,476	11,32,110,**415**,442	34,53,59,66,67,100,129,502
Env. Clone pAMC419	76,126,160,171,478	32,110,**415**,454	34,66,67,85,99,182,478
***Methylomonas*:**			
Methylomonas methanica S1 (this study)	76,126,644	11,32,360,443	53,66,67,85,129,**446**
Methylomonas rubra (this study)	26,76,126,618	11,32,110,250,443	53,66,67,85,129,**446**
Isolate LW13	76,126,644	32,360,454	53,66,67,85,129,**446**
Isolate LW15	76,125,644	11,32,360,442	66,67,85,181,**446**
Isolate LW16	76,126,644	11,32,360,443	53,66,67,85,129,**446**
Isolate LW19/LW21	76,126,644	11,32,110,250,443	66,67,85,182,**446**
Env. Clone pAMC434	76,126,644	11,32,360,443	66,67,85,182,**446**
Env. Clone pAMC435	76,126,316,329	11,32,361,443	66,67,85,182,**446**
Env. Clone pAMC462	76,126,644	11,32,110,250,443	67,182,151,**446**
***Methylosinus*:**			
Methylosinus trichosporium OB3b (this study)	37,62,115,172,**278**,280	8,86,151,**155**,255,289	80,85,100,186,193,300
Methylosinus sp. str. LAC (M95664)[2]	10,37,62,115,171,267,**280**	86,151,**155**,263,287	80,85,100,186,192,299
Isolate PW1	37,115,172,**280**,340	8,86,151,**155**,255,289	34,37,66,80,85,156,186,300
Isolate LW2	37,115,170,**280**,338	8,86,149,**155**,255,287	37,80,84,100,154,186,299
Isolate LW3/LW4/LW8	37,115,171,**280**,338	8,86,149,**155**,255,288	34,37,66,80,85,154,186,299
Env. Clone pAMC447	37,115,172,**280**,340	8,86,151,**155**,255,289	34,37,66,80,85,156,186,300
Env. Clone pAMC451	37,62,115,172,**278**,280	8,86,151,**155**,255,289	80,85,100,186,193,300
Env. Clone pAMC459	37,62,115,172,**278**,280	8,151,**155**,289,341	80,85,100,186,193,300
***Methylocystis*:**			
Methylocystis sp. str. M (U81595)[2]	37,112,114,172,**226**,279	8,149,289,494	80,85,100,184,191,300
Methylocystis parvus (this study)	37,112,115,172,**226**,289	8,86,149,155,255,289	37,80,85,100,154,186,300
Isolate LW5	37,112,115,172,**228**,280	8,86,151,155,255,289	37,80,85,100,156,186,300
***Methylococcus*:**			
Methylococcus str. Texas (X72770)[2]	2,75,126,160,165,**197**,278	177,340,**486**	34,35,59,66,151,182,476
Methylococcus str. Bath (X72771)[2]	2,76,126,160,165,**197**,279	177,341,**487**	34,35,59,67,151,182,477
***Methylocaldum*:**			
Methylocaldum tepidum (U89297)[2,3]	2,160,163,197,**208**,280	8,70,177,**340**,415	34,68,92,151,187,478
Methylocaldum szegediense (U89300)[2,3]	2,160,164,197,**208**,279	8,66,70,176,**341**,349	34,53,92,151,202,478

Table 3.2: Size (bp) of restriction fragments from PCR-amplified products of methanotroph 16S rDNA grouped by genus. Bold-faced values distinguish operational taxonomic units (OTU's) for each genus using only the portion of the 16S rRNA gene that would be PCR-amplified using the primers in this study. [1] From phylogenetic analysis of sequences. [2] Patterns predicted from sequences deposited in GenBank. [3] Restriction fragments for the first 1010 bp of 16S rDNA.

patterns of the new 16S rRNA gene sequences also fit clearly into the OTU's defined for their respective genera (Table 3.2). These corrected sequences are especially significant for the Type II *Methylosinus* and *Methylocystis* strains as only 10 total sequences are known for the Type II methanotrophs. It should be noted that many of the remaining 8 *Methylosinus* and *Methylocystis* 16S rRNA gene sequences in the database do not produce the correct OTU's when analyzed *in silico* and may contain sequence errors in addition to ambiguous bases. All reference sequences used in our analyses contained genera-specific OTU's and care was taken to choose the most accurate and complete sequence, when possible.

In most cases, the RFLP patterns observed with *Msp*I digests were sufficient to differentiate between each methanotroph genus. The genus *Methylomonas* was the only one to show a clearly distinct OTU within *Hae*III-digested sequences. In addition, the enzyme *Hha*I produced patterns that were useful in differentiating between the Type II methanotrophic genera, *Methylosinus* and *Methylocystis*. Within each genus, patterns for *Msp*I and *Hha*I digested sequences were often very similar. In these cases the patterns observed with *Hae*III digests were used to differentiate between different clones and pure cultures. The sequences included in Table 1 were analyzed using only those bases that would be amplified using the genus-specific primers used in this study. Non-methanotrophic representatives of the α- and γ-proteobacteria tested did not show any methanotrophic OTU's when digested *in silico* (data not shown).

pmoA PCR products were also analyzed both *in silico* and experimentally with *Msp*I, *Hae*III, and *Hha*I. Although these enzymes were useful in distinguishing between *pmoA* genes from different strains, no genus-specific OTU's could be identified.

3.4.2 Characterization of 16S rRNA and *pmoA* Genes in New Lake Washington Methanotrophic Isolates

Twelve pure cultures requiring methane for growth were obtained from enrichment cultures established using Lake Washington sediment (S. Stolyar, A. Auman, and M. Lidstrom, unpublished results). Sequencing of the 16S rRNA genes from these isolates revealed 1 *Methylobacter*, 5 *Methylomonas*, 1 *Methylocystis* and 5 *Methylosinus* strains. The OTU's predicted for the 12 Lake Washington (LW and PW) strains corresponded to the expected genera (Table 3.2). The *pmoA* genes from these isolates were also sequenced and screened by RFLP analysis. An analysis of existing *pmoA* sequences in the database in addition to our new *pmoA* sequences was used to design a primer specific to *pmoA* that should not amplify *amoA* (see Methods).

3.4.3 Characterization of 16S rRNA and *pmoA* Genes in Natural Methanotroph Populations

a. 16S rRNA Gene Sequences. 16S rRNA PCR products obtained using target DNA extracted from Lake Washington sediment samples were used in constructing gene libraries. A total of 200 randomly selected clones containing inserts were subjected to RFLP analyses and placed into groups based upon their representative RFLP pattern. The 200 clones fell into 38 groups, only 15 of which contained more than one clone each. All 38 groups were further examined for any of the defined methanotrophic OTU's (Table 3.2). Based on this parameter, a total of 6 groups were indicated to be methanotrophic sequences. Clones representing each of these 6 groups were selected for sequencing and the data suggested they were methanotroph 16S rRNA genes, based on comparison to other 16S rRNA genes. Ten clones not containing the defined methanotrophic OTU's

were also selected for partial sequencing. None of these sequences were methanotrophic 16S rRNA genes based on comparison with other sequences in the RDP, further supporting the validity of the OTU analysis.

The 16S rRNA gene sequences for the 6 methanotroph clones revealed 4 *Methylobacter* sequences and 2 *Methylomicrobium* sequences. No sequences were obtained for the remaining 6 genera. However, representatives of the *Methylomonas*, *Methylosinus* and *Methylocystis* genera were obtained as pure cultures isolated from the same sediment. Based on our sequence data from these isolates, new primers were designed to specifically amplify *Methylomonas* and *Methylosinus/Methylocystis* sequences. Using these primers, additional gene libraries were constructed. For each library, a total of 50 clones were used in the RFLP and OTU analyses. For the *Methylosinus/Methylocystis* library, a total of 6 groups were obtained and 3 of these had *Methylosinus* type OTU's. The 50 clones from the *Methylomonas* gene library fell into 5 groups of which 3 had the correct OTU's. The 6 clones from the *Methylosinus* and *Methylomonas* gene libraries were sequenced. For each of these libraries, the clones not containing the appropriate OTU's were partially sequenced. None of the clones without the appropriate OTU's contained methanotrophic 16S rRNA genes. Analysis of the environmental clones is summarized in Table 3.3. An environmental clone identical to a Lake Washington isolate was obtained for one *Methylomonas* strain, and a clone differing by only 2 nucleotides from a Lake Washington isolate was obtained for a *Methylobacter* strain. No other clones showed such close identity with any of the Lake Washington isolates.

16S rRNA Environmental Clones	RDP Similarity Rank (Organism and Value)	Pure Culture Representative?
1. pAMC405	*Methylobacter luteus* 0.861	No
2. pAMC415	*Methylobacter luteus* 0.875	LW1[1]
3. pAMC417	*Methylobacter luteus* 0.869	No
4. pAMC419	*Methylobacter whittenburyi* 0.714	No
5. pAMC421	*Methylomicrobium agile* 0.788	No
6. pAMC466	*Methylomicrobium agile* 0.782	No
7. pAMC434	*Methylomonas methanica* 0.925	LW15
8. pAMC435	*Methylomonas methanica* 0.925	No
9. pAMC462	*Methylomonas methanica* 0.830	No
10. pAMC447	*Methylosinus* str. B-3060 0.825	No
11. pAMC451	*Methylosinus* str. B-3060 0.841	No
12. pAMC459	*Methylosinus* str. B-3060 0.847	No

Table 3.3: Grouping of 16S rDNA environmental clones from Lake Washington sediment DNA. [1] Sequences differ at two nucleotides.

b. *pmoA* Gene Sequences. The new *pmoA*-specific primers were used to amplify partial *pmoA* gene products from DNA extracted from Lake Washington sediment and these PCR products were used in constructing gene libraries. A total of 200 clones containing inserts were subjected to RFLP analysis using the tetrameric restriction enzymes *Msp*I/*Hae*III and *Hha*I. The 200 clones fell into a total of 34 groups. Of the 34 groups, only 8 contained more than one clone. Clones representing 24 of the groups were sequenced and 15 of these were *pmoA* gene sequences. No *amoA* sequences were obtained. Pairwise comparisons of translated amino acid sequences for the *pmoA* PCR products obtained from environmental samples and from pure cultures showed identities ranging from 63.9-100%. Examination of the nucleotide sequences from the same region showed identities from to 63-99.6%. Analysis of this larger data set confirmed that it was not possible to identify OTU's for *pmoA* using these RFLP profiles.

The 15 environmental *pmoA* sequences were compared to known *pmoA* sequences and were found to class with those from known genera (Figure 3.3). These included 1 *Methylosinus*, 2 *Methylococcus*, 5 *Methylomicrobium*, 2 *Methylomonas* and 5 *Methylobacter* sequences. Of these sequences, 2 clones were identified that had 100% amino acid identity with a Type I methanotrophic isolate from Lake Washington (LW1). Some clones were identical in amino acid sequence but differed in nucleotide sequence. In all cases for the environmental clones and Lake Washington isolates, the *pmoA* obtained showed more identity with other *pmoA* genes than with the homologous gene, *amoA* from *Nitrosomonas europaea*. The nucleotide sequence identities to *amoA* ranged from 57.8%-62.6% while the amino acid identities to *amoA* were 47.5-56.1%.

3.4.4 Phylogenetic Analyses

The 16S rRNA and *pmoA* sequences from pure cultures and environmental clones were subjected to phylogenetic analyses using PHYLIP. In general, most of the new sequences grouped within the range of known sequences (Figures 3.1-3.3). However, one group of 16S rRNA sequences formed a distinct new cluster in the Type II methanotrophs as supported by BOOTSTRAP values (Figure 3.2). This group comprised isolates LW3, LW4, LW8, PW1 and clone pAMC447. The diversity of both the 16S rRNA and *pmoA* representatives was much broader than previously found in peat or marine environments, spanning the known diversity of methanotrophs, except that no 16S rRNA sequences were found that represent the genera *Methylococcus*, *Methylosphaera,* or *Methylocaldum.* However, 2 environmental *pmoA* clones were identified that grouped with *Methylococcus* although no *Methylocaldum*- or *Methylosphaera*- like *pmoA* sequences were found.

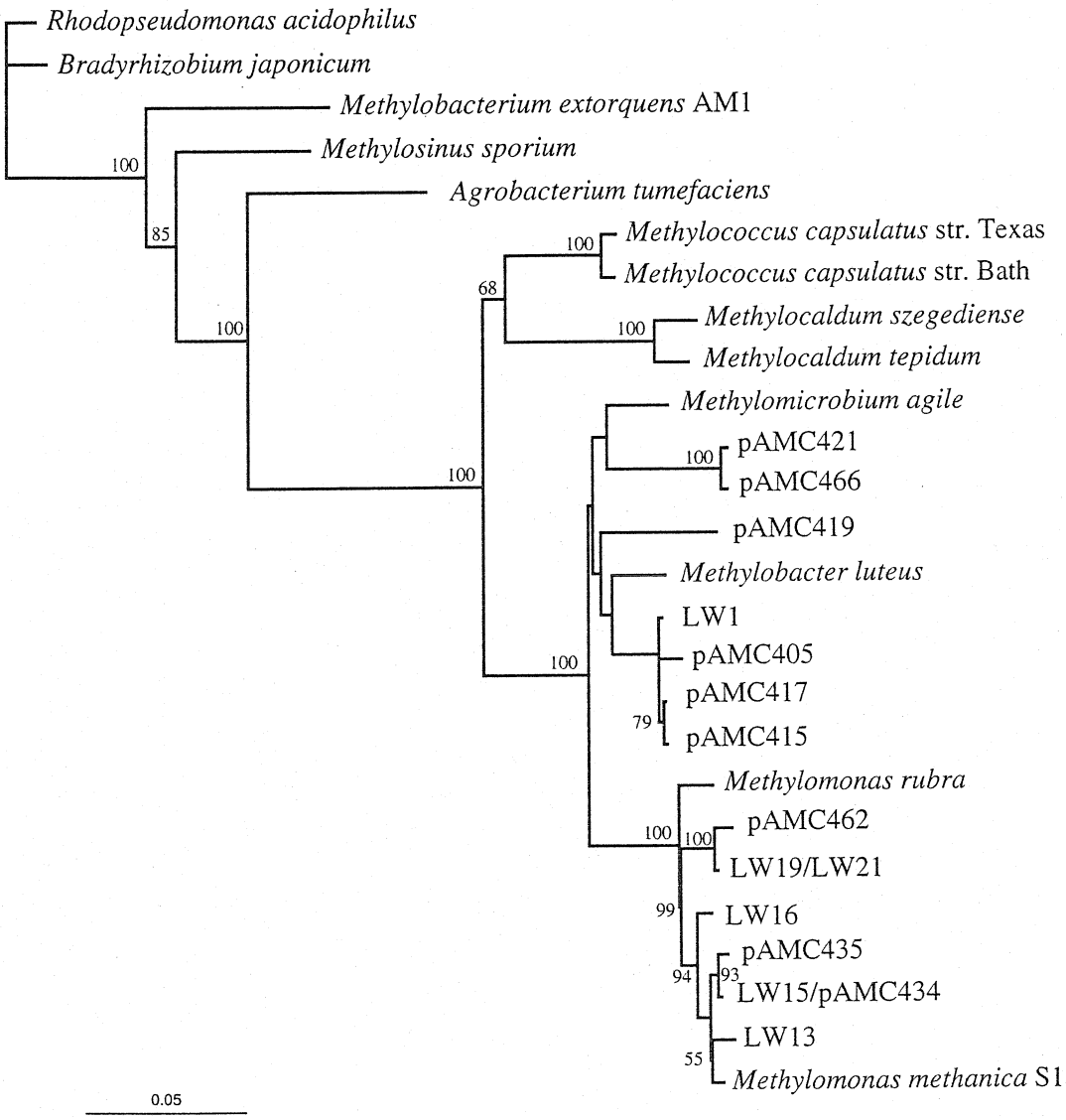

Figure 3.1: Phylogenetic analysis of 16S rRNA genes from Type I methanotrophs. The BOOTSTRAP values above 50% from 100 replicates are shown near the clades. The bar represents 5% sequence divergence, as determined by measuring the lengths of horizontal lines connecting any two species.

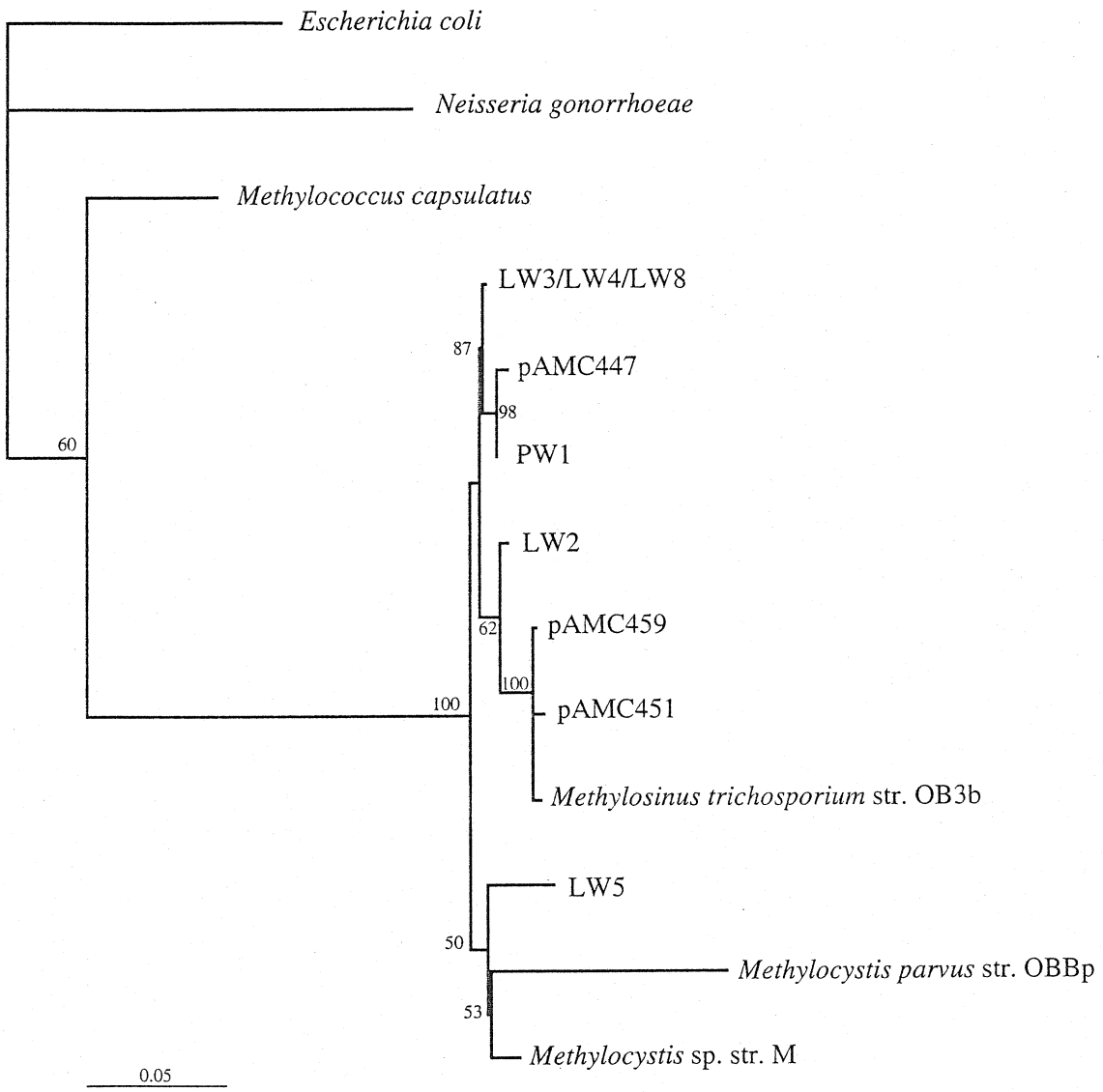

Figure 3.2: Phylogenetic analysis of 16S rRNA genes from Type II methanotrophs. The BOOTSTRAP values above 50% from 100 replicates are shown near the clades. The bar represents 5% sequence divergence, as determined by measuring the lengths of horizontal lines connecting any two species.

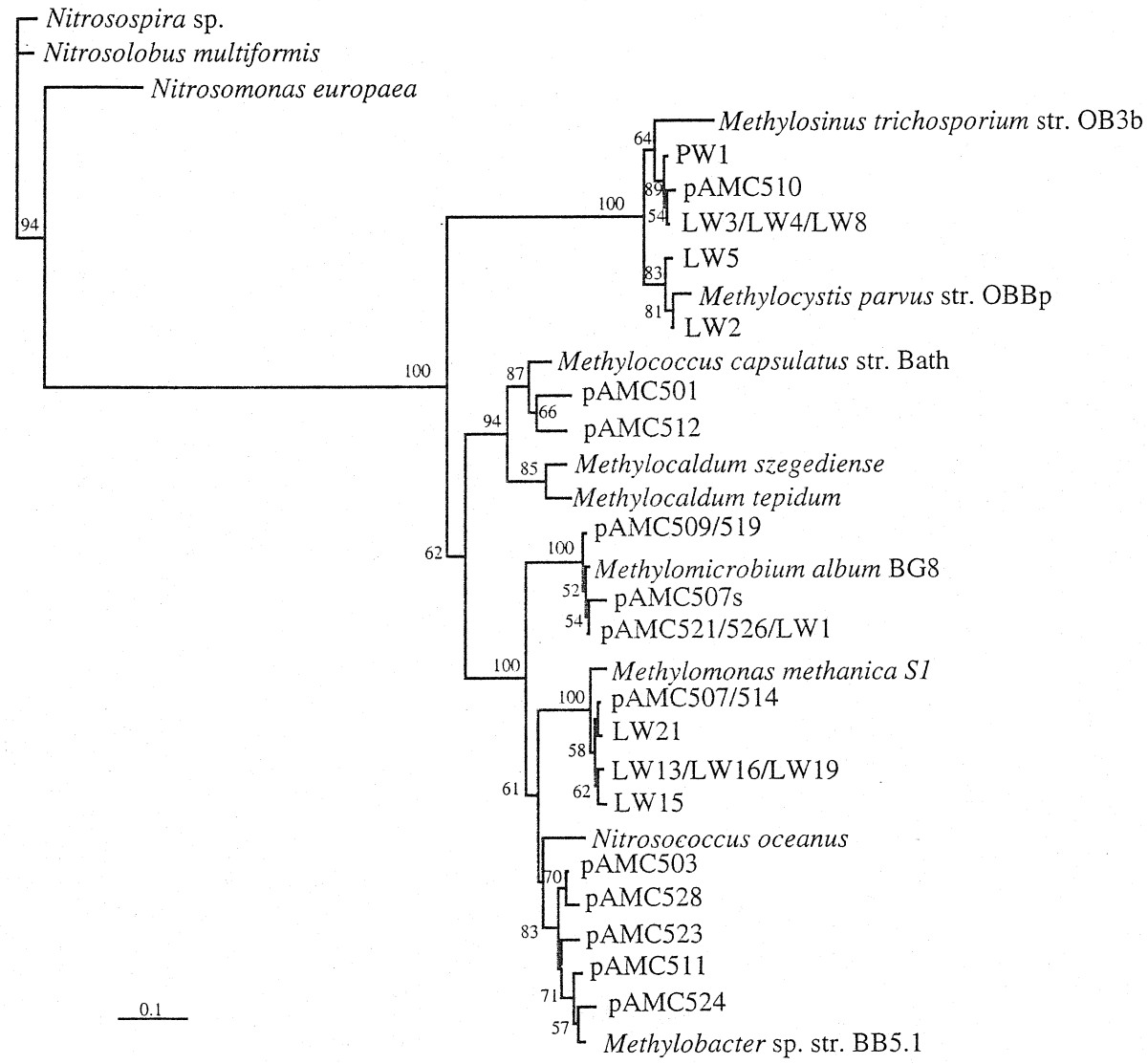

Figure 3.3: Phylogenetic analysis of the derived amino acid sequences of *pmoA* genes. The BOOTSTRAP values above 50% from 100 replicates are shown near the clades. The bar represents 10% sequence divergence, as determined by measuring the lengths of horizontal lines connecting any two species.

3.5 Discussion

Methanotrophic bacteria have environmental importance due to their role in carbon and oxygen cycling as well as their use in bioremediation strategies. In order to more fully apply molecular techniques associated with these important bacteria, more information is needed regarding the diversity of in situ populations in various environments. Molecular tools are especially important because many methanotrophs are difficult to isolate on agar plates, making growth-based assessment of natural populations problematic (14). The ability to rapidly assess and monitor natural populations of methanotrophs using molecular techniques holds great promise for understanding the complex role of these bacteria in nature.

Although primers currently exist for studying both 16S rRNA and *pmoA* genes from methanotrophs, they have some disadvantages for studying natural populations of these organisms. The 16S rRNA primers currently available have been based on a relatively small sequence database. In addition, our study has shown that some of the published sequences on which the primers were based contain errors that make accurate primer design difficult. In our study, these primers detected only a small subset of the existing methanotroph diversity in Lake Washington samples, with specific under-representation of the Type I *Methylomonas* strains and of all the Type II strains, both *Methylosinus* and *Methylocystis*. The available *pmoA* primers have the opposite disadvantage in that they amplify both *amoA* and *pmoA*, which makes them too non-specific for methanotroph-specific studies. Based on the sequences generated in this

study, we have designed new primers for methanotroph 16S rRNA and *pmoA* genes that appear to be more useful for studying methanotroph diversity in freshwater environments.

Using the newly developed primers, we analyzed the 16S rRNA and *pmoA* genes from pure cultures isolated from Lake Washington and from environmental clone banks from the same sediment. We identified a broad diversity of both of these genes including a total of 13 new Type I 16S rRNA genes, 7 new Type II 16S rRNA genes, and 18 new *pmoA* genes, 4 of which grouped with *pmoA* sequences from Type II strains. It is especially important to add to the database for the Type II genes, as the database contains fewer Type II sequences when compared to the Type I sequences. However, it is of equal importance to add to the database of environmental Type I sequences, as only two have been published to date, both from marine environments. We did not detect any 16S rDNA sequences that classed with the thermophilic methanotrophs of the genera *Methylococcus* and *Methylocaldum*, nor did we detect any *Methylosphaera*-like sequences. Since Lake Washington sediment is a freshwater environment that stays at moderately low temperature year-round (10-12°C), these results were not surprising.

So far, the phylogeny of *pmoA* genes that have been reported has mimicked the 16S rRNA phylogeny of the methanotrophs from which the *pmoA* genes were obtained. We have found that same correlation for the genes reported here from new Lake Washington isolates. These combined results suggest that *pmoA* gene sequences may be useful in inferring 16S rRNA phylogeny of methanotrophs in situ (26). A comparison of the sequences from the environmental banks of the methanotroph 16S rRNA and *pmoA* genes shows that both cover a similar breadth of diversity, except that we did detect two

pmoA sequences that are most similar to *Methylococcus pmoA*, even though no *Methylococcus* 16S rDNA sequences were detected.

In addition to the new methanotroph primers, we have also identified genus-level OTU's for methanotrophs. Since all of the strains and sequences tested in this study showed complete correlation with the OTU's, it seems likely that these will be useful tools for screening methanotrophic isolates and environmental clone banks from a wide range of environments. Even though all of the methanotroph-specific primers used in this study showed no other close matches to any other organisms in the database, non-methanotrophic sequences were obtained with all primers using environmental DNA templates. In our case, many of the non-methanotrophic 16S rRNA sequences obtained were chimeric. As yet, no reliable protocol is in use to circumvent these problems. However, in the case of the methanotrophs, our data suggest that the OTU's defined in this study can be used as an initial screen to distinguish between methanotroph and non-methanotroph sequences in 16S rRNA gene libraries constructed from environmental samples.

The use of the new tools, new sequences, primers, and OTU's developed in this study have demonstrated that the methanotrophs detectable by these methods in Lake Washington sediment samples demonstrate a diversity as broad as the known methanotrophs from all mesophilic environments. These results are in contrast with the studies of peat environments, which appear to contain only a limited group of Type II strains (21,23), and marine environments, which appear to be dominated by a limited group of Type I strains (16,31). The genes from two of the Lake Washington strains isolated from enrichments were also found in the environmental clone banks, suggesting

that these two strains may be significant in the in situ populations. This is especially true for strain LW1 since both a 16S rDNA sequence and a *pmoA* sequence with high identity to the same genes in this strain were found in the clone banks.

The types of analyses carried out in this study cannot provide information concerning the dominant groups of methanotrophs in situ due to the known problems associated with PCR-based approaches including differential amplification, artefactual PCR products and inhibition of PCR amplification by contaminants (33). However, we are now in a position to develop and test hybridization probes for assessing the relative importance of methanotroph subgroups and specific strains (such as LW1) within the detectable methanotroph populations.

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Chapter 4
The Abundance of Natural Populations of Methanotrophs in Lake Washington Sediment

4.1 Abstract

Total community DNA was extracted from Lake Washington sediments at the area of peak methane oxidation. The extracted lake DNA and control DNA were serially diluted and blotted onto nylon membranes. The membranes were hybridized with eubacterial and methanotroph-specific oligonucleotide probes homologous to portions of the 16S rRNA gene. The hybridization results were quantified using a phosphorimager and were used to estimate the total number of methanotrophs present in Lake Washington sediments. These results were compared to estimates obtained from sediment methane oxidation studies. The data suggest that the probing results agree with the V_{max}/cell estimates within a factor of 2. This information implies that the probes developed in this study detect the functionally significant populations of methanotrophs in Lake Washington and that they will be useful for obtaining accurate estimates of methanotrophs in this environment as well as in others.

4.2 Introduction

As discussed in Chapter 1, it has been shown that traditional culturing techniques are inadequate for examining diversity in natural populations of microbes (1). Recently, advances have been made in the development of culture-independent tools for examining natural populations of methanotrophs. These include PCR primers and hybridization probes for studying both functional and phylogenetic genes in these bacteria (see Chapter 3) and (3,5,6). Advances in molecular approaches for methanotrophs have been concomitant with advances in all areas of molecular biology. Technology has made DNA sequencing, PCR, and other molecular tools more accurate and commonly available.

Scientists and engineers have taken advantage of these advances and have established molecular characterization as the new "standard" for examining natural populations of microorganisms.

In this study, we have designed new hybridization probes for the molecular characterization of methanotrophs in lake sediments. The probes were based on 16S rDNA sequence data collected in Chapter 3, as well as additional data that have become available since this study was initiated in 1996 (2,12,13). The probes in this study represent significant advances over those that were previously available (3). We have used these probes to provide preliminary information on the methanotrophic populations in the sediments of Lake Washington. This study provides one of the first steps in developing well defined tools to use for analyzing the metabolic activities of in situ methanotrophs. The probes and methods developed herein have a wider application than just this study and can be utilized to examine natural populations of methanotrophs in a wide variety of natural and engineered systems. Information obtained from studies like these will provide data about environmental methanotrophs that are necessary to develop models for in situ bioremediation.

4.3 Materials and Methods:

4.3.1 Sediment Sampling

Sediment was collected from a 62 m deep station in Lake Washington on September 26, 1997, using a box core apparatus available aboard the Clifford Barnes research vessel at the University of Washington. The box core allows for the retrieval of sediment samples while preserving the in situ vertical stratification profile. Sediment

samples were obtained as subcores and these were sectioned at 0.5 cm intervals. The subsections were kept on ice during transport back to the laboratory (about 1-3 hours), where they were stored at -20°C until DNA extraction could be performed.

4.3.2 DNA Extraction and Purification

Total community DNA was extracted from Lake Washington sediment samples at the area of peak methane oxidation (Auman and Lidstrom, unpublished) and purified as described in Chapter 3. The DNA concentration was measured spectrophotometrically at 260 nm and compared to concentrations estimated visually from aliquots electrophoresed on a 1% agarose gel. Approximately 30 μg of DNA were obtained from 300 mg sediment (10^{-4} g DNA/ml sediment).

4.3.3 Slot Blots for DNA Immobilization

Purified Lake Washington DNA was blotted onto a Nytran Plus 0.45 μm membrane using a Minifold II Slot-Blot System following manufacturer's directions (Schleicher & Schuell). DNA from pure cultures of the methanotrophs *Methylomicrobium album* BG8 and *Methylosinus trichosporium* OB3b were also blotted as standard controls. A dilution series consisting of 0.9, 0.45, 0.22, 0.11, 0.056, 0.03, and 0.01 μg of each standard was blotted. Each sample was adjusted to 100 μl total volume using NaOH and EDTA to final concentrations of 0.4 N and 10 mM, respectively. Samples were incubated at 100°C for 10 minutes immediately before blotting. Previous work by Stan Grant in the Lidstrom Lab demonstrated that the degree of hybridization to blotted DNA in the first column of the Minifold II was significantly less than hybridization to DNA in the final two columns (4). Therefore, DNA was blotted using only the final two columns (columns B and C) when possible. After blotting, the

membrane was air dried and the DNA was fixed to the membrane by exposing it, DNA side down, to ultraviolet light for 5 minutes (15). The Lake Washington membrane was stored in an air-tight bag at room temperature before and after use.

4.3.4 Membrane Hybridization Experiments

The membrane was subjected to hybridization experiments with several oligonucleotide probes. One probe used was a eubacterial probe, f27, that was designed to hybridize to all bacterial 16S rRNA genes (8). In addition, two methanotroph-specific probes were developed in the course of this study. These probes were designed to hybridize to diagnostic regions of the 16S rRNA genes from Type I and Type II methanotrophs. The probes included the Type I probe, called type1b, and the Type II probe, called type2b. The type1b has the sequence 5' gtcagcgcccgaaggcct 3' that targets a region of the 16S rRNA gene from Type I methanotrophs. Another probe, type2b, has the sequence 5' cataccggrcatgtcaaaagc 3' and was designed to hybridize specifically to a portion of the 16S rRNA gene in Type II methanotrophs. All oligonucleotides were chemically synthesized by Gibco Life Technologies.

Prior to hybridization, the membrane was incubated for at least 1 hour in prehybridization buffer (20 mM $NaPO_4$ [pH 6.8], 2x SSC, 1.5% sodium dodecyl sulfate [SDS], 104 μg/ml Salmon testes DNA, 5x Denhardt's reagent). SSC is prepared as a 20x stock consisting of 3.0 M NaCl and 0.3 M sodium citrate and Denhardt's reagent is prepared as a 50x stock containing 10 g/l each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin (15). The oligonucleotide probes were labeled with γ^{32}P-ATP (New England Nuclear) by phosphorylation at the 5' end using polynucleotide kinase (Boehringer Mannheim Biochemicals [BMB]). For the end-labeling reaction, 20 pmoles

of each oligonucleotide were incubated with 2 µl 10x phosphorylation buffer (BMB), 10 units of polynucleotide kinase, 50 µCi γ^{32}P-ATP, and water to a total volume of 20 µl. The reaction was incubated at 37°C for 30 minutes and stopped by adding TE 50:50 (50 mM Tris buffer, 50mM EDTA) to a final volume of 200 µl. The labeled oligonucleotide was stored at -20°C. Prior to adding the probe to the membrane, the labeled oligonucleotide was denatured by incubation at 100°C for 10 minutes. 50 µl of denatured oligonucleotide was added to the prehybridization buffer containing the membrane and incubated overnight. The hybridization temperatures for the oligonucleotide probes were 45°C for f27, 50°C for type2b and 55°C for type1b. These temperatures were based on experimental optimizations using hybridization to Type I and Type II control methanotroph DNA. After overnight incubation, the membrane was washed in rinsing solution (0.5x SSC, 0.1% SDS) three times for 30 minutes each at the hybridization temperature. The membrane was air-dried and exposed to a phosphorimager cassette (Molecular Dynamics) for up to 4 days. The images were collected on a Molecular Dynamics Phosphorimager (Model SF) and processed using the manufacturer's imaging software, ImageQuant v.3.3.

4.4 Results

The results from the hybridization experiments are shown in Figures 4.1-4.3. In Figure 4.1, it can be seen that the eubacterial probe, f27, hybridizes to all the DNA that was blotted on the membrane, as expected. On the lower half of the membrane, known quantities of pure culture methanotrophic DNA were blotted, as described in Materials and Methods. From the known values for the amounts of DNA blotted, we were able to

calculate the total number of cells in the methanotroph standards that hybridized to the eubacterial probe. Estimates are known for the proportions of various cell components for the Gram-negative bacterium *Escherichia coli* (14). *E. coli* is approximately the same size and mass as the methanotrophs although the methanotrophs grow more slowly and therefore have less DNA per cell. *E. coli* has approximately 9.0×10^{-15} g DNA/cell based on 2.1 chromosomes/cell. While the number of chromosomes/cell for the methanotrophs is not known, best estimates are that they contain, on average, 1.25 chromosomes/cell. This amounts to approximately 5.1×10^{-15} g DNA/cell. Using the μg of control methanotroph DNA blotted, we were able to calculate the number of cells and relate this to the signal intensity for each standard blot, as determined by the phosphorimager software. From a standard curve of intensity vs. cells for the eubacterial probe, we calculated that the DNA from both depths of Lake Washington contains an average of 4.1×10^{11} cells/l sediment $\pm$ 1.5×10^{11} cells/l sediment.

Figure 4.2 shows the hybridization results using the oligonucleotide probe type1b. This probe hybridizes only to DNA from Type I methanotrophs, such as *Methylomicrobium album* BG8, and should not hybridize to DNA from Type II methanotrophs like *Methylosinus trichosporium* OB3b, as shown in Figure 4.2. The intensity data from the methanotroph standards on the Type I blot were used to calculate an additional plot of intensity vs. cells. Using this standard curve, we were able to calculate the total cells/l of Type I methanotroph DNA from Lake Washington sediment depths 1 and 2. Analysis of the Lake Washington DNA signals indicated that the Type I methanotrophs from both depths account for an average of 1.0×10^{11} cells/l sediment $\pm$ 1.6×10^{10} cells/l sediment.

Similar calculations were carried out for Figure 4.3 which shows hybridization of the oligonucleotide probe type2b to Type II methanotroph DNA. In this blot, signals can only be seen for depth 2, presumably because almost twice as much DNA was blotted for this depth as compared to depth 1 (see Figure 4.1). Analysis of the signals from the Lake Washington DNA at depth 2 indicates that the Type II methanotrophs account for approximately 9.1×10^9 cells/l sediment. The data from the blots can be used to obtain an estimate of the total number of methanotrophs in Lake Washington. Together, the calculations from the Type I and Type II blots indicate that the methanotrophs are present in Lake Washington sediment at roughly 1.1×10^{11} cells/l.

The estimate of total methanotrophs from the probing data was compared to the cell numbers determined from the methane oxidation rate calculated for Lake Washington sediment. Ann Auman in the Lidstrom lab performed $^{14}CH_4$ oxidation experiments using the same sediment as used in this study for DNA extraction and blotting. From her data, we calculated that the V_{max} value for the sediment was approximately 37.2 μM/hour. Estimates in the literature report V_{max}/cell values for methanotrophs between $0.5 - 3.0 \times 10^{-9}$ μmol/hr/cell (9,11,17). From the calculated V_{max} value, the sediment is thus estimated to have $1.2 \times 10^{10} - 7.4 \times 10^{10}$ methanotroph cells/l. This number agrees within a factor of 2 with the estimate of methanotrophs from the gene probing data.

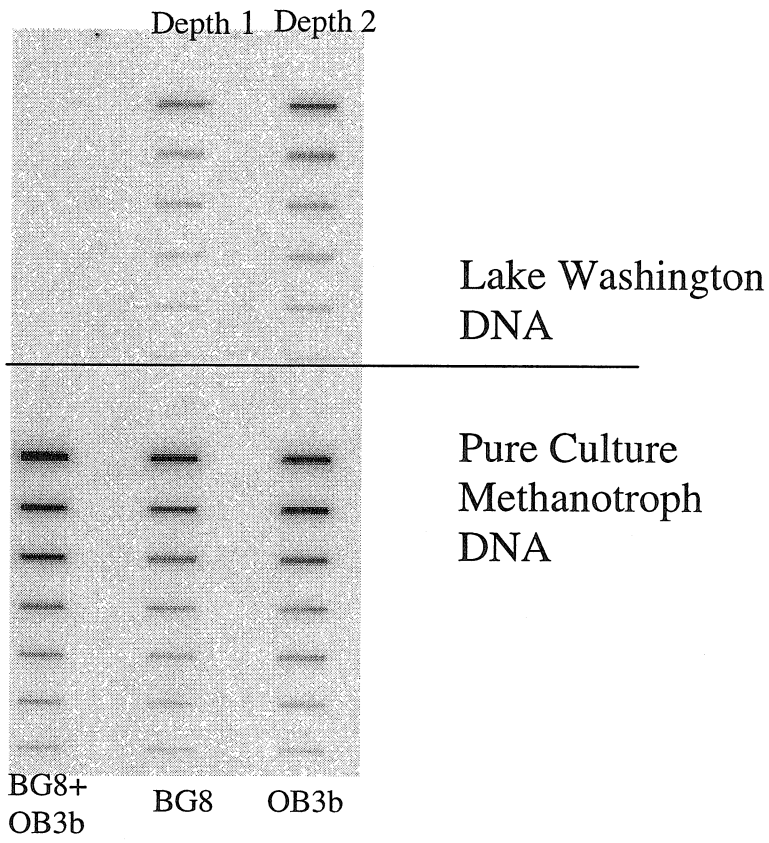

Figure 4.1: Phosphorimage of membrane hybridized with the f27 oligonucleotide, which detects all bacteria. Depth 1 and 2 are 0.5 cm sections encompassing the area of peak methane oxidation in Lake Washington sediments from the top down. Dilutions are 1:10. BG8 = *Methylomicrobium album* BG8, OB3b = *Methylosinus trichosporium* OB3b.

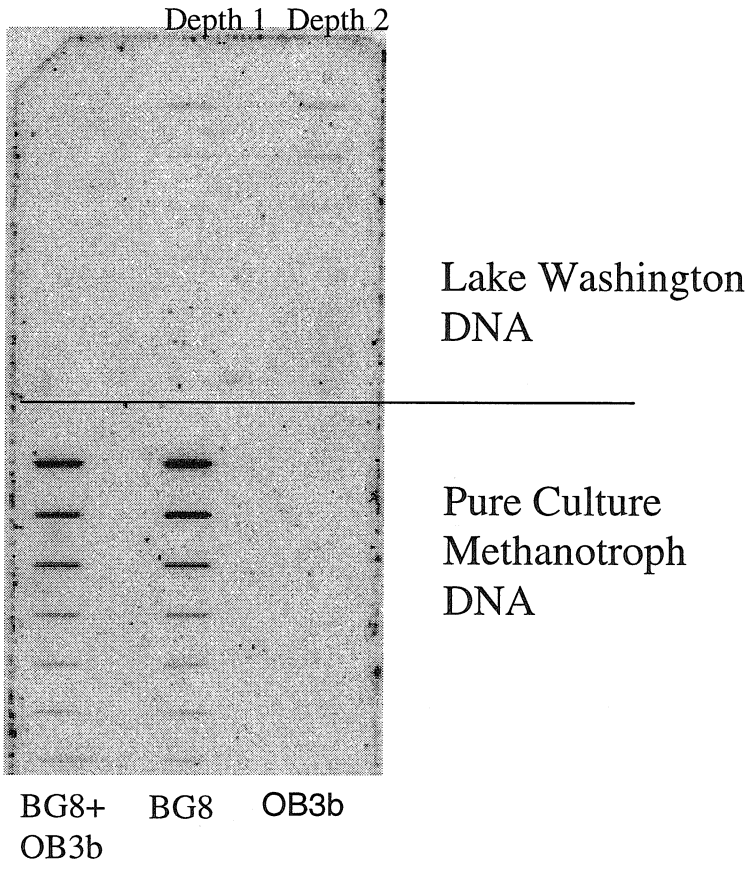

Figure 4.2: Phosphorimage of membrane hybridized with the type1b oligonucleotide, which detects Type I methanotrophs. Depth 1 and 2 are 0.5 cm sections encompassing the area of peak methane oxidation in Lake Washington sediments from the top down. Dilutions are 1:10. BG8 = *Methylomicrobium album* BG8, OB3b = *Methylosinus trichosporium* OB3b.

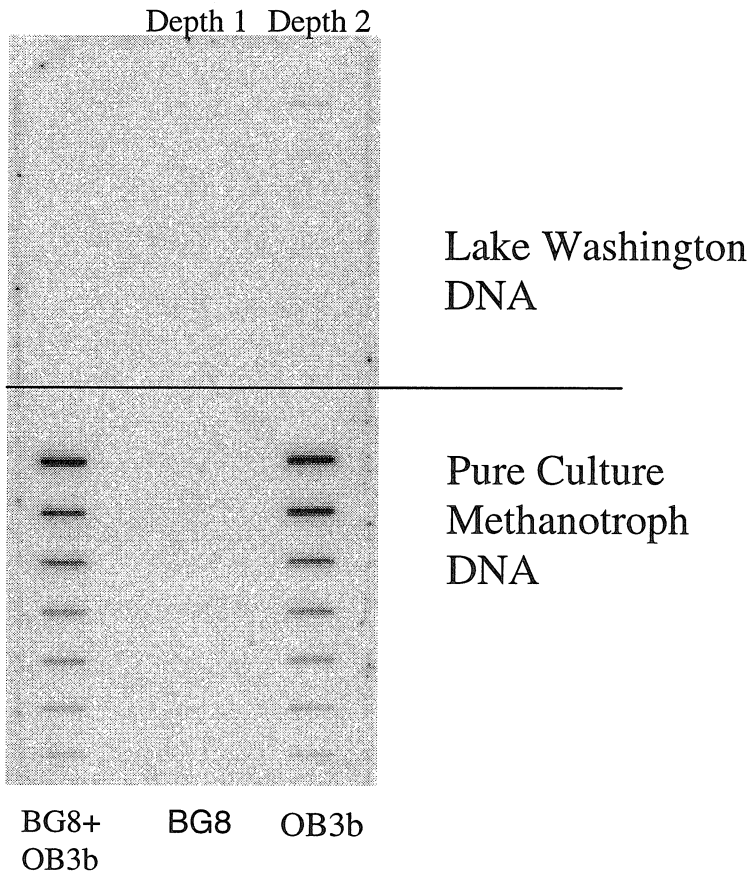

Figure 4.3: Phosphorimage of membrane hybridized with the type2b oligonucleotide, which detects Type II methanotrophs. Depth 1 and 2 are 0.5 cm sections encompassing the area of peak methane oxidation in Lake Washington sediments from the top down. Dilutions are 1:10. BG8 = *Methylomicrobium album* BG8, OB3b = *Methylosinus trichosporium* OB3b.

4.5 Discussion

A major goal of this part of the study was to develop robust hybridization probes that could detect the functionally significant methanotrophs in environments such as lake sediments that contain a broad strain diversity. The results from this study indicate that the probes developed detect the bulk of the natural populations of methanotrophs in the sediments of LakeWashington. Both Type I and Type II methanotroph signals were detected, suggesting that both of these populations are naturally present in the sediment. These results agree with those from Chapter 3 in which PCR was used to characterize functional and phylogenetic genes from methanotrophs in Lake Washington. In that study, as here, both Type I and Type II genes were detected in sediment samples taken from Lake Washington.

Since the rate of methane oxidation per cell is relatively constant, it is possible to obtain an estimate of the cell number from the V_{max} data, to compare to the number estimated from the probing. In this case, the estimates of methanotroph cells/l sediment from the probing data are approximately a factor of 2 higher than similar estimates from V_{max} data. These estimates agree within the level of uncertainty for these methods, which is about an order of magnitude for both (16). Previous studies in Lake Washington determined the V_{max} values to be between 26 – 45 μM/hour and the value here (37.2 μM/hour) falls well within this range (7,10). This suggests that the functional populations are relatively constant and have remained that way over a period of more than 10 years (7). Estimates of cell numbers from previous studies of methanotrophs in Lake Washington based on V_{max} values range from 2.6 – 4.5 x 10^{10} cells/l (9).

The preliminary data presented in this study suggest that probing techniques will be useful for quantifying natural populations of methanotrophs. Together with estimates of V_{max} values, the number of methanotroph cells/l sediment can be determined within an order of magnitude given the current state of knowledge. Furthermore, probing can differentiate between the two different types of methanotrophs and the abundance of each, providing more detailed information than V_{max} measurements. It is now possible to extend the probing studies initiated here to examine the metabolic activities of in situ methanotrophs in a wide variety of environmental situations. By examining natural populations of methanotrophs, especially those employed in bioremediation protocols, we can collect information important in elucidating the role of these organisms in nature. This information will help in developing models that assist in designing more effective strategies for methanotroph-mediated bioremediation.

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Chapter 5
Enriched Populations of Methanotrophs in Sediments: Applications for Bioremediation

5.1 Abstract

Sediment from Lake Washington was incubated in flask experiments simulating conditions of enhanced bioremediation. It was found that the $^{14}CH_4$ oxidation rates of the sediment populations increased 2-3 orders of magnitude above the intrinsic rates measured from unamended sediment. Correspondingly, the trichloroethylene (TCE) oxidizing capacity of the sediment was increased from non-detectable levels to greater than 7 μmol/liter/hour in certain flasks. The ratio of methane oxidation to TCE oxidation rates suggested that sMMO was not expressed. Hybridization with methanotroph specific oligonucleotide probes demonstrated that Type I methanotrophs were enriched in most flasks. In addition, probing indicated that Type II methanotrophs were enriched in all flasks approximately 2 fold, with select flasks showing 3-4 fold increases. However, the increases detected by the probes did not account for the increase in methane oxidation capacity. This suggests that the methanotrophs present were of an altered physiology compared to known methanotrophs or that a potentially novel group of methane oxidizers was enriched in these flasks.

5.2 Introduction

Methanotrophs have been suggested for use in bioremediation because they are able to oxidize trichloroethylene (TCE) by the methane oxidation system involving methane monooxygenase (MMO). TCE is a synthetic, halogenated compound that is a major contaminant of groundwater aquifers. Many of the studies of TCE degradation by methanotrophs have focused on well characterized mixed cultures or pure laboratory strains that may not represent in situ populations (7,8,10,17,22). However, a few studies

have focused on examining natural in situ populations of methanotrophs and their capacities for TCE degradation. The most notable of these studies have been conducted at the Westinghouse Savannah River Site in Aiken, SC, and the Moffett Naval Air Station in Mountain View, CA (3,11-13,19,21). At these sites, researchers have examined natural populations of methanotrophs by isolating pure cultures from enrichments and/or using traditional culturing techniques such as most probable number (MPN) studies (3,11,12). As was discussed in Chapters 1 and 3, these techniques can often lead to misrepresentations of the true in situ populations. In the few studies in which nucleic acid techniques have been applied, probes designed for one of the subunits of the sMMO were used as well as early probes designed against 16S rRNA genes in methylotrophs (2,19). While these early nucleic acid studies yielded valuable information regarding methanotrophs and other organisms, the results were not correlated directly with TCE degradation.

In this study, we have used sediment from Lake Washington to establish methanotroph enrichment cultures under conditions that simulate enhanced bioremediation. For enhanced in situ bioremediation, it is desirable to enrich the intrinsic methanotrophic populations. In addition, since the sMMO has about 1000 fold higher rate for TCE degradation than the pMMO, it is desirable to specifically enrich for sMMO containing methanotrophs (mainly Type II strains), and to develop conditions in which sMMO will be expressed. So far, in situ methane enhancement protocols have assumed that as populations increase after addition of methane and air, they will become copper-limited. If sMMO-containing strains are present, they should then become enriched and express sMMO, resulting in a dramatic increase in TCE degradation. We wanted to test

this idea directly using Lake Washington sediment and the molecular tools developed for studying methanotrophs in this environment.

We have investigated a number of different conditions for enrichment in flasks that should encourage sMMO expression. It has been shown that in standard media, approximately 10^6 copper atoms per cell are sufficient to repress expression of the sMMO (9). Methane oxidation kinetics in Lake Washington sediments suggest that the in situ methanotrophs are expressing pMMO. However, estimates of total copper and numbers of methanotrophs suggest the in situ populations should be nearly copper limited. Therefore, we should be able to increase the methanotrophic populations present in the sediment to a point at which they become copper-limited and a switch is made to sMMO expression. The approach taken was to add various nutrient additions to samples in flasks and follow the methane and TCE oxidation rates in the flasks over a period of 8-10 weeks. Using methanotroph-specific 16S rRNA gene probes, we have been able to assess the relative importance of the two different types of methanotrophs throughout the entire experiment. This study provides an in depth examination of the activities of the methanotrophic populations under conditions of enhanced bioremediation. It uses nucleic acid techniques to gain an understanding of the metabolic roles of these bacteria under different environmental conditions and the potential consequences that these factors have for in situ bioremediation.

5.3 Materials and Methods

5.3.1 Flask Experiments

Enrichment for methanotrophs in Lake Washington sediment was carried out in 250 ml Erlenmeyer flasks. Sediment was mixed 1:1 with filter sterilized lake water or

mineral salts medium as described in Table 5.1. The flasks were stoppered with rubber stoppers and incubated at 30°C with shaking at 200 rpm under a methane-air atmosphere (1:1 vol/vol). The gases in the flasks were replaced approximately every 2 days. The first flask experiment consisted of 5 flasks, each with a different diluent summarized in Table 5.1. All sediment was mixed 1:1 with the diluent to a final volume of 30 ml except for Flask 1 that contained only 15 ml of sediment with no diluent. The sediment for the first flask experiment was taken from a chamber incubation system that had been incubated with methane for 24 days at room temperature. The second flask experiment consisted of 4 flasks whose conditions are summarized in Table 5.1. All flask sediment was mixed 1:1 with the diluent for a final volume of 30 ml. The sediment for the second flask experiment had been incubated at 7°C (the measured in situ Lake Washington sediment temperature) for 60 days in closed polypropylene tubes. Sediment samples from each of the flasks were taken at regular intervals throughout the length of the experiment and kept at -20°C until DNA could be extracted.

5.3.2 Measurement of Methane Oxidation

Methane oxidation in sediment samples was calculated by incubation with $^{14}CH_4$ and measurement of ^{14}C-labelled products (carbonates and cell material) by scintillation counting (14). Sediment from the flask experiments was diluted (from 1:4 at the beginning of the experiments to 1:10 at the end of the experiments) with the same diluent as shown in Table 5.1. 2 ml aliquots of the diluted sediment were dispensed into sterile, 35 ml serum vials (Pierce) that were closed with gray butyl covered rubber stoppers (Wheaton) and aluminum crimp seals. Unlabeled methane was added through the stopper with syringes. The vials were then spiked with synthetic $^{14}CH_4$ (DuPont NEN, specific

Flask Experiment/Number	Depth Interval of Sediment	Diluent
Experiment 1/Flask 1	0-0.5 cm	none
Experiment 1/Flask 2	0-0.5 cm	distilled/deionized water and trace elements
Experiment 1/Flask 3	0-0.5 cm	NMS (trace elements/no vitamins) [a]
Experiment 1/Flask 4	0-0.5 cm	NMS (no trace elements/no vitamins) [a]
Experiment 1/Flask 5	0-0.5 cm	NMS ($FeSO_4$/formate) [b]
Experiment 2/Flask A	0.5-1 cm	filter sterilized Lake Washington bottom water
Experiment 2/Flask B	0.5-1 cm	NFMS[c]
Experiment 2/Flask C	0-0.5 cm	distilled/deionized water
Experiment 2/Flask D	0-0.5 cm	NFMS[c]

Table 5.1: Description of conditions for flask experiments. a) NMS = nitrate mineral salts media (23). b) FeSO4 was added to a final concentration of 500 μM and formate was added to a final concentration of 1 mM. c) NFMS = nitrate free mineral salts media (20).

activity = 55 mCi/mmol) using gas-tight syringes (Series A-2, Dynatech Precision Sampling). All partition coefficients for methane were calculated from the model of Duan, et al. (5). The vials were incubated at room temperature with shaking for up to 4 hours. At the end of the experiment, 7 N NaOH was added to a final concentration of 1 N. The NaOH serves to kill the bacteria by raising the pH and also to dissolve the gas phase CO_2 and $^{14}CO_2$ in the vial (14). After the addition of NaOH, the vials were flushed with air for 2 minutes to remove any excess methane. Next, 0.5 ml aliquots were placed in 4.5 ml scintillation fluid and counted on a liquid scintillation counter. All flask experiments were carried out in duplicate and controls were made for each flask by adding NaOH prior to the addition of $^{14}CH_4$. The raw data for Flask Experiments 1-2 are given in Appendix B.

5.3.3 Gas Chromatography

All gas chromatography was carried out using a Hewlett Packard 5890 Series II Gas Chromatograph equipped with a Merlin Microseal inlet (Cole Parmer), a DB-5 column, and a flame ionization detector (FID). The concentration of the unlabeled methane used for the methane oxidation experiments was checked by comparison to measurements of known methane standards. Headspace samples of 200 µl were injected into the GC. The injector, oven and detector temperatures were set at 120, 120, and 250°C, respectively. For the TCE degradation experiments, TCE was measured at several time points by removing 100 µl of headspace from sample vials and injecting the sample into the GC. A flame ionization detector was used based on a protocol developed by Lontoh and Semrau (8). The injector, oven and detector temperatures were set at 250, 120, and 250°C, respectively.

5.3.4 TCE Degradation Assays

TCE degradation in sediment samples from the flask experiments was assayed over time using 1.3 ml aliquots of sediment in 6 ml serum vials. The vials were capped with Teflon coated rubber butyl stoppers (Wheaton) and aluminum crimp seals. For each flask in each experiment, 2 sample vials were created in addition to 1 control vial. The control vial contained sediment to which 25 µl 5 N NaOH had been added. TCE (final concentration in liquid of 60µM) was added to the vials using Hamilton 1700 series gastight syringes from a bottle of TCE saturated water solution (1). TCE was measured every hour for 4 hours by injecting 100 µl headspace from each vial into the GC. A zero time point was taken 7 minutes after addition of TCE at which time it was found that the TCE had equilibrated between the liquid and gaseous phases. The vials were incubated at

room temperature with shaking at 200 rpm. A dimensionless Henry's constant of 0.42 was used for the partitioning of TCE between the sediment and the headspace (6). The raw data for Flask Experiments 1-2 are given in Appendix B.

5.3.5 DNA Extraction, Blotting, and Hybridization

DNA was extracted from flask sediment samples using the protocol described in Chapter 3. After extraction, the DNA was electrophoresed and purified by dialysis of agarose gel fragments using Spectra/Por® Membranes MWCO:2000 (Spectrum Labs). The products from dialysis were ethanol precipitated following standard protocols, resuspended in 50 µl water and stored at -20°C (15). Blotting and hybridization of the extracted DNA was carried out as described in Chapter 5 using the f27, type1b and type2b probes as described.

5.4 Results

5.4.1 Methane Oxidation Experiments

The results from the $^{14}CH_4$ oxidation experiments are shown in Figure 5.1 (Flask Experiment 1) and Figure 5.2 (Flask Experiment 2). The methane oxidizing capacity in all flasks increased in the first 40-50 days of both experiments, regardless of the nutrient additions used in each flask. After approximately 50-60 days, no further increase in methane oxidation was observed and all flasks analyzed decreased in their methane oxidation capacity.

Flasks in both experiments showed similar increases in methane oxidation over time. Two notable exceptions are Flasks B and D from Experiment 2. Flask B is the only flask that continued to increase in methane oxidation past 50 days. This flask reached its

maximum methane oxidation rate on day 64. Flask D is an interesting case because this flask showed an immediate increase in its methane oxidation rate and was an order of magnitude higher than the other flasks by day 20. The methane oxidation rate for this flask remained higher than all the other flasks at each time point measured although 2 flasks reached similar levels much after Flask D. Both Flasks B and D were treated with the same diluent but contained sediment taken from different depths. Estimates of cell numbers from the V_{max} values (see Chapter 4 for description) indicate that the cells in both flask experiments increased 2-3 orders of magnitude. These results are summarized in Table 5.2.

5.4.2 TCE Oxidation Experiments

TCE oxidation was measured over time using aliquots of sediment from each flask. The results are shown in Figures 5.3 and 5.4. Sediment from Lake Washington did not show any detectable TCE degradation in experiments performed on the days of sediment collection (data not shown). As early as 7 days after the beginning of methane enrichment, TCE degradation was detected in all flasks tested (Figures 5.3 and 5.4). For Experiment 2, TCE oxidation was only tested at 61 and 73 days, to assess sMMO expression. TCE degradation patterns roughly followed methane oxidation rates with the highest TCE degradation generally observed at the same time that the methane oxidation rates peaked.

5.4.3 Hybridization Membranes of Extracted DNA

DNA was extracted from sediment samples taken at regular intervals over time from each flask. The DNA from time points over 6 weeks of Flask Experiment 1 and 10

Experiment/Flask	Initial V_{max} [a]	Estimated Cells (cells/l) for Initial V_{max}	Highest V_{max} [a] (day of experiment)	Estimated Cells (cells/l) for High V_{max} [a]
Experiment 1/Flask 1	24.925	$0.83\text{-}5 \times 10^{10}$	7252.4 (42)	$0.24\text{-}1.45 \times 10^{13}$
Experiment 1/Flask 2	24.925	$0.83\text{-}5 \times 10^{10}$	11,909 (42)	$0.4\text{-}2.4 \times 10^{13}$
Experiment 1/Flask 3	24.925	$0.83\text{-}5 \times 10^{10}$	9574.6 (43)	$0.32\text{-}1.9 \times 10^{13}$
Experiment 1/Flask 4	24.925	$0.83\text{-}5 \times 10^{10}$	5237.6 (29)	$0.17\text{-}1 \times 10^{13}$
Experiment 1/Flask 5	24.925	$0.83\text{-}5 \times 10^{10}$	10,257 (42)	$0.34\text{-}2 \times 10^{13}$
Experiment 2/Flask A	12.2	$0.41\text{-}2.44 \times 10^{10}$	5964 (50)	$0.2\text{-}1.2 \times 10^{13}$
Experiment 2/Flask B	12.2	$0.41\text{-}2.44 \times 10^{10}$	10,241 (64)	$0.34\text{-}2 \times 10^{13}$
Experiment 2/Flask C	12.2	$0.41\text{-}2.44 \times 10^{10}$	5389.6 (50)	$0.18\text{-}1.1 \times 10^{13}$
Experiment 2/Flask D	12.2	$0.41\text{-}2.44 \times 10^{10}$	12,230 (20)	$0.41\text{-}2.4 \times 10^{13}$

Table 5.2: Summary of V_{max} methane oxidation rates and estimated cell numbers for Flask Experiments 1 and 2.
a) V_{max} (µmol/liter/hour).

weeks of Flask Experiment 2 were blotted along with control DNA from the methanotrophs *Methylomicrobium album* BG8, *Methylomonas rubra* S1 and *Methylosinus trichosporium* OB3b. The membranes were hybridized with 3 oligonucleotide probes, described in Chapter 4. The probes consist of a universal probe that detects all bacterial 16S rRNA genes, a Type I methanotroph probe and a Type II methanotroph probe. The hybridization data were quantified using a phosphorimager. The signals from the control DNA using the methanotroph probes were used to generate standard curves and the amount of DNA from the flasks that hybridized to the probes was calculated. The results, expressed as total increases in Type I and Type II DNA signals, are summarized in Table 5.3. The hybridization data indicate that the Type I methanotrophs in the flask experiments were enriched an average of 1.6-4.9 fold while the Type II methanotrophs were enriched approximately 1.3-4.4 fold. The largest increase in Type II methanotrophs was in Flask 2 of Experiment 1. Flask 2 showed an increase of 4.4x for Type II methanotrophs. Type I methanotrophs in this flask were enriched to a lesser (1.6x) extent. The values from Flask D of Experiment 2 could not be calculated due to lack of signal.

5.5 Discussion

The data above present the results from methane enrichment experiments performed with sediment samples from Lake Washington. The experiments were carried out under conditions that simulate an enhanced bioremediation scenario. The $^{14}CH_4$ oxidation data indicate that the methane oxidizing capacity in all of the flasks increased 2-3 orders of magnitude over the course of the experiments. Surprisingly, this includes

Flask Experiment/Number	Type I Methanotrophs	Type II Methanotrophs
Flask Experiment 1/Flask 1	4.9x increase	3.4x increase
Flask Experiment 1/Flask 2	1.6x increase	4.4x increase
Flask Experiment 1/Flask 3	1.7x decrease	3.7x increase
Flask Experiment 1/Flask 4	4x increase	1.8x increase
Flask Experiment 1/Flask 5	2.7x increase	1.7x increase
Flask Experiment 2/Flask A	1.6x decrease	1.5x increase
Flask Experiment 2/Flask B	1.9x increase	1.3x increase
Flask Experiment 2/Flask C	No change	1.9x increase
Flask Experiment 2/Flask D	Data not available	Data not available

Table 5.3: Changes in methanotroph populations in flask experiments, as determined by oligonucleotide probe hybridization data.

those flasks that contained only sediment in addition to those that contained sediment plus distilled/deionized water or filter sterilized lake water. These flasks showed increases similar to the flasks that were given additional nutrients in the form of media (NMS, NFMS) or trace elements. These data suggest that the methane oxidizing populations in Lake Washington are limited by methane and/or oxygen, but not by other nutrients.

In addition to the increase in methane oxidizing capacity, the flasks also increased in their abilities to degrade TCE. The increase in TCE degradation roughly followed the increase in methane oxidation with the highest rate for both observed on or around the same day. We know from previous data that the ratio of TCE:CH_4 oxidation is approximately 1:500 – 1:1,000 for methanotrophs expressing the pMMO and 1:1 for those expressing sMMO (16,18). The ratios of TCE:CH_4 oxidation for the flask sediment in the first week (Flask Experiment 1) was close to 1:500, suggesting a population of Type I methanotrophs expressing pMMO. However, in both flask experiments, the ratios of TCE:CH_4 oxidation rose to values up to 1:5379. At the end of both experiments, the

lowest ratio was 1:1355. These data suggest that no significant sMMO expression occurred in the flasks. Since Type II (sMMO containing) methanotrophs were present, we can conclude that contrary to our predictions, copper limitation was not achieved. Potentially, there could be a reservoir in the sediments for copper that we are unaware of or the populations in the sediment could contain more efficient copper utilization systems than laboratory cultures. These alternatives will need to be examined further in future studies.

A second unexpected result was that the hybridization studies with DNA extracted from the flasks indicate a much lower level of enrichment than calculated from the methane oxidation studies (Table 5.3). It is possible that the hybridization probes used in this study did not detect a major constituent of the enriched population. However, for this to be true, this population would have been a minor constituent of the original population. Analyses of the data from probing and methane oxidation experiments with the unamended sediment suggest that the two values agree and that these probes detect the major functional populations (Chapter 4). Another explanation for the apparent discrepancy is that methanotrophs were enriched with substantially higher methane oxidation rates per cell than most methanotrophs. A report of methanotrophs isolated from plant roots has shown that one strain has a 10-fold higher methane oxidation rate/cell than other methanotrophs and therefore a precedent exists (4). Further work will need to be carried out in this area to isolate and characterize the methane oxidizers enriched in these experiments. However, the probing results and the very high methane/TCE oxidation rates suggest that these may be novel methane oxidizers.

In summary, we have enriched flasks of Lake Washington sediment with methane and stimulated methane oxidizing populations and their TCE oxidizing capacity. We expected excess methane to stimulate the methanotrophic populations to a point where they would become copper limited and a switch would be made from pMMO to sMMO. However, we did not observe any sMMO expression or evidence for copper limitation in the flasks and further studies in this area will need to be conducted. In addition, the probing and rate data suggest the presence of a potentially novel group of methane oxidizers, a result that would not have been determined with conventional approaches. This study is one of the first to utilize molecular tools to monitor methanotrophic populations and correlate them to remediation of TCE. It is the first step towards understanding how methanotrophs and TCE interact under specific environmental conditions and it will pave the way for future studies aimed towards developing well defined systems to study the metabolic activities of organisms in situ.

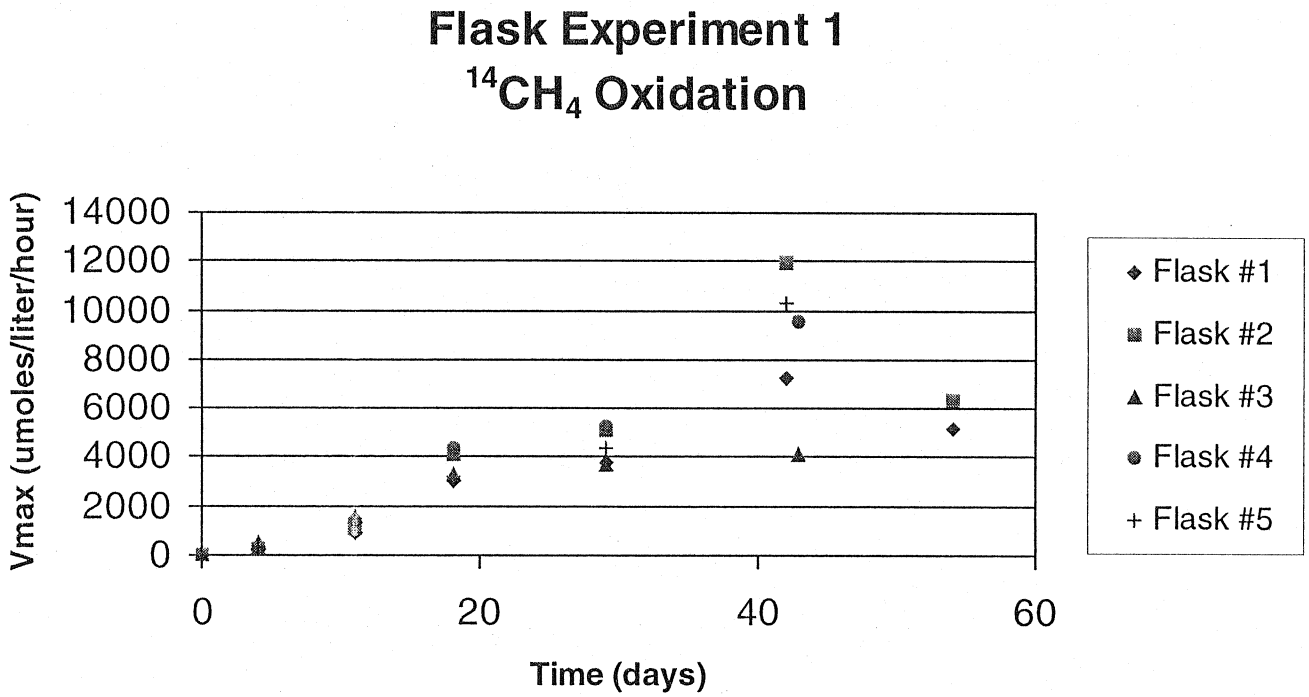

Figure 5.1: Summary of methane oxidation experiments for Flask Experiment 1.

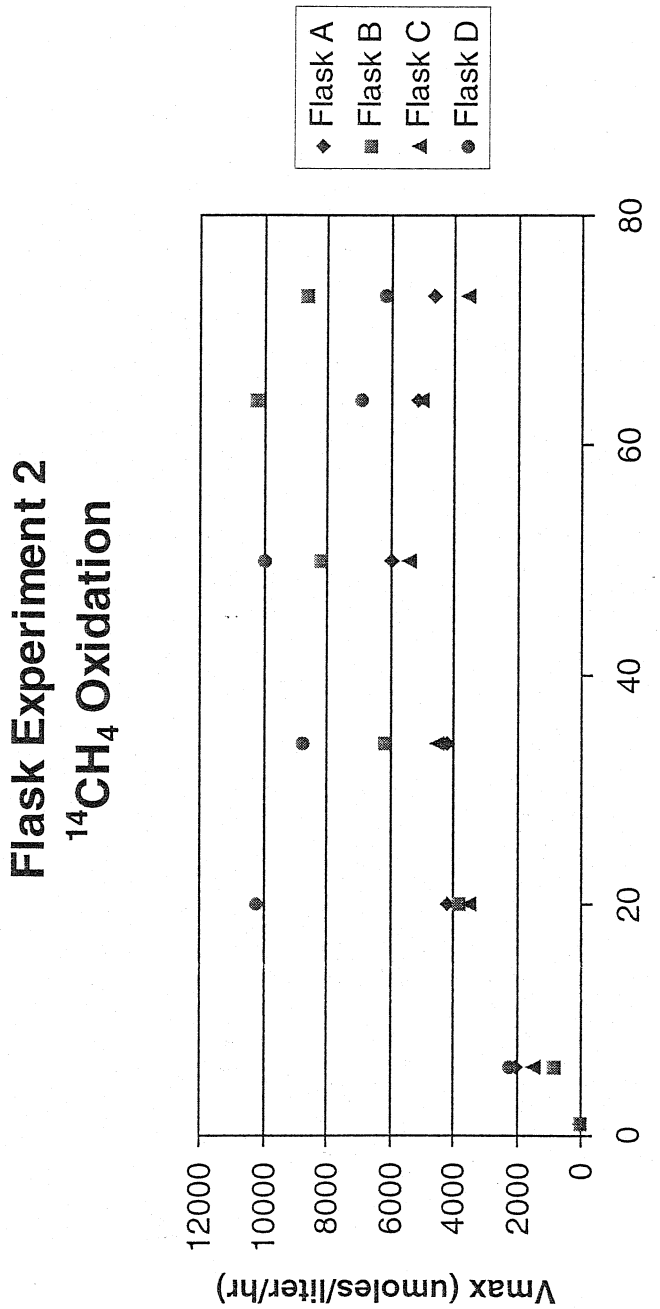

Figure 5.2: Summary of methane oxidation experiments for Flask Experiment 2.

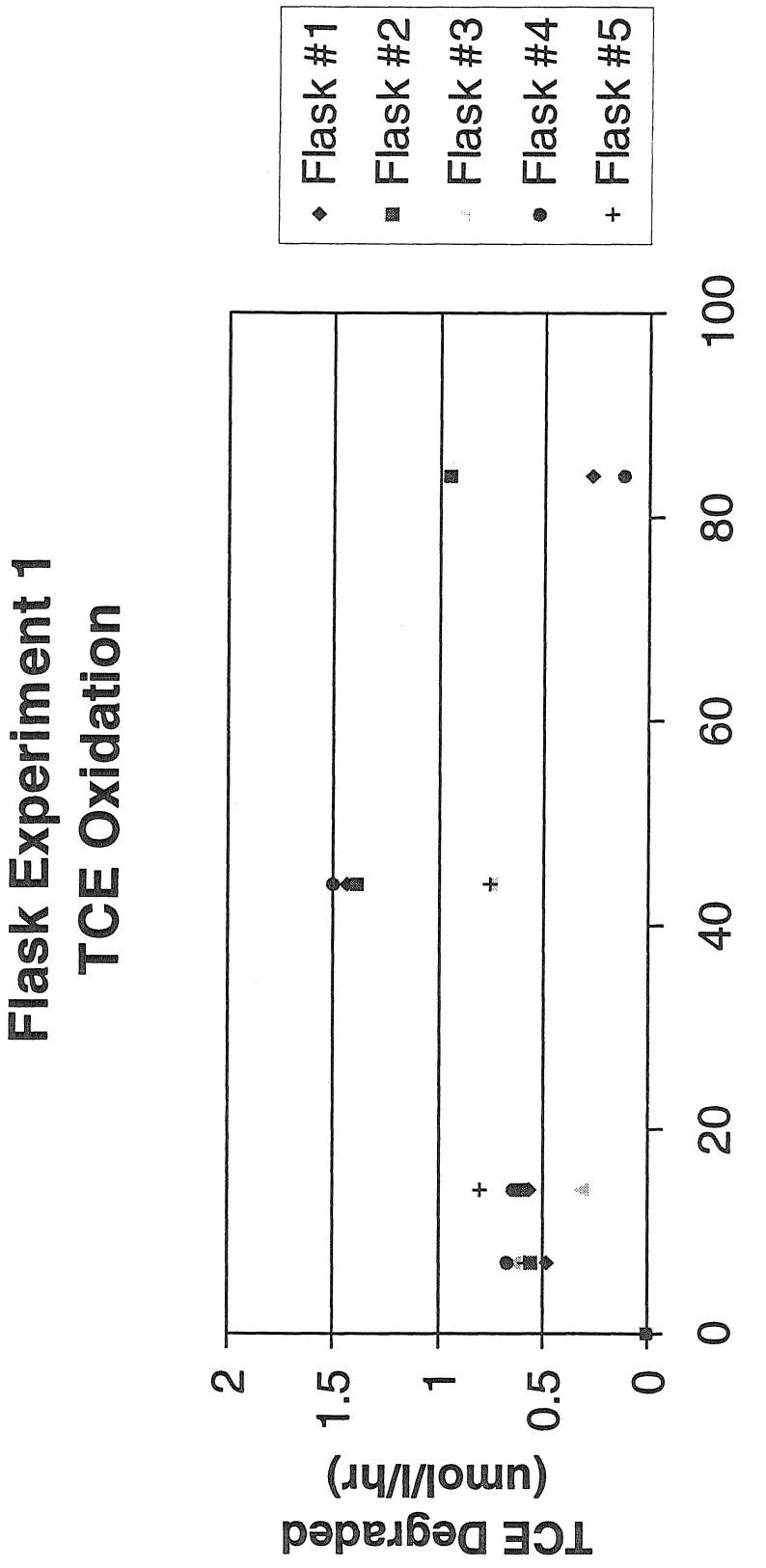

Figure 5.3: Summary of TCE oxidation experiments for Flask Experiment 1.

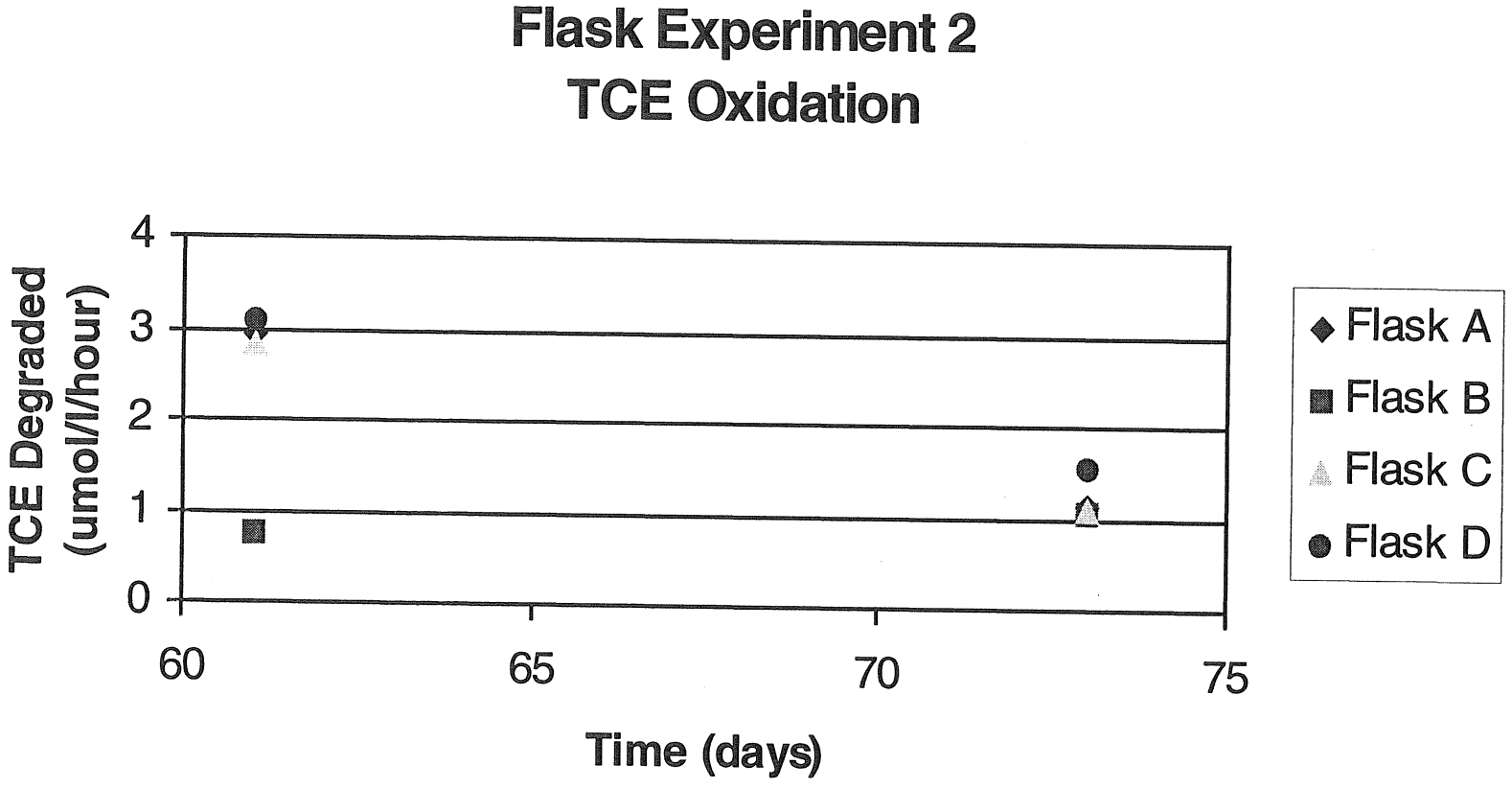

Figure 5.4: Summary of TCE oxidation experiments for Flask Experiment 2.

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Chapter 6
Conclusions and Future Work

In this thesis, molecular biological tools have been applied to examine methane oxidizing bacteria at the microbial level. Using these tools, we developed a greatly expanded database of functional and phylogenetic gene sequences for methanotrophs both from pure cultures and environmental DNA. This database was used to develop PCR primers and gene probes targeted to all known methanotrophs. We showed that these probes detect the major functional populations of methanotrophs in the sediments of Lake Washington, a freshwater habitat with broad methanotrophic diversity. Furthermore, we used these probes to determine the relative abundance of Type I and Type II methanotrophs in Lake Washington and were able to estimate the number of methanotrophs in the total bacterial populations. These probes were also used to follow enrichments in the sediment that mimic an enhanced in situ bioremediation protocol. In the enrichment experiments, we showed that 2-3 orders of magnitude increase in methane oxidation did not achieve significant relative enrichment of sMMO-containing strains or expression of sMMO. From these experiments, we obtained evidence suggesting that this enrichment protocol enriched for a population of methane oxidizers that were not detected by the phylogenetic probes.

The molecular tools for methanotrophs that existed prior to this study were based on a relatively small sequence database and had some disadvantages for studying natural populations of methanotrophs. We were able to use the probes developed in this study to demonstrate a surprisingly broad diversity of methanotrophs in Lake Washington. These results are in contrast to preliminary studies from peat and marine environments that suggest these environments contain very little methanotroph diversity. In Lake Washington, we detected the presence of a significant proportion of Type II strains, the

group of methanotrophs normally associated with the sMMO. This is the first time that Type II methanotrophs have been confirmed in a freshwater sediment by direct probing techniques. Because the sMMO is desirable for degrading halogenated solvents, the presence of these organisms has strong implications for bioremediation in this type of environment.

Our results from enrichment experiments that mimic enhanced bioremediation protocols suggest that in situ bioremediation protocols involving methanotrophs will probably involve solvent degradation by the pMMO. The pMMO has a lower rate of action towards solvents than the sMMO and is less desirable for bioremediation. The natural populations in our enrichment experiments did not express sMMO even after a 1000x increase in the total methane oxidation capacity. Our data suggest that the natural populations did not become copper limited and that copper limitation is the key to achieving high rate solvent consumption in these in situ enhancement protocols. This is in contrast to assumptions in the literature that an increase of this magnitude will always result in sMMO expression. The results from the methane enrichment experiments have great implications for modeling of bioremediation protocols involving methanotrophs. In addition, data from the enrichment experiments provided evidence for enrichment of a phylogenetically divergent group of methane oxidizers. At present, the identity of this putative new group of methanotrophs is not known, although they appear to be low rate solvent oxidizers. Future studies need to be conducted to determine the identity of these organisms and their kinetics of methane and TCE utilization.

In summary, this study has provided molecular tools useful for studying natural populations of methanotrophs and their responses to situations that mimic conditions of

intrinsic and enhanced bioremediation. We have begun to address the major issues that surround the use, monitoring, and optimization of natural methanotrophic populations for TCE bioremediation. This study is the first step in developing well-defined systems to study the metabolic activities of organisms in situ.

In the future, it will be necessary to expand the use of the molecular tools to study methanotrophic diversity and TypeI/TypeII populations in a wider range of environments, including contaminated sites. In addition, we need to study the factors that select for specific physiological groups in these different environments. These future studies will provide information necessary to develop predictive models enabling us to make more effective decisions about bioremediation.

Appendix A
A Chamber Incubation System for Population Enrichment Experiments

A chamber incubation system has been described previously to study the effects of methane oxidation on oxygen consumption and phosphate flux in sediment (1). Because of the success of these experiments, we proposed to use a modified chamber incubation system to study natural populations of methanotrophs in Lake Washington under a variety of conditions. Specifically, we hypothesized that the chamber system would be a useful tool to monitor population shifts of methanotrophs from Lake Washington sediments under experimental conditions that mimic those found in bioremediation protocols.

The chamber incubation system in this study (Figure A.1) consisted of two 1 liter compartments separated by a Teflon membrane (TE 36, pore size 0.45 μm, Schleicher & Schuell) and supported by a polypropylene grid. The Teflon membrane allowed gas exchange but was impervious to liquids. Sediment samples were placed on top of the grid and liquid in the form of filtered lake water or media was placed on top of the sediment samples. These conditions were chosen to approximate the in situ conditions as closely as possible.

Initial studies were conducted using the chamber incubation system and sediment collected from the area of peak methane oxidation in Lake Washington. In these studies, sediment and filtered lake water were placed on top of the membrane/grid and the chamber was sealed. Approximately 1 l of methane was placed in the bottom compartment while the top compartment was simultaneously flushed with air to prevent any pressure changes. The concentrations of methane in both compartments were measured over time using flame ionization detector gas chromatography (FID-GC). In addition, whole cell methane oxidation (V_{max}) rates were measured using on aliquots of

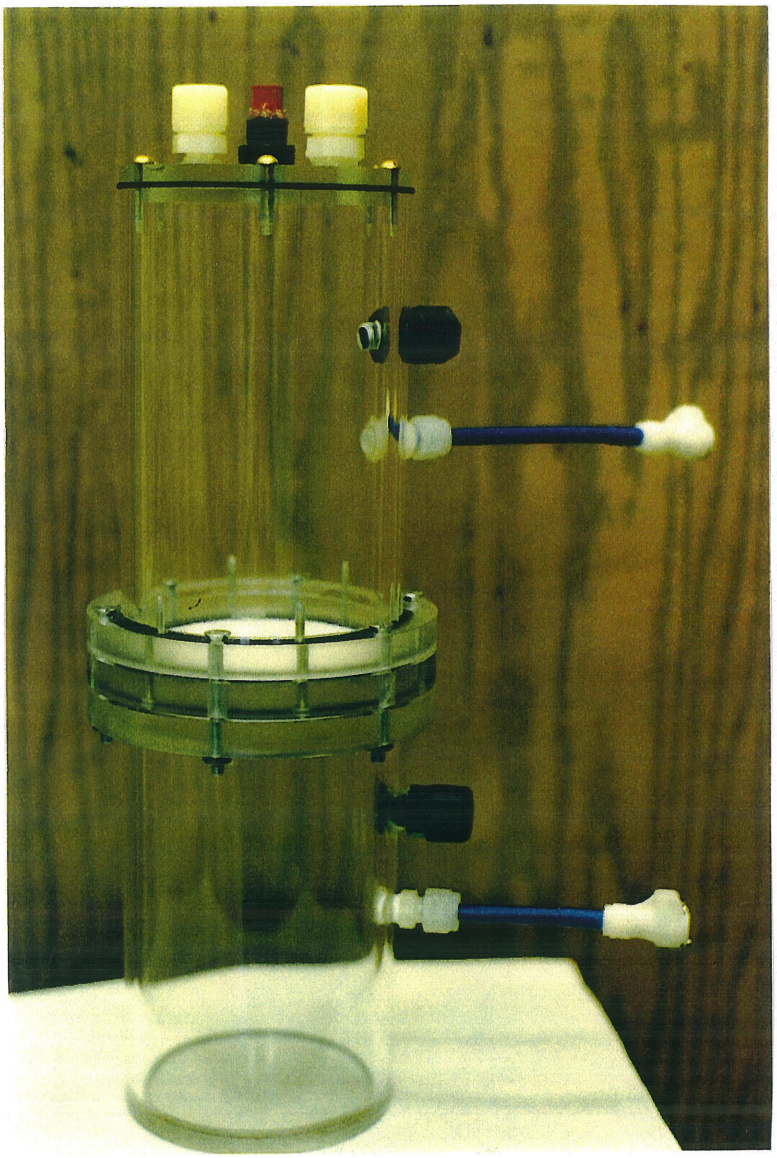

Figure A.1: Chamber incubation system used in initial population enrichment studies.

the sediment sample using $^{14}CH_4$. The goal of these experiments was to enrich the sediment sample with methane gas, simulating an enhanced bioremediation strategy. In theory, the methanotrophic populations present in the sediment should increase to a point at which they became copper limited and a switch would be made from pMMO to sMMO expression. sMMO expression is desirable during in situ bioremediation due to its high rate of activity towards halogenated solvents such as trichloroethylene (TCE).

Results from the first chamber study revealed by $^{14}CH_4$ oxidation analyses and FID-GC that the methanotrophic populations did not respond to the availability of additional methane. There was no statistically significant increase in the maximal rate of activity towards methane (V_{max}) over a period of 54 days. In fact, the population V_{max} dropped in half in the first 22 days of incubation.

Throughout the entire incubation experiment, a number of alterations were made in attempts to increase the methanotroph populations. One possibility for the lack of growth was that the methanotrophs were limited by the lack of an essential nutrient. Therefore, on day 18, nitrate mineral salts (NMS) medium with no added trace elements or vitamins was added on top of the sediment sample. This addition provided nutrients that are routinely given to pure methanotroph cultures in laboratory settings. Nonetheless, this amendment did not increase the populations. Another alteration addressed the possibility of low methane flux from the bottom to top compartments of the chamber. On day 22, methane gas was added to the top compartment and the disappearance over time measured by FID-GC. There appeared to be no significant consumption of methane in the top compartment. However, the V_{max} of the populations doubled returning the population to its initial V_{max} after 17 days of incubation with methane in the top compartment. This

suggested that methane limitation had occurred when the methane was present in only the bottom compartment.

One concern with adding methane to the top chamber was that this procedure did not resemble one that would be used in in situ bioremediation. There, methane is usually pumped from below the site of desired activity. Another concern with this alteration was that the populations would become oxygen limited. Care was taken to replace only a partial volume of air in the top compartment with methane. In addition, stirring was added on day 44 to increase the dissolved methane and air in the liquid overlying the sediment sample. From day 39 to day 54, the populations remained constant, i.e., no increase in the V_{max} was observed. Because the populations never increased in methane consumption beyond their initial capacity, we concluded that no net methane enrichment had occurred and this chamber incubation study was discontinued.

Two additional chamber incubation studies were established that increased the methane available to the sediment. The bottom compartment was altered to allow a continuous flow of methane, regulated by flow meters, and the extra gas vented through ports in the top compartment (Figure A.2). This change should assure that the sediment sample was receiving an ample supply of methane to determine whether methane limitation was one cause for the lack of enrichment.

The two chambers in the second set of experiments were set up identically except that chamber 1 had filtered lake water overlying the sediment sample while chamber 2 contained nitrate free mineral salts medium (NFMS). The lake water was to mimic natural conditions while the NFMS would provide nutrients but no additional nitrogen. We wanted to test whether limiting nitrogen availability would favor the enrichment of

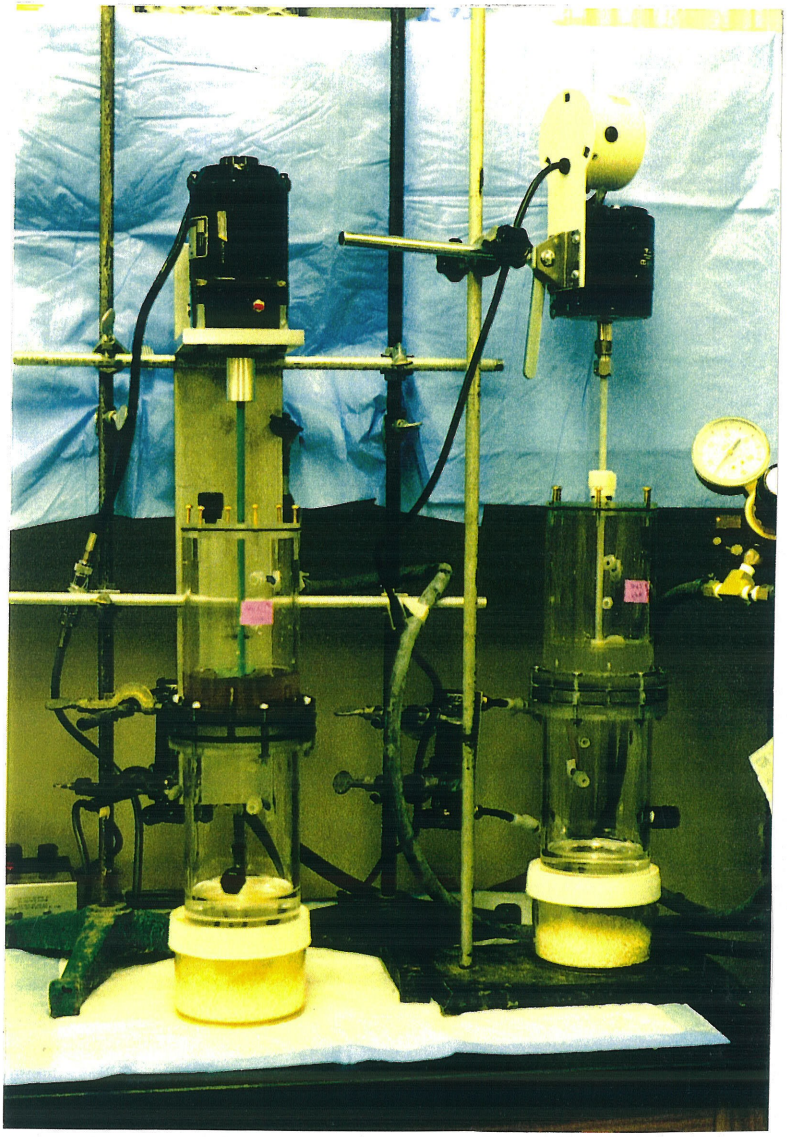

Figure A.2: Altered chamber incubation system used in population enrichment studies.

nitrogen fixing methanotrophs that are mainly Type II, sMMO expressing strains. The populations present in the sediment samples were followed over time by $^{14}CH_4$ oxidation analyses. During the first 22 days, the V_{max} of the populations in both chambers dropped by a factor of 2. On day 22, stirring was added to both chambers and ports in the top compartments were opened to the atmosphere. This alteration was made to increase the availability of oxygen to the populations in the sediment sample. An immediate (2 day) increase was observed in the V_{max} values of both chambers (Figure A.3). During this time, the V_{max} value of chamber 1 increased 3.9 fold while the V_{max} of chamber 2 increased 1.8 fold. This suggests that the populations present in the sediment samples had been oxygen limited, at least to some extent. Over the next 19 days, both chambers continued to increase in V_{max}. By the end of day 52, the V_{max} values of both chambers had leveled out at approximately 3.6 and 3.2 fold higher (respectively) than the starting V_{max} values. These data suggest that an increase of the methane oxidizing populations in the sediment samples had occurred, but they subsequently became nutrient-limited again. Since no significant difference was observed between the final response of the two chambers, it appears that the samples were not limited by nutrients present in NFMS.

TCE oxidation experiments were conducted on day 1 and day 53 with aliquots of sediment from both chambers. TCE oxidation experiments conducted on day 1 showed no detectable TCE degradation. However, the sediment samples from day 53 were able to degrade TCE at detectable but very low rates (0.38 μM/hr for chamber 1 and 0.44 μM/hr for chamber 2).

The increases in V_{max} values and TCE degradation indicate that the methane oxidizing populations in the sediment samples were enriched during the 52 days in both

chamber incubation systems. However, no sMMO expression was observed. This conclusion is based on the ratio of TCE oxidation:CH4 oxidation and the lack of enrichment of Type II methanotrophs from hybridization data (data not shown). In addition, the populations in the chamber incubation studies were not enriched as much as experiments carried out with the same sediment samples in flasks (see Chapter 5). Because of these factors, it was concluded that the chamber incubation system in its current configuration was not an appropriate tool to use to study population shifts in Lake Washington methanotrophs. Future experiments to explore alternate configurations will be carried out in my laboratory at Syracuse University.

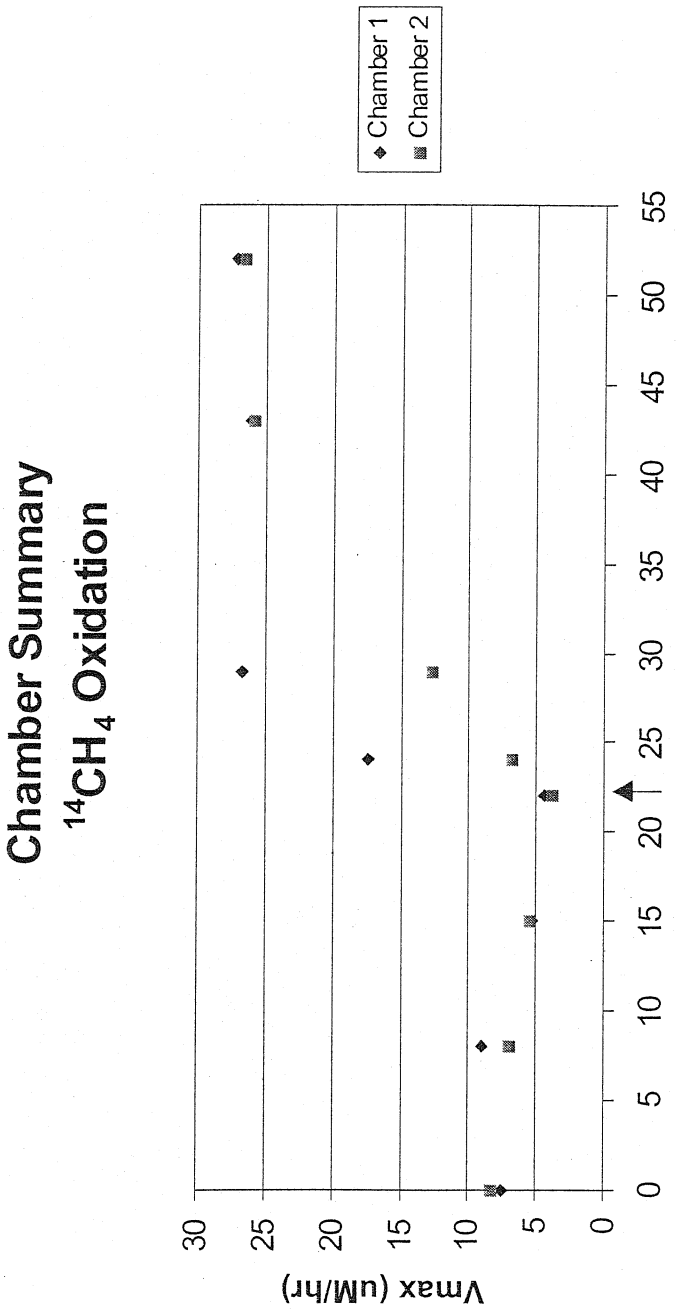

Figure A.3: Summary of V_{max} values calculated for Chambers 1 and 2.

Reference

1. **Sinke, A. J. C., F. H. M. Cottnaar, K. Buis, and P. Keiser**. 1992. Methane oxidation by methanotrophs and its effects on the phosphate flux over the sediment-water interface in a eutrophic lake. Microb.Ecol. **24**:259-269.

Appendix B
Raw Data for Chapter 5 Figures

Given below are the raw data for the $^{14}CH_4$ oxidation (Tables B.1 and B.2) and TCE degradation (Tables B.3 and B.4) experiments from Chapter 5. For each experiment, each flask was sampled in duplicate and the average value is given in the table along with the margin of error. Figure B.1 is a representative plot of a TCE degradation experiment over the time length of the experiment (4 hours). All experiments gave linear plots of TCE degradation similar to Figure B.1 with r^2 values typically ≥ 0.95.

	V_{max} (µmol/l/hour)				
days	**Flask 1**	**Flask 2**	**Flask 3**	**Flask 4**	**Flask 5**
0	24.9 ± 0.8	24.9 ± 0.8	24.9 ± 0.8	24.9 ± 0.8	24.9 ± 0.8
4	206.6 ± 6.6	219.4 ± n.d.	475.8 ± 11.4	288.6 ± 6.6	368.7 ± 39.1
11	945.2 ± 6.6	1094.4 ± 31.7	1594.7 ± 31.9	1423.2 ± n.d.	1473.8 ± 33.9
18	3056.9 ± 48.9	4153 ± 182.7	3288 ± 88.8	4379.8 ± 372.3	3193.9 ± 440.8
29	3823.6 ± 233.2	5065.7 ± 5.1	3703.5 ± 48.1	5237.6 ± 696.6	4397.3 ± 39.6
42	7252.4 ± n.d.	11909 ± n.d.	n.d.	n.d.	10257 ± n.d.
43	n.d.	n.d.	4110.1 ± 12.3	9574.6 ± 354.3	n.d.
54	5178.5 ± 82.8	6342.4 ± 120.5	n.d.	n.d.	n.d.

Table B.1: V_{max} data for $^{14}CH_4$ oxidation experiments from Flask Experiment 1. n.d. = not determined.

	V_{max} (μmol/l/hour)			
days	**Flask A**	**Flask B**	**Flask C**	**Flask D**
0	12.2 ± 0.9	12.2 ± 0.9	12.2 ± 0.9	12.2 ± 0.9
6	20226.5 ± 186.4	821 ± 31.2	1447.5 ± 208.4	2209.9 ± 185.6
20	4208 ± 223	3800.4 ± 64.6	3510.4 ± 77.2	10230 ± 327.4
34	4169.6 ± 58.4	6173.7 ± 179	4479.5 ± 62.7	8779.5 ± 61.4
50	5964 ± 190.8	8209.8 ± 566.5	5389.6 ± 264.1	9938.6 ± 149.1
64	5129 ± 169.2	10241 ± 10.2	5015.3 ± 120.4	6942.8 ± 243
73	4632.9 ± 171.4	8655.5 ± 285.6	3578.2 ± 153.9	6154.3 ± 258.5

Table B.2: V_{max} data for $^{14}CH_4$ oxidation experiments from Flask Experiment 2.

	V_{max} (µmol/l/hour)				
days	**Flask 1**	**Flask 2**	**Flask 3**	**Flask 4**	**Flask 5**
0	not detected	not detected	not detected	not detected	not detected
7	0.48 ± 0.17	0.55 ± 0.03	0.63 ± n.d.	0.67 ± 0.2	0.58 ± 0.09
14	0.56 ± 0.01	0.6 ± 0.14	0.31 ± n.d.	0.64 ± 0.02	0.8 ± 0.01
44	1.44 ± n.d.	1.39 ± 0.12	0.75 ± 0.21	1.5 ± 0.02	0.75 ± 0.14
84	0.27 ± n.d.	0.95 ± 0.02	n.d.	0.12 ± n.d.	n.d.

Table B.3: V_{max} data for TCE oxidation experiments from Flask Experiment 1. n.d. = not determined.

	V_{max} (μmol/l/hour)			
days	**Flask A**	**Flask B**	**Flask C**	**Flask D**
0	not detected	not detected	not detected	not detected
61	2.94 ± 0.03	0.75 ± 0.08	2.83 ± 0.04	3.1 ± 0.04
73	1.13 ± 0.02	1.06 ± 0.01	1.11 ± 0.08	1.56 ± 0.04

Table B.4: V_{max} data for TCE oxidation experiments from Flask Experiment 2.

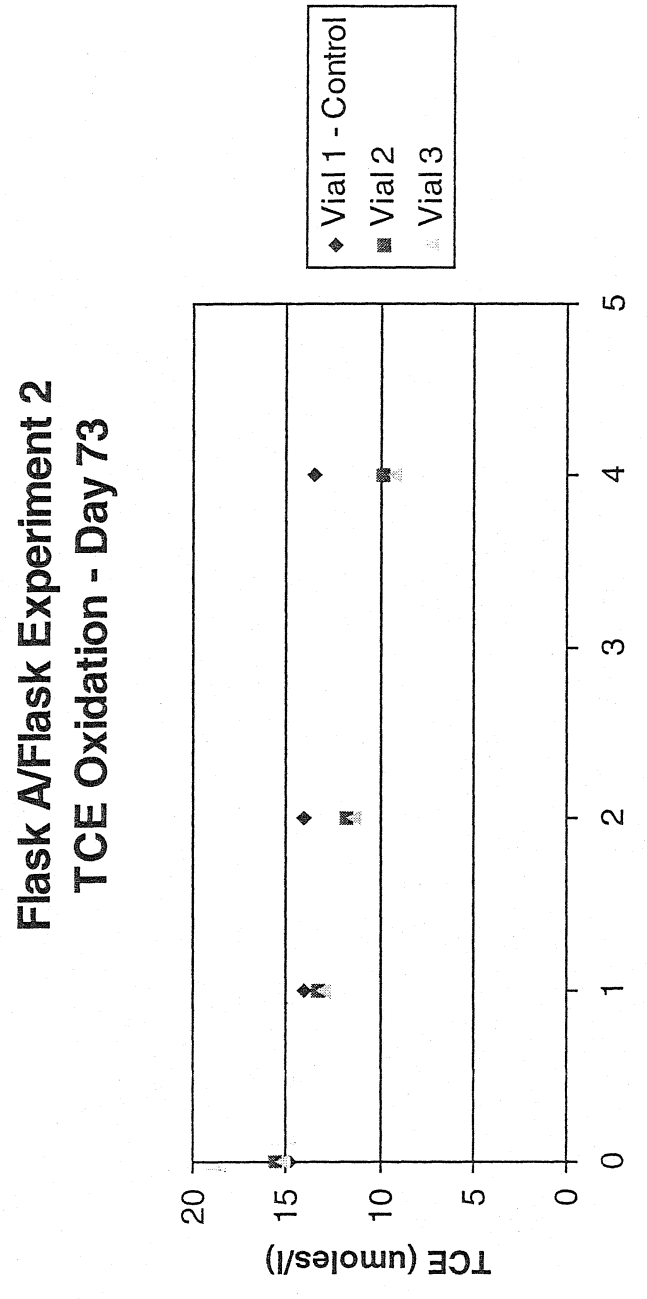

Figure B.1: Representative plot of TCE degradation during a 4 hour experiment.