Molecular Ecology Techniques for the Study of Aerobic Methanotrophs

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Methane oxidation can occur in both aerobic and anaerobic environments; however, these are completely different processes involving different groups of prokaryotes. Aerobic methane oxidation is carried out by aerobic methanotrophs, and anaerobic methane oxidizers, discovered recently, thrive under anaerobic conditions and use sulfate or nitrate as electron donors for methane oxidation (11, 104). This review will focus on the aerobic oxidation of methane.

Aerobic methanotrophs are a unique group of methylotrophic bacteria that utilize methane as a sole carbon and energy source (52). Based on their cell morphology, ultrastructure, phylogeny, and metabolic pathways, methanotrophs can be divided into two taxonomic groups: type I and type II. Type I methanotrophs include the genera Methylophilus, Methylosarcina, Methylosinus, Methylococcus, and Methylocella, which belong to the gamma subdivision of the Proteobacteria (Fig. 1). The type II methanotrophs Methylocystis, Methylosinus, Methylocella, and Methylocapsa are in the alpha subdivision of the Proteobacteria (52) (Fig. 1). Recently, two filamentous methane oxidizers have been described, Crenothrix polyspora (113), which has a novel pmocAB, and Clonothrix fusca (125), which has a conventional pmocAB. Both are gammaproteobacteria and are closely related to the type I methanotrophs. Most extant methanotrophs are cultured at 20 to 45°C and neutral pH but have also recently been isolated from extreme environments (reviewed in reference 122).

The first step in the oxidation of methane to CO2 is the conversion of methane to methanol by the enzyme methane monooxygenase. There are two forms of this enzyme: a particulate membrane bound form (pMMO) and a soluble cytoplasmic form (sMMO). The pMMO has been reported in all methanotrophs except for the genus Methylocella (121), whereas the sMMO is present only in certain methanotroph strains (94). The pMMO is a membrane bound copper and iron containing enzyme (reviewed in reference 49). The structural genes for this enzyme have been cloned and sequenced from Methylococcus capsulatus Bath (107, 114), Methylocystis sp. strain M, and Methylosinus trichosporium OB3b (45). They lie in a three-gene operon, pmocAB, which code for three integral membrane polypeptides of approximately 23, 27, and 45 kDa, respectively. These operons are present in duplicate copies in all three organisms. These duplicate copies of pmocAB are virtually identical and are transcribed from σ70-type promoters found upstream of the pmoc gene (45, 110).

The sMMO is a cytoplasmic enzyme containing a unique di-iron site at its catalytic center. It has a broad substrate range, including trichloroethylene, alkanes, alkenes, and aromatic compounds. The biochemistry of the sMMO has been studied in detail (reviewed in reference 75). It consists of three components: a hydroxylase, which is a dimer of three subunits, (αβγ); a regulatory protein (protein B); and a reductase (protein C). It is encoded by a six-gene operon (19, 111) containing the genes encoding the α, β, and γ subunits of the hydroxylase (mmoxYZ), the reductase (mmoc), and a regulatory or coupling protein (mmob). There is one other gene in the operon, orfY (mmoD), which has no known homologues in the public database but may play a role in assembly of the unique di-iron center of the sMMO enzyme (90).

Methanotrophs can be isolated from a wide variety of environments including air, the tissues of higher organisms, soils, sediments, and freshwater and marine systems and are all obligately aerobic, gram-negative bacteria. Methanotrophs play an important role in the oxidation of methane in the natural environment, oxidizing methane biologically produced in anaerobic environments by the methanogenic archaea and thereby reducing the amount of methane released to the atmosphere. Methane-oxidizing bacteria also oxidize methane from the atmosphere (i.e., at very low concentrations [ca. 1.7 ppm]), particularly in upland soils and forest soils, thereby mitigating global warming due to the effects of the greenhouse gas methane (25). Understanding this ability to oxidize atmospheric concentrations of methane, as well as the search for the organisms responsible, has occupied many researchers for the past decade, and many of the techniques developed to study the microbial ecology of the methanotrophs, which will be discussed here, have led to insights into atmospheric methane oxidation. Consequently, some of these studies into atmospheric methane oxidation are highlighted in this review.

**IDENTIFYING MOLECULAR MARKERS**

The key to identifying marker genes suitable for use in molecular ecology studies of organisms is the availability of sequences in a database from which to design primers. One obvious marker is the 16S rRNA gene due to the large database of sequences available, and also when new organisms are described, their 16S rRNA gene is always sequenced and thus becomes available for determining the origins of sequences obtained in molecular ecology studies. A complementary option for molecular ecology studies is a functional gene that is unique to the physiology and metabolism of the organisms being studied. Functional marker genes have two major advan-

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and, mmoX, and pmoA, and Methylomonas sequences as belonging to a methanotroph. In this group of molecules, available, can still be used to identify a particular gene sequence unique to the methanotrophs, but which have suitable datasets not congruent with the 16S rRNA phylogenies of the same organisms (59, 74). Other functional gene markers which are not advantages over housekeeping genes. First, they narrow down the investigation to the studied functional group, thus enabling a much higher sensitivity of detection in complex environments. Second, putative uncultivated members of the functional group can also be identified in most cases simply based on the presence of a homologous gene sequence. In contrast, with housekeeping genes, such as the 16S rRNA gene, a novel sequence may indicate the phylogenetic relatedness of the carrying bacterium but gives few clues about its physiology.

For the methanotrophs, their unique functional gene/enzyme is methane monoxygenase (pMMO and sMMO), with the enzyme methane monooxygenase (pMMO and sMMO), with the rate determining in the H₄MPT-linked C₁-transfer pathway (66) have been used to identify methanotrophs in environmental samples.

**DIVERSITY AND DISTRIBUTION: 16S rRNA GENES**

The first studies using 16S rRNA gene probes to target methanotrophs/methylotrophs were those of Hanson and co-workers, who studied the phylogeny of extant methanotrophs (16, 123). These researchers described probes 9α and 10y (Table 1), which targeted serine pathway and ribulose monophosphate (RuMP) pathway methylotrophs, respectively (123). These primers have been used in a number of PCR-based studies to detect methanotrophs (92) or used in denaturing gradient gel electrophoresis (DGGE) analysis of methanotroph communities (54, 55). However, the drawback of these probes is that they target methylotrophs and are not methanotroph specific. Probes were designed that targeted only methanotrophs (1034-Ser and 1035-RuMP) (16); however, these have received little use in studies of methanotroph diversity (92). The first genus-specific methanotroph primers designed (Mb1007, Mc1005, Mm1007, and Ms1020 [58]; see Table 1) targeted serine pathway and ribulose monophosphate (RuMP) pathway methylotrophs, respectively (123). These primers have been used in a number of PCR-based studies to detect methanotrophs (92) or used in denaturing gradient gel electrophoresis (DGGE) analysis of methanotroph diversity both as radiolabeled probes and as PCR primers (27, 67, 84). Type I and type II methanotroph-specific primers (MethT1dF, MethT1bR, and MethT2R; see Table 1) were designed to examine methanotroph diversity in landfill soil (129) and have been widely used (7, 38, 62). Recently, two studies have provided a large collection of group-specific 16S rRNA probes for the detection of methanotrophs (39, 48). Very recently, Chen et al. (21) designed new primer sets targeting type I and type II methanotrophs, respectively. These primer sets can amplify 16S rRNA genes from almost all known methanotrophs, including the genera Methylocaldum, Methylophthora, Methylocella, and Methylocapsa, which were not amplified using previous primer sets. These primers sets were used successfully to analyze methanotroph 16S rRNA genes and transcripts from a landfill cover soil (21). To date, only a few of these sets of methanotroph-specific 16S rRNA probes have been used in environmental studies (7, 39); however, this is a very useful resource for future studies (Table 1).

Primers targeting groups of methanotrophs not previously covered by 16S rRNA probes were also recently published, which included Methylophthora (67), Methyllobaculum (32), Methylocystis, Methylosinus, and Methylocapsa (33). Therefore, 16S rRNA gene probes and primers are now available that cover the majority of known methanotroph diversity. Methanotroph diversity has also been studied using universal bacterial 16S rRNA primers in clone library studies and in DGGE (42).

The majority of studies of methanotroph diversity, targeting the 16S rRNA gene, have involved the use of DGGE to examine sequence variation between environmental samples. Some studies have focused on new DGGE strategies for examining methanotroph diversity using different combinations of the existing 16S rRNA gene primers (7). In that study, 10 different
combinations of eubacterial and methanotroph-specific (48, 129) 16S rRNA gene primers were analyzed. For type I methanotroph communities, all of the combinations tested gave identical results, and the detection methods used either a nested approach with a first round of PCR with the MethT1dF/Methylomonas primer pair (129) and a second round with either MethT1bR primer pair (129) or else direct amplification with the primer pair 533f/Meth1bRGC, 357fGC/518r, or 533f/907rGC (129) or else nested strategies can be used (7).

Recently, 16S rRNA probes Gm705 and Am445 (48), specific for the Methylococcaceae and Methylocystaceae, were used in quantitative hybridizations to determine the relative abundance of methanotrophs involved in atmospheric methane oxidation in forest soils (77). That study revealed that methane oxidation in forest soils (77) was one order of magnitude more abundant than Methylococcaceae thanotrophic members of the Methylococcaceae (48, 38).

For both type I and II methanotrophs, the direct 16S rRNA gene PCR amplification strategy is recommended in environments where the abundance of methanotrophs is anticipated to be high. In environments being studied where methane oxidation activity is low (and thus the abundance of methanotrophs is low), the nested strategies can be used (7).

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DIVERSITY AND DISTRIBUTION: FUNCTIONAL GENES

Several functional genes have been used for the detection of methanotrophs in environmental samples. The first to be utilized were primers targeting the gene encoding the large subunit of methanol dehydrogenase (mxaF) (85), an enzyme unique to methanotrophs but which is found in all gram-negative methylotrophic bacteria. The primers (f1003 and r1561 [f1003/r1561]) were designed with sequence information from only three methylotrophic bacteria (Methylococcus capsulatus, Methylocystis umbricus, and Paracoccus denitrificans) but are still being used in their original form 10 years later. These primers were first used to investigate diversity in a study examining methane-oxidizing bacteria in peat bogs (87). In that study the database of mxaF sequences from extant methanotrophs and methylotrophs was extended, and a novel group of methanotroph-like sequences were identified in the peat environment. Since that study, these primers have been used to examine many different environments and in DGGE analyses (42, 54, 56). Recently, a new mxaF primer set (1003f/1555r) for methanotrophs and gram-negative methylotrophs has been designed and validated using fourteen mxaF sequences containing methylotrophs and/or methanotrophs and six negative control strains (98). Greater diversity of mxaF sequences has been retrieved from several environments using this primer set rather than the primer set 1003f/1561r (M. J. Cox and J. C. Murrell, unpublished data).

The second group of functional genes to be targeted were those encoding sMMO. Initial studies developed a probe to detect mmoB within the sMMO gene cluster (124). Subsequently, PCR primers were designed for five genes encoding the sMMO enzyme complex, mmoXYAB and -C (85). These were designed by using the only sMMO sequences available at the time from Methyllococcus capsulatus Bath and Methylosinus trichosporium OB3b (19, 111). In this study (85) the mmoXYB and mmoC genes were amplified from several samples including peat, soil, sediment, freshwater and estuarine environments. Sequencing of several more sMMO gene clusters (46, 89, 108) facilitated the development of different PCR primer sets for the amplification of mmoX from methanotrophs (2, 4, 5, 44, 62, 63, 86, 91, 108) (Table 2). However, all of these studies have shown the limited diversity of sMMO genes in environmental samples, possibly because the gene targeted, mmoX, is a highly conserved gene or because primers available are designed from a limited database of sMMO sequences and are therefore inherently biased toward the sequences seen in extant methanotrophs. However, the greatest drawback of using primers targeting sMMO genes to examine methanotroph diversity is that only a subset of methanotrophs contains these genes. On the other hand, the analysis of sMMO-containing methanotrophs in a given environment may be a necessity since a recent study demonstrated that the facultative methanotroph, Methylocella silvestris BL2, contains only sMMO and not pMMO (121).

The genes that have been studied most are those encoding particulate methane monooxygenase (pMMO), which is present in all methanotrophs with the exception of Methylocella silvestris (121). The first oligonucleotide primers designed to amplify internal fragments of the genes encoding pMMO and AMO (ammonia monoxygenase) enzyme complexes were the A189f/A682r primer set (57) (Table 3). The phylogeny of pmoA/amoA is reasonably congruent with the 16S rRNA gene phylogeny of the organisms from which the gene sequences were retrieved (57, 74). Therefore, retrieval of pmoA and amoa sequences provides information on the diversity of these organisms in the environment. The A189f/A682r primers have been used extensively in environmental studies to provide a molecular profile of the methane-oxidizing community (15, 56, 59, 60, 62, 67, 103) and have proved useful in detecting novel sequences (59, 71, 99). However, a new reverse pmoA-specific primer mb661r, used in conjunction with the A189f primer, was designed (27, 109) (Table 3) and demonstrated specificity in amplifying pmoA sequences but not amoa sequences. This primer has been used to determine in situ populations of methanotrophs in freshwater environments (27) and deep-sea hydrothermal vents (95).

The use of PCR primers to amplify amoA from ammonia oxidizers has been critically evaluated (100), with the amoA primers set of Rotthauwe et al. (106) recommended as the most suitable. A similar study was also carried out to evaluate the use of the available pmoA primer sets (15). In that study, a third reverse primer for PCR was designed, A650r, and three primer sets (A189f/A682r, A189f/mb661r, and A189f/A650r) were compared in studies of methanotroph diversity in Danish soils. The results indicated that the mb661r primer gave the best coverage of methanotroph diversity; however, the primers A682r and A650r detected novel groups of pmoA sequences, including the RA14 (Forest clone) group, which mb661r failed.

### Table 2. PCR primers used for amplification of mmoX genes from environmental samples

| Primer(s) | Sequence (5′-3′) | Product size (bp) | Reference |
|-----------|------------------|------------------|-----------|
| mmoX882/mmoXr1403 | GCCCTCAAAGTCTAGGCTAGGCGTTCGCCTCGGCT | 535 | 85 |
| mmoX1/mmoX2 | CCGTCCGCGTGGAGGCGATGACGCTCGCCCGTCG | 369 | 91 |
| 536f/1393r | CGCTGTGGAAGGGCATGAAAGGGGTTGTT | 341 | 44 |
| 534f/877r | CGCTGTGGAAGGGCATGAAACGCGTGGATGCTCGACCTTGAACTTTGAGCC | 1,230 | 4 |
| A166f/B1401r | ACCAAGGAGCGTGATGCAACTCGTGAGCGGCTCGGCTC | 863 | 62 |
| mae1/met4 | ACCAAGGAGCGTGTCCTGAGCGGCTCGGCTC | 5 | 5 |
| mmoX206f/mmoX886r | ATCGBAAACGGTTATCGGSGCAGCC/SACCCANGGCTGAGCTTGGAA | 719 | 63 |

* Primer mmoX1 was located at positions 2008 to 2037, and primer mmoX2 was located at positions 2347 to 2376. Primers A166f and B1401r are also known as mmoXa and mmoXd.

* **mmox901** GC is also used for DGGE analysis with primer mmoX1 (64).

* When used in PCR with the primer mmoX1.
to detect. Furthermore, primer A682r excludes Methyllocapsa, as well as genes from other uncultivated bacteria, which are indicated to be methanoxizers (99). Therefore, several recent studies have used both the A189f/A682r and the A189f/mb661r primer sets in order to obtain the best coverage of methanotroph diversity in Califormian upland grassland soil. The primer pair A189f/A682r was used in the first PCR, with A189f/mb661r or A189f/mb661r in the second round. This gave consistently high yields of pmoA amplicons (61). Another pmoA primer set, pmo1/pmor (23), was developed (Table 3), but these have not been widely used (5). Other primers have also been designed (42, 112, 120) but, to our knowledge, not used in other studies. The pmoA PCR primer set A189f/A682r has been adapted for DGGE analysis, with the GC clamp being added to A189f (15, 37, 54, 55, 71). The use of degenerate pmoA primers in DGGE may cause the appearance of multiple bands for individual organisms, which in complex environments may cause confusion in the interpretation of results. The A682r primer has four redundancies within its sequence that causes multiple banding problems in DGGE analysis, as can be seen from multiple bands with control organisms (54). Other primer sets, A189f/A650r (15, 37, 54, 55, 71), A189f/mc468 (15, 37, 54, 55, 71), A189f/f326 (15, 37, 54, 55, 71), and A189f/mc468 (15, 37, 54, 55, 71), have also been used for DGGE analysis of pmoA, but again both primer sets gave multiple bands with controls. Recently, a new nondegenerate primer (mb661r) has been designed, based on the methanotroph specific primer mb661r, and this has been successfully used for the detection of methanotroph pmoA genes in an alkaline soda lake (Mono Lake), without the production of multiple bands with controls (78), although these primers require further testing on other environmental samples in order to prove their value in determining methanotroph diversity.

Alternative approaches for analyzing pmoA diversity include terminal restriction fragment length polymorphism (T-RFLP), a rapid, sensitive method potentially avoiding the bias during cloning. T-RFLP is preferable in some cases since it also produces semiquantitative data. The first application of pmoA T-RFLP was reported by Horz et al. (62), and it has been widely used in a number of subsequent studies (18, 60, 61).

Besides the analyses using phospholipid fatty acids (PLFAs), which is discussed below, a number of studies of atmospheric methane oxidation have utilized the pmoA gene PCR primers to determine which groups of methanotrophs are involved. One of the first studies identified a novel pmoA group, RA14, closely related to type II methanotrophs (Alphaproteobacteria), which were suggested to represent the methanotrophic bacteria responsible for atmospheric methane oxidation (59). A second study also identified pmoA sequences from forest soils that were closely related to RA14 (55), which is usually called the “forest sequence cluster.” The novel cluster is commonly detected in acidic upland soils and forest soils and has now been called the USCα (for upland soil cluster α) (71). That study also detected a novel group of pmoA sequences distantly related to those of known type I methanotrophs (Gammaproteobacteria). This novel upland soil cluster γ (USCγ) was more likely to be detected in soils with pH greater than 6.0 than in more acidic soils. A study of a Californian upland grassland soil also identified pmoA sequences that clustered either with the USCα or with the USCγ (61), and a study of tropical upland soils from Thailand only identified USCα-related pmoA sequences in the two most acidic forested soils (pH 4.2), while these sequences were not detected in a third agricultural soil (pH 5.6), which also had lower atmospheric methane oxidation rates (72). Another study has revealed a novel group of Alphaproteobacterial methanotrophs from a methane-consuming neutral pH soil (105); these were referred to as cluster I and were previously detected in isolates from tundra soil (99). Subsequently, a study of two forest soils consuming atmospheric methane, one acidic and the other neutral pH, revealed the dominance of USCα pmoA sequences in the acidic soil and dominance of cluster I pmoA sequences in the neutral soil (73). A number of recent studies have focused on the analysis of

| Primer(s) | Sequence (5’→3’) | Product size (bp) | Reference |
|-----------|------------------|------------------|-----------|
| A189f/A682r | GGNGACTGGGACTTCTGG/GAASGCNGAGAAGAASGC | 525 | 57 |
| mb661r | CCGGMCACAAGCTTACC | 510* | 27 |
| pmo1/pmor | GGGGAACTTCTGGGTTGGAAGAGC/GGGGRCATCGCTTACCAGAA | 330 | 23 |
| pmo2/pmor | TCTTAYCCDDRCAACTGCGC | 178 | 23 |
| pmoA/mb661r | GGGNACTGGGACTTCTGGATCGACTTCAAGGATCG/GAASGCNGAGAAGAAGA | 530 | 120 |
| A650r | AGCTCCCTACCGAAGGT | 478* | 15 |
| mb661r/mb661r | CCGGMCACAAGCTTACC | 510* | 78 |
| pmoA/rev | TCTTGGGNTGGACNTAYTTCC/CGNGARTAYATHMGNATGGTNGA | 281 | 42 |
| Mb601 R* | ACRTAGTGTGAACCTTACC | 358 | 74 |
| Mc468 R* | GCSTGGAACAGGTAGCTGCC | 432* | 74 |
| II 223 F/II464 R* | CGTCTGATGTGCGCGAC/CGTGCCCGCTGACCATGYYG | 299* | 74 |
| Mcap630* | CTAGCAGTGCGGAGATATT | 444 | 74 |
| Forest675 R* | GCGGACGGAACGCTTACC | 416* | 74 |
| Mb661 R* | GCCTGCGGAGATATT | 491* | 74 |

* Primer A189f is also known as A189gc.
* Primer(s) designed for real-time PCR quantification of subsets of methanotrophs.
* Primers designed for real-time PCR quantification of subsets of methanotrophic bacteria.

Table 3. PCR primers used for amplification of pmoA genes from environmental samples
expression of methane monoxygenases in the environment using these \textit{pmaA} and \textit{mmoX} primer sets. This included the analyses of soil (51, 73), freshwater sediment (23, 96), landfill (21), and peatlands (22), providing direct in situ evidence of active methanotroph populations. It is interesting that only pMMO transcripts could be readily detected from all of these studies which may indicate that pMMO is largely responsible for methane oxidation in these environments.

\textbf{DIVERSITY AND ABUNDANCE: QUANTIFICATION OF METHANOTROPHS}

The traditional method for enumerating methanotrophs in environmental samples has been most-probable-number (MPN) cultivation. Although useful for isolation of methanotrophs this approach may be limited by cultivation bias but is still widely used. In fact, a study was carried out that looked at the effect of different media on the MPN counts of methanotrophs in lake sediment (18). These researchers found cell numbers four- to sixfold higher in a new medium (7 \times 10^6 per ml) than with conventional Whittenbury medium (128); another study in lake sediments found 10^6 to 10^6 methanotrophs per g (38). Several studies have measured methanotroph numbers in the rice root rhizosphere and found 10^4 to 10^5 g per g of soil (14). Methanotroph numbers have also been determined by MPN in trichloroethylene-contaminated aquifers (10^3 to 10^5 per g) (119), swamp sediment (10^6 to 10^6 per ml) (92), and wet meadow soil consuming atmospheric CH_4 (10^7 to 10^7 per g) (60). Methanotrophs have also been enumerated by slot blot hybridizations with methanotroph-specific probes (26, 115); however, this approach has not been widely used. Costello et al. (26) probed slot blots with type I (type 1b)- and type II (type 2b)-specific probes (Table 1) to estimate the abundance of methanotrophs in a freshwater lake sediment. The total number of type I methanotrophs was estimated to be 3.4 \times 10^5 to 6.7 \times 10^5, with the number of type II methanotrophs estimated to be 2.3 \times 10^6 to 6.8 \times 10^6, and these researchers also used a \textit{pmaA} probe and found 2.1 \times 10^6 to 9.0 \times 10^6 cells per g. These numbers are within the range estimated from methane oxidation rates of 1.3 \times 10^9 to 1.2 \times 10^9 cells per g of sediment and demonstrate the ability of this technique to estimate methanotroph abundance in environmental samples.

More recently, fluorescence in situ hybridization (FISH) targeting the 16S rRNA gene has been used to identify (39) and enumerate (32, 33) methanotrophs using many of the probes described earlier (see Table 1). Two studies of acidic peat using FISH to enumerate methanotrophs showed that the numbers of methanotrophs were around 10^6 cells per g of peat and that \textit{Methylocella} and \textit{Methylocystis} spp. were the predominant methanotrophs (60 to 95\%) in an acidic \textit{Sphagnum} peat bog (32, 33). One of the disadvantages of using FISH to enumerate methanotrophs is that it can only be used when the 16S rRNA genes of the target organisms are known. Due to the many diversity studies of methanotrophs using \textit{pmaA} phylogeny, many novel groups of methanotrophs can only be identified by \textit{pmaA} sequence; hence, FISH cannot be used to enumerate these organisms. However, a recent study has linked the use of functional genes in methanogens with FISH (76), a technique that may prove useful in the future for the detection of methanotrophs. Therefore, other techniques have been developed that target the \textit{pmaA} gene, including competitive reverse transcriptase PCR (51) and real-time PCR (50, 73, 74) assays. The competitive reverse transcription-PCR approach was developed for both \textit{pmaA} and \textit{mmoX} using internal RNA standards and capillary electrophoresis and was successfully used to quantify the amount of mRNA transcript in both whole cells and a model soil slurry system (51). To our knowledge, this has yet to be applied further to examine environmental samples. The quantitative real-time PCR assay for methanotrophs was developed from a method using SYBR green, previously used for detecting other bacteria, and \textit{pmaA} specific primers designed to target five different groups of methanotrophs in real-time PCR (74). This assay was successfully used to quantify the methanotroph community in a number of environments, including a flooded rice field soil (74), forest soils (73), and periodically water-saturated gleicy soils (70). More than 10^7 \textit{pmaA} molecules per g of soil were detected in the water-saturated gleicy soils (70), a result comparable to population sizes of methanotrophs found in other upland soils consuming atmospheric CH_4 by MPN analysis (60). Real-time PCR has also been used to monitor the population changes of type I methanotrophs during composting of organic matter using probe MB107 targeting the 16S rRNA gene, showing a maximum of 10^9 cDNA copies/g of compost, with the lowest numbers (10^7 cDNA copies/g) observed during the thermophilic phase (50).

\textbf{DIVERSITY AND FUNCTION-ACTIVITY: STABLE ISOTOPE PROBING}

Stable isotope probing (SIP) is a method that attempts to link the identity of an organism with its biological function under conditions approaching those in situ (101, 102) and has been recently reviewed (35, 43, 88). The principle of SIP is based on the natural abundance of \textit{13C} being approximately only 1\%. Consequently, the addition of \textit{13C}-labeled (>99\%) substrate to an environmental sample will result in \textit{13C} labeling of the actively dividing bacteria as the \textit{13C}-labeled substrate is being used as a carbon source and is only incorporated into DNA during DNA synthesis and replication. The \textit{13C}-labeled DNA can be separated from the \textit{13C}-labeled DNA of the bacteria that do not assimilate the labeled substrate by CsCl density gradient centrifugation (35, 43).

SIP can also be carried out with RNA (RNA-SIP). One key advantage of RNA-SIP is the natural amplification of the phylogenetic signature molecule rRNA in active cells. RNA also becomes \textit{13C} labeled much more rapidly than DNA in densely populated bioreactor samples, suggesting that RNA-SIP may have greater sensitivity than DNA-SIP (80, 81, 82, 127). A study of a soil ecosystem (81) successfully used \textit{13C}-labeled methanol (CH_3OH) to examine the diversity of methylotrophs, combining the use of both RNA-SIP and DNA-SIP. After just 6 days incubation, methylotroph-specific \textit{13C}-labeled rRNA was detected and contained high numbers of 16S rRNA gene clones from the genus \textit{Methylococcus} and also a novel group of \textit{Betaproteobacteria} which were close relatives of the genus \textit{Methylophilus}. After 42 days of incubation, the \textit{13C}-labeled DNA was dominated by 16S rRNA sequences closely related to \textit{Methylophilus}, suggesting that constant high availability of
methanol in the experiment had stimulated and enriched for this group of methy lok trophic organisms.

A number of studies have utilized SIP to study the functionally active methanotroph populations in environmental samples (20, 63, 79, 93, 103). The 16S rRNA gene libraries that were constructed in these studies from the [12C]DNA revealed that the methanotrophs made up a small percentage of the library (103) or were not detected (93), a fact which was common with other studies of 16S rRNA gene libraries looking at methanotroph diversity (84). However, 16S rRNA gene libraries constructed from [13C]DNA extracted from environmental samples incubated with 13CH4 contained a high percentage of methanotroph sequences, 32 to 96% of clones sequenced (63, 79, 93, 103). SIP studies with 13CH4 have also revealed a number of sequences whose role in methane oxidation is unclear. Sequences were detected that were closely related to Bdellovibrio sp. (63, 93) and Cytophaga sp. (93, 103), which may have resulted from the turnover of 13C due to predation. SIP can also reveal potentially novel groups of putative methane oxidizing bacteria that might reside within the Betaproteobacteria (63, 79, 93, 103).

SIP studies with 13CH4 have also looked at the diversity of methanotroph functional genes. In several studies, pmoA diversity was examined before and after SIP (79, 93, 103). In all of these environments, the pmoA diversity was lower in the [13C]DNA than in the [12C]DNA fraction. In peat soil using the pmoA A189f/A682r primer set, the majority of sequences in the [13C]DNA fraction were amoA sequences from ammonia oxidizing bacteria, while in the [13C]DNA fraction, the majority of sequences were pmoA sequences similar to those from extant type II methanotrophs, indicating that in this peat soil environment, the type II methanotrophs are the bacteria that are actively assimilating methane (93). In Russian soda lake sediments, both type I and type II methanotroph pmoA sequences, but no amoA sequences, were detected prior to SIP (79). However, after SIP all of the pmoA sequences isolated were closely related to pmoA sequences from extant type I methanotrophs, indicating that type I methanotrophs were actively assimilating methane in this high pH environment (around pH 10).

Recently, SIP has been used in a metagenomic analysis of a forest soil (36). In that study a complete methane monoxygenase operon (pmoCAB) was cloned into a bacterial artificial chromosome vector. Analysis of the pmoA sequence indicated that the clone was most similar to a Methylocystis sp. previously detected in this forest soil. This study demonstrated that reasonably large DNA fragments from uncultivated bacteria can be isolated using DNA-SIP and cloned for metagenomic analysis. This method was used by Neufeld et al. (97) to retrieve a 10-kb fragment containing mxaF from the metagenome. These authors demonstrated that long incubation and high substrate consumption used in early DNA-SIP experiments can be overcome by the use of short incubation times with low concentrations of substrates and subsequent multiple displacement amplification of the small amounts of 13C-labeled DNA obtained in such DNA-SIP experiments. Similar approaches were successfully applied to analyze uncultivated methanotrophs from acidic peatlands and demonstrated that gene clusters from uncultivated Methylocystis spp. could be retrieved by combining multiple displacement amplification, DNA SIP and metagenomics (Y. Chen and J. C. Murrell, unpublished data).

**MICROARRAYS**

DNA microarray (microchip, biochip, and gene chip) technology allows the parallel analysis of highly complex gene mixtures in a single assay and thus symbolizes the (post)genomic era of high-throughput science. Although microarrays initially emerged as tools for genome-wide expression analysis and are nowadays routinely used for this purpose, they are also increasingly being developed for diagnostic applications. Microbial diagnostic microarrays (MDMs) consist of nucleic acid probe sets, with each probe being specific for a given strain, subspecies, species, genus, or higher taxon (8).

The first MDM to target methanotrophs was a prototype functional gene array that targeted genes involved in nitrogen cycling, including nitrite reductase (nirS and nirK), ammonia monooxygenase (amoA), and particulate methane monooxygenase (pmoA) genes (130). The array contained 11 pmoA and 11 amoA gene fragments. That study indicated the potential of microarrays for revealing functional gene composition in natural microbial communities. A new version of this array was published recently (53) and included 174 probes for AMO, 241 probes from pMMO, and 57 probes for sMMO. An MDM was specifically developed for the detection and community analysis of methanotrophs (10). The microarray consisted of 59 oligonucleotide probes designed and fully validated against the pmoA genes of all known methanotrophs and amoA of the ammonia-oxidizing, nitrifying bacteria. The probes applied on this array were short oligonucleotides (i.e., 18 to 27 nucleotides), reliably discriminating a perfect match target and one with two mismatches, but in several cases also enabling single nucleotide discrimination. The potential of the pmoA microarray was tested with environmental samples, and the results were in close agreement with those of clone library sequence analysis (10). The microarray was then applied to analyze the methanotroph communities in landfill cover soils (20, 116). The results demonstrated that type II methanotrophs of the Methylocystis sp. were found to be the dominant methanotrophic member of this community, along with the type I methanotroph Methylocaldum sp., which had previously not been found in landfill soils (129). The results clearly reflected spatial trends in depth profiles of the landfill site samples. The pmoA MDM has also been used to monitor the success of methanotroph enrichments from hot water samples and as a first screening tool to assess the methanotroph diversity in various forest soils. Finally, an unanticipated result indicated the potential of the pmoA MDM to detect environmental perturbations by revealing an unexpected methanotroph community structure that was eventually shown to be linked to a disruption in the methane supply (116). Recently, an mRNA-based application of MDMs was successfully tested using a pmoA microarray for methanotrophs (9, 22) and may provide additional information on composition and functioning of microbial communities provided by DNA-based microarrays. The pmoA MDM has been upgraded (116), the latest version being comprised of 138 probes (L. Bodrossy, unpublished data).

An MDM targeting nifH genes was recently used to investigate the distribution and diversity of nitrogen-fixing microor-
organisms, and a number of the organisms identified were methanotrophs (65). MDMs allow for the rapid analysis of bacterial communities at a high resolution, with one researcher being able to analyze 40-plus samples per week, from DNA preparation through to analysis of results, a process which would take months with the clone library approach. Although the throughput of the MDM approach is comparable to that of DGGE or thermal gradient gel electrophoresis and T-RFLP, its phylogenetic resolution is much higher. The pmoA MDM currently resolves all known genera of methanotrophs, most of them down to the level of species or at least groups of species. The probe set on microarrays is limited to microbes with already sequenced genes, which means that data collected from environmental clone libraries have an important role in the continual development of the microarray. These studies have demonstrated that, by combining high resolution, high throughput and affordable overall costs, microarrays are a powerful tool for mapping the spatial and temporal variability and dynamics of microbial community structure in the environment.

OTHER MOLECULAR MARKERS: LIPIDS

Methanotrophs contain unique PLFAs (47). The unique PLFAs are 16:1ω5t, 16:1ω6c, and 16:1ω8c in type I methanotrophs and 18:1ω8c in type II methanotrophs. The measurement of these signature PLFAs has been widely used to estimate the biomass distribution of type I and II methanotrophs in environments well supplied with methane (12, 117). In environments where there is little methane, i.e., upland forest soils, it was suggested that these PLFAs should not be used as biomarkers (118). The sensitivity of PLFA-SIP makes it especially powerful for identifying active methanotrophs and their community structure in the environment (reviewed in reference 40). The use of 13C to isotopically label the PLFAs of methanotrophs in a soil from a temperate forest increased the sensitivity of detection of the PLFAs and provided evidence of methane assimilation at true atmospheric concentrations (17). The incorporation of 13C into PLFAs has been used in other studies of atmospheric methane oxidation (71, 83), with both studies suggesting the presence of novel type II methanotrophs. 13C-labeled PLFA analyses were also used to study methanotrophs in high-methane environments, including landfill cover soils (28), acidic peatland soils (22), and freshwater sediment (13). However, great care should be taken in interpreting PLFA data since the PLFA database for methanotrophs is much less extensive than the 16S rRNA and functional gene databases. For example, a recent study showed that Methylocystis heyeri strains (type II methanotrophs) contained large amounts of 16:1ω8c, a PLFA that was previously thought to be associated with type I methanotrophs only (31). In another study, Chen et al. suggested that acid tolerant, acidophilic type II methanotrophs may contain PLFAs that are different from their neutral pH counterparts (22).

Intact phospholipid profiles have also been used to identify methanotrophs, with two major classes of phospholipid being found (41). That study suggests that this technique can be very useful in bacterial chemotaxonomy, but this has yet to be applied to environmental samples. Finally, specific hopanoids produced by methane oxidizing bacteria were identified by labeling with 13C when forest soils were incubated with 13CH4 (29), as well as from pure cultures of Methylocaldum (30), providing potential new markers for identifying methanotrophs in environmental samples.

FUTURE OUTLOOK (GENOME AND PROTEOME)

Recently, the first complete genome sequence of a methanotroph, Methylococcus capsulatus Bath, was published (126), and the first draft of the genome sequence of the facultative methanotroph, Methylocella silvestris BL2, has just become available. The genomes of two other methanotrophs, Methylococcus capsulatus Bath and with other obligate and facultative methylothrophs may well reveal the molecular basis for the obligate nature of most extant methanotrophs. Proteomic analyses of regulation of methanotrophy by copper ions (68) and of the outer membrane subproteome (6) in Methylococcus capsulatus Bath have also recently been published. These genomic and proteomic analyses provide a wealth of information for studying the biology of methane oxidation (24, 69) and may provide insights into how methanotrophy is regulated under different environmental conditions. Future studies will undoubtedly use the information from the molecular ecology, genome, and proteome studies to aid in the isolation of new and novel methanotrophs and to reveal new and interesting physiology and biochemistry for this fascinating group of microorganisms.

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ADDENDUM

Two recent studies (37a, 99a) report the cultivation and characterization of two new extremely acidophilic methanotrophs from the Verrucomicrobia phylum. Unlike all other methanotrophs characterized previously, these acidophilic methanotrophs do not belong to Proteobacteria and contain three divergent pMMO clusters, indicating an ancient divergence of Verrucomicrobia and Proteobacteria methanotrophs rather than a recent horizontal gene transfer of pMMO. These recent findings, together with the discovery of the facultative methanotroph Methylocella silvestris, reopen the question “What are the taxonomic diversity of methanotrophs and the distribution of methanotrophy in the microbial world?,” and this question warrants further investigation.

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