The Methanol Dehydrogenase Structural Gene mxaF and Its Use as a Functional Gene Probe for Methanotrophs and Methylotrophs

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The methanol dehydrogenase gene mxaF, encoding the large subunit of the enzyme, was amplified from the DNA of a number of representative methanotrophs, methylotrophs, and environmental samples by PCR using primers designed from regions of conserved amino acid sequence identified by comparison of three known sequences of the large subunit of methanol dehydrogenase. The resulting 550-bp PCR products were cloned and sequenced. Analysis of the predicted amino acid sequences corresponding to these mxaF genes revealed strong sequence conservation. Of the 172 amino acid residues, 47% were conserved among all 22 sequences obtained in this study. Phylogenetic analysis of these MxaF sequences showed that those from type I and type II methanotrophs form two distinct clusters and are separate from MxaF sequences of other gram-negative methylotrophs. MxaF sequences retrieved by PCR from DNA isolated from a blanket bog peat core sample formed a distinct phylogenetic cluster within the MxaF sequences of type II methanotrophs and may originate from a novel group of acidophilic methanotrophs which have yet to be cultured from this environment.

In all gram-negative methylotrophic bacteria that have been studied, methanol oxidation is catalyzed by the pyrroloquinoline quinone-linked enzyme methanol dehydrogenase (MDH). This enzyme catalyzes the oxidation of methanol to formaldehyde and is distinct from the alcohol dehydrogenase of gram-positive methylotrophic bacteria (13) and methylotrophic yeasts (50). MDH carries out a key step in bacterial one-carbon (C1) metabolism since it catalyzes the production of formaldehyde, the intermediate of both assimilative and dissimilative methylene in aerobic soils (11, 12, 20, 27, 40). Methane has become one of the most important greenhouse gases, partly because until recently the concentration of methane in the atmosphere had been increasing at a rate of about 1% per year (10). Wetlands, for example, contribute an estimated 15 to 20% of the total methane emitted to the atmosphere each year (33). Functional gene probes targeted at the soluble MMO have been used to identify methanotrophs in blanket bog peat and several other environmental samples (34, 35, 38). However, the soluble MMO is not universal to all methanotrophs and is found predominantly in the genera Methylosinus and Methylococcus (44) (for a review, see reference 37). Therefore, we examined the use of a second functional gene probe, mxaF, which is found in all methanotrophs and also in the gram-negative methylotrophs. A third functional gene, pmoA, encoding one of the subunits of the particulate MMO, has also been examined recently (24). This gene has been found in all methanotrophs studied so far. The advantage of using the mxaF gene is that it is present in all methylotrophs and, therefore, the role in the environment of a larger group of organisms that can assimilate C1 compounds may be addressed.

In this study, we investigated whether regions of DNA specific to methanotrophs could be found which would allow the application of mxaF probes to discriminate methanotroph DNA sequences from those of other methylotrophs. MxaF contains the active site of the enzyme (6) and is likely to be highly conserved (46), and it is therefore easier to design...
Specified primers of low degeneracy. PCR primers have been designed that correspond to highly conserved regions of aligned MxaF sequences from *Methyllobacterium extorquens* AM1, *Methyllobacterium organophilum* XX, and *P. denitrificans* (34). These primers have previously been used to amplify MxaF genes from a variety of different environments, including freshwater, marine, soil, sediments, and blanket bog peat. However, the DNA sequences of the resultant PCR products have not been determined or analyzed (23, 25, 34). This paper reports the PCR amplification and sequencing of partial MxaF gene sequences from a large number of representative methanotrophs and methylotrophs from culture collections and environmental samples and the subsequent analysis of their gene sequences to affiliate the environmental MxaF clones with MxaF sequences from extant organisms.

**MATERIALS AND METHODS**

**Bacterial strains.** The following methanotrophic strains from the University of Warwick culture collection were used in this study: the type I methanotrophs (*γ*-Proteobacteria *Methyllobacterium capsulatus* (Bath) (NCIMB 11132), *Methyllobacterium extorquens* AM1 (NCIMB 11120), *Methyllobacterium organophilum* XX, and *P. denitrificans* (34). These primers have previously been used to amplify MxaF genes from a variety of different environments, including freshwater, marine, soil, sediments, and blanket bog peat. However, the DNA sequences of the resultant PCR products have not been determined or analyzed (23, 25, 34). This paper reports the PCR amplification and sequencing of partial MxaF gene sequences from a large number of representative methanotrophs and methylotrophs from culture collections and environmental samples and the subsequent analysis of their gene sequences to affiliate the environmental MxaF clones with MxaF sequences from extant organisms.

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FIG. 1. Alignment of predicted amino acid sequences corresponding to mxaF genes of methanotrophs and methylotrophs and to gene mxaF sequences retrieved from environmental samples by PCR. Mc.cap, Methylococcus capsulatus (Bath); LK6, methanotroph isolated from soil; Mn.met, Methylosomas methanicum S1; Mn.alb, Methylosomus album S1; Ms.spo, Methylosinus sporium 5; Mcy.par, Methylocystis parvus OBBP; Ms.tri, Methylosinus trichosporium OB3b; Mcy.stM, Methylocystis sp. strain M; Peat 1, Peat 3, Peat 4, Peat 5, and Peat 6, clones 1, 3, 4, 5, and 6, respectively; Enrich 1, peat methane enrichment clone 1; Xan.H4-14, Xanthobacter sp. strain H4-14; Msulf, Methylosulfonomonas methylovora M2; W3A1, Methylophilus methylotrophus W3A1; Par.den, Paracoccus denitrificans PD1207; Hyp.CM2, Hyphomicrobiurn sp. strain CM2; Mlb.rho., Methylobacterium rhodinum; Mlb.ext, Methylobacterium extorquens AM1; Mlb.org, Methylobacterium organophilum XX. Residues boxed in black are universally conserved in the sequences included in this study. Residues marked with a black dot are conserved residues of the active site (4–6), and those overlined are the two tryptophan docking motifs, W4 and W5 (17). Amino acids are numbered according to the published sequence for Methylobacterium organophilum XX MxaF (31).
<table>
<thead>
<tr>
<th>Organism</th>
<th>Organism</th>
<th>% Similarity to (or difference from) the sequence of organism:</th>
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<td></td>
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<tr>
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<td>90.7 85.9 90.1 88.9 11.6 12.9 12.9 11.0 11.0 12.2 11.6 11.0 10.5 18.0 14.5 25.7 20.3 12.8 14.5 14.0 14.5</td>
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<tr>
<td>Methylocystis parvus OBBP</td>
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<td>90.7 88.2 91.8 90.6 95.9 12.9 10.0 11.1 11.6 12.2 11.6 11.1 4.7 15.7 18.0 29.8 22.1 19.2 14.5 14.0 14.0</td>
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<tr>
<td>Methylosinus trichosporium OB3b</td>
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<td>89.5 90.0 95.3 94.2 93.6 94.1 8.2 9.9 11.1 11.1 11.7 9.9 11.7 21.1 17.5 26.3 22.2 17.5 17.0 16.4 16.4</td>
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<tr>
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<td>88.3 89.4 91.8 90.6 93.6 95.3 94.7 8.8 10.5 9.9 10.5 8.8 8.8 20.5 19.9 26.9 24.0 21.1 16.4 15.8 15.8</td>
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<td>Peat 1</td>
<td></td>
<td>90.1 88.2 91.2 90.1 94.8 97.1 94.7 95.9 2.9 1.2 1.8 1.2 9.9 19.8 18.0 27.5 22.7 16.9 15.7 15.1 16.3</td>
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<tr>
<td>Peat 3</td>
<td></td>
<td>88.4 87.6 90.6 89.5 94.8 96.5 94.7 95.3 98.2 4.1 4.7 2.9 10.5 20.3 19.2 27.5 23.8 18.0 16.9 16.3 17.4</td>
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<tr>
<td>Xanthobacter sp. strain H4-14</td>
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<td>85.3 88.9 87.7 90.7 90.7 88.9 88.3 91.3 90.7 91.3 90.1 91.9 91.3 14.5 31.0 19.2 20.3 15.7 15.1 16.3</td>
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<tr>
<td>Methylosulfonomonas methylovora M2</td>
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<td>88.9 86.5 89.5 88.3 93.0 90.7 89.5 88.3 90.7 90.7 90.7 89.5 91.3 91.9 93.0 25.7 15.1 15.1 15.7 15.1 16.3</td>
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<td>Methylophilus methylotrophus W3A1</td>
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<td>81.9 85.3 86.0 84.8 84.8 85.4 86.0 86.0 84.8 84.2 84.8 86.3 85.4 85.4 82.5 86.0 30.4 26.9 30.4 29.8 31.0</td>
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<td>P. denitrificans PD1207</td>
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<td>90.1 88.2 87.1 88.3 90.1 90.1 87.7 87.1 89.5 88.4 89.5 88.4 89.5 91.3 90.7 93.6 82.5 20.9 20.9 20.3 21.5</td>
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<td>Hyphomicrobium sp. strain CM2</td>
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<td>86.6 85.3 87.1 86.6 93.0 90.1 89.5 87.7 89.5 89.5 89.5 88.4 90.1 91.3 89.0 91.3 84.2 89.5 14.5 14.0 14.5</td>
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<tr>
<td>Methylobacterium rhodinum</td>
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<td>88.4 86.5 88.3 87.1 94.2 93.0 91.8 90.6 93.6 94.2 93.6 92.4 94.2 93.6 90.7 93.0 83.0 90.7 93.0 0.6 2.9</td>
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<tr>
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<td>87.8 85.9 88.6 96.7 94.2 92.4 93.6 94.2 93.6 93.0 92.4 94.2 93.6 90.7 93.0 93.0 83.0 90.7 93.0 98.3 98.3</td>
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Table 1. Matrix showing similarities and differences in identity derived from comparison of MxaF sequences of methanotrophs and methylotrophs generated by PCR amplification.

a Values in the upper triangle are percent identity differences, and those in the lower triangle indicate percent similarity.
363 to 373), corresponding to the W4 (residues 282 to 292) and W5 (residues 337 to 347) docking motifs in *Methylobacterium extorquens* (17), are also present (Fig. 1). Analysis of the MxaF sequences also identifies a gap in the amino acid alignment at residue 342. This gap is found in five of the eight methanotroph MxaF sequences and in the previously published sequence from *Methylophilus methylotrophus* W3A1 but could not be used to identify a particular group of organisms because it is not found in all the MxaF sequences from any group of organisms. However, this region of the alignment was not included in any phylogenetic analysis due to the uncertain position of the alignment gap.

Phylogenetic analysis of the amino acid sequences of the mxaF clones, derived by using the PROTDIST, PROTPARS, FITCH, and BOOTSTRAP programs of the PHYLIP package (16), has shown that the sequences form several clusters (Fig. 2). The sequences for the type I methanotrophs (*γ*-Proteobacteria) *Methylomicrobium album* BG8, *Methylomonas methanica* S1, *Methylococcus capsulatus* (Bath), and strain LK6 form a cluster that is distinct and separate from the type II methanotrophs (*α*-Proteobacteria) *Methylosinus sporium* 5, *Methylocystis parvus* OBBP, and *Methylocystis sp.* strain M sequences. There are two other clusters within the *α*-Proteobacteria, one consisting of the three *Methyllobacterium* sequences and the *Hyphomicrobium* sp. strain CM2 sequence and the other containing the sequences from the remaining *α*-Proteobacteria methanotrophs, *Paracoccus denitrificans* PD1207, *Methylosulphonomonas methylotrophus* M2, and *Xanthobacter* sp. strain H4-14. The MxaF sequence from *Methylophilus methylotrophus* W3A1, the only member of the *β*-Proteobacteria in this study, was distinct from these four clusters.

Analyses of nucleotide sequences performed with the DNA-DIST, DNAML, and DNAPARS programs of the PHYLIP package produced dendrograms (data not shown) that were very similar to those derived from the amino acid sequences (Fig. 2) and gave the same phylogenetic grouping of the various subdivisions of the class Proteobacteria.

The single MxaF sequence from a methane enrichment culture of a peat bog sample branches with the sequence from *Methylocystis parvus* OBBP, with a relatively high bootstrap value supporting this grouping. The methane enrichment culture (pH 5.8) was obtained with samples from the 10- to 12-cm section of the peat core and, as observed by microscopic examination, contained several different cell morphologies. They were all rod-shaped cells but had various lengths (0.7 to 4.0 μm) and diameters (0.7 to 2.0 μm). The five MxaF sequences, isolated directly by PCR from DNA extracted from three sections of a peat core (1 to 2, 10 to 12, and 28 to 30 cm), form a distinct group of their own within the MxaF sequences of the type II methanotrophs (within the *α*-Proteobacteria), suggesting that they may be MxaF sequences from novel methanotrophs.

**DISCUSSION**

The high level of conservation of the mxaF sequences among all the methylo trophs tested indicates that the primers are
likely to be extremely efficient for the detection of methanotrophs or methylotrophs in enrichments and environmental samples. However, the data obtained in this study show that it is not feasible to design a group-specific or genus-specific probe, for either methanotrophic \( \gamma \)-Proteobacteria, methanotrophic \( \alpha \)-Proteobacteria, or methylotrophic \( \alpha \)-Proteobacteria (i.e., methanol utilizers, rather than methane utilizers), due to the high degree of conservation at the nucleotide level. It is therefore necessary to analyze the derived amino acid or DNA sequences, using a phylogenetic analysis program such as PHYLIP (16), in order to identify the type of organisms from which these sequences have originated. Analysis of the sequences in this study demonstrated that this approach was very useful, with sequences from known organisms grouping as \( \alpha \)-, \( \beta \)-, and \( \gamma \)-Proteobacteria.

The MxaF sequences isolated directly from the peat environment appear to show some diversity (1.2 to 11.6% differences in identity). However, the only way to determine which, if any, of the bacteria from which the sequences originate is dominant within the peat environment would be to sequence a very large number of clones. It may have been possible to identify them by restriction fragment length polymorphism analysis, a technique which has been used to study diversity in several environments, including microbial mats (36), for which it was used to analyze 16S rRNA genes. However, this was not successful due to the high level of sequence conservation within the MxaF sequences and the restricted size of the PCR product, which allows for less sequence variation.

Analysis of the amino acid sequences showed that they include key amino acids at the active site of MDH (4–6) that are completely conserved: asparagine 287, aspartate 327, arginine 357, and asparagine 420. The tryptophan docking motifs (residues 308 to 318 and 363 to 373), corresponding to W4 (residues 282 to 292) and W5 (residues 337 to 347) in \( \text{Methylococcus extorquens} \) (17), are also present. The tryptophan docking motifs form a planar stabilizing girdle of interactions around the periphery of the \( \alpha \)-subunit of MDH (17). In \( \text{Methylococcus extorquens} \), the 11-residue tryptophan motifs, W4 and W5, fit the consensus sequence for tryptophan docking motifs the least (17). However, these motifs are highly conserved within the MxaF sequences analyzed in this study, suggesting that there is some reason for the difference in residues versus the consensus and that this difference is not a peculiarity in the MDH of \( \text{Methylococcus extorquens} \). The difference in this region in the MxaF sequences from the group of peat isolates, for which residue 315 is alanine and not the expected glutamine, is probably significant and suggests some relationship among the sequences isolated from organisms in the peat environment. This may be a way of distinguishing the MDH amino acid sequences of these acidophilic peat methanotrophs.

Sequence analysis shows that the environmental mxaF sequences (five from DNA extracted from a peat core and one from a methane enrichment culture) originate from a group of organisms with an mxaF sequence related to those of the type II methanotrophs. These sequences, which were the only sequence types to be detected in this study, are quite distinct for this environment. It is likely that they are from a group of novel methanotrophic organisms. This theory is supported by a previous study of 16S rRNA sequences from blanket bog peat samples (35). Novel 16S ribosomal DNA (rDNA) sequences were detected by probing 16S rDNA libraries made from the same sections of the peat core used in this study. These sequences grouped within the 16S rRNA sequences of the type II methanotrophs and were possibly from novel acidophilic organisms, since they were isolated from acidic (pH 3.6) blanket bog peat (35). Novel DNA sequences which group with DNA sequences from the type II methanotrophs have now been detected in two separate studies by using PCR primers specific for two different genes, one a functional gene (mxaF) and the other a phylogenetic gene (16S rRNA). This further strengthens the theory that the sequences detected by PCR are from novel acidophilic methanotrophs and also suggests that these organisms may predominate among the methanotrophs and methylotrophs in the blanket bog peat environment. However, work by Dunfield et al. (15) has suggested that neutrophilic methanotrophs can adapt to peat environments, since the methane consumption rates measured for peat samples showed optimum pH values which were about 2 pH units higher than the native peat pH in acidic peats. Therefore, it is probably more accurate to say that the sequences are from novel acid-tolerant methanotrophs. It may be possible to prove this theory by cloning much larger fragments of DNA from the environment by using a fosmid and identifying clones that contain both functional and phylogenetic genes. Previous work using a fosmid DNA library (45) retrieved a large fragment (40 kb) from a marine picoplankton assemblage and provided a view of the physiological potential and phylogenetic position of abundant but uncultivated organisms. A 40-kb clone was identified which contained an rRNA gene, and sequencing of this clone revealed both the 16S and 23S rRNA genes. Phylogenetic analysis identified it as being from the Crenarchaeota. Several functional genes were also sequenced, including an RNA helicase and a glutamate semialdehyde aminotransferase, indicating some of the physiological potential of the organism from which the clone originated. An alternative approach is to clone the 16S rDNA sequence of an organism from an enrichment culture and to design a fluorescently labelled oligonucleotide probe for this sequence, using the database of methanotroph and methylotroph 16S rRNA sequences produced by Bowman et al. (7) and Hanson and coworkers (9, 47). The organism could then be isolated by following the manipulation of enrichment conditions by monitoring the abundance of these bacteria in the enrichment with the fluorescently labelled probe (2), an approach that has been previously used in this laboratory for the detection of novel marine methanotrophs (23). The genes encoding the soluble and particulate MMOs and MxaF could then be analyzed, and quantitative PCR could be used to determine the abundance of this organism in the blanket bog peat environment.

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