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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 (C_1) metabolism since it catalyzes the production of formaldehyde, the intermediate of both assimilative and dissimilative metabolism in methylotrophs. In methane-oxidizing bacteria (methanotrophs), MDH is the second enzyme in the methane oxidation pathway, and it oxidizes the methanol produced from the oxidation of methane by methane monooxygenase (MMO).

Studies on the genetics of methanol oxidation in *Methylobacterium extorquens* AM1 have revealed that at least 17 genes play a role in this process (18, 28, 29). Three of these genes encode the structural proteins of the methanol oxidation complex: *mxaF* and *mxaI* encode the large (α) and small (β) subunits of MDH, respectively, and *mxaG* encodes cytochrome c_L, the primary electron acceptor for MDH. The *mxaF* gene is approximately 1.8 kb in size and encodes a 66-kDa polypeptide, while the *mxaI* gene is 290 bp in size and encodes an 8.5-kDa polypeptide. The *mxaF* genes from four methylotrophic bacterial strains—*Methylobacterium extorquens* AM1 (3), *Methylobacterium organophilum* XX (31), *Paracoccus denitrificans* PD1207 (21), and *Methylophilus methylotrophus* W3A1 (51)—have previously been cloned and sequenced. Although the *mxaF* genes of two type I methanotrophs, *Methylococcus capsulatus* (Bath) and *Methylomicrobium album* BG8, have been cloned, no complete sequences for *mxaF* genes from methanotrophs have been reported. Complementation studies suggested broad functional conservation of the methanol oxidation system among gram-negative methane and methanol utilizers (46), since cloned *mxaF* genes from a methanotroph were able to restore MDH activity in an *mxaF* mutant of *Methylobacterium extorquens* AM1. The *mxaF* genes of the type I methanotroph *Methylomonas* sp. strain A4 (48) and the type II methanotroph *Methylosinus trichosporium* OB3b (1) have also been cloned, and expression and immunoblotting experiments have determined that the order of the methanol oxidation genes (*mxaF*, *mxaJ*, *mxaG*, *mxaI*, and *mxaR*) is conserved among distantly related methylotrophs which are members of the α and γ subdivisions of the class *Proteobacteria* (α- and γ-Proteobacteria) (48).

Methanotrophs are widespread in natural habitats, play an important role in carbon cycling, and have attracted attention because they are probably the largest biological sink for methane 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 C_1 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

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mxaF-specific primers of low degeneracy. PCR primers have been designed that correspond to highly conserved regions of aligned MxaF sequences from *Methylobacterium extorquens* AM1, *Methylobacterium 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 *Methylococcus capsulatus* (Bath) (NCIMB 11132), *Methylomonas methanica* S1 (NCIMB 11130), *Methylomicrobium album* BG8 (NCIMB 11123), and LK6, a soil isolate and a member of the *Methylococcaceae*; and the type II methanotrophs (α-Proteobacteria) *Methylosinus sporium* 5 (NCIMB 11126), *Methylocystis parvus* OBBP (NCIMB 11129), and *Methylosinus trichosporium* OB3b (NCIMB 11131). *Methylocystis* sp. strain M (a type II methanotroph and member of the α-Proteobacteria) was obtained from H. Uchiyama (National Institute for Environmental Studies, Tsukuba, Japan). Chromosomal DNAs were prepared as described by Oakley and Murrell (39). *Xanthobacter* sp. strain H4-14 (a member of the α-Proteobacteria) (30), *Methylosulfonomonas methylovora* (a methanesulfonate utilizer and member of the α-Proteobacteria [26]), and *Hyphomicrobium* sp. strain CM2 (a member of the α-*Proteobacteria*) (14) were from the University of Warwick culture collection. *Methylobacterium rhodinum* (a member of the α-Proteobacteria) (ATCC 14821) was obtained from Y. Izumi (University of Tottori, Tottori, Japan), and *Methylophilus methylotrophus* (a member of the β-Proteobacteria) (NCIMB 10515) was obtained from C. Jones (University of Leicester, Leicester, United Kingdom). DNA was extracted from these organisms as described by Marmur (32). The methylotrophic bacteria used in this study represent a broad range of α-, β-, and γ-Proteobacteria.

DNA extraction from environmental samples and enrichment cultures. Samples of blanket bog peat were obtained from the Moorhouse National Nature Reserve (grid reference, NY 758333) which covers an area of nearly 4,000 ha straddling the Pennine Hills in the north of England (22). Intact core samples of peat (30 cm depth) were obtained by the methods of Hall et al. (19). The core was carefully extruded from the sample tube and sectioned into 1.0-cm-thick (between 0 and 10 cm) and 2.0-cm-thick (between 10 and 30 cm) slices with a sharp serrated knife. Total DNA was extracted from peat core sections by the methods of Selenska and Klingmuller (43) and Bruce et al. (8). This protocol had been previously demonstrated to lyse all methanotrophs and methylotrophs in enrichment cultures and the University of Warwick culture collection (35a). Peat samples consistently yielded high-quality DNA which could be digested with restriction endonucleases and was suitable for use as a template in PCR amplification experiments.

Methane-oxidizing bacteria were isolated from blanket bog peat samples in 50-ml enrichment cultures that were established in 250-ml conical flasks sealed with rubber Suba seals. The basal medium was the ammonium nitrate mineral salts medium of Whittenbury et al. (49) adjusted to pH 5.8 with phosphate buffer (200 mM potassium phosphate, pH 5.8). Flasks were supplied with methane by removing 50 ml of air from the headspace and injecting 60 ml of a methane-carbon dioxide mixture (95:5). All cultures were incubated at 30°C for 4 to 6 weeks at 200 rpm. DNA was extracted from enrichment cultures, which had grown to a final optical density at 540 nm of 0.1 to 0.2, by the method described by Marmur (32).

PCR amplification. *mxaF* genes were amplified from all DNA samples in 50-μl reaction mixtures in 0.5-ml microcentrifuge tubes. Amplification reactions were performed in 1.5 mM Mg^{2+} with the reagents supplied in GIBCO-BRL (Paisley, Scotland) *Taq* polymerase kits and with 20 ng of template DNA and 100 pmol each of primers *mxa* f1003 (GCGGCACCAACTGGGGCTGGT) and *mxa* r1561 (GGGCAGCATGAAGGGCTCCC). Primers are numbered according to the published sequence for *Methylobacterium organophilum* XX (31). Reactions were carried out in a Hybaid TR2 thermocycler (Hybaid, Teddington, Middlesex, United Kingdom) with 30 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 5 min. Reaction products were checked for size and purity on 1% (wt/vol) agarose gels (41) and then ligated into the pCR II vector supplied with the TA cloning kit (Invitrogen, San Diego, Calif.) according to the manufacturer's instructions.

DNA sequencing and analysis. Small-scale preparations of plasmids were done by the method of Saunders and Burke (42). DNA sequencing reactions were carried out by cycle sequencing with the Dye Terminator Kit of PE Applied Biosystems (Warrington, Cheshire, United Kingdom). Primers used for the sequencing reactions were the M13 −40 forward and M13 reverse primers (GIBCO-BRL). Nucleotide and inferred peptide sequences were aligned manually with sequences obtained from the GenBank database, and dendrograms were constructed by using the programs PROTDIST, PROTPARS, DNADIST, DNAPARS, DNAML, FITCH, and BOOTSTRAP from the PHYLIP version 3.5c package (16).

Nucleotide sequence accession numbers. The partial *mxaF* gene sequences have been deposited in the GenBank database under accession no. U70511 to U70527 and U85503.

RESULTS

DNA of a high molecular mass was extracted from the 1- to 2-cm, 10- to 12-cm, and 28- to 30-cm peat core sections, with 2 g of peat yielding up to 100 μg of DNA. The *mxaF*-specific PCR primers amplified a single DNA fragment of the predicted size (550 bp) from all methanotroph, methylotroph, and environmental DNA samples tested. PCR primers were also tested against a range of bacteria which do not oxidize methanol (*Escherichia coli*, *Erwinia carotovora*, and *Thiobacillus halophilus*). No products were obtained from any of these organisms. This specificity of the PCR for methanol oxidizers was confirmed by Southern blotting and hybridization (41) of the products to a probe corresponding to the same portion of *mxaF* generated from *Methylosinus trichosporium* OB3b at a stringency allowing approximately 35% base pair mismatching (data not shown).

The PCR-amplified regions of the *mxaF* genes from eight methanotrophs, four methylotrophs, a methane enrichment culture inoculated with a sample of peat bog from the 10- to 12-cm section of a core (one *mxaF* sequence), and total DNA extracted from three sections, 1 to 2, 10 to 12, and 28 to 30 cm, of a peat core (two *mxaF* sequences from each section) were cloned and sequenced. The MxaF sequences derived from these 19 new *mxaF* sequences were aligned with four previously published MxaF sequences (GenBank accession numbers are given in parentheses): those of *Methylobacterium extorquens* AM1 (M31108), *Methylobacterium organophilum* XX (M22629), *Methylophilus methylotrophus* W3A1 (U41040), and *Paracoccus denitrificans* PD1207 (M17339). Two of the MxaF sequences obtained directly from DNA extracted from peat were found to be identical, and therefore only one sequence was used in the amino acid and DNA sequence analyses.

Overall amino acid sequence conservation among MxaF sequences can be seen in the amino acid alignment (Fig. 1); 47% of the amino acid sequence is conserved throughout the 22 sequences in the alignment. The four published sequences exhibit 61% amino acid sequence conservation in the 172 amino acids of the PCR product and 56.5% conservation throughout the complete *mxaF* gene (which codes for 582 amino acids), indicating that the region chosen for PCR amplification is no more highly conserved than the rest of the *mxaF* gene. Values for identity and similarity between the MxaF sequences in this work (Table 1) ranged from 0.6 to 31.0% for identity difference and from 82.4 to 100% for similarity. This provides further evidence for the high degree of conservation within the MxaF protein because even though there is 47% conservation among all the sequences, the lowest identity value obtained when comparing any two sequences is 69%.

Analysis of the amino acid sequences (Fig. 1) shows that the key amino acids at the active site, asparagine 287, aspartate 327, arginine 357, and asparagine 420 (17), are completely conserved among all MxaF sequences. The tryptophan docking motifs that form a planar stabilizing girdle of interactions around the periphery of the subunit (residues 308 to 318 and

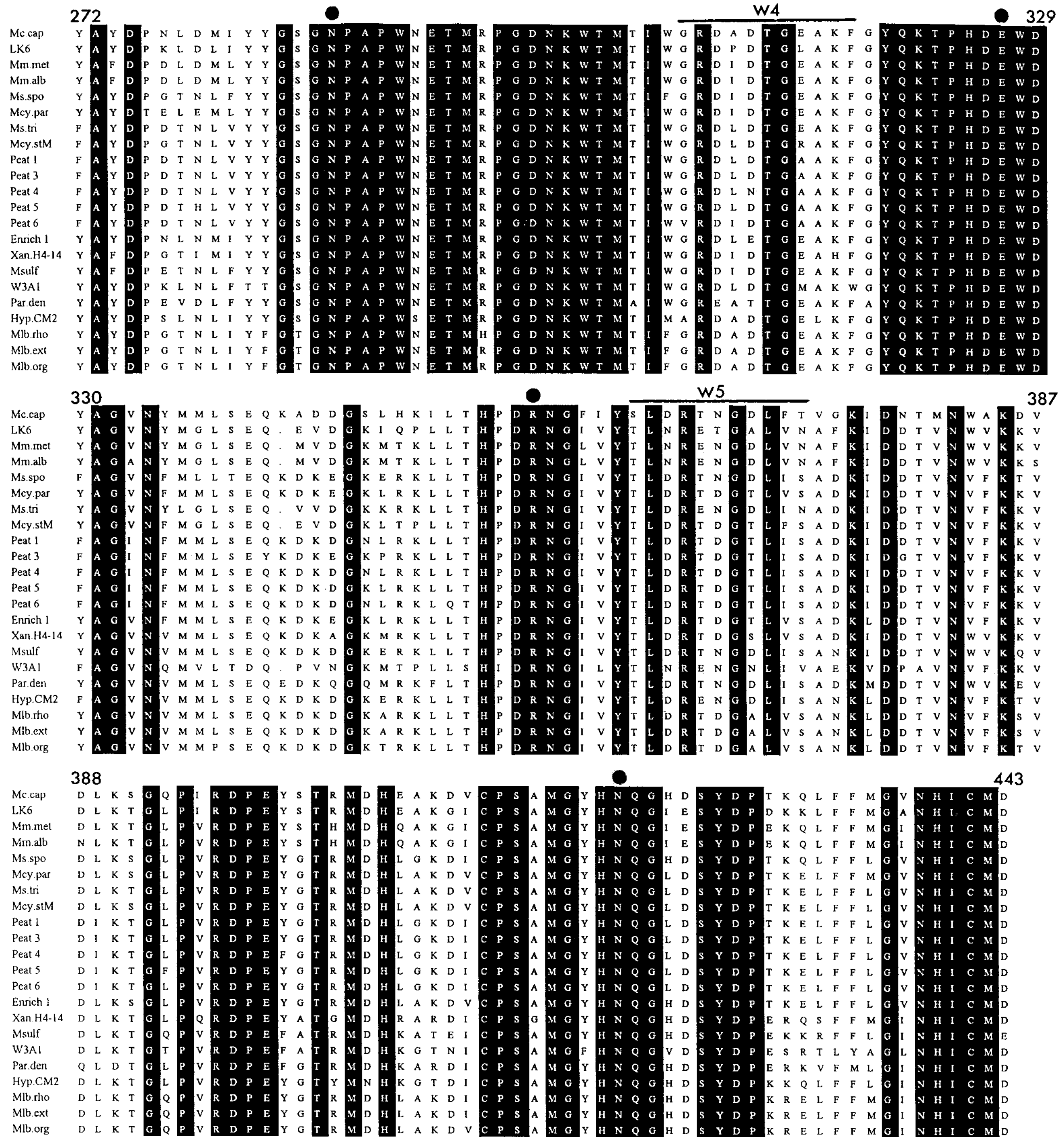

FIG. 1. Alignment of predicted amino acid sequences corresponding to *mxaF* genes of methanotrophs and methylotrophs and to gene *mxaF* sequences retrieved from environmental samples by PCR. Mc.cap, *Methylococcus capsulatus* (Bath); LK6, methanotroph isolated from soil; Mm.met, *Methylomonas methanica* S1; Mm.alb, *Methylomicrobium album* BG8; 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, *Hyphomicrobium* 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 1. Matrix showing similarities and differences in identity derived from comparison of MxaF sequences of methanotrophs and methylotrophs generated by PCR amplification[a]

Organism	% Similarity to (or difference from) the sequence of organism:																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. *Methylococcus capsulatus* (Bath)		21.2	18.6	22.2	21.5	20.5	21.1	21.1	23.8	25.6	25.0	25.0	23.8	17.5	23.3	22.7	34.5	26.8	24.4	22.7	22.1	22.1
2. LK6	86.5		11.2	12.9	22.9	20.0	17.1	17.6	21.8	22.9	22.9	22.9	21.8	20.0	21.2	21.2	27.1	25.3	23.5	21.2	20.6	21.8
3. *Methylomonas methanica* S1	89.5	94.1		1.8	21.0	18.1	16.4	20.5	22.8	24.0	24.0	23.4	22.8	19.9	17.0	18.1	27.5	24.0	21.6	22.2	21.6	22.8
4. *Methylomicrobium album* BG8	83.3	92.9	98.8		22.8	19.9	18.1	22.2	24.0	25.1	25.1	24.6	24.0	21.7	18.7	19.9	29.2	25.1	23.4	24.0	23.4	24.6
5. *Methylosinus sporium* 5	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
6. *Methylocystis parvus* OBBP	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
7. *Methylosinus trichosporium* OB3b	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
8. *Methylocystis* sp. strain M	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
9. Peat 1	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
10. Peat 3	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
11. Peat 4	90.1	88.2	91.2	90.1	94.8	97.1	94.7	95.9	100	98.3		2.9	2.3	10.5	20.9	18.0	27.5	22.1	18.0	16.9	16.3	17.4
12. Peat 5	88.9	87.1	90.1	88.9	93.6	95.9	93.6	94.7	98.8	97.1	98.8		2.9	11.6	20.3	18.6	29.2	23.8	17.4	16.9	16.3	17.4
13. Peat 6	90.1	88.8	91.8	90.6	95.3	97.7	95.3	96.5	99.4	98.8	99.4	98.3		9.9	19.8	17.4	26.9	23.3	16.9	15.1	14.5	15.7
14. Enrichment 1	91.9	88.2	91.8	90.6	96.5	98.8	94.2	94.7	97.1	96.5	97.1	95.9	97.7		15.7	17.4	28.7	20.9	16.9	13.4	12.8	12.8
15. *Xanthobacter* sp. strain H4-14	85.5	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
16. *Methylosulfonomonas methylovora* M2	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
17. *Methylophilus methylotrophus* W3A1	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
18. *P. denitrificans* PD1207	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
19. *Hyphomicrobium* sp. strain CM2	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
20. *Methylobacterium rhodinum*	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
21. *Methylobacterium extorquens* AM1	88.4	86.5	88.3	87.1	94.2	93.0	91.8	90.6	93.6	94.2	96.3	92.4	94.2	93.6	90.7	93.0	83.0	90.7	93.0	100		2.3
22. *Methylobacterium organophilum* XX	87.8	85.9	87.7	86.6	94.2	92.4	91.2	90.1	93.0	93.0	93.0	91.9	93.6	93.0	90.1	92.4	82.5	90.1	93.0	98.3	98.3	

[a] Values in the upper triangle are percent identity differences, and those in the lower triangle indicate percent similarity.

Methylomicrobium album BG8
Methylomonas methanica S1
LK6
Methylococcus capsulatus (Bath)
Gamma-*Proteobacteria*
Methylophilus methylotrophus W3A1
Beta-*Proteobacteria*
Paracoccus denitrificans PD1207
Methylosulfonomonas methylovora M2
Xanthobacter strain H4-14
Hyphomicrobium strain *CM2*
Methylobacterium organophilum XX
Methylobacterium extorquens AM1
Methylobacterium rhodinum
Methylosinus sporium 5
Methylocystis parvus OBBP
Enrichment clone 1
Peat clone 3
Peat clone 5
Peat clone 6
Peat clone 4
Peat clone 1
Methylocystis sp. strain M
Methylosinus trichosporium OB3b
Alpha-*Proteobacteria*
100, 89, 79, 51, 56, 100, 94, 65, 91, 51, 100, 53, 65, 54
1%

FIG. 2. Phylogenetic analysis of derived amino acid sequences corresponding to *mxaF* genes of methanotrophs, methylotrophs, and environmental samples. The dendrogram shows the results of an analysis with PROTDIST (16); the BOOTSTRAP values above 50% from 100 replicates are also shown. The bar represents 1% sequence divergence, as determined by measuring the lengths of the horizontal lines connecting any two species.

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, *Methylosinus trichosporium* OB3b, *Methylocystis parvus* OBBP, and *Methylocystis* sp. strain M sequences. There are two other clusters within the α-Proteobacteria, one consisting of the three *Methylobacterium* sequences and the *Hyphomicrobium* sp. strain CM2 sequence and the other containing the sequences from the remaining α-Proteobacteria methylotrophs, *Paracoccus denitrificans* PD1207, *Methylosulfonomonas methylovora* 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 DNADIST, 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 methylotrophs 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 γ-Proteobacteria, methanotrophic α-Proteobacteria, or methylotrophic α-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 α-, β-, and γ-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 *Methylobacterium extorquens* (17), are also present. The tryptophan docking motifs form a planar stabilizing girdle of interactions around the periphery of the α-subunit of MDH (17). In *Methylobacterium 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 just a peculiarity in the MDH of *Methylobacterium 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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