The Soluble Methane Monoxygenase Gene Cluster of the Trichloroethylene-Degrading Methanotroph Methylocystis sp. Strain M

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In methanotrophic bacteria, methane is oxidized to methanol by the enzyme methane monooxygenase (MMO). The soluble MMO enzyme complex from Methylocystis sp. strain M also oxidizes a wide range of aliphatic and aromatic compounds, including trichloroethylene. In this study, heterologous DNA probes from the type II methanotroph Methylosinus trichosporium OB3b were used to isolate soluble MMO (sMMO) genes from the type II methanotroph Methylocystis sp. strain M. sMMO genes from strain M are clustered on the chromosome and show a high degree of identity with the corresponding genes from Methylosinus trichosporium OB3b. Sequencing and phylogenetic analysis of the 16S rRNA gene from Methylocystis sp. strain M have confirmed that it is most closely related to the type II methanotroph Methylosinus parvus OBBP, which, unlike Methylocystis sp. strain M, does not possess an sMMO. A similar phylogenetic analysis using the pmoA gene, which encodes the 27-kDa polypeptide of the particulate MMO, also places Methylocystis sp. strain M firmly in the genus Methylocystis. This is the first report of isolation and characterization of methane oxidation genes from methanotrophs of the genus Methylocystis.

The methanotrophic bacteria oxidize methane to methanol with the enzyme methane monooxygenase (MMO). This enzyme can be of two different types: a soluble, cytoplasmic enzyme complex (sMMO) or a membrane-bound, particulate enzyme (pMMO). pMMO is found in all methanotrophs during copper-sufficient growth conditions and requires copper ions for both expression and activity (58). It has a relatively narrow substrate specificity but will, in addition to oxidizing methane, cooxidize a number of short-chain alkanes, alkenes, and ammonia (7, 52). By contrast, sMMO has a very broad substrate specificity and is able to cooxidize a wide range of alkanes, alkenes, and aromatic compounds (8). There is no apparent advantage for methanotrophs to possess the additional sMMO system other than to have the capacity to grow under copper-deficient conditions, and the ecological significance of having this additional system is not known.

There has been considerable interest in methanotrophs, since it has been recognized that they are a major sink for atmospheric methane (40). The methanotrophs have also attracted considerable attention, since they are able to degrade a number of important groundwater pollutants, such as trichloroethylene (TCE) and other halogenated hydrocarbons (1, 39). The enzyme responsible for biodegradation of TCE and other pollutants is MMO. Both types of MMO can degrade TCE; however, pMMO (10) degrades TCE at very low rates compared with sMMO, e.g., that from Methylosinus trichosporium OB3b (38). Previously, a new type II methanotroph, Methylocystis sp. strain M, capable of degrading TCE at high concentrations was isolated (54, 55) and its sMMO was purified and characterized (29). The concentration of TCE which strain M was able to degrade (77 μM) is similar to that which can be degraded by Methylosinus trichosporium OB3b (145 μM) (39) but considerably higher than those that can be degraded by other methanotrophs (39). Not all methanotrophs contain an sMMO, and generally this enzyme has been observed only in strains of Methylococcus or Methylosinus. Only one Methylomonas (18) and Methylocystis sp. strain M (29) have been reported to possess an sMMO (53). The most extensively characterized of these are the sMMOs from Methylococcus capsulatus (8, 42) and Methylosinus trichosporum (20). The DNA sequence of the gene cluster that codes for the sMMO proteins has been determined for two methanotrophs, Methylococcus capsulatus (Bath) (49, 50) and Methylosinus trichosporum OB3b (5, 6, 26). The sMMO gene cluster of Methylococcus capsulatus (Bath) has been used to probe for sMMO genes in a number of representative strains of methanotrophs (51). sMMO homologies were detected only in Methylococcus and Methylosinus strains. sMMO appeared to be absent from the type II methanotroph Methylocystis parvus OBBP and type I representatives of Methylocladium album, Methylocomonas methanica, Methylocomonas agile, and Methylobacter capsulatus. sMMO consists of three components, proteins A, B, and C. Protein A is made up of three subunits, α, β, and γ, arranged in an α2β2γ2 configuration, which are coded for by the mmoX, mmoY, and mmoZ genes, respectively, and is the hydroxylase component of the enzyme complex (57). Protein B acts as an effector of electron transfer in the catalytic mechanism (13) and is coded for by mmoB. Protein C is the reductase component of the enzyme (21) and is coded for by mmoC. In contrast, pMMOs of methanotrophs have proved far more refractory to biochemical analysis (31, 32, 48). However, the enzyme has recently been purified in an active form (58) and the genes encoding pMMO have been cloned from Methylococcus capsulatus (Bath) (45). The genes encoding the 27- and 45-kDa pMMO polypeptides, pmoA and pmoB, respectively, are linked on the chromosome. The ammonia-oxidizing bacteria contain an enzyme evolutionarily related to pMMO, the ammonia monooxygenase, for which two genes have been identified, amoa and amob (45). The sequence data available from sMMO and pMMO genes have allowed the design of PCR primers which amplify the corresponding genes from...
other methanotrophs in culture and in environmental samples (16, 17, 24, 25).

The methanotrophs have been classified, on the basis of the results of chemotaxonomic studies and 16S rRNA phylogenetic analyses, into six genera: Methylococcus, Methylobacter, Methylophilomonas, Methylosinus, and Methylocystis (2–4, 15). These six genera fall into two phylogenetically distinct, exclusively methanotrophic groups. Methanotrophs with type I intracelluar membranes include the genera Methylophilomonas, Methylophilomonas, Methylobacter, and Methylococcus, which are all related to bacteria of the γ subdivision of the class Proteobacteria (γ-Proteobacteria). Methanotrophs with type II membranes include Methylosinus and Methylocystis, which belong to the α subdivision of the class Proteobacteria. At present, Methylocystis sp. strain M is being used as a test organism for bioremediation in reactors and in aquifers (36, 37, 46); therefore, techniques to rapidly detect Methylocystis sp. strain M in the environment need to be developed. Here we describe the molecular characterizations of the sMMO genes from Methylocystis sp. strain M and compare their sequences with sMMO sequences from other genera of methanotrophs. We also establish that Methylocystis sp. strain M does belong to the genus Methylocystis, since the only other well-characterized Methylocystis species, Methylocystis parvus OB2B, does not contain an sMMO (53).

MATERIALS AND METHODS

sMMO gene cluster cloning and sequencing. Methylocystis sp. strain M DNA was isolated by the method of Marmur (23). Restriction fragments of strain M DNA were generated by partial digestion with Sau3AI, and 15–25-kb fragments were excised from an agarose gel. The DNA fragments were then ligated into pSK31 by in vitro packaging with a Gigapack II packaging kit (Stratagene) and transfected into Escherichia coli. The clone library was then probed with 32P-labeled mmoX and mmoC PCR products generated with the primers of McDonald et al. (24) to identify clones containing both mmoX and mmoC genes, and thus the whole sMMO gene cluster. A suitable clone was then subcloned and sequenced with an Autocycle sequencing kit and Automated Laser Fluorescent DNA sequencer (Pharmacia Biotech) and also by cycle sequencing with a dye-terminator kit from Applied Biosystems.

PCR amplification. The 16S rRNA gene (rDNA) was amplified with the bacterium-specific primers 27F and 1492F (92). Amplification reactions were performed with the reagents supplied with Gibco-BRL (Paisley, Scotland) Taq polymerase kits at a magnesium ion concentration of 1.5 mM and 20 ng of template DNA and 100 pmol of each primer were added. The reactions were carried out in a Perkin-Elmer thermocycler with 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. DNA amplification was also carried out with primers specific for the methanotroph gene mmoA (which encodes the 27-kDa subunit of pMMO) (17), A189 (GGNGACTGGACTTCTGG) and A682 (GAASGCNGAGAAGAASGC). Reaction conditions were the same as for the 16S rDNA PCR, except for an annealing temperature of 56°C.

PCR products were checked for size and purity on 1% (wt/vol) agarose gels (43) and then ligated into the PCR II vector supplied with a TA cloning kit (Invitrogen, San Diego, Calif.) according to the manufacturer's instructions.

DNA sequencing of 16S rDNA and mmoA clones. Small-scale preparations of plasmids were made by the method of Saunders and Burke (44). DNA sequencing reactions were carried out by cycle sequencing with the dye-terminator kit from Applied Biosystems. Primers used for sequencing the 16S rDNA clone were the M13 forward, M13 reverse, A189, and A682 primers.

Phylogenetic analysis. 16S rDNA sequences were aligned manually to represent protobacterial sequences obtained from the GenBank database, and dendrograms were constructed with the programs DNADIST, DNAML, DNAPARS, FITCH, and BOOTSTRAP from the PHYLIP version 3.4 package (11). Only regions of the 16S rDNA which could be unambiguously aligned were included in the analyses (bases 220 to 450, 482 to 836, 850 to 1133, 1140 to 1284, and 1287 to 1430, numbered according to the E. coli numbering system). Analyses were also performed with partial sequences to check for the presence of chimeric sequences (22). Secondary structure predictions for methanotroph 16S rRNAs were constructed manually, based on the published models of Neefs et al. (30) and Gutell et al. (14). The mmoA-derived amino acid sequence was also analyzed, and dendrograms were constructed with the programs PRODIST, PROTPARS, FITCH, and BOOTSTRAP from the PHYLIP version 3.4 package (11).

RESULTS

Nucleotide sequence accession numbers. The complete sequence of the Methylocystis sp. strain M sMMO gene cluster, the 16S rDNA sequence of Methylocystis sp. strain M, and the mmoA gene sequence of Methylocystis sp. strain M have been deposited in the GenBank database under the accession no. U81594, U81595, and U81596, respectively.

Alignment of the derived amino acid sequences of the mmoX, mmoB, and mmoC proteins of the sMMO gene cluster (Fig. 2) and amino acid and DNA sequence comparisons (Table 1) were made with Methylocystis sp. strain M and the two previously sequenced sMMO gene clusters from Methylosinus trichosporium OB3b and Methylococcus capsulatus (Bath). The results from the comparisons clearly demonstrate that Methylocystis sp. strain M is most closely related to Methylosinus trichosporium OB3b, with amino acid sequences between 80.5 and 96.4% identical and nucleotide sequences 80.8 to 94.7% identical, while Methylocystis sp. strain M and Methylococcus capsulatus (Bath) are between 51.3 and 82.3% identical and Methylosinus trichosporium OB3b are between 48.7 and 80.2% identical. However, the degree of amino acid sequence conservation of all three sequences varies among proteins. Proteins are universally conserved as follows: MmoX, 79.5%; MmoY, 53.4%; MmoB, 62.4%; MmoZ, 46.5%; MmoC, 44.3%; and OrfY, 23.1%. The iron binding sites for the two iron atoms of the binuclear iron center in MmoX (42) (Fig. 2) and amino acid and DNA sequence comparisons (Table 1) were made with Methylocystis sp. strain M and the two previously sequenced sMMO gene clusters from Methylosinus trichosporum OB3b and Methylococcus capsulatus (Bath). The results from the comparisons clearly demonstrate that Methylocystis sp. strain M is most closely related to Methylosinus trichosporum OB3b, with amino acid sequences between 80.5 and 96.4% identical and nucleotide sequences 80.8 to 94.7% identical, while Methylocystis sp. strain M and Methylococcus capsulatus (Bath) are between 51.3 and 82.3% identical and Methylosinus trichosporum OB3b are between 48.7 and 80.2% identical. However, the degree of amino acid sequence conservation of all three sequences varies among proteins. Proteins are universally conserved as follows: MmoX, 79.5%; MmoY, 53.4%; MmoB, 62.4%; MmoZ, 46.5%; MmoC, 44.3%; and OrfY, 23.1%. The iron binding sites for the two iron atoms of the binuclear iron center in MmoX (42) (Fig. 2a), located at amino acid positions 147 and 242 to 246, and the cysteine residues typical of a MhoX (42) (Fig. 2a), located at amino acid positions 147 and 242 to 246, and the cysteine residues typical of a
proteins. Proteins are universally conserved as follows: MmoX, the degree of amino acid sequence conservation varies between described from OB3b (5, 6). The sequence T^{563}GGCACN_{5}TTGCCN_{11}G^{390} is identical has shown the genes to be most similar to those previously ported. Sequencing of the sMMO gene cluster from strain M experiments (51), and no sequence information has been re- been shown to contain an sMMO but only in hybridization (49, 50). A fourth genus of methanotrophs, Methylomonas vus, and branches closest to the other Methylocystis trichosporium (5, 6) and branches closest to the other Methylocystis parvus sp. strain M chromosome contains...
Nielsen and colleagues (33, 34) found that it was likely that transcription of the \( \text{mmoX} \) gene is initiated at position 424 (a G, which corresponds to G390 in this work) and directed from the \( \sigma^{34} \) promoter. The putative \( \sigma^{34} \) promoter may be involved in expression of sMMO, which is mediated by copper ions by a mechanism which inactivates an activator protein, since all known \( \sigma^{34} \) promoters are regulated.

FIG. 2. Alignments of derived amino acid sequences of the \( \text{mmoX} \) (a), \( \text{mmoB} \) (b), and \( \text{mmoC} \) (c) genes from \textit{Methylosinus trichosporium} OB3b, \textit{Methylocystis} sp. strain M, and \textit{Methylococcus capsulatus} (Bath). Residues in filled boxes are universally conserved, and residues in stippled boxes are the Fe binding sites on MmoX (a) and the conserved cysteine residues of MmoC (c). The lines above the sequences of MmoC (c) indicate the 92-amino-acid N terminus. Amino acids are numbered according to the published sequences for \textit{Methylococcus capsulatus} (Bath) (47, 48). Ms.tri, \textit{Methylosinus trichosporium} OB3b; Mcy.stM, \textit{Methylocystis} sp. strain M; Mc.cap, \textit{Methylococcus capsulatus} (Bath).
by modulation of abundance or states of activity of activator proteins. The activators positively control transcription of genes under the control of \(\sigma^54\) in response to a diverse set of physiological signals, such as nitrogen availability, oxygen tension, energy limitation, and availability of different metabolic compounds (47). Therefore, it would not be too surprising if a mechanism involving copper and possibly methane controls sMMO expression via a \(\sigma^54\)-dependent promoter.

The most characterized of type II methanotrophs of the Methylocystis genus, Methylocystis parvus OBBP, does not contain an sMMO (53), and both of the other type II methanotrophs, Methylosinus trichosporium OB3b and Methylosinus sporium, contain sMMO. It has been suggested that Methylocystis sp. strain M may be a Methylosinus species. Therefore, the 16S rDNA from Methylocystis sp. strain M was cloned and sequenced in order to determine its true phylogenetic position. Phylogenetic analysis (Fig. 3) confirmed that strain M is a type II methanotroph, distinct from but most closely related to the Methylocystis sequence from Methylocystis parvus OBBP. Previously, strain M had been shown to have characteristics typical of type II methanotrophs (54) and especially of the Methylocystis species. It contains intracellular membranes arranged around the periphery of the cell, the predominant fatty acid is C18:1,\(\alpha\)-esterified with a \(\alpha\) content of 64.5 mol%, and it is nonmotile. However, strain M does differ from other strains of Methylocystis in its cell morphology, since it is rod shaped while the other Methylocystis species are ellipsoid or curved. To further strengthen the placing of Methylocystis sp. strain M as a Methylocystis species, the pmoA gene was cloned and sequenced. Phylogenetic analysis of the pmoA gene from Methylocystis sp. strain M in Methylocystis parvus OBBP.

### Table 1. Comparison of derived amino acid sequences of the sMMO proteins of Methylocystis sp. strain M, Methylosinus trichosporium OB3b, and Methylococcus capsulatus (Bath)\(^a\)

<table>
<thead>
<tr>
<th>Sequence compared</th>
<th>Compared organisms(^b)</th>
<th>% DNA sequence identity</th>
<th>% Amino acid sequence identity</th>
<th>% Amino acid sequence similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmoX</td>
<td>M. caps. and M. stM</td>
<td>76.9</td>
<td>82.3</td>
<td>89.9</td>
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<tr>
<td></td>
<td>M. tric. and M. stM</td>
<td>91.4</td>
<td>95.4</td>
<td>97.3</td>
</tr>
<tr>
<td></td>
<td>M. tric. and M. caps.</td>
<td>76.0</td>
<td>80.2</td>
<td>88.9</td>
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<tr>
<td>mmoY</td>
<td>M. caps. and M. stM</td>
<td>67.9</td>
<td>61.0</td>
<td>76.2</td>
</tr>
<tr>
<td></td>
<td>M. tric. and M. stM</td>
<td>91.0</td>
<td>88.3</td>
<td>92.4</td>
</tr>
<tr>
<td></td>
<td>M. tric. and M. caps.</td>
<td>66.7</td>
<td>57.1</td>
<td>72.6</td>
</tr>
<tr>
<td>mmoZ</td>
<td>M. caps and M. stM</td>
<td>64.8</td>
<td>51.8</td>
<td>68.5</td>
</tr>
<tr>
<td></td>
<td>M. tric. and M. stM</td>
<td>86.9</td>
<td>87.0</td>
<td>94.7</td>
</tr>
<tr>
<td></td>
<td>M. tric. and M. caps.</td>
<td>63.8</td>
<td>50.0</td>
<td>67.9</td>
</tr>
<tr>
<td>mmoB</td>
<td>M. caps. and M. stM</td>
<td>71.9</td>
<td>66.0</td>
<td>80.4</td>
</tr>
<tr>
<td></td>
<td>M. tric. and M. stM</td>
<td>94.7</td>
<td>96.4</td>
<td>100</td>
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<tr>
<td></td>
<td>M. tric. and M. caps.</td>
<td>71.1</td>
<td>66.0</td>
<td>81.1</td>
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<td>orfY</td>
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<td>64.5</td>
<td>42.9</td>
<td>65.5</td>
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<td></td>
<td>M. tric. and M. stM</td>
<td>79.2</td>
<td>59.8</td>
<td>72.5</td>
</tr>
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<td>M. tric. and M. caps.</td>
<td>64.4</td>
<td>40.0</td>
<td>55.0</td>
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<tr>
<td>mmoC</td>
<td>M. caps. and M. stM</td>
<td>62.8</td>
<td>51.3</td>
<td>71.0</td>
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<tr>
<td></td>
<td>M. tric. and M. stM</td>
<td>80.8</td>
<td>80.5</td>
<td>86.4</td>
</tr>
<tr>
<td></td>
<td>M. tric. and M. caps.</td>
<td>61.1</td>
<td>48.7</td>
<td>67.6</td>
</tr>
</tbody>
</table>

\(^a\) Data were obtained by using the Genetics Computer Group sequence analysis software package, version 7.2.

\(^b\) M. caps., Methylococcus capsulatus (Bath); M. stM, Methylocystis sp. strain M; M. tric., Methylosinus trichosporium OB3b.

**FIG. 3.** Phylogenetic analysis of the 16S rDNA from Methylocystis sp. strain M. The dendrogram shows the results from analysis with DNADIST; the BOOTSTRAP values 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.
FIG. 4. Phylogenetic analysis of the derived amino acid sequence of pmOA from Methylocystis sp. strain M. The dendrogram shows the results from analysis with PROTDIST; the BOOTSTRAP values from 100 replicates are also shown. The bar represents 3% sequence divergence, as determined by measuring the lengths of the horizontal lines connecting any two species.

Methylocystis sp. strain M has also shown it to be a type II methanotroph most closely related to Methylocystis parvus OB3b (Fig. 4). The pmOA gene has recently been shown to be effective for the phylogenetic analysis of methanotrophs and nitrifiers containing the pmOA or amoA gene (28). The sequencing of the pmOA gene has also provided further sequence information of the pmOA genes from the type II methanotrophs, providing a larger database from which to design functional gene probes for methanotrophs.

The generation of sequences of the sMMO gene cluster, pmOA, and 16S rDNA will now allow the designing of probes and PCR primers to be used to identify and track Methylocystis sp. strain M in bioreactors and aquifers (36, 37). Further information from the analysis of sequence data has shown that the sMMO from Methylocystis sp. strain M has a G+C content of 61.3%, with a third-codon GC bias of 88.6%, a fact which will help in the designing of degenerate oligonucleotide probes and primers. The cloning and sequencing of the sMMO gene cluster will also help in future work to establish the complete degradation of TCE by cloning the sMMO gene cluster into Xanthobacter sp. strain DA4, which already degrades the TCE degradation products produced by strain M (56). The fact that the efficiency of degradation of TCE by Methylocystis sp. strain M is similar to that of Methylosinus trichosporium OB3b may be explained by the high similarity of their sMMO gene cluster sequences.

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