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 methanol 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 *Methylococcus* (18) and *Methylosinus* 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 trichosporium* (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 trichosporium* 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 *Methylomicrobium album*, *Methylococcales anamachica*, *Methylomonas agile*, and *Methylomicrobium capsulatum*. 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*, *mmod*, and *mmod* 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 *mmod*. Protein C is the reductase component of the enzyme (21) and is coded for by *mmodC*. 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: *Methyllococcus*, *Methylbacter*, *Methylocoribium*, *Methylomonas*, *Methylosinus*, and *Methylocystis* (2–4, 15). These six genera fall into two phylogenetically distinct, exclusively methanotrophic groups. Methanotrophs with type I intracellular membranes include the genera *Methylococcus*, *Methylocoribium*, *Methylbacter*, and *Methyllococcus*, which are all related to bacteria of the γ subdivision of the class Proteobacteria (γ-Proteobacteria). Methanotrophs with type II membranes include *Methyloomonas* 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* OBPP, 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 *SacI* and 15- to 25-kb fragments were excised from an agarose gel. The DNA fragments were then ligated into pSK13 by in vitro packaging with a Gigapack II packaging kit (Stratagene) and transfected into Escherichia coli. The clone library was then probed with a 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 Autoseq Cycle 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 E7 and r1902 (12). 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 92°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR product was further subcloned into three clones, pUPEP1, pUPEP2, and pUPEP3. Four of the clones were then sequenced: pBMS1, which contains most of the *mmox* gene; pUPEP1, which contains the 5′ end of *mmox* and the 5′ end of *mmoy*; pUPEP2, which contains a central part of *mmoy*; and pUPEP3, which contains the 5′ end of *mmoy* and the *mmob*, *mmoz*, *ormy*, and *mmoc* genes. The larger clones, pUPEH3 and pPWM3, were used to provide overlapping sequence where the smaller clones join.

Alignments 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) (Table 1). 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. 2c), located at amino acid positions 143 to 147 and 242 to 246, and the cysteine residues typical of a ferrodoxin-like Fe₂S₂ center in MmoC (Fig. 2c), located at amino acids 42, 47, 50, and 82, are also conserved universally.

**RESULTS**

sMMO gene cluster, cloning, and sequencing. Probes for *mmox* and *mmoc* genes were generated by PCR from *Methylosinus trichosporium* OB3b with primers designed previously (24) and used to probe a *Methylocystis* sp. strain M Sau3A library. With both the *mmox* and *mmoc*-specific probes, three clones were identified; however, all showed the same digestion pattern with several restriction enzymes. A single clone (pWM3) which contained the complete sMMO gene cluster (Fig. 1) on a 18.7-kb DNA fragment from strain M was studied further. This DNA insert was excised from the vector on a BamHI fragment. The presence of the sMMO genes on pWM3 was confirmed by PCR amplification with the *mmox*, *mmoy*, *mmob*, and *mmoz* primers (24) and sequencing of the resultant products. The cluster was subcloned into two further clones, a 1.6-kb BamHI fragment (pBMS1) and a 6.2-kb BamHI-HindIII fragment (pUMH3). The larger, 6.2-kb clone was further subcloned into three clones, pUPEP1, pUPEP2, and pUPEP3. Four of the clones were then sequenced: pBMS1, which contains most of the *mmox* gene; pUPEP1, which contains the 5′ end of *mmox* and the 5′ end of *mmoy*; pUPEP2, which contains a central part of *mmoy*; and pUPEP3, which contains the 5′ end of *mmoy* and the *mmob*, *mmoz*, *ormy*, and *mmoc* genes. The larger clones, pUPEH3 and pPWM3, were used to provide overlapping sequence where the smaller clones join.

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

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**Phylogenetic analysis.** 16S rDNA sequences were aligned manually to the representative protococbacterial sequences obtained from the GenBank database, and dendrograms were constructed with the programs PROTDIST, PROTPARS, FITCH, and BOOTSTRAP from the PHYLIP version 3.4 package (11).
**FIG. 1.** Strategy for cloning the sMMO gene cluster from *Methylocystis* sp. strain M.

*Methylocystis* sp. strain M was shown to branch with the type II methanotroph cluster (*Methylocystis parvus, Methylosinus trichosporum, and Methylosinus sporum*) (see Fig. 3), branching closest to the other *Methylocystis* sequence from *Methylocystis parvus* OBBP. This branching was found with all analysis programs (DNADIST, DNAML, and DNAPARS) and was supported by BOOTSTRAP values (Fig. 3).

**pmoA gene cloning and phylogenetic analysis.** The *pmoA* specific PCR primers amplified a single DNA fragment of the predicted size (525 bp) from *Methylocystis* sp. strain M. The PCR product was cloned with the TA cloning kit (Invitrogen) and sequenced. Phylogenetic analyses of the derived amino acid sequence of the *Methylocystis* sp. strain M *pmoA* clone with the PROTDIST, PROTPARS, FITCH, and BOOTSTRAP programs of the PHYLIP package (Fig. 4) have shown that the *pmoA* sequence of *Methylocystis* sp. strain M clusters with the other type II methanotroph *pmoA* sequences, i.e., those from *Methylosinus trichosporum* and *Methylocystis parvus*, and branches closest to the other *Methylocystis* sequence, *Methylocystis parvus*. This branching is confirmed by both PROTDIST and PROTPARS analysis and supported by BOOTSTRAP values.

**DISCUSSION**

Using probes specific for *mmoX* and *mmoC*, we have cloned a region of the *Methylocystis* sp. strain M chromosome containing the complete sMMO gene cluster. This is only the third sMMO gene cluster to be sequenced, and it is from a different group of methanotrophs, the other two being from *Methylosinus trichosporum* (5, 6) and *Methylococcus capsulatus* (Bath) (49, 50). A fourth genus of methanotrophs, *Methylomonas*, has been shown to contain an sMMO but only in hybridization experiments (51), and no sequence information has been reported. Sequencing of the sMMO gene cluster from strain M has shown the genes to be most similar to those previously described from *Methylosinus trichosporum* OB3b (5, 6). The degree of amino acid sequence conservation varies between 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 derived amino acid sequence of the N-terminal 90-amino-acid region of protein C exhibited significant homologies with ferrodoxins from plants and bacteria. This region also contains the four conserved cysteine residues which are a common feature of Fe₂S₂ iron-sulfur centers of ferrodoxins, as was previously shown for *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporum* OB3b (6, 50). Also, the derived amino acid sequence of the α subunit of protein A has shown that the iron binding domains for the two irons in the R2 protein of ribonucleotide reductase identified in *Methylococcus capsulatus* (49) and *Methylosinus trichosporum* (5), which contain specific aspartate, glutamate, and histidine residues, are also highly conserved in *Methylocystis* sp. strain M. Previously, the two published sequences of the sMMO gene cluster were aligned with the sequences of the iron-coordinating four-helix bundle in the R2 protein of ribonucleotide reductase to obtain a predicted model for the active site of MMO (35). X-ray crystal structure data from *Methylococcus capsulatus* (Bath) have now been produced for the hydroxylase component of the sMMO, which agree with this prediction (41). The elucidation of a third sMMO cluster sequence in this work will allow further structural predictions for the components of sMMO. The open reading frame *orfY* has been identified in the sequences of the sMMO gene cluster of all three organisms; however, there is only 23% conservation between the derived putative amino acid sequences. Ribosome binding sites are found upstream of *orfY* in all three clusters, which provides further support for the validity of this open reading frame, but searches of the GenBank database have failed to identify any sequences which may suggest a role for *orfY*. Work to try to identify the role of *orfY* will continue, if indeed there is a functional open reading frame.

Analysis of the sequence upstream of *mmox* identified a putative promoter sequence very close to the consensus sequence recognized by E. coli RNA polymerase containing $\sigma^{54}$ (9). The sequence T$^{63}$GCACN$^{TTGCCN}_{7}G^{990}$ is identical to the sequence identified in *Methylosinus trichosporum* OB3b by transcription analysis (33, 34). The putative $\sigma^{54}$ promoter is
located in an optimal position with respect to transcriptional initiation at G$_{390}$. Nielsen and colleagues (33, 34) found that it was likely that transcription of the mmoX gene is initiated at position 424 (a G, which corresponds to G$_{390}$ in this work) and directed from the s$^54$-like promoter. The putative s$^54$ 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 s$^54$ promoters are regulated by copper ions. 

FIG. 2. Alignments of derived amino acid sequences of the mmoX (a), mmoB (b), and mmoC (c) genes from Methylosinus trichosporium OB3b, Methylocystis sp. strain M, and 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 Methylococcus capsulatus (Bath) (47, 48). Ms.tri, Methylosinus trichosporium OB3b; Mcy.stM, Methylocystis sp. strain M; Mc.cap, 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 \( s^{54} \) 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 \( s^{54} \)-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,ω9C with a saG 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 Meth-
ylocytsis sp. strain M has also shown it to be a type II methanotroph most closely related to Methylocystis parva OBBP (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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REFERENCES


