

Role of multiple gene copies in particulate methane monooxygenase activity in the methane-oxidizing bacterium *Methylococcus capsulatus* Bath

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Genes for the subunits of particulate methane monooxygenase, *PmoABC*, have been sequenced from the γ -proteobacterial methanotroph *Methylococcus capsulatus* Bath. *M. capsulatus* Bath contains two complete copies of *pmoCAB*, as well as a third copy of *pmoC*. The two *pmoCAB* regions were almost identical at the nucleotide sequence level, differing in only 13 positions in 3183 bp. At the amino acid level, each translated gene product contained only one differing residue in each copy. However, the *pmoC3* sequence was more divergent from the two other *pmoC* copies at both the far N-terminus and far C-terminus. Chromosomal insertion mutations were generated in all seven genes. Null mutants could not be obtained for *pmoC3*, suggesting that it may play an essential role in growth on methane. Null mutants were obtained for *pmoC1*, *pmoC2*, *pmoA1*, *pmoA2*, *pmoB1* and *pmoB2*. All of these mutants grew on methane, demonstrating that both gene copies were functional. Copy 1 mutants showed about two-thirds of the wild-type whole-cell methane oxidation rate, while copy 2 mutants showed only about one-third of the wild-type rate, indicating that both gene copies were necessary for wild-type particulate methane monooxygenase activity. It was not possible to obtain double null mutants that were defective in both *pmo* copies, which may indicate that some expression of pMMO is important for growth.

Keywords: methane monooxygenase, methanotroph, duplicate genes, *pmo*

INTRODUCTION

Methanotrophs are a group of Proteobacteria that utilize methane as a sole source of carbon and energy (Hanson *et al.*, 1990). The initial transformation involves the conversion of methane to methanol by methane monooxygenase (MMO). Two types of MMO are known, a cytoplasmic or soluble form (sMMO) and a membrane-bound or particulate form (pMMO). All known meth-

anotrophs contain the pMMO, but the sMMO is found in only a few methanotrophs, as a second enzyme (Hanson *et al.*, 1990; Koh *et al.*, 1993; Nakajima *et al.*, 1992). In those strains that contain both enzymes, sMMO is expressed only under copper-limitation (Prior & Dalton, 1985a; Smith & Dalton, 1989). The sMMO is an NADH-linked iron enzyme with a broad substrate specificity (Colby & Dalton, 1976; Fox *et al.*, 1989; Green & Dalton, 1989). The genes for the subunits of sMMO have been cloned and sequenced from *Methylococcus capsulatus* Bath (Stainthorpe *et al.*, 1990), *Methylosinus trichosporium* OB3b (Cardy *et al.*, 1991) and *Methylocystis* sp. strain M (McDonald *et al.*, 1997). Insertion mutants in sMMO genes in *Methylosinus trichosporium* OB3b still express pMMO normally (Martin & Murrell, 1995). Recent studies have suggested that in *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b, transcription of the sMMO

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Abbreviations: pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase; NMS, nitrate mineral salts medium; NMSF, NMS medium containing iron and formate.

The GenBank accession numbers for the sequences reported in this paper are L40804 (for the fragment containing *pmoC1*), U94337 (for the copy 2 *pmo* gene cluster) and AF091320 (for the fragment containing *pmoC3*).

genes is negatively regulated by copper (Nielsen *et al.*, 1997).

The pMMO is a copper enzyme with a relatively narrow substrate specificity, and it contains at least three subunits, of approximately 45, 26 and 23 kDa (Smith & Dalton, 1989; Nguyen *et al.*, 1994, 1998; Zahn & DiSpirito, 1996). The 26 kDa subunit apparently contains the active site, as it binds the suicide inhibitor acetylene (Prior & Dalton, 1985b; DiSpirito *et al.*, 1992). Hybridization studies have suggested that methanotrophs contain multiple copies of genes encoding the 45 and 26 kDa subunits (*pmoB1* and *pmoA1*, respectively) (Semrau *et al.*, 1995). One copy of the genes encoding these two subunits was cloned and sequenced, and the genes were found to be adjacent to each other in the order *pmoA1*–*pmoB1* (Semrau *et al.*, 1995). In addition, a gene encoding the 23 kDa subunit (*pmoC1*) has been shown to be present upstream of *pmoA1* (Nguyen *et al.*, 1996). *pmoA1* and *pmoB1* showed substantial similarity to genes encoding the analogous subunits of a related enzyme, ammonia monooxygenase (Semrau *et al.*, 1995), which are also present in multiple copies in nitrifying bacteria (McTavish *et al.*, 1993; Norton *et al.*, 1996). In the nitrifiers, the genes are also organized in a cluster in the order *amoCAB* with an additional, single copy of *amoC* (Klotz *et al.*, 1997; Sayavedra-Soto *et al.*, 1998). In *Nitrosomonas europaea*, an insertion mutant defective in one of the *amoA* genes showed slower growth than the wild-type, while a mutant containing an insertion in the other *amoA* gene showed normal growth (Hommes *et al.*, 1998). However, mutants are not available in the other *amo* genes. A 3.3 kb transcript covering *pmoA*, *pmoB* and *pmoC* as well as smaller transcripts are detectable in *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b expressing pMMO, but it is not known which of the sets of genes is responsible for these transcripts (Nielsen *et al.*, 1997). Likewise, analogous transcripts have been detected in nitrifying bacteria, and it has been shown that the *amoA* mutant showing slower growth also has decreased amounts of these transcripts (Hommes *et al.*, 1998; Sayavedra-Soto *et al.*, 1998).

In this paper, we present the entire sequence of both *pmoCAB* gene clusters and a third copy of *pmoC* from *Methylococcus capsulatus* (Bath), as well as the phenotypes of insertion mutants generated in each of the seven genes.

METHODS

Bacterial strains, plasmids and growth conditions. *Escherichia coli* strains DH5 α , DH5 α MCR (Bethesda Research Laboratories), Inv α F' (Invitrogen) and S17-1 (Simon *et al.*, 1983) were used in this study (Table 1). They were grown in LB medium in the presence of appropriate antibiotics as described by Sambrook *et al.* (1989). *Methylococcus capsulatus* Bath was grown on nitrate mineral salts medium (NMS) (Whittenbury & Dalton, 1981) in batch culture at 42 °C with or without copper added as CuSO₄·5H₂O (10 μ M final concentration), with a 1:1 (v/v) CH₄/air ratio in the head space, or on agar plates with the same medium. In some cases

NMS without copper, with 0.5 mM ferrous sulfate and 1 mM formate (NMSF) was used to grow bacteria with sMMO expressed. All *M. capsulatus* Bath mutants were grown from frozen cultures and tested by diagnostic PCR after growth to ensure that the insertion mutations were correct.

DNA manipulations. Plasmid isolation, *E. coli* transformation, restriction enzyme digestion, ligation, blunting ends with T4 DNA polymerase, or filling in ends with Klenow enzyme were carried out as described by Sambrook *et al.* (1989). The chromosomal DNA of *M. capsulatus* Bath grown in batch culture was isolated by the procedure of Marmur (1961). For the insertion mutants, chromosomal DNA was isolated from cells grown on agarose plates. In this case, cells were washed from the agarose surface using 3 ml TEN (50 mM Tris/EDTA + 150 mM NaCl) and the liquid was collected in 25 ml Corex tubes. Tubes were spun for 5 min at 10000 *g* and 4 °C and the supernatant poured off. The pellet was resuspended by adding 3 ml TEN with 4 mg lysozyme ml⁻¹ and incubated at 37 °C for 1 h. Next, 3 ml TEN with 4% (w/v) SDS was added to the tubes and incubated in a 45–50 °C water bath until the solution was clear (approximately 30 min). DNA was extracted using phenol and precipitated using ethanol by standard procedures (Sambrook *et al.*, 1989).

DNA sequencing. DNA sequencing was carried out by the Caltech Sequencing Facility and the University of Washington Sequencing Facility, with an Applied Biosystems automated sequencer, from both strands.

DNA–DNA hybridization. DNA–DNA hybridizations were carried out with dried agarose gels as described by Meinkoth & Wahl (1984) at 68 °C for DNA fragment probes or 42 °C for oligonucleotide primer probes. For hybridizations, 6 \times SSC (1 \times SSC is 0.15 M NaCl and 0.015 M sodium citrate) was used; for washes, 0.5 \times SSC was used. Dried gels are not appropriate for DNA fragments less than 0.8 kb, and for these gels, nucleic acids were transferred to a nylon membrane (HybondN⁺) by capillary blotting and treated according to the vendor's recommendation. For DNA labelling, a random-primed DNA labelling kit (Amersham) or direct nucleic acid labelling and detection systems (Amersham) were used for radioactive or non-radioactive labelling, respectively. T4 polynucleotide kinase (Boehringer Mannheim) was used for oligonucleotide labelling.

PCR. PCR was performed in 50 μ l reaction mixtures in 0.5 ml microcentrifuge tubes. Reactions were carried out in a Perkin-Elmer GeneAmp PCR System 9600 thermal cycler. Thirty cycles of 92 °C for 1 min; 55 °C for 1 min; 72 °C for 1 min were performed followed by a final extension of 5 min at 72 °C to amplify fragments up to 3 kb. To amplify fragments more than 3 kb, the program recommended by Boehringer Mannheim for the Expand PCR System was used: 2 min at 92 °C, 10 cycles of 92 °C for 15 s, 57 °C for 30 s, 68 °C for 4 min and 15 cycles with elongation times of 20 s, with a final step of 72 °C for 7 min. *Taq* polymerase (Boehringer Mannheim) was used. Primers used were as follows. For the Km-cassette (accession no. X06404): KBF1 (5'-CGATAGATTGTCGCACCTG-3') and KBR2 (5'-CACTTTCTGGCTGG-ATG-3'). For amplification of *pmo* genes (accession no. L40804): CSS1F (5'-CAAACAATGGCAGCAACAAC-3') 118 to 138 bp, CSS2F (5'-CCTGTGGGTGCGGTGGTAC-3') 222 to 241 bp, CSSK4F (5'-GGCACTGGCCGTCATGGGCAC-3') 799 to 820 bp, CSS9F (5'-GCCACACCTTCTGGTTC-ATGG-3') 727 to 747 bp, CSS9R (5'-GCCTTCGTCCACG-GCTTC-3') 875 to 893 bp, CSS10R (5'-AGATGAACGCC-GCAAAAC-3') 601 to 618 bp, CSS16R (5'-GTGAGCCAC-TTCTTGTCACG-3') 172 to 190 bp, CSS17R (5'-GAGCT-

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant trait(s)	Source or reference
<i>E. coli</i>		
DH5 α	(ϕ 80 <i>dlacZ</i> Δ M15) Δ (<i>lacZY-argF</i>)U169 <i>deoR</i> <i>recA1 endA1 hsdR17</i> (r _k ⁻ m _k ⁺) <i>supE44 gyrA96</i> <i>relA</i>	Bethesda Research Laboratories
DH5 α MCR	DH5 α <i>mcrA</i> Δ (<i>mmr-hsdRMS-msrBC</i>)	Bethesda Research Laboratories
Inv α F'	(ϕ 80 <i>lacZ</i> Δ M15) Δ (<i>lacZYA-argF</i>) <i>deoR</i> ⁺ F' <i>recA1 endA1 hsdR17</i> (r _k ⁻ m _k ⁺) <i>supE44 gyrA96</i> <i>relA</i>	Invitrogen
S17-1	<i>thi pro recA hsdR</i> RP4-2- <i>tet</i> ::Mu <i>aphA</i> ::Tn7	Simon <i>et al.</i> (1983)
<i>M. capsulatus</i> Bath		
T401	Uncharacterized Tn5 chromosomal insertion	This study
MAK5	<i>pmoA1</i> :: Km ^r	This study
MAK16	<i>pmoA2</i> :: Km ^r	This study
MBK1-1	<i>pmoB1</i> :: Km ^r	This study
MBK206	<i>pmoB2</i> :: Km ^r	This study
MCK60	<i>pmoC1</i> :: Km ^r	This study
MCK62	<i>pmoC2</i> :: Km ^r	This study
MBS2	<i>pmoB1</i> :: Sm ^r	This study
MBS202	<i>pmoB2</i> :: Sm ^r	This study
Plasmids		
pUC18/19Ap ^r	<i>lacZ</i>	Yanisch-Perron <i>et al.</i> (1985)
pCR II	Ap ^r Km ^r <i>lacZ</i> α	Invitrogen
pCR2.1	Ap ^r Km ^r <i>lacZ</i> α	Invitrogen
pUC4K	Ap ^r Km ^r	Vieira & Messing (1982)
pAYC61	Ap ^r Tc ^r	Chistoserdov <i>et al.</i> (1994)
pBR325	Ap ^r Cm ^r Tc ^r	Bolivar (1978)
pBR325 Ω	pBR325:: Ω Sm ^r /Sp ^r	C. Murrell, University of Warwick, UK
pSUP2021	Ap ^r Km ^r	Simon <i>et al.</i> (1983)
pRK2013	Km ^r	Figurski & Helinski (1979)
pAMC100	pUC19 with 2.9 kb <i>KpnI</i> insert	Semrau <i>et al.</i> (1995)
pAMC101	pUC19 with 1.8 kb <i>KpnI</i> PstI insert	Semrau <i>et al.</i> (1995)
pAMC102	pUC19 with 2.5 kb <i>EcoRI</i> insert	Semrau <i>et al.</i> (1995)
pAMC103	pUC19 with 1.0 kb <i>EcoR</i> insert	This study

GCACCACCAATGGTTG-3') 135 to 155 bp, CSS19R (5'-GTACCACCGCACCTCAGG-3') 222 to 241 bp, ASS1F (5'-CTGGGACTTCTGGTCGGACTG-3') 1202 to 1223 bp. Copy 2 specific (accession no. U94337): ESSR (5'-CCTGACGTCTAAAATCCAGC-3') 3242 to 3262 bp. For amplification of *pmoC3* (accession no. AF091320): C3F1 (5'-GGCATCAATCCAACACC-3') 1 to 17 bp, C3R1 (5'-GCGGACGGTGGCTCCCTGG-3') 855 to 874 bp.

All sequence generated by PCR was confirmed from a second, separate amplification reaction.

Computer analysis. Translation and analyses of DNA and DNA-derived polypeptide sequences were carried out using Genetic Computer Group (Wisconsin) programs and the National Center for Biotechnology Information BLAST server using the BLAST algorithm (Altschul *et al.*, 1990, 1997).

Construction of insertion mutants affected in pMMO genes and a control strain. The Km^r cassette from pUC4K and the

Ω Sm^r/Sp^r cassette from pBR325 Ω were used as selective inactivating markers. pBR325 Ω was constructed in the laboratory of J. C. Murrell (University of Warwick, UK) using the omega fragment from pHP45-omega (Prentki & Kirsh, 1984). The constructs with mutated genes were subcloned into the suicide vector pAYC61 (Chistoserdov *et al.*, 1994) and introduced into *M. capsulatus* Bath by conjugation. *E. coli* S17-1 was used as donor. Matings were conducted as described by Stoljar *et al.* (1995). Donor and recipient cells (ratio 1/5–1/10) were mixed and resuspended in NMS medium, then cells were pelleted, resuspended by pipetting in 30–40 μ l NMS and plated as a spot on NMS agar. Plates were incubated at 37 °C for 48 h under methane/air, and then cells were transferred to selective plates. Mutants of *M. capsulatus* Bath were selected on plates with 50 μ g Km ml⁻¹ or 100 μ g Sm and Sp ml⁻¹ and screened on plates with 75 μ g Ap ml⁻¹ to test for vector absence. Ap-sensitive clones were examined by hybridization to DNA blots with gene-specific and vector-specific

probes to verify recombinational events, as well as by PCR amplification using primers for diagnostic regions.

A control strain was generated for comparative phenotypic studies in which a Tn5 (*Km^r* gene) was inserted into a random chromosomal location by using pSUP2021 and pRK2013 (as a helper plasmid). This strain shows normal growth on methane, and is able to grow on methane in the presence of kanamycin.

sMMO assay. sMMO activity was detected on plates using the naphthalene plate assay described by Graham *et al.* (1992). Maximum sMMO activity on plates was achieved when cells were grown on NMSF medium.

Whole-cell methane oxidation assay. Rates of whole-cell methane-dependent O₂ uptake were determined polarographically using an oxygen electrode chamber at 42 °C, as described by McNerney & O'Connor (1980). Cells were washed and resuspended in 50 mM HEPES buffer pH 7.0.

RESULTS

Sequencing of *pmoC1*

A 2.9 kb *KpnI* fragment of *M. capsulatus* Bath DNA had been previously cloned (pAMC100), and sequencing of the 3' region revealed *pmoA1* and part of *pmoB1* (Fig. 1; Semrau *et al.*, 1995). A 1.8 kb *KpnI-PstI* fragment containing most of *pmoA1* and 1 kb of the upstream region was subcloned into pUC19 to generate pAMC101 (Fig. 1), and the insert was sequenced. One ORF was found upstream of *pmoA1*. A region near the N-terminus starting 10 residues after the first methionine revealed an almost perfect match to an amino acid sequence (AAAEAPLKDKKWLTF) that had been determined by Edman degradation for the 23 kDa pMMO subunit (Nguyen *et al.*, 1998). The only discrepancy was that the underlined lysine was predicted by the nucleotide sequence to be a leucine. This ORF was designated *pmoC1* and is predicted to encode a polypeptide of 260 amino acids with a molecular mass of 29 000 Da, assuming it starts at the position determined by Edman degradation. A hydrophathy analysis of *pmoC1* predicts a minimum of three and a maximum of six membrane-spanning regions with no classical leader sequence at the N-terminus. A search of both protein and DNA databases revealed significant identity with *amoC* sequences from nitrifiers (Klotz *et al.*, 1997; Sayavedra-Soto *et al.*, 1998) (44–54% identity at the amino acid level).

Sequencing and analysis of the second copy of *pmo* genes

Hybridization experiments had suggested that part of the second copy of *pmoB* was located on a 1.0 kb *EcoRI* fragment (Semrau *et al.*, 1995). This fragment was cloned into pUC19 to generate pAMC103 (Fig. 1), and the insert was sequenced. This sequence showed high identity to the *pmoB1* sequence, and an ORF was present whose predicted product showed high identity to PmoB1. All attempts to clone the upstream *KpnI* and *EcoRI* fragments were unsuccessful, even with low-

copy-number vectors. It has been suggested that these gene products may be toxic in alternative hosts (Semrau *et al.*, 1995). Hybridization analysis of chromosomal DNA digested with different restriction enzymes and probed with *pmoA1*, *B1* and *C1* sequences demonstrated that the segment of chromosomal DNA upstream of *pmoB2* contained *pmoA* and *pmoC* sequences similar to the genes previously characterized. Therefore, the sequence of this region was obtained by PCR, using *Km*-insertion strains to target the second copy.

To obtain the missing sequences for the second copy of *pmoAB*, strain MBK206 containing a *Km* insertion in the *KpnI* site of *pmoB2* (Fig. 1; and see below) was used for amplification from the chromosome. The primers targeted the 5' end of *pmoA2* (ASSF1) and the *Km*-resistance cassette (KBF1), to amplify a fragment overlapping the unsequenced region. The use of the *Km*-resistance insertion mutant ensured that the amplified fragment was from copy 2, not copy 1. A PCR product with the expected size of 1.9 kb was obtained. This DNA fragment was cloned into the Invitrogen vector pCR2.1 and sequenced. As expected, it contained most of *pmoA2* and the 5' portion of *pmoB2*.

For the region containing *pmoC2*, probing experiments had shown that *pmoC2* and the 5' end of *pmoA2* were present on a 1.4 kb *Sall* fragment (data not shown). Assuming that *pmoC2* was similar in size to *pmoC1*, this *Sall* fragment should contain all of *pmoC2* and 0.4 kb of DNA 5' to *pmoC2*. Attempts to clone this *Sall* fragment were unsuccessful. Therefore, an inverse PCR procedure was used that involved the insertion mutant MCK62, which contains a *Km*-resistance gene in the *BbsI* site of *pmoC2* (Fig. 1; see below for details of mutant construction). First, the appropriate size fraction of *Sall*-digested MCK62 DNA was isolated from a gel. This pool of DNA was then ligated, which joined the *Sall* fragments into a circle. This DNA was then digested with *HindIII*, which cuts within the *Km*-resistance gene but not within the *pmoC2* region. This procedure separated *pmoC2* and its upstream region into two inverted parts flanked by portions of the *Km*-resistance cassette. It was then possible to amplify the entire region using appropriate primers targeted to the ends of the *Km*-resistance cassette (KBF1 and KBR2). A fragment of the correct size (2 kb) was obtained, and it was sequenced directly as a PCR product. Based on the known sequence for the first copy of *pmoCA* and for the *Km*-resistance cassette, we identified *pmoC2*, the *pmoC2*–*pmoA2* intergenic region and 170 bp of *pmoA*. The sequence of the 5' end of *pmoC2* and the region immediately upstream of the translational start site was confirmed by direct PCR from chromosomal DNA, using appropriate primers. These data completed the sequence of all three subunits of the second copy of the *pmo* genes.

We have also amplified 3.5 kb and 3.1 kb fragments containing the entire *pmoCAB2* gene cluster. The 3.5 kb fragment containing all three genes plus the known upstream region was amplified using a primer specific to

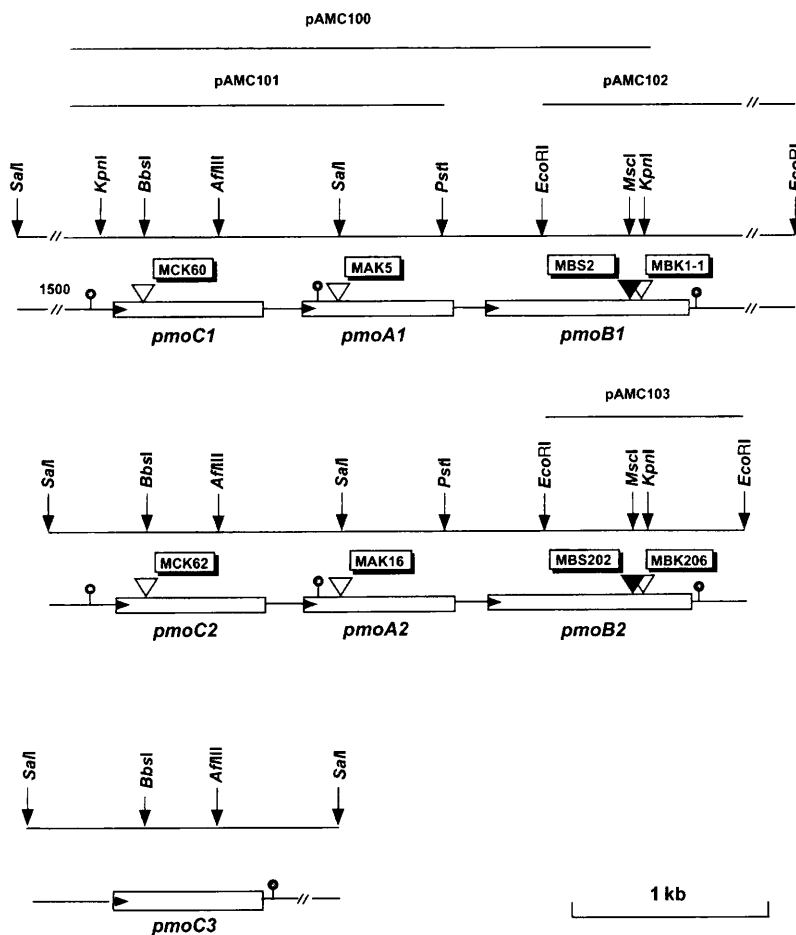


Fig. 1. Physical and genetic map of the fragments containing the *pmo* genes of *M. capsulatus* Bath. Triangles indicate sites of the Km (open) and Sm (filled) resistance genes insertions; the names of each insertion mutant are noted above each triangle. Lines above depict cloned fragments used in this study. Spheres indicate putative hairpin structures.

a region just inside the *SalI* site upstream of *pmoC2* and the ESSR1 primer complementary to the region immediately downstream of *pmoB2*. The primers used in amplifying the 3.1 kb fragment were CSS1F and ESSR1. This produced a fragment with the same 3' end as the 3.5 kb fragment, but with a shorter 5' end that begins 6 nucleotides upstream of the start codon for *pmoC2*. Therefore, both fragments contain all three ORFs, but the smaller one lacks a 0.4 kb 5' region. These fragments were the expected size, confirming that the *pmoCAB2* genes that have been sequenced are linked on the chromosome. We were able to clone the 3.1 kb fragment using the pCR2.1 vector but were unable to clone the larger fragment. In addition, we were able to clone a PCR product containing a 400 bp region upstream of the *pmoC2* translational start site. These results suggest that the difficulties in cloning these genes are related to expression of *pmoC2*.

The nucleotide sequence of the region containing the second copy of *pmoCAB* was almost identical to the first copy. Starting with the ATG identified for *pmoC1*, the sequences of *pmoC1* and *pmoC2* differ only in 3 nucleotides, which result in only one amino acid change, at residue 239, where PmoC1 contains an arginine and PmoC2 contains a serine (Fig. 2). However, the 125 bp sequence upstream of the start site for *pmoC1* showed

only 40% identity to the same region for *pmoC2*. The *pmoC*–*pmoA* intergenic region showed more divergence than the *pmoC* coding region, with differences in 5 of 164 nucleotides.

The two regions containing *pmoAB* were even more similar at the nucleotide sequence level, differing in only 5 out of 2236 nucleotides. The predicted amino acid sequence of PmoA1 differs from that of PmoA2 only at residue 173, where PmoA1 contains an asparagine while PmoA2 contains a tyrosine. Likewise, the predicted amino acid sequences of PmoB1 and PmoB2 differ only at residue 385, where PmoB1 contains a serine instead of an arginine in PmoB2. The *pmoA1*–*pmoB1* and *pmoA2*–*pmoB2* intergenic regions are identical. The sequences of the two gene copies begin to diverge 80 nucleotides downstream of the termination codon for *pmoB*, after which the identity drops to 35%. Immediately before this divergent point are conserved sequences that could form a double-hairpin structure with a calculated ΔG° of $-194.6 \text{ kJ mol}^{-1}$ ($-46.5 \text{ kcal mol}^{-1}$) for copy 1 and $-204.3 \text{ kJ mol}^{-1}$ ($-48.8 \text{ kcal mol}^{-1}$) for copy 2. This structure is a candidate for a transcriptional terminator. Because of the similarity in the sequences of the two copies, in all cases they were sequenced at least twice from different DNA preparations, and in each case the sequences agreed exactly.

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PmoC3 MATTTAGGIAAIDRLLLDKKWLVFAIGIYTVFYLVWRWYEGVYGSAGLDSFAPEFETYW
PmoC2  . . A . . I . . A . . AEAP . . . . . T . . LA . . . . .
PmoC1  . . A . . I . . A . . AEAP . . . . . T . . LA . . . . .

MNFlyTEIVLEIVTASILWGylWkTRDRNLAAALTPREELRRNFTHLVWLVAYAWAIYWGA
.....
.....

SYFTEQDGtWHQTIIVRDtDFTPSHIIeFYLSYPIYIITGFAAFIYAKTRLPPFFAKGISLP
.....
.....

YLVLVVGPFMILPNVGLNEWGHtFwFMEELFVAPLHYGFVIFGWLALAVMGLTQTfYSF
.....
.....R.

241  SHL FERD-LCPDIR 253
      AQGGLGQS . . EAVDEGLIAK 260
      AQGGLGQS . . EA

```

Fig. 2. Comparison of the three translated products of the *pmoC* genes. Periods denote residues identical to the PmoC3 sequence; a dash denotes a gap.

Construction of *pmo* gene disruptions and phenotypic characterization of mutants

For the *pmoA* mutants, Km-resistance insertions were generated in the *Sall* site of *pmoA1* in pAMC101, and were used in allelic exchange experiments to obtain insertions in the chromosome for both *pmoA1* and *pmoA2*. For the *pmoC* insertions, Km-resistance insertions were generated in the *BbsI* site of *pmoC1* in pAMC101, and were used to obtain insertions in the chromosome for both *pmoC1* and *pmoC2*. For the *pmoB* mutants, Km-resistance insertions were generated in the *KpnI* site of *pmoB2* in pAMC103, but in this case chromosomal insertions were only obtained in *pmoB2*. Therefore, a second construction was generated with a Km-resistance insertion in the *KpnI* site of *pmoB1* in pAMC102 and was used to generate chromosomal insertions in *pmoB1*. Each of these recombination events was confirmed by hybridization and/or by PCR of specific *pmo*-Km cassette fragments. The hybridization experiments also confirmed previous results (Semrau *et al.*, 1995) showing that two copies of *pmoA* and *pmoB* were present.

Mutants were isolated on plates designed either for pMMO or for sMMO expression, and tested for growth on both types of media. For pMMO expression, plates of NMS medium plus copper were used. For sMMO expression, several types of medium were tested with the wild-type, and it was determined by the naphthalene colorimetric assay that maximal sMMO expression could be achieved with NMS plates containing no added copper, but with added formate and iron (NMSF medium; see Methods). All of these mutants grew on methane on both types of media. On plates of NMSF medium, it was shown by the naphthalene colorimetric assay that the wild-type and the mutants exhibited substantial sMMO activity, which appeared similar by visual examination. On plates of NMS medium containing copper, none of the strains showed detectable sMMO activity, and were assumed to be expressing pMMO.

All of the mutants showed some genetic instability, due to recombination between the gene copies. Therefore, for phenotypic characterization, transfers were kept to a

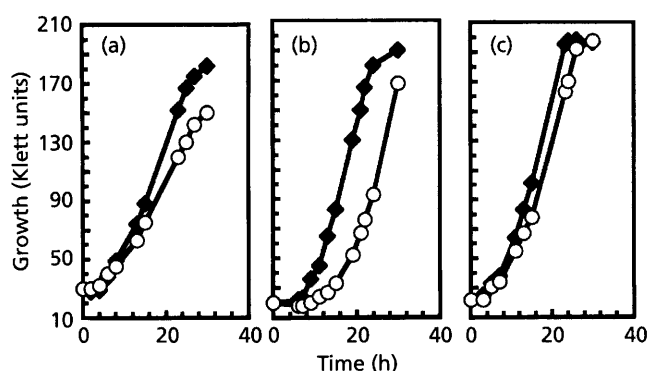


Fig. 3. Growth curves, determined from Klett readings with a no. 6 filter, of the insertion mutants for the *pmo* genes. (a) *pmoC1*:Km (◆) and *pmoC2*:Km (○); (b) *pmoA1*:Km (◆) and *pmoA2*:Km (○); (c) *pmoB1*:Km (◆) and *pmoB2*:Km (○). Growth curves for the T401 strain (wild-type containing a Tn5 insertion) were the same as for the copy 1 mutants.

minimum and cultures were inoculated from plates that had been streaked directly from frozen stocks. To ensure that the cultures used for phenotypic characterization carried the appropriate mutation, all cultures were tested after growth for the correct insertion by diagnostic PCR.

In broth culture, the growth rate of the mutants defective in the copy 1 genes in the presence of kanamycin was not significantly different from that of a wild-type strain containing a Km-resistance marker, strain T401 (Fig. 3; Table 2). However, the growth rate of the mutants with insertions in the copy 2 genes was consistently 15–20% slower than the wild-type or the copy 1 mutants, and longer lag phases were common. Methane-dependent O₂ uptake rates were determined in mutant and wild-type strains, as a measure of pMMO activity. Mutants in the copy 1 genes had rates that were 65–85% of the wild-type rate. However, mutants in copy 2 genes showed lower rates, 37–50% of the wild-type rate (Table 2).

Attempts were made to generate double mutants in *pmoB* using both Km- and Sm-resistance cassettes. A

Table 2. Doubling time and methane oxidation rates for wild-type and *pmo* mutants

Strain	Affected gene	Doubling time (h)*	CH ₄ oxidation [nmol (mg protein) ⁻¹ min ⁻¹]
T401 (Km ^r wild-type)		7.8 ± 1.0	351
MCK60	<i>pmoC1</i>	6.6 ± 2.1	299
MCK62	<i>pmoC2</i>	9.2 ± 1.3	177
MBK1-1	<i>pmoB1</i>	8.0 ± 0.4	232
MBK206	<i>pmoB2</i>	9.3 ± 2.1	130
MAK5	<i>pmoA1</i>	5.5 ± 2.1	225
MAKF016	<i>pmoA2</i>	8.8 ± 1.2	165

* Measurements are means and standard deviations for triplicate cultures grown in the presence of kanamycin.

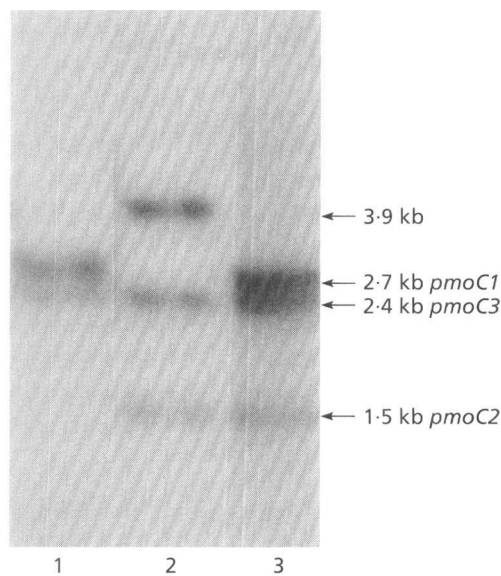


Fig. 4. DNA blot showing hybridization of *SalI*-digested *M. capsulatus* Bath chromosomal DNA to a *pmoC1* probe (the *KpnI*–*SmaI* fragment of pAMC101). 1, MCK62 (*pmoC2* insertion strain); 2, MCK60 (*pmoC1* insertion strain); 3, wild-type.

Sm-resistance cassette was inserted into the *MscI* site within *pmoB2* in pAMC103, and an *EcoRI* fragment with the disrupted *pmoB2* sequence was recloned into the suicide vector pAYC61. The MBK1-1 mutant carrying a Km insertion in *pmoB1* was used as a recipient, and selection was carried out on both NMS and NMSF plates. Although colonies were obtained on both types of media that were resistant to both Sm and Km, most of them showed decreased growth on plates with each transfer, and were not viable after three to four transfers. Those that were able to grow through multiple transfers were Ap-resistant, suggesting they were single-crossover recombinants containing a complete *pmoB* gene. Hybridization analysis confirmed the suspected recombination events (data not shown). When the same Sm-resistance construction was used in wild-type, double-crossover recombinants were obtained

separately in *pmoB1* and *pmoB2*, demonstrating that the Sm-resistance plasmid was functional for generating insertion mutations. These mutants were also used as recipients for constructions containing Km insertions into each *pmoB* gene, and in these cases also, no Ap-sensitive clones were obtained.

Third copy of *pmoC*

As noted above, all of the insertion mutants were analysed by hybridization with probes for the genes that were disrupted and for the Km-resistance cassette. In the case of the *pmoA* and *pmoB* mutants, these experiments produced the expected results and confirmed the presence of two copies. However, hybridization of *SalI*-digested *M. capsulatus* Bath chromosomal DNA to a *pmoC1* probe (the *KpnI*–*SmaI* fragment of pAMC101) revealed three bands of hybridization of 1.5, 2.4 and 2.7 kb (Fig. 4). Because it was known that neither *pmoC1* nor *pmoC2* contained a *SalI* site, these results suggested the presence of a third copy of *pmoC* in the genome of *M. capsulatus* Bath. This idea was supported by results from hybridization of *SalI*-digested DNA from the insertion mutants MCK60 and MCK62, which contain a Km-resistance insertion in *pmoC1* and *pmoC2*, respectively. In the *pmoC1* mutant, hybridization with the *pmoC1* probe showed that the 2.7 kb band was increased in size about 1.3 kb, as expected for the insertion, while the other two bands remained the same. In the *pmoC2* mutant, hybridization with the same probe showed that the 1.5 kb band increased to about 2.8 kb, as expected, and the other two bands were not well resolved (Fig. 4). These results suggested that the 2.4 kb band contained a third copy of *pmoC*. *SalI*-digested chromosomal DNA from the MCK60 mutant (containing an insertion in *pmoC1*) was hybridized with primers specific for *pmoC* sequence (CSS16R, CSS17R, CSSK4F, CSS9R and CSS10R) as well as the *pmoA*-specific primer ASS1F. As expected, all of the primers hybridized to the bands corresponding to copy 1 and copy 2, and most of the *pmoC*-specific primers hybridized to the 2.4 kb band containing the putative *pmoC3*. However, CSS17R and ASS1F did not hybridize

to the 2.4 kb *Sall* fragment or to any new fragments. These results suggested that a third copy of *pmoA* is not present and that significant divergence of the *pmoC3* sequence occurs in the N-terminal region. Therefore, we used an inverse PCR strategy similar to that used for the copy 2 genes to obtain the N-terminal, C-terminal and upstream regions of *pmoC3*.

Sall-digested DNA from MCK60 was ligated and redigested with *Afl*III. This DNA was used as a template for PCR with the CSS19R and CSS9F primers. A PCR product of the predicted size was obtained, cloned into pCR2.1 vector and sequenced, providing the sequence of the flanking regions. PCR primers (C3F1 and C3R1, see Methods) were then designed to amplify the whole ORF of *pmoC3* and complete the sequence. An ORF of 253 amino acids was identified with high identity to the other copies of *pmoC* in the central region but containing divergence at the N- and C-termini (Fig. 2).

The entire ORF for *pmoC3* was generated by PCR and cloned into the pCR2.1 vector. However, only plasmids containing *pmoC3* in the orientation opposite to the direction of transcription from the pCR2.1 *lac* promoter were obtained. Attempts were made to generate insertion mutations in *pmoC3*, using the *Bbs*I site for a Km^r cassette and the cloned PCR product containing the entire *pmoC3*. Matings were tested on both NMS plates with copper (for pMMO expression) and NMSF plates (for sMMO expression). However, only single-cross-over insertions were obtained, which generated an intact *pmoC3*. These mutants grew normally on methane.

DISCUSSION

The results in this report complete the sequence of two sets of *pmoCAB* genes, and provide the sequence of a third copy of *pmoC*. For the first two sets, the sequences are almost identical within the coding and intergenic regions, but they diverge outside the *pmoCAB* regions. For *pmoC3*, the sequence diverges in the N- and C-terminus of the encoded protein (Fig. 2). The genetic organization and numbers of gene copies for this *pmo* system are highly similar to those observed for *amo* genes in *Nitrosomonas europaea* (Hommes *et al.*, 1998; Sayavedra-Soto *et al.*, 1998), but other nitrifiers have three sets of *amo* genes (Norton *et al.*, 1996; Sayavedra-Soto *et al.*, 1998). The role of these multiple gene copies has not been well understood in either methanotrophs or nitrifiers. Methanotrophs such as *M. capsulatus* Bath have two alternative methane oxidation systems, the pMMO and the sMMO. Therefore, the physiological role of pMMO is different in these methanotrophs from that of ammonia monooxygenase in nitrifiers, since nitrifiers do not contain an alternative ammonia oxidation system.

In this study, double-crossover insertion mutants were isolated for each *pmo* gene found in the two sets of complete *pmoCAB* clusters, to assess the contribution of each gene copy to pMMO activity. These mutants all grew on methane in the presence of copper and in the absence of detectable sMMO expression, suggesting

that pMMO must be produced from each set of genes under these growth conditions. This conclusion is confirmed by the results of the methane oxidation rate experiments in mutants and wild-type, which showed that about a third of the whole-cell methane oxidation is due to copy 1 genes, while about two-thirds is due to copy 2 genes. The presence of the two copies of *pmo* genes apparently gives the cells excess methane oxidation capacity, since impairment of the copy 1 genes did not affect growth. However, impairment of the copy 2 genes did result in a small growth defect, suggesting that cells need the copy 2 gene expression for normal growth.

These results are similar in some respects to the results obtained so far for the *amo* system in nitrifying bacteria. In *N. europaea*, mutants have only been generated in *amoA* gene copies, so no information is available for *amoB* or *amoC* mutants. For *amoA*, mutants defective in each gene were viable, but mutants in one copy showed decreased growth while the other mutants showed normal growth (Hommes *et al.*, 1998), similar to the results we report here for *pmoA* mutants. However, in the nitrifier the whole-cell ammonia oxidation rate did not change significantly in the mutant with normal growth rate, suggesting that under these conditions the wild-type ammonia monooxygenase activity could be sustained by the other gene copy. This is in contrast to our results, in which both copies are required to maintain wild-type pMMO activity.

Since the two clusters of *pmo* genes are so similar in sequence, it seems likely that the enzymes produced from each copy have a similar function. Therefore, the role of the duplicate gene clusters may be simply to provide increased gene expression, although it appears that this higher level of pMMO activity is not necessary for maximal growth under laboratory conditions. It may be that the two copies are important for different types of growth conditions, for instance under stress conditions that might be encountered in the natural environment. It is also possible that the two copies are differentially expressed under different growth conditions, since the sequences upstream of *pmoC1* and *pmoC2* are highly divergent. Further work involving promoter identification and expression will be required to resolve these questions.

We have also examined *pmoC3*, since the role of this separate *pmoC* gene is unknown and in *M. capsulatus* Bath it has a more divergent sequence than the other two *pmoC* copies. However, we were unable to isolate a null mutant in *pmoC3*. We cannot be certain whether this failure is due to an unknown technical problem that caused double-crossover recombination to occur at extremely low frequencies, or whether it is due to the essential nature of *pmoC3* under the tested growth conditions. However, since we were able to isolate null mutants in *pmoC1* and *pmoC2* using a similar procedure and similar constructions, it is likely that *pmoC3* null mutants are not viable. Therefore, our results suggest that *pmoC3* has an essential function in methane oxidation separate from the function of *pmoC1* and

pmoC2. The role of this separate copy of *pmoC* is not yet clear, but it probably fulfils a function necessary for generating active pMMO from both *pmo* clusters. It is unlikely that this function is to provide PmoC for pMMO, since the major transcript from the two clusters contains all three genes (*pmoCAB*) and it is likely that all three are translated (Nielsen *et al.*, 1997).

Since *M. capsulatus* Bath contains an alternative methane oxidation system (sMMO), it should be possible to obtain a pMMO-negative strain under conditions in which sMMO is expressed. We developed plate growth conditions that maximized expression of sMMO, and attempted to generate a double mutant that contained double-crossover (null) mutations in both *pmoB* genes. However, all isolates always contained one wild-type *pmoB*, the result of a single-crossover recombination event. It is unlikely in this case that a technical problem occurred, because null mutants were readily obtained in each *pmoB* copy individually using both markers. Similar attempts to obtain double mutants in *pmoA* were also unsuccessful (unpublished data). It is known that *M. capsulatus* Bath can grow quite well in broth culture under conditions in which sMMO is the dominant methane oxidation enzyme, with only a small amount of pMMO present (Prior & Dalton, 1985a; Zahn & DiSpirito, 1996). Therefore, it is not clear why null mutants could not be obtained on plates. It is possible that the sMMO levels achieved on plates were too low to allow for growth, although they appeared to be substantial as judged by the colorimetric plate assay. Alternatively, it is possible that low level pMMO expression is required for growth on methane on plates even when sMMO is expressed.

ACKNOWLEDGEMENTS

This research was supported by a grant from the NSF (MCB9630645) and a University Research Initiative grant from ARPA (N00014-92-J-1901). The plasmid pBR325 Ω was kindly provided by Dr J. C. Murrell. We thank Drs L. Chistoserdova, R. Meima and H. Toyama for helpful discussions.

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Received 13 January 1998; accepted 28 January 1999.