Genes involved in the copper-dependent regulation of soluble methane monooxygenase of *Methylococcus capsulatus* (Bath): cloning, sequencing and mutational analysis

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The key enzyme in methane metabolism is methane monooxygenase (MMO), which catalyses the oxidation of methane to methanol. Some methanotrophs, including *Methylococcus capsulatus* (Bath), possess two distinct MMOs. The level of copper in the environment regulates the biosynthesis of the MMO enzymes in these methanotrophs. Under low-copper conditions, soluble MMO (sMMO) is expressed and regulation takes place at the level of transcription. The structural genes of sMMO were previously identified as *mmoXYBZ, mmoD* and *mmoC*. Putative transcriptional start sites, containing a **σ**70- and a **σ**N-dependent motif, were identified in the 5′ region of *mmoX*. The promoter region of *mmoX* was mapped using truncated 5′ end regions fused to a promoterless green fluorescent protein gene. A 9–5 kb region, adjacent to the sMMO structural gene cluster, was analysed. Downstream (3′) from the last gene of the operon, *mmoC*, four ORFs were found, *mmoG, mmoQ, mmoS* and *mmoR*. *mmoG* shows significant identity to the large subunit of the bacterial chaperonin gene, *groEL*. In the opposite orientation, two genes, *mmoQ* and *mmoS*, showed significant identity to two-component sensor–regulator system genes. Next to *mmoS*, a gene encoding a putative **σ**N-dependent transcriptional activator, *mmoR* was identified. The *mmoG* and *mmoR* genes were mutated by marker-exchange mutagenesis and the effects of these mutations on the expression of sMMO was investigated. sMMO transcription was impaired in both mutants. These results indicate that *mmoG* and *mmoR* are essential for the expression of sMMO in *Mc. capsulatus* (Bath).

**INTRODUCTION**

Methanotrophs are a subgroup of methylotrophic bacteria, which have the distinctive property of utilizing methane as their sole source of carbon and energy. The key enzyme in methane metabolism is methane monooxygenase (MMO), which catalyses the oxidation of methane to methanol. Some methanotrophs, including *Methylococcus capsulatus* (Bath), possess two distinct MMOs (Lipscomb, 1994). The biosynthesis of the MMO enzymes in these methanotrophs is regulated by the level of copper available to the cells. When methanotrophs are grown at a high copper-to-biomass ratio, the membrane-bound, particulate MMO (pMMO) is synthesized. pMMO (Zahn & Dispirito, 1996) is present in all known methanotrophs (reviewed by Hanson & Hanson 1996). At a low copper-to-biomass ratio, some methanotrophs produce a soluble, cytoplasmic methane-oxidizing enzyme (sMMO), e.g. *Mc. capsulatus* (Bath) (Stanley et al., 1983), *Methylosinus trichosporium* OB3b (Lipscomb, 1994) and *Methylomonas* (Shigematsu et al., 1999).

Regulation of copper-dependent MMO expression takes place at the level of transcription (Nielsen et al., 1997; Murrell et al., 2000). The intriguing and strong copper regulation of the expression of MMO enzymes has been the subject of several studies, but details of the mechanism of regulation remain to be elucidated. A DNA fragment was isolated from *Mc. capsulatus* (Bath) (Stainthorpe et al.,...
1990), containing the gene cluster encoding sMMO, consisting of \textit{mmoXYBZDC}. It occurs in a single copy in the genome of \textit{Mc. capsulatus} (Bath) and its transcription is initiated from a putative promoter possessing \(\sigma^70\) and \(\sigma^3\) recognition sites (Nielsen \textit{et al}., 1997). pMMO is encoded by the \textit{pmoCAB} gene cluster. \textit{Mc. capsulatus} (Bath) harbours two almost identical sets of the \textit{pmoCAB} cluster (Semrau \textit{et al}., 1995). In addition, a third \textit{pmo} gene has been detected (Stolyar \textit{et al}., 1999). The physiological significance of multiple gene copies is unclear. However, the expression and activity of pMMO in \textit{Mc. capsulatus} (Bath) depends on the availability of copper (Stanley \textit{et al}., 1983; Nielsen \textit{et al}., 1997; Stolyar \textit{et al}., 1999). The upstream (3') regions of the \textit{pmo} gene clusters appear to contain \(\sigma^70\)-binding sites (Stolyar \textit{et al}., 2001; Gilbert \textit{et al}., 2000). In addition to down-regulation of the biosynthesis of sMMO, copper ions also inhibit the activity of sMMO in the cell (Green \textit{et al}., 1985; Jahng \& Wood, 1996). A completely copper-deficient environment, which would favour the expression of sMMO, rarely exists in nature (Forstner \& Wittman, 1979), therefore pMMO is likely to be functional in most natural environments (Phelps \textit{et al}., 1992; Hanson \& Hanson, 1996). This is disadvantageous for potential bioremediation applications since sMMO has much wider substrate specificity than pMMO (Burrows \textit{et al}., 1984). sMMO is capable of degrading a number of recalcitrant and carcinogenic hazardous chemicals (reviewed by Hanson \& Hanson, 1996), hence for biotechnological exploitation, control over the copper-regulated expression of MMOs would be desirable. In this study we aimed to identify the genes involved in the copper-dependent regulation of sMMO expression in \textit{Mc. capsulatus} (Bath). \textit{mmoG}, \textit{mmoQ}, \textit{mmoS} and \textit{mmoR} were identified downstream (3') from \textit{mmoXYBZDC}, and the phenotypic characterization of mutant strains deficient in \textit{mmoG} and \textit{mmoR} genes was carried out.

**METHODS**

**Growth media and strains.** Bacterial strains used in this work are listed in Table 1. Strains were routinely grown in nitrate mineral salts (NMS) medium (Whittenbury \& Dalton, 1981) containing 5-0 \(\mu\)M CuSO\(_4\). Low-copper medium was prepared without adding CuSO\(_4\). Growth of \textit{Mc. capsulatus} (Bath) under these low-copper conditions allows sMMO expression in \textit{Mc. capsulatus} (Bath). Solid medium was prepared by the addition of agar (Gibco) (1\%, w/v) and plates were incubated in anaerobic jars under a CH\(_4\)/air/CO\(_2\) (50:48:2\%, by vol.) gas mixture for 5–7 days. Liquid cultures (50 ml) were grown in 500 ml flasks fitted with rubber Suba Seals. The headspace was filled with the same gas mixture. All cultures of \textit{Mc. capsulatus} (Bath) were grown at 45\(^\circ\)C. \textit{Escherichia coli} strain DH5\(\alpha\) was used for propagating plasmids. Antibiotic resistance was selected by the addition of kanamycin (Km, 25 \(\mu\)g ml\(^{-1}\)) or gentamicin (Gm, 10 \(\mu\)g ml\(^{-1}\)), as required.

**Conjugation and construction of mutant strains.** For conjugation, the \textit{E. coli} S17-1 \textit{pir} mobilizer strain (Herrero \textit{et al}., 1990) was used as the donor strain after transformation with the appropriate plasmid. Reporter constructs and plasmids for mutagenesis were introduced into \textit{Mc. capsulatus} (Bath) by conjugation. Donor (\textit{E. coli}) and recipient (\textit{Mc. capsulatus}) cells were harvested when the cell densities reached 5 \times 10\(^8\) cells ml\(^{-1}\). Two millilitres of donor and 30 ml recipient culture were mixed and resuspended in NMS medium. Cells were then pelleted (13000 \(g\), 4 \(\degree\)C, 3 min) and resuspended in 200 \(\mu\)l NMS. The resulting cell suspension was plated as a spot on a 0.2 \(\mu\)m pore size nitrocellulose filter disc (Reanal) and placed onto an NMS plate containing 0-02% (w/v) protease peptone. Plates were incubated for 16 h at 37 \(\degree\)C under a CH\(_4\)/air/CO\(_2\) (50:48:2\%, by vol.) gas mixture. Transconjugants were subsequently selected on NMS agar plates, supplied with the appropriate antibiotics (Gm, 10 \(\mu\)g ml\(^{-1}\); Km, 25 \(\mu\)g ml\(^{-1}\)). Correct insertion of the Gm resistance gene (aac\(\text{C-I}\)) was tested by Southern blotting and DNA–DNA hybridization. Genomic DNA from Km-sensitive and Gm-resistant clones was purified and digested with appropriate restriction enzymes. Southern blotting (Sambrook \textit{et al}., 1989) was used to transfer the DNA onto Hybond-N+ (Amersham) membrane. DNA–DNA hybridizations were carried out with gene-specific and vector-specific probes as described by Sambrook \textit{et al}., 1989). The correct insertion and recombination events were confirmed by PCR, using primers specific for the appropriate DNA fragments (Table 1).

**DNA manipulations.** DNA manipulations were performed according to Sambrook \textit{et al}., 1989). Dynazyme polymerase (Finnzymes) was used in routine PCR experiments and Pfu polymerase (Fermentas) was used when required (due to its proof-reading ability), according to the manufacturers’ instructions. The identities of cloned PCR products were confirmed by DNA sequencing. Sequencing was done on both strands with an Applied Biosystems 373 Stretch automatic DNA sequencer.

**In silico analysis.** Homology searches in the GenBank database were carried out using the BLAST, BLASTX and BLASTP programs (http://www.ncbi.nlm.nih.gov/). Protein motifs were identified by PROSCAN (http://npsa-pbil.ibcp.fr). Promoters were predicted by Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html). Amino acid sequences were aligned using CLUSTAL W software (Thompson \textit{et al}., 1997). Homology searches in the TIGR unfinished microbial genomes database were carried out using the TIGR BLAST search engine for unfinished microbial genomes: http://tigrblast.tigr.org/umfg/ Preliminary sequence data were obtained from the TIGR website at http://www.tigr.org. Membrane topologies of polypeptides were predicted by the TMHMM (version 2.0) server (http://www.cbs.dtu.dk/services/TMHMM/).

**Isolation of total RNA.** Total RNA was isolated from 4 ml aliquots of \textit{Mc. capsulatus} (Bath) batch cultures grown to a cell density of 10\(^6\) cells ml\(^{-1}\). Cells were centrifuged at 12000 \(g\) and resuspended in 300 \(\mu\)l SET buffer [20\% (w/v) sucrose, 50 mM EDTA (pH 8-0), 50 mM Tris/HCl (pH8-0)]. Lysis buffer (300 \(\mu\)l) [20\% (w/v) SDS, 1\% (w/v) (NH\(_4\))\(_2\)SO\(_4\), pH 4-8] was added and tubes were briefly mixed by inversion until the solution become clear. Cell debris and most of the DNA was precipitated by the addition of 300 \(\mu\)l saturated NaCl solution, followed by centrifugation (12000 \(g\) for 30 min). RNA was precipitated from the supernatant by the addition of 500 \(\mu\)l 2-propanol (Sigma) and collected by centrifugation at 12000 \(g\) for 30 min. The pellet was washed twice with 70\% (v/v) ethanol, resuspended in H\(_2\)O and analysed on a 1-5\% (w/v) agarose gel.

**RT-PCR.** Total RNA extracts from \textit{Mc. capsulatus} (Bath) were treated with DNase I (Amersham). Total RNA (1 \(\mu\)g) was added to 30 pmol reverse primer mxatgrev (Table 1) in 9 \(\mu\)l water. Samples were incubated at 70 \(\degree\)C for 15 min then cooled on ice. Four microlitres dNTPs, 4 \(\mu\)l M-MLV 5 \(\times\) buffer, 0-5 \(\mu\)l RNasin ribonuclease inhibitor (Promega) and 1 \(U\) M-MLV reverse transcriptase (Promega) were mixed with 11 \(\mu\)l DNase-treated RNA and incubated at 37 \(\degree\)C for 1 h, followed by 5 min at 70 \(\degree\)C to inactivate the enzymes. A sample (2-5 \(\mu\)l) of the reverse transcription reaction was used as
Table 1. Bacterial strains, plasmids and primers used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid/primer</th>
<th>Characteristics</th>
<th>Reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<td><em>Mc. capsulatus</em> (Bath)</td>
<td>recA thi pro hsdR&lt;sup&gt;+&lt;/sup&gt; RP4-2-Tc::Mu Km::Tn7 &lt;i&gt;spir&lt;/i&gt;</td>
<td>Whittenbury et al. (1970)</td>
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<tr>
<td><em>E. coli</em> S17-1 &lt;i&gt;spir&lt;/i&gt;</td>
<td>recA1 endA1 gyrA96 thi-1 relA1 hsdR17 (supE44 ΔlacU169) (Φ80lacZΔM15)</td>
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<td><em>E. coli</em> DH5α</td>
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<td><strong>Plasmids</strong></td>
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<td>Ap&lt;sup&gt;R&lt;/sup&gt; cloning vector</td>
<td>Stratagene</td>
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<td>pK18mob</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt; RP4 mob, mobilizable cloning vector</td>
<td>Schäfer et al. (1994)</td>
</tr>
<tr>
<td>pK18mob sac</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt; RP4 mob, mobilizable cloning vector with sac&lt;sup&gt;B&lt;/sup&gt; gene for positive selection</td>
<td>Schäfer et al. (1994)</td>
</tr>
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<td>p34S-Gm</td>
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<td>Dennis &amp; Zylstra (1998)</td>
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<td>pPROBE-NT</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;, promoter-probe vector with EGFP</td>
<td>Miller et al. (2000)</td>
</tr>
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<td>Stainthorpe et al. (1989)</td>
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<td>HKrev</td>
<td>5′-ATCCGCGAGATCCTACCAACGGC-3′</td>
<td>mmox&lt;sup&gt;S&lt;/sup&gt;</td>
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</table>

template in the PCR reaction (one cycle of 96 °C for 1 min, followed by 30 cycles of 96 °C for 30 s; 60 °C for 30 s; 72 °C for 30 s followed by a final extension of 10 min at 72 °C). PCR products were analysed by agarose gel electrophoresis.

**SDS-PAGE and Western hybridization.** Cells were harvested at 13 000 <i>g</i> for 2 min, resuspended in 1 ml 50 mM Tris/HCl, pH 7-0, and were broken by repeated freeze–thaw cycles in liquid nitrogen. Intact cells were removed by centrifugation (13 000 <i>g</i>, 15 min). The cell-free extract was separated into membrane and soluble fractions by centrifugation at 100 000 <i>g</i> for 1 h. The soluble fraction was removed and the membrane fraction was resuspended in 100 µl 50 mM Tris/HCl, pH 7-0. Samples were stored at −20 °C. Protein concentration was determined using the Bio-Rad protein assay kit. Samples were analysed on 12 % (w/v) SDS-polyacrylamide gels stained with Coomassie brilliant blue. Western blots (Sambrook et al., 1989) were challenged with an antiserum raised against the hydroxylase component of sMMO.

**sMMO activity measurement.** Approximately 50 mg crushed naphthalene crystals was added to a 3 ml batch culture of *Mc. capsulatus* (Bath) and this mixture was shaken at 45 °C for 1 h. The cells were removed by centrifugation at 12 000 <i>g</i> for 2 min; 50 µl 5 mg <i>o</i>-dianisidine ml<sup>−1</sup> solution (Sigma) was added to the supernatant and colour formation was monitored by measuring the absorbance change at 530 nm. All measurements were done in triplicate. The sMMO activity of *Mc. capsulatus* (Bath) colonies was visualized by the addition of naphthalene crystals to the plates. Plates were incubated at 45 °C for 1 h and then 50 µg <i>o</i>-dianisidine ml<sup>−1</sup> (Sigma) solution was layered onto the surface of the plate. The deep purple colour of the colonies indicated sMMO activity.
Green fluorescent protein (GFP) activity measurement. GFP fluorescence was measured using a Quanta Master QM-1 fluorimeter (Photon Technology International) at an excitation wavelength of 490 nm, an emission wavelength of 510 nm and emission/excitation slit widths of 8 nm. Intensity readings are represented by arbitrary units and were normalized to a cell density of 10⁸ cells ml⁻¹. The mean±SD values of three independent assays are indicated in the Results.

Cloning and sequencing of smmo gene downstream region. The 5·4 kb sequence downstream from smmoC was already cloned on pCH4 (Stainthorpe et al., 1989, 1990). pCH4 was digested with Bsp120I and the resulting 1·1 and 4·2 kb fragments were isolated. The 1·1 kb fragment was ligated into Bsp120I-digested pBluescript2SK++, resulting in pBX2. The 4·2 kb fragment was ligated into Bsp120I-digested pBLR1MCS-4, resulting in pMCS441 (Fig. 1). Nucleotide sequences were determined using universal sequencing primers. Additional sequencing was performed on both strands by primers complementary to the ends of the known sequences. The upstream (5′) region of smmoQ was cloned from genomic DNA of Mc. capsulatus (Bath). The restriction map of the unknown genomic region was constructed by Southern hybridization analysis of genomic DNA digested by AclI, Bsp120I, BamHI, HindIII, KpnI, PaeI, SalI and XhoI. The labelled DNA fragment amplified from pCH4 by PCR, using primers HKfw and HKrev (Table 1, Fig. 1), was used as the probe in Southern hybridization.

An approximately 27 kb genomic fragment was cloned into pK18mob as follows. The 991 bp EcoRI–BglII fragment from the upstream region of smmoX was cloned into EcoRI/BamHI-digested pK18mob and designated pKPS. pKPS was introduced into Mc. capsulatus (Bath) via conjugation. The vector inserted into the smmoX upstream region by homologous recombination. The Km-resistant Mc. capsulatus (Bath) colonies were selected and proper insertion was tested by diagnostic PCR. Genomic DNA was purified, digested with HindIII and self-ligated, resulting in pKPS (Fig. 1). pKHKg was constructed on a similar basis. The DNA fragment of smmoS was amplified by PCR using primers HKfw and HKrev. The PCR product was ligated into HincII-digested pK18mob. The proper orientation of the insert was selected and this plasmid was named pKHK.

The genomic DNA, which contained the inserted pKHK, was digested with BamHI, ligated and named pKHKg (Fig. 1). pKHKg was sub-cloned by digestion with EcoRI, Pael, Psrl and KpnI. The fragments which contained the vector were isolated and self-ligated, resulting in pKHKg1, pKHKg2, pKHKg4 and pKHKg6 respectively (Table 1, Fig. 1).

Construction of plasmids. The pPROBE-NT promoter-probe vector (Miller et al., 2000) was used for testing the promoter activity of the smmoX 5′ region. Four promoter regions of smmoX (Fig. 2) were amplified by PCR using the MX1f, MX2f, MX3f and MX4f forward primers and the MXr reverse primer (Table 1). The primers were designed to place restriction sites at each end of the PCR product to facilitate insertion into the promoter-probe vector pPROBE-NT containing enhanced GFP (EGFP) as a reporter gene, resulting in plasmids pMX1, pMX2, pMX3 and pMX4 (Fig. 2). Vectors for inactivation of smmoG and smmoR in Mc. capsulatus (Bath) by marker-exchange mutagenesis were constructed as follows. The 1128 bp Bsp120I fragment of pCH4 was ligated into Bsp120I-digested pBluescript2SK+, resulting in plasmid pBX2. A 314 bp fragment was deleted from pBX2 by digestion with NcoI and BseII. The 3735 bp fragment containing the whole vector and the 5′ and 3′ end of the smmoG insert was isolated. The non-compatible ends of this fragment were filled in with Klenow polymerase and ligated with the 855 bp Smal fragment containing the Gm resistance gene, accC1, from p34S-Gm (Dennis & Zylstra, 1998), resulting in pBXGm1. pBXGm1 was digested with Xbal and PstI (Fig. 1). The 1844 bp fragment was ligated into XbaI/Smal-cut pK18mobsac (Schäfer et al., 1994), resulting in plasmid pKmoG.

For the marker-exchange mutagenesis of smmoR, pKmoR was constructed. pKHKg2 was digested with EcoRI and SalI, then filled in with Klenow polymerase. The 4346 bp fragment was isolated and ligated with the SalI fragment of p34S-Gm containing the Gm resistance gene, in pKmoR (Fig. 1).
RESULTS

Organization of the groEL, mmoQ, mmoS and mmoR gene cluster

The approximately 9.5 kb region downstream (3′) from the gene cluster harbouring mmoXYBZDC (Stainthorpe et al., 1989, 1990) was analysed in this study. Of this non-sequenced region 5.5 kb had already been cloned on pCH4 (Stainthorpe et al., 1989, 1990). An ORF was found oriented in the same direction as mmoC and adjacent to it. This hypothetical gene was named mmoG. Further downstream from mmoG, another ORF was identified, which was oriented in the opposite orientation. The putative product of this gene (mmoQ) resembles the regulator part of a two-component sensor–regulator system (Stock et al., 2000). Next to mmoQ, a sequence with a high degree of identity to the 3′ end of the sensor part of the two-component signal transduction system was found at the end of the fragment. The complete mmoS gene was isolated from the genome of Mc. capsulatus (Bath). Upon sequencing this newly isolated DNA fragment, the missing part of mmoS and a gene encoding a putative σN-dependent transcriptional activator (mmoR) (soluble methane monoxygenase regulator) were found (Fig. 1).

Analysis of the mmoX promoter region

Previous analysis of the transcription of the mmo genes in Mc. capsulatus (Bath) has revealed that the transcription starts from a single promoter upstream (5′) from mmoX. In the mmoX promoter region, sequences resembling both σ70 and a weak σN-binding site were identified and a σ70-type promoter was predicted to be the likely promoter (Nielsen, 1996).

Upon closer examination, a σN recognition site (Reitzer & Schneider, 2001; Humberto et al., 1999) was found 12 bp upstream (5′) from mmoX. In the mmoX promoter region: TGGCAC-N6-TGCNNt (Fig. 2a). Further upstream (5′) an integration host factor (IHF)-binding site (WATCAA-N4-TTR) could be recognized, which is typical
for σ^N-type promoters (Collado-Vides et al., 1991). Genes encoding IHF have been identified in the unfinished genome of *Mc. capsulatus* (Bath) (TIGR; http://www.tigr.org); they show 77% sequence identity to those of *E. coli*. It is also a general property of σ^N promoters that they have a distant upstream (5') activating region to bind a σ^N transcription activator protein, which then regulates the start of transcription by assuming an active or an inactive conformation (Buck et al., 2000). The presence of such a distant activator region was tested using a promoter-probe vector. To design the necessary construct, a suitable reporter gene had to be selected, which was not available for *Mc. capsulatus* (Bath). GFP has been shown to be applicable in various systems (Siegle et al., 2000). It requires only oxygen and Ca^2+ for activity. The pPROBE-Nt vector is known to function in a wide range of hosts, including *Mc. capsulatus* (Bath) (R. Csáki and others, unpublished results). Various lengths of the upstream region of *mmoX* were amplified by PCR and cloned into a transcription fusion vector (Fig. 2b). The GFP activities of cultures grown in the presence and absence of copper were compared. The results (Fig. 2) clearly indicate that a distant upstream region between 358 and 280 bp (from the ATG codon of *mmoX*) contains the upstream activator sequence for a σ^N-dependent transcription activator and is necessary for copper regulation of the *mmoX* promoter. Taken together, the analysis of the upstream region suggests that a σ^N-type promoter governs transcription of the sMMO structural gene cluster.

Sequence analysis of the new ORFs

*MmoG*, the putative chaperonin. *MmoG* encodes a putative protein of 559 aa with a predicted molecular mass of 59.5 kDa. *MmoG* (methylene-monooxygenase-associated GroEL) has 39% identity and 60% similarity to the large subunit of the *cpn60*-type chaperonins (GroEL) (Braig 1998; Ranson et al., 1998), including GroEL of *Mc. capsulatus* (Bath). Usually the gene encoding the small subunit of the complex (GroESL) is located immediately upstream (5') from *mmoX* (Segal & Ron, 1996). However, no GroES-like sequence was identified 3' or 5' to *mmoG* in *Mc. capsulatus* (Bath). A gene with significant identity to *mmoG* has also been found in *Ms. trichosporium* OB3b (Stafford et al., 2003). The putative MmoG of *Ms. trichosporium* OB3b has 41% identity and 59% similarity to MmoG from *Mc. capsulatus* (Bath). There was a putative Shine–Dalgarno sequence (CGGAG) 10 bp upstream (5') from the ATG start codon of *mmoG*. A typical σ promoter motif was not recognizable. A characteristic component of heat-shock gene promoters is the CIRCE (controlling inverted repeat of chaperone expression) motif, the binding site for the HrcA protein, which regulates the expression of the chaperone. Such a CIRCE motif (TTAGACCTC-N_9-GAGTGCAA; Lemos et al., 2001) was not present in the 351 bp region separating *mmoC* and *mmoG*, although it was found in both *groESL* operons identified elsewhere in the *Mc. capsulatus* genome (TIGR unfinished microbial genomes, http://tigrblast.tigr.org/ufmg/). ρ-independent transcription terminal signals were missing from the intergenic region between *mmoC* and *mmoG*. It should also be noted that at 90 bp downstream (3') from the *mmoG* stop codon there is a potential high energy (−20.2 kcal = 84.5 kJ) stem–loop structure, which may serve as a ρ-independent terminator signal. These properties suggest that *mmoG* and *mmoC* might be co-transcribed and thus MmoG may play a significant role in the maturation or assembly of the sMMO complex or may be a transcription factor required for the initiation of transcription of sMMO.

*MmoS-MmoQ*, the putative two-component signal transduction system. The genes of the sensor–regulator system, *mmoS* and *mmoQ*, were transcribed in the opposite orientation to *mmoG* (Fig. 1). A putative σ^70 promoter motif was identified 5' to the consensus Shine–Dalgarno sequence (Fig. 3). The putative gene product of *mmoS* was 1177 aa long (predicted molecular mass of 128.6 kDa). The putative regulator protein MmoQ was 634 aa long (69.8 kDa). The start codon of *mmoQ* overlaps with the stop codon of *mmoS*. The putative peptides encoded by *mmoS* and *mmoQ* showed approximately 50% identity to two-component regulatory systems, composed of a sensor and a regulator protein (Stock et al., 2000). In these systems, the environmental signal detected by the sensor protein is transmitted to the regulator protein via transphosphorylation (reviewed by West & Stock, 2001).

**Fig. 3.** Nucleotide sequence of the *mmoS-mmoR* intergenic region. Putative σ^70−35 and −10 sequences are indicated by underlining; the start codons of MmoS and MmoR are shown in open boxes; the 267 bp internal sequence between the two putative promoters is not shown.
Two PAS-PAC domains (Ponting & Aravind, 1997; Anantharaman et al., 2001) were detected in the N-terminal sensor region of MmoS (Fig. 4a). These domains are usually found in sensor proteins responding to the redox state of the environment (Taylor et al., 1999). Predicted membrane topology (Sonnhammer et al., 1998) suggested that MmoS from *Mc. capsulatus* (Bath) contains a transmembrane region between the 19th and 41st amino acids. Such a transmembrane arrangement is also characteristic of the PAS domains of redox-sensing two-component systems (Bauer et al., 1999). In addition, there was a GAF domain (Aravind & Ponting, 1997) adjacent to the PAS-PAC domain in MmoS of *Mc. capsulatus* (Bath). A GAF domain is characteristic for sensor proteins binding small molecules, e.g. cGMP (Anantharaman et al., 2001). The occurrence of both kinds of domains within a single sensor molecule is a rare phenomenon (Anantharaman et al., 2001). The typical metal-binding motif MxCxxC was not recognizable in the N-terminal region of MmoS.

A typical sensor protein element, the His-kinase phosphoacceptor domain, is present in MmoS. MmoS from *Mc. capsulatus* (Bath) also contains two receiver domains (REC), which receive the phosphoryl group at a conserved Asp residue. Interestingly, the sensor contains a His-containing phosphotransfer domain, which is usually present on a separate protein (West & Stock, 2001). The domain organization of the putative MmoQ is distinct from the typical regulator proteins. The regulator protein also contains a receiver domain, the phosphorylation of which changes the activity of the regulator. Most regulators are transcription factors. Their DNA-binding activity changes upon phosphorylation, thereby controlling the promoter activity and thus the biosynthesis of the participating protein(s) in response to the environmental stimulus (Reitzer & Schneider, 2001). Some regulators do not contain a DNA-binding domain. These regulators transfer their phosphate groups to a target protein, thereby changing its activity (Bourret et al., 1989; Robinson et al., 2000; Stock et al., 2000). The putative MmoQ protein of *Mc. capsulatus* (Bath) does not display any DNA-binding motifs. The C-terminal region of MmoQ contains a GGDEF sequence element, which is associated with the receiver domain (Stock et al., 2000). In fact, the predicted N-terminal protein sequence of MmoQ from *Mc. capsulatus* (Bath) shows no significant similarity to any other proteins in current databases.

**MmoR**, the putative σ^N^-dependent transcription activator. The *mmoR* gene encodes a 581 aa polypeptide of 63.4 kDa carrying every feature described for σ^N^-dependent transcription activators (Stock et al., 2000). In addition to the σ^N^-activator domain and the ATPase motif, a DNA-binding helix–turn–helix domain is located towards the C

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**Fig. 4.** Domain organization of the putative two-component phosphorelay system, MmoS-MmoQ. (a) The multi-component phosphorelay system often begins with a hybrid histidine protein kinase. The transmembrane segment is indicated by Tm. The HATPase domain catalyses the ATP-dependent autophosphorylation (thick black arrow) of a specific conserved His residue in the HisKA domain. Dashed arrows indicate the putative phosphotransfer scheme when a specific environmental signal is sensed by the PAS-PAC and GAF domains. The phosphoryl group is then transferred to a specific Asp residue located within the receiver (REC) domain. The His-containing phosphotransfer domain (HPT) (usually located on a separate protein) receives the phosphate moiety at a conserved His residue; (b) This phosphate moiety is then transferred to the receiver domain of the regulator protein, MmoQ. X indicates a putative effector molecule(s), which may influence the transcription of the *mmo* operon.
terminus. The mmoR gene is preceded by $\sigma^{70}$ and ribosome-binding elements (Fig. 3). MmoR also lacks the metal-binding MxCxxC motif (Koch et al., 1987), indicating that, as with MmoS, MmoR probably does not bind copper ions directly. MmoR from Mc. capsulatus (Bath) has 40% identity and 54% similarity to MmoR from Ms. trichosporium OB3b (Stafford et al., 2003).

**Phenotypic characterization of $\Delta$mmoG, $\Delta$mmoR mutants.** $\Delta$mmoG and $\Delta$mmoR mutants of Mc. capsulatus (Bath) were constructed to investigate the influence of MmoG and MmoR on the expression of sMMO. The growth rate of the $\Delta$mmoG and $\Delta$mmoR mutant strains was indistinguishable from the wild-type controls on copper-containing NMS plates, but both mutants grew more slowly on 'copper-free' plates. Unlike the wild-type Mc. capsulatus (Bath), the $\Delta$mmoG and $\Delta$mmoR mutants showed no measurable naphthalene-oxidizing sMMO activity (data not shown) under any growth conditions. In line with the activity assays, the mutant strains did not contain detectable amounts of sMMO hydroxylase polypeptides when analysed by blotting and probing with a serum specific for the sMMO hydroxylase components of Mc. capsulatus (Bath) (Fig. 5a). The complete absence of these sMMO proteins was confirmed using cells grown in liquid culture, both in the presence and absence of copper ions. Finally, the mutants were subjected to RT-PCR analysis to detect the transcripts of the mmo genes. The PCR primers mxatgfw and mxatgrev (Table 1), which amplified the N-terminal of mmoX, were used. As shown in Fig. 5(b), mmoX transcripts were only present in wild-type Mc. capsulatus (Bath) cells grown in the absence of copper.

The pMX1 promoter-probe plasmid, with the functional mmoX promoter, was introduced into the $\Delta$mmoG and $\Delta$mmoR mutants. On copper-containing medium, GFP was not expressed in any of these strains. On low-copper medium, only the wild-type strain with plasmid pMX1 showed GFP expression, while the mutants did not (data not shown).

These various, independent methods unequivocally demonstrated that there was no functional sMMO enzyme (neither transcribed nor translated gene products) produced in the $\Delta$mmoG and $\Delta$mmoR mutant strains of Mc. capsulatus (Bath). These results indicate that both MmoG and MmoR are indispensable for the correct biosynthesis of sMMO as has also been recently demonstrated in Ms. trichosporium OB3b (Stafford et al., 2003).

The sMMO-deficient mutant strains were also capable of growing on low-copper medium, apparently without possessing functional sMMO. Hence, these cells had no perceptible sMMO activity to support their growth on methane. The growth rates of the mutants did not show significant differences compared to the wild-type cells expressing either sMMO or pMMO (data not shown).

Presumably the mutant cells were scavenging Cu$^{2+}$ and pMMO sustained their growth on low-copper medium.

**DISCUSSION**

Four genes ($\text{mmoG, mmoR, mmoQ}$ and $\text{mmoS}$) immediately downstream (3') from the mmmo gene cluster were identified, sequenced and characterized. The fact that an IHF recognition site is present in the promoter region of mmoX and that there are known IHF genes in the Mc. capsulatus (Bath) genome (data obtained from the TIGR website), suggest that the expression of sMMO may not be regulated by a simple copper
Switch. It has been demonstrated that an activator region at about 335 bp upstream (5') from the start codon of \textit{mмоX} is required for copper-dependent activity of the promoter. The presence of the IHF-binding site and the requirement of an upstream activator region suggest the existence of a $\sigma^{N}$-dependent promoter. The requirement of $\sigma^{N}$ for the expression of sMMO of \textit{Ms. trichosporium} OB3b was also demonstrated by the analysis of an \textit{rpoN} mutant of \textit{Ms. trichosporium} OB3b which was also unable to express sMMO (Stafford \textit{et al}, 2003). Several attempts were made in our study to mutate \textit{rpoN} of \textit{Mc. capsulatus} (Bath) without any success.

Deletion mutagenesis was applied to generate ‘knock-out’ mutants of both \textit{mмоG} and \textit{mмоR}. Analysis of these mutants provided evidence that these genes are required for the expression of active sMMO. The data do not provide straightforward evidence for the participation of these genes in the copper-dependent regulation of sMMO expression, although mutation analysis indicates an sMMO$^{-}$ phenotype under ‘low-copper’ growth conditions, $\Delta mмоR$, and interestingly the $\Delta mмоG$ strain with the pMX1 plasmid, did not show GFP activity under ‘low-copper’ growth conditions. This indicates that the transcriptional activation of sMMO was abolished in these mutants. It has also been found that on copper-deficient medium, these mutants are unable to express sMMO and the promoter activity of \textit{mмоX} is impaired without any significant effect on the growth rate as compared to the wild-type strain. MмоG may therefore participate in the maturation and assembly of the sMMO polypeptides or in the biosynthesis of a protein regulating the expression of the sMMO structural gene cluster (e.g. MмоR).

The MмоR protein likely participates in $\sigma^{N}$-dependent promoter activation, although this promoter is copper-sensitive and MмоR contains no copper-binding motifs.

Thus the exact role of MмоR needs further investigation. If MмоR does indeed activate the expression of \textit{smмо} genes, additional proteins (possibly MмоQ and MмоS), which may sense the changes in copper concentration in the cell environment and mediate this information to MмоR, must be involved (Fig. 6). Low-level transcription of \textit{pmоCAB} operons was detected by Stolyar \textit{et al} (2001) under sMMO expressing conditions. We presume that traces of copper ions in the medium may allow active pMМО formation. Under laboratory conditions, where methane is supplied in vast excess, this low amount of pMМО is sufficient for growth.

The only other system where the putative regulatory genes \textit{mмоR} and \textit{mмоG} have been studied is that of \textit{Ms. trichosporium} OB3b, a Type II methanotroph found in the $\alpha$-subdivision of the Proteobacteria (Stafford \textit{et al}, 2003). A comparison with our results reveals several similarities and differences. In both cases the \textit{mмоR} and \textit{mмоG} genes are found in the neighbourhood of the \textit{smмо} operon, although the arrangement of the genes relative to the structural genes is dissimilar: \textit{mмоR} and \textit{mмоG} are positioned 5’ to \textit{mмоX} in \textit{Ms. trichosporium} OB3b. mмоR has a putative $\sigma^{70}$-like promoter in \textit{Mc. capsulatus} (Bath), but there is no recognizable promoter motif for \textit{mмоR} in \textit{Ms. trichosporium} OB3b. No promoter-like sequences can be identified for \textit{mмоG} either in \textit{Mc. capsulatus} (Bath) or in \textit{Ms. trichosporium} OB3b (Stafford \textit{et al}, 2003). The deduced amino acid sequences of the MмоR and MмоG proteins in the two bacteria show a considerably high degree of identity (40 and 40 %, respectively) and similarity (50 and 55 %, respectively). Mutation of \textit{mмоR} or \textit{mмоG} yields the same phenotype in both strains, thus it is reasonable to assume that the functions of MмоR and MмоG are the same in \textit{Mc. capsulatus} (Bath) and \textit{Ms. trichosporium} OB3b.

It intends to say that the MмоR sequences in the two bacteria show 40 % identity (50 % similarity) and the MмоG

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\textbf{Fig. 6.} Model for the regulation of the \textit{mмо} operon. The model involves an unknown mechanism for sensing copper ions. MмоS transduces this signal by changing the phosphorylation stage of MмоQ. This signal is then transferred from MмоQ to MмоR, which activates the transcription of the \textit{mмо} operon via $\sigma^{N}$. A hypothetical signal pathway is indicated by dotted arrows. The exact function of MмоG cannot be determined from the data obtained to date. It may be required for the correct folding of MмоR and/or for the maturation of active sMMO (indicated by hatched arrows).
sequences show the same 40% identity (55% similarity) in the two strains compared.

Sequences homologous to mmQ and mmS have not yet been identified in the sMMO-related gene cluster of Ms. trichosporium OB3b. The precise functions of MmoQ and MmoS in Mc. capsulatus (Bath) remain unknown and require further detailed analysis. The 530th residue of MmoS is the single conserved histidine in the His-kinase domain, which may receive the phosphate from ATP (Bilwes et al., 1999). Generally, only the regulator protein contains the receiver domain in bacteria. This receives the phosphate group from the phosphoacceptor domain after the appropriate environmental signal. A minority of the two-component systems consist of a hybrid sensor in which the sensor polypeptide contains a receiver domain at the C terminus (West & Stock, 2001). Remarkably, in Mc. capsulatus (Bath), the hybrid sensor contains two receiver domains, both located at the C-terminal portion of the protein. Usually with these types of sensor proteins, more than one His-Asp phosphoryl transfer reaction takes place and the scheme involves a His-containing phosphotransfer (HPT) protein that serves as a His-phosphorylated intermediate (West & Stock, 2001). The phosphate moiety is transferred from the receiver domain of the sensor to the HPT and then onto the receiver domain of the regulator. In these cases, the additional step in the phosphotransfer implicates an additional possible regulation point (Robinson et al., 2000). Interestingly, the C-terminal end of MmoS contains an HPT (Fig. 3a), which may be an intermediate step of phosphorylation between the receiver domain of the sensor and the receiver domain of the regulator protein.

MmoS does not apparently contain the sequence element MxCxxC, usually required for metal binding; therefore it may be that an altered redox balance, rather than the binding of copper ions directly, may be the environmental signal for the two-component regulatory system. Surprisingly, the putative MmoQ protein does not display any DNA-binding motifs. In our hypothetical model for sMMO regulation in Mc. capsulatus (Bath), MmoS is attached to the cytoplasmic side of the membrane and senses copper ions via an unknown mechanism. The signal is transferred by phosphorylation to MmoQ. MmoQ is assumed to regulate the transcription of the mno operon through an interaction with MmoR. MmoG is indispensable for the expression of sMMO. MmoG brings MmoR into a transcriptionally competent form and/or it is required for the folding of sMMO (Fig. 6).

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