INTRODUCTION

Methanotrophs are bacteria capable of using methane as their sole carbon and energy source. The first step in the oxidation of methane to CO₂ is the conversion of methane to methanol by the enzyme methane monooxygenase (MMO). There are two forms of this enzyme, a particulate membrane-bound form (pMMO) and a soluble cytoplasmic form (sMMO). pMMO is a membrane-bound copper- and iron-containing enzyme and is present in virtually all known methanotrophs (Nguyen et al., 1994; Zahn & Dispirito, 1996; Takeguchi et al., 1999). The structural genes for this enzyme have been cloned and sequenced from Methylococcus capsulatus Bath (Semrau et al., 1995; Stolyar et al., 1999), Methylocystis sp. strain M and Methylosinus trichosporium OB3b (Gilbert et al., 2000). They lie in a three-gene operon, pmoCAB, believed to encode three integral membrane subunits of approximately 47, 27 and 25 kDa, respectively. These operons are present in duplicate, almost identical, copies in all three organisms and are transcribed from σ⁰-type promoters found upstream of the pmoC gene (Semrau et al., 1995; Gilbert et al., 2000; Stolyar et al., 2001; G. P. Stafford & J. C. Murrell, unpublished data).

sMMO is a cytoplasmic enzyme containing a unique di-iron site at its catalytic centre. It has a broad substrate range, including trichloroethylene, alkanes, alkenes and aromatic compounds. The biochemistry of sMMO has been studied in detail (reviewed by Lipscomb, 1994). It consists of three components: a hydroxylase, which is a dimer of three identical, copies in all three organisms and are transcribed from σ⁰-type promoters found upstream of the pmoC gene (Semrau et al., 1995; Gilbert et al., 2000; Stolyar et al., 2001; G. P. Stafford & J. C. Murrell, unpublished data). The methanotrophic bacterium Methylosinus trichosporium OB3b converts methane to methanol using two distinct forms of methane monooxygenase (MMO) enzyme: a cytoplasmic soluble form (sMMO) and a membrane-bound form (pMMO). The transcription of these two operons is known to proceed in a reciprocal fashion with sMMO expressed at low copper-to-biomass ratios and pMMO at high copper-to-biomass ratios. Transcription of the smmo operon is initiated from a σ⁰ promoter 5’ of mmoX. In this study the genes encoding σ⁰ (rpoN) and a typical σ⁰-dependent transcriptional activator (mmoR) were cloned and sequenced. mmoR, a regulatory gene, and mmoG, a gene encoding a GroEL homologue, lie 5’ of the structural genes for the sMMO enzyme. Subsequent mutation of rpoN and mmoR by marker-exchange mutagenesis resulted in strains Gm1 and JS1, which were unable to express functional sMMO or initiate transcription of mmoX. An rpoN mutant was also unable to fix nitrogen or use nitrate as sole nitrogen source, indicating that σ⁰ plays a role in both nitrogen and carbon metabolism in Ms. trichosporium OB3b. The data also indicate that mmoG is transcribed in a σ⁰- and MmoR-independent manner. Marker-exchange mutagenesis of mmoG revealed that MmoG is necessary for smmo gene transcription and activity and may be an MmoR-specific chaperone required for functional assembly of transcriptionally competent MmoR in vivo. The data presented allow the proposal of a more complete model for copper-mediated regulation of smmo gene expression.
the public databases, but it may play a role in assembly of the unique di-iron centre of the sMMO enzyme (Merkh & Lippard, 2002).

The mechanism controlling the expression of the \textit{mmo} genes in response to copper, the copper switch, has long been a topic of study since its discovery by Stanley \textit{et al.} (1983). Nielsen \textit{et al.} (1997) showed that transcription of the \textit{smmo} and \textit{pmmo} operons was reciprocal, with expression at low and high copper-to-biomass levels respectively. In contrast to the promoters identified 5’ of the \textit{pmoCAB} operon in \textit{Ms. trichosporium OB3b}, the promoter present 5’ of the \textit{mmo} operon possesses high similarity to a \(\sigma^N\) consensus promoter: TGGCA-N\textsubscript{e}TTGCA/t (Barrios \textit{et al.}, 1999; Nielsen \textit{et al.}, 1997). Initiation of transcription from these promoters requires the involvement of the \(\sigma^N\) subunit of RNA polymerase and a transcriptional activator protein (Merrick, 1993). These activator proteins are often called enhancer-binding proteins (EBPs) due to the fact that they facilitate initiation of transcription from remote enhancer regions, upstream activator sequences (UASs), which lie 5’ of the promoter to which \(\sigma^N\) binds (Morrett & Segovia, 1993). This mode of transcriptional regulation is widespread among bacteria and is known to control diverse functions, such as C4-dicarboxylate transport (Huala \textit{et al.}, 1992), toluene-\(\omega\)-xylene metabolism (Arenghi \textit{et al.}, 1999), acetoin catabolism (Krüger & Steinbüchel, 1992) and nitrogen fixation (Michiels \textit{et al.}, 1998).

The presence of an \(\sigma^N\)-type promoter 5’ of \textit{mmoX} suggested the involvement of the alternative sigma factor, \(\sigma^N\), and an EBP in the regulation of the \textit{mmo} cluster. Thus, in this study we sought to establish the presence of an \textit{rpoN} gene, encoding \(\sigma^N\), and to determine if the regulation of the \textit{mmo} operon was controlled in a \(\sigma^N\)-dependent manner. We present here a molecular analysis of the \textit{rpoN} gene from \textit{Ms. trichosporium OB3b} and show that it plays a role in regulation of the copper switch. We also describe the cloning and sequencing of two new genes of the \textit{mmo} gene cluster and show that one of these encodes MmoR, a member of the EBP family, and the other encodes a putative chaperone, MmoG, polypeptides which control regulation of the \textit{mmo} operon.

\section*{METHODS}

\textbf{Growth media and strains.} The bacterial strains used in this study are described in Table 1. Methanotrophs were grown at 30 °C in shaking (200 r.p.m.) batch culture supplied with a headspace of methane and air (1:5) in nitrate mineral salts (NMS) medium as described by Whittenbury \textit{et al.} (1970). Methanotrophs were maintained on NMS agar plates and incubated in gas-tight jars under the same gas conditions. For ammonia mineral salts medium, potassium nitrate was replaced by 1 g ammonium chloride \(\text{g}^{-1}\). Mineral salts medium contained no fixed nitrogen source. Where necessary, glutamine (filter-sterilized) was added as an aqueous solution to a final concentration of 0-05% (w/v) and served as a nitrogen source. Low-copper media were prepared using Milli-Q water in acid-washed glassware using trace elements solution lacking added copper (Stanley \textit{et al.}, 1983). Low-copper liquid cultures were grown in acid-washed flasks and solid media were prepared using Noble agar instead of Bacto agar.

\textit{Escherichia coli} strains were grown in Luria-Bertani medium. Antibiotics were used at the following final concentrations: for \textit{E. coli}, ampicillin (Ap, 50 \(\mu\text{g mL}^{-1}\)); gentamicin (Gm, 5 \(\mu\text{g mL}^{-1}\)); kanamycin (Km, 25 \(\mu\text{g mL}^{-1}\)); and for \textit{Ms. trichosporium OB3b}, Ap (50 \(\mu\text{g mL}^{-1}\)), Gm (2-5 \(\mu\text{g mL}^{-1}\)), Km (10 \(\mu\text{g mL}^{-1}\)), unless otherwise stated.

\textbf{DNA manipulation.} Preparation of plasmid DNA and standard DNA manipulations were essentially performed according to the methods of Sambrook \textit{et al.} (1989). DNA was extracted from \textit{Ms. trichosporium OB3b} using the method of Oakley & Murrell (1988). Plasmids used in this study are detailed in Table 1.

\textbf{PCR.} PCR was performed in 50 \(\mu\text{l}\) reaction mixtures in 0-5 ml microcentrifuge tubes using a Hybaid Touchdown Thermal cycler. \textit{Taq} polymerase (Gibco-BRL) was used. After an initial denaturation step at 94°C (5 min), the \textit{Taq} polymerase was added. Amplification was performed using 30 cycles of 94°C for 1 min, varying the annealing temperature as required for 1 min, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. In the case of the PCR primers for amplification of \textit{mmoG} (Cpn60F3498, CTGCGGAGAAGAATTGTC; Cpn60R3941, GCGATT-CATAGGCTCGTA) (Cpn60F5, CGGTCGCAATGTGGTGAT, was used for mutant JS2) from \textit{Ms. trichosporium OB3b} and \textit{rpoN} (SS4MELF, CACACCGAGATCCGCTTGTC; SS4MELR, CTCATATCCGGCTGTTAC) from \textit{Sinorhizobium melloti} 1021, a touchdown protocol, with annealing temperature decreasing from 70 to 50°C (\textit{mmoG}) or 73 to 53°C (\textit{rpoN}) at 1°C per cycle with a final extension step of 15 cycles and an annealing temperature of 51°C (\textit{mmoG}) or 54°C (\textit{rpoN}), before a final extension step as described above, was performed. These reaction mixtures also contained 25 \(\mu\text{l}\) DMSO-betaine solution (2-6 M betaine, 2-6%, v/v, DMSO).

\textbf{Random-priming and Southern hybridization.} DNA probes were labelled by random priming according to Feinberg & Vogelstein (1984). PCR-generated probe (50 ng) was labelled with 50 \(\mu\text{Ci}\) [\(\text{32P}\)]dGTP. Unincorporated label was removed using a MicroSpin Column (Amersham Pharmacia Biotech), according to the manufacturer’s instructions. Probes were denatured by the addition of NaOH to a final concentration of 0-4 M immediately prior to hybridization. Southern blotting (Sambrook \textit{et al.}, 1989) was used to transfer DNA onto nylon Hybond-N membranes (Amersham). DNA was fixed to the membrane with an Ultraviolet (UV) Stratalinker (Stratagene). Hybridizations were carried out in hybridization solution (0-5 M \(\text{NaHPO}_4/0-5\) M \(\text{Na}_{2}\text{HPO}_4\), pH 6-8, 7%, w/v, SDS) for 16 h at 65°C. Initial washes were performed at 21°C in 2x SSC (NaCl, 173-3 g \text{l}^{-1}; tri-sodium citrate, 88-2 g \text{l}^{-1}; pH 7-0) and stringency was gradually increased by raising the temperature and lowering the SSC concentration. Probes were generated by PCR using the primers listed above or from the appropriate restriction fragments of plasmids listed in Table 1 that had been purified from an agarose gel using the GeneClean II kit (Bio 101).

\textbf{DNA sequencing and analysis.} DNA sequencing was performed by L. Ward (University of Warwick) by cycle sequencing with the Dye Terminator Kit (PE Applied Biosystems) and analysis using a model 373A automated sequencing system (PE Applied Biosystems). DNA sequences and derived amino acid sequences were analysed using the DNASTAR package. Similarity searches were performed using the BLAST program (Altschul \textit{et al.}, 1990) against public protein and gene databases (http://www.ncbi.nlm.nih.gov). Deduced amino acid sequences were aligned using the CLUSTAL W program. Alignment positions where sequence ambiguity existed and where data were not available for all sequences were excluded from the analysis. The phylogeny of the \textit{rpoN} sequences was determined with programs available within PHYLIP (Felsenstein, 1993). An evolutionary distance matrix prepared using the Dayhoff PAM
parameter model (PROTDIST) was used for construction of phylogenetic trees with the FITCH program. The significance of the branch points was assessed by bootstrap resampling of the dataset (CONSENSE) of 100 trees generated by evolutionary distance (PROTDIST) analysis. Preliminary sequence data for the rpoN gene from Mc. capsulatus (Bath) were obtained from The Institute for Genomic Research website at http://www.tigr.org. The nucleotide sequence of the rpoN cluster and the extended mno cluster have been deposited in GenBank under accession numbers AY148878 and X55394, respectively.

Conjugations. The procedure for conjugating plasmids from E. coli into methanotrophs was based on the method developed by Martin & Murrell (1995). The RP4-mob-containing plasmid to be conjugated (Table 1) was first transferred into E. coli S17-1. Next, a 10 ml overnight culture of the donor E. coli strain was collected on a sterile 47 mm nitrocellulose filter (0-2 μm pore size; Millipore). The E. coli donor strain was washed on the filter with 50 ml sterile NMS (or appropriate methanotroph medium). A 50 ml culture of the methanotroph was grown to an OD540 of 0.3 and collected on the same filter as the E. coli donor strain and again washed with 50 ml medium. The filter was placed (cells up) on an NMS (or other) agar plate containing 0-02 % (w/v) nalidixic acid and incubated for 24 h at 30℃ in the presence of methane. After 24 h, cells were resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centrifugation and the pellet was resuspended in 10 ml sterile (NMS) medium before being concentrated by centr...
mno gene-specific DNA fragments was tested by PCR using primers specific for these genes. RNA was quantified in Quartz cuvettes in a Beckman DU-70 spectrophotometer at a wavelength of 260 nm.

RT-PCR. The reverse transcription step was essentially performed as per the manufacturer’s instructions (Roche). Briefly, 1 μg RNA was added to 50 pmol reverse primer in 4·5 μl water and denatured at 65 °C for 15 min in a Hybaid Thermocyclers Thermal cycling system, followed by cooling on ice for 2 min. Two microlitres each of 10 mM DNTP, 2 μl 100 mM DTT, 4 μl 5X Expand buffer, 0·5 μl water and 1 μl Expand Reverse Transcripase (40 units μl⁻¹) were added before incubation at 42 °C for 1 h. The mno-specific RT-PCR experiments used primers 206F (ATCGGCAARGAATAYGCSG) and 886R (ACCCANGCTGCACTTGGAA) (numbered from the start of mnoX; accession no. X55394). PCR reactions to determine the presence of mno-specific cDNA in the samples were performed as described above at an annealing temperature of 60 °C, producing a product of 720 bp. RT-PCR was performed on the mnoG region using the primers used for PCR. The PCR step for these experiments was performed using a Touchdown protocol (70–50 °C) followed by 15 cycles at 51 °C using a reaction mixture containing DMSO-betaine (see above). The mnoG PCR product was 444 bp.

Preparation of whole-cell extracts. Total cell protein was extracted from 50 ml cultures (OD₆₅₀=0·5) harvested by centrifugation or scraped from agar plates and resuspended in 400 μl 20 mM Tris/HCl (pH 7·3) and boiled for 5 min in sample buffer containing no bromophenol blue. The cells and protein were quantified using the Bio-Rad Protein assay reagent according to the manufacturer’s instructions.

SDS-PAGE and Western blotting. Protein samples (120 μg) were separated by 10% (w/v) SDS-PAGE (Laemmli, 1970) using an X-Cell II Mini-Cell apparatus (Novex) and stained with Coomasie brilliant blue R250. Molecular masses were estimated against low-molecular-mass markers from Amersham. Antisera against the hydroxylase subunit of Ms. trichosporium OB3b was a gift from Dr Thomas Smith, University of Warwick. Pure hydroxylase subunit of Ms. trichosporium OB3b was provided by Ms Sue Slade, University of Warwick. Western blotting was performed with an X-Cell II blot module (Novex) and Hybond-C nitrocellulose membrane (Amersham). Secondary horseradish-peroxidase-conjugated goat anti-rabbit IgG (Sigma) was used for visualization of cross-reacting protein bands.

Naphthalene plate and liquid assays. The activity of sMMO was routinely assayed using a qualitative naphthalene oxidation assay (Brusseau et al., 1990). Methanotrophs were grown on low-copper agar for 7–10 days before incubation at 30 °C in the presence of naphthalene crystals for 30 min. A solution of 10 mg tetrazotized o-dianisidine ml⁻¹ (Sigma) was then dropped onto the colonies. After depletion of copper ions from these low-copper agar plates, sMMO-positive colonies were deep purple, but sMMO-negative colonies remained orange. A similar procedure was followed for liquid cultures. One millilitre of a 50 ml culture was incubated with one crystal of naphthalene for 30 min at 30 °C in an Eppendorf tube before addition of tetrazotized o-dianisidine to 10 mg ml⁻¹. Again the appearance of a purple colour was indicative of sMMO activity.

RESULTS AND DISCUSSION

Cloning of the rpoN gene cluster from Ms. trichosporium OB3b

To clone rpoN from Ms. trichosporium OB3b, Southern blots of chromosomal DNA were probed with the rpoN gene from S. meliloti. The rpoN probe was prepared using primers S54MELF and S54MELR to amplify the S. meliloti rpoN gene from pSMEL5 (Table 1). Using this approach, the S. meliloti-derived probe allowed the identification of a putative rpoN-containing 3·7 kb Srl fragment (data not shown). Partial genomic libraries of Srl-restricted DNA fragments from Ms. trichosporium OB3b (3·0–5·0 kb) were prepared in the pUC18 vector. Due to the presence of an rpoN gene in the chromosome of the E. coli host strain, a pooled-miniprep method was employed. This allowed the isolation of this 3·7 kb Srl fragment in clone pGPS519.

DNA sequence analysis of the rpoN gene cluster from Ms. trichosporium OB3b

The nucleotide sequence of the 3·7 kb Srl fragment from pGPS519 was determined. It contained 1272 bp of the rpoN gene and four other ORFs (Fig. 1a). This clone is missing approximately 250 bp of the rpoN gene at its 5′ end. The derived sequence of RpoN from Ms. trichosporium OB3b is most closely related (~55% amino acid identity) to the RpoN from several members of the family Rhizobiaceae (identified using the BLASTX tool). It contains the highly conserved rpoN-box motif (ARRVATKYPRE; Merrick, 1993) except that the final glutamate residue is replaced by an aspartate in the Ms. trichosporium OB3b RpoN sequence. A phylogenetic distance tree compiled from an alignment of the deduced amino acid sequences of 35 rpoN genes revealed that the RpoN from Ms. trichosporium OB3b groups with other members of the ς₃₃-subclass of the Proteobacteria, loosely following 16S rRNA phylogeny (Fig. 2). The alignment was based on 307 aa and excluded the hyper-variable region II (Studholme & Buck, 2000). The deduced amino acid sequence of the putative rpoN gene from Mc. capsulatus Bath (preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org) was also included in this analysis and falls within a group formed by the ς₃₃-subclass of the Proteobacteria.

Immediately adjacent to the Ms. trichosporium OB3b rpoN gene lie two ORFs (Fig. 1a), orf1 appears to be a new member of a group of genes found adjacent to rpoN genes in many bacteria (Ronson et al., 1987; Michiels et al., 1998; Warrelmann et al., 1992; Merrick, 1993). It encodes a polypeptide of 192 aa which is 48% identical to the derived amino acid sequence from the corresponding gene (ORF203) from Bradyrhizobium japonicum (Kullik et al., 1991). The only other polypeptides with significant identity to the cloned sequence of orf1 are a spinach ribosomal protein (Merrick & Edwards, 1995) and the sequence derived from a gene (ORF113) upstream of the pheA gene from E. coli (Powell et al., 1995). A putative Shine–Dalgarno sequence with high similarity to the E. coli consensus (AGGAGG) was located 7 bp from the start codon of orf1: AGGTGG. In addition, a DNA sequence (CGATCGAACG-N₄-CGTTGCATG) with the potential to form a stable stem–loop structure (ΔG = -10.9 kcal = 45·6 kJ) was found 10 bp downstream (3′) from the stop codon of rpoN, which may function as a transcriptional terminator.
orf2 encodes a polypeptide of 186 aa with a high degree of identity (64%) to the sequence derived from a gene encoding the phosphotransferase system enzyme II from *Mesorhizobium loti* (ptsN) (Kaneko et al., 2000). A putative Shine–Dalgarno sequence (ATGGGA) was located 10 bp 5’ of the ATG codon of this ORF. It is possible that this gene is co-transcribed with orf1 since no potential stem–loop structures are present between orf1 and orf2. orf3 is transcribed in the opposite direction and encodes a protein of 92 aa. Its derived amino acid sequence possesses significant identity (44–57%) to conserved hypothetical proteins of unknown function found in the genome sequences of

![Diagram](https://example.com/diagram.png)

**Fig. 1.** Organization of the *mmo* and *rpoN* gene clusters from *M. trichosporium* OB3b. (a) The putative transcriptional terminator between *rpoN* and ORF1 is shown. Restriction sites used in the construction of pGPS103Gm are also labelled. (b) The promoter sequences identified by Nielsen et al. (1997) are shown as P2N and P2α. Putative transcriptional terminators are shown 3’ of *mmoG* and *mmoC*. The restriction sites used in the construction of pJS102 and pJS104 are also labelled.

![Diagram](https://example.com/diagram.png)

**Fig. 2.** Phylogenetic analysis of *σN*. Amino acid sequences were obtained from the Entrez protein database (accession numbers in parentheses). The alignment was based on 307 aa from positions 171 to 616. Analyses used programs available within PHYLIP (Felsenstein, 1993). Pairwise distances were calculated with PROTDIST (Dayhoff PAM matrix) and a tree generated with FITCH. The RpoN of *Aquifex aeolicus* was chosen as the outgroup. Bootstrap analysis (SEQBOOT; 100 trees) was used to determine the reliability of branch points. Only values above 70% from the consensus tree (CONSENSE) are shown, as these are considered to support branch points (Zharkikh & Li, 1992).
organisms such as *Caulobacter crescentus*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* (GenBank accession numbers AE05795, AE004645 and AAL20464.1, respectively). Finally, the partial ORF, *orf4*, encoding 99 aa has 69% identity with a putative two-component regulator identified from the genome sequence of *Mesorhizobium loti* (Kaneko et al., 2000).

Of the four ORFs associated with the *rpoN* gene from *Ms. trichosporium* OB3b, *orf1* and *orf2* are almost always found in *rpoN* operons (Merrick, 1993). The genetic linkage of these genes with *rpoN* in many organisms and now *Ms. trichosporium* OB3b supports the proposal that they may play a role in co-regulation of *σN*-dependent operons (Merrick, 1993; Michiels et al., 1998; Powell et al., 1995). However, this linkage is not observed in those organisms which contain two *rpoN* genes, such as *Rhodobacter sphaeroides* (Meijer & Tabita, 1992) where the second *rpoN* copy lies adjacent to nitrogen fixation genes. Southern analysis using the *rpoN* gene from *Ms. trichosporium* OB3b as a homologous probe revealed that there is probably only one copy of the *rpoN* gene in *Ms. trichosporium* OB3b (data not shown). The clear phenotypes observed for an *rpoN* mutant presented in this paper also support this suggestion.

**Sequencing and analysis of two new ORFs 5’ of the mmo cluster of *Ms. trichosporium* OB3b**

The genes encoding SMMO lie in a six-gene operon in the chromosome of *Ms. trichosporium* OB3b (Cardy et al., 1991). Sequencing of the region 5’ of *mmoX* has revealed the presence of two ORFs (Fig. 1b). The first of the ORFs encoded a new member of the *σN*-dependent EBP family of transcriptional activators (649 aa, 70-3 kDa), which are involved in the regulation of a wide range of physiological processes, including nitrogen fixation, nitrate assimilation, dicarboxylic acid transport, toluene oxidation and *o*-xylene degradation (Morrett & Segovia, 1993; Merrick, 1993). Its derived amino acid sequence possesses 27% identity to AcoR from *Ralstonia eutrophus* (formerly *Alcaligenes eutrophus*), a positive regulator of transcription of the acetoin catabolism operon, which responds to acetoin levels (Krüger & Steinbüchel, 1992). Analysis of the sequence 5’ of the acoR-like gene reveals the presence of a possible Shine-Dalgarno sequence (GGGA), but no putative promoters or regulatory sequences have been identified. It may be that such elements are present further upstream from these genes than the sequenced region. Since the acoR-like gene lies 5’ of the mmo gene cluster and it is a member of the EBP family, we propose the name mmoR (soluble methane monooxygenase regulator). The EBP family of proteins is characterized by their conserved modular structure, comprising a highly conserved central domain and a DNA-binding C-terminal domain (Morrett & Segovia, 1993; Buck et al., 2000). An alignment of the central and C-terminal domains of MmoR from *Ms. trichosporium* OB3b revealed striking similarity with 10 other members of this family of regulators (data not shown). The central domain contains the characteristic Walker A and Walker B ATPase domains, a nucleotide-binding sequence and a conformational change switch motif (Morrett & Segovia, 1993; Buck et al., 2000). The C-terminal domain contains a putative DNA-binding helix-turn-helix motif.

Conversely, the N-terminal domains of these EBP proteins are highly variable in both sequence and length and are proposed to confer effector specificity to these proteins. The derived amino acid sequence of the MmoR gene reveals an extended N-terminal domain. These extended N-terminal regions contain the sensory domains, which in the case of AcoR from *Ralstonia eutrophus* H16 and TouR from *Pseudomonas stutzeri* are believed to bind the aromatic compounds acetoin and toluene-*o*-xylene, the substrates for the metabolic gene clusters which they control (Krüger & Steinbüchel, 1992; Arenghi et al., 1999). In fact, a recent study by Garmendia et al. (2001) succeeded in shuffling the N-terminal domain of XylR from *Pseudomonas putida* with the corresponding domains from other regulators to expand the range of aromatic compounds to which it was able to respond. It therefore seems likely that the extended N-terminal domain of MmoR also serves such a function in signal sensing and transduction. The MmoR protein exerts positive regulation over the mmo operon under conditions of copper starvation and thus it is likely that MmoR is in some way modified in response to low-copper conditions. An analysis of the N-terminal region does not reveal a typical copper-binding motif, such as the multiple Cys-X-X-Cys motifs observed in copper metallochaperones or copper ATPases of both prokaryotes and eukaryotes (Koch et al., 1997; Strausak et al., 1999). Neither does MmoR contain the four conserved cysteine residues believed to bind a metal ion in the region between the central and C-terminal regions of oxygen-sensitive NifA proteins (Morrett & Segovia, 1993).

In the following article, Csáki et al. (2003) also report the cloning and sequencing of an EBP located in close proximity to the *smmo* gene cluster from *Mc. capsulatus* Bath (see Fig. 1 in Csáki et al., 2003). A comparison of the amino acid sequences of MmoR from *Ms. trichosporium* OB3b and *Mc. capsulatus* Bath reveals that they possess 40% identity (54% similarity) over the whole polypeptide and that the central region is very highly conserved (53% identity, 71% similarity). Interestingly, an alignment of the N-terminal regions reveals significant homology from amino acid 1 to 184. In contrast the derived amino acid sequence of the N-terminal region of AcoR from *Ralstonia eutrophus* possesses only 20% identity with MmoR from *Mc. capsulatus* Bath and 24% with MmoR from *Ms. trichosporium* OB3b, and lacks any runs of identical sequence motifs, similar to those observed in an alignment between MmoR from the two methanotrophs. However, the predicted amino acid sequence of MmoR from *Mc. capsulatus* Bath is 37 aa longer (at the N-terminal end) and MmoR from *Ms. trichosporium* OB3b contains three sections absent from MmoR of *Mc. capsulatus* Bath in the region between the N terminus and the conserved central region (of 43, 26 and 36 aa). It is
possible that the observed common motifs may play a role in receiving the low-copper signal and allow activation of the smmo genes.

To the best of our knowledge, the only example of a metal-regulated EBP is ZraR from *E. coli*, which is believed to play a role in zinc homeostasis. However, ZraR is a member of a two-component sensor pair with ZraS, where ZraS is the actual sensor of zinc levels (Reiter & Schneider, 2001). Thus MmoR may represent a novel copper-responsive member of the EBP family.

The ORF immediately 5' of mmoX encodes a member of the GroEL (Cpn60) protein chaperone family. Since this groEL homologue lies in an apparent operon with *mmoR*, upstream (5') of the structural genes encoding sMMO, we propose the name *mmoG* (methane monooxygenase-associated GroEL homologue). It encodes a polypeptide of 581 aa, approximately 40 aa longer than most GroEL polypeptides. There is a putative Gly-Gly-Met (GGM) repeat at the extreme C terminus of the polypeptide. This repeat of chaperone expression: TTAGCACTC-N9-GAG-GmR (from Neisseria meningitidis (Parkhill et al., 2000). Csáki et al. (2003) also report the sequencing of a GroEL homologue, also designated *mmoG*, located close to the mmo gene cluster from *Ms. capsulatus* Bath whose derived amino acid sequence is 41% identical to MmoG from *Ms. trichosporium* OB3b. The MmoG protein from *Ms. capsulatus* Bath also lacks a groES companion gene, but in contrast to *Ms. trichosporium* OB3b, *mmoG* in *Mc. capsulatus* is oriented 3' of *mmoC*. However, it seems unlikely that their location in close proximity to the *smmo* gene cluster and the lack of a groES companion gene is a coincidence and they may both play a key role in smmo regulation.

Sequence analysis of MmoG from *Ms. trichosporium* OB3b revealed the presence of a region of identity with the F1α ATPase subunits of ATP synthases and a valline proposed to be involved in GroEL oligomerization (Tanaka et al., 1997; Lund, 2001). Many GroEL proteins contain multiple Gly-Gly-Met (GGM) repeats at the extreme C terminus (Tanaka et al., 1997; Lund, 2001) which are absent from the MmoG sequence. It is worth noting that the predicted length of this polypeptide is 581 aa, approximately 40 aa longer than most GroEL polypeptides. There is a putative Shine–Dalgarno sequence (GAGGA) 17 bp 5' of the start codon and a putative stable stem–loop structure (AAAG-CGCTGCGGCAGA-N4-TTCGCGCAGCGUUUU) (ΔG = −23·2 kcal = −97·1 kJ) 28 bp downstream (3') of the end of *mmoG*. The presence of several U residues after the predicted stem–loop indicates that this may be a ρ-independent transcriptional terminator and that *mmoG* is transcribed independently of the *smmo* operon. The absence of putative terminators between *mmoR* and *mmoG* coupled with RT-PCR results shown later indicated that they may be transcribed together. Therefore, in contrast to many GroEL genes, the *mmoG* gene from *Ms. trichosporium* OB3b is not found in an operon with a GroES gene. Several bacteria contain individual groEL genes and always one or more complete groESL operon (Lund, 2001). The presence of a lone *mmoG* gene indicates that *Ms. trichosporium* OB3b may possess one or more groESL operons in its genome. Many groEL genes are regulated in a negative manner by a repressor, HrcA, via binding to a 9 bp conserved inverted repeat sequence or CIRCE element (controlling inverted repeat of chaperone expression): TTAGCACTC-N9-GAG-TGAAA (Lemos et al., 2001; Segal & Ron, 1996). Neither a CIRCE element nor putative promoter sequences is found 5' of *mmoG* or *mmoR* in *Ms. trichosporium* OB3b. Attempts to identify the transcriptional start site for the *mmoR* and *mmoG* genes by primer extension were unsuccessful (data not shown).

The presence of a σ^N^-dependent transcriptional activator in close proximity to the mmo cluster indicated that it probably played an important role in transcription of the *mmo* operon. Several bacterial operons are regulated in a σ^N^-dependent manner by a positive activator proximal to the cognate gene cluster, e.g. *E. coli* S17-1 to the recipient organism by conjugation, but are not maintained in methanotrophs (Martin & Murrell, 1995).

For mutation of the rpoN gene, plasmid pGPS103Gm was constructed as follows. A 563 bp *NcoI*-HindIII fragment was deleted from the chloramphenicol resistance gene of pBR329mob. Into this modified version of pBR329mob, a 2·3 kb *NcoI*-HindIII fragment from pGPS519, containing rpoN and ORF1, was ligated. The rpoN gene was then disrupted by the insertion of the Km resistance cassette (GmR^R^) from p34S-Gm into a BglIII site which lies in the middle of rpoN. This was achieved by digestion of pGPS103 with BglIII, followed by ligation of an 863 bp *BamHI* fragment containing GmR^R^ into this site to create pGPS103Gm (Fig. 3a).

Similarly, pJS102 was constructed for mutagenesis of mmoR (Fig. 3b). A 4424 bp HindIII–*BamHI* fragment containing mmoR and mmoG was ligated into pK18mob (to give pK18mob-mmo) before deletion of a 1014 bp *SalI* fragment which was subsequently replaced by a GmR^R^ cassette from p345-Gm to create pJS102 (Fig. 3b).

**Marker-exchange mutagenesis of rpoN and mmoR**

To inactivate rpoN and mmoR from *Ms. trichosporium* OB3b, a double crossover homologous recombination event between pGPS103Gm or pJS102 and the wild-type version of rpoN or mmoR in the chromosome must occur. This

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would result in the Gm<sup>R</sup> cassette residing in the chromosomal copy of rpoN/mmoR. Plasmids pGPS103Gm and pJS102 were transferred into Ms. trichosporium OB3b by filter mating. Exconjugants were selected on NMS agar plates containing 5 µg Gm ml<sup>−1</sup>. After transfer of pGPS103Gm into Ms. trichosporium OB3b, strain Gm1 was obtained. It was later discovered that the inability to use nitrate as a nitrogen source is a phenotype of the rpoN mutant, Gm1. Therefore, this mutant was probably using alternative nitrogen sources that were released from the dead or dying bacteria on the NMS selection plates. Strain Gm1 had a Gm<sup>R</sup> Ap<sup>S</sup> phenotype which indicated the loss of the plasmid backbone from the cell and that a double crossover event had occurred. Analysis of rpoN from strain Gm1 by PCR and Southern blotting (data not shown) indicated that a double crossover event had occurred, which was subsequently confirmed by the phenotype of this mutant, described later. Analysis of the DNA sequence between rpoN and ORF1 revealed a potential stem–loop terminator sequence, indicating that ORF1 and rpoN may be transcribed independently. This information, coupled with the absence of terminator sequences in the Gm<sup>R</sup> cassette (from p34S-Gm), indicates that this mutation in rpoN should not have a polar effect on ORFs 1 and 2.

Strain JS1 was isolated following the transfer of pJS102 into Ms. trichosporium OB3b. This strain had a Gm<sup>R</sup> Km<sup>S</sup> phenotype, indicating that a double crossover event had occurred, resulting in the loss of the pJS1 plasmid backbone from the chromosome. Analysis of strain JS1 by PCR and Southern blotting using the mmoR gene as a probe confirmed a double crossover event had occurred between pJS1 and the chromosomal copy of mmoR (data not shown).

**Inactivation of rpoN and mmoR abolishes sMMO expression at the level of transcription**

Activity of sMMO in rpoN and mmoR mutant strains was tested qualitatively using the naphthalene assay (Brusseau et al., 1990). Colonies expressing sMMO (wild-type) appeared purple on the addition of tetrazotized o-dianisidine, whereas those not expressing sMMO remained orange. It was clear

**Fig. 3.** Plasmids constructed for marker exchange mutagenesis of (a) rpoN (pGPS103Gm), (b) mmoR (pJS102) and (c) mmoG (pJS104). (a) pGPS103 was constructed as follows. A 563 bp Ncol–HindIII fragment was deleted from the chloramphenicol resistance gene of pBR329mob, followed by insertion of a 2.3 kb Ncol–HindIII fragment from pGPS519, containing rpoN and ORF1. The rpoN gene was then disrupted by the insertion of an 863 bp BamHI fragment containing the Gm resistance cassette (Gm<sup>R</sup>) from p34S-Gm into the BglII site, which lies within rpoN. (b) pJS102 was constructed as follows. A 4424 bp HindIII–BamHI fragment containing mmoR and mmoG was ligated into pK18mob before deletion of a 1014 bp SalI fragment from mmoR, which was subsequently replaced by insertion of an 89 bp SalI fragment containing the Gm<sup>R</sup> cassette from p34S-Gm to create pJS102. (c) Plasmid pJS104 was constructed in a similar manner to pJS102. The same HindIII–BamHI fragment was cloned into the vector pK18mobSacB before removal of a 635 bp SphI fragment from the mmoG gene. This was then replaced by a 913 bp SphI fragment containing the Gm<sup>R</sup> cassette from p34S-Gm to create pJS104.
from these plate assays that there was no sMMO activity in strain Gm1 or JS1 (Fig. 4). Cell-free extracts from low-copper-grown cells of strains Gm1, JS1 and wild-type Ms. trichosporium OB3b were prepared. These extracts were then analysed by SDS-PAGE, which clearly showed that the levels of sMMO subunits were significantly reduced in cell-free extracts of strains Gm1 and JS1 (Fig. 5a). A Western blot of an identical gel was probed with antisera to the sMMO-hydroxylase complex. Although there were problems with non-specific binding of the antisera to the other proteins, including the large subunit of methanol dehydrogenase, it was clear that the levels of α, β and γ subunits were dramatically reduced in cell-free extracts prepared from strains Gm1 and JS1 (Fig. 5b).

The absence of the sMMO-hydroxylase subunits and lack of sMMO activity indicated that initiation of transcription from the σ^N-promoter 5' of mmoX may be defective in both mutants. Thus, the presence of mmoX-specific transcripts was tested using RT-PCR. Total RNA was extracted from cultures of wild-type, strain Gm1 and strain JS1 grown in low-copper medium. No mmoX-specific transcripts were detected by RT-PCR in RNA extracted from either the JS1 or Gm1 strains (Table 2). Therefore, when the sMMO activity assay, SDS-PAGE, Western blots and RT-PCR data are considered as a whole, it is clear that both the rpoN and mmoR gene products are indispensable for the regulation of the mmo operon and that they act at the level of transcriptional initiation. The confirmation of a role for σ^N in the regulation of the mmo operon from Ms. trichosporium OB3b, coupled with the identification of putative σ^N-type promoters upstream of the mmo operons of Methylacystis sp. strain M (McDonald et al., 1997), Mc. capsulatus Bath (Nielsen et al., 1996), Methylohomas sp. KSPII and KSWIII (Shigematsu et al., 1999) implies that this mechanism of regulation may be widespread among methanotrophs. Indeed a similar EBP gene lies 3’ of the mmo gene cluster in Mc. capsulatus Bath and is thought to be involved in regulation of mmo expression (Csáki et al., 2003).

**mmoG is transcribed in a σ^N-independent manner**

RT-PCR was used to assess the presence of mmoG transcripts in strain JS1 using primers CPN60F3498 and CPN60R3941. In addition, transcription of mmoG was tested in strain Gm1 (rpoN mutant) to determine if its expression was σ^N-dependent. Transcripts for mmoG from strains JS1, Gm1 and the wild-type were detected in cells grown on low-copper media (Table 2). (We were unable to assess transcription of the mmoR gene due to problems in amplification of mmoR by PCR.) The presence of an mmoG transcript in strains Gm1 and JS1 showed that mmoG is transcribed in a σ^N- and MmOR-independent manner. The RT-PCR data also suggest that mmoG may be transcribed constitutively, i.e. under high- and low-copper conditions (data not shown). groEL genes are not always expressed (Segal & Ron, 1996). For example, a transcript for the groESL2 operon of *Rhodobacter sphaeroides* is not detectable under heat shock or normal growth conditions (Lee et al., 1997). In contrast, the five groESL operons from *Bradyrhizobium japonicum* are expressed to different degrees under certain conditions with the groESL3 operon being regulated in a σ^N-dependent manner (Fischer et al., 1993). Preliminary data suggest that both the genetic location and regulation of this mmoG may be unique and warrant further investigation.

**A marker-exchange mutant of mmoG does not express functional sMMO**

To examine if mmoG is involved in the regulation/assembly of the sMMO enzyme, a mutant lacking a functional mmoG was constructed using the suicide plasmid pJS104. Plasmid pJS104 was constructed in a similar manner to pJS102 (Fig. 3). The same HinIII–BamHI fragment was cloned into the vector pK18mob8SacB before removal of a 635 bp *Sphl* fragment from the mmoG gene. This was then replaced by the Gm 8 cassette from p34S-Gm to create pJS104. After conjugation and selection of Gm 8 Km^- clones, PCR and Southern blotting were used to confirm that a double recombination event had occurred, resulting in mutagenesis of mmoG (data not shown). The activity of the sMMO enzyme was assayed using the naphthalene assay and showed that strain JS2 was incapable of producing a functional sMMO enzyme under low-copper conditions (Fig. 4). Although it was clear that the mmoG mutant lacked a
functional sMMO it was not possible to determine whether this was due to an absence of the sMMO polypeptides or the presence of non-functional (presumably incorrectly folded) sMMO polypeptides. SDS-PAGE and Western analysis using sMMO-hydroxylase-specific antisera clearly showed that the sMMO subunits were absent from strain JS2 under high- and low-copper growth conditions (Fig. 5a, b). To determine whether the absence of the sMMO polypeptides was due to a lack of transcription we performed mmoX-specific RT-PCR. Total RNA was extracted from strain JS2 grown under the presence and absence of copper. No mmoX-specific transcripts were detected from RNA from strain JS2 (Table 2), indicating that MmoG, in addition to MmoR and RpoN is required for transcription of the mmo operon.

This was surprising, since if MmoG was an sMMO-specific chaperone then the polypeptides might be present, but unable to form a functional sMMO. However, it is possible that MmoG is a co-regulator of sMMO, acting via interaction with MmoR, or that it is required for the folding of MmoR into a transcriptionally competent conformation. The latter possibility would be unusual since GroEL chaperones are believed to be relatively promiscuous in

**Table 2. Summary of RT-PCR results from mutant strains of Ms. trichosporium OB3b**

RNA was extracted from late-exponential cultures of relevant strains of Ms. trichosporium OB3b in the presence of high (+Cu) or low (−Cu) copper as described in Methods. The plus or minus signs refer to the presence or absence, respectively, of the relevant RT-PCR product using the primers described in Methods.

<table>
<thead>
<tr>
<th>RT target</th>
<th>WT +Cu</th>
<th>−Cu</th>
<th>Gm1 rpoN::GmR +Cu</th>
<th>−Cu</th>
<th>JS1 mmoR::GmR +Cu</th>
<th>−Cu</th>
<th>JS2 mmoG::GmR +Cu</th>
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<td>mmoX</td>
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the range of proteins over which they assist folding (reviewed by Lund, 2001). However, an unfolded variant of DmpR, an EBP controlling the dmp operon responsible for phenol catabolism in Pseudomonas species, containing deletions in the N-terminal phenol-binding effector region co-purified with GroEL when expressed in E. coli (Skärstad et al., 2000), suggesting that GroEL may be required for the folding of the native DmpR regulator. At present, it is not possible to distinguish between these (or any other) possibilities for the mechanism of regulation of the mmo operon by Mmog and MmOR, but adds further interest to the discovery of a GroEL homologue in this region of the chromosome of Ms. trichosporium OB3b.

**Mutation of rpoN abolishes the ability of Ms. trichosporium OB3b to utilize N₂ or nitrate as sole nitrogen source**

It is known that Ms. trichosporium OB3b is capable of growth on several nitrogen sources, including N₂, nitrate and ammonium (Murrell & Dalton, 1983a, b). In many organisms, several facets of nitrogen metabolism are under the control of σN (Merrick, 1993; Merrick & Edwards, 1995). Therefore, to assess the role which σN plays in the regulation of these processes in Ms. trichosporium OB3b, the ability of the rpoN⁻ strain Gm1 to grow on several nitrogen sources was tested. In contrast to the wild-type strain, strain Gm1 was unable to grow under N₂-fixing conditions indicating that σN was required for the transcription of the N₂-fixation (nif) genes in Ms. trichosporium OB3b. However, it was possible that the inability of the Gm1 strain to use N₂ as a nitrogen source was due to an inability to assimilate ammonia.

Strain Gm1 was originally isolated on NMS agar, but grew very poorly on this medium. As NMS agar may contain trace amounts of alternative nitrogen sources, the ability of strain Gm1 to utilize nitrate (10 mM) as sole nitrogen source in NMS liquid medium was tested. These growth experiments showed conclusively that the Gm1 strain could not use nitrate as sole nitrogen source (data not shown). However, the product of nitrate reduction by nitrate reductase and nitrite reductase is ammonia. Therefore, a defect in the ammonia assimilation process may also be the cause of an inability to use nitrate as nitrogen source.

Previous work by Murrell & Dalton (1983b) had shown that Ms. trichosporium OB3b possessed both glutamine synthetase (GS) and glutamate synthase (GOGAT). These enzymes enable assimilation of ammonia by conversion to glutamine and then glutamate. Cell extracts from cells grown with nitrate (10 mM), ammonium (18 mM) and N₂ all possessed similar GS and GOGAT activities (Murrell & Dalton, 1983b). The absence of glutamate dehydrogenase was noted and indicated that Ms. trichosporium OB3b assimilated ammonia solely via the GS/GOGAT pathway. Thus, the observation in this study that strain Gm1 can utilize ammonia (18 mM) and glutamine (39 mM) as sole nitrogen sources at growth rates similar to the wild-type strain (data not shown) indicates that its GS/GOGAT pathway is intact and constitutively expressed. Thus, the σN of Ms. trichosporium OB3b appears to be essential to regulation of nitrogen metabolism and is probably required for the initiation of transcription of the enzyme systems responsible for nitrogen fixation and assimilation of nitrate, but not the GS/GOGAT pathway.

In contrast, strain JS1 was capable of growth on all nitrogen sources tested, indicating that mmoR plays no role in the regulation of the aspects of nitrogen metabolism examined in this study.

**Final conclusions and future prospects**

We have shown the importance of rpoN in at least three processes: (1) expression of the sMMO enzyme; (2) growth on nitrate as sole nitrogen source; and (3) N₂ fixation. We also report the identification of a new member of the EBPs, MmOR, which is responsible for transcriptional regulation of the mmo operon and may provide the first example of an EBP directly responsive to a metal ion. This mode of regulation may represent a general mechanism of smmo gene regulation in methanotrophs since σN promoters are found 5’ of the mmoX genes in several methanotrophs (Nielsen et al., 1996; McDonald et al., 1997; Shigematsu et al., 1999). In addition, mmoR and mmoG homologues are present in the mmo gene cluster of Mc. capsulatus Bath (Csáki et al., 2003). Interestingly, insertion mutations in both mmoR and mmoG from Mc. capsulatus Bath also inactivate sMMO activity and abolish transcription in a manner similar to that reported in this paper (Csáki et al., 2003). However, the factors controlling the transcription of the pmmo operon remain unknown. Recent findings regarding the pmmo operon indicate that it is transcribed from σN promoters in Methylocystis sp. strain M (Gilbert et al., 2000), Mc. capsulatus Bath (Stolyar et al., 2001) and Ms. trichosporium OB3b (G. P Stafford & J. C. Murrell, unpublished) and is not controlled by σN or MmOR since strains Gm1 and JS1 can still grow on methane in high-copper medium. The copper-induced switch between mmo and pmmo expression must involve a copper-sensing system that transmits the signal to MmOR and the pmmo operon. At this stage, the mechanism of how MmOR senses copper remains an enigma. Does MmOR bind copper directly? Is MmOR itself regulated by copper levels? Where does MmOR bind upstream of mmoX? Does MmOR sense copper or relay the signal to MmR? Some of the answers to these questions may be revealed by studies in Mc. capsulatus Bath, where Csáki et al. (2003) report a possible signal relay mechanism through a sensor–regulator pair found in this region of the chromosome in Mc. capsulatus Bath (mmoQS). It is also possible that a similar pair of genes is present 5’ of mmoR in Ms. trichosporium OB3b and attempts are currently under way to identify these genes in our laboratory.

We also report the discovery of an MmOR-specific GroEL-type chaperone (Mmog), which may be required for the
correct assembly of MmoR, or another as yet unknown regulatory protein, into a transcriptionally competent state under low-copper conditions.

In light of the data presented here, we propose a new model for the regulation of the smmo system in Ms. trichosporium OB3b (Fig. 6). Transcription of the smmo cluster under copper-limitation occurs from a σ^N-dependent promoter located 5' of mmoX, since mmo transcription is abolished in the rpoN::Gm strain Gm1. Transcription from this promoter also requires the smmo-specific EBP MmoR and the GroEL homologue MmoG, as shown by mutation of mmoR (strain JS1, mmoR::Gm) and mmoG (strain JS2, mmoG::Gm). This work has also revealed that mutation of the mmoG gene results in a strain (JS2, mmoG::Gm) which fails to produce a functional sMMO complex. Thus we propose that production of a functional sMMO-enzyme complex under low-copper conditions requires the transcription factors MmoR and RpoN in addition to the putative MmoR-specific chaperone, MmoG.

We also propose that σ^N is an integral component of a regulon, including the nitrogen fixation (nif) and assimilatory nitrate utilization (nas) systems in addition to the smmo operon of Ms. trichosporium OB3b.

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