Regulation of bacterial methane oxidation: transcription of the soluble methane mono-oxygenase operon of *Methylococcus capsulatus* (Bath) is repressed by copper ions

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Methane is oxidized to methanol by the enzyme methane mono-oxygenase (MMO) in methanotrophic bacteria. In previous work, this multicomponent enzyme system has been extensively characterized at the biochemical and molecular level. Copper ions have been shown to irreversibly inhibit MMO activity *in vivo* and *in vitro*, but the effect of copper ions on transcription of the genes encoding the soluble (cytoplasmic) MMO (sMMO) has not previously been investigated. To examine more closely the regulation of bacterial methane oxidation and to determine the role of copper in this process, we have investigated transcriptional regulation of the sMMO gene cluster in the methanotrophic bacterium *Methylococcus capsulatus* (Bath). Using Northern blot analysis and primer extension experiments, it was shown that the six ORFs of the sMMO gene cluster are organized as an operon and the transcripts produced upon expression of this operon have been identified. The synthesis of these transcripts was under control of a single copper-regulated promoter, which is as yet not precisely defined.

Keywords: *Methylococcus capsulatus* (Bath), methanotrophs, *smmo* genes, mRNA, copper ions

INTRODUCTION

The obligate methane-oxidizing bacteria, methanotrophs, utilize methane as their sole source of carbon and energy. The first step in the methane oxidation pathway is catalysed by the enzyme methane mono-oxygenase (MMO), which converts methane to methanol in an oxygen- and NADH-dependent reaction. Methanol is then oxidized by methanol dehydrogenase to formaldehyde, which is assimilated into cellular biomass. A proportion of the formaldehyde is further oxidized via formate to carbon dioxide to provide reducing power for biosynthesis and the initial oxidation of methane (Anthony, 1986).

Whereas all methanotrophs appear to express a membrane-bound, particulate MMO (pMMO), a soluble enzyme complex (sMMO) has been found in only a few methanotrophs. The type of enzyme expressed seems to

be dependent upon the availability of copper ions in the growth medium; pMMO activity is observed when organisms are grown at a high copper ion:biomass ratio, whereas organisms subjected to copper deficiency contain sMMO (Scott *et al.*, 1981a, b; Stanley *et al.*, 1983; Koh *et al.*, 1993; Nakajama *et al.*, 1992; Pilkington & Dalton, 1991). sMMO has a broader substrate range than the pMMO. It catalyses the oxidation of a wide range of aliphatic, alicyclic and aromatic hydrocarbons (Colby & Dalton, 1978; Stirling & Dalton, 1979).

The sMMO complex from the type X methanotroph *Methylococcus capsulatus* (Bath) has been extensively characterized (Colby & Dalton, 1979; Green & Dalton, 1985; Woodland & Dalton, 1989; Dalton *et al.*, 1993). sMMO consists of three components. Protein A, the hydroxylase component, comprises three subunits, α , β and γ , of 60, 45 and 20 kDa, respectively (Woodland & Dalton, 1989). Protein B is a protein of 16 kDa and is thought to act as a modulator of the MMO reaction by forming a specific complex with the hydroxylase component (Green & Dalton, 1985; Liu & Lippard, 1991; Fox *et al.*, 1991; Lipscomb, 1994). Protein C is a 39 kDa iron-sulfur

Abbreviations: s/pMMO, soluble/particulate methane mono-oxygenase; NMS, nitrate mineral salts.

flavoprotein responsible for shuttling electrons from NADH to Protein A of sMMO (Lund & Dalton, 1985; Lund *et al.*, 1985). The sMMO from the type II methanotroph *Methylosinus trichosporium* OB3b has also been extensively characterized. The sMMO from this related organism was resolved into three components with similar properties to the components of the sMMO from *M. capsulatus* (Bath) (Fox *et al.*, 1989, 1990).

The structural genes encoding the sMMO components of *Methylococcus capsulatus* (Bath) (*smmo*) have been cloned and sequenced (Stainthorpe *et al.*, 1989, 1990). These genes were found to be clustered in the order *mmoX*, *mmoY*, *mmoB*, *mmoZ*, *orfY* and *mmoC* and encode α , β , Protein B, γ , a protein of unknown function and Protein C, respectively. This clustering of the sMMO structural genes has also been found in the type II methanotroph *Methylosinus trichosporium* OB3b (Cardy *et al.*, 1991a, b).

Relatively little is known, however, about the regulation of this gene cluster since the genetics of methanotrophs is relatively poorly developed and there are few efficient systems for gene transfer or mutagenesis in these organisms (reviewed in Murrell, 1994). Similarly, expression studies on sMMO using primer extension and Northern blot analyses have been hampered by difficulties in the preparation of good quality mRNA from methanotrophs (J. C. Murrell & D. L. N. Cardy, unpublished observations).

The switch from expression of the soluble to the particulate enzyme complex in *Methylococcus capsulatus* (Bath) has been shown to depend on the concentration of copper ions in the growth medium. The switch is correlated with the formation of extensive intracytoplasmic membranes and the MMO activity of the cells becomes entirely membrane-associated. sMMO activity is no longer detectable (Prior & Dalton, 1985).

It is not fully understood how copper ions mediate this switch. The regulation of sMMO and pMMO, and the role of copper in this metabolic switch, is complicated by the pleiotropic effect of this ion in methane oxidation. Copper ions are essential for pMMO activity and are believed to reside in the active site of the enzyme (Chan *et al.*, 1993; Nguyen *et al.*, 1994). Copper ions have also been shown to inhibit sMMO activity both *in vivo* and *in vitro* (Stanley *et al.*, 1983; Dalton *et al.*, 1984; Prior & Dalton, 1985; Green *et al.*, 1985). Finally, in addition to the inhibitory effect of copper ions on the soluble enzyme itself, it has been suggested that copper ions may have a potential role as a cofactor in transcriptional inhibition of the sMMO gene cluster of *Methylosinus trichosporium* OB3b (Tsien *et al.*, 1989).

METHODS

Bacterial strains. The type X methanotroph *Methylococcus capsulatus* (Bath) was obtained from the University of Warwick culture collection. The *Escherichia coli* K12 strain CSH50 [$\Delta(lac pro)rpsL$; Miller, 1972] was used as the host in DNA cloning experiments.

Growth of *M. capsulatus* (Bath). *M. capsulatus* (Bath) was grown in continuous culture in a 2.5 l working volume fermenter (LH Engineering) on a nitrate mineral salts (NMS) medium (Dalton & Whittenbury, 1976). The chemostat was operated under oxygen limitation with methane (20%, v/v, in air, at a flow rate of 250 ml min⁻¹) as carbon source. Temperature and pH were maintained at 45 °C and 6.8, respectively. A dilution rate (*D*) of 0.05 h⁻¹ was used and at steady-state, the dry weight of the culture was 1.5–2.0 mg ml⁻¹. To ensure that *M. capsulatus* cells were only expressing sMMO, Cu²⁺ was omitted from the trace elements solution of NMS. To confirm the presence or absence of sMMO, the assay of Brusseau *et al.* (1990), which measures the oxidation of naphthalene to naphthol by sMMO, but not pMMO, was used.

Biochemical methods. Preparation of plasmid DNA and DNA manipulations were according to Maniatis *et al.* (1982). *In vitro* RNA synthesis was performed according to the manufacturer (Promega).

Construction of plasmids

Plasmid pMX2. The upstream 2.67 kb *EcoRI*–*Bam*HI fragment of pCH4 was purified from an agarose gel and ligated to the large *EcoRI*–*Bam*HI fragment of pBR322 (Bolivar *et al.*, 1977), resulting in pMO1. Thus, pMO1 contains the first 1193 nucleotides of the *mmoX* gene preceded by the 1477 upstream nucleotides. The *mmoX* intragenic *Pst*I–*Bam*HI fragment of 0.58 kb was isolated from pMO1 and inserted into pGEM3 cut with the same enzymes, resulting in pMX2. Transcription from the T7 RNA polymerase promoter, pT7, produced a single-stranded transcript complementary to *mmoX*-encoding mRNAs.

Plasmids pMY2, pMB1 and pMZ1. The *smmo*-specific fragments of plasmids pMY2, pMB1 and pMZ1 were generated by PCR using plasmid pCH4 as template. The primers used for PCR amplifications contained recognition sequences for the restriction enzymes *Bam*HI (upstream primer) and *Eco*RI (downstream primer) and after digestion with these enzymes, the DNA fragments were ligated to pGEM3 (Promega) also digested with these enzymes. The transcripts produced upon transcription from pT7 of these plasmids are described in Table 1.

Plasmid pMC2. The region covering the last 16 nucleotides of *orfY*, the *orfY*–*mmoC* intergenic region and the first 194 nucleotides of *mmoC* was amplified by PCR using plasmid pCH4 as template and primers with *Eco*RI (upstream primer) and *Pst*I (downstream primer) sites. After restriction of the amplified DNA fragment with these enzymes, the resulting *Eco*RI–*Pst*I DNA fragment was ligated to pGEM4 (Promega) digested with the same enzymes, resulting in pMC2. Prior to use as template for *in vitro* generation of an *orfY*–*mmoC*-specific riboprobe, plasmid pMC2 was restricted with *Aat*II. Thus, the resulting riboprobe covers the region from 5725 to 5831 (the last 16 nucleotides of *orfY*, the *orfY*–*mmoC* intergenic region and the first 76 nucleotides of *mmoC*).

Isolation of total RNA. Total RNA was isolated from 1.5 ml aliquots of *M. capsulatus* (Bath) cells taken from a chemostat culture (OD₅₄₀ of approximately 8.0). The cells were pelleted by centrifugation in 1.5 ml Eppendorf tubes and resuspended in 300 μ l of a solution containing 0.3 M sucrose, 5 mg lysozyme ml⁻¹ and 0.01 M sodium acetate, pH 4.5. These aliquots were kept on ice for 10 min and then frozen in liquid nitrogen for 10 s. A solution (300 μ l) containing 2% (w/v) SDS and 0.01 M sodium acetate, pH 4.5, was added and the aliquots were incubated for 1 min at 65 °C. The preparations were mixed with 0.5 ml phenol and incubated with vigorous shaking for 3 min at 65 °C and then frozen in liquid nitrogen for 10 s. The

Table 1. Plasmids used in this study

Plasmid	Type of replicon	Relevant genotypes	<i>smmo</i> co-ordinates present*	Riboprobe complementary to:	Source
pMX2	pGEM3	<i>bla</i> pT7	2087–2670	<i>mmoX</i>	This study
pMY2	pGEM3	<i>bla</i> pT7	3511–4326	<i>mmoY</i>	This study
pMB1	pGEM3	<i>bla</i> pT7	4343–4698	<i>mmoB</i>	This study
pMZ1	pGEM3	<i>bla</i> pT7	4884–5232	<i>mmoZ</i>	This study
pMC2	pGEM4	<i>bla</i> pT7	5725–5949†	<i>orfY-mmoC</i>	This study
pCH4	pBR325	<i>bla tet</i>	1–7187	–	Stainthorpe <i>et al.</i> (1990)
pGEM3	–	<i>bla</i> pT7	–	–	Promega
pGEM4	–	<i>bla</i> pT7	–	–	Promega

* Gene EMBL accession number, M90050.

† The riboprobe generated from pMC2 covers the region from 5725 to 5831 (see Methods).

supernatants were recovered by centrifugation for 5 min and the phenol extraction repeated. Finally, the RNA was extracted with 0.5 ml phenol/chloroform (1:1) at room temperature, precipitated with 1.2 ml 96% (v/v) ethanol and 75 µl sodium acetate, pH 4.5. The pellet was resuspended in 30 µl H₂O and the concentration of nucleic acid determined by measuring the A_{260} on an Ultraspec III spectrophotometer (Pharmacia).

Northern blot analysis. RNA (10 µg) was dried in a vacuum drier and resuspended in 4.5 µl H₂O, 2 µl 5× formaldehyde gel running (FGR) buffer (0.1 M MOPS, pH 7, 40 mM sodium acetate, 5 mM EDTA), 3.5 µl formaldehyde (7%, v/v, final concentration) and 10 µl formamide (50%, v/v, final concentration). The RNA molecules were denatured by incubation for 15 min at 60 °C and separated in a 1.2% (w/v) agarose gel containing 2.2% formaldehyde which was run at 5 V cm⁻¹ with FGR buffer as the electrophoresis buffer. The gel was then washed in H₂O for 20 min at room temperature. Nucleic acids were transferred to a Zeta-Probe GT membrane (Bio-Rad) by overnight capillary blotting using 50 mM NaOH as the transfer buffer. The membranes were air-dried and prehybridized for 2 h at 42 °C in hybridization buffer (1 mM NaCl, 4 mM Na₄P₂O₇·10H₂O, 5× Denhardt's solution, 1% SDS, 10%, w/v, PEG 6000, 50 mM Tris/HCl (pH 7.5) and 250 µg yeast tRNA ml⁻¹) before the ³²P-labelled riboprobe was added. After overnight hybridization at 42 °C, membranes were washed two times for 5 min at room temperature in 2× SSC, two times for 30 min at 65 °C in 0.2× SSC, 1% SDS and two times for 30 min at 65 °C in 0.1× SSC before being air-dried and used for autoradiography. The 0.24–9.5 kb RNA ladder from Gibco BRL was used as molecular size standard in Northern blot experiments.

Primer extension experiments. RNA (5 µg) and ³²P-labelled primer (15 ng) were heated for 2 min at 80 °C in 5 µl hybridization buffer (100 mM KCl, 50 mM HEPES, pH 7.0) followed by gradual cooling to 30 °C over a 60 min period. Three microlitres of a solution containing 250 mM Tris/HCl, pH 8.4, 20 mM MgCl₂, 20 mM DTT, 0.1 mM (each) dNTP and 0.75 units AMV reverse transcriptase µl⁻¹ (Life Sciences) was added and the mixture was incubated at 40 °C for 30 min. The extension products were precipitated with ethanol and resuspended in 6 µl formamide loading buffer, preheated at 85 °C for 3 min and loaded onto a 7% polyacrylamide gel alongside a set of dideoxy sequencing reactions (Sanger *et al.*, 1977) of plasmid pCH4 DNA with the same primer. The sequences of the primers used (in the 5'–3' direction) were:

Xp1, ATGCCCGATGACGTCACGG; Xp2, AGCCATCGG-ACATAACGG; Xp3, GCGGCCGCCGGAACCTG; Xp4, TCGATCCGACCACTGCGG; Xp5, ACGCGGCGGTGC-CGCTG; Yp1, TCGGTCAGACCGCGCGTC; orfYp1, TGGCTGAAATGCCGATTTCG; MC5781, CGCCATCCTC-CGTCACCG.

RESULTS

Identification of SMMO-specific transcripts

sMMO-specific transcripts were investigated by Northern blot analysis of total RNA isolated from *M. capsulatus* (Bath) cells grown in continuous culture on a nitrate mineral salts medium (Dalton & Whittenbury, 1976). The chemostat was operated under oxygen limitation with methane as sole source of carbon and energy. sMMO-expressing cells (at high cell density) were established on copper-deficient NMS medium (i.e. with no added copper) and pMMO expressing cells were established on NMS medium containing 50 µM copper sulphate. Gene-specific antisense riboprobes were obtained by subcloning of intragenic regions of *mmoX*, *mmoY*, *mmoB*, *mmoZ* and *mmoC* into pGEM3 or pGEM4 vectors, followed by *in vitro* transcription using the T7 RNA polymerase. The DNA fragments used for the construction of the probe plasmids are described in detail in Table 1.

Total RNA was fractionated on an agarose gel, transferred to a nylon membrane and hybridized to the ³²P-labelled gene-specific riboprobes. Total RNA isolated from *M. capsulatus* (Bath) cells grown in the presence of 50 µM copper sulphate did not hybridize to any of the probes used (not shown), which suggests that in the presence of copper, sMMO structural gene expression is switched off or repressed. Total RNA isolated from copper-depleted cultures (e.g. no added copper sulphate), however, yielded transcripts complementary to the specific gene probes used.

When the *mmoX*-specific gene probe was used to probe total RNA from *M. capsulatus* (Bath) grown in the absence of copper, two transcripts of approximately 1.7 and 4.0 kb

Fig. 1. Organization of the *smmo* operon (Stainthorpe *et al.*, 1990), origin of the *smmo* antisense riboprobes and identification of *smmo* transcripts. ORFs of the *smmo* operon are indicated with hatched boxes and DNA fragments used for construction of probe plasmids are indicated by black boxes below the *sMMO* gene cluster (see also Table 1). Messenger RNAs are indicated with arrows pointing in the 5'-3' direction. The 5' end is located at nucleotide 1391 and the approximate termination points are indicated at the end of each mRNA. The (not precisely defined) copper-regulated promoter region is indicated upstream of *mmoX*. ^{32}P -labelled antisense riboprobes were transcribed from the probe plasmids and used to generate the Northern blots shown below the vertical arrows. The molecular mass standard was a commercial RNA ladder (Gibco BRL).

exhibited homology to this probe (Fig. 1). We designated these transcripts mRNA-1 and mRNA-2, respectively. Assuming that there was transcription of the *smmo* gene cluster from a promoter not far upstream (5') of the *mmoX* gene, mRNA-1 may encode the *mmoX* gene product alone, and mRNA-2 may encode the *mmoX*, *mmoY*, *mmoB* and *mmoZ* gene products on one polycistronic mRNA.

This is consistent with the results obtained when the *mmoY*-, *mmoB*- and *mmoZ*-specific gene probes were used to probe *M. capsulatus* (Bath) total RNA: each of these gene probes only hybridized to one transcript of approximate size 4.0 kb, which we believe is identical to mRNA-2 (Fig. 1). Upon lengthy over-exposure of the autoradiographs, a faint band of approximate size 5.5–6.0 kb was detectable (not visible in Fig. 1). This may be a full-length transcript encoding the gene products of the entire *sMMO* gene cluster (see below). We designated this transcript mRNA-3.

M. capsulatus (Bath) total RNA, however, when hybridized to the *orfY*–*mmoC*-specific gene probe clearly yielded a transcript of approximately 5.5–6.0 kb (Fig. 1). This mRNA had the same mobility on 1.2% agarose gels as the weakly detectable mRNA-3 described above and, taken together with the primer extension analysis described below, we believe that these transcripts are identical.

Thus, transcription of the *sMMO* gene cluster appears to be initiated from a single promoter upstream of the *mmoX* gene.

Transcription of the *sMMO* gene cluster is copper-regulated

While it has been firmly established that increasing the amount of copper in the growth medium of *M. capsulatus* (Bath) induces the expression of pMMO and irreversibly inhibits the activity of the *sMMO* complex (Stanley *et al.*,

Fig. 2. The effect of copper addition to a chemostat culture of *M. capsulatus* (Bath). A steady-state culture of the organism was established on NMS medium at $D = 0.05 \text{ h}^{-1}$ with no added copper in the NMS medium. Copper sulphate was then added to a final concentration of $50 \mu\text{M}$, at time zero, directly into the culture and samples were removed for RNA isolation and sMMO activity determination.

1983; Dalton *et al.*, 1984; Prior & Dalton, 1985), the role of copper at the level of transcription still remains to be clarified. We therefore investigated the effect of copper ions on the transcription of the *smmo* gene cluster by adding copper ions to an sMMO-expressing culture of *M. capsulatus* (Bath). Actively growing sMMO-expressing cells [as determined by the ability of cells to oxidize naphthalene to naphthols (pMMO does not oxidize naphthalene) which can then be detected in a colorimetric assay (Brusseau *et al.*, 1990)] were maintained in a copper-depleted chemostat. Copper ions were added to a final concentration of $50 \mu\text{M}$, and samples were harvested before and at several time points after addition. The relative sMMO activity in the different samples was determined spectrophotometrically by measuring their ability to oxidize naphthalene.

The effect of copper ions on whole cell sMMO activity, as revealed by this experiment, is seen in Fig. 2. Sixty minutes after the addition of copper, the sMMO activity decreased to 50%, and after 120 min, the activity was only a few percent of the initial level (i.e. before the addition of copper).

To investigate if the cells responded to the copper treatment by switching off the transcription of the sMMO gene cluster, total RNA was isolated from the samples described above and used in Northern blot experiments. When the *mmoX*-specific gene probe was used to probe total RNA isolated from *M. capsulatus* (Bath) before and after the addition of copper, the levels of mRNA-1 and mRNA-2 were found to decrease with a half-life of 5–10 min (Fig. 3a). The disappearance of mRNA-2 was further visualized by probing of the RNA samples with the *mmoY*-, *mmoB*- and *mmoZ*-specific gene probes. As seen in Fig. 3(b), the copper-induced reduction in the

Fig. 3. Northern analysis of the sMMO mRNAs before and after addition of copper sulphate. Time-points of sampling are indicated above each lane (min). mRNA-1, mRNA-2 and mRNA-3 are described in Fig. 1. (a) RNA probed with *mmoX*; (b) RNA probed with *mmoB*; (c) RNA probed with *orfY-mmoC*. The molecular mass standard was a commercial RNA ladder (Gibco BRL). The riboprobes used were generated using T7 RNA polymerase with (a) plasmid pMX2, (b) plasmid pMB1 and (c) plasmid pMC2 (*Aat*II-digested) as templates.

level of *smmo* transcripts was also confirmed in these experiments (since the results of *mmoY*, *mmoB* and *mmoZ* probings were identical, only the *mmoB* probing is shown).

Data presented above suggested that the transcription of the three *smmo*-specific mRNAs is initiated from the same promoter upstream of the *mmoX* gene. Fig. 3(c) shows the result of an *orfY-mmoC*-specific probing of total RNA isolated from *M. capsulatus* before and after addition of copper sulphate. As expected, this transcript also disappeared within a similar time scale after addition of copper ions.

Primer extension analysis of sMMO mRNAs

To determine the exact location of the 5' ends of the sMMO transcripts, primer extension analysis was carried out on *M. capsulatus* (Bath) total RNA samples extracted from cells taken before and after addition of copper sulphate. The primer Xp1 (see Methods for sequence) was used to direct the synthesis of complementary cDNA in the region immediately upstream of *mmoX*. When total RNA isolated from the copper-depleted chemostat culture of *M. capsulatus* (Bath) was used as template for reverse transcriptase, a unique extension product was obtained (Fig. 4, lane 2). This extension product mapped to nucleotide C_{1391} which is located 87 nucleotides upstream of the *mmoX* start codon. No extension products were detected, however, when total RNA isolated from *M. capsulatus* (Bath) cells taken after addition of copper sulphate to the chemostat were used as templates for cDNA synthesis (Fig. 4, lanes 10 and 30). Since no obvious similarities to other known promoters are found just upstream of C_{1391} , this 5' end could either result from trimming of a longer transcript or from transcriptional initiation at this site. No specific 5' ends were found, however, when the primer extension experiments were performed with four different primers which anneal

Fig. 4. Primer extension analysis. RNA isolation and primer extension were carried out as described in Methods. The primer used (Xp1 – see Methods for sequence) is homologous to nucleotides 1422–1440, 37 bp upstream of the *mmoX* start codon. RNA was isolated from samples taken before and after the addition of copper sulphate. Time points of sampling after addition of copper sulphate are indicated above each lane (min). The nucleotide sequence ladder was obtained by sequencing of plasmid pCH4 using the Xp1 primer.

further upstream of Xp1 (data not shown). The primers used were Xp2, Xp3, Xp4 and Xp5 (see Methods for sequences).

Intergenic regions between *mmoX* and *mmoY* and between *mmoZ* and *orfY* are of sizes that would allow the presence of promoter elements without interfering with the expression of the adjacent genes (Stainthorpe *et al.*, 1989, 1990). For this reason, and to complement the Northern analysis, primer extension analyses were performed using primers complementary to these regions. The primers Yp1 and orfYp1 (see Methods for sequences) are complementary to the regions immediately downstream of the *mmoY* and *orfY* start codons, respectively, and when cDNA synthesis was directed by these primers, no extension products were detected (data not shown). Nor was an extension product obtained when an *mmoC*-specific primer (MC5781, see Methods) was used in an identical experiment (data not shown). Hence, the data obtained in the primer extension experiments are consistent with the Northern analyses.

DISCUSSION

Previous reports on DNA sequence analysis of the sMMO gene cluster of *M. capsulatus* (Bath) have shown that the genes encoding the components of sMMO are all linked on the chromosome in the order *mmoX*, *mmoY*, *mmoB*,

mmoZ, *orfY* and *mmoC*. Intercistronic regions were found to be relatively small, the largest are located between *mmoX* and *mmoY* (78 bp) and between *mmoZ* and *orfY* (155 bp) (Stainthorpe *et al.*, 1989, 1990). Only the *mmoX* gene was found to be preceded by putative promoter sequences showing homology to *E. coli* –35 and –10 consensus sequences. Weak homology to the –12 and –24 promoter elements of *Pseudomonas* (Deretic *et al.*, 1987) and to *nif*- and *ntr*- (*RpoN*) like promoters (Dixon *et al.*, 1986) was also found in this region. Since it has been extremely difficult to obtain high quality mRNA samples from methanotrophs, previous attempts to identify *smmo*-specific transcripts were unsuccessful (J. C. Murrell and others, unpublished results).

Using Northern blot and primer extension analyses, we undertook a closer examination of the RNAs produced from the sMMO gene cluster. Primer extension analysis of four regions likely to contain promoter elements (i.e. upstream of *mmoX*, between *mmoX* and *mmoY*, between *mmoZ* and *orfY*, and within *orfY*) indicated that transcription is initiated from a single promoter located 87 nucleotides upstream of the *mmoX* start codon. No specific 5' ends were found in other regions investigated in this study. A background of non-specific cDNAs, probably due to premature stops by reverse transcriptase, was obtained when primers complementary to regions downstream of the *mmoX* ORF were used. This indicates that a target for the *smmo*-specific primers was present (i.e. *smmo*-specific mRNA) but the 5' end was too far upstream to be reached by reverse transcriptase or simply too large to be seen on a sequencing gel.

The Northern analyses yielded three transcripts apparently differing only in their 3' ends: mRNA-1 encoding *mmoX*; mRNA-2 encoding *mmoX*, *mmoY*, *mmoB* and *mmoZ*; and mRNA-3 encoding all the *mmo* cistrons. Whether mRNA-1 and mRNA-2 originates from endonucleolytic cleavage of mRNA-3 or from transcriptional termination events is at present unknown. However, motifs resembling ρ -independent transcriptional terminators are present in the *mmoX*/*mmoY* (T_1) and *mmoZ*/*orfY* (T_2) intercistronic regions (Stainthorpe *et al.*, 1989).

To investigate the role, if any, of copper ions in transcriptional regulation of the sMMO gene cluster, we followed the pattern of *smmo*-specific mRNAs after addition of copper to a copper-depleted culture. We found that the level of *smmo*-specific mRNAs in *M. capsulatus* (Bath) decreased in response to the sudden availability of copper ions when all the other growth parameters (e.g. pH, oxygen, methane and nitrogen) were constant. This important observation may indicate that transcription of the *smmo* genes is specifically repressed by copper ions. Since the growth of the cells was unaffected by the copper treatment and no general RNA degradation was observed (as visualized on agarose gels), it is likely that a specific inhibition of the *smmo* promoter was provoked by the sudden availability of copper ions. Post-transcriptional control, however, such as copper-dependent changes in stability of sMMO mRNAs, cannot be ruled out at this stage.

We also investigated the effect of copper ions on whole-cell sMMO activity. As revealed in Fig. 2, the copper treatment resulted in loss of sMMO activity, although the time taken for this negative effect of copper on sMMO was a little delayed compared to earlier reports (Green *et al.*, 1985). Whether this loss of sMMO activity was due to specific inhibition of the enzyme or simply due to protein turnover cannot be ruled out from our data.

The molecular mechanism by which copper ions regulate the *smmo* promoter is not yet understood. Metal ions have, however, in several cases been identified as cofactors in the regulation of bacterial gene expression. The metal ions act via receptor proteins, which, upon binding of the metal, become activated as transcriptional repressors or activators. This kind of regulation has been described for a number of iron assimilation genes in *E. coli*. In this organism, transcription of genes involved in iron uptake is under the negative control of a regulator protein called Fur (reviewed in Silver & Walderhaug, 1992). In the presence of divalent ferrous ions, Fur recognizes and binds to operator sequences located in promoters of iron-regulated genes, preventing transcription. When the iron concentration drops, the Fur-iron complex dissociates resulting in derepression of the genes (de Lorenzo *et al.*, 1987).

Copper ions might act on the *smmo* promoter via such a receptor protein according to the following model. The RNA polymerase transcribes the *smmo* genes in the absence of copper ions. When copper ions are abundant, however, it complexes with the transcriptional regulator which then becomes capable of binding to an operator sequence in the *smmo* promoter region. Consequently, transcription of the *smmo* genes is repressed. We cannot, however, exclude the possibility that copper ions inactivate a transcriptional activator of the *smmo* promoter.

This is the first example of a metal-regulated promoter in methanotrophs and may provide a valuable tool for gene expression in these ecologically important organisms. The molecular basis for copper-mediated gene regulation in methanotrophic bacteria warrants further study, although this may prove difficult in a group of bacteria for which there are few genetic systems (Murrell, 1992).

In conclusion, our results imply that the six ORFs of the sMMO gene cluster are organized as an operon transcribed by a single copper-regulated promoter upstream of *mmoX*.

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