Homologous expression of soluble methane monooxygenase genes in *Methylosinus trichosporium* OB3b

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An homologous expression system has been developed for soluble methane monooxygenase (sMMO) genes from *Methylosinus trichosporium* OB3b. sMMO-minus mutants were previously obtained after marker-exchange mutagenesis, by the insertion of a kanamycin-resistance cassette into the *mmoX* gene of the sMMO operon. Complementation of the sMMO-minus genotype was achieved by conjugation with broad-host-range plasmids containing the native promoter and sMMO operon from *Ms. trichosporium* OB3b (pVK100Sc and pHM2). In wild-type methanotrophs, copper ions present in the growth medium at concentrations greater than 0.25 μ M inhibit transcription of sMMO genes. The stable maintenance of pVK100Sc resulted in transconjugant methanotrophs with a decreased sensitivity to copper, since expression of sMMO occurred at copper sulphate concentrations of 7.5 μ M. sMMO activity was only detected in soluble extracts after the addition of purified sMMO reductase component, which is inhibited by copper ions *in vitro*. This phenomenon could have arisen due to the increased number of sMMO gene copies (derived from pVK100Sc) in the cell. Transconjugants obtained from conjugations with pHM2 expressed sMMO at copper concentrations of 0–2.5 μ M only and sMMO activity was not restored by the addition of purified reductase component at copper concentrations higher than 2.5 μ M. Southern hybridization showed that the plasmid had integrated into the chromosome, probably by a single homologous recombination event. This is the first report of homologous sMMO expression in a methanotroph with enzyme activities that are comparable to the activity reported in wild-type strains. This expression system will be useful for site-directed mutagenesis of active-site residues of sMMO from *Ms. trichosporium* OB3b.

Keywords: methanotroph, methane monooxygenase, recombinant, site-directed mutagenesis

INTRODUCTION

Methylosinus trichosporium OB3b is a methanotrophic bacterium which utilizes methane as a sole source of carbon and energy (Hanson & Hanson, 1996). Methane is oxidized to methanol, which is then oxidized further to formaldehyde by methanol dehydrogenase. Formaldehyde is subsequently assimilated into biomass or further oxidized to CO₂, providing reducing equivalents

for biosynthesis. Methane oxidation is catalysed by methane monooxygenase (MMO) (EC 1.14.13.25) in a reaction that requires oxygen and NADH. *Ms. trichosporium* OB3b expresses two types of MMO: a membrane-bound, particulate MMO (pMMO) (Zahn & DiSpirito, 1996; Nguyen *et al.*, 1998) and a cytoplasmic, soluble MMO (sMMO) (Dalton *et al.*, 1993; Lipscomb, 1994). The MMO enzymes of *Ms. trichosporium* OB3b are able to degrade important groundwater pollutants including trichloroethylene and other halogenated hydrocarbons (Oldenhuis & Janssen, 1993). The highest rates of trichloroethylene degradation have been reported with *Ms. trichosporium* OB3b expressing sMMO (Oldenhuis *et al.*, 1989; Brusseau *et al.*, 1990).

Abbreviations: MMO, methane monooxygenase; sMMO, soluble methane monooxygenase; pMMO, particulate methane monooxygenase; NMS, nitrate mineral salts.

Expression of the two distinct types of MMO is dependent upon the availability of copper ions in the growth medium: a high copper-to-biomass ratio ($>0.25 \mu\text{M}$ copper ions) results in pMMO expression and a low copper-to-biomass ratio ($<0.25 \mu\text{M}$ copper ions) causes expression of sMMO (Stanley *et al.*, 1983). Using Northern blotting and primer extension analysis, Nielsen *et al.* (1996, 1997) showed that sMMO-specific mRNAs were transcribed under conditions of low copper-to-biomass ratios. In the presence of copper, the level of sMMO-specific mRNAs decreased and pMMO transcripts were detected. Furthermore, copper ions inhibit activity of the reductase component *in vitro* in *Methylococcus capsulatus* (Bath) (Green *et al.*, 1985) and *Ms. trichosporium* OB3b (Fitch *et al.*, 1993).

sMMO has been purified and characterized from a number of methanotrophs, but most is known about the enzymes from *Ms. trichosporium* OB3b (Fox *et al.*, 1989) and *Mc. capsulatus* (Bath) (Colby & Dalton, 1978). Three protein components, the hydroxylase, the reductase and a regulatory protein B, are required for activity of sMMO. The hydroxylase comprises three subunits, arranged in an $\alpha_2\beta_2\gamma_2$ configuration of about 250 kDa (Woodland & Dalton, 1989; Dalton *et al.*, 1993). The α subunits contain a di-iron centre (Lip-scomb, 1994) where methane and dioxygen interact to form methanol at the active site (George *et al.*, 1996). The reductase is a single polypeptide and contains an FAD group and one Fe_2S_2 centre per molecule. It accepts electrons from NADH_2 and transfers them one at a time to the di-iron site of the hydroxylase (Lund *et al.*, 1985). Protein B is a single-subunit protein containing no metals, prosthetic groups or cofactors (Green & Dalton, 1985), the activity of which may be regulated by proteolysis (Lloyd *et al.*, 1997).

The X-ray crystal structure of the hydroxylase from *Ms. trichosporium* OB3b has been reported (Elango *et al.*, 1997), demonstrating the high level of conservation within the active site of the hydroxylase from *Mc. capsulatus* (Bath) (Rosenzweig *et al.*, 1993). From these studies, active-site residues located within the α subunit (encoded by *mmoX*) of sMMO have been implicated as having key roles in the mechanism of methane oxidation, including Glu243, Thr213 and Cys151 (Rosenzweig *et al.*, 1993, 1995, 1997). Site-directed mutagenesis experiments at these positions are important to understand how methane, a stable and relatively unreactive molecule, is hydroxylated.

The genes encoding sMMO from *Ms. trichosporium* OB3b have been cloned and sequenced (Cardy *et al.*, 1991a, b). The α , β and γ subunits of the hydroxylase are encoded by *mmoX*, *mmoY* and *mmoZ*, respectively, protein B by *mmoB* and the reductase by *mmoC*. *mmoB* and *mmoC* from *Mc. capsulatus* (Bath) were active when expressed in *Escherichia coli*, but the recombinant hydroxylase was inactive (West *et al.*, 1992). sMMO from *Ms. trichosporium* OB3b was inactive when expressed in *E. coli* (Jahng *et al.*, 1996). Active recombinant sMMO from *Ms. trichosporium* OB3b was reported in *Pseudomonas putida* F1, *Agrobacterium*

tumefaciens and *Rhizobium meliloti* (Jahng & Wood, 1994; Jahng *et al.*, 1996). However, when the recombinant strains were assayed using the propylene oxidation assay (Pilkington & Dalton, 1990; Fox *et al.*, 1989), negligible sMMO activity was detected (Jahng *et al.*, 1996).

Methanotrophs are difficult organisms to work with in terms of mutant isolation and gene transfer (Murrell, 1994). Marker-exchange mutagenesis has been used for the isolation of nitrogen-fixation mutants in methanotrophs (Toukdarian & Lidstrom, 1984). A similar strategy was used to construct stable sMMO-minus mutants of *Ms. trichosporium* OB3b by the insertion of a 1.5 kb kanamycin-resistance gene into the chromosome (Martin & Murrell, 1995). Six mutants were isolated that could not grow in the absence of copper, confirming an sMMO-minus pMMO-positive phenotype. Using a colorimetric assay based on the oxidation of naphthalene (Brusseau *et al.*, 1990), sMMO activity was not detected and Southern hybridization confirmed the location of the kanamycin-resistance gene in the chromosome.

To overcome the lack of sMMO expression in an active form in heterologous hosts, the aim of this work was to complement the sMMO-minus genotype of *Ms. trichosporium* OB3b marker-exchange mutants with plasmid-encoded sMMO genes. Complementation of the *mmoX*-minus genotype with plasmid-encoded genes will allow active-site mutants to be expressed using this homologous expression system.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are described in Table 1. Growth of methanotrophs was as described by Colby & Dalton (1978) except that cultures were incubated at 30°C . For the growth of cultures in minimal-copper, $0.1 \times$ nitrate mineral salts (NMS) was used, from which trace metal ions were removed using the methods described by Tsien *et al.* (1989). Cultures were grown in copper-plus media using $1 \times$ NMS from which trace metal ions were not removed. *Ms. trichosporium* OB3b was also grown in batch culture using a 2 litre fermenter under the following conditions: temperature, 30°C ; agitation speed, 500 r.p.m.; pH, 6.8, maintained via the automatic addition of 0.5 M HCl or 0.5 M NaOH ; methane supply, 60 ml min^{-1} ; air supply, 80 ml min^{-1} . For continuous culture, the same conditions were used as for batch culture except that the culture was oxygen limited. The dilution rate was $0.02\text{--}0.06 \text{ h}^{-1}$ to maintain the OD_{540} at 2.2–3.8. The switch from expression of sMMO to expression of pMMO was followed by the sequential addition of copper sulphate to 2.5, 5 and $7.5 \mu\text{M}$. Cells were harvested at steady state, which was reached after approximately 4–5 volume changes (6–8 d).

Construction of plasmids. The plasmids used in this study (Table 1) were constructed in *E. coli* DH1 via methods described by Sambrook *et al.* (1989). pHM1 (Martin, 1994) was previously constructed by subcloning the streptomycin-resistance gene (Ω) from pHP45 Ω (Prentki & Krisch, 1984) as a *SalI* fragment and ligating it into pDSK509 (Keen *et al.*, 1988) digested with the same enzyme. The sMMO operon from *Ms. trichosporium* OB3b was excised from pMTL1000 (Martin, 1994) as a *KpnI* fragment and subcloned into *KpnI*-digested

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
<i>E. coli</i>		
DH1	F ⁻ <i>recA1 endA1 gyrA96 thi1 hsdR17</i> (<i>r_k⁻ m_k⁺</i>) <i>supE44 λ⁻</i>	Low (1968)
S17-1	RP4 2-Tetracycline::Mu-Kanamycin::Tn7 <i>pro res mod⁺</i>	Simon <i>et al.</i> (1983)
Methanotrophs		
<i>Ms. trichosporium</i> OB3b	Type II possessing sMMO and pMMO	University of Warwick culture collection
OB3b mutant A	Kanamycin-resistant sMMO-minus single-crossover mutant of OB3b	Martin & Murrell (1995)
OB3b mutant F	Kanamycin-resistant sMMO-minus double-crossover mutant of OB3b	Martin & Murrell (1995)
RF100	Kanamycin- and tetracycline-resistant sMMO-minus single-crossover mutant of OB3b harbouring pVK100	This study
RF100Sc	Kanamycin- and tetracycline-resistant sMMO-minus single-crossover mutant of OB3b complemented with pVK100Sc	This study
JLHM1	Kanamycin- and streptomycin-resistant sMMO-minus double-crossover mutant of OB3b harbouring pHM1	This study
JLHM2 strains A-E	Kanamycin- and streptomycin-resistant sMMO-minus double-crossover mutant of OB3b complemented with pHM2	This study
Plasmids		
pDSK509	Kanamycin-resistant Mob ⁺ conjugative broad-host-range vector	Keen <i>et al.</i> (1988)
pHM1 (formerly pDSK509Ω)	pDSK509 containing a streptomycin-resistance cassette as a <i>Bam</i> HI fragment	Martin (1994)
pHM2 (formerly pDSK509ΩSc)	pHM1 containing the sMMO operon of <i>Ms. trichosporium</i> OB3b as a <i>Kpn</i> I fragment	Martin (1994)
pDCV200	pBR325 containing a 7.6 kb <i>Hind</i> III fragment of <i>Ms. trichosporium</i> OB3b genomic DNA encoding <i>mmoX</i> and the 5' region of <i>mmoY</i>	Cardy <i>et al.</i> (1991a)
pDCV202	pBR325 containing a 2.6 kb <i>Hind</i> III fragment of <i>Ms. trichosporium</i> OB3b genomic DNA encoding the 3' region of <i>mmoZ</i> , <i>orfY</i> and <i>mmoC</i>	Cardy <i>et al.</i> (1991b)
pMLT24	Ampicillin-resistant pBR322-based cloning vector	Chambers <i>et al.</i> (1988)
pMLT24-202	pMLT24 containing a <i>Hind</i> III fragment from pDVC200, encoding the 5' region of the <i>Ms. trichosporium</i> OB3b sMMO operon	Martin (1994)
pMTL-1000	pMLT24 containing the sMMO operon subcloned from pDVC200 and pDVC202	Martin (1994)
pVK100	Kanamycin- and tetracycline-resistant IncP1, broad host range	Knauf & Nester (1982)
pVK100Sc	pVK100 containing the <i>Ms. trichosporium</i> OB3b sMMO operon as a <i>Bam</i> HI fragment	Martin (1994)

pHM1, to generate pHM2 (Martin, 1994) (Fig. 1). pVK100Sc (Martin, 1994) was previously constructed by subcloning DNA encoding the 5' region of the *Ms. trichosporium* OB3b sMMO operon from pDCV200 (Cardy *et al.*, 1991a) as a *Hind*III fragment into pMLT24 (Chambers *et al.*, 1988), to generate pMLT24-202 (Martin, 1994). The sMMO gene cluster from pDCV202 (Cardy *et al.*, 1991b) was previously cloned into pMLT24-202 (Martin, 1994) to generate pMLT-1000 containing the entire sMMO operon from *Ms. trichosporium* OB3b. The complete sMMO operon was excised from pMTL1000 (using the multiple cloning sites in pMLT24) as a *Bam*HI fragment. This was subcloned into pVK100 (Knauf & Nester, 1982) linearized with *Bgl*II, generating pVK100Sc (Fig. 2). pHM2 and pVK100Sc both contain the native sMMO promoter sequence.

Isolation of plasmid DNA. Plasmid DNA was isolated from *Ms. trichosporium* OB3b using a method devised by Saunders & Burke (1990) with the following modifications. A 50 ml

culture (OD₅₄₀ 0.5–0.8) was pelleted and resuspended in 1.8 ml solution 1 (50 mM glucose, 20 mM Tris/HCl, 10 mM EDTA, pH 8.0) plus 200 µl lysozyme (10 mg ml⁻¹). The suspension was incubated at 37 °C for 15 min, followed by addition of 4 ml solution 2 (0.2 M NaOH, 1%, w/v, SDS) and incubation for 15 min on ice. Then 4 ml solution 3 (3 M potassium acetate, pH 4.8) was added and the mixture inverted every 2 min for 10 min (on ice).

Nick-translation and Southern hybridization. These methods were performed according to Sambrook *et al.* (1989). The following radiolabelled DNA fragments derived from pHM2 were used to probe JLMH2-C: the 1.7 kb *Pvu*II kanamycin-resistance cassette; the 2 kb *Bam*HI streptomycin-resistance cassette; and the 2 kb *Sac*I–*Sal*I fragment of *mmoX* (Fig. 1).

Conjugation. The technique used for conjugating plasmids from *E. coli* into methanotrophs was based upon a method developed by Martin (1994). Batch cultures of methanotrophs

Fig. 1. Restriction map of pHM2 (Martin, 1994) containing the sMMO operon from *Ms. trichosporium* OB3b. Km^R, kanamycin-resistance gene; Sm^R, streptomycin-resistance gene; B, *Bam*HI; C, *Cl*ai; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sa, *Sac*I; X, *Xho*I. Probes used for Southern hybridizations are also shown.

at an OD₅₄₀ of approximately 0.2 were used; 50 ml of methanotroph recipient and 10 ml of overnight donor *E. coli* S17-1 culture containing the plasmid to be conjugated were washed with NMS. They were collected on a 47 mm sterile nitrocellulose filter (0.2 µm pore size). The filter was placed on NMS agar containing 0.02% (w/v) Proteose Peptone and incubated for 24 h at 30 °C in the presence of methane (50% in air). Cells from the conjugation plates were resuspended in 10 ml NMS and concentrated by centrifugation at 7000 *g* for 5 min. The cell pellet was resuspended in 1 ml NMS and 100 µl aliquots plated onto selective agar, followed by incubation for 2–3 weeks with methane/air (1:1, v/v) until single colonies appeared.

Preparation of soluble and insoluble extracts. Methanotrophs were lysed by four passages through a French pressure cell at 137 MPa and centrifuged at 50 400 *g* for 60 min at 4 °C. Soluble extracts (containing sMMO) were prepared as previously described (Fox *et al.*, 1989). Insoluble extracts (con-

taining pMMO) were prepared by resuspending the insoluble material in a minimum volume of 25 mM MOPS, pH 7.0, containing 40 µM copper sulphate.

Protein purification. Purifications of sMMO from *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b were undertaken as described by Colby & Dalton (1978) and Fox *et al.* (1989), respectively.

Enzyme assay. sMMO activity in soluble and insoluble extracts was determined by the propylene oxidation assay of Pilkington & Dalton (1990). The colorimetric naphthalene oxidation assay was used to detect sMMO activity in whole cells (Brusseau *et al.*, 1990). Naphthalene oxidation plate assays for sMMO were performed as described by Bodrossy *et al.* (1995).

Determination of protein concentration. Protein concentration was assayed using Bradford protein assay reagent (Bio-Rad) with bovine serum albumin as the standard.

Fig. 2. Restriction map of pVK100Sc (Martin, 1994) containing the sMMO operon from *Ms. trichosporium* OB3b. Y, *orfY*; Km^R , kanamycin resistance gene; Tc^R , tetracycline resistance gene; *rlx*, conjugal transfer locus of RK2; *cos*, *cos* site of phage λ ; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I; X, *Xho*I. See Fig. 1 for a detailed map of the sMMO operon.

Antibodies. Antiserum against the hydroxylase component of sMMO from *Ms. trichosporium* OB3b was raised as previously described (Woodland & Dalton, 1989), except that the pure hydroxylase was denatured by heating for 5 min at 80 °C before mixing with Freund's adjuvant.

SDS-PAGE and Western blotting. SDS-PAGE was performed with an X-Cell II apparatus (Novex). Acrylamide gels (12%, w/v) (Laemmli, 1970) were stained with Coomassie brilliant blue. Molecular masses were calculated using Mark 12 wide-range protein standards (Novex). Western blotting was performed with an X-Cell II Blot Module (Novex) and Hybond C nitrocellulose membranes (Amersham).

RESULTS

Growth of *Ms. trichosporium* OB3b sMMO-minus mutants

Ms. trichosporium OB3b mutants A and F were used in this study, which both have a kanamycin-resistance cassette inserted into *mmoX* (Martin & Murrell, 1995). Mutant A arose from a single homologous recombination event, while mutant F arose from a double homologous recombination event. Since naphthalene is oxidized by sMMO and not pMMO, the naphthalene oxidation assay was used to screen for the absence of sMMO activity in the sMMO-minus mutants. Three hundred single colonies of each mutant were subcultured onto minimal-copper and copper-plus agar both containing 50 μ g kanamycin ml^{-1} . Wild-type *Ms. trichosporium* OB3b was used as the positive control. After 3 weeks growth, naphthalene oxidation plate assays confirmed that sMMO activity was absent in the *Ms. trichosporium* OB3b sMMO-minus mutants grown on copper-plus or minimal-copper agar. Wild-type *Ms. trichosporium* OB3b gave a positive naphthalene oxidation assay result on minimal-copper agar only. Using

minimal-copper 0.1 $\times$ NMS batch cultures, sMMO expression was detected in the wild-type organism only. Although sMMO expression was not detected in the *Ms. trichosporium* OB3b sMMO-minus mutants under the same conditions, growth was evident, possibly due to scavenging of trace amounts of copper (necessary for the expression and activity of pMMO) from the medium by these cells. Western blotting with antiserum against the hydroxylase of *Ms. trichosporium* OB3b confirmed that the α , β and γ subunits of the hydroxylase were expressed. Western blot analysis of soluble extracts from *Ms. trichosporium* OB3b mutant F demonstrated cross-reactivity with the β and γ subunits of the hydroxylase, which are probably still expressed since there is not a transcriptional terminator within the kanamycin-resistance cassette inserted into *mmoX* (Fig. 3a). Therefore, in *Ms. trichosporium* OB3b mutant F at least, sMMO expression could be restored by complementation of *mmoX* only.

Conjugation of broad-host-range plasmids into *Ms. trichosporium* OB3b sMMO-minus mutants

To select for *Ms. trichosporium* OB3b mutant A transconjugants harbouring pVK100 and pVK100Sc, 50 μ g kanamycin ml^{-1} and 20 μ g tetracycline ml^{-1} were used. *Ms. trichosporium* OB3b mutant A [pVK100] transconjugants (designated RF100) were isolated at a frequency of 2×10^{-8} per recipient. *Ms. trichosporium* OB3b mutant A [pVK100Sc] transconjugants (designated RF100SC) were isolated at a frequency of 1×10^{-8} per recipient. To select for *Ms. trichosporium* OB3b mutant F transconjugants harbouring pHM1 and pHM2, 50 μ g kanamycin ml^{-1} and 25 μ g streptomycin ml^{-1} were used. *Ms. trichosporium* OB3b mutant F [pHM1] transconjugants (designated JLHM1) were selected on copper-plus agar at a frequency of 1.7×10^{-7} per recipient. *Ms. trichosporium* OB3b mutant F [pHM2] transconjugants (designated JLHM2) were selected on copper-plus agar at a frequency of 1.2×10^{-7} per recipient. Spontaneous resistance to kanamycin and tetracycline and/or kanamycin and streptomycin was observed at frequencies of $< 1 \times 10^{-10}$ in the sMMO-minus mutants.

Identification of sMMO-positive transconjugant methanotrophs

The putative transconjugant methanotrophs isolated above were replica-plated onto copper-plus and minimal-copper agar. After growth for 3 weeks in the presence of methane, the naphthalene oxidation plate assay was used to screen for the sMMO activity. No sMMO-positive JLHM1 or RF100 transconjugants were isolated, despite repeated attempts. sMMO activity was identified in RF100SC, and the activity continued to be maintained on minimal-copper agar containing 50 μ g kanamycin ml^{-1} and 20 μ g tetracycline ml^{-1} . Three sMMO-positive JLHM2 transconjugants, designated JLHM2-A, JLHM2-B and JLHM2-C, were identified on minimal-copper agar. Replica-plating all of the trans-

Fig. 3. (a) Extracts of *Ms. trichosporium* OB3b mutant F (lane 1) and wild-type *Ms. trichosporium* OB3b (lane 2), both grown in minimal-copper 0.1 × NMS batch culture, Western blotted and probed with antiserum against the hydroxylase of *Ms. trichosporium* OB3b. Each lane contained 40 µg soluble extract. (b) Extracts of RF100SC probed with antiserum against the hydroxylase from *Ms. trichosporium* OB3b. Lane 1, 5 µg purified hydroxylase from *Ms. trichosporium* OB3b; lanes 2, 3, 4 and 5, 30 µg soluble extract of RF100SC grown with 0, 2.5, 5 and 7.5 µM copper sulphate, respectively. At 0 µM copper, the medium would contain a minimal amount of contaminating copper.

conjugants harbouring pHM2 onto minimal-copper agar for a second time, followed by naphthalene oxidation plate assays, identified two more sMMO-positive transconjugants, designated JLMH2-D and JLMH2-E. These five sMMO-minus mutants that had been complemented with pHM2 were maintained on minimal-copper agar containing 50 µg kanamycin ml⁻¹ and 25 µg streptomycin ml⁻¹.

Growth of RF100SC and JLMH2-C in batch and continuous culture

RF100SC and JLMH2-C were each grown in a 2 litre fermenter containing 1 × NMS with 1 µM copper sulphate. This medium was used to ensure that the transconjugant methanotrophs could grow using pMMO, prior to the depletion of copper and expression of sMMO. This occurred as the cell density rose above an OD₅₄₀ of 2 and a biomass concentration of 0.86 g dry cell weight l⁻¹ (assuming a dry cell weight of 0.429 g l⁻¹ at an OD₅₄₀ of 1.0; Pilkington, 1986). The positive control used in all cases was wild-type *Ms. trichosporium* OB3b. When cells were expressing pMMO, the growth rate of *Ms. trichosporium* OB3b was 0.05 h⁻¹ and during expression of sMMO, the growth rate was 0.052 h⁻¹. The corresponding growth rate of RF100SC was approximately 0.05 h⁻¹ when expressing either pMMO or sMMO. The growth rate of JLMH2-C was 0.024 h⁻¹ when cells were expressing pMMO; it was 0.034 h⁻¹ during expression of sMMO. The switch from expression of pMMO to expression of sMMO occurred

at a biomass concentration of 0.8–1.1 g dry cell weight l⁻¹, in all cases.

Expression and activity of sMMO in RF100SC and JLMH2-C

Continuous culture was used to determine the enzyme activity of MMO in the different methanotroph mutants complemented with sMMO genes. This also allowed the switch from expression of sMMO to pMMO to be monitored by the addition of increasing concentrations of copper sulphate. Propylene oxidation assays of soluble and insoluble extracts were performed at increasing copper sulphate concentrations, to determine the location and activity of MMO (Table 2). Specific activities of MMO from wild-type *Ms. trichosporium* OB3b grown in NMS containing between 0 and 1 µM copper sulphate in continuous culture have been reported in the range of 20–80 nmol propylene oxidized min⁻¹ (mg protein)⁻¹ for the soluble fraction (containing sMMO) and 0–8 nmol propylene oxidized min⁻¹ (mg protein)⁻¹ for the insoluble fraction (containing pMMO) (Burrows *et al.*, 1984; Park *et al.*, 1991; Fox *et al.*, 1991; Martin, 1994). The MMO activities detected during this work in soluble extracts of wild-type *Ms. trichosporium* OB3b and RF100SC were comparable to values given in these reports. Specific activities of MMO of 140 ± 13 nmol propylene oxidized min⁻¹ (mg protein)⁻¹ were obtained for the soluble fraction of JLMH2-C. Such high activities had only previously been observed in wild-type *Ms. trichosporium* OB3b by Stirling & Dalton

Table 2. Enzyme activities during a switch from expression of sMMO to the expression of pMMO in wild-type and transconjugant methanotrophs

Soluble, 1 to 5 mg soluble extract; insoluble, 1 mg insoluble extract. sMMO activity resides in the soluble fraction and pMMO activity in the insoluble fraction. *In vitro* activity of pMMO is likely to be lower than *in vivo* pMMO activity. The results are means of triplicate assays $\pm$ SD. ND, Not detected.

Strain	Copper sulphate concn (μ M)	Propylene oxidation activity [nmol min^{-1} (mg protein^{-1}) ⁻¹]	
		Soluble extract	Insoluble extract
Wild-type OB3b	0	42 $\pm$ 9	3 $\pm$ 1
	2.5	10 $\pm$ 4	18 $\pm$ 10
	5	ND	57 $\pm$ 8
	7.5	ND	63 $\pm$ 12
JLHM2-C	0	140 $\pm$ 13	ND
	2.5	29 $\pm$ 3	21 $\pm$ 4
	5	4 $\pm$ 0.5	49 $\pm$ 9
	7.5	ND	74 $\pm$ 15
RF100SC	0	54 $\pm$ 0.6	ND
	2.5	12 $\pm$ 0.2	62 $\pm$ 0.3
	5	3 $\pm$ 0.4	65 $\pm$ 0.2
	7.5	50 $\pm$ 0.9*	66 $\pm$ 0.4

* Activity was detected in the soluble fraction by the addition of saturating amounts of purified reductase from *Mc. capsulatus* (Bath).

(1979), who observed very high MMO activity in the soluble fraction: 84–146 nmol propylene oxidized min^{-1} (mg protein^{-1})⁻¹. Propylene oxidation activity was not detected in soluble extracts of RF100 or JLHM1.

The soluble and insoluble extracts that were assayed for MMO activity were also analysed by SDS-PAGE and Western blotting using antiserum against the hydroxylase component of *Ms. trichosporium* OB3b as probe. In wild-type *Ms. trichosporium* OB3b and JLHM2-C grown in continuous culture, the hydroxylase of sMMO was detected in the soluble fraction of cells grown in the absence of copper only. At 2.5 and 5 μ M copper sulphate, it was possible that the enzyme activity detected in the soluble extracts of JLHM2-C (Table 2) was derived from sMMO polypeptides present at a concentration too low to be detected by Western blotting. Constitutive expression of the hydroxylase was observed in RF100SC, grown in continuous culture at unusually high copper sulphate concentrations of 7.5 μ M, as determined by Western blotting (Fig. 3b). At a copper sulphate concentration of 7.5 μ M, sMMO activity could be detected in soluble extracts only by the addition of saturating amounts of purified sMMO reductase from *Mc. capsulatus* (Bath). Purified sMMO reductase from *Ms. trichosporium* OB3b was not available. The restoration of sMMO activity *in vitro* suggested that the lack of sMMO activity in whole cells grown in medium with copper sulphate added to 7.5 μ M was due to the inactivation of the reductase component. Inactivation of the reductase from *Mc. capsulatus* (Bath) by copper has previously been demonstrated by Green *et*

al. (1985). This result also confirmed functional complementation between the reductase of *Mc. capsulatus* (Bath) and the other sMMO components of *Ms. trichosporium* OB3b as previously reported (Stirling & Dalton, 1979).

Analysis of plasmid and chromosomal DNA from RF100SC and JLHM2-C

Southern blot analysis was used to elucidate how complementation of the sMMO-minus genotype in *Ms. trichosporium* OB3b mutants A and F had occurred. Using the modified alkaline lysis method developed in this work, attempts were made to isolate plasmid DNA from *Ms. trichosporium* OB3b, RF100, RF100SC, JLHM1 and JLHM2-C. Plasmid DNA was successfully isolated from all the methanotrophs except JLHM2-C. Southern blotting of the plasmid DNA isolated from RF100 and RF100SC confirmed that the DNA isolated was pVK100 and pVK100Sc, respectively, and that no significant deletions or modifications to these plasmids had occurred. It therefore appeared that sMMO expression had been restored in *Ms. trichosporium* OB3b mutant A via transcription of sMMO genes carried by pVK100Sc. Since repeated attempts to isolate plasmid DNA from JLHM2-C were unsuccessful, restriction digests of chromosomal DNA were performed and used in Southern hybridizations. Positive hybridization was identified with kanamycin- and streptomycin-resistance gene probes and a *mmoX* probe (see Methods). Analysis of the hybridization fragments obtained suggested that,

Fig. 4. Proposed homologous recombination event in JLHM2-C. Km, kanamycin-resistance cassette; Sm, streptomycin-resistance cassette.

in JLHM2-C, sMMO expression had been restored due to the homologous recombination of pHM2 into the chromosome. This could have occurred by single homologous recombination between the inactivated *mmoX* gene of *Ms. trichosporium* OB3b mutant F and the intact copy of *mmoX* present on pHM2 (Fig. 4).

DISCUSSION

The active expression of recombinant sMMO genes has previously been unsuccessful due to the expression of inactive hydroxylase in heterologous hosts. Using the homologous expression system developed in this study, sMMO expression was restored in sMMO-minus mutants by complementation with plasmid-encoded genes. This system has the advantage of a high level of active expression, arising from the natural sMMO promoter(s). In all methanotrophs so far studied, these appear to be very strong owing to the accumulation of the hydroxylase component of sMMO to about 60% of the total soluble cell protein. A major problem in developing genetic systems for the methanotrophs has been the lack of a suitable way of transforming these organisms and identifying plasmids that are stably maintained (Murrell, 1994). The best way of transforming methanotrophs with plasmids appears to be by conjugal transfer, which was used in the work presented here. Problems with slow growth of methanotrophs on plates and low conjugation frequencies (10^{-5} to 10^{-8}) have, however, yet to be overcome.

Although *Ms. trichosporium* OB3b mutant A was derived from a single homologous recombination event

(Martin & Murrell, 1995), spontaneous reversion to an sMMO-positive phenotype was not detected during this study, even at high cell densities in a fermenter. However, a problem was encountered when using pVK100 and pVK100Sc since only tetracycline resistance could be used to select for transconjugants containing this plasmid. Methanotrophs appear to be partially resistant to this antibiotic and combined with the instability of tetracycline over the extended growth periods of these organisms (typically 1–3 weeks on plates), this made the selection of transconjugant methanotrophs unreliable. Indeed, sMMO-positive *Ms. trichosporium* OB3b mutant F transconjugants harbouring pVK100Sc could not be isolated, possibly because of this problem. The problem of tetracycline selection with other methanotrophs (*Methylocystis parvus* OBBP and *Methylocystis pyriformis*) has also been reported (Jahng *et al.*, 1996).

Wild-type *Ms. trichosporium* OB3b and JLHM2-C switched from the expression of sMMO to expression of pMMO at a copper sulphate concentration of 0–2.5 μ M. In contrast, expression of sMMO was still observed in the complemented mutant strain RF100SC at a copper sulphate concentration of 7.5 μ M. This could have been caused by multiple copies of pVK100Sc in the cell, and hence multiple copies of the gene encoding the 'active regulator protein' proposed by Nielsen *et al.* (1997). Therefore, a higher concentration of the active regulator protein may be present in cells of RF100SC and so a higher concentration of copper could be required to repress transcription of the sMMO genes. Alternatively, the increased sMMO promoter copy number could

require a higher concentration of copper for the repressor to bind and cause a net repression. At copper sulphate concentrations of 7.5 µM, sMMO activity could only be detected in RF100SC by the addition of purified reductase from *Mc. capsulatus* (Bath) to soluble extracts. This suggests that sMMO activity was lost due to inactivation of the reductase component by the higher concentration of copper as previously shown for the same component of the *Mc. capsulatus* (Bath) enzyme (Green *et al.*, 1985). Presumably RF100SC was able to utilize methane as a sole carbon and energy source when sMMO was inactive by co-expressing pMMO. Fitch *et al.* (1993) reported that sMMO activity in soluble extracts of wild-type *Ms. trichosporium* OB3b decreased by 25% in the presence of 15 µM copper and that complete inactivation occurred with 75 µM copper. Inactivation of sMMO activity by copper has also been studied in *Mc. capsulatus* (Bath) (Green *et al.*, 1985). Copper ions at 10 µM caused a 27% reduction in whole-cell sMMO activity whilst 80 µM copper ions caused complete inactivation of purified sMMO proteins.

Using this homologous system for the expression of hydroxylase mutants requires that transconjugants are grown under conditions of low copper-to-biomass ratios so that sMMO is expressed. If some of the mutations rendered the sMMO inactive, then the organism would not grow in conditions of low copper-to-biomass ratios and the mutated hydroxylase proteins would not be expressed. However, results obtained in this study suggest that when sMMO-minus mutants containing a kanamycin-resistance cassette in *mmoX* are cultured in minimal-copper medium, the inactivated sMMO operon is still transcribed. The most likely explanation for this is that the organism scavenges sufficient copper for the concomitant expression of active pMMO and inactive sMMO, to utilize methane as a sole carbon and energy source. This result suggests that previous ideas about the lack of expression of pMMO in cells expressing sMMO are incorrect. Consequently, the hydroxylase which may be inactivated by mutations introduced into *mmoX* by site-directed mutagenesis would still be expressed under growth conditions of minimal copper.

ACKNOWLEDGEMENTS

This work was supported through Biotechnology and Biological Sciences Research Council (BBSRC) studentships to John Lloyd and Ruth Finch. We thank Howard Martin for constructing some of the plasmids used in this work, as well as Robert Titmus and Susan Slade for excellent technical assistance. We also thank the reviewers for their helpful comments.

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Received 19 June 1998; revised 22 September 1998; accepted 28 October 1998.