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Molecular genetics of methane oxidation

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Abstract

Biological methane oxidation is carried out by methanotrophs, bacteria that utilize methane as their sole carbon and energy source. The enzyme they contain that is responsible for methane oxidation is methane monooxygenase, the most well studied being the soluble methane monooxygenase enzyme complexes from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b. In both organisms, the genes encoding soluble methane monooxygenase have been found to be clustered on the chromosome in the order *mmoX, mmoY, mmoB, mmoZ, orfY* and *mmoC*. These genes encode the α and β subunits of Protein A, Protein B, the γ subunit of Protein A, a protein of unknown function and Protein C respectively of the soluble methane monooxygenase complex. The complete DNA sequences of both gene clusters have been determined and they show considerable homology. Expression of soluble methane monooxygenase genes occurs under growth conditions where the copper-to-biomass ratio is low. Transcriptional regulation of the gene cluster from *Methylosinus* occurred at an RpoN-like promoter, 5' of the *mmoX* gene. *mmoB* and *mmoC* of *Methylococcus* have been expressed in *E. coli* and the proteins obtained were functionally active. Soluble methane monooxygenase mutants have been constructed by marker-exchange mutagenesis. They were found to be more stable than those generated using the suicide substrate dichloromethane. Soluble methane monooxygenase probes have been used to detect both methane monooxygenase gene-specific DNA and methanotrophs in natural environmental samples.

Introduction

Methylotrophic bacteria that can grow on methane as their sole source of carbon and energy are usually known as methanotrophs. They are generally regarded as obligate in nature, growing well on methane and usually on methanol. Methanotrophs have attracted considerable interest over the past twenty or so years due to their potential for the production of bulk chemicals, single cell protein and use in biotransformations (e.g. see Lidstrom & Stirling 1990; Leak 1992; Large & Bamford 1988). More recently, their ability to degrade the groundwater pollutant trichloroethylene and other chlorinated compounds has also been examined in detail (reviewed in Oldenhuis & Janssen 1993; Alvarez-Cohen 1993). Currently there is considerable interest in methanotrophs since they appear to be ubiquitous in nature, (Whittenbury et al. 1970; Hanson et al. 1991; Hanson & Wattenberg 1991; Hanson et al. 1993) and have recently been recognized as a major sink for atmospheric methane (reviewed in Reeburgh et al. 1993; King 1993; Cicerone & Oremland 1988; Topp & Hanson 1991).

Methanotrophs can be classified into three groups. Type I methanotrophs such as *Methylomonas* utilize the ribulose monophosphate pathway for the assimilation of formaldehyde into cell carbon, possess bundles of intracytoplasmic membranes and are related to bacteria in the γ-subdivision of the purple bacteria (class Proteobacteria). Type II methanotrophs, such as *Methylosinus*, utilize the serine pathway for formaldehyde fixation, possess intracytoplasmic membranes arranged around the periphery of the cell and are generally related to the bacteria in the beta-subdivision of the *Proteobacteria*. Type X methanotrophs such as *Methylococcus capsulatus* (Bath) appear to have some

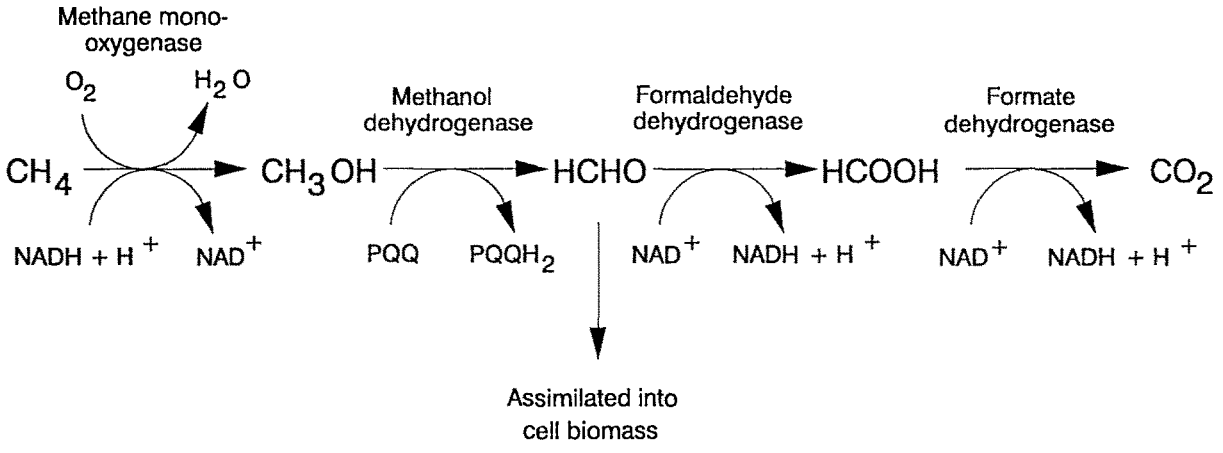

Fig. 1. The pathway of methane oxidation by bacteria.

properties of both Type I and Type II methanotrophs. The taxonomy of methanotrophs has been recently reviewed in detail (Green 1992, 1993, Hanson et al. 1993). Advances in the molecular taxonomy of these organisms will be summarized in this review.

Methanotrophs grow by oxidizing methane using the pathway described in Fig. 1. Since the physiology and biochemistry of methylotrophy has been described in detail (see Anthony 1982, 1986; de Vries et al. 1990; Dijkhuizen et al. 1992; Goldberg & Rokem 1991) methanol oxidation and formaldehyde metabolism will not be discussed here. Methane oxidation is carried out by the enzyme methane monooxygenase (MMO). A membrane-bound, particulate methane monooxygenase (pMMO) appears to be present in all methanotrophs grown in the presence of relatively high concentrations of copper ions (Dalton 1992). However, studies on this enzyme have been hampered by its lability in cell-free extracts and difficulties in purification from the membranes of methanotrophs (Smith & Dalton 1989; Akent'eva & Gvozdev 1990). Biochemical and biophysical studies have shown that pMMO activity is directly related to the copper/total membrane protein ratio in membrane fractions from *Methylococcus capsulatus* (Bath) grown at different copper concentrations. pMMO appears to consist of at least 3 polypeptides of 46, 35 and 26 kDa, and EPR studies suggest the presence of a trinuclear copper centre (Chan et al. 1993). In some methanotrophs, a second form of MMO, a soluble, cytoplasmic form (sMMO) is synthesized when the growth conditions are such that the culture copper-to-biomass ratio is low (less than 1 μM per gram dry weight). This sMMO is structurally and catalytically distinct from the pMMO (Burrows et al. 1984; Stanley et al. 1983; Park 1991, 1992; reviewed in Dalton 1992) and has a broad substrate specificity, oxidizing a wide range of aliphatic and aromatic compounds. Probably the most extensively characterized sMMOs are those isolated from the Type II methanotroph *Methylosinus trichosporium* OB3b (Fox et al. 1989, 1991; Burrows et al. 1984; Froland et al. 1993) and the Type X methanotroph *Methylococcus capsulatus* (Bath) (Coby & Dalton 1989; Green & Dalton 1985; Woodland & Dalton 1984; reviewed by Dalton 1992; Dalton 1993). sMMO has also been characterized from the Type II strains *Methylosinus sporium* 5 (Pilkington & Dalton 1991) and *Methylocystis* strain M (Nakajima et al. 1992). sMMO has also been reported recently in the Type I methanotroph *Methylomonas methanica* 68-1 (Koh et al. 1993).

The sMMO complex of *M. capsulatus* (Bath) and *M. trichosporium* OB3b have very similar properties, and both consist of three components A, B and C. Protein A is made up of two copies of each of three subunits α, β and γ of molecular masses of 60, 45 and 20 kDa and is the hydroxylase component of the enzyme complex. Protein A contains a binuclear iron-oxo centre believed to be the reactive centre for catalysis (Fox et al. 1988; de Witt et al. 1991; Dalton et al. 1993; Froland et al. 1993). Protein B, 16 kDa, is a single polypeptide containing no metal ions or cofactors and functions as a regulatory protein (Green & Dalton 1985; Fox et al. 1991; Liu et al. 1991; Froland et al. 1993). Protein C is a 39 kDa NADH reductase containing 1 mol each of FAD and a 2Fe2S cluster which accepts electrons from NADH and transfers them to the diiron site of the

hydroxylase component of the sMMO, Protein A (Fig. 2). The mechanism of action of sMMO has recently been reviewed (Dalton et al. 1993; Froland et al. 1993).

Since methane monooxygenase is the unique step in the pathway of methane oxidation it is natural that the majority of biochemical studies on obligate methanotrophs have centred on this enzyme, while work on methanol oxidation and formaldehyde metabolism has largely been done using methanol and methylamine utilizers. One other area of metabolism explored in the methanotrophs is that of nitrogen metabolism. Both nitrogen fixation and ammonia assimilation by these organisms has been studied at the physiological and biochemical level. Due to the abundance of heterologous probes for genes involved in nitrogen assimilation, the majority of the early studies on the molecular biology of methanotrophs were done using nitrogen metabolism as a model system (reviewed in Murrell 1992). This review will focus on recent developments in the molecular genetics of methane oxidation and will summarize the genetic techniques available for mutagenesis and gene transfer in methanotrophs. Data on molecular biology of methanol oxidation, specifically by methanotrophs, will also be included for completeness.

Gene transfer systems for methanotrophs

A significant problem in the development of genetic systems for methanotrophs has been the lack of suitable ways of introducing and maintaining heterologous vector DNA into these organisms. Coupled with their relatively slow growth on plates, this has meant that fairly slow progress has been made in this area.

Bacteriophages and plasmids in methanotrophs

Bacteriophages specific for methanotrophs have been isolated from a wide range of environments, including groundwater, fermentors, soils, rumen of cattle, fish and oil and gas installation waters (Tyutikov et al. 1976, 1980, 1983; Tikhonenko et al. 1982; Wunsche et al. 1988). Bacteriophages obtained have been characterized in terms of their plaque morphology, ultrastructure, antigenic properties, nucleic acid content and restriction enzyme analysis and lytic spectrum. *Methylosinus*- and *Methylocystis*-specific phages are particularly prevalent in nature but few attempts to use them for transduction experiments with methanotrophs have been reported.

A number of large plasmids, ranging from around 50–190 kb have been detected in a number of methanotrophs including *Methylomonas, Methylocystis, Methylosinus* and *Methylobacter*. Of ten representatives examined, only *M. capsulatus* (Bath) did not contain any detectable plasmid DNA (Lidstrom & Wopat 1984). Some cross-hybridization with plasmid DNA from different *Methylosinus* species was noted and a rudimentary restriction endonuclease map of the 55 kb plasmid from *M. albus* was constructed. However, difficulties in the large scale isolation of plasmid DNA from these organisms have hampered their development as potential cloning vectors. There is currently no evidence for the presence of antibiotic or metal resistance genes, nitrogen fixation genes or methane oxidation genes on these plasmids but since these plasmids represent 'significant potential coding' regions, their presence should not be disregarded in any molecular genetics work with these organisms.

Transformation and conjugation studies

A transformation system was first described for *Methylococcus* (Williams & Bainbridge 1971) although the high concentrations of DNA required made this a very inefficient system. The lack of suitable plasmids or genetic markers has hampered further studies. Limited success has been achieved by the use of electroporation (Murrell et al. unpublished; Gerdes & Nielsen pers. comm.) but a concerted effort using this technique in conjunction with some of the plasmids described below is likely to be the best route to the development of a quick, simple transformation system for methanotrophs.

Conjugal transfer of plasmid R68.45 into *M. trichosporium* OB3b from *Pseudomonas aeruginosa* PAO8 by filter matings using kanamycin resistance as the selectable marker has proved successful. Frequencies of transfer of 10^{-2} to 10^{-3} per donor were reported (Warner et al. 1980). Lidstrom and colleagues have also transferred the IncP1 broad host range cosmid cloning vector into *Methylomonas, Methylocystis* and *Methylosinus* species at frequencies ranging from 10^{-2} to 10^{-8}. The mobilizing plasmid used in these filter matings was pRK2013 (Lidstrom et al. 1984). Similar results using this plasmid have been achieved in the author's laboratory with *M. trichosporium* OB3b and *M. capsulatus* (Bath).

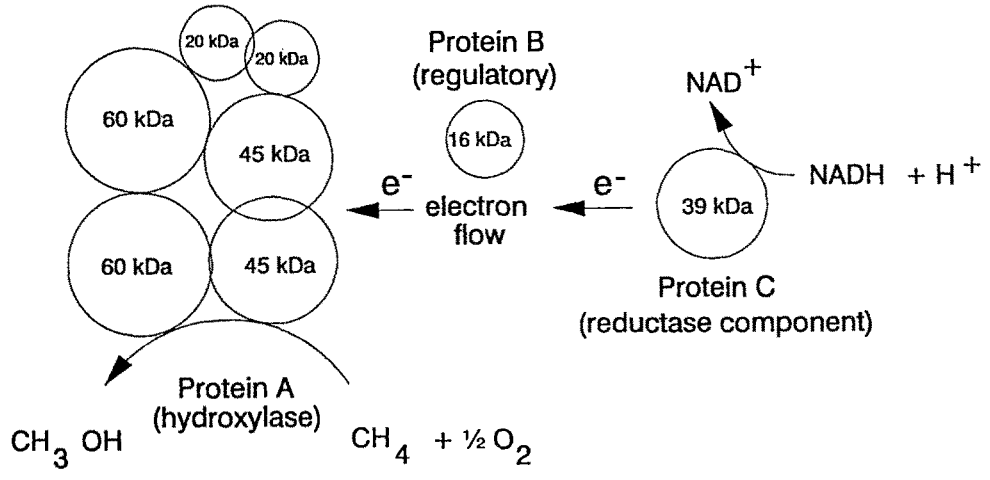

Fig. 2. The soluble methane monooxygenase enzyme complex of *Methylococcus capsulatus* (Bath). The sites of interaction of polypeptides is not known at present.

A number of broad-host-range plasmids have also been transferred to *Methylomonas albus* BG8. Using filter matings the IncP plasmids R68.45, R751 and derivatives carrying bacteriophage Mu and/or transposons and pS-1 (Inc W plasmids) were transferred at frequencies of 10^{-7} to 10^{-8}. RP4 and R300B transferred at higher frequencies (10^{-3}) (McPheat et al. 1987a). Plasmid pULB113, a derivative of RP4 into which a deleted fragment of bacteriophage Mu has been inserted, has been transferred by filter matings into *M. trichosporium* OB3b (Al-Taho & Warner 1987) and *Methylococcus capsulatus* (Bath) (Davidson 1993) at frequencies of around 10^{-5} per recipient. Kanamycin and streptomycin appear to be good genetic markers for methanotrophs since the rate of spontaneous mutation for resistance to these antibiotics is very low (less than 10^{-8}). Plasmids carrying kanamycin resistance (pDSK509, RP4, pJFF350, pSUP2021) and streptomycin resistance (pGSS33, RSF1010) have been successfully transferred to *M. capsulatus* (Bath) at frequencies of 10^{-3} to 10^{-9} per recipient (Davidson 1992). A number of cosmids such as pLAFR1, pCP13 and pJRD215 have also been conjugated at frequencies of around 10^{-4} to 10^{-5} into *M. trichosporium* and *M. capsulatus* using pRK2013 as helper plasmid (Khan 1992).

A long-awaited, detailed study on the effects of growth media composition, conjugation and selection media, conjugation methods, temperature, donor: recipient ratios on the conjugation of transposon Tn5 delivery vectors into *M. capsulatus* (Bath) and *M. trichosporium* OB3b has recently been carried out. The plasmids used for transposon delivery were pSUP 2021 (Simon et al. 1983) pUW964 (Weiss et al. 1983) and pME9 (Rella et al. 1985). Conjugation media supplemented with glucose and selected amino acids and filter conjugations gave the best transfer frequencies at around 10^{-5} to 10^{-6} transconjugants per recipient. Generally 12 hours of conjugation gave the best results. For *M. capsulatus/E. coli* crosses, a conjugation temperature of 37° C rather than 45° C (the optimum growth temperature for the methanotroph) was optimum. No significant differences in conjugation frequencies for temperatures of 30–37° C were observed with *M. trichosporium/E. coli* crosses. Best conjugation frequencies were obtained with a ratio of donor to recipient in filter conjugations of between 1 : 4 and 1 : 10 (Khan 1992; Leak & Khan, manuscript in preparation).

Mutagenesis techniques

The major drawback to the development of mutagenesis procedures for the obligate methanotrophs is probably due to their relatively slow growth on agar plates and the fact that they rely on methane as their sole source of carbon and energy. A consequence of this is that selection of methane oxidation mutants may be difficult. This can usually be overcome since most methanotrophs can be adapted to grow on methanol, thus allowing the selection of an MMO^- phenotype.

The first studies using the mutagens nitrosoguanidine, ethyl methane sulphonate and ultraviolet light showed that it was difficult to increase the spontaneous mutation frequencies of *Methylococcus* and *Methylomonas* to amino acid analogues and antibiotics (Harwood et al. 1972; Williams et al. 1977). However, one leaky p-amino benzoic acid-requiring mutant of *Methylococcus* was isolated and it was also noted that methanotrophs became filamented after ultra violet treatment and in this filamented state they became more susceptible to DNA damaging agents (Williams & Shimmin 1978). Difficulties in mutant induction due to the lack of an error-prone SOS repair process was suggested by these workers (Williams & Bainbridge 1976; Bainbridge 1983).

More recently, methanotroph mutants lacking the ability to grow on methane have been isolated using the 'suicide-substrate' dichloromethane (DCM). This is cooxidized by MMO to the potentially toxic product carbon monoxide and therefore methanotrophs growing on methanol agar plates that are DCM resistant are potential methane monooxygenase mutants. Methanol-adapted *Methylosinus trichosporium* (Nicolaidis & Sargent 1987) and *Methylomonas albus* (McPheat et al. 1987b) were incubated on methanol plates in a DCM atmosphere. This gave rise to DCM-resistant colonies at frequencies of around 10^{-4} to 10^{-5}. When tested, some of these mutants failed to grow on methane and tested negative in a standard MMO assay. The medium used for growth of *M. trichosporium* contained 0.2 μg/l copper, therefore it was likely that only pMMO - minus mutants would have been selected in these experiments and the possibility that DCM-resistant mutants could have retained a functional sMMO, unfortunately was not tested (Nicolaidis & Sargent 1987). As *M. albus* only contains a particulate MMO, it was assumed that mutations lay within the structural gene(s) for pMMO (McPheat et al. 1987b).

Since the above studies, a number of researchers have re-examined the use of DCM as a selective agent for the isolation of MMO mutants and more attention has been given to the growth regime in terms of the copper-to-biomass ratio of cultures in order to identify MMO mutants. Screening of methanotrophs for the presence or absence of a functional sMMO has also been made easier due to the development of a quick reliable plate assay. This is based on the work of Hanson and colleagues (Brusseau et al. 1990) and relies on the conversion of naphthalene to naphthols by soluble MMO but not particulate MMO. The naphthols thus formed can be reacted with tetrazotized O-dianisidine to form purple diazo dyes with intense colour. This method now allows the assay of soluble MMO activity on plates. The procedure does not kill potential mutants which can subsequently be recovered and streaked onto fresh agar plates under the appropriate growth conditions (Graham et al. 1992).

A similar naphthalene assay, coupled with DCM selection has recently been used to isolate and characterize MMO mutants of *M. trichosporium* OB3b (Phelps et al. 1992). *M. trichosporium* OB3b mutants defective in only the particulate form of MMO were isolated by

- suppressing sMMO expression by the presence of high concentrations of copper in selection plates;
- adding excess methanol with DCM to inhibit possible sMMO-mediated activation of DCM to toxic products in any colonies where sMMO might be expressed;
- adding low concentrations of yeast extract to enhance growth and plating efficiency on methanol;
- carrying out mutagenesis selection over a long period (of five weeks).

Although DCM-resistance was unstable in many mutants (a phenomenon observed in other laboratories, including the authors), five stable mutants that were deficient in pMMO and expressed sMMO constitutively in the presence of elevated copper concentrations (up to 12 μM, which inhibits expression of sMMO in the wild-type organism) were found. These mutants that had lost the ability to express pMMO activity concomitantly failed to form the intracytoplasmic membranes characteristic of wild-type OB3b expressing pMMO. Subsequent detailed analysis of these sMMO-constitutive OB3b mutants has suggested that either a defect in copper metabolism affecting copper assimilation causes lack of pMMO expression or that a more general copper-dependent regulatory system that may control pMMO induction, sMMO repression, copper uptake and production of the intracytoplasmic membranes associated with pMMO, has been inactivated (Fitch et al. 1993). The possibility that one of the mutants, which expresses higher levels of sMMO than the wild-type organism, has an altered promoter or regulator for sMMO, is currently being investigated using *xylE* transcriptional fusion vectors (Barta & Hanson 1993).

Molecular biology of the methane oxidation pathway

Methanol dehydrogenase genes

Research, primarily in the laboratories of Lidstrom, Hanson, Goodwin & Harms, has resulted in the identification and cloning of a number of genes involved in methanol oxidation from the Gram negative methylotrophs *Methylobacterium extorquens* and *Paracoccus denitrificans* (reviewed in Lidstrom 1992; Barta & Hanson 1993; Harms 1993). One of these genes, *moxF* from *Methylobacterium*, encoding the large subunit structural gene of methanol dehydrogenase, has been used as a heterologous hybridization probe to identify and clone the corresponding *moxF* genes from *M. capsulatus* (Bath) and *M. albus* BG8 (Stephens et al. 1988). Expression studies of subclones of methanotroph *moxF* genes in the T7 expression vector pTZ18R (Tabor & Richardson 1985) resulted in the production of polypeptides which cross-reacted with antibody to MDH from *Methylobacterium*. Plasmids containing *moxF* from *M. capsulatus* and *M. albus* were also used to complement MDH mutants of *Methylobacterium*, however resulting methanol dehydrogenase activities obtained were lower than those seen in the wild-type *Methylobacterium*.

The *moxF* gene from a λgt11 genomic library of *M. trichosporium* OB3b has been identified and isolated using antibody raised against purified MDH from this organism. Western blotting of lysates from this library and analysis of subsequent subclones of the *moxF* gene suggested that expression was from an unidentified methanotroph promoter (Al-Taho et al. 1990).

A third strategy has been used to isolate MDH genes from methanotrophs. MDH has been purified from the marine methanotroph *Methylomonas* sp. strain A4 and was found to be very similar to the corresponding enzyme from methylotrophs. Using a degenerate oligonucleotide derived from the N-terminal amino acid sequence of MDH from *Methylomonas*, in conjunction with a heterologous *moxF* gene probe from *M. extorquens* AM1, a DNA fragment of approximately 10.5 kb containing *moxF* was isolated from *Methylomonas* A4. Subsequent analysis using protein expression experiments in *E. coli* indicated the presence of the genes encoding the small subunit of MDH (*moxI*), the methanol- dehydrogenase specific cytochrome C_{552} (*moxG*) and two other genes designated *moxJ* and *moxR* whose functions are unknown. The order of these genes is *moxFJGIR*, the same as for facultative methylotrophs. The transcriptional start site for *moxF* was mapped by primer extension analysis and the sequences 5' to the transcriptional start of *moxF* from strain A4 appear to be different to the putative *moxF* promoter sequences of the facultative methyltrophs (Waechter-Brulla et al. 1993).

Methane monooxygenase genes

Purification and biochemical characterization of the sMMO of *M. capsulatus* (Bath) by Dalton and coworkers has generated N-terminal amino acid sequence information on the β and γ subunit polypeptides of Protein A. This has been used to identify and clone the sMMO gene cluster from *M. capsulatus* (Bath) (Stainthorpe et al. 1989, 1990). DNA sequencing of this gene cluster has revealed that the genes encoding the α, β and γ subunits of Protein A (*mmoX, Y* and *Z*), Protein B (*mmoB*) and Protein C (*mmoC*) are all linked on the chromosome. Codon usage in these genes shows that there is a marked preference for either C or G in the third position of the codon (Murrell 1992a). The sMMO gene cluster of *M. capsulatus* (Bath) has been used to probe for sMMO genes in a number of representative strains of methanotrophs (Stainthorpe et al. 1990). sMMO homologues were only detected in *Methylococcus* and *Methylosinus* strains. sMMO appeared to be absent from the Type II methanotroph *Methylocystis parvus* OBBP or Type I representatives of *Methylomonas albus, Methylomonas methanica, Methylomonas agile* and *Methylobacter capsulatus*.

sMMO gene probes from *Methylococcus* were used to clone the sMMO gene cluster from *M. trichosporium* OB3b (Cardy et al. 1991a, b). This gene cluster has also been DNA sequenced and the genes are arranged in the same order as in *Methylococcus* (Fig. 3). The function of orfY, which lies between *mmoZ* and *mmoC*, is unknown at present. Deduced polypeptide sequences of *M. trichosporium* and *M. capsulatus* sMMO components showed a high degree of similarity (Table 1) and codon usage in OB3b was similar to that found for *Methylococcus* (Murrell 1992a). The α subunits of Protein A exhibited a remarkably high degree of sequence similarity (94%). The Glu-x-x-His sequence motif, which is known to coordinate the two irons in the R2 protein of ribonucleotide reductase, was found in two regions of the α-subunit. The x-ray crystal structure of the R2 protein is known (Nordlund et al. 1990) and the geometry of amino acids at the diiron site is fairly well defined. Since two regions of the α-subunits

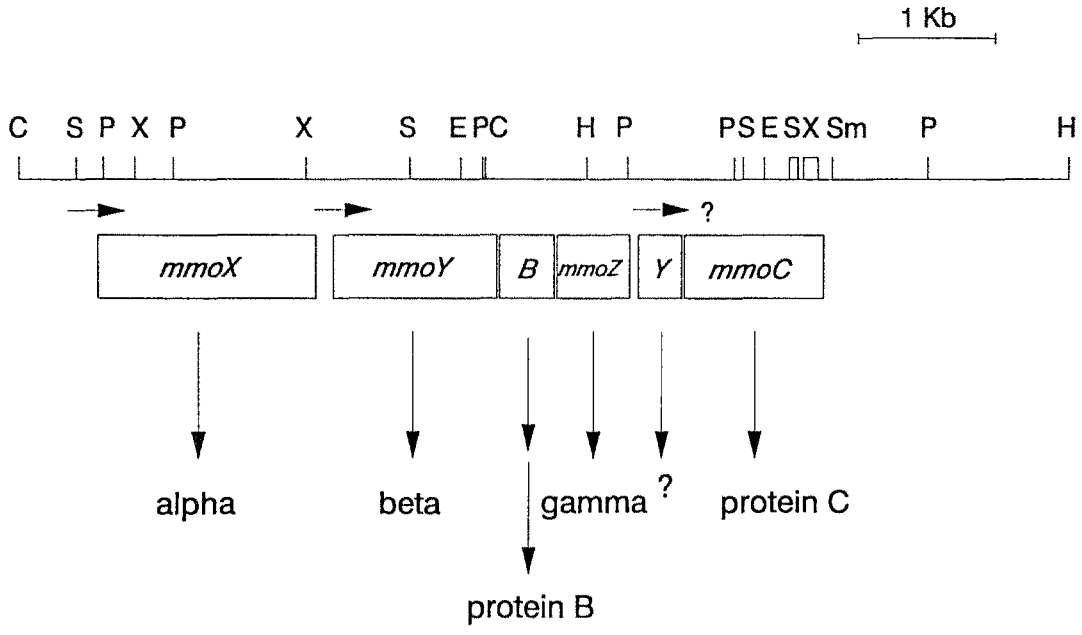

Fig. 3. The soluble methane monooxygenase gene cluster of *Methylosinus trichosporium* OB3b. Arrows indicate putative transcriptional initiation sites (Reproduced with permission of Intercept Ltd. © 1993, from Microbial Growth on C_1 Compounds by J.C. Murrell & D.P. Kelly).

Table 1. Comparative analysis of the deduced polypeptide sequences of *M. trichosporium* OB3b and *M. capsulatus* sMMO components.

M. capsulatus (Bath) sMMO			*M. trichosporium* OB3b sMMO			Percentage similarity
Component	No. of aa's	Mwt (kDa)	Component	No. of aa's	Mwt (kDa)	
Protein A:			Protein A:			
α	527	60.6	α	525	60	94
β	387	44.7	β	394	45	83.5
γ	170	19.8	γ	169	19.3	85
Protein B	141	16	Protein B	138	14.9	89.4
Protein C	348	38.5	Protein C	340	38	78

of the *M. capsulatus* and *M. trichosporium* hydroxylase components (Protein A) align very well with the four helix iron coordination bundle of R2 (see Fig. 4) it has been possible to propose an active site model for the sMMO hydroxylase (Murrell 1992; Dalton 1992; Nordlund et al. 1992; Dalton et al. 1993). It should now be possible to test this model by site specific mutagenesis of the putative Fe binding residues at the proposed active site of sMMO.

The N-terminal 90 amino acids of Protein C from both methanotrophs exhibited significant homologies with ferredoxins from plant and bacteria (Stainthorpe et al. 1990a) confirming biochemical evidence for a ferredoxin-like 2Fe-2S centre in Protein C (Lund & Dalton 1985; Fox et al. 1989). Three of the four conserved cysteine residues of the 2Fe-2S centre lie within a highly conserved domain centred around amino acid residues 36-51 of Protein C. The fourth cysteine is in a second domain around residues 66-83. These are again key residues for future site-directed mutagenesis studies.

```
   glu   his     tyr                glu   his

   115   118     122                238   241
- - E T I H S R S Y T H - - - - R D E A L H - - E.coli
- - E V V H S R V Y N I - - - - R D E A V H - - HSV2
- - E N V H G E T Y A N - - - - R D E L L H - - EBV
- - E N I H S E M Y S L - - - - R D E G L H - - Clam
- - E T I H S R S Y T H - - - - R D E Q L H - - T4
- - E N I H S E M Y S L - - - - R D E G L H - - Mouse
- - E N I H S E T Y S L - - - - R D E G L H - - Yeast
- - E N I H S E M Y S L - - - - R D E G L H - - Vaccinia
- - E V V H A R V Y S Q - - - - R D E A I H - - Varicella

- - E I R H T H Q C A Y - - - - T D E L R H - - OB3b
- - E I R H T H Q C A Y - - - - T D E L R H - - MC
   144   147 149                243   246

   glu   his his              glu   his
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Fig. 4. Alignment of regions of the α subunit of Protein A from *Methylococcus capsulatus* (Bath) (MC) and *Methylosinus trichosporium* (OB3) with two of the iron coordinating regions of the R2 protein of ribonucleotide reductase. (Ribonucleotide reductase data taken from Nordlund et al. 1990.) (Reproduced with permission of Intercept Ltd. © 1993, from Microbial Growth on C_1 compounds by J.C. Murrell & D.P. Kelly.)

The derived amino acid sequences of polypeptides β, γ and B of sMMO do not have any significant homologies with any protein sequences in current databases. There were no homologies found with the predicted orfY gene product to indicate a possible function. The presence of *orfY* in the sMMO gene cluster is intriguing and it is an ideal candidate for future mutagenesis experiments.

An *E. coli*-based, Zubay-type in vitro transcription/translation system has been used to express sMMO genes from *Methylosinus* (Cardy et al. 1991a) although the amounts of protein obtained by this method were small. However, a DNA fragment containing *mmoB*, the gene encoding Protein B from *M. capsulatus* (Bath) was subcloned into the T7 RNA polymerase expression vector pT7-5 (Tabor & Richardson 1985) to yield plasmid pEB51. On induction, *E. coli* containing this plasmid expressed Protein B to approximately 2% of its total soluble protein. This 'recombinant' Protein B, when tested in reconstitution experiments with purified Protein A and Protein C from *M. capsulatus* (Bath), had similar specific activities to those of the purified wild-type protein B (West et al. 1992). Protein C of *Methylococcus* has also been expressed in *E. coli* using the T7 polymerase system. Similar levels of expression and activity close to the 'wild-type' protein were observed.

Thus far there has only been limited success in the expression of Protein A from *M. capsulatus* (Bath) in *E. coli*. The presence of sequences capable of forming stem-loop secondary structures in mRNA 5' to the *mmoY* gene and immediately 3' of *mmoZ* (Stainthorpe et al. 1989) may be a factor in this. Also the presence of the Protein B gene between *mmoY* and *mmoZ* (see Fig. 3) might have limited successful expression but a recombinant plasmid containing only *mmoX, Y* and Z in pT7.7 only gave poor expression in the T7 system (West et al. 1992). The reasons for the poor expression of Protein A are unclear and experiments are now underway to try to express these genes using other expression systems.

M. trichosporium OB3b is a better organism than *M. capsulatus* (Bath) in which to study the regulation of expression of sMMO since OB3b will also grow well on methanol agar plates. The physiological growth conditions under which OB3b switches between using pMMO and sMMO, in response to the Cu^{2+}-to-biomass ratio are also now well defined and have been the subject of several studies (Burrows et al. 1984; Park et al. 1991; Park et al. 1992; Tsien et al. 1989). In order to determine the basis for differential expression of sMMO in the presence or absence of Cu^{2+}, mRNA was extracted from OB3b grown in 'high Cu^{2+}' or 'Cu^{2+} free' medium. This mRNA was then used to generate cDNAs by reverse transcriptase using *mmo* gene-specific primers. mRNA from OB3b grown in the presence of Cu^{2+} (i.e. containing pMMO) did not yield any cDNA products. Northern blotting exper-

iments using these mRNAs and sMMO gene probes have confirmed that sMMO is transcribed only during growth of OB3b in the absence of Cu^{2+} (Murrell et al.; Hanson et al., unpublished).

Primer extension analysis has indicated where the major start points of mRNA synthesis are from the sMMO gene cluster of OB3b (see Fig. 5). Upstream of *mmoX*, the major start of mRNA synthesis is a C-residue at position 423. Preceding this are sequences which have a high degree of homology to the -12, -24 conserved regions of promoters recognised by RNA polymerase δ^{54} (NtrA, RpoN). A further set of promoter sequences with good homologies to the consensus -10, -35 sequences of *E. coli* exist 9bp 'upstream' of the -24 hexamer (indicated in Fig. 5 by overlining) but this putative promoter does not appear to be functional under any of the 'high-' or 'low-Cu^{2+}' growth conditions so far tested.

Upstream of *mmoY* is a second point of initiation of transcription (a G residue at 2161) which is preceded by a putative -10, -35 *E. coli* consensus sequence. Further primer extension analysis suggests a third transcription initiation site upstream of *orfY* although its precise position is not known yet. Again this maybe preceded by both a putative RpoN-like -12, -24 promoter and consensus -10, -35 promoter elements. Northern analysis indicated a single transcript encoding *orfY* and *mmoC* (Murrell et al. unpublished). Further evidence to support the hypothesis that the synthesis of sMMO is regulated at the level of transcription and that more than one mRNA species is produced from the sMMO cluster has come from the work of Hanson and colleagues who have also noted the lack of sMMO-specific mRNA in OB3b cells grown in the presence of 'high-Cu^{2+}' (1 mM) and detected a transcript of approximately 2 Kb in size when probing sMMO-specific mRNA with an *mmoC*-specific oligonucleotide. The same transcript did not hybridize to an *mmoX*-specific oligonucleotide (Barta & Hanson, unpublished).

A broad host-range promoter probe vector pAA182 (Lodge et al. 1990) has been used to analyse the *Methylococcus* sMMO gene cluster. Results indicated that there was promoter activity, as determined by β-galactosidase activity, of subclones of the sMMO cluster in pAA182, between *mmoX* and *mmoY* and upstream of *orfY* or *mmoC*. The second promoter is likely to lie upstream of *orfY* due to the larger intergenic region between *mmoZ* and *orfY*. However, promoter activity was not observed in cloned fragments containing regions of the sMMO cluster including the 5' region and upstream of the start of *mmoX* (Murrell 1993). This last result with *Methylococcus* contradicts earlier findings of a promoter upstream of *mmoX* in sMMO from OB3b and needs to be reexamined in *M. capsulatus* by both primer extension analysis and the use of other promoter probe vectors such as the promoter-probe vector pHX200 (Xu et al. 1993) containing a promoterless *xylE* gene of *Pseudomonas* as the reporter gene.

Finally, examination of sMMO gene sequences from both *Methylococcus* and *Methylosinus* has revealed DNA sequences which would result in the formation of potential stem-loop 'terminator' sequences upon transcription, between *mmoX* and *mmoY* and immediately 3' of *mmoZ*, which together with the primer extension analysis described above suggests that the sMMO gene cluster is expressed as several transcriptional units rather than one long polycistronic mRNA (Murrell 1993).

Use of the cloned sMMO genes has also been made in the construction of sMMO mutants of *M. trichosporium* OB3b. A reverse-genetics approach has been taken using marker exchange mutagenesis, a method first used successfully by Toukdarian & Lidstrom (1984) for the construction of nitrogen fixation mutants of *Methylosinus* sp. strain 6. A DNA fragment containing *mmoX* of OB3b was cloned into pBR325. An *XhoI* fragment internal to the *mmoX* gene was replaced with a kanamycin resistance cassette and the *mob* fragment was also inserted into pBR325 to facilitate transfer by conjugation of the resulting recombinant plasmid pHM32 from *E. coli* S17-1 to OB3b (Fig. 6). Homologous recombination of the mutated *mmoX* gene with the wild-type *mmoX* gene in the chromosome and subsequent loss of the unstable pBR325-based plasmid allowed the isolation of kanamycin resistant OB3b colonies. These were DCM resistant on methanol-agar plates and grew only in the presence of Cu^{2+} on methane i.e. they had the expected $pMMO^{+}$, $sMMO^{-}$ phenotype (Murrell 1993). Attempts are now being made to complement these *mmoX*::kan mutants with the wild-type *mmoX* gene.

Molecular taxonomy and ecology of methanotrophs

Until recently, the taxonomy of methanotrophs has largely been based on pheno- and chemo-taxonomic studies. Current classification schemes (Green 1992, 1993; Hanson et al. 1991) have been strengthened as a result of the nucleotide sequencing of both 5S

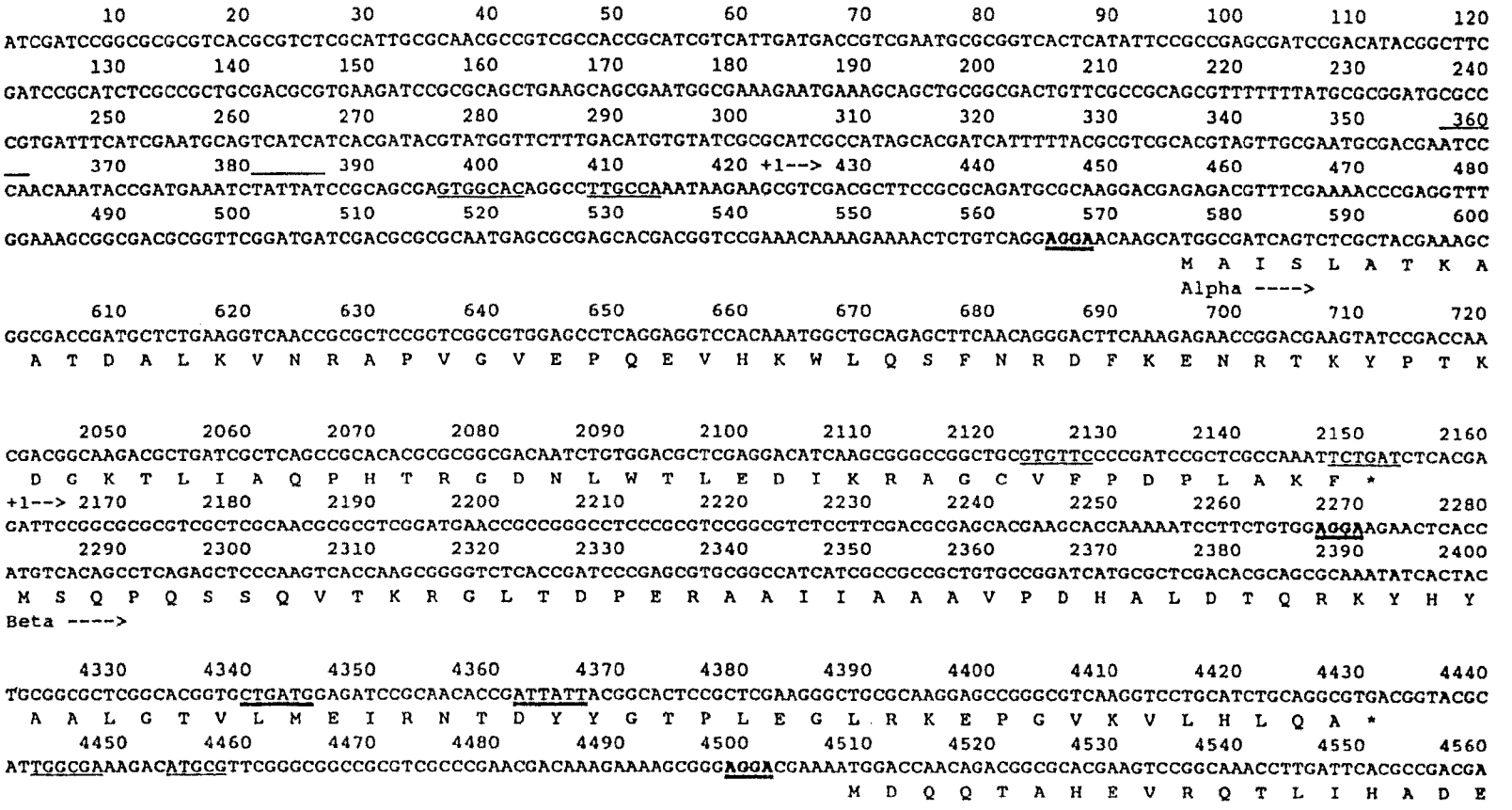

Fig. 5. The major transcription initiation sites and putative promoter elements of the sMMO gene cluster from *Methylosinus trichosporium* OB3b. Transcriptional start sites, determined by primer extension analysis are indicated by + 1. Putative promoter elements are underlined or doubled underlined. Potential Shine-Dalgarno sequences are double underlined and in bold type face. The N-terminal amino acid sequences of the α and β polypeptides of Protein A are given (derived from nucleotides 575–721 and 2281–2400 respectively). Nucleotides 4321–4560 encode the C-terminal region of the γ-polypeptide of Protein A and the N-terminal of OrfY. (Reproduced with permission of Intercept Ltd. © 1993, from Microbial Growth on C_1 compounds by J.C. Murrell & D.P. Kelly.)

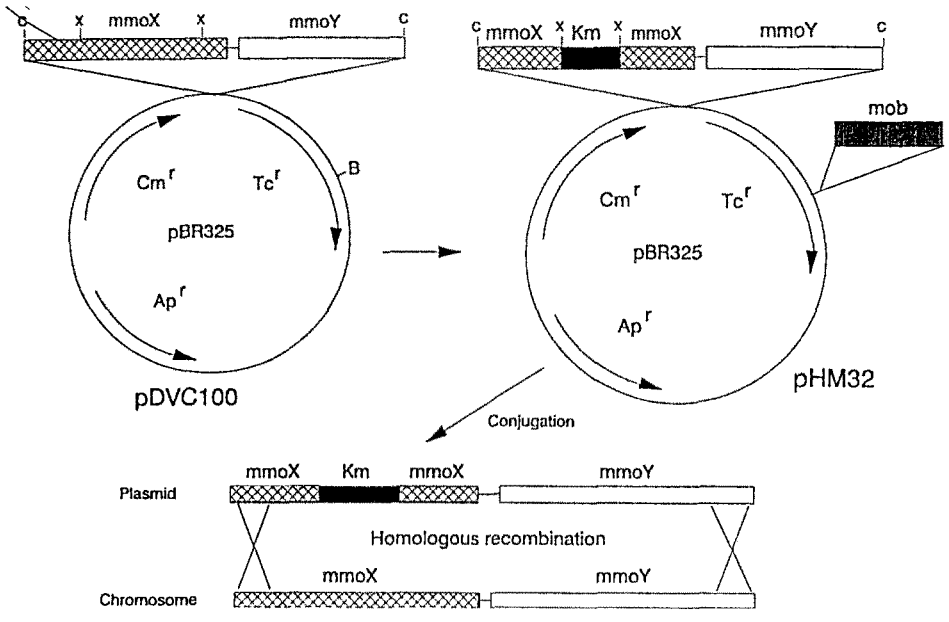

Fig. 6. Marker exchange mutagenesis using the *mmoX* gene of *M. trichosporium* OB3b. A *Cla*1 DNA fragment of OB3b containing *mmoX* and *mmoY* was subcloned into pBR325. A kanamycin resistance cassette then replaced an internal *Xho*1 fragment of the *mmoX* gene. To facilitate conjugation back into OB3b, a mob fragment was cloned into the *Bam*H1 site of pBR325 to give pHM32. Homologous recombination of the 'mutated' *mmoX* gene on pHM32 with the 'wild type' sMMO on the OB3b chromosome yielded a kanamycin resistant, sMMO[PI]AM, pMMO$^+$ OB3b strain. (Reproduced with permission of Intercept Ltd. © 1993, from Microbial Growth on C_1 compounds by J.C. Murrell)

and 16S ribosomal RNA (rRNA) from a large number of methanotrophs and methylotrophs (Tsuji et al. 1990; Boulygina et al. 1990; Bratina et al. 1992; Bowman 1992). Type II methanotrophs such as *Methylocystis parvus, Methylosinus trichosporium* and *Methylosporovibrio methanica* appear to cluster in the α-2 subdivision of the class Proteobacteria and form a separate cluster from other serine pathway methylotrophs. Type I methanotrophs such as *Methylomonas methanica, Methylomonas albus, Methylomonas rubra* and *Methylomonas luteus* together with the Type X methanotroph *Methylococcus capsulatus* are found in the γ-subdivision of the purple bacteria. (reviewed by Hanson et al. 1993; Boulygina et al. 1993).

In addition to the important information these RNA sequences provide on the molecular taxonomy of methane-oxidizing bacteria, they also provide the basis upon which to design oligonucleotide probes specific for methanotrophs (and methylotrophs) in the natural environment. Hanson and colleagues have designed oligonucleotide probes from 16S rRNA data which can be end-labelled with ^{32}Phosphorous or dyes such as fluoresceine and rhodamine for fluorescence microscopy work. The first probe designated 9α was complementary to target sequences in 16S rRNA of serine pathway methylotrophs and is specific for these organisms. The second probe 10γ was specific for RuMP pathway methylotrophs. These two probes, when labelled with two different fluors have been successfully used to differentiate two different groups of methanotrophs (Tsien et al. 1990; Hanson & Wattenberg 1991; Hanson et al. 1993). Hanson and colleagues have in a detailed study, subsequently constructed phylogenetic trees, using 16S rRNA sequences from 36 methylotrophic bacteria and 10 representative organisms from subdivisions within the *Proteobacteria*, on the basis of sequence similarities. From the cluster analysis it was possible to distinguish bacteria using either the ribulose monophosphate pathway or the serine pathway and also between methylotrophs and methanotrophs within these groups. These sequence data were used to design five new oligonucleotide probes which when coupled to a non-radioactive detection system could be used to identify different groups of methylotrophs at sensitivities of as little as 1 ng of RNA (Brusseau, Bulygina & Hanson 1994). These probes will be extremely useful for the detection and identification of methanotrophs in environmental samples.

Restriction fragment length polymorphism (RFLP) has proved useful to distinguish between different

methane oxidizers (Tsuji et al. 1989). Although the methanol dehydrogenase structural gene *moxF* is highly conserved in methanotrophs (and other Gram negative methylotrophs), RFLP analysis allows the detection of small differences in *moxF* sequences between organisms. A similar approach has been successfully used to detect trichloroethylene-degrading methanotrophs in bioreactor and environmental samples using a sMMO Protein B gene probe from *M. trichosporium* OB3b (Tsien & Hanson 1992). Another example of the power of molecular ecology techniques, combining both rRNA based oligonucleotide probing and sMMO gene probing has been used to characterize a methanotroph from a bacterial consortium in a bioreactor that was rapidly degrading trichloroethylene and chloroform (Alvarez-Cohen et al. 1992). Similarly an sMMO gene probe has been used to confirm the production of soluble methane monooxygenase in a Type I methanotroph *Methylomonas methanica* 68-1 (Koh et al. 1993). Comparison with *M. trichosporium* sMMO gene probes, in Southern blotting experiments indicated some degree of evolutionary diversification of sMMOs. These molecular ecology techniques have recently been reviewed in detail (Hanson et al. 1993).

The highly conserved nature of the methanol dehydrogenase gene *moxF* and sMMO genes in methanotrophs has also been utilized in order to detect methanotrophs in environmental samples. Pairs of polymerase chain reaction (PCR) primers have been designed for each of the five sMMO genes, based on these sequences from *M. capsulatus* (Bath) and *M. trichosporium* OB3b and for *moxF* from *Methylobacterium* and *Paracoccus*. PCR amplification of individual sMMO genes is now possible using control template DNA from any methanotroph known to contain sMMO. This technique has been used to identify and clone fragments of the sMMO gene cluster from *Methylocystis* strain M, a methanotroph from which soluble MMO has recently been purified and characterized (Nakajima et al. 1992). The cloning and DNA sequencing of the sMMO genes from this organism is now underway and preliminary analysis suggests that there is a high degree of homology with the corresponding genes from *Methylococcus* and *Methylosinus* (Murrell & Uchiyama unpublished).

When sMMO primers were used in PCR reactions using total bacterial DNA isolated from a variety of freshwater, marine, soil and peat samples, PCR products of the predicted size were obtained. When probed with the corresponding sMMO gene from *Methylococcus* or *Methylosinus*, a high degree of homology was observed. Finally, to prove that methanotroph-specific DNA had been amplified from environmental DNA samples, the PCR fragments were cloned and DNA sequenced. Sequencing of putative sMMO gene fragments indicated that they were very similar to, but not identical to known sMMO gene sequences, illustrating the biodiversity of methanotrophs in the environment (McGowan et al. 1992; Murrell et al. unpublished).

Concluding remarks

The cloning and expression of the genes encoding sMMO from methanotrophs should now allow the predicted active site model for this enzyme to be analysed by site-directed mutagenesis. Perhaps the biotechnological potential of this enzyme can be further enhanced by altering its catalytic utility. The regulation of this enzyme by copper ions can also be investigated at the molecular level and it will be intriguing to find out exactly at which level and by which mechanism, metal ion regulation of sMMO occurs. The next year or so will undoubtedly see the cloning and sequencing of genes encoding the particulate methane monooxygenase and again it will be interesting to learn how this set of genes is regulated, particularly in those methanotrophs that contain the two forms of MMO. Another area of research on methanotrophs that is likely to receive much attention in the future is the use of 16S rRNA- and *mmo*- gene specific probes to detect and identify methanotrophs in natural environmental samples. It has only been recently recognized that methanotrophs may play an important role as a sink for atmospheric methane in a wide variety of environments and both traditional and molecular ecology techniques can be used to reexamine the ecology of this fascinating group of organisms.

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