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Trichloroethylene oxidation by the membrane-associated methane monooxygenase in type I, type II and type X methanotrophs

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Abstract

Trichloroethylene (TCE) oxidation was examined in 9 different methanotrophs grown under conditions favoring expression of the membrane associated methane monooxygenase. Depending on the strain, TCE oxidation rates varied from 1 to 677 pmol/min/mg cell protein. Levels of TCE in the reaction mixture were reduced to below 40 nmolar in some strains. Cells incubated in the presence of acetylene, a selective methane monooxygenase inhibitor, did not oxidize TCE.

Cultures actively oxidizing TCE were monitored for the presence of the soluble methane monooxygenase (sMMO) and membrane associated enzyme (pMMO). Transmission electron micrographs revealed the cultures always contained the internal membrane systems characteristic of cells expressing the pMMO. Naphthalene oxidation by whole cells, or by the cell free, soluble or membrane fractions was never observed. SDS denaturing gels of the membrane fraction showed the polypeptides associated with the pMMO. Cells exposed to ^{14}C-acetylene showed one labeled band at 26 kDa, and this protein was observed in the membrane fraction. In the one strain examined by EPR spectroscopy, the membrane fraction of TCE oxidizing cells showed the copper complexes characteristic of the pMMO. Lastly, most of the strains tested showed no hybridization to sMMO gene probes. These findings show that the pMMO is capable of TCE oxidation; although the rates are lower than those observed for the sMMO.

Introduction

Methanotrophs are a restricted group of bacteria which utilize methane as their sole carbon and energy source. The first enzyme in the methane utilization pathway, the methane monooxygenase (MMO), catalyzes the oxidation of methane to methanol. In some methanotrophs, the cellular location of the MMO has been shown to depend on the copper concentration during growth (Dalton et al. 1984; Scott et al. 1981; Stanley et al. 1983). At low copper to biomass ratios, the enzyme activity is observed in the soluble (cytoplasmic or periplasmic) fraction and is referred to as the soluble MMO (sMMO). The sMMO has been purified from four different methanotrophs and is a complex three component enzyme (Fox et al. 1989; Green & Dalton 1985; Lund & Dalton 1985; Patel & Savas 1987;

Pilkington & Dalton 1991; Woodland & Dalton 1984). Component A is the hydroxylase, which is composed of three subunits with molecular weights of 54,000, 42,000 and 17,000 in a $\alpha_2\beta_2\delta_2$ subunit structure. The hydroxylase component contains two binuclear iron clusters (Fox et al. 1988). Component B (molecular weight 16,000) contains no redox active prosthetic groups and is proposed to be a regulatory protein (Green & Dalton 1985). Component C is an iron sulfur (Fe_2S_2) flavoprotein (FAD) of molecular weight 44,000 and is the reductase component mediating electron transfer from NADH to the hydroxylase (Lund & Dalton 1985).

At higher copper to biomass ratios, methane oxidation activity is observed in the cell membrane fraction and this enzyme is referred to as the membrane associated or particulate MMO (pMMO) (Stanley et al. 1983). Little is known of the molecular structure of the pMMO due to the lability of the enzyme after disruption of the cells. There is indirect evidence that the enzyme contains copper, and inhibitor studies indicate the enzyme is coupled to the electron transport chain (Stanley et al. 1983). In addition, three proteins with molecular weights of 46 kDa, 35 kDa and 26 kDa are induced when cells expressing the sMMO convert to expression of pMMO under altered growth conditions (Stanley et al. 1983). The purification of membrane associated methane oxidizing complexes has been reported for *Methylosinus trichosporium* OB3b (Tonge et al. 1977) and *Methylococcus capsulatus* (strain M) (Akant'eva & Gvozdev 1988). However, reported characteristics of these complexes differ, and attempts to reproduce these procedures in other laboratories have not been successful.

In addition to their molecular properties, the substrate specificity of the sMMO differs from that of the pMMO. The sMMO is a very non-specific enzyme and will oxidize straight-chain, branched-chain alkanes or alkenes up to 8 carbons long, as well as cyclic and aromatic compounds, in addition to methane (Brusseau et al. 1990; Colby et al. 1977; Green & Dalton 1989; Stirling et al. 1979). The membrane associated enzyme is more specific and will oxidize alkanes and alkenes up to 5 carbons long, but will not oxidize cyclic or aromatic compounds (Brusseau et al. 1990; Stirling et al. 1979). With the exception of methane, the oxidation of these substrates is unable to support growth and have been termed co-oxidations.

Recent studies have also shown that pure and mixed cultures of methanotrophic bacteria can co-oxidize trichloroethylene (TCE) (Henry & Grbic-Galic 1991; Little et al. 1988; Neilson 1990; Oldenhuis et al. 1989; Strandberg et al. 1989; Tsien et al. 1989). In most of these studies it is not known which form of the MMO was responsible for TCE oxidation. In *M. trichosporium* OB3b it has been shown that the soluble form of the enzyme is responsible for the high rates of TCE degradation (Fox et al. 1990; Green & Dalton 1989; Oldenhuis et al. 1989; Tsien et al. 1989). However, in many of the laboratory and field studies the cells have been cultured under conditions in which the membrane associated MMO should be expressed. In these studies the cells did oxidize TCE, although the reported rates of TCE degradation were orders of magnitude slower than those expected if the cells were expressing the sMMO.

The purpose of the present work was to determine whether the membrane associated form of the MMO is capable of oxidizing TCE. Until active preparations of the purified enzyme are available, it is not possible to determine this directly. However, indirect evidence has been obtained by culturing cells in medium containing high concentration of copper and then examining these cells for the presence of the sMMO, pMMO and TCE oxidation. These results show that cells expressing only the pMMO do oxidize TCE.

Materials and methods

Enrichment and isolation

Three different isolates, *Methylomonas* sp. GD1, GD2 and GD3 were enriched from a TCE contaminated groundwater aquifer located in General Dynamics Plant No.4, Ft. Worth, TX, with methane as previously described (Lidstrom 1988) with the omission of NaCl from the culture medium.

Strains and growth conditions

The marine methanotrophs, *Methylomonas* A1, A4, A45 and C1 (Lidstrom 1988) were cultured at 37° C in nitrate mineral salts medium (NMS; Whittenbury & Dalton 1981) plus a vitamin mixture described by Lidstrom (1988). *Methylomonas* sp. MN (Lidstrom, unpublished results) and *Methylococcus capsulatus* Bath (Whittenbury et al. 1970) were grown in NMS plus vitamins at 37° C. The methanotrophs isolated from TCE-contaminated groundwater aquifers, *Methylomonas* sp. MM2 (Henry & Grbic-Galic 1990), GD1 and GD2 were grown on NMS plus vitamin mixture at room temperature. *Methylocystis* LWY (Lidstrom, unpublished results), *Methylocystis parvus* OBBP and *M. parvus* OBBPM and *Methylomonas albus* BG8 (Woodland & Dalton 1984) were cultured on NMS plus vitamins at 30° C. *Methylosinus trichosporium* OB3b (Woodland & Dalton) was grown in the nitrate mineral salts medium (NSM) described by Cornish et al. (Cornish et al. 1984) at 30° C. The copper concentrations in NMS and NSM were varied between 0 and 20 μM. Cells grown in shake flasks were cultured on either methane (under an atmosphere of 20% methane: 80% air(v/v)) or 0.2% methanol. Cells grown in a 10-liter fermentor were sparged at flow rates of 80–150 ml/minute methane and 2000–2500 ml/minute air.

Methylomonas sp. A4 was 'adapted' to growth on methanol by gradually increasing the concentration of methanol in the growth medium as described by Hou et al. (1978). The methanol adapted strain is called *Methylomonas* sp. A45. *Methylocystis parvus* OBBP was adapted to growth on methanol in chemostat culture as previously described (Linton & Vokes 1978; Prior & Dalton 1985). The methanol adapted strain is called *M. parvus* OBBPM.

Isolation of cells, soluble and membrane fractions

Cells were harvested by centrifugation at 13,200 × g for 10 minutes at 4° C and washed in either 25 mM piperazine-N,N'bis [2-ethane sulfonic acid] (PIPES), pH 7.0, buffer containing 20 μM $CuSO_4$ (Drummond et al. 1989) for the pMMO or 25 mM 3-(N-morpholino)propanesulfonic (MOPS), pH 7.0, buffer containing 200 μM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 2 mM cysteine (Fox et al. 1989) for the sMMO, 250 mM NaCl was added for the marine strains. The cells were resuspended in the respective washing buffers. Cells were lysed by passage through a French pressure cell three times at 20,000 psi and the unlysed cells and cell debris removed by centrifugation at 13,200 × g for 15 minutes at 4° C. The pellet was discarded and the supernate centrifuged at 150,000 × g for 90 minutes. The pellet from this preparation was used as the membrane fraction, and the supernate was used as the soluble fraction.

Electron microscopy

In preparation for electron microscopy, cells were pelleted by centrifugation at 4,330 × g for 10 minutesand washed twice in 10 ml of 0.1 M sodium cacodylate buffer, pH 7.3 overnight at 4° C. The postfixed cells were centrifuged and washed once in 0.1 M sodium cacodylate buffer. The pellet was resuspended in 2% molten agar. The agar enrobed cells were postfixed in 1% OsO_4 for 1.5 h at room temperature. The samples were dehydrated and embedded in Lufts Epon and polymerized overnight at 60° C (Dawes 1979). The samples were sectioned on a Sorvall MT-6000 and viewed in a JEOL 100 CX at 80 KeV.

Incubation of cells with ^{14}C-labeled acetylene

Cell suspensions (3–7 ml total volume; 2–5 mg cell protein/ml) were incubated in 35 ml serum vials capped with Tuf-Bond teflon/silicon discs, Pierce Chemical Co., at room temperature. Reactions were initiated by either injecting 0.25 ml of ^{14}C labeled acetylene (approximately 10 μCi, 150 nM; New England Nuclear, Boston, MA) or by addition of 50 μl of a ^{14}C-acetylene/DMSO solution and shaken at ambient temperature for 10–15 minutes

on a reciprocating shaker. Generation of the ^{14}C-acetylene/DMSO solution was prepared by the method of Hyman & Arp (1990). Following the incubation period, 1 ml of unlabeled acetylene was injected and the mixture was incubated at room temperature for 15 minutes. Cells were harvested and washed and the soluble and membrane fractions isolated as described above.

Electrophoresis and fluorography

Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis was performed by the Laemmli (1970) method on 15% gels. Samples for electrophoresis were either boiled in sample buffer for 1 minute with 1.5% (v/v) β-mercaptoethanol or incubated in sample buffer and 1.5% β-mercaptoethanol for approximately 10 minutes at room temperature. Gels were stained for total protein with Coomassie brilliant blue R.

Fluorographic detection of radioactivity in polyacrylamide gels was determined by the method of Chamberlain (1979) using sodium salicylate.

Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance spectra were recorded at X-band on a Varian E-109 spectrometer. Samples of membrane suspensions were placed in 5mm OD quartz tubes, degassed with argon and rapidly frozen in liquid nitrogen. During data acquisition the samples were held in an Oxford Instruments liquid helium cryostat at temperatures of 10 K.

Methane monooxygenase assay

Whole cell MMO activity was determined at room temperature by measuring the epoxidation of propylene using 20 mM sodium formate as a reductant (Prior & Dalton 1985). The product (propylene oxide) was measured by gas chromatography using either a Porapak Q or HayeSep A column in either a Hach GC or Antex 300 gas chromatograph with a flame-ionization detector. Expression of soluble or membrane associated MMO was determined by assaying the respective fractions for propylene epoxidation using NADH (2 mM) as the reductant. Cells assayed for sMMO were resuspended in 25 mM MOPS, pH 7.0, buffer containing 200 μM Fe $(NH_4)_2 \cdot 6H_2O$ and 2 mM cysteine (Fox et al. 1989) while cells assayed for pMMO were resuspended in 25 mM PIPES, 20 μM $CuSO_4$, pH 7.0, buffer (Smith & Dalton 1989).

Trichloroethylene degradation assay

TCE oxidation was monitored by its disappearance from the reaction mixture by gas chromatography. Assays were performed in sealed 6 ml serum vials containing 1 ml reaction mixtures at room temperature. Reaction mixtures contained 1 to 15 mg protein in 20 mM PIPES, 20 mM sodium formate, 25 μM copper sulfate, pH 7.0, buffer. The reaction was started by the addition of TCE. The vials were immediately sealed with an unused (not punctured) Tuf-Bond teflon septum after the addition of TCE. Reactions were stopped at time intervals varing between 5 minutes and 24 h. The reaction was stopped and the TCE extracted from the aqueous phase by the addition of 1 ml of pentane with a gas tight syringe and the vials centrifuged at 4,000 × g for 10 minutes at room temperature. Preparation of TCE stock solutions and monitoring of TCE in the reaction mixtures were performed as described by Tsien at al. (1989).

Other assays

Oxidation of naphthalene to naphthol was determined by the method of Brusseau et al. (1990). The concentration of protein was determined by the method of Lowry et al. (1951). Hydroxypyruvate reductase and 3-hexulose-6-phosphate synthase were performed by the methods of Stafford et al. (1954) and Dahl et al. (1972), respectively.

DNA manipulations

Isolation of total cellular DNA from the methanotrophic strains were preformed as described by Fulton et al. (1984). The sMMO specific DNA probe used contained the 3′ end of the *mmo* X gene and all of the *mmo* Y, *mmo* Z and *mmo* C genes from *M. capsulatus* Bath as described by Stainthorpe et al.(1990). Preparation of the sMMO-specific DNA probe, hybridization conditions and probing were determined as described by Stainthorpe et al. (1990). Stringencies of hybridization allowed for up to 60% base-pair mismatching of probe and target sequences.

Results

Characterization of methanotrophs isolated from TCE contaminated sites

Four strains of methanotrophs isolated from TCE contaminated groundwater sites were used in this study. *Methylomonas* sp. MM2 was isolated from a Moffett Field Navel Air Station groundwater aquifer sample and supplied by S.M.Henry (Henry & Grbic-Galic 1991). *Methylomonas* sp. GD1, GD2 and GD3 were isolated from a groundwater aquifer from General Dynamics Air Force Plant No.4. Before cultures were isolated from the General Dynamics site, the native methanotrophic community was tested for the ability to degrade TCE. The original field samples contained approximately 6 μM TCE. These samples were incubated in an atmosphere of 40% methane/60% air. After one week the TCE concentration was less than 0.04 mM. Samples incubated in the presence of 40% methane/10% acetylene/50% air or cyanide killed controls showed TCE concentrations between 4.9 and 6.2 μM TCE. Three methanotrophic cultures were isolated from this groundwater sample. Like *Methylomonas* sp. MM2, the General Dynamics strains were all pink pigmented Gram negative motile rods and showed typical Type I internal membrane arrangement (Whittenbury & Dalton 1981). The three strains all grew well on methane and methanol but were not capable of growth on substrates containing C-C bonds, i.e. glucose and succinate. All strains contained activity of the ribulose monophosphate pathway enzyme 3-hexulose-6-phosphate synthase and no detectable activity for the serine pathway enzyme hydroxypyruvate reductase. Based on these results the General Dynamics strains are tentatively classified as *Methylomonas* sp. GD1, GD1 and GD3.

Acetylene binding proteins

Acetylene has been shown to be an irreversible inhibitor of both the soluble and membrane associated MMO (Colby et al. 1975; Dalton & Whittenbury 1976; De Bont & Mulder 1976). Using labeled ^{14}C-acetylene, Prior & Dalton (Prior & Dalton 1985) found the label bound to a single polypeptide of 54 kD in *Methylococcus capsulatus* (Bath) expressing the sMMO and this was shown to be the 54 kDa subunit of component A. Cells of *M. capsulatus* (Bath) grown under conditions in which only the membrane associated MMO was expressed showed only one ^{14}C-acetylene labeled polypeptide with a molecular weight of 26,000 (Prior & Dalton 1985). This labeled polypeptide is similar in size to one of the membrane proteins induced when the cells are grown under high copper to biomass condition, and has been presumed to be a component of the pMMO. Therefore, we tested several strains of methanotrophs for acetylene binding proteins.

Total inhibition of MMO activity was also observed in whole cells of all strains tested following treatment with ^{14}C-labeled and unlabeled acetylene (see 'Materials and methods') and in most cases a single major polypeptide was labeled (Table 1). The fluorogram of *Methylomonas* sp. A4, *Methylomonas* sp. MM2 and *Methylococcus capsulatus* (Bath) expressing the membrane associated MMO and treated with ^{14}C acetylene is shown in Fig. 1. Of the strains tested only *Methylococcus capsulatus* Bath showed a specifically labeled polypeptide band (Fig. 1I) when the samples were heated (100° C) before loading. The labeled polypeptide had a molecular weight of 26,000 which was identical to the results obtained by Prior & Dalton (1985). In all other strains tested the label appeared

to aggregate and not enter the gel (Fig. 1A and 1E). To test whether the labeled membrane polypeptides were precipitating in the sample buffer, samples were boiled for 30 to 60 seconds in sample buffer with 1.5% β-mercaptoethanol (Fig. 1A, 1E and 1I), or incubated at room temperature in sample buffer with 1.5% β-mercaptoethanol for 10–15 minutes (Fig. 1B, 1F and 1J). The different sample treatments had little effect on the single labeled polypeptide in *M. capsulatus* Bath. However, when cell samples of *Methylomonas* sp. A4 (Fig. 1B), *Methylomonas* sp. MM2 (Fig. 1F) as well as the other methanotrophs tested were not heated before loading on SDS-denaturing gels, one specifically labeled, major polypeptide with a molecular weight of 25,500–26,000 was observed. The fluorograph of the membrane fraction of *Methylomonas* sp. A4 also showed a second labeled polypeptide with a molecular weight of 17,000. The 17 kDa polypeptide appears to be a breakdown product of the 26 kDa radiolabeled polypeptide and is observed in all the strains tested except *Methylomonas* sp. MN and *M. capsulatus* (Bath) in stored samples. In the case of *Methylomonas* sp. A4, the appearance of this peptide has been correlated with freezing and thawing of the sample (B.Speer & M.Lidstrom, unpublished). Heat induced aggregation of membrane proteins in SDS-gel electrophoresis buffers has been observed in other systems (Briggs

Table 1. Membrane associated and acetylene binding polypeptides in type I, type II and type X methanotrophs.

	Methanotroph	pMMO Associated Polypeptides (kDa)	*Cells Expressing*		^{14}C-Labeled Polypeptide (kDa)
			sMMO	pMMO	
Type I	Freshwater				
	Methylomonas albus BG8	26, 43	–	+	26
	Methylomonas sp. MN	36, 46	–	+	36
	Methylomonas sp. MM2	26, 35, 45	–	+	26
	Methylomonas sp. GD1	42	–	+	ND[1]
	Methylomonas sp. GD2	26, 42	–	+	ND
	Methylomonas sp. GD3	26, 42	–	+	ND
	Marine				
	Methylomonas sp. A1	25.5, 35, 43	–	+	25.5
	Methylomonas sp.A4	25.5, 34, 43	–	+	25 5
	Methylomonas sp. A45[2]	25,5, 34, 43	–	+	25.5
	Methylomonas sp. C1	25.5, 34, 43	–	+	25.5
Type II					
	Methylosinus trichosporium OB3b	25.5, 42	–	+	25.5
	Methylocystis parvus OBBP	25.5, 43	–	+	25.5
	Methylocystis parvus OBBPM[3]	25.5, 43	–	+	25.5
	Methylocystis LWY	26, 43	–	+	26
Type X					
	Methylococcus capsulatus Bath	26, 35, 46	–	+	26
	Methylococcus capsulatus Bath	–	+	–	54

[1] Not determined.

[2] Methanol adapted strain of *Methylomonas* sp. A4.

[3] Methanol adapted strain of *Methylocystis parvus* OBBP.

Only the membrane associated acetylene binding proteins from *Methylomonas* sp Mn and *Methylococcus capsulatus* Bath entered SDS denaturing gels when boiled (60 seconds) in Laemmli sample buffer

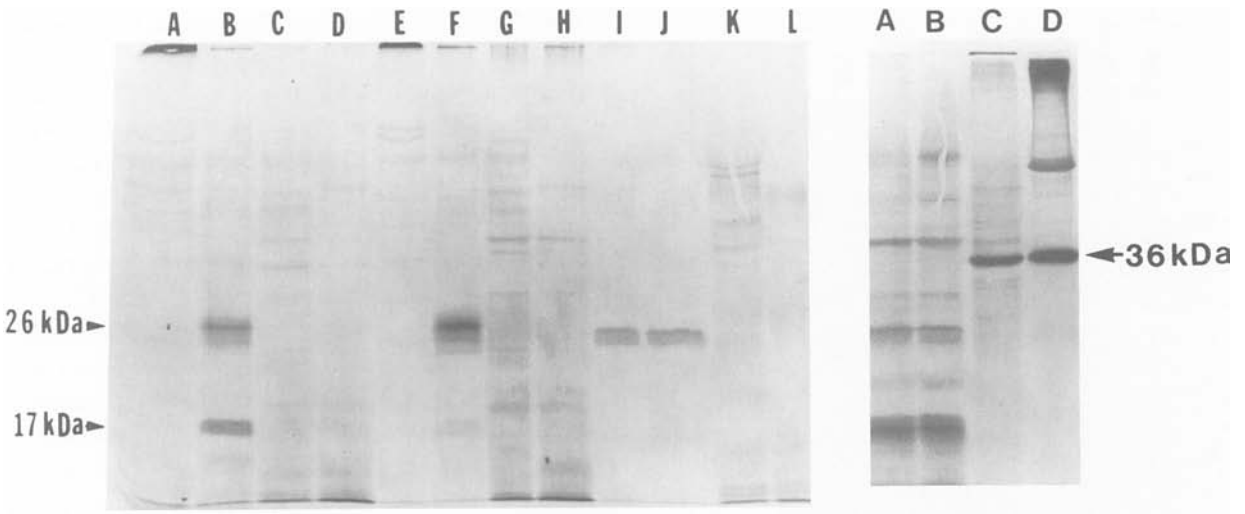

Fig. 1. Fluorogram of a 15% SDS-polyacrylamide gel of cell membrane (lanes A, B, E, F, I and J) and soluble (C, D, G, H, K and L) fractions of *Methylomonas* sp. A4 (lanes A–D), *Methylomonas* sp. MM2 lanes (E–H) and *M. capsulatus* (Bath)(lanes I–J) labeled with ^{14}C-acetylene. Samples were either incubated at room temperature (lanes B, D, F, H, J and L) or boiled (lanes A, C, E, G, I and K) before loading. Samples contained 50 μg of total protein.

Fig. 2. Fluorogram of a 15% SDS-polyacrylamide gel of cell soluble (lanes A and B) and membrane (lanes C and D) fractions of *Methylomonas* sp. MN labeled with ^{14}C-acetylene. Samples were either incubated at room temperature (lanes A and C) or boiled (lanes B and D). Samples contained 50 μg protein

et al. 1975; DiSpirito et al. 1986; Kadenbach et al. 1983; Merle & Kadenbach 1980).

In contrast to the other stains tested, in *Methylomonas* sp. MN two major radiolabeled protein bands at approximately 36,000 and 75,000 d were observed on SDS-PAGE in the membrane fraction when the samples are boiled before loading (Fig. 2D). However, if the sample is not heated only one radiolabeled polypeptide was observed with a molecular weight of 36,000 (Fig. 2C), suggesting that the high molecular weight material observed after heat treatment is a dimer of the acetylene binding protein. In *Methylomonas* sp. MN several major radiolabled polypeptides with molecular weights of 82, 46, 22, and 14 kDa were consistently observed in the soluble fraction (Fig. 2A and 2B). These polypeptides do not appear to represent a new form of the sMMO since neither methane or naphthalene activity was ever observed in the soluble fraction.

pMMO associated proteins

In addition to the acetylene binding polypeptide, two other membrane associated proteins were induced in *M. capsulatus* (Bath) when cultures were switched to high copper medium, with molecular weights of 46,000 and 35,000 Da (Prior & Dalton 1985). In *M. trichosporium* OB3b the only observed change in the polypeptide profile when cells were switched to high copper medium was a 42,000 Da membrane associated polypeptide (Burrows et al. 1984). Lastly, in *Methylomonas albus* BG8 addition of copper to the growth medium increased the cell yield, MMO activity and concentrations of two polypeptides with molecular weights of 41,000 and 22,000 (Collins et al. 1991). In the strains tested in this study variability in the major membrane polypeptides associated with the pMMO was observed (Table 1, Fig. 3). Of the strains tested, 13 out of 15 showed a major 25,000 to 26,000 d polypeptide, 6 out of 15 showed a major 34,000 to 35,000 d poly-

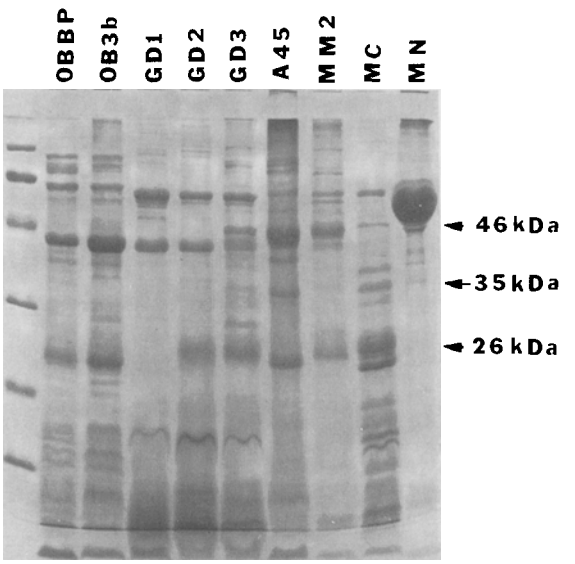

Fig. 3. SDS-polyacrylamide gel of the membrane fraction from *Methylocystis parvus* OBBP (OBBP), *Methylosinus trichosporium* OB3b (OB3b), *Methylomonas* sp. GD1 (GD1), *Methylomonas* sp. GD2 (GD2), *Methylomonas* sp. GD3 (GD3), *Methylomonas* sp. A45 (A45), *Methylomonas* sp. MM2 (MM2), *Methylococcus capsulatus* (Bath)(MC) and *Methylomonas* sp. MN (MN) grown in culture medium containing 2.5 mM copper sulfate. Protein content of membrane samples were 50 mg. The standards (unlabeled lane) correspond to molecular masses 92,000, 66,200, 45,000, 31,000, 21,500 and 14,400 Da.

peptide and all showed a major 42,000 to 46,000 d polypeptide.

Presence of pMMO

Several criteria were used to ensure that the cells tested for the ability to degrade TCE were expressing the pMMO and that the sMMO was absent. First, all of the strains were examined for the capacity to express the soluble form of the enzyme. To test for this ability, the cells were cultured in copper free medium in a fermentor to late stationary phase. Under these growth conditions the sMMO was observed only in the two strains know to contain it, *M. trichosporium* OB3b and *M. capsulatus* Bath. During studies with *M. capsulatus* Bath we observed that the sMMO can be induced in a fermentor at lower cell densities (OD_{600nm} of 1.0 to 1.5) if a vitamin mixture (see 'Materials and methods') is added to the culture medium and if the cells are cultured at 37° C instead of 42° C.

The potential to express the sMMO was also examined by probing genomic DNA from these strains with the sMMO gene probes constructed by Stainthorpe et al. (1990). Again, of the strains used in this study only *M. trichosporium* OB3b and *M. capsulatus* Bath showed positive hybridization (Table 2).

The cells used in the TCE degradation studies were cultured under conditions that should not allow expression of the sMMO, that is, in medium containing 2.5 to 20 μM copper sulfate and grown to an optical density (600 nm) of 0.7 to 1.5 before harvesting. With the exception of *M. capsulatus* Bath and *M. trichosporium* OB3b, the cultures were grown in a 10 liter fermentor. Cells of *M. capsulatus* Bath and *M. trichosporium* OB3b were cultured in shake flasks to an optical density (600 nm) of 0.5 to 0.7 to impede the expression of the sMMO. In all cells tested, the following criteria were used to confirm the presence of the membrane associated MMO and the absence of the sMMO (Table 2). Electron micrographs of cells cultured under these conditions showed the internal membranes characteristic of cells expressing the membrane associated MMO. MMO activity was never observed in the soluble fractions even in samples concentrated with a stirred cell (YM5 filter) to over 50 mg/ml protein. Low levels (1–5% of the whole cell rates) of MMO activity were observed in the membrane samples. SDS-denaturing gels of the cell extract or soluble fractions never showed the major polypeptides characteristic of the sMMO. SDS-denaturing gels of the cell extract and membrane fractions always showed the polypeptide(s) characteristic of the pMMO (Tables 1 and 2; Fig. 3). Cells exposed to ^{14}C-acetylene showed one major labeled polypeptide with a molecular weight of 25,500–26,000 or 36,000, that was present in the membrane fraction (Table 2, Fig. 1). As a positive control, *M. trichosporium* OB3b and *M. capsulatus* Baths were cultured in low copper medium to late stationary phase to induce the sMMO. In both strains over 80% of the whole cell methane oxidation rates were observed in the soluble fractions. In addition, cells of *M. capsulatus* Bath

grown under low copper conditions and exposed to ^{14}C-acetylene showed one labeled polypeptide with a molecular weight of 54,000 that was present in the soluble fraction (Table 1). The oxidation of naphthalene to naphthol, a characteristic of the sMMO (Brusseau et al. 1990), was never observed in whole cells or in the cell free, soluble or membrane fractions, although it was easily detected in cells of *M. trichosporium* OB3b and *M. capsulatus* Bath grown under copper-limiting conditions. Low temperature electron paramagnetic resonance spectra of the membrane fractions of TCE oxidizing culturres showed the copper complexes associated with the pMMO (Fig. 4; Shiemke et al., submitted). These copper complexes are never observed in the membrane fraction from cultures of *M. trichosporium* OB3b and *M. capsulatus* Bath grown under low copper conditions. From these results we conclude that only the membrane associated MMO was present in the cells used in the following TCE oxidation studies.

TCE degradation by methanotrophs

The TCE oxidation rates by the eight methanotrophs expressing the membrane associated MMO were monitored at room temperature. The four strains isolated from groundwater aquifer samples, *Methylomonas* sp. MM2, *Methylomonas* sp. GD1, *Methylomonas* sp. GD2 and *Methylomonas* sp. GD3 showed poor TCE degradation activities. Of the four strains isolated from groundwater aquifer samples only *Methylomonas* sp. MM2 show significant and consistent TCE oxidation rates of 2 to 10 pmol/min/mg cell protein. Following 12 h incubation periods the amount of TCE remaining in the reaction mixtures was 2.1 to 1.9 μM. Low (less than 1 pmol/min/mg cell protein) degradation rates were

Table 2. Presence of membrane associated methane monooxygenase in trichloroethylene oxidizing cultures of type I, type II and type X methanotrophs.

Methanotrophs	Assay				
	Electron micrographs	Acetylene binding protein (kDa)	Naphthalene oxidation	EPR spectra	sMMO gene probe
Type I					
Methylomonas sp. A45	IM[1]	25.5[+]	–	+[1]	–
Methylomonas sp. MN	IM[1]	36[1*]	–	nd[2]	–
Methylomonas sp. MM2	IM	26	–	+	–
Methylomonas sp. GD1	IM	26	–	nd	–
Methylomonas sp. GD2	IM	26	–	nd	–
Methylomonas sp. GD3	nd	nd	–	nd	–
Type II					
Methylosinus trichosporium OB3b	IM[1]	25.5	–	nd	+[1]
Methylocystis parvus OBBP	IM[1]	25.5	–	nd	–
Type X					
Methylococcus capsulatus Bath	IM[1]	26[1]	–	+[1]	+[1]

[1] Determined on cells grown in high copper medium but cells were not tested for the ability to oxidize TCE.
[2] Not determined.
* acetylene binding polypeptides in the soluble fraction are not listed.
Codes used in table: IM, cells showed internal membrane systems, (–) for naphthalene oxidation refers to the lack of naphthalene oxidation to naphthol by whole cell or by the cell free, membrane, or soluble fractions; positive (+) under EPR spectra refers to the presence of the copper signals associated with the presence of the pMMO; positive (+) or negative (–) for sMMO gene probe refers to positive or negative hybridization to the DNA probes specific for the sMMO genes.

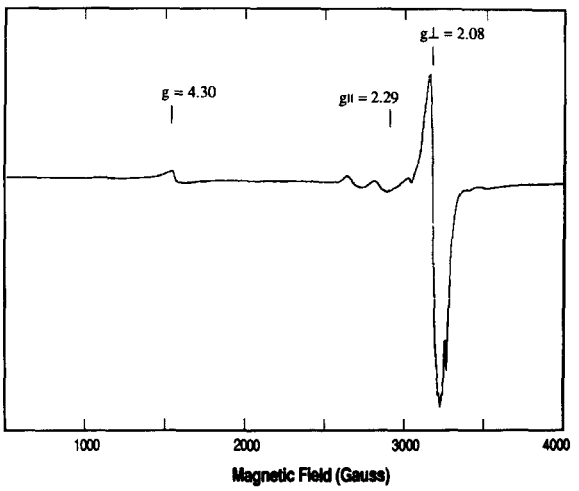

Fig. 4. X-band electron paramagentic resonance spectrum of a membrane. suspension from *Methylomonas* sp. MM2: 5.2 mg protein/ml in 20 mM PIPES buffer (pH 7.0) at 6K. Instrumental conditions were as follows: microwave power: 0.2 mW, microwave frequency: 9.217 GHz, modulation amplitude: 10 gauss, modulation frequency: 10 kHz, gain: 1.25×10^3, time constant: 0.064 second.

observed in *Methylomonas* spp. GD1 and GD2. However, the oxidation rates of TCE by these strains were inconsistent and never lasted more than 12 h after the cells were harvested. TCE degradation was never detected in *Methylomonas* sp. GD3.

The TCE degradation rates in the 'standard' laboratory strains were much higher than those observed in the methanotrophs isolated from TCE contaminated sites. We refer to 'standard' laboratory strains as those isolated on the basis of methane oxidation not on the basis of TCE oxidation. The degradation rates in these strains were 296 pmol/min/mg protein for *Methylomonas* sp. A45, 233 pmol/min/mg cell protein for *Methylomonas* sp. MN, 58 pmol/min/mg cell protein for *Methylosinus trichosporium* OB3b, 202 pmol/min/mg cell protein for *Methylococcus capsulatus* (Bath) and 677 pmol/min/mg cell protein for *Methylocystis parvus* OBBP (Fig. 5). The levels of TCE remaining in the reaction mixtures after 12 h incubation periods was less than 0.04 μM for *Methylomonas* sp. A45, 3.1 μM for *Methylomonas* sp. MN, 0.05 μM for *Methylosinus trichosporium* OB3b, 0.05 μM for *Methylocystis parvus OBBP* and 1.7 μM for *Methylococcus capsulatus* (Bath). The higher rates of TCE oxidation in these strains was correlated with high whole cell MMO activities, which ranged from 75 to 200 nmol/min/mg cell protein. Whole cell MMO activities for the four isolates from TCE contaminated ground water sites were much lower, varying between 2.7 and 12 nmol/min/mg cell protein.

No significant loss (less than 3%) of TCE was observed in the buffer controls or in the acetylene or cyanide killed controls. If the septum used in the reaction vial was not punctured, there was no significant loss of TCE from the reaction mixture of the acetylene or cyanide killed controls (Fig. 5). If the teflon seal is punctured the loss of TCE from the reaction mixture can be as high as some of the oxidation rates, probably due to adsorption of TCE by the silicon septum. This result may explain why others have not detected TCE oxidation above background rates in some of these strains. When loss of TCE in the killed controls was observed it was subtracted from the live samples, although this was never significant.

Discussion

The membrane associated MMO is found in all known strains of methanotrophs, while the sMMO has so far been detected only in *Methylobacterium*, *Methylosinus* and *Methylococcus* strains (Patel & Savas 1987; Pilkington et al. 1991; Stainethorp et al. 1990). In addition, the sMMO is only expressed under conditions of extreme copper limitation, which are difficult to achieve in the laboratory and may be found only rarely, if at all, in nature. Thus, it is important to understand the membrane associated MMO in these organisms. However, the membrane associated MMO remains a poorly characterized enzyme due to the lability of the enzyme activity after cell lysis. Although two reports of enzyme purification have appeared (Akent'eva & Gvozdev 1988; Tonge et al. 1977) this work has not been repeated. Active enzyme has been solubilized from the membrane of *M. capsulatus* Bath by Smith & Dalton (1989) but they have been unable to purify the enzyme further. The

enzyme is believed to contain copper and may be composed of at least three subunits (Prior & Dalton 1985; Stanley et al. 1983).

Recent studies have correlated high rates of TCE oxidation by *M. trichosporium* OB3b with expression of the sMMO, suggesting that only the soluble MMO can oxidize TCE (Oldenhuis et al. 1989; Tsein et al. 1989). However, other studies (Henry & Grbic-Galic 1991; Little et al. 1988) have shown that methanotrophs isolated from TCE contaminated groundwater aquifer samples oxidized TCE when grown under conditions in which the cells should be expressing the membrane associated MMO. Electron micrographs of these isolates always showed that the cells contained the internal membrane systems characteristic of cells expressing the membrane associated MMO. In order to address the question of whether the membrane associated MMO can oxidize TCE we have developed a series of indirect measurements for the presence of the enzyme. First, we assessed the acetylene binding polypeptides. In the sMMO, the acetylene binding polypeptide is part of the hydroxylase component, and it seems likely that in the membrane-associated MMO the acetylene binding polypeptide is also a part of the enzyme complex. We surveyed 10 different methanotrophs to determine whether a common membrane-associated acetylene binding polypeptide was present in cells expressing the membrane-associated MMO. When cells from the different methanotrophs were grown under conditions in which they express the membrane associated MMO and exposed to ^{14}C-acetylene, the major labeled polypeptide was located in the membrane and with one exception, had a molecular mass of 25,500–26,000 d. In *Methylomonas* sp. MN the labeled polypeptide had a molecular of 36,000 d (Table 1). Since only one major polypeptide was labeled and in most cases it was similar in molecular mass, the labeling of this polypeptide served as a likely indicator of expression of the membrane-associated MMO.

Examination of the acetylene binding polypeptides along with all other tests listed in Table 2 showed strong expression of the membrane associated MMO, with no detectable expression of the soluble MMO. The naphthalene oxidation assay is

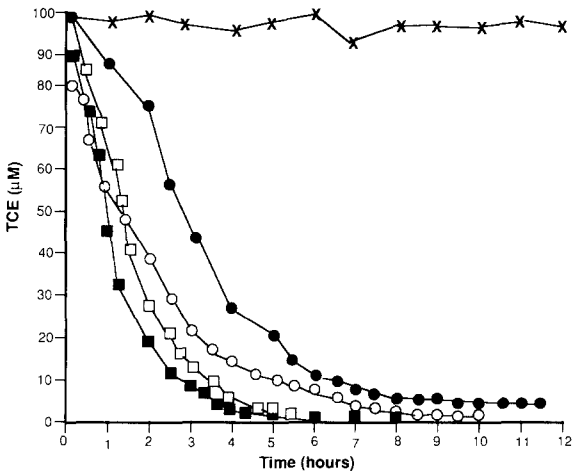

Fig. 5. Time course of TCE oxidation by *Methylocystis parvus* OBBPM (■), *Methylomonas* sp. A45 (□), *Methylococcus capsulatus* Bath (O) and *Methylomonas* sp MN and cyanide killed *Methylomonas* sp. A45 (×). The reaction mixtures contained 80–100 mM TCE and washed cells (*M. parvus* OBBPM, 4.5 mg; *Methylomonas* sp. A45, 10.7 mg protein; *M. capsulatus* Bath , 17.0 mg protein; *Methylomonas* sp. MN, 8 2 mg protein) in 20 mM PIPES, pH 7 0, buffer containing 20 μM copper sulfate and 20 mM sodium formate.

an especially sensitive indicator of soluble MMO activity, and it was always negative. In addition, all attempts to induce the soluble MMO failed except in the two species known to contain it, *M. trichosporium* OB3b and *M. capsulatus* Bath. Finally, no hybridization was observed to a gene probe for the soluble MMO, although this probe hybridizes to DNA from all strains known to contain the soluble MMO (Stainethorp 1990). If the TCE degrading methanotrophs tested do have a 'sMMO' it must differ considerably from that of *Methylococcus* and *Methylosinus* species since there is no detectable homology at the DNA level and the sMMO from *M. trichosporium* OB3b and *M. capsulatus* Bath is so highly conserved (Cardy et al. 1991).

These data all strongly suggest that the cells used for the TCE oxidation assay did not contain soluble MMO. We cannot rule out the presence of a third, unknown TCEoxidation system, but this seems unlikely. TCE oxidation was always inhibited completely by acetylene, and so any alternative systems should bind acetylene. Therefore, unless the acetylene binding polypeptide of a third system were 25–26 kDa, we would have detected it. With the

exception of *Methylomonas* sp. GD3, all of the methanotroph strains tested here oxidized TCE in the absence of expression of the soluble MMO. The rates of TCE oxidation observed in the standard laboratory strains were higher than those observed in the strains isolated from TCE contaminated sites, which reflected higher whole cell MMO activities in the standard strains.

Even the highest rates observed (677 pmol/min/mg protein for *M. parvus* OBBP) are much lower than the rates found in *M. trichosporium* OB3b expressing the soluble MMO (456 nmol/min/mg protein; Brusseau et al. 1990; 290 nmol./min/mg protein; Oldenhuis et al. 1991). However, in some cases the TCE was removed to a level near that required for drinking water (38 nM; US EPA 1980). Consideration for treatment of contaminated groundwater using methanotrophs must include both rates and affinities for the co-substrate. The data presented here suggest that viable treatment programs could be developed in the absence of bacteria containing the soluble MMO.

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