

Soluble Methane Monooxygenase Production and Trichloroethylene Degradation by a Type I Methanotroph, *Methylomonas methanica* 68-1

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A methanotroph (strain 68-1), originally isolated from a trichloroethylene (TCE)-contaminated aquifer, was identified as the type I methanotroph *Methylomonas methanica* on the basis of intracytoplasmic membrane ultrastructure, phospholipid fatty acid profile, and 16S rRNA signature probe hybridization. Strain 68-1 was found to oxidize naphthalene and TCE via a soluble methane monooxygenase (sMMO) and thus becomes the first type I methanotroph known to be able to produce this enzyme. The specific whole-cell sMMO activity of 68-1, as measured by the naphthalene oxidation assay and by TCE biodegradation, was comparatively higher than sMMO activity levels in *Methylosinus trichosporium* OB3b grown in the same copper-free conditions. The maximal naphthalene oxidation rates of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b were 551 ± 27 and 321 ± 16 nmol h⁻¹ mg of protein⁻¹, respectively. The maximal TCE degradation rates of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b were $2,325 \pm 260$ and 995 ± 160 nmol h⁻¹ mg of protein⁻¹, respectively. The substrate affinity of 68-1 sMMO to naphthalene (K_m , 70 ± 4 μ M) and TCE (K_m , 225 ± 13 μ M), however, was comparatively lower than that of the sMMO of OB3b, which had affinities of 40 ± 3 and 126 ± 8 μ M, respectively. Genomic DNA slot and Southern blot analyses with an sMMO gene probe from *Methylosinus trichosporium* OB3b showed that the sMMO genes of 68-1 have little genetic homology to those of OB3b. This result may indicate the evolutionary diversification of the sMMOs.

Soluble methane monooxygenase (sMMO) present in certain methanotrophic bacteria has been found to be capable of oxidizing a wide range of carbon substrates (9). These include halogenated aliphatic compounds (32) such as trichloroethylene (TCE), a significant groundwater pollutant (46). Degradation of TCE by pure methanotrophic cultures has been well documented (11, 18, 22, 32, 39, 40). The rapid oxidation and degradation of TCE by sMMO (5) and the ready availability of natural gas as a growth substrate make methanotrophs attractive for bioremediation applications (17).

Methanotrophs are divided into three types or groups (I, II, and X) on the basis of intracytoplasmic membrane ultrastructure, enzymatic characteristics, fatty acid carbon chain length (48), G+C values (moles percent) (24), and 16S rRNA sequences (41). It is believed that sMMO is found only in type II and X methanotrophs in copper-limiting conditions (26), and thus relatively high TCE degradation rates appear to be limited to a few species of type II and X methanotrophs (5, 32, 39, 40). A recent study reported that particulate (membrane-associated) MMO (pMMO) can degrade TCE but degradation rates are several orders of magnitude lower than those with sMMO (11). Likewise, pMMO cannot oxidize naphthalene, which is readily oxidized to naphthol by sMMO (5, 11). The naphthalene oxidation assay has been established as an indicator of sMMO activity (5, 11, 40).

Southern blotting with a 5.8-kb *Bam*HI fragment of the sMMO gene cluster (*mmoX*, *mmoY*, *mmoZ*, *mmoB*, and *mmoC*) of the type X methanotroph *Methylococcus capsulatus* Bath was used to find homologous genes in various

methanotrophic species (26, 36, 40). The probe hybridized to the type II methanotrophs *Methylosinus trichosporium* and *Methylosinus sporium* in conditions allowing 30% mismatch. No homologous genes were detected in the type II methanotroph *Methylocystis parvus* and in the type I methanotrophs tested, including *Methylomonas* spp. and *Methylobacter* spp. Another probe derived from a 2.2-kb *Eco*RI fragment of the sMMO gene cluster of *Methylosinus trichosporium* OB3b hybridized to other *Methylosinus* species (40), but no hybridization was found with *Methylococcus capsulatus* Bath in similar hybridization conditions. No sMMO has been observed in type I methanotrophs to date. Therefore, it appears that the sMMO genes are limited to a few species of type II and type X methanotrophs.

The objective of this investigation was to confirm the type I taxonomy of strain 68-1 and to determine whether TCE degradation by this strain was conferred by the existence of a unique sMMO production in a type I methanotroph.

This study reports the existence of sMMO activity in a type I methanotroph (identified as *Methylomonas methanica* 68-1) isolated from a TCE-contaminated aquifer. The sMMO of 68-1 was compared with that of *Methylosinus trichosporium* OB3b in terms of TCE degradation kinetics and genetic homology.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. Bacterial strains used in this study are listed in Table 1. Methanotrophic bacteria were grown on a nitrate mineral salts (NMS) medium (8) under an atmosphere of 4:1 air/methane. Most methanotrophic and heterotrophic bacteria were grown at 26 to 28°C except *Methylococcus capsulatus* Bath and *Methylococcus capsulatus* ATCC 19069^T, which were grown at

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TABLE 1. Bacterial strains used in this study and hybridization of their total RNA with 16S rRNA signature probes 10- γ and 9- α

Type and species of bacterial strain ^a	Source ^b or reference	Hybridization with given 16S rRNA probe	
		10- γ	9- α
Type I methanotrophs			
" <i>Methylomonas agile</i> "	ATCC 35068	ND ^c	ND
" <i>Methylomonas alba</i> "	ATCC 33003 (= BG8)	+	-
<i>Methylomonas methanica</i>	ATCC 35067 ^T	+	-
" <i>Methylomonas rubra</i> "	NCIMB 11913	+	-
Strain 68-1	22	+	-
Type X methanotrophs			
<i>Methylococcus capsulatus</i>	ATCC 19069 ^T (= Texas)	ND	ND
<i>Methylococcus capsulatus</i>	ATCC 33009 (= Bath)	+	-
Type II methanotrophs			
" <i>Methylocystis parvus</i> "	ATCC 35066 (= OBBP)	ND	ND
" <i>Methylosinus sporium</i> "	ATCC 35069 (= 5)	-	+
" <i>Methylosinus trichosporium</i> "	ATCC 35070	-	+
RuMP pathway methyloprophs			
" <i>Methylomonas clara</i> "	ATCC 31226	ND	ND
<i>Methylophilus methyloprophus</i>	ATCC 53528 ^T	ND	ND
Serine pathway methyloprophs^d			
<i>Hyphomicrobium</i> sp.	ATCC 43129	ND	ND
<i>Methylobacterium extorquens</i>	ATCC 14718 (= AM1)	-	+
<i>Methylobacterium organophilum</i>	ATCC 27886 ^T (= XX)	-	+
<i>Methylobacterium rhodesianum</i>	ATCC 43882 ^T	ND	ND
Heterotrophs			
<i>Agrobacterium tumefaciens</i>	ATCC 23308	-	-
<i>Bacillus subtilis</i>	ATCC 6051 ^T	ND	ND
<i>Escherichia coli</i>	ATCC 23716 (= K-12)	ND	ND
<i>Rhizobium leguminosarum</i>	Strain 248 (19)	ND	ND
<i>Pseudomonas putida</i>	Strain F1 (44)	ND	ND

^a Quotation marks indicate that the species has a taxonomically invalid status (20).

^b ATCC, American Type Culture Collection, Rockville, Md.; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

^c ND, not done.

^d Serine, serine transhydroxymethylase.

37°C. The ribulose monophosphate (RuMP) pathway methyloprophs were grown in NMS containing 0.1% (vol/vol) methanol, and the serine pathway methyloprophs were grown in NMS containing methylamine-HCl (6.75 g/liter) except *Methylobacterium rhodesianum* ATCC 43882^T, which was grown in nutrient broth. All heterotrophs were grown in nutrient broth unless otherwise mentioned. Copper-free NMS media were prepared with double glass-distilled water in glassware washed by the procedure of Tsien et al. (39). Growth of cultures and density of cell suspensions were monitored at 600 nm with a spectrophotometer DU-70 (Beckman, Palo Alto, Calif.).

Electron microscopy. Strain 68-1 was grown to late log phase in NMS medium containing either 0 or 1 μ M CuSO₄.

Cells were harvested by centrifuging the cultures at 5,000 \times g. Cell pellets were fixed in 3% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.0) for 1 h at room temperature. After being washed three times in 0.1 M phosphate buffer for 1 h, the cells were postfixed in 2% (wt/vol) OsO₄ for 1 h. The cells were dehydrated in a graded series of alcohol-water mixtures and then embedded in Epon 812 (Electronmicroscopy Sciences, Fort Washington, Pa.) as described before (23). The embedded cells were cut with a diamond knife and stained with uranyl acetate (45) and basic lead citrate (34) prior to observation with a Hitachi H-600 electron microscope (Hitachi Co., Tokyo, Japan).

Phospholipid fatty acid and lipopolysaccharide hydroxy fatty acid analyses. Strain 68-1 was grown as described above. The total lipid of the lyophilized cell material was extracted and fractionated with activated silicic acid (29, 47). The phospholipid fraction was transesterified to the corresponding methyl esters by mild alkaline methanolysis and analyzed by gas chromatography (29). Lipid isomers and double-bond position were determined by dimethyl-disulfide derivitization (12, 28).

16S rRNA signature probe hybridization. Total RNAs were extracted from strain 68-1 and nine other strains, including methanotrophs, methyloprophs, and one heterotroph, by the hot-phenol extraction method (30). Complementary 16S rRNA oligonucleotide probes specific for RuMP pathway-containing (10- γ probe: 5'-GGTCCGAAGATCCCCCGCTT 3') and serine pathway-containing (9- α probe: 5'-CCCT GAGTTATTCGAAAC-3') methyloprophs (38) were utilized and were labeled with [γ -³²P]ATP (2). RNA was immobilized for slot blot hybridization (35) and hybridized to the 16S rRNA probes by the procedure of Tsien et al. (38).

DNA slot blotting and Southern hybridization. Both chromosomal and plasmid DNA isolations were carried out by the procedure of Ausubel et al. (2). For DNA slot blot hybridization, 2 μ g of each DNA sample dissolved in 25 μ l of water was added to 25 μ l of 1 M NaOH and denatured in a 95°C water bath for 10 min. The denatured DNA solution was then cooled rapidly on ice, and 50 μ l of 0.5 M NaOH and 100 μ l of water were added. A nucleic acid slot blot system (BioDot SF Microfiltration Apparatus; Bio-Rad Laboratories, Richmond, Calif.) was assembled and mounted with a 6 \times SSC (0.9 M sodium chloride and 0.09 M trisodium citrate, pH 7.0)-prewetted Biotrans nylon membrane (ICN Biomedicals, Cleveland, Ohio). The denatured DNA samples were loaded into wells of the slot blot apparatus and transferred to the filter by applying a vacuum. The filter was then air dried and baked at 80°C for 1 to 2 h to immobilize the DNA. For DNA Southern blot hybridization, 10 μ g of each genomic DNA was digested with *Eco*RI enzyme and separated by electrophoresis on 0.8% agarose gels in 1 \times TBE buffer (50 mM Tris base, 50 mM boric acid, 1 mM EDTA [pH 8.3]). The electrophoresed DNA was transferred to Biotrans nylon membranes by the Vacuum-Blotting System (Pharmacia Biotechnology AB, Uppsala, Sweden) in accordance with the manufacturer's instruction. A DNA probe complementary to a 2.2-kb *Eco*RI fragment of the *Methylosinus trichosporium* OB3b sMMO gene cluster (carrying the *mmoB*, *mmoZ*, *orfY*, and part of the *mmoC* genes [40]) was labeled with the Genius Nonradioactive Labeling and Detection Kit (Boehringer Mannheim Corp., Indianapolis, Ind.). All subsequent prehybridization, hybridization, and detection of the labeled DNA probe were performed by following the Genius System User's Guide for Filter Hybridization (Boehringer Mannheim). Detection in this experiment was

carried out by using a chemiluminescent detection system with Lumi-Phos 530 provided by the manufacturer.

Quantitative measurement of sMMO activity. A slightly modified version of the naphthalene oxidation assay of Brusseau et al. (5) was used to quantitatively measure sMMO activity. Ten milliliters of each culture was degassed in 58-ml serum bottles sealed with rubber stoppers by evacuating the headspace and backfilling with air to remove residual methane. Cultures were then diluted to an A_{600} of 0.2 (0.08 to 0.09 mg of dry cell weight per ml) with NMS media. The diluted cultures were transferred in 1-ml aliquots to 10-ml screw-cap test tubes, and 1 ml of prefiltered saturated naphthalene solution (234 μ M at 25°C [42]) was added to each aliquot. The samples were prepared in triplicate. The reaction mixtures were incubated at 200 rpm on a rotary shaker at 25°C for 1 to 3 h. A heat-killed control and a sterile medium control as blanks were also prepared and processed in the same way as the tests. After incubation, 100 μ l of freshly prepared tetrazotized-*o*-dianisidine solution (4.21 mM) was added to each tube, and the formation of colored diazo-dye complexes was immediately monitored by recording the A_{525} by spectrophotometry. The intensity of diazo-dye formation is proportional to the naphthol concentration (1-naphthol and 2-naphthol). Wackett and Gibson (43) determined the molar extinction coefficient (ϵ) of the diazo-dye to be 38,000 $M^{-1} cm^{-1}$. The protein content of the cell suspension was determined by the microbiuret procedure (25). The specific activity of sMMO was expressed as nanomoles of naphthol formed per milligram of cell protein per hour.

Quantitative measurement of TCE degradation by whole cells. Diluted cell suspensions (A_{600} of 0.2) were prepared as described in the above procedure and transferred in 1-ml aliquots to screw-cap septum vials (14 ml; Pierce Chemical, Rockford, Ill.). Sodium formate was then added to each vial to a final concentration of 20 mM. Heat-killed control samples were prepared for all experiments. TCE degradation was initiated by adding 5 μ l of a TCE aqueous stock solution (1,100 ppm at 25°C [42]) to each vial to a final concentration of 5.5 ppm (42 μ M). The vials were then sealed with Teflon-lined silicone septa, inverted, and incubated on a rotary shaker (200 rpm) at room temperature (25°C) for various time periods. Triplicate samples and their corresponding heat-killed controls were sacrificed at designated time intervals by adding 2 ml of *n*-hexane containing 1,2-dibromoethane (1 ppm) as an internal control. The ungraded TCE was extracted into the organic phase by shaking and centrifugation (2,000 $\times g$ for 20 min). The degradation analyses were performed on a Shimadzu GC 9AM gas chromatograph (Shimadzu Analytical Instruments Co., Kyoto, Japan) equipped with a split injection port (1:1) run at 220°C, an RTX volatiles capillary column (15 m by 0.53-mm inside diameter; Restek Corp., Bellefonte, Pa.) run isothermally at 80°C, and an electron capture detector run at 220°C. Nitrogen was used as the carrier gas (flow rate, 10 ml/min). The peak areas were calculated with a Shimadzu integrator, C-R6A Chromatopac. A 5- or 10- μ l portion of the organic phase was sampled by using a gas-tight syringe (Hamilton Co., Reno, Nev.) and injected into the sample injection port. Under these conditions, TCE and 1,2-dibromoethane exhibited retention times of 4.3 and 1.7 min, respectively.

Quantitative measurement of TCE degradation by cell fractions. Cell extracts were prepared by the modified method of Nakajima et al. (27). Cells, grown up to early stationary phase, were harvested by centrifugation at 13,200

$\times g$ for 10 min at 4°C and washed in 100 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] buffer (pH 7.0) containing 5 mM sodium thioglycolate as a reducing agent. The washed cells were resuspended in the buffer and sonicated (20 30-s pulses with 1.5-min cooling on ice between each pulse) at an output of 0.4 to 0.6, using a Virsonic 300 sonicator (The VirTis Co., Gardiner, N.Y.). Cell debris was removed by centrifugation at 20,000 $\times g$ for 15 min at 4°C, and then the pellets were discarded and the supernatants were centrifuged at 150,000 $\times g$ for 1.5 h. The supernatants from this preparation were used as the soluble fractions, and the pellets were resuspended in the PIPES buffer (pH 7.0) containing 5 mM sodium thioglycolate and used as the particulate fractions. For the assay of TCE degradation activity by sMMO, 1 ml of the soluble fraction was dispensed into each vial and then NADH (3.5 mM) and FeCl₃ (200 μ M) were added, while the particulate fraction containing NADH (3.5 mM) and CuSO₄ (20 μ M) was used for the assay of TCE degradation activity by pMMO. Triplicate vials were prepared for each fraction, and the subsequent TCE degradation assay was performed as described above.

Determination of kinetic parameters of naphthalene oxidation and TCE degradation. Strain 68-1 and *Methylosinus trichosporium* OB3b were grown to early stationary phase in 500 ml of copper-free NMS (in 2-liter flasks). The cell suspensions were diluted to an A_{600} of 0.2, and 1 ml of each of the suspensions was added to screw-cap septum vials (14 ml; Pierce). Various concentrations of naphthalene (7.3 to 234.0 μ M) and TCE (5.0 to 500.0 μ M) were added to the vials. In addition, 20 mM sodium formate was added to ensure that NADH pools were saturated (5). The vials were incubated for 1 h at 25°C. Two independent experiments with triplicate vials for each concentration were performed to determine K_m and V_{max} values. It was found that the Lineweaver-Burke plots were not statistically adequate because inversion distorted the error span in lower substrate concentrations. Thus, rectangular hyperbolic curve fits were performed by using the computer program DeltaGraph (DeltaPoint Inc.). The maximum substrate concentrations for naphthalene and TCE were assumed to be 234 μ M and 8.365 mM, respectively. These concentrations are the points at which these compounds are saturated in water at 25°C (42).

RESULTS AND DISCUSSION

Identification of strain 68-1. Strain 68-1, a methanotroph originally isolated by Little et al. (22) from a TCE-contaminated aquifer, when grown under copper limitation, was found to produce sMMO as indicated by the naphthalene oxidation assay of Brusseau et al. (5). It was suspected that this strain was *Methylomonas methanica* by virtue of its prominent pink carotenoid pigmentation (48). This was confirmed through the use of 16S rRNA signature oligodeoxynucleotide probes, electron microscopy, and phospholipid fatty acid analysis.

The RNA of strain 68-1 was found to hybridize to the 10- γ 16S rRNA oligodeoxynucleotide probe which is specific for RuMP pathway-containing methylotrophs (38), which include both type I and type X methanotrophs (14). No hybridization occurred with the 9- α 16S rRNA probe, which is specific for serine pathway-containing methanotrophs. Other methanotrophs whose RNAs were hybridized to probe 10- γ were *Methylomonas methanica*, *Methylomonas rubra*, *Methylomonas alba* BG8, and *Methylococcus capsulatus* Bath, while probe 9- α hybridized to *Methylosinus trichosporium*,

TABLE 2. Growth and naphthalene oxidation and TCE degradation kinetic parameters of *Methylomonas methanica* 68-1 and *Methylosinus trichosporium* OB3b grown in copper-free conditions

Strain	NMS +				Naphthalene oxidation ^a		TCE degradation ^b	
	1 μM CuSO_4		No CuSO_4		K_m (μM)	V_{\max} (nmol h ⁻¹ mg of protein ⁻¹)	K_m (μM)	V_{\max} (nmol h ⁻¹ mg of protein ⁻¹)
	μ (h ⁻¹) ^c	Biomass (mg liter ⁻¹)	μ (h ⁻¹)	Biomass (mg liter ⁻¹)				
<i>Methylomonas methanica</i> 68-1	0.074	202.9 \pm 8.1	0.060	93.0 \pm 10.1	70 \pm 4	551 \pm 27	225 \pm 13	2,325 \pm 260
<i>Methylosinus trichosporium</i> OB3b	0.060	160.1 \pm 8.0	0.053	178.8 \pm 10.1	40 \pm 3	321 \pm 16	126 \pm 8	995 \pm 160

^a The stationary-phase culture was used. Naphthalene concentrations used were in the range of 7.3 to 234.0 μM .

^b The stationary-phase culture was used. TCE concentrations used were in the range of 5.0 to 500.0 μM .

^c Indicates specific growth rate; cultures were grown in 500 ml of NMS medium in 2-liter flasks under 4:1 air/methane. Flasks were shaken at 200 rpm at 25°C.

Methylosinus sporium, *Methylobacterium organophilum*, and *Methylobacterium extorquens*. Neither probe hybridized to *Agrobacterium tumefaciens*, which was used as a negative control (Table 1).

Strain 68-1 exhibits intracytoplasmic membrane (ICM) ultrastructures that are typical of type I and X methanotrophs, not type II (21). Strain 68-1 was found to contain 14:0 (30%), 16:1 ω 8c (18%), 16:1 ω 7c (15%), 16:1 ω 6c (13%), and 16:1 ω 5t (13%) as its major phospholipid fatty acids. This profile is quite distinctive among methanotrophs and has only been observed in the species *Methylomonas methanica*, *Methylomonas aurantiaca*, and *Methylomonas fodinarum* (3, 16). Since 68-1 possessed a pink carotenoid pigmentation, it was highly likely that 68-1 is a *Methylomonas methanica* strain, whereas *Methylomonas fodinarum* and *Methylomonas aurantiaca* produce a distinctively orange carotenoid pigmentation (4).

Effects of copper absence on strain 68-1. When strain 68-1 was grown in a state of copper absence, the biomass level and specific growth rate declined significantly. *Methylosinus trichosporium* OB3b, however, showed no significant differences in the parameters (Table 2). A decline in ICM was observed in 68-1; however, many cells still showed obvious ICM formation (Fig. 1B). This is unlike the effect of copper limitation on the type I methanotroph *Methylomonas alba*, which only produces pMMO (40): i.e., a complete loss of ICMs and a drastically decreased specific growth rate (7). Similar phenomena were also observed in *Methylococcus capsulatus* Bath grown on methanol in the absence of copper (33). The methanotroph lost ICM, and the membranes only occurred at the cell periphery. There was a gradual increase in ICM formation as the copper ion concentration increased to 4.8 μM , at which ICMs were fully recovered. In the methanotroph "*Methanomonas margaritae*," ICM forma-

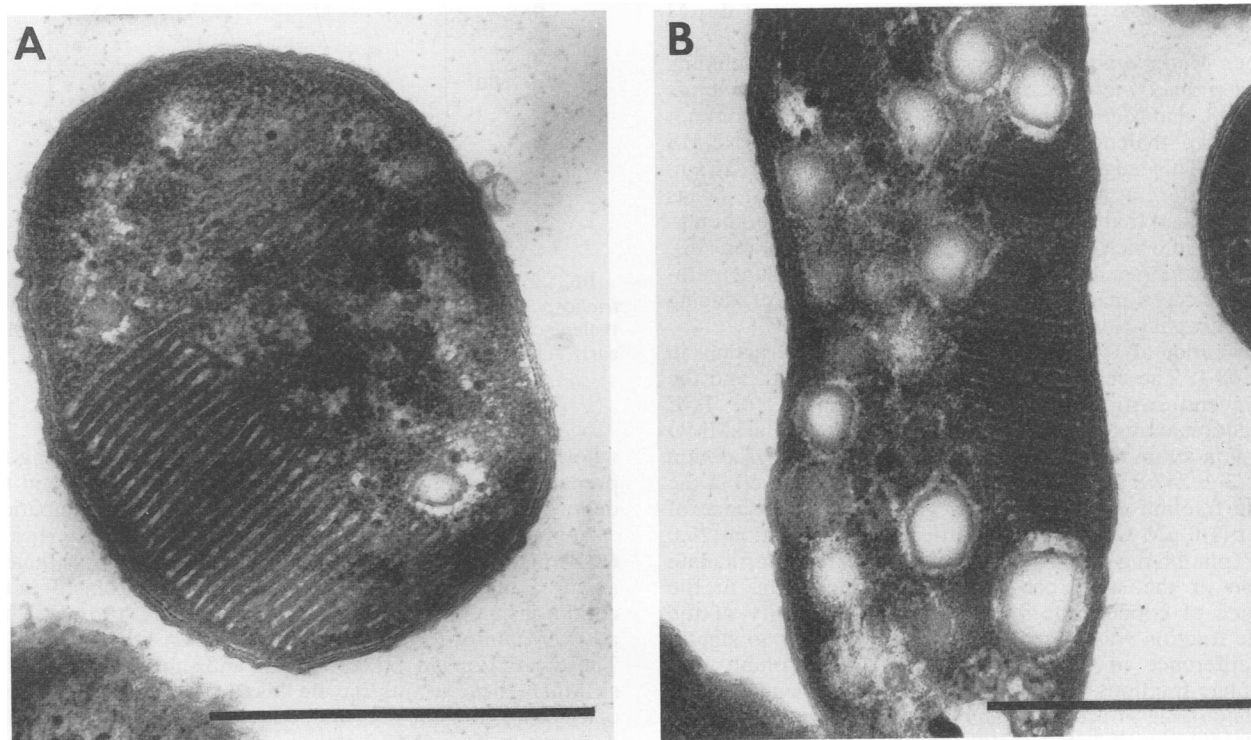


FIG. 1. Electron micrographs of thin sections of strain 68-1 grown in the presence (1.0 μM CuSO_4) (A) and absence (B) of copper. Bars, 1 μm .

tion was evident during copper limitation; however, its arrangement was altered (37). Here the addition of copper ions strongly accelerated the growth of the cells. By contrast, no change was observed in the ICM of *Methylomonas* sp. strain MM2 regardless of copper concentration (18). In this study some cells of 68-1 seemed to lack ICM when they were grown in the absence of copper. The increase in poly- β -hydroxybutyrate storage granules was striking in most cells (Fig. 1B). This has also been observed in *Methylomonas alba* (7) and *Methylococcus capsulatus* (33). No obvious change was observed in the phospholipid fatty acid profile of 68-1 during copper limitation. Similar results were obtained by Guckert et al. (16) with *Methylosinus trichosporium* OB3b.

Naphthalene oxidation by 68-1 whole cells. When grown in copper-free conditions, strain 68-1 readily oxidized naphthalene. Naphthalene oxidation in 68-1 and *Methylosinus trichosporium* OB3b was found to be highest in early stationary growth phase (Fig. 2). The naphthalene oxidation rate of 68-1 was at least two times as high as that of OB3b. In a state of methane limitation, enzyme activity declined. This may be due to reduced electron transfer through sMMO as an NADH conservation mechanism in these organisms (10). The activity of naphthalene oxidation in strain 68-1 was totally repressed when it was grown in the presence of CuSO_4 (1 μM). A similar phenomenon has been observed in *Methylosinus trichosporium* OB3b (5) in which naphthalene oxidation and TCE degradation were directly attributed to the sMMO expression in the absence of copper. This was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis. Significant but low amounts of TCE degradation were observed in *Methylosinus trichosporium* OB3b grown in the presence of 1 μM CuSO_4 (5, 11). In our flask batch culture experiments, no detectable sMMO activity was observed in strain 68-1 whole cells grown in the presence of 1 μM CuSO_4 .

Other *Methylomonas* sp. strains investigated, including *Methylomonas methanica* ATCC 35067^T, *Methylomonas alba* BG8, and *Methylomonas rubra* NCIMB 11913, did not oxidize naphthalene and grew poorly in copper-free NMS medium. This suggests that sMMO activity is a strain-specific feature at least in *Methylomonas methanica*. It has been theorized that sMMO may result from an adaptation to conditions in which there is long-term copper limitation (10). Habitats lacking readily available copper but abundant methane and oxygen may provide a niche for sMMO-producing methanotrophs.

Distribution of TCE degradation activity of cell fractions in strain 68-1. The cells of strain 68-1 were ruptured, and the soluble and particulate fractions were assayed for TCE degradation activity to confirm the localization of the sMMO activity in strain 68-1. Significantly higher TCE degradation (1,092.2 \pm 247.8 pmol/h/mg of protein) was observed in the soluble fraction of the 68-1 cells grown in the absence of copper (1.0 μM CuSO_4), while little TCE degradation (26.0 \pm 3.2 pmol/h/mg of protein) occurred in the particulate fraction of the same cell preparation. However, in the presence of copper, the TCE degradation activity of the soluble fraction was reduced to a point at which no significant difference in the activity between the soluble and particulate fractions was observed: 88.6 \pm 29.8 and 125.4 \pm 54.5 pmol/h/mg of protein, respectively. This indicated that the sMMO was induced in the absence of copper and is responsible for the high rates of TCE degradation. The overall TCE degradation rates were lower than those of

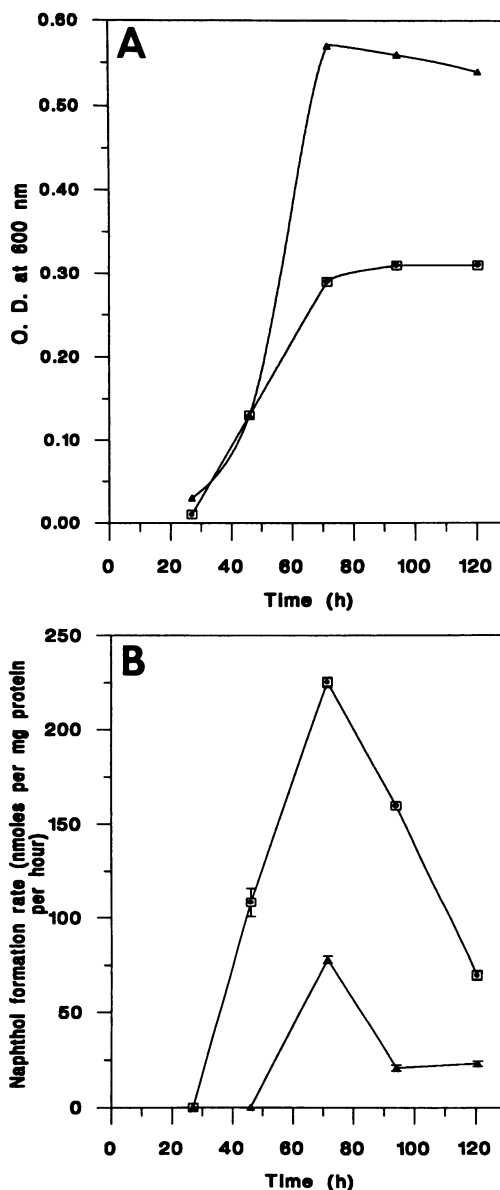


FIG. 2. Growth curves (A) of strain 68-1 (□) and *Methylosinus trichosporium* OB3b (▲) grown under copper-free conditions. Naphthalene oxidation rates (B) of strains 68-1 (□) and OB3b (▲) peak in early stationary growth phase. O.D., optical density.

whole cells because MMO activity loss occurred during the preparation of cell fractions following cell rupture and the degradation rates were determined after 22 h of incubation.

Naphthalene oxidation and TCE degradation kinetics of 68-1 whole cells. Strain 68-1 was able to oxidize naphthalene and TCE more rapidly than OB3b (Fig. 2 and Table 2) in the conditions employed. By contrast, however, strain 68-1 had a lower affinity for these compounds than OB3b (Table 2). These variations in oxidation rates and substrate affinities of sMMO in these strains may be linked to variations in the rate of electron transfer and the active-site structure. The TCE degradation rate of 68-1 shown as V_{\max} (2,325 \pm 260 nmol/h/mg of protein) was higher than the V_{\max} of OB3b (995 \pm 160 nmol/h/mg of protein). This appears to be because 68-1

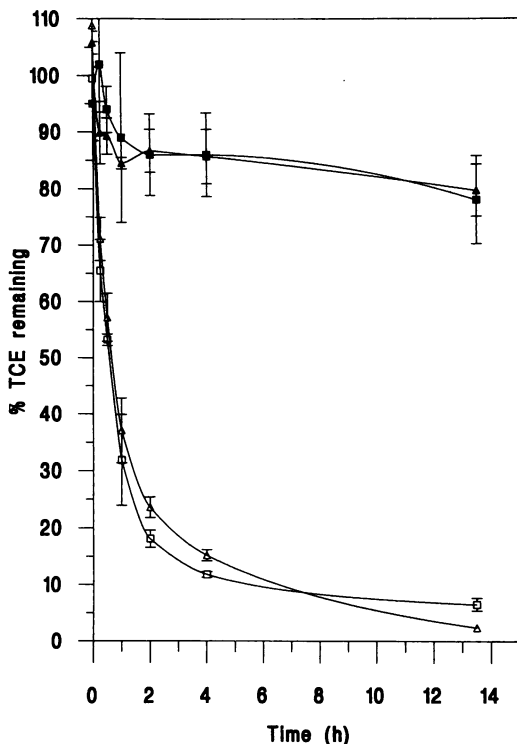


FIG. 3. TCE degradation by viable cell suspensions of 68-1 (\square) and *Methylosinus trichosporium* OB3b (\triangle). Each diluted cell suspension (0.08 to 0.09 mg [dry weight] of cells per ml) of 68-1 and OB3b in the early stationary phase was incubated with 42 μ M TCE and 20 mM sodium formate, and the remaining TCE concentrations were determined at the given time points within 24 h. Heat-killed controls of 68-1 (\blacksquare) and OB3b (\blacktriangle) are also shown.

has higher sMMO protein content in unit total protein than OB3b. However, the higher V_{\max} (290 ± 99 nmol/min/mg of cells) of OB3b was observed in different TCE assay conditions in which initial degradation rates were determined within a short period of time (0 to 7 min) (31).

The time course of TCE degradation is shown in Fig. 3. The remaining TCE concentration in the 68-1 reaction mixture decreased more rapidly than that in OB3b. This appears to be due to the rapid TCE degradation rate of 68-1 as reflected by the higher V_{\max} of TCE degradation by 68-1. In heat-killed controls in both strains, at least about 80% of the initial TCE was maintained and thus extractable. Changes of TCE degradation rates of 68-1 and OB3b are also monitored (data not shown). The TCE degradation of 68-1 decreased to 47% of the initial degradation rate in 1 h, whereas the degradation rate of OB3b decreased to a similar level in 2 h. The rapid decrease in TCE degradation rates of the both strains may be attributed to the substrate depletion and sMMO inactivation. The enzyme inactivation by TCE could be associated with loss of iron from the hydroxylase component of the enzyme (15) or could occur by reaction with hydrolysis products of TCE epoxide (13).

DNA slot blot and Southern blot hybridization by sMMO DNA probe. Though studies have indicated that the amino acid sequence homology of sMMO between type X and II methanotrophs is high (78 to 94%) (6, 26), codon usage variation (26) critically affects gene probe hybridization results. Using conditions in which approximately 30% nucleotide mismatch was accounted for, the *Methylosinus*

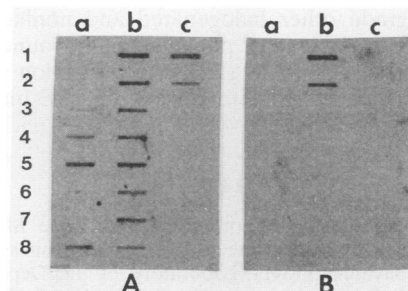


FIG. 4. DNA slot blot hybridization of strain 68-1 and other methyloprophs with an sMMO B-gene probe from *Methylosinus trichosporium* OB3b. Hybridizations with 2 μ g of each genomic DNA were performed at low (A) and high (B) stringency. a1, 68-1; a2, *Methylosinus methanica*; a3, *Methylosinus rubra*; a4, *Methylosinus alba* BG8; a5, *Methylosinus clara*; a6, *Methylosinus agile*; a7, *Methylophilus methyloprophus*; a8, *Methylobacterium rhodesianum*; b1, *Methylosinus trichosporium* OB3b; b2, *Methylosinus sporium* 5; b3, *Methylocystis parvus* OBBP; b4, *Methylobacterium organophilum* XX; b5, *Methylobacterium extorquens* AM1; b6, *Methylococcus capsulatus* Bath; b7, *Methylococcus capsulatus* Texas; b8, *Hyphomicrobium* sp.; c1, *Rhizobium leguminosarum* 248; c2, *A. tumefaciens*; c3, *Pseudomonas putida*; c4, *Bacillus subtilis*.

trichosporium OB3b sMMO gene probe did not hybridize to the homologous genes in *Methylococcus capsulatus* Bath (40). In our experiments employing low-stringency hybridization conditions allowing approximately 40% nucleotide mismatch, this probe nonspecifically bound to a wide range of bacterial species, many not capable of producing either sMMO or pMMO (Fig. 4). When more stringent conditions were applied allowing for 30% nucleotide mismatch, the probe only bound to its strain of origin (OB3b) and to *Methylosinus sporium* ATCC 35069. The probe, however, did not bind to the DNA of 68-1 in either the low- or the high-stringency conditions. Southern blot hybridization performed in the stringent conditions has shown that 2.2- and 1.6-kb *EcoRI* fragments of OB3b and ATCC 35069 hybridized to the sMMO probe but the gene probe did not hybridize to any *EcoRI* restriction fragment of the type I and X methanotrophs and heterotrophs tested (e.g., 68-1, *Methylosinus methanica*, *Methylosinus rubra*, *Methylosinus alba*, *Methylosinus agile*, *Methylococcus capsulatus* Bath, *Methylococcus capsulatus* Texas, *Rhizobium leguminosarum*, and *Agrobacterium tumefaciens*) (data not shown). Further studies will be necessary to determine exactly how the 68-1 sMMO differs from the more well-studied sMMOs of *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath in terms of DNA and protein sequences. This will elucidate the evolutionary diversity of sMMOs in methanotrophs.

Both type I and II methanotrophs, including *Methylosinus* spp. and *Methylosinus* spp., have been isolated from TCE-contaminated aquifers (1, 11, 18, 22). Therefore, type I methanotrophs should also be considered potential organisms in in situ TCE bioremediation. To date, no previous study has reported sMMO production in a type I methanotroph. In this study a typical type I methanotroph, *Methylosinus methanica* 68-1, has the ability to produce sMMO and rapidly degrade TCE, producing a relatively high biomass under the apparent nonoptimal state of copper limitation. The strain may have considerable bioremediation potential as a result. Further studies on this strain will examine its

ability to degrade other halogenated and nonhalogenated aliphatic, heterocyclic, and polycyclic compounds. Strain 68-1 will also be utilized in sMMO optimization studies in bioreactors dedicated to methanotrophic TCE biodegradation.

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