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Characterization of the Methanotrophic Bacterial Community Present in a Trichloroethylene-Contaminated Subsurface Groundwater Site

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Groundwater, contaminated with trichloroethylene (TCE) and tetrachloroethylene (PCE), was collected from 13 monitoring wells at Area M on the U.S. Department of Energy Savannah River Site near Aiken, S.C. Filtered groundwater samples were enriched with methane, leading to the isolation of 25 methanotrophic isolates. The phospholipid fatty acid profiles of all the isolates were dominated by 18:1ω8c (60 to 80%), a signature lipid for group II methanotrophs. Subsequent phenotypic testing showed that most of the strains were members of the genus *Methylosinus* and one isolate was a member of the genus *Methylocystis*. Most of the methanotroph isolates exhibited soluble methane monooxygenase (sMMO) activity. This was presumptively indicated by the naphthalene oxidation assay and confirmed by hybridization with a gene probe encoding the *mmoB* gene and by cell extract assays. TCE was degraded at various rates by most of the sMMO-producing isolates, whereas PCE was not degraded. Savannah River Area M and other groundwaters, pristine and polluted, were found to support sMMO activity when supplemented with nutrients and then inoculated with *Methylosinus trichosporium* OB3b. The maximal sMMO-specific activity obtained in the various groundwaters ranged from 41 to 67% compared with maximal rates obtained in copper-free nitrate mineral salts media. This study partially supports the hypothesis that stimulation of indigenous methanotrophic communities can be efficacious for removal of chlorinated aliphatic hydrocarbons from subsurface sites and that the removal can be mediated by sMMO.

Subsurface microbial communities are metabolically active and capable of conducting diverse biochemical reactions (12). Regardless of the depth, subsurface microorganisms carry out the cycling of carbon, nitrogen, sulfur, manganese, iron, and phosphorus. Although microbial activity does seem to be higher in sandy sediments, it does not decrease with depth or with geological formation (1, 12). Different geological formations are inhabited by different bacterial phenotypes and genotypes (1).

Contamination of subsurface environments with chlorinated hydrocarbons, in particular trichloroethylene (TCE) and tetrachloroethylene (PCE) is a potentially serious threat to drinking-water sources (11). It has been demonstrated that subsurface microbial communities degrade a wide variety of chlorinated hydrocarbons in the laboratory. Additionally it has been shown that methane enrichments of subsurface samples stimulate the in situ degradation of TCE and other chlorinated aliphatic compounds (13).

The enzyme methane monooxygenase (MMO) oxidizes methane to methanol and also can cometabolize many other compounds (8). Both types of MMO, the soluble (sMMO) and the particulate (pMMO) forms, are capable of TCE degradation (9). However, sMMO not only possesses a broader substrate specificity but also can also degrade TCE many times faster than pMMO (30, 39), as well as other monooxygenases and dioxygenases able to carry out TCE cometabolism (11). The biochemical and genetic structure of sMMO has been extensively characterized (8, 27). Several gene probes have been developed for the detection of sMMO genes in environmental samples (25, 37, 40).

The complete mineralization of TCE to CO_2 appears to be carried out most efficiently by the combined action of methanotrophic and heterotrophic microbial communities (41). Since methanotrophic bacteria are relatively ubiquitous in nature (18), they may serve as an instrument in the in situ bioremediation of contaminated sites. The feasibility of in situ bioremediation with methanotrophs has been investigated by Semprini et al. (36) at the Moffet Naval Air Station, Mountain View, Calif., and a similar project has been undertaken at the U.S. Department of Energy Savannah River Site near Aiken, S.C. (19). Between February and April 1992, air was injected into TCE- and PCE-contaminated subsurface sediments at the Area M site, Savannah River Laboratory, by using horizontal-well technology (23). Subsequently (between April and October 1992), air-methane mixtures (100:1 and 25:1) were successively injected into the site with the intention of stimulating methanotrophic TCE degradation (19).

The analysis of methanotrophic population dynamics in response to the air-methane injection series is an ongoing project involving DNA extraction and gene probing of subsurface core materials (21). Initial results suggest that *mmoB* genes, part of the sMMO gene cluster, are extensively distributed throughout the entire site and that following the air injection period, *mmoB* gene frequency increased, with approximately 50% of all core samples demonstrating positive signals for the *mmoB* gene (21). Likewise, studies by Pfiffner et al. (31) have reported a substantial increase in methanotroph populations in the Area M groundwater. In this investigation the goal was to confirm and document the

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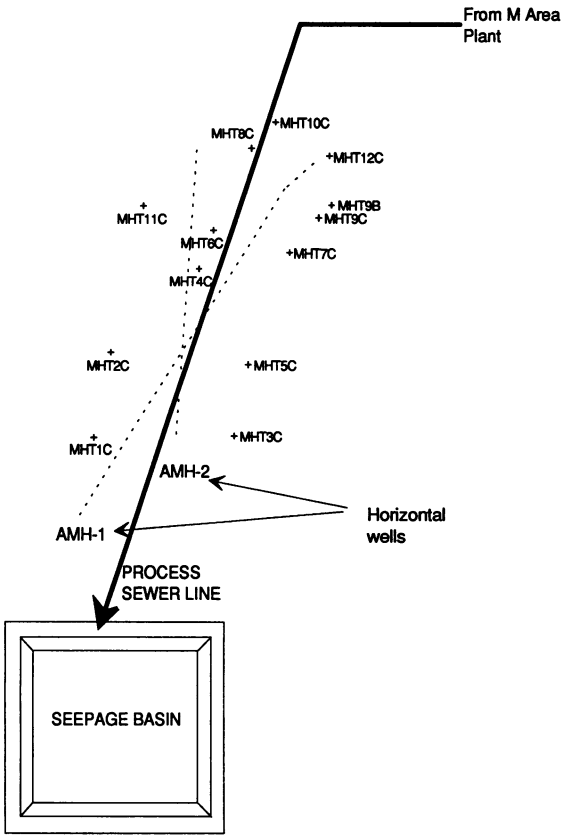

FIG. 1. Location of the Area M groundwater monitoring wells (MHT) at the Savannah River Laboratory. The TCE and PCE groundwater plume originated from the process sewer line carrying chemical wastes to a seepage basin. Dashed lines represent the injection (AMH-1) and extraction (AMH-2) horizontal wells, with the primary area of effect centered on wells MHT-1 to MHT-6.

distribution and characteristics of the group II methanotrophic population in the groundwater at the Area M site prior to the onset of the in situ bioremediation effort. The hypothesis of this investigation is that the groundwater methanotroph community is dominated by sMMO-producing methanotrophs, as suggested by the distributed of sMMO genes in the aquifer sediments. It also is postulated that since sMMO is extensively distributed, it is likely that methanotrophs are able to express sMMO in the groundwater and that groundwater isolates are capable of TCE biodegradation.

MATERIALS AND METHODS

Groundwater samples and chemical analysis. Groundwater samples were collected from 13 monitoring wells at the U.S. Department of Energy Savannah River Site near Aiken, S.C. (Fig. 1). See references 10 and 19 for details of the sampling procedures. Chemical analyses of the Area M groundwater samples (Table 1) were carried out as previously described (10, 19). An artificial groundwater, polluted with various aromatic and chlorinated aliphatic hydrocarbons, was derived from chemical analyses of artesian water at the K-25 facility, Oak Ridge National Laboratories, Oak Ridge, Tenn. The chemical analysis for this groundwater (Table 1) was performed by Steven Herbes, Environmental Sciences Division, Oak Ridge National Laboratories. K-25 samples could not be obtained directly for security reasons. Additionally, a pristine groundwater was studied and was obtained from a 30-m well near Blaine, Tenn. Chemical analysis (Table 1) of the Blaine groundwater was performed by U.S. Environmental Protection Agency standard methods (14).

Isolation and purification of methanotrophic bacteria. Area M groundwater samples (250 ml) were filtered through 0.22-μm-pore-size filters. The filters were placed into 60-ml enrichment vials (Wheaton Inc., Millville, N.J.) containing 10 ml of sterile nitrate mineral salts (NMS) liquid medium. The NMS medium contained 2 mM $NaNO_3$, 2 mM phosphate buffer (pH 6.8), 10 mM $NaHCO_3$, 150 μM $MgSO_4$ ·

TABLE 1. Characteristics of groundwaters studied in this experiment

Groundwater[a]	Sample depth (m)	pH	Temp (°C)	Dissolved O_2 concn (mg/liter)	Organic carbon concn (mg/liter)	Nitrate concn (μM)	Phosphate concn (μM)	Iron concn (μM)	Pollutants (concn, μg/liter)[b]	Methanotrophs (cells/ml)[a]
Area M wells[c,d]										
MHT-1C	41.3	6.3	19	8	<1	15	<1	1	TCE (225), PCE (56)	0
MHT-2C	41.7	5.4	19	9	<1	17	<1	4	TCE (658), PCE (46)	<0.1
MHT-3C	41.2	5.3	19	9	<1	20	<1	6	TCE (459), PCE (69)	0
MHT-4C	42.7	6.7	20	8	<1	17	<1	18	TCE (47), PCE (2)	200
MHT-5C	41.6	5.3	19	11	<1	30	<1	1	TCE (300), PCE (256)	<0.1
MHT-6C	43.3	6.4	20	10	<1	21	<1	6	TCE (160), PCE (210)	0
MHT-7C	42.8	8.4	20	9	<1	15	<1	21	TCE (221), PCE (226)	0
MHT-8C	43.3	6.0	20	8	<1	17	<1	1	TCE (327), PCE (16)	0
MHT-9B	45.5	5.9	19	11	<1	20	<1	3	TCE (479), PCE (270)	<0.1
MHT-9C	43.3	6.5	20	8	<1	14	<1	4	TCE (492), PCE (60)	0
MHT-10C	43.1	6.5	20	8	<1	19	<1	6	TCE (450), PCE (11)	0
MHT-11C	42.3	8.0	20	9	<1	16	<1	7	TCE (40), PCE (2)	ND
K-25	ND[e]	6.6	ND	10	8	3	<1	74	Mixed waste[f]	ND
Blaine	30.0	7.8	ND	ND	10	16	3	29	None	ND

[a] Methanotroph bacterial counts are based on most-probable-number counts determined prior to air injection (31).
[b] TCE and PCE concentrations were determined before air injection.
[c] The ammonia and copper content of the groundwaters was below the analytical detection limit (5 and 0.1 μM, respectively).
[d] No data are available for monitoring well MHT-12C.
[e] ND, not determined.
[f] K-25 groundwater pollutants (in micrograms per liter): naphthalene, 90; 2-methylnaphthalene, 90, -1,1-dichloroethylene, 300; trichloroethylene, 86; 1,1-dichloroethane, 1,300; 1,1,1-trichloroethane, 3,500; benzene, 300; toluene, 600; ethylbenzene, 270; xylene, 250.

$7H_2O$, 50 μM ferric EDTA, 50 μM $CaCl_2 \cdot 2H_2O$, 4 μM $ZnSO_4 \cdot 7H_2O$, 4 μM $MnSO_4 \cdot 7H_2O$, 1 μM $CuSO_4 \cdot 6H_2O$, 1 μM KI, 1 μM K_2SO_4, 1 μM H_3BO_3, 0.65 μM $CoCl_2 \cdot 6H_2O$, and 0.4 μM $Na_2MoO_4 \cdot 2H_2O$. The vials were sealed with butyl rubber stoppers and aluminum caps, and the headspace (50 ml) within the vials was altered to create either a 1:1 or a 1:19 methane-air atmosphere. The vials were then incubated at 25, 28, or 37°C for up to 21 days. Liquid samples from the enrichment vials were spread plated onto NMS medium solidified by the addition of 1.5% (wt/vol) agarose type V (Sigma Chemical Co., St Louis, Mo.) and incubated under a 1:4 methane-air atmosphere for 7 days at the appropriate enrichment temperature. Colonies appearing on the spread plates were then transferred to NMS liquid medium in 60-ml serum vials and reincubated for 7 days. Contents of vials showing turbidity were again spread plated onto agarose-solidified NMS medium. Subsequent transfers were performed by 16-streak dilution. The purity of the methanotrophic cultures was ascertained by microscopy and by plating onto nutrient agar (CM26; Difco Laboratories, Detroit, Mich.) and NMS agar containing 0.3% (vol/vol) methanol incubated with and without methane (45). Pure cultures were stored on NMS agar supplemented with 0.25% (wt/vol) activated charcoal.

Strain characterization. The Gram stain reaction and the correlative tests, i.e., aminopeptidase production and lysis in 10% (wt/vol) sodium dodecyl sulfate (SDS) and 3% (wt/vol) KOH (15), were determined with 3-day-old cultures. Microcolonies on 24-h NMS Noble agar (no. 0142; Difco) plates were observed by oil immersion phase-contrast microscopy. Rosette and exospore formation was verified by this procedure. Growth from 14-day NMS cultures was stained with the Ziehl-Neelsen stain (15) to detect *Methylocystis* lipid cysts (46). The presence of exospores was ascertained by the viability of strains after suspensions of 7-day NMS cultures were heated at 80°C for 30 min on a heating block.

The lipids of the isolates were quantitatively extracted by the modified one-phase chloroform-methanol method of Bligh and Dyer (44). After phase separation, the lipids were recovered in the chloroform layer. Total lipid was fractionated into neutral lipid, glycolipid, and phospholipid with activated silicic acid (32). The fatty acids in the phospholipid-containing fraction were transesterified to the corresponding fatty acid methyl esters by mild alkaline methanolysis (44). Gas-chromatographic analyses were performed with a no. 5890 gas chromatograph equipped with a cross-linked methyl silicone fused-silica capillary column (50 m by 0.32 mm [inner diameter]) (Hewlett Packard, Denver, Colo.), a flame ionization detector, and a split/splitless injector under the conditions described by Ringelberg et al. (32). The identity of the phospholipid fatty acids (PLFA) was confirmed by gas chromatography-mass spectrometry (32). The fatty acid double-bond position and geometry were confirmed by gas chromatography-mass spectrometry analysis of the dimethydisulfide adducts of monounsaturated PLFA (29).

Nitrogen fixation was determined by the acetylene reduction assay. The strains were grown in 10 ml of nitrogen-free NMS medium in 60-ml serum vials to the late logarithmic growth phase. To the vials were added 2 ml of acetylene and 50 μl of 1 M sodium formate, and the vials were incubated for 2 h. Headspace samples were analyzed for ethylene by using a Shimadzu GC9-AM chromatogram equipped with a flame ionization detector and an AT-1 (1.2-μm) capillary column (15 m by 0.53 mm [inner diameter]) (Alltech, Deerfield, Ill.) maintained at 60°C with nitrogen as the carrier gas (flow rate, 1 ml/min).

sMMO assays. A modified version of the naphthalene oxidation assay (22) was used to determine the specific activity of sMMO. The methanotroph isolates were grown in copper-free NMS medium in 60-ml serum vials. The cultures were then diluted to an A_{600} of 0.2 with NMS medium. The diluted cultures were transferred in 1-ml aliquots to 10-ml screw-cap test tubes, and 1 ml of saturated naphthalene solution (234 μM at 25°C [42]) was added to each tube. The samples were prepared in triplicate. The reaction mixtures were incubated at 200 rpm on a rotary shaker at 25°C for 1 h. Controls for these assays included heat-killed cultures as well as cultures grown in the presence of 1 μM $CuSO_4 \cdot 6H_2O$, which completely represses sMMO synthesis (30, 39). After incubation, 100 μl of freshly prepared 0.2% (wt/vol) tetraotized *o*-dianisidine solution was added to each tube, and the formation of diazo dye complexes was immediately monitored by recording the A_{525} by spectrophotometry. The intensity of diazo dye formation was proportional to the naphthol concentration. Wackett and Gibson (43) have previously determined the molar extinction coefficient (ε) of the naphthol diazo dye to be 38,000 M^{-1} cm^{-1}. The protein content of cell suspensions was determined by the microbiuret procedure (26). The specific activity of sMMO was then expressed as nanomoles of naphthol formed per milligram of cell protein per hour.

sMMO activity (or lack of it) was confirmed by using cell extracts fractionated by ultracentrifugation. Isolates were grown in the absence of $CuSO_4$ and in the presence of 5 μM $CuSO_4$ and then centrifuged at 10,000 × *g* for 10 min. The pellet was resuspended in 10 ml of 25 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0) containing 1 mM thioglycolate (28). Cell extracts were then obtained by sonication (three 30-s bursts at maximum intensity with 1 min of cooling on ice between pulses; VirSonic 300; VirTis Instruments, Gardiner, N.Y.) followed by centrifugation at 12,500 × *g* for 10 min at 4°C. The supernatant was then centrifuged at 150,000 × *g* for 90 min. The resultant supernatant represented the sMMO-containing soluble fraction. The pellet representing the pMMO-containing particulate fraction was discarded. The sMMO activity in the soluble fraction was assayed by using the TCE degradation assay (see below).

TCE and PCE degradation analyses. Diluted suspensions of each isolate (A_{600}, 0.2) were prepared and transferred in 1-ml aliquots to Teflon-lined screw-cap septum vials (14 ml; Pierce, Rockford, Ill.). Heat-killed control samples were prepared for each strain studied. TCE degradation was initiated by adding TCE (saturated aqueous stock solution, 8.36 mM at 25°C [42]) or PCE (saturated aqueous stock solution, 0.91 mM at 25°C [42]) to each vial to a final concentration of 10 μM. The vials were then incubated for 5 to 15 min at 25°C with shaking at 200 rpm. The reactions were terminated by the addition of 2 ml of *n*-hexane containing 1,2-dibromoethane (1 mg/liter) as an internal control. Undegraded TCE or PCE was extracted from the organic phase by shaking and centrifugation (3,000 × *g* for 20 min). The TCE and PCE were quantified by using a GC-9AM gas chromatogram (Shimadzu Analytical Instruments Co., Kyoto, Japan) equipped with a split injection port run at 220°C, an R_{TX}-volatiles capillary column (30 m by 0.53 mm [inner diameter]) (Restek Corp., Bellefonte, Pa.) run isothermically at 120°C, and an electron capture detector run at 220°C. Nitrogen was used as the carrier gas (flow rate, 10 ml/min). The peak area were integrated with a Shimadzu C-R6A

Chromatapac. Under the conditions used, TCE, PCE, and 1,2-dibromoethane exhibited retention times of 2.8, 3.9, and 4.2 min, respectively.

[^{14}C]TCE mineralization analyses. Diluted suspensions of each isolate were prepared as described above. Acid-killed control samples were prepared for each strain studied. [^{14}C]TCE mineralization was initiated by the addition of [^{14}C]TCE (to obtain 250,000 dpm) to each vial. Reactions were incubated for 24 h at 25°C. Mineralization was determined by measuring the evolution of $^{14}CO_2$ from [^{14}C]TCE. After incubation, the samples were acidified with 2 M H_2SO_4 to release $^{14}CO_2$ from the assay medium. Radioactive CO_2 was trapped in 0.5 ml of 0.4 M NaOH. The NaOH was added to 10 ml of Ready Safe liquid scintillation counter fluid (Beckman Instruments, Inc., Fullerton, Calif.), and $^{14}CO_2$ levels were determined with a Beckman LS3801 liquid scintillation counter.

Gene probe analyses. Chromosomal DNA was extracted from each individual methanotrophic isolate by a modification of the Marmur procedure as described by Bowman et al. (4). DNA (6 μg) was immobilized on nylon filters as previously described (33). The filters were then baked at 80°C for 1 h and stored at room temperature until needed.

Primers for the amplification of a 0.4-kb internal fragment of the *mmoB* gene from the sMMO gene cluster of *Methylosinus trichosporium* OB3b (ATCC 35070) (5) consisted of chemically synthesized 18-base oligomers, sense 5′-ATGTC CAGCGCTCATAAC-3′ and antisense 5′-TCAGATGTCG GTCAGGGC-3′ (Genosys, The Woodlawns, Tex.). A single-stranded *mmoB* gene probe was generated by asymmetric amplification with *Taq* polymerase (33) and [α-^{32}P]dCTP (ICN Radiochemicals, Irvine, Calif.). A 2.5-kb *Sma*I fragment equivalent to *moxF*, coding for the methanol dehydrogenase large subunit of *Methylobacterium organophilum* XX (ATCC 27886) (24), was labeled with [α-^{32}P]dCTP by random priming (Stratagene, La Jolla, Calif.). Plasmids pJC286 (7), containing *nifHDK* genes of *Klebsiella pneumoniae*, and pJP99, containing an *Xba*I-*Bal*I fragment of the *dhlA* gene, encoding the haloalkane dehalogenase of *Xanthobacter autotrophicus* GJ10 (ATCC 43050) (20), were labeled with [α-^{32}P]dCTP by nick translation (33). Unincorporated nucleotides were removed by using a commercially available size inclusion column (Stratagene). DNA hybridization was carried out as described by Sayler et al. (35). The hybridization solution consisted of 7% SDS, 1 mM EDTA, and 0.5 M Na_2HPO_4 (6). Hybridization and washing of filters were carried out at 65°C.

Groundwater sMMO activity experiment. Area M (12 wells), K-25, and Blaine groundwaters (Table 1) were amended with the various constituents of NMS medium and sterilized by autoclaving. The groundwater samples (in 60-ml serum vials) were then inoculated with 10^5 *Methylosinus trichosporium* OB3b cells and incubated under methane-air (1:4) at 25°C. The sMMO specific activity was regularly measured by the naphthalene oxidation assay as the cultures grew. In addition, NMS medium containing 0, 0.25, and 1.0 μM $CuSO_4 \cdot 6H_2O$ was inoculated and assayed in the same way as the groundwater samples.

RESULTS

Isolation and characterization of methanotrophic bacteria. Enrichment cultures of the Area M groundwater with 50% methane showed visible turbidity within 10 days. Twenty-five different methanotrophic bacterial isolates were obtained from the methane enrichments established from 11 of the 13 groundwater well samples (Table 2). The selection of isolates was based on differences in colony morphology as they appeared on the initial plating of positive enrichments. Following purification, microscopic observation of individual isolates showed that most, 24 in all, were motile, straight, or slightly curved gram-negative rods, which formed a small bud at the nonflagellated end and often occurred in rosette-like formations. Isolate 9BB, however, was a nonmotile, short, curved rod which did not bud and produced large, cyst-like inclusions which were acid-alcohol fast according to the Ziehl-Neelsen staining procedure. Since the budding isolates remained viable after heat treatment at 80°C for 30 min, whereas isolate 9BB did not, it appears likely that most of the isolates were forming exospores typical of the genus *Methylosinus* (46), whereas strain 9BB appeared to be a typical example of a member of the genus *Methylocystis*. The phenotypic identification of the strains was confirmed by PLFA analysis. The isolate PLFA profiles were very similar, with the predominating PLFA being 18:1ω8c (60 to 80%). The minor PLFA components (content exceeding 1%) were also present at relatively consistent levels among the isolates and included 16:1ω7c (1 to 11%), 16:0 (1 to 3%), and 18:1ω7c (15 to 35%). This PLFA profile is essentially unique to group II methanotrophs (3, 17). All the strains were found to actively fix nitrogen and grew abundantly in nitrogen-free NMS liquid medium (Table 2). Nitrogen fixation is known to be carried out by all group II methanotrophs tested so far (27, 45).

sMMO activity, TCE and PCE degradation, and [^{14}C]TCE mineralization studies. The oxidation of naphthalene to naphthol and TCE degradation activity was used to ascertain the presence of putative sMMO activity in the subsurface methanotrophic isolates. Most of the isolates were capable of oxidizing naphthalene to naphthol and degrading TCE at various rates. No strain was able to appreciably degrade or transform PCE even after 24 h of incubation. Only isolates 3CA and 8CB showed no detectable naphthol formation. Additionally, these strains did not degrade or mineralize TCE (Table 2). When grown in the absence and presence of copper, certain strains, including 1CA, 2CA, 2CB, 5CA, 5CB, and 9CB, exhibited a detectable but relatively low naphthalene oxidation rate (<1 to 12 nmol/h/mg of protein) and an undetectable TCE degradative capacity (Table 2).

The remaining groundwater isolates, when grown under copper-free conditions, possessed significant naphthalene oxidation and TCE degradation rates (Table 2). These strains also mineralized TCE to the greatest extent, with <1 to 4.6% of the added [^{14}C]TCE being converted to $^{14}CO_2$ (Table 2). When these strains were grown in the presence of 1 μM $CuSO_4$, naphthalene oxidation and TCE degradation rates were negligible. sMMO activity was confirmed in these strains by assaying the soluble fractions of cells grown in the presence and absence of copper. TCE degradation was undetectable in the soluble fractions obtained from copper-grown cultures. Significant TCE degradation was, however, detected in soluble fractions from the copper-free cultures.

Gene probe analysis. Four different gene probes were hybridized with chromosomal DNA isolated from the subsurface methanotrophic isolates as well as from *Methylosinus trichosporium* OB3b. High-stringency hybridization conditions which allowed for about 30 to 35% nucleotide mismatch were used for all the probes.

A 0.4-kb single-stranded gene probe containing the *mmoB* gene of the sMMO gene cluster was used to determine whether any homologous genes of the sMMO gene cluster were present in the methanotrophic isolates. We found that

TABLE 2. Biochemical and genetic attributes of the Area M groundwater methanotroph isolates[a]

Area M well	Isolate[b]	Naphthalene oxidation (nmol/h/mg of protein)	TCE degradation (nmol/min/mg of protein)	[^{14}C]TCE to $^{14}CO_2$ (% mineralization)	*mmoB* present	*moxF* present	Nitrogen fixation	
							nifHDK	Acetylene reduction
MHT-1C	1CA	<1	ND[c]	ND	–[d]	–	–	+
MHT-2C	2CA	3 ± 1	ND	ND	–	–	–	+
	2CB	6 ± 2	ND	ND	+	–	–	+
	2CC	307 ± 13	9 ± 1	2.9	+	–	–	+
	2CD	328 ± 22	18 ± 4	2.8	+	–	–	+
MHT-3C	3CA	ND	ND	ND	–	–	–	+
MHT-4C	4CA	247 ± 14	23 ± 4	4.2	+	–	–	+
	4CB	217 ± 3	19 ± 2	4.4	+	–	–	+
	4CC	69 ± 5	2 ± 1	<1.0	+	–	–	+
MHT-5C	5CA	8 ± 2	ND	ND	–	–	–	+
	5CB	6 ± 1	ND	ND	–	–	–	+
	5CC	536 ± 14	30 ± 1	4.0	+	–	–	+
	5CD	678 ± 14	30 ± 2	3.0	+	–	–	+
MHT-6C	6CA	594 ± 20	41 ± 5	4.6	+	–	–	+
	6CB	422 ± 19	20 ± 3	3.9	+	–	–	+
MHT-7C	7CA	256 ± 30	11 ± 2	3.9	+	–	–	+
	7CB	434 ± 25	29 ± 1	4.0	+	–	–	+
MHT-8C	8CA	173 ± 16	6 ± 1	1.5	+	–	–	+
	8CB	ND	ND	ND	–	–	–	+
MHT-9B	9BA	137 ± 8	9 ± 1	2.8	–	–	–	+
	9BB	48 ± 6	0.9 ± 0.3	<1.0	–	–	–	+
	9BSPa	37 ± 4	<0.1	<1.0	+	+	–	+
MHT-9C	9CA	41 ± 1	0.5 ± 0.1	1.2	–	+	–	+
	9CB	12 ± 2	ND	ND	+	+	–	+
MHT-10C	10CA	21 ± 3	0.8 ± 0.3	<1.0	+	+	–	+
M. trichosporium OB3b		315 ± 7	21 ± 2	3.3	+	–	+	+

[a] The *dhlA* gene probe did not hybridize to any of the isolates or to *Methylosinus trichosporium* OB3b.
[b] No isolates were obtained from monitoring wells MHT-11C and MHT-12C.
[c] ND, not detectable.
[d] –, no hybridization.

64% of the isolates showed *mmoB* homologs (Table 2). The *mmoB* gene probe hybridized to many of the strains exhibiting putative sMMO activity. In contrast, isolates 9BA and 9BSPa and *Methylocystis* strain 9BB did not hybridize with the *mmoB* probe but possessed sMMO activity as suggested by the enzyme assay results (Table 2). In addition, strain 2CB, although lacking directly identifiable sMMO activity, hybridized to the *mmoB* gene probe. Other strains, including 1CA, 2CA, 5CA, 5CB, and 9CB, which, like 2CB, showed weak naphthalene oxidation activity, did not hybridize with the *mmoB* gene probe (Table 2).

It has been shown previously that the *moxF* gene from *Methylobacterium organophilum* XX hybridized with various methanotrophs and methylotrophs under conditions allowing 45% nucleotide mismatch (38). We decided to find whether *moxF* was also conserved among the group II methanotrophs isolated in this study. Under conditions allowing 35% mismatch, only 4 of the 25 isolates, strains 9BSPa, 9CA, 9CB, and 10CA, hybridized with the *moxF* gene probe. The presence of *nifHDK* genes was also ascertained by probing with plasmid pJC286 (7). *Methylosinus trichosporium* OB3b was found to strongly hybridize with the *nif* genes on pJC286; however, none of the isolates hybridized with this probe, even though the acetylene reduction technique confirmed that all the strains isolated were capable of fixing nitrogen. Additionally, no homologs of the *dhlA* gene of *X. autotrophicus* GJ10 were detected in any of the isolates. This gene encodes a part of the enzyme haloalkane dehalogenase, which catalyzes hydrolytic dehalogenation of various chlorinated aliphatic hydrocarbons such as 1,2-dichloroethane (20).

sMMO activity in groundwater. By using the naphthalene oxidation assay, it was possible to detect significant sMMO specific activity in *Methylosinus trichosporium* OB3b in the amended groundwaters. The OB3b specific growth rate (0.02 to 0.11 h^{-1}) and sMMO specific activity (105 to 243 nmol/h/mg of protein) varied considerably in the amended Area M groundwater samples. The lower values were found in the more acidic and alkaline samples from wells MHT-2C, MHT-3C, MHT-5C (pH 5.3 to 5.4), MHT-7C, and MHT-11C (pH 8.0 to 8.4). Figure 2 shows the combined averaged sMMO specific activity of OB3b grown in the Area M

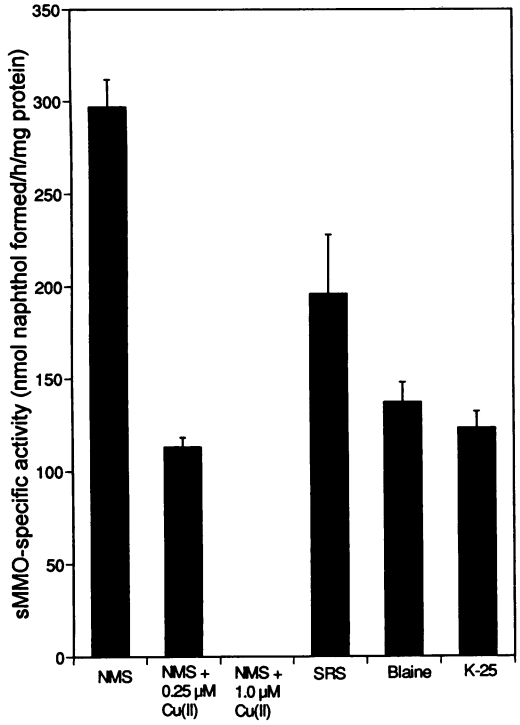

FIG. 2. Level of sMMO specific activity of *Methylosinus trichosporium* OB3b when cultivated in various polluted and pristine groundwaters. The value for Area M (SRS) groundwater is the averaged result of samples drawn from 12 monitoring wells.

groundwaters. OB3b exhibited low growth rates but significant sMMO activity in the relatively alkaline Blaine groundwater (0.03 h^{-1}; 123 nmol/h/mg of protein) and in the heavily polluted K-25 groundwater (0.02 h^{-1}; 136 nmol/h/mg of protein) (Fig. 2).

DISCUSSION

The U.S. Department of Energy Savannah River site includes a region designated Area M, which has become extensively polluted with TCE and PCE as a result of leaking process sewer lines (Fig. 1) (19). Since the pollutant plume was oriented linearly in the vadose and saturated zones, it was possible to initially use in situ air stripping with horizontal wells (23). However, significant levels of TCE and PCE still remain in the groundwater and sediments. An in situ bioremediation project involving a series of injections of air-methane mixtures into the subsurface sediments, stimulating the indigenous methanotrophic community and hopefully leading to TCE degradation, is under way (19). Initial gene probe results from sediment core samples (21) and this study demonstrate that viable populations of methanotrophs able to produce sMMO and degrade TCE are extensively distributed throughout the Area M aquifer and also suggest that sMMO can be expressed in the Area M groundwater and in other groundwaters.

Methanotroph community structure of the Area M aquifer. Relatively little information exists on methanotroph ecology, although methanotrophs are thought to be ubiquitous in most aerobic terrestrial habitats (18). The best information on methanotrophs in natural habitats is found for lake systems. Various studies suggest that in oligotrophic lakes group II methanotrophs tend to dominate the methanotroph community whereas in eutrophic lake metaliminions, where methanotroph populations concentrate, group I methanotrophs dominate (18, 34). The present study indicates that this also seems to hold true for the rather oligotrophic groundwaters of Area M. The methanotrophs we isolated were all group II methanotrophs, which all possessed the signature lipid 18:1ω8c (3, 17). Although the presence of methanotrophs from Area M has been shown previously (13), the cultures obtained were never characterized and were simply complex mixtures or consortia which exhibited TCE degradative activity. Since only group II methanotroph isolates were obtained in the present study, there was the suggestion that the conditions used during the enrichments could be biased to isolation of group II but not group I methanotrophs. The batch culture procedure used for isolation tends to favor the dominant methanotroph in a given habitat (45). The incubation temperature has been used as the primary means of selecting a wide variety of methanotroph species from environmental samples (4). In the present study, enrichments of groundwater performed at 37°C, which tends to favor moderately thermophilic *Methylococcus* species, were all negative. There is no known specific culture procedure selective for only group II or only group I methanotrophs. Recently, results of bioreactor studies have suggested that group I methanotrophs can outcompete group II methanotrophs when methane levels approach limitation (16). In the present study, enrichments were also performed with only a 5% methane headspace; however, growth failed to develop in any of these enrichments, possibly because of the inherently low methanotroph population in the groundwater samples (Table 1). Different nitrogen sources likewise do not appear to favor methanotroph groups specifically; however, they may be species selective. Hypothetically, nitrogen fixation could be used as a useful selective trait for group II methanotrophs, since relatively few group I methanotrophs possess nitrogenase (18). It therefore appears that group II methanotrophs are the dominant type of methanotroph present in Area M groundwater and that if there are any group I methanotrophs, they are present in only extremely small numbers. It is possible that after the injection of methane into the site, group I methanotrophs will become isolatable. This may be significant, since recent evidence has been obtained that sMMO activity is not limited to group II methanotrophs but has been observed in certain strains of the group I methanotroph *Methylomonas methanica* isolated from K-25 artesian water samples (22).

sMMO activity of the methanotroph isolates. In the present study we found that many of the isolates obtained from Area M groundwater, identified as mostly *Methylosinus* species, have a high TCE degradative capacity. The naphthalene oxidation assay represents an effective presumptive test for sMMO activity and correlates quite well with the TCE degradation rate (2). In the presence of 1 μM $CuSO_4$, naphthalene oxidation was completely absent in all the strains examined here. In the absence of copper, the rates of naphthalene oxidation varied among the strains. The addition of an artificial electron donor for sMMO, 10 mM sodium formate, did not alter this variation among the strains. The variations could be due to differences in sMMO naphthalene K_m values among the various strains. Koh et al. (22) have shown that the sMMO K_m values for naphthalene in *Methylosinus trichosporium* OB3b and in *Methylomonas methanica* 68-1 differ, being 40 and 67 μM, respectively. The variation in naphthalene oxidation rates was also reflected in TCE degradation rates. Several strains (5CC, 5CD, and

6CA) showed a higher specific TCE degradation rate than did *Methylosinus trichosporium* OB3b. The sMMO in these strains possibly makes up a higher proportion of the whole cell protein. TCE degradation rates in these strains were also higher than that in *Methylosinus trichosporium* OB3b and other strains with comparable sMMO activity levels. Overall, a significant capacity for TCE degradation was found for methanotrophs isolated from 9 of the 13 monitoring wells at Area M (Fig. 1). Therefore it is apparent that methanotrophs with sMMO activity are extensively distributed throughout the water table of the groundwater aquifer. The methanotrophs possibly originate from sediment near the surface, which supports higher microbial populations (10), and have been washed down by rainfall trickling into and through the aquifer sediments.

Gene probe analysis. The *mmoB* gene was selected as the probe for sMMO genes since it represented one of the more relatively conserved regions of the sMMO gene cluster. The *mmoB* genes of *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath have approximately 67% nucleotide homology (27). Recently, McGowan et al. (25) performed polymerase chain reaction amplification of *mmoB* genes from several methanotroph pure cultures as well as from DNA extracted from filtered seawater. In the present study the *mmoB* gene hybridized to most but not all of the apparently sMMO-producing isolates. The absence of sMMO was confirmed in this strain after the soluble fraction was assayed directly for TCE degradative activity. Certain strains did not hybridize with *mmoB* but possessed significant naphthalene oxidation and TCE degradation activity. The presence of sMMO in these strains was confirmed by directly assaying their soluble extracts (Table 2). A possible reason for this is that these strains may contain *mmoB* genes with significant differences in nucleotide sequence. Recently, Nakajima et al. (28) described a 35- to 40-kDa regulatory protein that made up part of the sMMO protein complex of *Methylocystis* sp. strain M. The *mmoB* gene in *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath, encoding the regulatory B protein of sMMO, is about 15 to 16 kDa (27). These differences in the molecular mass of the regulatory protein suggest that other variations may be possible among other methanotrophs producing sMMO.

None of the groundwater isolates were found to hybridize to the *K. pneumoniae nifHDK* on pJC286. Only the control strain, *Methylosinus trichosporium* OB3b, was found to hybridize these genes under the relatively stringent conditions used here. Under relatively low-stringency conditions, most but not all group II methanotrophs hybridize to the *nif* gene cluster; however, all known nitrogen-fixing methanotrophs including *Methylococcus capsulatus* Bath and the non-nitrogen fixer *Methylomonas methanica* hybridized to the *nifH* gene (27).

Potential for in situ bioremediation of Area M by methanotrophs. sMMO activity with correspondingly significant TCE degradation rates was observed among a high proportion of the indigenous methanotroph population from the Area M groundwater. The methanotroph community appears totally dominated by group II methanotrophs, which seem well adapted to oligotrophic conditions. In addition, it appears that these organisms are able to express sMMO in the Area M groundwater since copper levels appear low enough to prevent induction of pMMO. This supports the results of gene probe analyses of Area M sediments in which *mmoB* homologs were found to be distributed extensively, albeit without any pattern, throughout the site (21). Recent evidence shows that methanotroph populations have actually increased in the groundwater by at least 4 orders of magnitude following the methane injection series (31). Likewise, Jiménez et al. (21) report that an increase in the frequency of *mmoB* homologs has occurred in sediment samples taken throughout a depth profile following the air injection; this increase is from a frequency of 16% ± 10% to 47% ± 14%. Overall, this suggests that methane pulses have the potential for stimulation of in situ bioremediation in the Area M TCE-polluted groundwaters by allowing native sMMO-producing methanotroph populations to increase. However, it is still unknown whether sMMO is expressed in the sediments effectively enough for in situ bioremediation. A study is now in progress in which antisense *mmoB* probes will be used to detect and quantify *mmoB* transcripts in the sediment material.

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