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Degradation of Chlorinated Aliphatic Hydrocarbons by *Methylosinus trichosporium* OB3b Expressing Soluble Methane Monooxygenase

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Degradation of trichloroethylene (TCE) by the methanotrophic bacterium *Methylosinus trichosporium* OB3b was studied by using cells grown in continuous culture. TCE degradation was a strictly cometabolic process, requiring the presence of a cosubstrate, preferably formate, and oxygen. *M. trichosporium* OB3b cells degraded TCE only when grown under copper limitation and when the soluble methane monooxygenase was derepressed. During TCE degradation, nearly total dechlorination occurred, as indicated by the production of inorganic chloride, and only traces of 2,2,2-trichloroethanol and trichloroacetaldehyde were produced. TCE degradation proceeded according to first-order kinetics from 0.1 to 0.0002 mM TCE with a rate constant of 2.14 ml min^{-1} mg of $cells^{-1}$. TCE concentrations above 0.2 mM inhibited degradation in cell suspensions of 0.42 mg of cells ml^{-1}. Other chlorinated aliphatics were also degraded by *M. trichosporium* OB3b. Dichloromethane, chloroform, 1,1-dichloroethane, and 1,2-dichloroethane were completely degraded, with the release of stoichiometric amounts of chloride. *trans*-1,2-Dichloroethylene, *cis*-1,2-dichloroethylene, and 1,2-dichloropropane were completely converted, but not all the chloride was released because of the formation of chlorinated intermediates, e.g., *trans*-2,3-dichlorooxirane, *cis*-2,3-dichlorooxirane, and 2,3-dichloropropanol, respectively. 1,1,1-Trichloroethane, 1,1-dichloroethylene, and 1,3-dichloropropylene were incompletely converted, and the first compound yielded 2,2,2-trichloroethanol as a chlorinated intermediate. The two perchlorinated compounds tested, carbon tetrachloride and tetrachloroethylene, were not converted.

Chlorinated aliphatic hydrocarbons such as trichloroethylene (TCE), tetrachloroethylene (PCE), 1,1,1-trichloroethane, and chloroform are frequent constituents of industrial wastewaters. These suspected carcinogens are resistant to biodegradation in aerobic subsurface environments, and this contributes to their persistence in polluted groundwaters (8). Evidence for anaerobic biodegradation of TCE and PCE in the laboratory has been reported (1, 19, 20), but these transformations are often incomplete and occasionally result in the accumulation of equally harmful metabolites, such as dichloroethylenes and vinyl chloride.

Recent research has shown that mixed cultures of methane-utilizing bacteria can cometabolically degrade low concentrations (1 to 5 μM) of chloroform (18) and TCE (5, 23). A pure culture of a methane-oxidizing bacterium that degrades TCE was isolated from TCE-contaminated aquifer material by Little et al. (12). Methanotrophs are considered to be capable of performing oxidative conversions of halogenated aliphatic hydrocarbons as a result of the broad substrate range of their methane monooxygenases (MMOs) (2, 3, 7, 17). Similar cooxidations can be carried out by some bacteria that oxidize aromatic compounds, as indicated by the work of Nelson et al. (14), who reported the isolation of an aerobic bacterium capable of oxidizing TCE to CO_2 when the organism was simultaneously exposed to phenol. Wackett and Gibson (21) have demonstrated bacterial degradation of TCE induced by toluene.

The above experiments were performed with cultures isolated from polluted environments. Since it is unclear what selective advantage the ability to degrade TCE would have for microorganisms, it could well be that these activities are also exhibited by bacteria obtained from samples with no history of exposure to chlorinated compounds and that the ability to degrade TCE is caused only by the lack of substrate specificity of the particular oxidative enzyme.

In methanotrophic bacteria, at least two classes of MMO can be distinguished on the basis of their intracellular localization (4). All methanotrophs tested are able to form a particulate or membrane-bound enzyme, whereas some cultures, such as *Methylococcus capsulatus* and *Methylosinus trichosporium* OB3b (2, 4, 15), are capable of producing a soluble type of MMO (sMMO) with a broader substrate range when copper becomes limiting. It is not known which type of MMO would be favorable for TCE degradation, although TCE was not degraded by two strains of *Methylomonas methanica* that produce particulate MMO (pMMO) (4) and can degrade *trans*-1,2-dichloroethylene (9). There is also little information about kinetic aspects, such as degradation rates, toxicity, and percent dehalogenation of these contaminants.

We previously described the degradation of *trans*-1,2-dichloroethylene by a mixed methanotrophic culture and by pure cultures of *Methylomonas methanica* and *M. trichosporium* OB3b (9). The purpose of the work presented here was to determine whether a well-characterized methanotroph is able to cometabolically degrade TCE and other chlorinated aliphatic compounds and whether this ability is related to a specific type of MMO. The results show that *M. trichosporium* OB3b is capable of TCE biodegradation when cultivated under conditions that allow the formation of its soluble type of MMO and that, apart from perchloro compounds, one- and two-carbon chlorinated hydrocarbons are converted by this system.

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MATERIALS AND METHODS

Organism and growth conditions. *M. trichosporium* OB3b (NCIB 11131) was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom. The organism was grown in batch culture in closed flasks as well as in continuous cultures. In all cases, methane was the sole carbon source. Mineral medium (MMF) was the same as that described by Janssen et al. (9), except for the trace metals solution, from which copper sulfate was omitted to allow derepression of the sMMO. When cells were cultivated in the presence of copper, 4.8 μM $CuSO_4$ was added to the MMF. Solid media, which were used for maintaining pure cultures of *M. trichosporium* OB3b, had the same composition (MMF without copper in all cases) and were supplemented with 1.5% agar. During batch culture, the flasks were filled to 30% of their volume with growth medium. The gas phase consisted of 15% natural gas and 85% air (vol/vol).

Continuous cultivation of cells of *M. trichosporium* OB3b was carried out in 1-liter fermentors, as previously described (R. G. Lageveen, Ph.D. thesis, University of Groningen, Groningen, The Netherlands, 1987). MMF, phosphates, and the trace metals solution were sterilized separately, and the medium was acidified to pH 2.5 with HNO_3 before mixing, to prevent the formation of precipitates. Air and natural gas were saturated with water before they were blown through the medium. The pH was regulated at 6.8 with 0.5 N KOH and 0.5 N H_2SO_4. Other conditions were as follows: working volume, 650 ml; temperature, 30°C; impeller speed, 400 rpm; natural gas flow rate, 10 to 14 ml min^{-1}; air flow rate, 50 to 70 ml min^{-1}; methane/oxygen ratio, 0.8; dissolved-oxygen tension, 10 to 20% air saturation; dilution rate, 0.03 to 0.045 h^{-1}; biomass density, 1.8 to 2.5 mg of cells ml^{-1}.

Preparation of suspensions of *M. trichosporium* OB3b. Cells grown in fermentors and in shake flasks were harvested by centrifugation (6,000 × *g* for 5 min at 4°C) and suspended in MMF supplemented with 20 mM phosphate (pH 7.0). Suspensions prepared in this way were used throughout this work for degradation experiments. Cells for MMO assays (see below) were prepared similarly in MMF containing 20 mM phosphate (pH 7.0) and 5 mM $MgSO_4$.

TCE degradation with different cosubstrates. Unless stated otherwise, cells from continuous cultures grown in MMF not supplemented with copper sulfate were used for degradation experiments. For TCE degradation experiments with different cosubstrates, we used 500-ml closed flasks which had Teflon-lined screw caps and contained 200 ml of cells (0.15 to 0.2 mg ml^{-1}) in MMF with 1 mM phosphate buffer (pH 7.0). The cosubstrate added was 40% (vol/vol in the gas phase) methane, 20 mM methanol, or 20 mM sodium formate. Incubations took place in a reciprocal shaker (200 rpm) at 30°C, and samples were taken at different times for analyses.

For optimizing TCE degradation with formate as the cosubstrate, testing the toxicity of TCE, and determining the minimal concentration of cells and formate, experiments were performed by using 100-ml closed flasks which had Teflon-lined screw caps and contained 50 ml of 20 mM phosphate buffer (pH 7.0) with increasing concentrations of TCE, cells, or sodium formate, respectively. Incubations were performed as described above, and samples were analyzed after 24 h.

TCE degradation kinetics. Degradation kinetics of TCE were estimated by using a 1-liter fermentor that was totally filled with MMF supplemented with 20 mM phosphate buffer (pH 7.0) and 20 mM formate. The temperature (30°C) and impeller speed (400 rpm) were the same as in the fermentors used for continuous cultivation of the cells. Before TCE and cells were added, the medium was saturated with air, and oxygen concentrations were monitored with a pO_2 electrode during the experiments to ensure that sufficient oxygen was present for degradation. TCE and cells were added to concentrations of 0.05 to 0.1 mM and 0.1 to 0.14 mg ml^{-1}, respectively.

Degradation of other chlorinated aliphatic compounds. Chlorinated-aliphatic-hydrocarbon degradation assays were performed by using 100-ml closed flasks equipped with Teflon-lined screw caps. The flasks contained 50 ml MMF supplemented with 20 mM phosphate buffer (pH 7.0) and 20 mM sodium formate. Cells were added at 0.35 mg of cells ml^{-1}. Incubations took place in a reciprocal shaker (200 rpm) at 30°C, and samples were taken for analysis after 24 h. Controls, containing no cells, were used in all cases.

Analytical methods. Concentrations of chlorinated hydrocarbons were measured by capillary gas chromatography (GC) of pentane extracts. Samples (4.5 ml) were extracted with 1.5 ml of double-distilled pentane containing PCE as an internal standard. Extracts were analyzed on CP-Wax 52 CB fused-silica capillary columns (length, 25 m; inner diameter, 0.25 mm; Chrompack, Middelburg, The Netherlands) installed in a Chrompack type 438S gas chromatograph equipped with a flame ionization detector and an electron capture detector. Split injection was used (vent flow, 10 ml min^{-1}; column pressure, 50 kPa) with nitrogen as the carrier gas, and the oven temperature was kept at 43°C for 3 min followed by a temperature rise of 10°C min^{-1} to 200°C. Quantitation was carried out with a computing integrator (no. CR6A; Shimadzu, Kyoto, Japan). The ECD was used in all assays, except when high concentrations of TCE (>0.2 mM) were being measured. PCE and chloroform, which had the same retention time, were extracted with pentane containing 1,1,1-trichloroethane as the internal standard.

For analysis of propylene oxide, cyclohexanol (see below), and chlorinated intermediates, diethyl ether extracts were used. Conditions for extraction and GC were similar to those described above. Diethylether contained 1 mM TCE as an internal standard, and the concentrations of propylene oxide and cyclohexanol were detected with a flame ionization detector.

Chlorinated intermediates were analyzed by GC, both on the polar column mentioned above and on a CP-Sil 5 CB column (length, 25 m; inner diameter, 0.22 mm; Chrompack) that was used with helium as the carrier gas (column pressure, 120 kPa; vent flow, 15 ml min^{-1}) and connected to a flame ionization detector.

The intermediates found were concentrated 200-fold by evaporation of the solvent used for extraction and positively identified by GC-mass spectrometry. GC conditions and columns were as above, but helium was used in all cases. The GC-mass spectrometer was a Ribermag R10-10, operated at an electron beam energy of 70 eV and a source temperature of 180°C.

Chloride production was determined with an ion-selective electrode (Orion type 97-17) and by a colorimetric assay (10). These methods gave essentially the same results.

Enzyme assays. Total MMO (pMMO plus sMMO) activities were determined by measuring propylene oxidation to propylene oxide, as described by Colby et al. (3). sMMO activity alone was determined by measuring cyclohexane oxidation to cyclohexanol. Cyclohexane is a substrate for sMMO but not for pMMO, as shown by Burrows et al. (2).

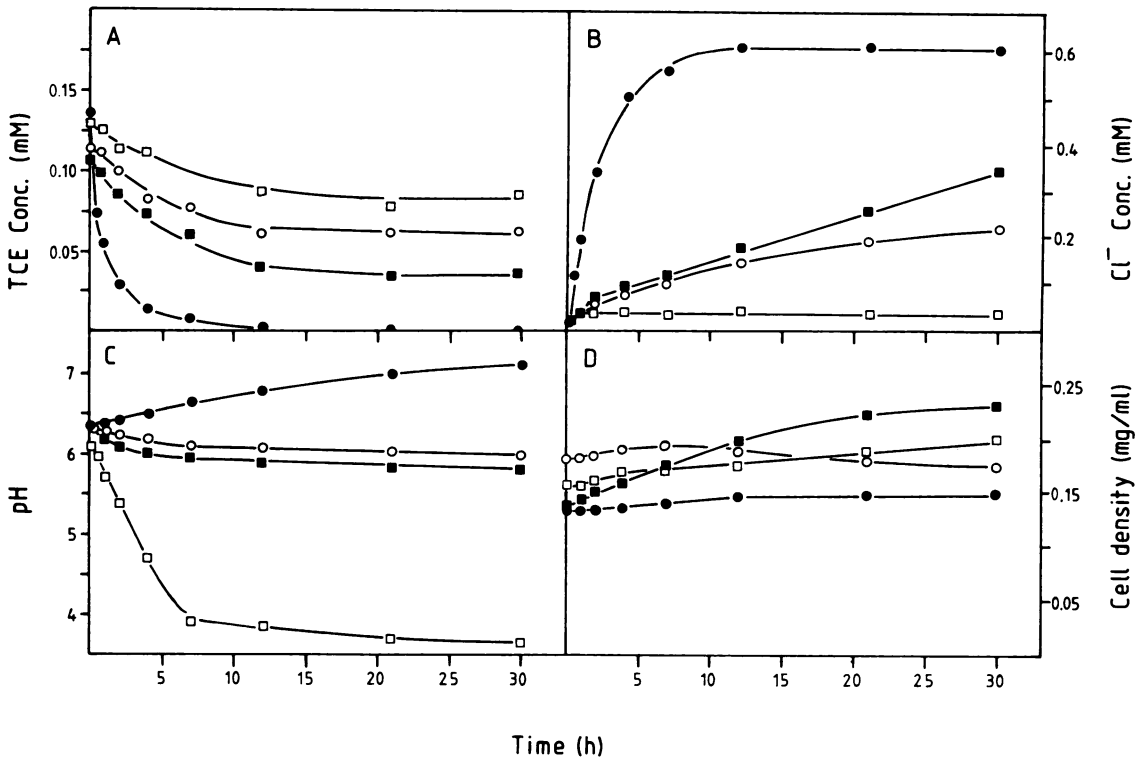

FIG. 1. Effects of cosubstrates on the degradation of TCE by *M. trichosporium* OB3b (0.15 to 0.2 mg ml^{-1}) in MMF, incubated at 30°C. The concentration of TCE in the medium (A) and the chloride production (B) were measured, the pH was determined (C), and the cell density (D) was monitored. Symbols: ■, 40% (vol/vol in gas phase) methane; □, 20 mM methanol; ●, 20 mM formate; ○, no cosubstrate.

Both assays were carried out in 100-ml closed glass bottles equipped with Viton septa and containing 20 ml of a cell suspension of 1.5 to 2 mg ml^{-1} in 20 mM potassium phosphate buffer (pH 7.0)–5 mM $MgSO_4$–20 mM sodium formate. Assays were started by injecting the substrate (20 ml of propylene or 20 μl of cyclohexane) into the suspension. Samples (1 ml) were withdrawn for product determination at 5- to 10-min intervals over a period of 30 min.

Chemicals. Organic liquid chemicals were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany, and from Janssen Chimica, Beerse, Belgium. The purity of these chemicals was checked by GC. Natural gas was obtained from Air Products, Waddinxveen, The Netherlands, and propylene was a gift of DSM, Geleen, The Netherlands.

RESULTS

Degradation of TCE with different cosubstrates. Cells of *M. trichosporium* OB3b were grown in continuous culture in MMF containing no copper and subsequently tested for their ability to degrade TCE. Different cosubstrates that can supply reducing equivalents required for the monooxygenase reaction were added. TCE disappeared completely within 20 h, and all inorganic chloride was released when formate was added as the cosubstrate (Fig. 1). Methane gave slightly more degradation than when no cosubstrate was used. No degradation was observed when methanol was used as the electron-donating substrate. During degradation experiments the pH was monitored, and a lowering of the pH was observed when methanol was used (Fig. 1C). This decrease in pH was at least partially responsible for differences in activity, since a lower pH alone also resulted in decreased TCE removal (data not shown). During these degradation experiments the growth of the cells was monitored, and only with methane was a significant increase of cell mass found (Fig. 1D).

The amount of formate needed for complete degradation of 0.2 mM TCE within 24 h by suspensions of 0.2 mg (dry weight) of cells ml^{-1} was 1 mM (Fig. 2A). TCE degradation appeared to be strictly dependent on the presence of oxygen, since no further decrease of TCE occurred when oxygen was depleted (data not shown).

Toxicity of TCE. The tolerance of cells to TCE was tested by incubating cell suspensions of 0.42 mg of cells ml^{-1} with different concentrations of TCE and then measuring the TCE concentrations remaining after 24 h. At elevated concentrations of TCE (more than 0.35 mM TCE added), inhibition occurred and TCE was recovered (Fig. 2B). Because of the partitioning of TCE between the gas phase and the liquid phase, the actual concentrations of TCE in the medium, as measured, were 0.57 times lower than the values that would have been obtained if all the TCE added had been present in the liquid phase. Thus, the critical concentration of TCE was 0.2 mM in the liquid phase in a cell suspension of 0.42 mg ml^{-1}.

Because tolerance was assumed to depend on the ratio between TCE concentration and cell mass, the minimal cell concentration for degrading 0.2 mM TCE added within 24 h was determined (Fig. 2C). With less than 0.2 mg of cells ml^{-1}, TCE was not completely degraded and chloride production decreased.

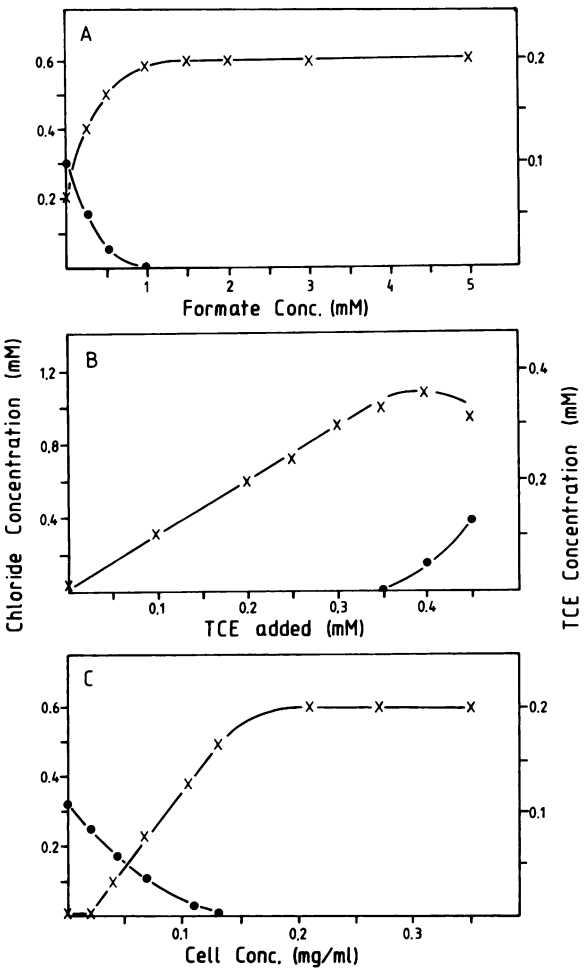

FIG. 2. Optimization of TCE degradation by *M. trichosporium* OB3b cells incubated at 30°C, with samples analyzed after 24 h. (A) Effect of increasing formate concentrations on the degradation of 0.2 mM TCE with 0.2 mg of cells ml^{-1}. (B) Effect of increasing TCE concentrations on TCE degradation, with a cell suspension of 0.42 mg ml^{-1} and 20 mM formate. (C) Effect of increasing cell densities on the degradation of 0.2 mM TCE with 5 mM formate. Symbols: ×, chloride concentration; ●, TCE concentration.

Influence of copper on TCE degradation. To study whether the ability to degrade TCE is influenced by growth conditions, particularly the availability of copper, *M. trichosporium* OB3b was cultivated both in flasks and in continuous culture with medium containing no copper and medium

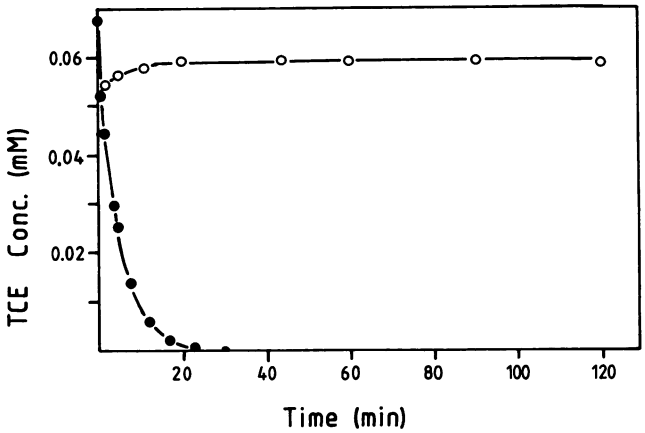

FIG. 3. Effect of copper on the degradation of 0.06 mM TCE by *M. trichosporium* OB3b cells (0.1 to 0.14 mg ml^{-1}) with 20 mM formate at 30°C. Symbols: ●, cells cultivated without copper; ○, cells cultivated with 4.8 μM copper in the growth medium.

containing 4.8 μM copper sulfate (Table 1). It was found that 0.2 mM TCE was completely degraded and that stoichiometric amounts of chloride (0.6 mM) were released with cells from continuous cultures as well as with cells from batch cultures when no copper was used in the growth medium. When cultivation was carried out in the presence of copper, no degradation of TCE or liberation of chloride took place with cells from batch cultures or continuous cultures (Table 1).

It is known that under copper stress the sMMO from *M. trichosporium* OB3b is expressed and that only this MMO is able to use cyclohexane as a substrate (2). Therefore, cyclohexanol production rates by our cultures were measured. Although all cells possessed active MMO (propylene oxide production, 40 to 100 nmol min^{-1} mg of $cells^{-1}$), only cells grown in medium to which no copper was added oxidized cyclohexane (cyclohexanol production, 6 to 24 nmol min^{-1} mg of $cells^{-1}$), showing that sMMO was expressed in both batch and continuous culture during copper limitation. Since only these cells gave rise to TCE degradation (Table 1; Fig. 3), as mentioned above, it was concluded that TCE degradation is mediated exclusively by sMMO.

TCE degradation kinetics. The course of TCE degradation was monitored over time in a reactor that was completely filled with medium and cells to exclude effects of mass transfer from the gas phase to the liquid phase (Fig. 3). The degradation of TCE by cells of *M. trichosporium* OB3b

TABLE 1. MMO activities and TCE biodegradation by cultures of *M. trichosporium* OB3b cultivated under different conditions[a]

Growth conditions	Production (nmol/min per mg of cells) of:		Amt (mM) of TCE left[b]	Amt (mM) of chloride released[b]	Initial TCE degradation rate[c] (nmol/min per mg of cells)
	Propylene oxide	Cyclohexanol			
Continuous culture, no copper	52	24	5×10^{-5}	0.62	150
Continuous culture, 4.8 μM Cu^{2+}	95	<0.1	0.12	<0.02	<0.1
Batch culture, no copper	43	6	1×10^{-4}	0.58	27
Batch culture, 4.8 μM Cu^{2+}	43	<0.1	0.11	<0.02	<0.1

[a] Growth conditions of the cells were as described in Materials and Methods.
[b] Biodegradation of 0.2 mM TCE by 0.3 to 0.4 mg of cells ml^{-1} with 20 mM sodium formate as a source of reducing equivalents. TCE and chloride concentrations were measured after 24 h.
[c] TCE degradation rate was determined with an initial concentration of 0.07 mM TCE and 0.1 mg of cells ml^{-1}.

TABLE 2. Biodegradation of chlorinated aliphatics by cells expressing sMMO[a]

Halogenated compound	Remaining concn (mM)		Degradation (%)[b]	Chloride release (mM)	
	Without cells	With cells		Without cells	With cells
Dichloromethane	0.167	$<10^{-5}$	100	0.03	0.41
Chloroform	0.138	$<10^{-5}$	100	0.04	0.59
Carbon tetrachloride	0.046	0.045	<10	0.04	0.04
1,1-Dichloroethane	0.041	$<10^{-5}$	100	0.05	0.39
1,2-Dichloroethane	0.112	$<10^{-5}$	100	0.06	0.40
1,1,1-Trichloroethane	0.069	0.026	62	0.04	0.10
1,1-Dichloroethylene	0.030	0.018	40	0.02	0.16
trans-1,2-Dichloroethylene	0.143	$<10^{-5}$	100	0.06	0.33
cis-1,2-Dichloroethylene	0.110	$<10^{-5}$	100	0.08	0.41
Tetrachloroethylene	0.069	0.072	<10	0.03	0.03
1,2-Dichloropropane	0.162	$<10^{-5}$	100	0.04	0.13
trans-1,3-Dichloropropylene	0.128	0.019	85	0.03	0.24

[a] Biodegradation experiments were carried out at 30°C with suspensions of 0.3 to 0.4 mg of *M. trichosporium* OB3b cells ml^{-1}; the cells were grown continuously in medium without copper; 20 mM formate and 0.2 mM halogenated substrate were added. Actual concentrations in the cell suspensions were lower because of partitioning between the gas phase and the liquid phase. Concentrations of remaining compounds and inorganic chloride were measured after 24 h.
[b] Percent degradation compared with controls with no cells.

proceeded according to first-order kinetics from 0.1 to 0.0002 mM TCE, with a rate constant of 2.14 ml min^{-1} mg of $cells^{-1}$ (Fig. 3).

The effect of methane on the degradation of TCE was tested. Degradation of 0.02 mM TCE by cells with a density of 0.04 mg ml^{-1} was monitored, and the initial degradation rates were determined. Without methane the degradation rate was 40 nmol min^{-1} mg of $cells^{-1}$, and with 0.02 mM methane the rate was 15 nmol min^{-1} mg of $cells^{-1}$.

Detection of intermediates. During the degradation of TCE, more than 90% of the organic chlorine was liberated as inorganic chloride in all cases, implying that only low levels of chlorinated intermediates or products may be expected. In pentane or ether extracts, traces of two chlorinated intermediates were found by GC. These compounds were identified as trichloroacetaldehyde and 2,2,2-trichloroethanol by GC and GC-mass spectrometry, respectively. These degradation products of TCE were formed in very small amounts, since the conversion of 0.2 mM TCE by cells with a density of 0.4 mg ml^{-1} resulted in the accumulation of 1.6 μM 2,2,2-trichloroethanol and 1.4 μM trichloroacetaldehyde, as measured after 24 h of incubation.

Preliminary experiments showed that *M. trichosporium* OB3b cells stimulate a partial conversion of trichloroacetaldehyde to 2,2,2-trichloroethanol and vice versa. Cells at a density of 0.4 mg ml^{-1} were incubated with 0.2 mM concentrations of these substrates at 30°C and analyzed after 48 h. When trichloroacetaldehyde was the substrate, 12 μM 2,2,2-trichloroethanol was produced. From 2,2,2-trichloroethanol, 4.5 μM trichloroacetaldehyde was produced. These conversions were not found in the absence of cells.

Degradation of other chlorinated substrates. Degradation of other chlorinated aliphatic hydrocarbons was tested with cells of *M. trichosporium* OB3b grown in continuous culture with and without copper in the medium. When added at a concentration of 0.2 mM, dichloromethane, chloroform, 1,1-dichloroethane, 1,2-dichloroethane, *trans*-1,2-dichloroethylene, *cis*-1,2-dichloroethylene, and 1,2-dichloropropane were consumed to below our detection limit within 24 h by cells grown under copper limitation (Table 2). Conversion rates were at least 0.4 nmol min^{-1} mg of $cells^{-1}$. Of these compounds, dichloromethane, chloroform, 1,1-dichloroethane, and 1,2-dichloroethane were degraded, with the release of stoichiometric amounts of inorganic chloride. Under the

TABLE 3. Biodegradation of chlorinated aliphatics by cells expressing pMMO[a]

Halogenated compound	Remaining concn (mM)		Degradation (%)[b]	Chloride release (mM)	
	Without cells	With cells		Without cells	With cells
Dichloromethane	0.173	0.024	86	<0.02	0.27
Chloroform	0.144	0.006	96	<0.02	0.44
Carbon tetrachloride	0.044	0.040	<10	<0.02	0.03
1,1-Dichloroethane	0.049	0.045	<10	<0.02	0.03
1,2-Dichloroethane	0.117	$<10^{-5}$	100	<0.02	0.27
1,1,1-Trichloroethane	0.073	0.070	<10	<0.02	<0.02
1,1-Dichloroethylene	0.031	0.032	19	<0.02	0.02
trans-1,2-Dichloroethylene	0.135	$<10^{-5}$	100	<0.02	0.18
cis-1,2-Dichloroethylene	0.102	0.045	56	<0.02	0.16
Tetrachloroethylene	0.069	0.073	<10	<0.02	<0.02
1,2-Dichloropropane	0.170	0.083	51	<0.02	0.02
trans-1,3-Dichloropropylene	0.115	0.060	48	<0.02	0.02

[a] Biodegradation experiments were carried out at 30°C with suspensions of 0.3 to 0.4 mg of *M. trichosporium* OB3b cells ml^{-1}; the cells were grown continuously in medium with 4.8 μM copper, and 20 mM formate and 0.2 mM halogenated substrate were added. Actual concentrations in the cell suspensions were lower because of partitioning between the gas phase and the liquid phase. Concentrations of remaining compounds and inorganic chloride were measured after 24 h.
[b] Percent degradation compared with controls with no cells.

same conditions, 1,1,1-trichloroethane, 1,1-dichloroethylene, and 1,3-dichloropropylene were partially degraded, with conversions of 62, 40, and 85%, respectively (Table 2). Carbon tetrachloride and PCE were not degraded.

Degradation products were detected by GC analyses for 1,1,1-trichloroethane, *trans*- and *cis*-1,2-dichloroethylene, 1,3-dichloropropylene, and 1,2-dichloropropane. Intermediates of 1,1,1-trichloroethane and 1,2-dichloropropane were further identified by GC-mass spectrometry and turned out to be 2,2,2-trichloroethanol and 2,3-dichloro-1-propanol, respectively. Of the added 1,1,1-trichloroethane, 40% was converted to 2,2,2-trichloroethanol. 2,3-Dichloro-1-propanol was found in only small amounts (<5% of added 1,2-dichloropropane).

Only 1,2-dichloroethane and *trans*-1,2-dichloroethylene were totally converted by cells grown with copper in the medium (Table 3). For all compounds tested, chloride production by cells grown with copper was far below the levels found when cells grown without copper, and hence expressing sMMO, were used for degradation.

DISCUSSION

Cometabolic oxidative conversion by microorganisms producing nonspecific monooxygenases could be a feasible alternative for biological degradation of halogenated organic compounds that are not utilized by organisms as carbon sources. Methanotrophs are good candidates to perform this conversion. This is suggested by observations made on the degradation of TCE, other chlorinated ethylenes, and chloroform with methane-enriched soil columns (18, 23), mixed cultures (5), and pure cultures (12).

In this report we describe the degradation of TCE by a pure culture of *M. trichosporium* OB3b. The results show that TCE can be rapidly degraded when cells are grown under copper limitation. It is known that the sMMO of *M. trichosporium* OB3b has a much broader substrate range than the pMMO (2) and that copper stress influences the type and intracellular location of MMO in this organism (16). From the data presented here, this appears to be crucial to the capability of this microorganism to degrade TCE and other chlorinated aliphatic hydrocarbons. TCE was only degraded by cells possessing sMMO due to growth under copper stress, as confirmed by their ability to oxidize cyclohexane (2). When 4.8 μM copper was added to the medium, no sMMO was formed and no degradation of TCE occurred. The total values for the MMO activities (sMMO plus pMMO) of cells grown in fermentors are comparable to the data found by Burrows et al. (2). It is understandable that the activities of cells grown in batch culture are lower than those of cells cultivated continuously, since higher cell densities can be obtained, which will result in more strict copper limitation and efficient derepression of sMMO (4). Cyclohexanol production by continuously cultivated cells was the same as found by Burrows et al. (2), but Burrows et al. measured a higher propylene production rate for cells grown in low-copper medium (1 μM) as well as in high-copper medium (5 μM). This was probably because we did not use any copper, and hence a lower activity of total MMO was found.

The above data lead us to conclude that sMMOs are much more suitable for the degradation of TCE than are pMMOs, which are produced during copper sufficiency in *M. trichosporium* (2) and in *Methylococcus capsulatus* (16) and are probably the only form of MMOs in some other organisms, such as *Methylomonas methanica*, *Methylomonas albus* BG8, and *Methylocystis parvus* OBBP (4). Methanotrophs that exclusively produce sMMO have not been described.

The maximal rates for TCE conversion found here were rather high, with values up to 150 nmol min^{-1} mg of $cells^{-1}$ at 70 μM TCE. TCE degradation proceeded according to first-order kinetics, with a rate constant of 2.14 ml min^{-1} mg of $cells^{-1}$. This is similar to the results of Wackett and Gibson (21), who also found first-order degradation from 80 to 8 μM TCE by toluene-induced *Pseudomonas putida* F1 cells. However, *M. trichosporium* OB3b appears to convert TCE at a 100-fold-higher rate at the same concentrations. Wackett and Gibson also tested the degradation of TCE by *M. trichosporium* OB3b and observed that addition of formate did not enhance TCE degradation and that the initial degradation rate was significantly lower than with *P. putida* F1 (21). In their experiments, however, *M. trichosporium* OB3b was grown in the presence of copper, which does not yield active cells, as we have shown here.

At a TCE concentration of 4 μM, we found a degradation rate of 8.5 nmol min^{-1} mg of $cells^{-1}$ (Fig. 3), which is about 1,000-fold higher than the degradation rate of TCE found by Little et al. (12) with the methanotrophic bacterial strain 46-1. Like Wackett and Gibson, Little et al. cultivated their methanotrophs in the presence of copper. Furthermore, they used methane as the electron-donating cosubstrate, which is far from optimal. We found that the degradation rate is highest with formate.

Methane, the normal substrate of MMO, presumably inhibits TCE conversion. The K_m values for CH_4 in *Methylococcus capsulatus* (6) and *M. trichosporium* OB3b (11) were estimated to be 3 and 2 μM, respectively. The deviation of TCE degradation kinetics from Michaelis-Menten kinetics may be caused by a half-saturation constant which is much higher than the concentrations actually used ($K_m \gg S$). If this is true, then the K_m must be at least 0.2 mM, which would imply that the affinity of the enzyme for its natural substrate, methane, is at least 2 orders of magnitude higher than its affinity for TCE. The V_{max}, on the other hand, would be in the same range or even higher, because this value was estimated at 26 nmol of methane min^{-1} mg of $cells^{-1}$ by Joergensen (11).

When methanol was used as the cosubstrate, TCE degradation was inhibited. *M. trichosporium* OB3b cells (type II membrane) did not grow on 1 mM methanol in batch culture, and no degradation of TCE occurred when methanol was used as the cosubstrate. This feature agreed with observations of Whittenbury et al. (22) that methanol was extremely toxic to most methanotrophs (with the exception of the *Methylomonas* group) when added to the medium at a concentration as low as 0.01% (vol/vol). Only Little et al. (12) reported a methane-utilizing bacterium (type I membrane) that degrades TCE during growth on methane or methanol.

Although two chlorinated intermediates were found and identified, no further attempts were made to elucidate the degradation route for TCE. MMO has been shown to convert propylene and *trans*-1,2-dichloroethylene to their corresponding epoxides (2, 9), making it plausible that TCE is converted to TCE oxide. The degradation of this epoxide has been studied by Miller and Guengerich (13), who found it to have a half-life of 12 s at neutral pH, with formic acid and carbon monoxide, but not trichloroacetaldehyde, as the degradation products. Since trichloroacetaldehyde was a major product of TCE conversion by

cytochrome P-450, however, TCE epoxide was proposed not to be an obligate intermediate (13). Instead, chlorine shift and hydrolysis of an enzyme-bound carbonium ion were suggested to be involved. It is not yet possible to conclude which TCE degradation route is followed by *M. trichosporium* OB3b. Our observation that TCE is extensively dechlorinated during its degradation is more in agreement with a TCE oxide than a trichloroacetaldehyde intermediate. We assume that 2,2,2-trichloroethanol, which was identified in some experiments, was formed by the reduction of trichloroacetaldehyde, a conversion demonstrated to be possible under the conditions used for TCE degradation experiments.

Besides TCE, a number of other environmentally important chlorinated compounds were degraded by cells of *M. trichosporium* OB3b. In fact, all chlorinated C_1, C_2, and C_3 hydrocarbons tested were at least partially oxidized and some chloride production was detected with each compound. Intermediates formed from chlorinated alkenes were probably the corresponding chlorinated epoxides. 1,1,1-Trichloroethanol and 1,2-dichloropropane were partially converted to chlorohydrins. We propose that apart from the perchloro compounds, all chlorinated aliphatic hydrocarbons (C_1 to C_3) may be converted by a methanotroph, producing sMMO, and that for most of these compounds, the sMMO of organisms such as *Methylosinus* and *Methylomonas* spp. is much more active than the pMMO.

The ability to cooxidize TCE and other chlorinated hydrocarbons appears not to be a specific property of a bacterium adapted to the presence of these compounds in its environment. Instead, methanotrophs seem to have the general ability to cometabolically oxidize halogenated aliphatic compounds. These conversions are carried out fortuitously owing to the broad substrate range of their MMOs. It is improbable that selective enrichment of TCE-degrading methanotrophs under field conditions can be achieved, because there seems to be no selective advantage for the organisms that can convert it. However, toxic reactive metabolites such as aldehydes may be produced. Furthermore, the oxidation state of the first product of some compounds (TCE, chloroform) does not allow energy gain by oxidative processes.

The applicability of methanotrophs for the removal of TCE from waste streams or polluted material from the environment will be restricted by two main factors. First, the degradation proceeds according to first-order kinetics, which implies that long treatment periods or high cell concentrations will be essential if low final concentrations are required. Second, not all methanotrophs can degrade TCE, since some organisms produce exclusively pMMO (4), and organisms that can form sMMO do so only under appropriate growth conditions. More insight into the ecophysiology of methanotrophs is necessary for establishing what conditions could lead to a selective advantage of TCE-degrading strains. Possibly, copper availability will also be an important factor in determining the types of methanotrophs enriched in nonsterile systems exposed to methane. It will be more difficult to manipulate this factor in field situations, e.g., for the decontamination of aquifers.

Another observation that is important for the applicability of methanotrophs is that methane inhibits TCE degradation. During methane-supported growth this could limit TCE degradation, whereas under conditions of methane limitation or depletion another electron-donating substrate would be required. Formate could be a candidate, but it will also be utilized by other organisms in mixed cultures. For treatment of waste streams, a two-step system, in which the first step involves methane (natural gas) used under controlled conditions to support the growth of the desirable methanotrophs and the second step involves rapid degradation supported by another electron donor, may be required.

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