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Kinetics of Chlorinated Hydrocarbon Degradation by *Methylosinus trichosporium* OB3b and Toxicity of Trichloroethylene

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The kinetics of the degradation of trichloroethylene (TCE) and seven other chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b were studied. All experiments were performed with cells grown under copper stress and thus expressing soluble methane monooxygenase. Compounds that were readily degraded included chloroform, *trans*-1,2-dichloroethylene, and TCE, with V_{max} values of 550, 330, and 290 nmol min^{-1} mg of cells^{-1}, respectively. 1,1-Dichloroethylene was a very poor substrate. TCE was found to be toxic for the cells, and this phenomenon was studied in detail. Addition of activated carbon decreased the acute toxicity of high levels of TCE by adsorption, and slow desorption enabled the cells to partially degrade TCE. TCE was also toxic by inactivating the cells during its conversion. The degree of inactivation was proportional to the amount of TCE degraded; maximum degradation occurred at a concentration of 2 μmol of TCE mg of cells^{-1}. During conversion of [^{14}C]TCE, various proteins became radiolabeled, including the α-subunit of the hydroxylase component of soluble methane monooxygenase. This indicated that TCE-mediated inactivation of cells was caused by nonspecific covalent binding of degradation products to cellular proteins.

Like many other chlorinated hydrocarbons, trichloroethylene (TCE) has become an important environmental pollutant because of its toxic properties and its widespread occurrence as a soil, air, and water contaminant. Chlorinated hydrocarbons tend to resist degradation in conventional biological waste treatment systems and in natural ecosystems. Apart from some of the lesser chlorinated compounds, such as dichloromethane, vinyl chloride, and 1,2-dichloroethane, they do not generally serve as growth substrates for microbial cultures.

Cometabolic conversions of several chlorinated aliphatics have been found both under aerobic and anaerobic conditions. Under methanogenic conditions, degradation was found to be relatively slow and mostly resulted in the accumulation of vinyl chloride, which is even more toxic (2, 4, 10, 35, 36). Laboratory studies have shown that TCE can be transformed aerobically by several oxygenase-producing bacterial cultures, including toluene-oxidizing strains (12, 23, 37, 41), the ammonia-oxidizer *Nitrosomonas europaea* (1, 34), and a propane-oxidizing *Mycobacterium vaccae* (38).

Most of the studies on aerobic TCE degradation were done with methane-utilizing mixed and pure cultures (7, 20, 24, 28, 30, 31, 40). With methanotrophs, much higher conversion rates could be obtained than with other cometabolic TCE degraders such as *Pseudomonas* strain G4. Depending on the substrate concentrations used and the presence of reducing cosubstrates, rates of 20 (30) up to 200 (24) nmol min^{-1} mg of cells^{-1} were found. In these latter studies, *Methylosinus trichosporium* OB3b was used, and it was demonstrated that only the soluble methane monooxygenase (sMMO) was capable of degrading TCE. The synthesis of sMMO is known to be derepressed when cells are grown under copper stress (5, 26).

There is still little information available about the kinetics of the degradation of chlorinated aliphatic compounds by cultures of methanotrophic bacteria. Kinetic parameters have not been determined, and it is unknown to what extent TCE degradation is affected by possible toxic effects. Toxicity was observed not only in experiments with methanotrophs (24, 30, 31) but also in experiments with toluene-oxidizing bacteria (39) and propane-oxidizing bacteria (38). Since these aspects will be of great importance for judging the applicability of methanotrophs for cleanup purposes, we decided to investigate this in more detail. In this study, we examined the kinetics of the degradation of TCE and other chlorinated aliphatic compounds by cell suspensions of *M. trichosporium* OB3b and investigated the toxicity of TCE during TCE degradation.

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 composition of the medium (MMF) and the conditions for maintaining and cultivating the cells were the same as described previously (14, 24). For all TCE degradation studies, the organism was grown in continuous cultures under copper stress (24). Cells expressing particulate MMO (pMMO), as used in [^{14}C]TCE incorporation assays, were obtained by cultivation in batch culture with MMF medium containing 0.07 μM copper.

Degradation kinetics. Degradation kinetics of TCE and other chlorinated aliphatic compounds were estimated by using a jacketed elimination vessel (JEV). This JEV consisted of a double-walled glass cylinder, closed at the bottom and equipped with a side port for sampling. At the top, the vessel was closed with a Teflon piston, which could move freely down to prevent formation of a gas phase when samples were withdrawn. The volume could vary from 90 to 30 ml. The contents were mixed with a magnetic stirrer. Samples of 1 ml were taken with a syringe through a Viton septum in the side port.

The JEV was filled with aerated MMF medium supplemented with 20 mM phosphate buffer (pH 6.8) and 20 mM

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formate. The temperature was held at 30°C by recycling water of 30°C through the double wall of the glass tube. Cells were taken directly from the fermentor without washing and added to concentrations of 0.07 to 0.3 mg (cell dry weight) ml^{-1}. Chlorinated compounds were added at different concentrations ranging from 0.2 to 750 μM to determine the initial degradation rates at various substrate levels. The degradation of substrates was monitored by taking samples at different time points (0, 0.25, 1, 2, 4, and 7 min) followed by gas chromatographic (GC) analysis. The same method was used for monitoring methane conversion, using mixtures of MMF medium and methane-saturated MMF medium.

For the determination of kinetic parameters, initial estimations were made with five different concentrations between 5 and 250 μM. The results were used for a precise determination with fresh cells, using seven to eight concentrations between 0.2 and 5 times the initial estimate for the K_m. Results of initial estimates and precise determinations were in good agreement, showing that the data were reproducible.

Kinetic parameters were estimated by transforming the data in different ways (Lineweaver-Burk, Eadie-Hofstee, Hanes-Woolf, and Eisenthal-Cornish-Bowden plots [6]), and consistency between these different plots was checked. If toxicity occurred, as indicated by a deviation from Michaelis-Menten kinetics at higher concentrations, only the rates obtained at lower substrate concentrations were used for establishing kinetic constants.

The effect of methane on TCE degradation was tested by replacing a part of the medium in the JEV with medium saturated with methane. Controls received the same amount of MMF medium saturated with nitrogen.

Activated carbon experiments. Three different types of powdered activated carbon (PAC) were tested for their capacity to adsorb TCE. Two types (D10 and SA4) were made available by Norit, Amersfoort, The Netherlands. Another type of PAC was obtained from J. T. Baker Chemicals B.V., Deventer, The Netherlands. D10, SA4, and PAC from Baker all had a diameter of 10 to 50 μm and total internal surface areas of 600, 650, and 780 m^2 g^{-1}, respectively. These three types were tested for TCE adsorption activity in 100-ml flasks that contained 0.5 g of PAC. Various amounts of TCE were added, and the flasks were completely filled with MMF medium.

TCE degradation experiments were performed in 250-ml flasks containing 50 ml of MMF medium, 20 mM phosphate, 20 mM formate, 5 g of PAC from Baker $liter^{-1}$, and 10 mM TCE. Cells of *M. trichosporium* OB3b were added at 1 and 25 h at densities of 0.33 and 0.4 mg ml^{-1}, respectively. Flasks were closed with Teflon-lined screw-caps and incubated at 30°C under rotary shaking. Chloride concentrations in the medium were measured to monitor TCE degradation.

Experiments with ^{14}C-labeled TCE. Incubations with ^{14}C-labeled TCE ([^{14}C]TCE) were done with whole cells of *M. trichosporium* OB3b, either grown in a fermentor under copper deficiency or grown in batch with 10 μg of $CuCl_2$ $liter^{-1}$ in the medium. The conditions (buffer and temperature) for incubations were the same as used in the JEV experiments. For cells expressing sMMO, 1 ml of cells at a concentration of 0.5 mg ml^{-1} was incubated in 3-ml vials which were sealed with Viton septa. [^{14}C]TCE was added from a stock solution in portions of 100 μl at 0, 15, 45, and 105 min. The [^{14}C]TCE stock solution in water had an activity of 6.6 mCi $liter^{-1}$ and a TCE concentration of 1.6 mM. After 205 min of incubation, the cells were centrifuged (10 min, 12,000 × *g*) and washed twice with mineral medium. The cells were lysed by ultrasonic disruption, and a crude extract (2 to 2.5 mg of protein ml^{-1}) was obtained by centrifugation (15 min, 12,000 × *g*). For comparison, incubations with unlabeled TCE were performed as described above.

The same procedure was followed with cells grown in batch with 0.07 μM copper and therefore expressing pMMO, except that 1.5 ml of these cells at a concentration of 2.5 mg ml^{-1} was incubated with the [^{14}C]TCE stock solution and the centrifugation time was 3 min instead of 15 min.

Radioactive samples were analyzed by counting with a liquid scintillation counter and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and fluorography. Protein samples were run on vertical slab gels of 12.5% polyacrylamide-SDS (SDS-PAGE) by the method of Laemmli (18). Marker proteins were ovotransferrin (78 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), myoglobin (17 kDa), and cytochrome *c* (12 kDa).

Gels were stained with fast green, destained by using methanol-acetic acid-water (45:10:45, vol/vol/vol), and then washed with 100% acetic acid with two changes. The gels were placed in acetic acid containing 20% (wt/vol) 2,5-diphenyloxazole (PPO) for 1.5 h. After being washed three times with water and drying down onto Whatman filter paper, the gels were placed in an X-ray cassette with Kodak X-ray film. The cassettes were stored at −70°C for 1 and 4 weeks before the fluorographs were developed.

Analytical methods. Concentrations of chlorinated hydrocarbons were measured by capillary GC of pentane extracts. Samples (mostly diluted samples) of 4.5 ml were extracted with 1.5 ml of double-distilled pentane containing 0.05 mM 1-bromohexane as an internal standard. GC conditions and quantitation were the same as previously described (24). The electron capture detector was used in all experiments. Methane concentrations were estimated by directly injecting 2-μl samples from the incubation mixture in the JEV into the GC. A Poraplot Q column (50 m by 0.32 mm) (Chrompack) was operated isothermally at 100°C with nitrogen (100 kPa; split flow, 50 ml/min) as the carrier gas and a flame ionization detector.

Chloride production was determined by a colorimetric assay and with an ion-selective electrode, both previously described (16, 24).

Oxygen consumption rates were determined with an electrode at 30°C in MMF medium, using cells at 0.1 to 0.15 mg (dry weight) ml^{-1} and 5 mM methanol or 0.15 mM methane as the substrate.

Protein concentrations in cell suspensions as well as in cell extracts were estimated by the Folin phenol method, with bovine serum albumin as the standard.

Chemicals. Organic liquid chemicals were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany, and from Janssen Chimica, Beerse, Belgium. [^{14}C]TCE had a specific activity of 4.1 mCi $mmol^{-1}$ and was obtained from Sigma Chemical Co., St. Louis, Mo. The purity of these chemicals was checked by GC. Natural gas was obtained from Air Products, Waddinxveen, The Netherlands.

RESULTS

Kinetic parameters of chlorinated hydrocarbon degradation. Whole cells of *M. trichosporium* OB3b were taken from a copper-free continuous culture and used immediately for degradation experiments. For eight different chlorinated aliphatic compounds, the initial degradation rates at various

TABLE 1. Kinetic parameters for the degradation of methane and chlorinated aliphatics by *M. trichosporium* OB3b cells expressing sMMO

Substrate	K_m (μM)	V_{max} (nmol min^{-1} mg of $cells^{-1}$)	k_1[a] (ml min^{-1} mg of $cells^{-1}$)	Ci[b] (μM)	Cell mass[b] (mg of cells ml^{-1})	Ci[c] (μM)
Methane	92 ± 30	363 ± 50	3.9	ND[d]	0.14	ND
Dichloromethane	4 ± 1	33 ± 1	8	>100	0.11	>90
Chloroform	34 ± 6	548 ± 55	16	160–230	0.10	150
1,2-Dichloroethane	77 ± 9	65 ± 6	1	50–70	0.09	55
1,1,1-Trichloroethane	214 ± 18	24 ± 1	0.1	>700	0.85	>80
1,1-Dichloroethylene	5 ± 2	6 ± 1	1	20–30	0.09	22
trans-1,2-Dichloroethylene	148 ± 61	331 ± 89	2	90–150	0.12	75
cis-1,2-Dichloroethylene	30 ± 10	182 ± 29	7	130–260	0.07	185
Trichloroethylene	145 ± 61	290 ± 99	2	70–100	0.13	70

[a] First-order rate constant, V_{max}/K_m.
[b] Maximum allowable substrate concentration for the cell densities used in the experiments.
[c] Maximum allowable substrate concentration standardized for cell densities of 0.1 mg ml^{-1}.
[d] ND, Not determined.

concentrations were measured and plotted by the Lineweaver-Burk, Eadie-Hofstee, Hanes-Woolf, and Eisenthal-Cornish-Bowden methods for the determination of kinetic parameters. Linear curves were found with the first three plots, which indicated that Michaelis-Menten kinetics was followed. From these data, the K_m and V_{max} were estimated (Table 1).

K_m values for the chlorinated hydrocarbons tested differed over a range of 50-fold. Dichloromethane and 1,1-dichloroethylene showed the lowest K_m value, and with chloroform and *cis*-1,2-dichloroethylene, a lower K_m was found than with methane. Apart from chloroform, *trans*-1,2-dichloroethylene, and TCE, all compounds showed V_{max} values that were much lower than those with methane. The ratios (k_1) between V_{max} and K_m values are also given (Table 1); these are a measure for rate at low substrate concentration and selectivity of the enzyme. Dichloromethane, chloroform, and *cis*-1,2-dichloroethylene showed higher k_1 values than methane, while the k_1 values with TCE and *trans*-1,2-dichloroethylene were only twofold lower. 1,1,1-Trichloroethane was a very poor substrate.

Table 1 also shows the concentration range above which the chlorinated substrates showed toxic effects. These concentration ranges were the thresholds for acute toxicity, which means that at and below the lower value indicated Michaelis-Menten kinetics was found but that a significant deviation occurred above the higher value of the range shown. The K_m values of *trans*-1,2-dichloroethylene and TCE had to be determined with concentrations below the K_m values estimated and resulted in less reliable values compared with the others (Table 1). Because toxicity was linked to cell densities, maximum allowable concentrations were calculated for densities of 0.1 mg of cells ml^{-1} (Table 1, last column). These values are nearly the same except for 1,1-dichloroethylene, which already showed toxicity at very low concentrations (20 to 30 μM), and for *cis*-1,2-dichloroethylene and chloroform, which were not toxic up to high concentrations (185 and 150 μM, respectively). Dichloromethane and 1,1,1-trichloroethane did not show any acute toxicity at the highest substrate levels used (100 and 700 μM, respectively).

Effect of methane. The effect of methane on the degradation of TCE was tested by the addition of methane-saturated MMF medium. In the presence of 0.27 mM methane with 0.42 μM TCE, the TCE degradation rate decreased from 0.50 to 0.15 nmol min^{-1} mg of $cells^{-1}$ (Fig. 1). With different initial TCE concentrations at a fixed methane level, a deviation from Michaelis-Menten kinetics occurred, which made

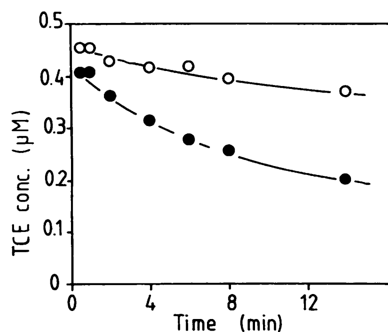

FIG. 1. Effect of 0.27 mM methane on the degradation of 0.42 μM TCE by *M. trichosporium* OB3b cells (0.058 mg ml^{-1}). Symbols: ○, with methane; ●, no methane added.

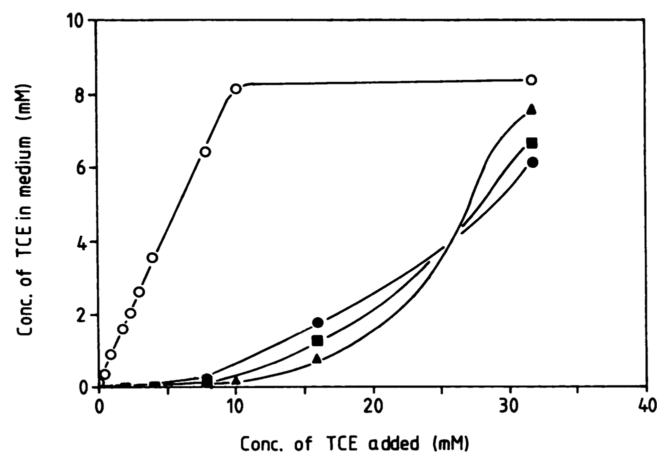

FIG. 2. Effect of addition of different types of PAC (5 g $liter^{-1}$) on the adsorption of TCE under degradation conditions, as described in Materials and Methods. Symbols: ■, PAC type SA4; ●, PAC type D10; ▲, PAC from Baker; ○, no PAC added.

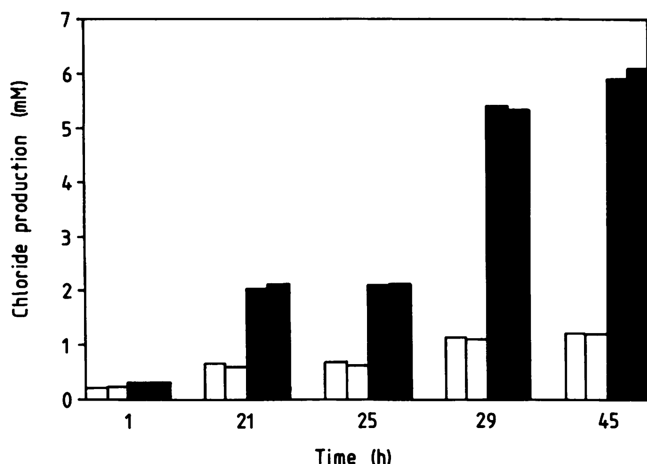

FIG. 3. Effect of PAC on the degradation of TCE by cell suspensions of *M. trichosporium* OB3b in 20 mM phosphate buffer–20 mM formate incubated at 30°C. After adsorption of TCE (10 mM) to PAC (5 g liter^{-1}), cells were added at 1 h (0.33 mg ml^{-1}) and 25 h (0.4 mg ml^{-1}). Degradation was monitored by taking duplicate samples and measuring the chloride concentration. Open bars, no PAC present; solid bars, PAC present.

it impossible to determine an inhibition constant (data not shown).

Activated carbon experiments. We tested whether the toxicity could be overcome by reducing the concentration of TCE in the medium by the addition of PAC. Three different types of PAC were tested. Without PAC, all TCE added was recovered from the medium until maximum solubility of TCE was reached, which was 8 mM. In the presence of PAC, the TCE concentration in the liquid remained below 0.2 mM for TCE additions up to 10 mM. Above these values, the PACs became saturated and most of the TCE added remained in the liquid (Fig. 2). All three types of PAC had nearly the same adsorption capacity, which was 1 to 2 mmol of TCE g of activated carbon^{-1}. PAC from Baker was chosen for further experiments because of its low chloride content.

After adsorption of 10 mM TCE to PAC (5 g liter^{-1}), cells were added at 1 and 25 h at 0.33 and 0.4 mg of cells (dry weight) ml^{-1}, respectively. Duplicate samples were taken at 1, 21, 25, 29, and 45 h, and chloride concentrations were measured. When no PAC was present, very poor degradation was observed (Fig. 3). About 0.2 mM TCE was degraded after the first addition of cells, which increased to 0.4 mM TCE after the second addition of cells. In the presence of PAC, TCE degradation was about 0.7 mM and readdition of cells resulted in degradation of an additional 1.3 mM TCE (Fig. 3). It was obvious that desorption of TCE occurred during TCE degradation and that by adding PAC acute toxicity could be overcome. The data further suggest that cells became rapidly inactivated under these conditions, since degradation apparently stopped after 0.7 and 1.3 mM of TCE was degraded, although enough oxygen and formate were present for the oxidation of 10 mM TCE.

Inactivation of cells or MMO during TCE conversion. We investigated whether the TCE degradation rate decreased during its conversion as a result of inactivation of cells or MMO. The cause of inactivation of MMO was tested by monitoring TCE depletion rates after repeated additions of TCE to a suspension of cells in MMF medium plus formate. The cell suspension had a density of 0.11 mg ml^{-1}, and TCE was added at 30-min intervals to a concentration of 40 to 45 μM. Immediately before each addition of TCE, the suspension was aerated by gentle shaking with air. The rate of TCE degradation was lower after each subsequent addition (Fig. 4). The decrease of the rate of TCE degradation with time did not occur with a control culture to which no TCE was added (Fig. 4A). Cells to which TCE was repeatedly added showed a methanol-dependent oxygen consumption rate of 33 nmol min^{-1} mg of cells^{-1}, while a value of 91 nmol min^{-1} mg of cells^{-1} was found with cells incubated similarly without TCE. Methane-dependent oxygen consumption decreased from 51 to 17 nmol min^{-1} mg of cells^{-1} upon TCE conversion.

The activity of the cells with respect to the rate of TCE degradation (V_{max}) could be given by $V_{max,\,t} = V_{max,\,0} - p\,(S_a - S_t)$, where $V_{max,\,0}$ and $V_{max,\,t}$ represent the activity of the cells at time points 0 and t, respectively; S_a is the total amount of substrate added; S_t is the amount of substrate left at time t; and p is the inactivation constant, which means the amount of activity (nanomoles minute^{-1} milligram^{-1}) inactivated per amount of substrate converted (micromoles). From the slope of the curve in Fig. 4B, an inactivation constant of 0.48 was calculated.

[^{14}C]TCE experiments. The substrate conversion-linked inactivation of MMO could be due to specific covalent binding of TCE during its conversion to the hydroxylase

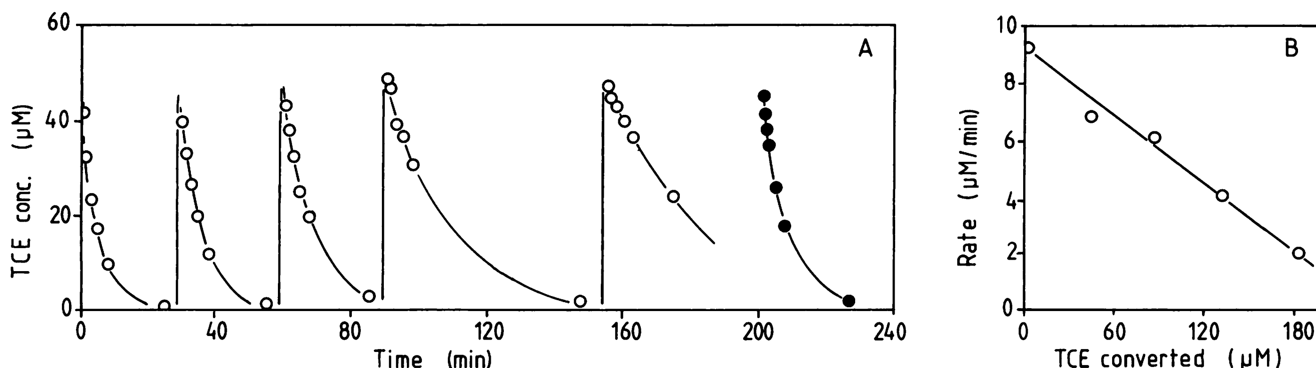

FIG. 4. (A) Effect of repeated addition of 40 to 45 μM TCE on degradation rate of TCE (○) by *M. trichosporium* OB3b cells (0.11 mg ml^{-1}). As a control, degradation was also measured with cells to which TCE was added for the first time after 200 min (●). (B) Degradation rate versus amount of TCE converted.

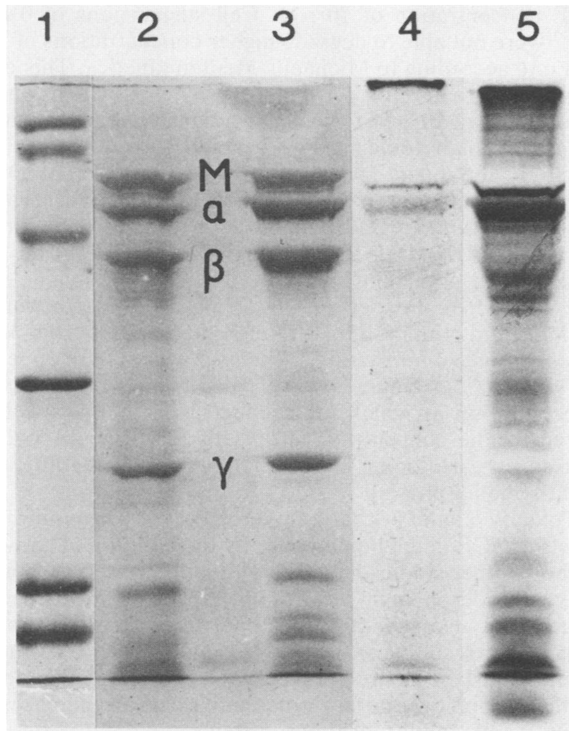

FIG. 5. [^{14}C]TCE binding to proteins of crude extracts of *M. trichosporium* OB3b grown under copper deficiency. The figure shows the protein banding pattern on SDS-PAGE and the corresponding fluorograph. Lanes: 1, molecular weight marker proteins; 2, crude extract from cells not incubated with TCE; 3, crude extract from cells incubated with [^{14}C]TCE; 4 and 5, fluorographs of proteins corresponding to lane 3, developed after 1 and 4 weeks, respectively. Bands representing the α-, β-, and γ-subunits of the hydroxylase component of sMMO and the methanol dehydrogenase (M) are indicated.

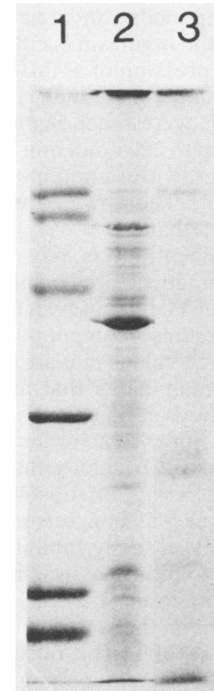

FIG. 6. [^{14}C]TCE binding to proteins of crude extracts of *M. trichosporium* OB3b grown in batch culture with 0.07 μM copper. The figure shows the protein banding pattern on SDS-PAGE and the corresponding fluorograph. Lanes: 1, molecular weight marker proteins; 2, crude extracts from cells incubated with [^{14}C]TCE; 3, fluorograph of proteins corresponding to lane 2, developed after 4 weeks.

component of MMO or to a nonspecific reaction of products of TCE degradation with cellular components. To elucidate this phenomenon, we did degradation experiments with [^{14}C]TCE.

Cells of *M. trichosporium* OB3b grown under copper deficiency were incubated with [^{14}C]TCE and formate. After a total addition of 460 μM labeled TCE with a total activity of 2.6 μCi, a crude extract was prepared. Extracts were analyzed for covalent binding of TCE and/or its conversion products to proteins by scintillation counting, SDS-PAGE, and fluorography (Fig. 5). Of the total amount of ^{14}C label added, 0.4% was recovered in the crude cell extract, yielding a specific activity of 56 nCi mg of protein^{-1}.

The fluorographs showed that the ^{14}C remained bound to cellular proteins, even after boiling in SDS, which indicated that TCE or the products of TCE conversion formed a covalent bond with proteins.

Analysis of cell extracts of *M. trichosporium* OB3b grown under conditions in which sMMO activity was expressed showed that the α-subunit (54.4 kDa [9]) of the hydroxylase component of sMMO was labeled, and scintillation counting showed that 10% of the label present in crude extract was represented by this band (Fig. 5). One other band, just below the methanol dehydrogenase band, was also strongly labeled, as was the top of the gel. The fluorograph showed that methanol dehydrogenase itself and the β-subunit and γ-subunit (43.0 and 22.7 kDa, respectively [9]) of the hydroxylase were not labeled.

The incorporation of [^{14}C]TCE in cells grown in the presence of copper is shown in Fig. 6. The fluorograph shows that the label remained bound to the proteins during boiling but that none of the proteins were specifically labeled. A low overall incorporation of label in cells with pMMO was observed on fluorographs (Fig. 5 and 6). Liquid scintillation counting of the radioactive protein samples also showed that the incorporation of ^{14}C in cellular material was much lower than that of the cells expressing sMMO. The specific activities were 56 and 4 nCi mg of protein^{-1} for cells with sMMO and pMMO, respectively.

DISCUSSION

Methanotrophic bacteria have the potential to degrade halogenated organic compounds that are not utilized by organisms as carbon sources. Methane-oxidizing bacteria could therefore become increasingly important for the application of biological techniques for environmental protection, soil cleanup, and groundwater treatment, provided that suitable treatment technologies for employing their cometabolic degradative capacity can be developed. An effective process requires the organisms to be active under conditions that can be achieved by a technically and economically feasible approach. Important factors include substrate specificity, reaction rates, and stability of the organisms. Several aspects related to these points were studied in the work presented here.

Previous work (24, 30) showed that rapid degradation of

TCE and related compounds can be achieved by *M. trichosporium* OB3b when the organism is cultivated under copper limitation, causing expression of sMMO with higher activity toward more chlorinated compounds. In these studies, degradation rates of TCE were much higher than found earlier (20, 37) and rates of up to 200 nmol min^{-1} mg of $cells^{-1}$ were observed with cells grown in continuous culture and incubated with formate (24). These rates are in the same range as the rate found with methane.

Since substrate concentrations may vary strongly, which may influence the practical applicability, we studied the degradation kinetics of TCE and seven related compounds in more detail. Most of these compounds had not only high V_{max} values but also K_m values that are much higher than the concentrations of contaminants that are often observed in practice (Table 1). The kinetic constants found with chlorinated aliphatics were in the same order of magnitude as with methane, apart from 1,1,1-trichloroethane and 1,1-dichloroethylene, which were very poor substrates. The low affinity of the cells for methane was in agreement with the observation that methane only partially inhibited TCE conversion. The K_m for methane found by us was much higher than the value reported by Joergensen (2 μM) (15), probably because we used cells grown in the absence of copper and thus expressing only the sMMO. The observed differences between the various chlorinated compounds were not related to variations in culture conditions since all experiments were performed with cells from the same steady state in the chemostat. It may well be, of course, that changes in growth conditions such as growth rate (29) or copper content of the medium (24) result in different values.

For some of the compounds that we tested, kinetic parameters were recently determined with purified sMMO from the same organism (8). Fox et al. (8) observed only minor differences in V_{max} and K_m values for dichloroethylenes and TCE. Furthermore, K_m values were substantially higher for TCE and *trans*-1,2-dichloroethylene with whole cells than with purified enzyme. This could point to uptake, transport, or diffusion of these compounds being a rate-limiting step in their conversion. Since practical applications of methanotrophs for environmental clean-up will involve the use of whole organisms rather than purified enzymes, it is important that parameters obtained with cells are used for determination of rates that can be achieved.

At low substrate concentrations, the rate of transformation (micromolar $minute^{-1}$) can be given by $dC/dt = -V_{max}/K_s \cdot X \cdot C$, where X represents cell density (milligrams $liter^{-1}$), C the substrate concentration (micromolar), and $-V_{max}/K_s$ is the first-order rate constant (milliliters $minute^{-1}$ milligram of $cells^{-1}$). Table 1 shows that the rate constants varied from 0.1 ml min^{-1} mg of $cells^{-1}$ for 1,1,1-trichloroethane to 16 ml min^{-1} mg of $cells^{-1}$ for chloroform. With these kinetic parameters it is possible to predict half-lives at certain cell densities. For example, if a half-life of 30 days is wanted, minimal cell densities of 16×10^{-4} (about 5×10^5 cells) and 1×10^{-6} mg ml^{-1} would be needed for 1,1,1-trichloroethane and chloroform, respectively. These values are probably too optimistic since the kinetic constants were determined at 30°C with formate as the electron donor. According to Jain and co-workers (13), cell densities of 10^5 cells $liter^{-1}$ could be maintained when contaminated soil or groundwater was inoculated with specialized microorganisms.

A serious problem is the stability of the bacteria. Because of the toxicity of some chlorinated hydrocarbons, inactivation of the cells occurred. Acute toxicity was apparent at a TCE concentration of 70 μM. Cell suspensions of 0.1 mg ml^{-1} were not able to degrade higher concentrations of TCE at a rate according to Michaelis-Menten kinetics. This effect was even greater for 1,1-dichloroethylene, which was already toxic at 22 μM for cells at a density of 0.1 mg ml^{-1}. Relatively high toxicity of 1,1-dichloroethylene was also found by Green and Dalton (11), who observed that 90% of the activity of protein A of purified sMMO from *Methylococcus capsulatus* (Bath) was lost in 10 min at 45°C in the presence of NADH and 1,1-dichloroethylene. The high toxicity and poor conversion of 1,1-dichloroethylene were not noticed by Fox et al. (8), who worked with purified sMMO from strain OB3b, although the sMMOs from *Methylococcus capsulatus* and *M. trichosporium* OB3b appear to be similar (27). Note, however, that with whole cells the concentration at which toxic effects appear may be influenced by the concentrations of cells (24), which could be caused by partitioning of the compounds between the cells and the liquid phase.

Toxicity could be partly overcome by preventing high levels of TCE in the liquid phase by the addition of activated carbon. Other studies have shown that activated carbon not only provides a high adsorption capacity for toxic compounds but also is a suitable supporting material for microorganisms, resulting in stable systems (3, 17, 22). Further studies must be done to reveal whether immobilization of methanotrophic bacteria on activated carbon increases the activity and stability of those systems.

Another problem is that only a relatively small amount of TCE added could be degraded per amount of active cells. About 2 μmol of TCE was converted per mg of cells. These data are in good agreement with the inactivation constant, p, obtained by monitoring TCE depletion rates after repeated additions of TCE. From this experiment, it was calculated that 0.48 mg of cells was inactivated per μmol of TCE converted.

Leak and Dalton (19) investigated the efficiency of carbon assimilation into biomass of *Methylococcus capsulatus* (Bath) under different conditions and found a growth yield (Y_{CH_4} = gram of cells gram of $substrate^{-1}$) of 0.50 under the same growth conditions that we used for *M. trichosporium*. Assuming this Y_{CH_4} of 0.5, it can be calculated that 53 μmol of CH_4 (1.3 ml at 1 atm [101 kPa]) is necessary for replacing the cells inactivated by conversion of 1 μmol of TCE. This suggests that for cleaning up groundwater (100 μg of TCE $liter^{-1}$), a minimal amount of 1 ml of CH_4 liter of $groundwater^{-1}$ is required under optimal conditions (30°C, 20 mM formate, plenty of oxygen, etc.).

The TCE toxicity was due not only to reactions of TCE degradation products with MMO (Fig. 5) but also to general metabolic inactivation of the cells, as indicated by the decrease in methanol-stimulated oxygen consumption rates. We have also found that TCE conversion decreases viability (data not shown).

Toxicity of TCE related to its conversion was also found by Wackett and Householder (39) and Fox et al. (9). Toluene dioxygenase in *Pseudomonas putida* F1 was found to be responsible for growth inhibition in the presence of TCE (39). As with the sMMO of *M. trichosporium* OB3b, the inhibitory effects may stem from metabolic activation of TCE by toluene dioxygenase to form reactive intermediates that modify intracellular molecules. This is also suggested by the recent work of Fox et al. (8), who observed that about 0.83 μmol of TCE could be converted per mg of hydroxylase component and that all MMO proteins present in their incubation system were labeled.

In our experiments, despite nonspecific binding, some proteins of cells expressing sMMO were clearly not labeled. Methanol dehydrogenase was probably not labeled because it is a periplasmic protein, which could indicate that the reactive products of TCE degradation do not reach the periplasmic space or medium. The observation that the β-subunit and the γ-subunit of the hydroxylase component of sMMO were hardly labeled deviates from the data of Fox et al. (8), who, working with purified proteins, found labeling of both these subunits. Conceivably, the organization of the MMO components in the cell affects their relative sensitivity toward reaction with TCE oxidation products.

The cause of TCE toxicity is the nonspecific reaction of conversion product(s) with cell components, including the sMMO hydroxylase component. Good candidates are TCE epoxide or acylchlorides that are produced after its hydrolysis. Experiments with [^{14}C]TCE in mice and rat hepatic microsomes support the view that to bind to protein, it is necessary for TCE to be metabolized to its epoxide (32, 33). TCE epoxide is expected to be highly reactive toward cellular nucleophiles, e.g., proteins and nucleic acids, and binds covalently. Other possible reactive metabolites that might bind irreversibly are chloral (2,2,2-trichloroacetaldehyde), dichloroacetyl chloride, and formyl chloride (21). Organisms that are more suitable for the conversion of large amounts of TCE thus should possess active detoxification systems for these compounds.

Our results showed nonspecific binding of [^{14}C]TCE degradation products with cells expressing sMMO and, to a very small extent, also with cells expressing pMMO. This shows that TCE is not a suicide substrate of MMO, as was found with [^{14}C]acetylene, which with both sMMO and pMMO was specifically bound to proteins associated with methane-oxidizing activity (25). TCE thus does not seem to react immediately during its conversion. Furthermore, the lack of activity of pMMO with TCE does not appear to be caused by an increased sensitivity toward inactivation by TCE or its conversion products. We propose that TCE is just a very poor substrate for pMMO of *M. trichosporium* OB3b, although several other halogenated aliphatics can be converted by pMMO (14, 24).

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