

Biodegradation of Trichloroethylene by *Methylosinus trichosporium* OB3b

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The methanotroph *Methylosinus trichosporium* OB3b, a type II methanotroph, degraded trichloroethylene at rates exceeding 1.2 mmol/h per g (dry weight) following the appearance of soluble methane monooxygenase in continuous and batch cultures. Cells capable of oxidizing trichloroethylene contained components of soluble methane monooxygenase as demonstrated by Western blot (immunoblot) analysis with antibodies prepared against the purified enzyme. Growth of cultures in a medium containing 0.25 μ M or less copper sulfate caused derepression of the synthesis of soluble methane monooxygenase. In these cultures, the specific rates of methane and methanol oxidation did not change during growth, while trichloroethylene oxidation increased with the appearance of soluble methane monooxygenase. *M. trichosporium* OB3b cells that contained soluble methane monooxygenase also degraded vinyl chloride, 1,1-dichloroethylene, *cis*-1,2-dichloroethylene, and *trans*-1,2-dichloroethylene.

Trichloroethylene (TCE) is a significant environmental pollutant contaminating soil and groundwater (29). While this compound has a large number of industrial applications as a solvent and degreaser, inadequate disposal techniques or accidental spillage have threatened drinking water supplies (26). The presence of TCE and other chlorinated aliphatic hydrocarbons in drinking water threatens human health because of their toxicity and carcinogenicity (21). These compounds are persistent in several environments. When biodegradation does occur, the potential for accumulation of more recalcitrant and toxic intermediates arises with incomplete mineralization. For example, anaerobic bacteria transform TCE to the carcinogen vinyl chloride by reductive dechlorination (23, 32).

Several studies have revealed that TCE could be transformed aerobically by consortia of microorganisms (11, 12, 34), some soil microorganisms including the ammonia-oxidizing bacteria *Nitrosomonas europaea* (3), toluene-oxidizing bacteria (22, 33), and cultures of methanotrophic bacteria (19). Methanotrophs are a group of microorganisms which grow on methane as a sole source of energy and as a major source of carbon (2, 15). While obligate methanotrophs derive energy solely from the oxidation of one-carbon compounds, they can oxidize a variety of compounds including many alkanes, alkenes, and aromatic hydrocarbons (9, 16, 17).

The methane monooxygenase (MMO) systems of methanotrophic bacteria catalyze the incorporation of one oxygen atom from molecular oxygen into methane to produce methanol. These enzymes exist as a soluble or a particulate (membrane-bound) form (10, 27). The two forms of the enzyme are thought to differ structurally and catalytically (5, 28).

Only the soluble MMO has been purified, having been isolated from both type I and type II methanotrophs. Chromatography resolves soluble MMO into three components: hydroxylase, reductase, and component B (7). The hydroxylase from the type II methanotroph *Methylosinus trichosporium* OB3b is composed of three polypeptides with molecular masses of 54 kilodaltons (kDa) (alpha subunit), 43 kDa

(beta subunit), and 23 kDa (gamma subunit) (13). The molecular masses of the reductase and component B are 39 and 15.8 kDa, respectively (13, 14). Reports have linked the availability of copper during the growth of the methanotrophs *Methylococcus capsulatus* and *M. trichosporium* to the expression of the soluble form of MMO. The soluble form of MMO is synthesized under copper-limited growth conditions (28). The soluble form of MMO has a broader substrate specificity than the membrane-bound (particulate) enzyme (5). However, the particulate enzyme also oxidizes many compounds other than methane. It has been suggested that the particulate MMO is responsible for the oxidation of TCE (S. M. Henry, A. A. Dispirito, M. E. Lidstrom, and D. Grbic-Galic, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, K69, p. 256). In the present report, evidence is presented that the expression of soluble MMO activity is correlated with high rates of TCE degradation by *M. trichosporium*.

MATERIALS AND METHODS

Bacteria and culture condition. *M. trichosporium* OB3b obtained from R. Whittenbury, Warwick University, Coventry, United Kingdom, was maintained on a mineral salts medium (8) at 30°C with shaking at 200 rpm (2.5-cm stroke length) under methane-air (1:4 [vol/vol]) at 1 atm. The liquid volume did not exceed 25% of the total volume of the baffled Erlenmeyer flasks in which the cultures were grown. The A_{600} of cultures and cell suspensions was measured with a Spectronic 20 spectrophotometer (Milton Roy Co., Rochester, N.Y.).

M. trichosporium was grown in an Omni-culture benchtop fermentor (The VirTis Co., Inc., Gardiner, N.Y.) for several experiments. The 2-liter glass incubation chamber was inoculated with 200 ml of *M. trichosporium* culture grown in a flask to an optical density of 1.00. The initial absorbance of the chemostat culture was 0.10. Methane and air were sparged into the fermentor at the following rates: methane, 25 ml/min; air, 500 ml/min. The agitation of the chemostat was initially 300 rpm and was increased to 450 rpm after the culture reached an absorbance greater than 0.80.

Experiments were conducted to modify levels of soluble MMO by varying the concentration of copper in the growth

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media. The media were prepared in baffled glass Erlenmeyer flasks with purified water with copper concentrations ranging from 0.00 to 1.00 μM copper sulfate. The inoculum was grown to an optical density of 1.0 in the mineral salts medium containing 1.00 μM copper sulfate. The cells were harvested by centrifugation at $5,000 \times g$ and washed in media containing 0.00 μM added copper sulfate. The cells were suspended in a minimal amount of 0.00 μM copper medium and evenly distributed in 0.5-ml aliquots to the flasks containing media with different levels of copper. The initial absorbance values of the cultures were 0.20. All transfers were made with disposable serological pipettes (Falcon; Becton Dickinson Labware, Oxnard, Calif.). The flasks were fitted with rubber stoppers containing gas ports (25) through which methane-air (1:4 [vol/vol]) was added and replenished every 8 h.

Elimination of trace metal ions from water and glassware.

Glass-distilled deionized water was purified by passage through a column of Chelex-100 resin (4.5 by 12.0 cm). The resin was precycled according to the instructions of the manufacturer (Bio-Rad Chemical Division, Richmond, Calif.), equilibrated with 0.1 M citrate buffer (pH 7.0) followed by 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-NaOH (pH 7.0), and washed extensively with distilled deionized water. Purified water was stored in vessels treated to remove trace metal ions.

All glassware was washed with detergent, rinsed with distilled water, and soaked overnight in a mixture of H_2SO_4 and HNO_3 (3:1, vol/vol). The acid-treated glassware was subsequently rinsed with distilled deionized water and soaked overnight in 25 mM EDTA (pH 8.0). After EDTA treatment, the glassware was washed with large quantities of distilled deionized water and rinsed several times with the purified, Chelex-100-treated water.

Preparation of antibodies for soluble MMO components.

Purified soluble MMO components were obtained from B. Fox and J. Lipscomb, Department of Biochemistry, Medical School, University of Minnesota. Young adult female New Zealand rabbits were used for antibody preparation. Components of soluble MMO (hydroxylase, component B, and reductase) at concentrations of 300 to 500 $\mu\text{g}/\text{ml}$ in phosphate-buffered saline (pH 7.0) were each emulsified immediately before injection with an equal volume of Freund incomplete adjuvant. Each protein component was injected in 1-ml aliquots into the fore- and hindquarters of the rabbits. The process was repeated in 2 weeks. Rabbits were bled at week 6 to test the effectiveness of the procedure. Thereafter, booster injections and bleedings were performed alternately each week until sufficient antibody production was observed. Immunoglobulin G (IgG) fractions of antibodies were purified by passing antisera through a protein A-Sepharose CL-4B column (Pharmacia Fine Chemicals, Piscataway, N.J.).

SDS-PAGE and Western blot analysis (immunoblotting). A discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system described by Laemmli (18) and this laboratory (20) was used to separate cell proteins. A 10% resolving gel was utilized for all separations. Immunoassays for soluble MMO components were standardized by adding 200 μg of cell extract to gels for detection of hydroxylase components and 400 μg of cell extract for detection of components B and reductase. Culture (5 ml) normalized to a constant absorbance of 1.00 was harvested from the various experimental growth conditions by centrifugation in a Microfuge (Beckman Instruments, Inc., Irvine, Calif.) and stored as a pellet at -20°C for analysis.

Each gel was run with molecular weight protein standards purchased from Sigma Chemical Co. (St. Louis, Mo.). Electrophoresis was performed at 15 mA for 6 h at 16°C . Coomassie blue (0.125% [wt/vol] Coomassie brilliant blue R in 10% acetic acid-30% methanol) was used to stain the gels. Proteins resolved on the SDS-polyacrylamide gel were transferred to a nitrocellulose membrane with a 0.45- μm pore size in a Trans-Blot cell (Bio-Rad Laboratories, Richmond, Calif.). The membranes were incubated at room temperature with IgG fractions that contained antibodies against soluble MMO components at a dilution of $1:10^4$. This process was followed by reactions with goat anti-rabbit IgG conjugated with alkaline phosphatase (Promega Biotec, Madison, Wis.). Each component was detected by a colorimetric assay as previously described (4, 20).

In Ouchterlony double-gel-diffusion analyses (31), a single precipitation band was formed when antisera against the hydroxylase, reductase, and B components of MMO were reacted with the respective antigens. Immunological cross-reactivity between components was not detected. Western blot analysis was performed after separating components by denaturing SDS-PAGE. By this method, the three hydroxylase subunits were readily separated on the gel and could be detected. The reductase and B components of soluble MMO in whole-cell extracts were not detected unless more than 400 μg of sample was used. However, purified reductase and component B were readily apparent in 1- μg quantities. Antiserum diluted $1:10^4$ was routinely used in Western blot analysis. Dilutions up to 80,000-fold were still effective in detecting each antigen on Western blots. At dilutions of antisera greater than $1:10^4$, the intensities of protein bands decreased proportionally to the dilutions of the antisera. At the optimal dilution of $1:10^4$, color intensities of protein bands were proportional to the amount of antigens applied to the gel. As little as 50 ng of the hydroxylase component could be detected by this procedure, and the color intensity of the bands was proportional to the amount of antigen over the range of 50 ng to 1 μg .

Quantitative determination of TCE degradation. The ability of *M. trichosporium* to degrade TCE after growth under various conditions was determined by measuring the disappearance of that compound by gas chromatography. All assays were performed in sealed serum bottles containing media that had been preincubated at 30°C . The culture sample to be tested was diluted to an optical density of 0.20 with prewarmed medium containing the same amount of copper sulfate as the original sample. The dilution was prepared in 120-ml glass serum bottles sealed with 20-mm Teflon-lined rubber septa (Baxter/American Scientific Products, Plymouth, Minn.). The resulting cell suspension was degassed to remove residual methane by evacuating the headspace with a vacuum pump and back filling with air. This process was repeated three times. The bottle was opened, and the culture was then transferred with disposable serological pipettes (Falcon) in 2-ml aliquots to 10 ml (total volume) glass serum bottles sealed with 20-mm Teflon-lined rubber septa.

The assay was initiated by adding 10 μl of a 4 mM aqueous stock solution of TCE to each cell suspension with a gas-tight syringe (The Hamilton Co., Reno, Nev.) to give a final concentration of 20 μM . The TCE stock solution was prepared in vessels containing no headspace to prevent the unwanted partitioning of that compound into the gas phase. The amount of TCE added to the assay bottles yields a concentration of 20 μM assumed to be in the aqueous phase

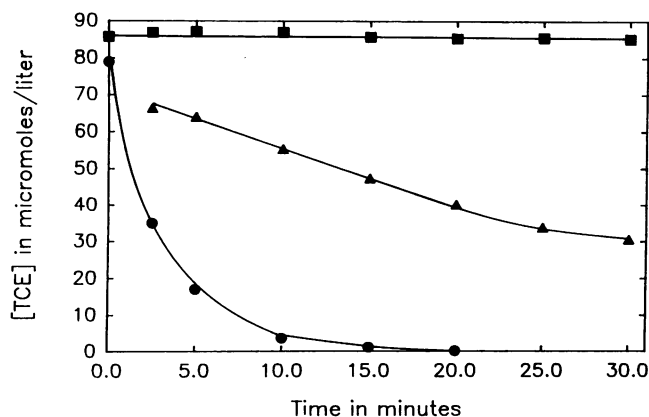


FIG. 1. Oxidation of TCE by an *M. trichosporium* culture. *M. trichosporium* cells were removed from a fermentor at a cell density of 0.8 g/liter for TCE oxidation assays. Symbols: ●, cell density of 0.8 g/liter; ▲, cell density of 0.08 g/liter; ■, heat-killed cell control.

as previously described (33). The initial concentration of TCE added to the vials was varied in some experiments.

Samples were inverted and incubated at 30°C on a platform shaker (200 rpm, 2.5-cm stroke length). Triplicate samples were sacrificed at designated intervals by adding 1 ml of *n*-pentane containing 1 mg of 1,2-dibromoethane per liter as an internal standard with a 1-ml gas-tight syringe (Hamilton). In all cases, a set of samples were sacrificed at time zero to serve as a control for monitoring TCE loss over time.

The bottles were centrifuged at $5,000 \times g$ for 10 min. A portion of the organic phase from each sample was transferred to 1-ml glass autosampling vials and diluted with *n*-pentane. Analyses were performed on a Hewlett-Packard 5790A series gas chromatograph equipped with a 30-m Non-Pakd RSL-160 polydimethylsiloxane thick-film capillary column (Alltech Associates, Inc., Deerfield, Ill.) and electron capture detection. Splitless injections of 1- μ l samples were made by a Hewlett-Packard 3390A injector. The following operating conditions were used: injector temperature, 150°C; column temperature, 35°C (1 min) to 120°C at 15°C/min; detector temperature, 250°C; hydrogen carrier gas flow, 8 ml/min. Under these conditions, TCE and 1,2-dibromoethane had retention times of 4.0 and 5.4 min, respectively.

Under conditions in which potential rate limitation due to the mass transport of TCE between the medium and air was a concern, the preceding method was modified to eliminate the headspace. The culture was adjusted to an optical density of 0.20, degassed, and saturated with air. Following aeration, the culture to be tested was added in a volume sufficient to completely fill glass vials (1.8-ml total volume). The vials were sealed with 11-mm Teflon-lined rubber septa. The assay was initiated by delivering TCE in an aqueous stock solution with a gas-tight syringe to the bottom of the upright vials. A second syringe was used to withdraw an equivalent volume of cell suspension from the top of the vials. This step maintained the pressure of the samples. Incubation was again performed with the vials inverted on a platform shaker operated at 200 rpm and 30°C.

Assays were terminated at the desired time points, usually every 2.5 min, by injecting *n*-pentane containing 1 mg of 1,2-dibromomethane per liter as an internal standard. This liquid-liquid extraction technique used 0.6 ml of *n*-pentane delivered via a gas-tight syringe to the inverted assay vials,

TABLE 1. TCE degradation by *M. trichosporium* cell suspensions^a

Sample no.	Culture density (A_{600})	TCE degradation (μ mol/h per g of cells [dry wt])
1	1.8	0
2	2.7	0
3	4.1	180
4	4.4	210
5	5.0	410
6	3.3	ND ^b
7	3.6	725
8	5.8	370

^a Cell growth and TCE degradation were measured every 24 h.

^b ND, Not determined.

while a second syringe was inserted to collect displaced solution. The organic layer was analyzed as described above.

Analysis by direct injection of the headspace was conducted to determine the kinetic course of TCE disappearance. Headspace gas samples of 50 μ l were removed with a gas-tight syringe at the desired times. The samples were injected immediately into a Hach Carle AGC 100 gas chromatograph equipped with a 1.8-m 0.1% AT-1000 on Graphpac-GC (80/100 mesh) column (Alltech Associates) and flame ionization detection. The instrument was operated isothermally at 150°C with a nitrogen carrier gas flow of 30 ml/min. TCE had a retention time of 2.1 min under these conditions. Quantitative determination of TCE was made by measuring peak heights, which were compared with the response obtained with standard TCE injections. This method yielded results comparable to those of the pentane extraction protocol.

Quantitative determination of other chlorinated aliphatic hydrocarbons. Incubations with other substrates were con-

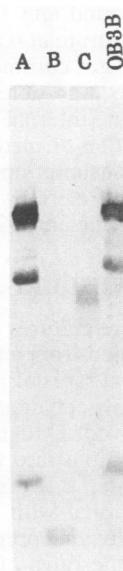


FIG. 2. Detection of soluble MMO components from *M. trichosporium* by Western blotting. Hydroxylase (2 μ g) (lane A), 1 μ g of component B (lane B), 1 μ g of reductase (lane C), and 200 μ g of cell extracts (lane OB3b) were each applied to lanes of an SDS-polyacrylamide gel. Mixed IgGs prepared against hydroxylase, reductase, and component B proteins at a dilution of 1:10,000 were used to detect the proteins.

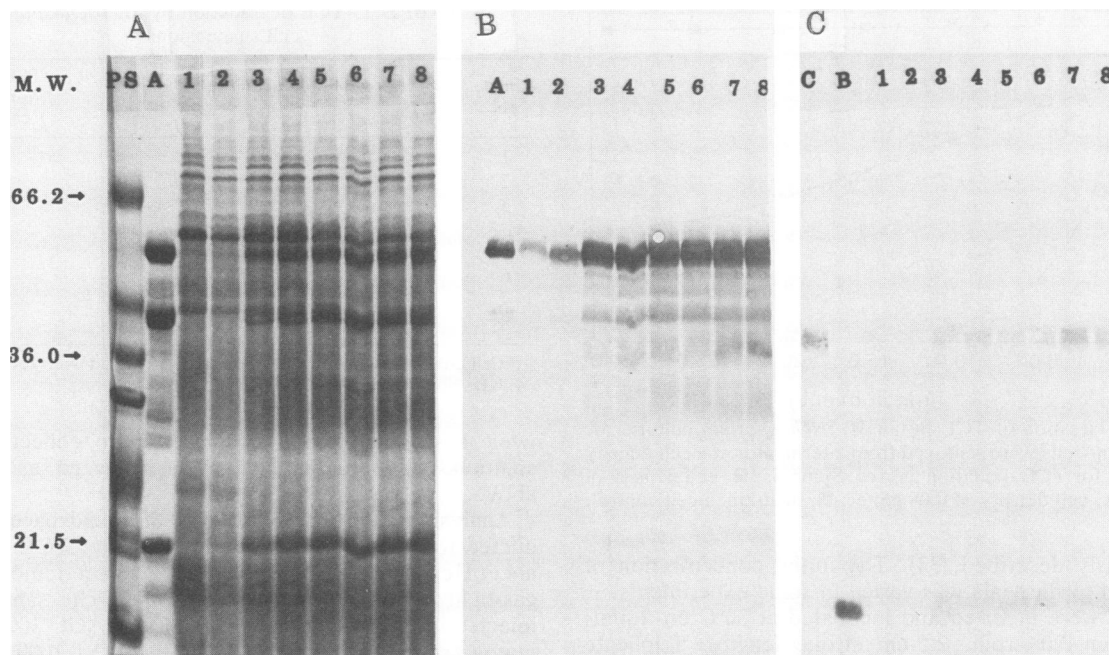


FIG. 3. SDS-PAGE and Western blot analysis of hydroxylase, reductase, and component B from a fermentor culture of *M. trichosporium*. (A) SDS-PAGE of solubilized proteins (200 μg) stained with Coomassie blue. (B and C) Western blot analysis of the same samples as in panel A and in Table 1, probed with antibodies prepared against purified hydroxylase (B) and reductase and component B (C). Characteristics of the cells extracted for each sample listed are indicated in Table 1. Approximately 200 μg of cell extracts was used in panels A and B, and 400 μg of cell extracts was used in panel C. Sigma low-molecular-weight (m.w.) protein standards (kilodaltons, 10^3) as well as hydroxylase, component B, and reductase standards are indicated as lanes PS, A, B, and C, respectively.

ducted as described for TCE. However, all compounds were delivered into incubation mixtures in methanol stock solutions prepared to concentrations of 40 mM. The compounds were delivered in 1- μl aliquots to give an initial concentration of 20 μM test compound and 12.4 mM methanol. The concentration of each substrate at 0 and 1 h was determined by pentane extraction and gas chromatography. The concentration of the compounds was determined by comparison with the 1,2-dibromoethane internal standard.

Quantitative determination of methane and methanol oxidation rates. Oxygen consumption due to methane and methanol utilization was measured with an oxygen electrode (Rank Bros., Bottisham, England) (25).

RESULTS

TCE degradation by pure cultures of *M. trichosporium*. *M. trichosporium* cells obtained from a pure culture grown in a fermentor degraded TCE at rates of 1.2 mmol/h per g of cells (dry weight) (Fig. 1). At low cell densities (0.08 g/liter), TCE was not completely removed. Under these conditions, TCE was removed at a rapid initial rate which declined after 25 min. In these experiments, a decrease in TCE oxidation rates over time was observed with both dilute resting cell suspensions and purified enzyme preparations. At higher cell densities, the decline in the rates of TCE degradation was not obvious. TCE degradation rates ranging between 500 $\mu\text{mol/h}$ per g of cells (dry weight) and 1.2 mmol/h per g of cells (dry weight) were routinely obtained.

Correlation of soluble MMO and TCE degradation. Samples from a fermentor culture were harvested daily, and TCE degradation activities were determined. In this experiment, specific rates of TCE degradation varied from undetectable

levels to 727 $\mu\text{mol/h}$ per g of cells (Table 1). All degradation rates were determined at a cell density of 0.20 and an initial TCE concentration of 20 μM . Figure 2 shows the methods used to detect components of soluble MMO in extracts of *M. trichosporium* cells. Samples of the same culture were used for SDS-PAGE and Western blot analysis (Fig. 3). A Western blot was conducted with antihydroxylase IgG, showing alpha, beta, and gamma subunits of the soluble MMO hydroxylase component (Fig. 3B). Relatively low levels of hydroxylase components were detected in samples 1 and 2 (Fig. 3B). No TCE degradation activity was observed in these two samples (Table 1). In samples 3 to 8, TCE degradation activities were approximately proportional to the amount of the hydroxylase subunits present in the cells. Hydroxylase subunit bands were observed in SDS gels stained with Coomassie blue (Fig. 3A) in samples 3 to 8. These bands were not visible in stained gels in samples 1 and 2. Bands representing component B and reductase were not apparent in the same gel, probably owing to the lower levels of these proteins in cells (Fig. 3A). However, these two components are clearly visible in samples 3 to 8 of a Western blot reacted with the IgG fractions of antisera prepared against component B and reductase (Fig. 3C). The amount of samples applied to these gels was twice as much as that used for the detection of hydroxylase components. Component B and reductase antigens were not detected in samples 1 and 2 (Fig. 3C).

Similar results were observed in a shake flask culture. Only samples taken from stationary-phase cultures and at high cell densities were able to degrade TCE. Soluble MMO components were detected by immunoblotting only in cells that were observed to oxidize TCE (data not shown).

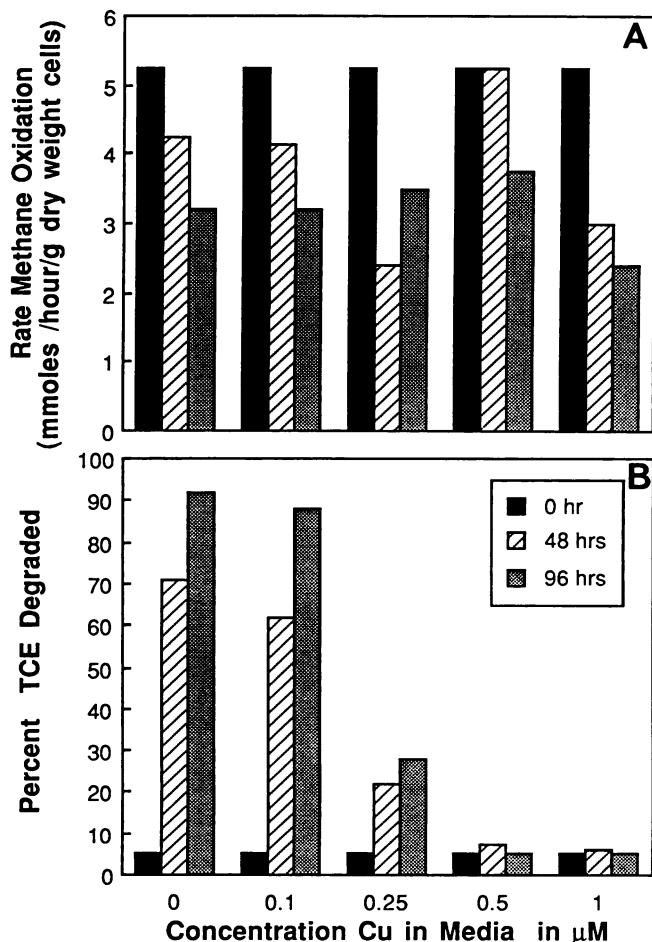


FIG. 4. Rate of methane oxidation and extent of TCE degradation by *M. trichosporium*. Cultures with copper concentrations varying from 0 to 1 μM were harvested at 0, 48, and 96 h after inoculation and assayed for TCE and methane oxidation. (A) Rates of methane oxidation; (B) percent TCE degradation.

A set of shake flask cultures containing mineral salts media with various copper concentrations were analyzed for TCE degradation activity and specific rates of methane oxidation. Cells were harvested during exponential growth at cell densities of 0.35 to 0.45 g/liter, and the relative amount of soluble MMO as determined by Western blot analysis indicated that soluble MMO was detectable in cells grown in the presence of $\leq 0.25 \mu\text{M}$ copper (data not shown). TCE removal was determined after a 3-h incubation period.

The specific rates of methane oxidation were not significantly different in cultures grown in the presence of different amounts of copper over the range of 0.00 to 1.00 μM (Fig. 4A). The extent of TCE oxidation was significantly less in cultures grown with $\geq 0.25 \mu\text{M}$ added copper (Fig. 4B). It was apparent that the ratio of the specific rates of TCE oxidation to methane oxidation increased with culture age (and cell densities) in cultures grown in media containing 0.25 μM or lower concentrations of copper (Fig. 4A and B).

The data presented in Fig. 5 showed that TCE oxidation occurred during growth of *M. trichosporium* at densities of 1.0 g/liter and greater in the mineral salts medium containing an initial copper concentration of 0.25 μM . The specific rates of methane and methanol oxidation did not vary significantly during this experiment. The hydroxylase components were

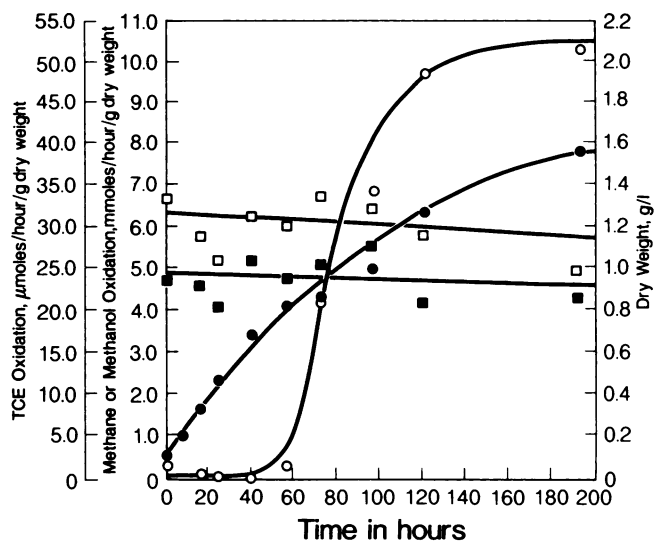


FIG. 5. Rates of TCE, methane, and methanol oxidation during growth of an *M. trichosporium* culture containing 0.25 μM copper. Specific rates of TCE (\circ), methane (\blacksquare), and methanol oxidation (\square) were plotted against time of culture growth. The growth curve (\bullet) was also plotted. l, Liter.

detected in Coomassie blue-stained SDS-polyacrylamide gels in samples removed at 96 h and later (Fig. 6A). The hydroxylase subunit antigens were apparent at 72 h (0.8 g of cells [dry weight] per liter). Hydroxylase components were detected with antihydroxylase serum in the inoculum (Fig. 6B), which is probably due to the inoculum culture being grown to an elevated cell density. Furthermore, the cells used for the inoculum oxidized TCE at low rates. As the culture grew, soluble MMO was diluted out. Because the same amount of cell material was used to prepare each sample, the amounts of hydroxylase components in samples 3 to 5 were below detection limits. The antigens were detectable later during the growth of the culture when they made up an increased proportion of the biomass.

Degradation of chlorinated aliphatic hydrocarbons by *M. trichosporium*. The capacity of *M. trichosporium* to degrade the full range of chlorinated ethylenes was tested. This organism was capable of degrading vinyl chloride, 1,1-dichloroethylene, *cis*-1,2-dichloroethylene, *trans*-1,2-dichloroethylene, and TCE over the 1-h incubation period (Table 2). However, tetrachloroethylene proved to be refractory under these test conditions. The presence of methanol reduced the efficiency of TCE degradation by approximately 40%.

DISCUSSION

In these studies, *M. trichosporium* degraded TCE at rates exceeding 1.2 mmol/h per g of cells (dry weight), a rate more than 10-fold higher than data published in previous reports (3, 19, 33). For example, in a previous report in which initial rate and cell dry weight, or protein used, are available, *Pseudomonas putida* F1 was shown to oxidize TCE at a maximal rate of 0.06 mmol/h per g of cells (dry weight) (33). Other comparisons are complicated by the lack of uniformity in experiments. Factors such as cell mass, TCE concentration, and the solvent used to prepare TCE stock solutions influence rate determinations. There have been previous reports that pure cultures of methanotrophs degrade TCE,

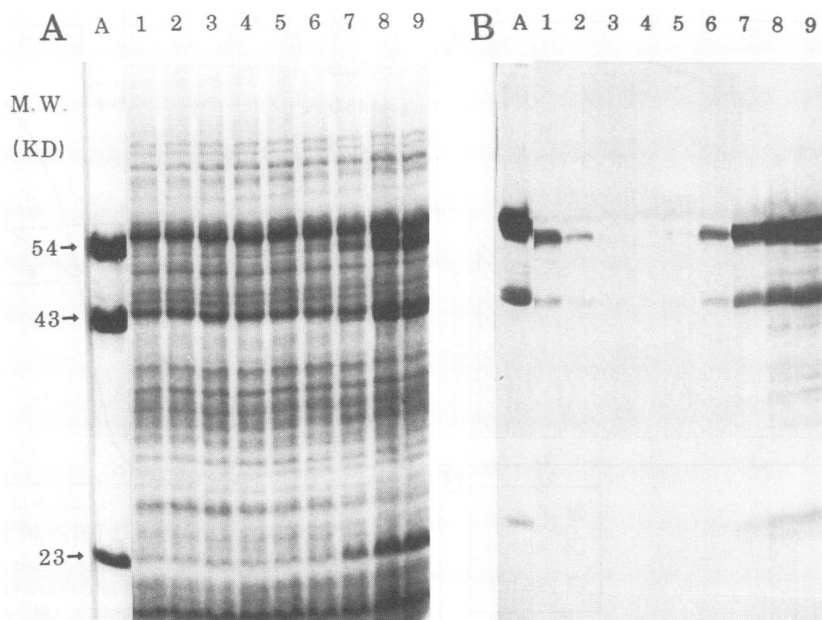


FIG. 6. SDS-PAGE and Western blot analysis of hydroxylase of samples from a culture of *M. trichosporium* grown with 0.25 μM copper. Samples were taken from the culture described in the legend to Fig. 5. (A) SDS-polyacrylamide gel stained with Coomassie brilliant blue R showing protein profile of each sample. (B) Western blot analysis of hydroxylase, indicated as lane A, was used as the standard. The molecular weights (m.w.) of hydroxylase subunits are indicated at the left of panel A. Samples were harvested at 0, 16, 24, 40, 56, 72, 96, 120, and 192 h after inoculation, corresponding to samples 1 through 9 respectively. KD, Kilodaltons.

but the microorganisms used had not been previously characterized (19). This is the first detailed study employing a methanotroph that has been extensively investigated by genetic, biochemical, and immunological methods.

The sensitivity of the Western immunoblotting assay, which detected MMO proteins at a level of 50 ng to 1.0 μg , allowed the detection of soluble MMO components in approximately 1 to 2 mg of cells. By simultaneously conducting TCE degradation assays and immunoblotting of MMO components, we were able to demonstrate that the rapid degradation of TCE by *M. trichosporium* occurred only when soluble MMO was detectable. These data did not permit us to discern whether the particulate MMO was inactive in TCE degradation or whether it catalyzed a low rate of TCE oxidation. However, the results indicated that soluble MMO was required for the high rates of TCE degradation that we observed in *M. trichosporium*.

Immunoblotting also permits the determination of specific enzymes without relying on molecular weight values as the sole characteristic for differentiating proteins. The molecular masses of soluble MMO hydroxylase subunits from different methanotrophs ranged from 53 to 60 kDa for alpha, 40 to 45 kDa for beta, and 17 to 23 kDa for gamma (6, 7, 14, 24, 35).

TABLE 2. Degradation of chlorinated ethylenes by resting cell suspensions of *M. trichosporium*

Substrate (20 μM initial concn)	% Decrease after 1 h
Tetrachloroethylene	<5
TCE	56
<i>cis</i> -1,2-Dichloroethylene	100
<i>trans</i> -1,2-Dichloroethylene	30
1,1-Dichloroethylene	42
Vinyl chloride	100

The molecular masses of major particulate MMO components have been reported as 35 ± 3 and 45 ± 3 kDa for *Methylococcus capsulatus* and 47 kDa for *M. trichosporium* (1, 30). Without applying immunoblotting techniques, it would be difficult to determine the identity of protein bands in the 35- to 45-kDa range in a stained SDS-polyacrylamide gel and to assign them to the soluble or particulate MMO enzyme systems.

The synthesis of soluble MMO in cultures of *M. trichosporium* can be manipulated by varying the copper concentration in minimal medium. Because the effect of copper concentration on the synthesis of soluble MMO can only be observed at concentrations between 0.00 and 0.50 μM , great care must be exercised to minimize contamination of water, culture flasks, and chemicals by exogenous copper that can mask the effects reported here. In cultures of *M. trichosporium* with different copper concentrations, TCE degradation activities over time correlated very well with the amounts of soluble MMO components.

These studies suggest that TCE or an oxidation product may be toxic to *M. trichosporium*. Alternatively, the supply of reductant available to the cells may be limiting. The rate of TCE removal decreases during the metabolism of that compound. This effect is readily apparent when low cell densities are used in assays. In this case, the total amount of TCE removed by the culture is limited despite high initial rates of oxidation. Further progress in the development of methanotrophs as a means of degrading chlorinated solvents is predicated on maintaining high rates of biodegradation over extended periods.

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