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Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity

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Abstract

Methylosinus trichosporium OB3b biosynthesizes a broad specificity soluble methane monooxygenase that rapidly oxidizes trichloroethylene (TCE). The selective expression of the soluble methane monooxygenase was followed in vivo by a rapid colorimetric assay. Naphthalene was oxidized by purified soluble methane monooxygenase or by cells grown in copper-deficient media to a mixture of 1-naphthol and 2-naphthol. The naphthols were detected by reaction with tetrazotized *o*-dianisidine to form purple diazo dyes with large molar absorptivities. The rate of color formation with the rapid assay correlated with the velocity of TCE oxidation that was determined by gas chromatography. Both assays were used to optimize conditions for TCE oxidation by *M. trichosporium* OB3b and to test several methanotrophic bacteria for the ability to oxidize TCE and naphthalene.

Abbreviations: A_{600} – absorbance due to cell density measured at 600 nm, HPLC – high pressure liquid chromatography, NADH – reduced nicotinamide adenine dinucleotide, SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis, sMMO – soluble methane monooxygenase, TCE – trichloroethylene

Introduction

Trichloroethylene (TCE) is an Environmental Protection Agency Priority Pollutant that is found widespread in groundwater (Storck 1987). It is suspected carcinogen (Miller & Guengerich 1983). Concern over TCE contamination of drinking water is heightened by the observation that TCE can be transformed to vinyl chloride by some anaerobic bacteria (Vogel & McCarty 1985). Vinyl chloride is mutagenic and carcinogenic in humans (Maltoni & Lefemine 1974).

In contrast to anaerobes that reductively dehalogenate TCE, several aerobic bacteria have been demonstrated to utilize oxygenases in the biodegradation of TCE. Both monooxygenase and dioxygenase enzymes have been implicated in TCE oxidation. Toluene dioxygenase (Nelson et al. 1988; Wackett & Gibson 1988; Zylstra et al. 1989), toluene monooxygenases (Shields et al. 1989; Winter at al. 1989), ammonia monooxygenase (Arciero et al. 1989), propane monooxygenase (Wackett et al. 1989), and methane monooxygenase (Oldenhuis et al. 1989; Tsien et al. 1989; Fox et al. 1990) have all been demonstrated to oxidize TCE. The rate of TCE degradation by *Methylosinus trichos-*

porium OB3b, which synthesizes methane monooxygenase, exceeded that of currently known bacteria containing other oxygenase systems (Tsien et al. 1990). The rapid oxidation rates of several chlorinated alkenes and alkanes, and the availability of natural gas as a growth substrate combine to make methanotrophs attractive for bioremediation.

M. trichosporium OB3b and other methanotrophs have the capacity to synthesize both soluble and membrane-bound forms of methane monooxygenase. Investigations with membrane and soluble cell fractions indicate that soluble methane monooxygenase (sMMO) has a wider substrate specificity, suggesting that this form of the enzyme has broader applications in bioremediation. Recently, it has been shown that high rates of TCE oxidation by *M. trichosporium* OB3b are correlated with the production of sMMO (Oldenhuis et al. 1989; Tsien et al. 1989). In these studies, the biosynthesis of sMMO was derepressed by limiting the amount of $CuSO_4$ available to the bacterium in the growth medium. The formation of sMMO by *M. trichosporium* OB3b was monitored directly using antibodies raised against purified protein components in a Western blotting protocol (Tsien et al. 1989). Independently, Oldenhuis and coworkers (1989) followed the biosynthesis of sMMO by assaying the oxidation of cyclohexane to cyclohexanol, a reaction reported to be catalyzed exclusively by sMMO and not by the membrane-bound MMO (Burrows et al. 1984). In the former case, the rigorous detection of sMMO antigens required SDS-PAGE, immunoblotting, and a colorimetric assay for antigen-antibody complexes. The detection of in vivo activity with cyclohexane required diethyl ether extraction of whole cell reaction mixtures and the further analysis of ether extracts for the presence of cyclohexanol by gas chromatography. Both of these procedures are time-consuming and require equipment that would preclude their easy application in the field. In this report, we describe the use of a rapid technique that requires no instrumentation for detecting the presence of sMMO, thus facilitating studies on TCE biodegradation by methanotrophs. The protocol is based on the ability of sMMO to oxidize the bicyclic aromatic hydrocarbon naphthalene to 1-naphthol and 2-naphthol which react spontaneously with tetrazotized *o*-dianisidine to form intense purple-colored products. The colorimetric and gas chromatography assays were used to determine which methanotrophs synthesize sMMO and degrade TCE at rapid rates. In addition, optimum conditions for TCE oxidation by *M. trichosporium* OB3b were rapidly obtained using the colorimetric assay and confirmed by other analytical methods.

Materials and methods

Materials

Trichloroethylene and naphthalene were purchased from Adrich Chemical Company, Milwaukee, Wisconsin. 2-Naphthol was obtained from Allied Chemical Company, Morristown, New Jersey. 1-Napththol and tetrazotized *o*-dianisidine were acquired from Sigma Chemical Company, St. Louis, Missouri. All other chemicals were of the highest purity available and used without further purification.

Organisms and culture conditions

The microorganisms and their sources are described in Table 1. All strains were grown in 500 ml triple baffled Erlenmeyer flasks on the same mineral salts media (Cornish et al. 1984) containing 0 μM or 1 μM copper sulfate. Trace metal ions were eliminated from water and glassware (Tsien et al. 1989) prior to inoculation. The liquid volume did not exceed 25% of the total flask volume. Cultures were incubated at 30° C with shaking at 200 rpm (2.5 cm stroke length) under methane-air (1 : 4 [vol/vol]) at 1 atm. The gas mixture was replenished twice daily. The A_{600} of cultures and cell suspensions was measured with a Spectronic 20D spectrophotometer (Milton Roy Co., Rochester, NY). All cultures were grown to an A_{600} of 1.00 before the assays were performed. *M. trichosporium* OB3b was also grown in chemostat culture for use in the optimization studies (Tsien et al. 1989).

Colorimetric assay

The ability of the bacterial strains to transform naphthalene was tested by incubating each culture with that compound and reacting the products with tetrazotized *o*-dianisidine. The reaction of tetrazotized *o*-dianisidine with an aqueous solution of 1- or 2-naphthol produces a violet adduct (Wackett & Gibson 1983). Each culture sample was diluted to an A_{600} of 0.20 with prewarmed medium containing the same amount of copper sulfate as the original sample in a 120 ml (total volume) glass serum bottle sealed with a 20 mm Teflon-lined rubber septum (Baxter/American Scientific Products, Plymouth, MN). The resulting cell suspension was degassed to remove residual methane (Tsien et al. 1989). The culture was transferred with disposable serological pipettes (Falcon; Becton Dickinson Labware, Oxnard, CA) in 1 ml aliquots to 10 ml (total volume) glass serum bottles containing crystalline naphthalene. The naphthalene was provided in amounts sufficient to give a saturated aqueous solution. The bottles were sealed with 20 mm Teflon-lined rubber septa.

Samples were inverted and incubated at 30° C on a platform shaker (200 rpm, 2.5 cm stroke length).

Table 1. Correlation of colorimetric assay and GC assay for TCE degradation.

Methanotroph[1]	Source	Type	Naphthalene oxidation[2]	TCE oxidation[3]	SDS-PAGE[4]
M. trichosporium OB3b	R. Whittenbury[5]	II			
0 μM $CuSO_4$			+	+	+
1 μM $CuSO_4$			–	–	–
Methylosporovibrio methanica 81Z	This laboratory	II			
0 μM $CuSO_4$			+	+	+
1 μM $CuSO_4$			–	–	–
Methylosinus sporium #27	Y. Trotsenko[6]	II			
0 μM $CuSO_4$			–	–	–
1 μM $CuSO_4$			–	–	–
Methylocystis parvis OBBP	R. Whittenbury[5]	II			
0 μM $CuSO_4$			–	–	–
1 μM $CuSO_4$			–	–	–
Methylocystis pyriformis #14	Y. Trotsenko[6]	II			
0 μM $CuSO_4$			–	–	–
1 μM $CuSO_4$			–	–	–
Methylomonas methanica	R. Whittenbury[5]	I			
0 μM $CuSO_4$			No growth	No growth	No growth
1 μM $CuSO_4$			–	–	–
Methylomonas albus BG8	R. Whittenbury[5]	I			
0 μM $CuSO_4$			No growth	No growth	No growth
1 μM $CuSO_4$			–	–	–

[1] All strains were grown to an A_{600} of 1.00 before analysis.
[2] As evidenced by a purple color with the diazonium reagent.
[3] Greater than 10% TCE oxidized over 1 h with a starting TCE conc. of 20 μM.
[4] Evidence for presence or absence of sMMO by SDS-PAGE as described in Methods.
[5] R. Whittenbury, University of Warwick, Coventry, England.
[6] Y. Trotsenko, USSR Academy of Sciences, Puschino, Moscow Region, 142292, USSR.

Samples were sacrificed at time intervals by adding 100 μl of freshly hydrated tetrazotized *o*-dianisidine (4.21 mM). Heat-killed and sterile media controls were also tested. If formed, the colored product was clearly visible to the naked eye or readily monitored by recording the absorption spectrum over the range of 430 to 650 mm with a Beckman DU-70 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). This adduct, as well as azo dyes formed from synthetic 1-naphthol and 2-naphthol, proved to be unstable in the mineral salts media, and phosphate and organic buffers used in these studies. However, the intensity of color formation immediately following the addition of tetrazotized *o*-dianisidine was proportional to the naphthol concentration (Wackett & Gibson 1983).

Identification of products from naphthalene metabolism

In order to determine the compounds which reacted to form the colored adduct, cell suspensions of chemostat-grown *M. trichosporium* OB3b incubated with naphthalene were extracted with 1.0 ml of ethyl acetate rather than adding the diazonium salt reagent. High-pressure liquid chromatography (HPLC) was used to separate the metabolites. HPLC was performed on a component system consisting of a two-position electric actuator (Valco Instruments Company, Inc., Houston, TX), a SP8800 ternary LC pump (Spectra-Physics, San Jose, CA), a model 204 UVIS variable/dual wavelength programmable detector (Linear Instruments Corporation, Reno, NV) and a SP4400 integrator (Spectra-Physics). Each 20 μl sample was analyzed using a 250 mm × 4.6 mm Adsorbosphere C18 5U column (Alltech Associates, Deerfield, IL). The metabolites were separated by gradient elution. Following injection, the initial solvent composition of 60% methanol in water was maintained for 5 min. Then, a 10 min linear gradient was run to render a final methanol concentration of 95%. The flow rate of the solvent was 1.0 ml/min. The wavelength used in detection was 270 nm.

Enzyme reaction conditions

The three soluble MMO components, purified to homogeneity from *M. trichosporium* OB3b (Fox & Lipscomb 1988; Fox et al. 1989), were the generous gift of B. Fox and J. Lipscomb, Department of Biochemistry, Medical School, University of Minnesota. Naphthalene crystals were added to a 10 ml glass serum bottle containing 1 nmol hydroxylase, 0.2 nmol component B, and 2 nmol reductase in 1.0 ml of 25 mM MOPS (3-[N-morpholino] propane sulfonic acid), pH 7.5. The bottle was sealed with a 20 mm Teflon-lined rubber septum and the reaction initiated by adding 15 μl of 10 mM NADH. The bottle was shaken at 30° C for 15 min. In some experiments, the products were analyzed by HPLC as described in the preceding section. Control experiments were conducted for the colorimetric assay *in vitro* by omitting each of the purified sMMO components or NADH.

Parameters affecting TCE degradation

TCE degradation assays were performed as described by Tsien et al. (1989). Analysis by direct injection of the headspace from 10 ml (total volume) glass serum bottles into a gas chromatograph was conducted to determine the kinetic course of TCE disappearance.

When determining the effect of cooxidizable substrates on TCE degradation, culture samples were diluted to an A_{600} of 0.20, degassed to remove residual methane, transferred in 2 ml aliquots to the reaction bottles, and provided with TCE (usually 200 μM) and a cosubstrate to supply the reductant required for the reaction. Sodium formate or methanol were delivered from 200 mM aqueous stock solutions. Methane was provided as a gas to fill a percentage of the total headspace volume. TCE disappearance was determined at intervals over a 2-hour time period.

The effect of pH was determined by harvesting chemostat-grown *M. trichosporium* OB3b cells at 10,000 × g in a centrifuge, resuspending the cells in a minimal amount of 50 mM potassium phosphate

buffer (pH 7.0), diluting this dense suspension to an A_{600} of 0.20 in 50 mM potassium phosphate buffer of the desired pH and transferring the samples to glass reaction bottles. The reaction vials were provided with 0 or 5 mM sodium formate and 200 μM TCE. The TCE remaining in the headspace was measured over a 2-hour time period.

The effect of sodium chloride was determined by suspending chemostat-grown *M. trichosporium* OB3b cells in 50 mM potassium phosphate buffer (pH = 7.0) containing NaCl from 0 to 1.0 M. TCE degradation was tested with 0 or 5 mM sodium formate as a cosubstrate.

The effect of cell concentration was determined by harvesting chemostat-grown *M. trichosporium* OB3b, resuspending the cells in a small amount of minimal salts media and diluting the dense suspension to an A_{600} of 0.05 to 8.00. TCE was provided and the rate of its disappearance measured. No substrate other than TCE was present in order to test the effect of endogenous reductants in the cells.

To test the effect of temperature on reaction rates, bottles were preincubated at the desired temperature in a constant temperature bath for 30 minutes prior to initiating the assay with the addition of cells suspended in minimal salts media. The rate of TCE disappearance was measured over a 15 minute period.

In the determination of V_{max} and K_m, the conditions of 30° C and pH 7.0 were maintained. Sodium formate was provided at a concentration of 25 mM to ensure that TCE oxidation would not become reductant-limited. The amount of TCE provided was varied by delivering different volumes of the 4 mM aqueous stock solution to the reaction vials. Chemostat-grown *M. trichosporium* OB3b cells diluted in minimal salts media to A_{600} of 0.20 were delivered in 2 ml aliquots and sealed in 10 ml glass serum bottles. The disappearance of TCE was monitored by headspace analysis. The concentration of TCE available to the cells in the aqueous phase was calculated from Henry's Law (Gossett 1987).

Different methanotrophs were anlyzed for TCE degradation at TCE concentrations of 20 and 200 μM, with 0 and 25 mM sodium formate in minimal salts media (pH 7.0) at 30° C.

Quantitative determination of methane, methanol and formate oxidation rates

All methanotrophs tested for TCE and naphthalene oxidation exhibited oxygen consumption due to methane, methanol and formate measured with an oxygen electrode (Rank Bros, Bottisham, England) (Patt et al. 1974). This procedure ensured that cells used in different experiments were in a similar metabolic state.

SDS-PAGE and Western blot analysis (immunoblotting)

The presence of soluble MMO in all methanotrophs was examined after separating cell proteins with discontinuous SDS-PAGE (Laemmli 1970). Soluble MMO in type II methanotrophs was detected by immunoblotting with antibodies prepared against sMMO components from *M. trichosporium* OB3b (Tsien et al. 1989). Soluble MMO in type I methanotrophs was determined by SDS-PAGE without further manipulation (Dalton et al. 1984; Tsien & Hanson, unpublished data).

Results

Colorimetric assay for soluble methane monooxygenase

In preliminary experiements, *M. trichosporium* OB3b was grown on media containing 0 or 1 μM $CuSO_4$ and each culture was incubated with naphthalene for 30 min. Only the copper-deficient culture was observed to turn deep purple following the addition of tetrazotized *o*-dianisidine. In a subsequent test to monitor TCE oxidation by gas chromatography, the copper-deficient culture alone was shown to be active. Previously, Dalton and coworkers (1981) had demonstrated that cell-free

extracts obtained from *Methylococcus capsulatus* oxidized naphthalene yielding 1-naphthol and 2-naphthol. Thus, the observations above with *M. trichosporium* OB3b suggested that naphthalene was being selectively oxidized to naphthol(s) by the sMMO and the naphthol(s) reacted with tetrazotized *o*-dianisidine to yield purple diazo dyes. This hypothesis was tested directly using purified sMMO enzyme components from *M. trichosporium* OB3b. The three sMMO protein components were incubated in MOPS buffer with NADH and naphthalene for 15 minutes and then developed with tetrazotized *o*-dianisidine as described in the Materials and methods. A purple color was observed. No color was formed in any of the controls lacking one or more of the MMO components or NADH.

The purple product(s) formed in the enzyme reaction mixture above was analyzed spectrophotometrically and shown to have an absorption maximum at 528 nm. When *M. trichosporium* OB3b cells were incubated with naphthalene and the oxidation products were reacted with tetrazotized *o*-dianisidine, the resultant product(s) also absorbed maximally at 528 nm. It is known that phenols couple with diazonium salts to generate azo dyes (Saunders 1949; Parsons et al. 1955). Synthetic 1-naphthol and 2-naphthol each reacted with the diazonium salt to produce compounds with absorption maxima at 528 nm and 527 nm, respectively. Thus, either 1-naphthol, 2-naphthol or a mixture were likely to be the enzyme products responsible for the color reaction.

The products of naphthalene oxidation by sMMO were further analyzed by HPLC. *M. trichosporium* OB3b cell suspensions or purified sMMO components were incubated with naphthalene, extracted with ethyl acetate, and the organic phase was analyzed by reverse phase chromatography. Two polar peaks were observed in chromatograms derived from extracts of cells or purified enzyme components but not in a control with an incomplete enzyme system. The faster migrating compound ($R_t = 7.9$ min) was identified as 2-naphthol and the peak eluting second ($R_t = 8.8$ min) was shown to correspond to 1-naphthol. The products were identified on the basis of their chromatographic properties by HPLC and by identical properties with authentic standards using ultraviolet spectroscopy. Zero time cell incubations and incomplete enzyme controls demonstrated that the products are derived from naphthalene and required a fully active sMMO enzyme system for their formation. Furthermore, a similar ratio of 1-naphthol and 2-naphthol was observed with whole cell and with purified enzyme incubations suggesting that sMMO alone is responsible for naphthol formation in vivo.

The data indicate that coupled azo dyes from both 1-naphthol and 2-naphthol contribute to the absorption band at 528 nm observed in whole cell and enzyme reaction mixtures. The azo dyes are sufficiently unstable during chromatography to preclude their isolation and physical characterization. The extinction coefficient of the diazo product from 1-naphthol is known to be 38,000 cm^{-1} M^{-1} (Wackett & Gibson 1983), allowing for highly sensitive determination of in vivo sMMO activity.

Correlation of TCE and naphthalene oxidation by methanotrophs

The utility of rapid method described above was extended by the observation that methanotrophs reacting positively in the naphthalene assay also oxidized TCE rapidly as evidence by gas chromatography (Table 1). In these experiments, both type I and type II methanotrophs were grown on media containing 0 or 1 μM copper sulfate to express selectively sMMO or membrane-bound MMO, respectively. *M. trichosporium* OB3b has previously been shown to biosynthesize sMMO under copper-limited growth conditions and the correlation with the naphthalene assay was established in this study. An additional type II methanotroph, *Methylosporovibrio methanica* 81Z, also oxidized both TCE and naphthalene when grown on copper-deficient media. Other type II organisms, *Methylocystis parvis* OBBP, *Methylosinus sporium* #27 and *Methylocystis pyriformis* #14 grew equally well in both media but failed to oxidize TCE or

naphthalene. The culture conditions required for different type II methanotrophs to derepress sMMO biosynthesis may not be identical, necessitating a direct assay for sMMO proteins by SDS-PAGE as described below. *Methylomonas* strains are thought to produce a particulate MMO only (Anthony 1986). As expected, both tested strains were negative in the TCE and naphthalene assays when grown with copper, and they failed to grow when copper was not supplied to the cultures.

The TCE and naphthalene assays, that appear to be specific for sMMO in a broad spectrum of methanotrophs, were further calibrated by using gel electrophoresis to probe directly for the sMMO protein components. In these experiments, SDS-PAGE of whole cell protein extracts were visualized by Coomassie Blue staining and using the Western blotting technique with antibodies raised against purified sMMO components from *M. trichosporium* OB3b. As shown in Table 1, there was complete agreement between positive and negative results in the TCE and naphthalene assays, and the ability to visualize sMMO protein or antigen bands by electrophoretic methods.

These data further suggest that only the soluble methane monooxygenase will catalyze appreciable oxidation of TCE and naphthalene, allowing the colorimetric assay to be used as a rapid method to monitor biodegradation potential in a broad range of methanotrophs.

Environmental parameters affecting TCE degradation by M. trichosporium *OB3b*

The optimization of TCE biodegradation can be facilitated by monitoring the presence of sMMO in cells using the colorimetric test. The data can then be applied to the degradation of TCE monitored directly by gas chromatography. As shown in Table 2, the effects of pH, sodium chloride concentration, temperature and cell density on TCE degradation were examined. In general, the conditions examined for in vivo activity showed broad optimum ranges. The rate was observed to increase over the full range of cell densities examined. High initial rates of TCE oxidation were observed over a temperature range of 28–40° C. However, at 40° C the high rate declined rapidly, perhaps due to enzyme or general metabolic instability at the elevated temperature.

Effect of cooxidizable substrates on TCE oxidation

In a previous study (Tsien et al. 1989), the rate of TCE oxidation was observed to decline over time at high TCE concentrations. The hypothesis that cells might become limited for intracellular NADH to support sMMO was investigated. Experiments were conducted with the cooxidizable C_1 substrates methane, methanol and formate in admixture with cells and naphthalene. The colorimetric assay indicated that intermediate concentrations of methane and methanol, and high concentrations of formate significantly stimulated soluble methane monooxygenase activity in vivo. The effect of methane, methanol and formate on TCE oxidation was also investigated by gas chromatography. As shown in Fig. 1, methane stimulated TCE degradation when added as 5 or 10% of the gas phase above the cell suspension but the rates declined at higher concentrations. Similarly, methanol was observed to show significant stimulation of TCE oxidation at concentrations of 0.1–1.0 mM but revealed inhibitory effects at higher concentrations. Formate, in contrast, stimulated TCE degradation significantly at concentrations of ≥ 5 mM.

Effect of TCE concentration on reaction velocity

Purified sMMO from *M. trichosporium* OB3b exhibits hyperbolic saturation kinetics with TCE as the substrate with a K_m of 35 μM and a V_{max} of 682 nmol/min per mg protein (Fox et al. 1990). The in vivo kinetic properties were investigated using cells stimulated with 25 mM sodium formate, which should maintain the intracellular NADH pools at saturating levels. As observed with purified enzyme, the in vivo system exhibits saturation behavior, although the concentrations are higher than in

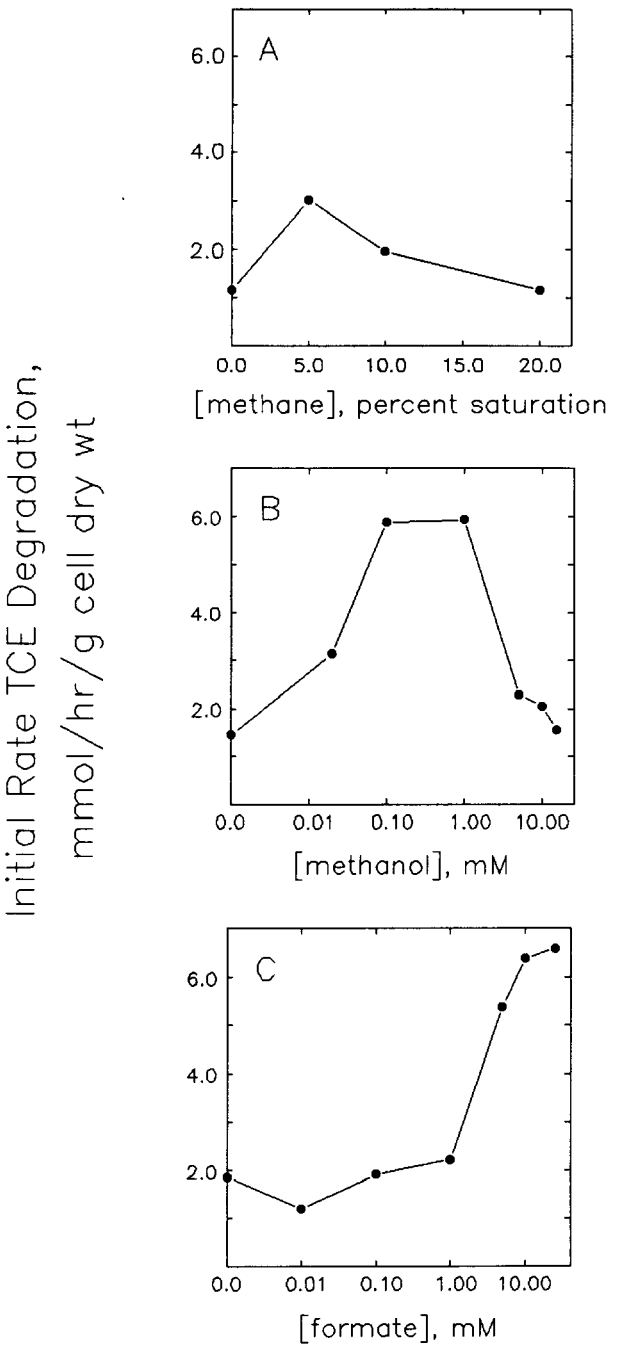

Fig. 1. Optimization of TCE degradation of *M. trichosporium* OB3b cell suspensions by addition of a cooxidizable C_1 substrate. Methane saturation in water at 30° C is 1.19 mM (Dean 1985).

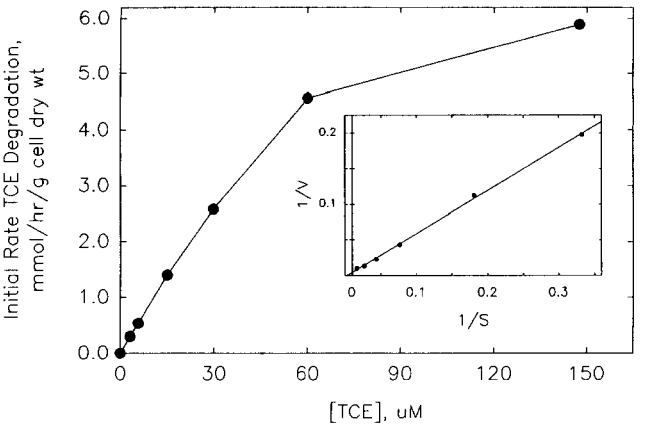

Fig. 2. Substrate saturation curve for initial rates of TCE oxidation by *M. trichosporium* OB3b. Henry's Law was used to calculate the concentration of TCE in the aqueous phase.

vitro (Fig. 2). The apparent K_m was determined to be 138 μM and the apparent V_{max} was 13.2 mmol of TCE per h per g dry weight of cells.

Discussion

Previous studies demonstrated that methanotrophs can oxidize monocyclic aromatic hydrocarbons (Colby et al. 1977; Higgins et al. 1980) and bicyclic aromatic hydrocarbons (Dalton et al. 1981). Burrows et al. (1984) showed that sMMO is responsible for aromatic hydrocarbon oxidation, while the particulate MMO is inactive with aromatic substrates. In the present study, the bicyclic aromatic hydrocarbon naphthalene was oxidized by purified sMMO from *M. trichosporium* OB3b to 1-naphthol and 2-naphthol. Methanotrophs expressing sMMO also produces 1-naphthol and 2-naphthol. The reaction of naphthols with tetrazotized *o*-dianisidine was utilized as a sensitive and rapid colorimetric indicator of sMMO activity to monitor the biodegradative potential of methanotrophs. The high extinction coefficient per mol of 1-naphthol (38,000 cm^{-1} M^{-1}) reported for a naphthol azo dye imparts the sensitivity to detect sMMO activity even under suboptimal conditions. The color reaction had been used previously to detect 1-naphthol produced during the fungal oxidation of naphthalene (Wackett & Gibson 1983).

Naphthalene and TCE assays were used to dem-

onstrate that some methanotrophs express sMMO and oxidize TCE at rapid rates. The method of growth may preclude the derepression of sMMO biosynthesis in some strains. However, the results were shown to correlate with the previously described use of SDS-PAGE and Western immunoblotting to detect sMMO (Tsien et al. 1989) further validating the colorimetric assay.

M. trichosporium OB3b is a well-studied, easy to grow type II methanotroph that has been demonstrated to oxidize TCE at rapid rates by two independent groups (Oldenhuis et al. 1989; Tsien et al. 1989). Thus, it was selected for use in optimization experiments. The versatile response of *M. trichosporium* OB3b, maintaining high TCE degradation rates under a broad range of conditions (Table 2), indicates great potential in bioremediation applications. Previous studies raised the issue of rate diminution at high TCE concentrations or low cell concentrations, suggesting that enzyme inactivation or depletion of intracellular NADH might preclude TCE degradation over extended periods of time (Tsien et al. 1989). This question was addressed (Oldenhuis et al. 1989 and this study) by demonstrating that oxidizable C_1 substrates stimulate the initial rate and extent of TCE oxidation by *M. trichosporium* OB3b. Methane, methanol and formate are oxidized by methanotrophs with the potential to generate NADH which is a required cosubstrate for sMMO activity. Other potential substrates, such as acetate, succinate, β-hydroxybutyrate and glucose, did not markedly stimulate TCE degradation (Brusseau, Hanson & Wackett, unpublished data). The maintenance of intracellular NADH pools probably underlies the stimulatory effects observed here. Methane and methanol are also substrates for sMMO. Thus, they can compete with TCE for occupancy of the enzyme active site and cause inhibition of TCE oxidation (Fig. 1). This interpretation is supported by the observation that inhibition occurs at concentrations just above the K_m of the *M. trichosporium* sMMO for methane (25 μM, Fox et al. 1989) and above the K_m of the *M. capsulatus* sMMO for methanol (0.95 mM) (Colby et al. 1977).

Unlike methane and methanol, formate stimulated TCE oxidation markedly but did not inhibit at high concentrations up to 100 mM. Soluble MMO oxidizes many hydrophobic compounds but formate (pKa = 3.8) (Dean 1985) exists as an anion at neutral pH and would likely be excluded from the lipophilic active-site of the enzyme. The data suggest that formate is an ideal substrate for providing electrons to support sMMO-dependent TCE oxidation. However, methanotrophs in large-scale bioreactors supported by formate may be quickly overrun with faster growing non-methanotrophic formate-oxidizers (Gottschalk 1986). Methane will only support the growth of methanotrophs. Thus, an optimum large-scale bioremediation strategy might involve fine-tuning methane and TCE concentrations to maximize the maintenance of reducing equivalents while simultaneously minimizing inhibition effects.

The in vivo steady-state kinetic parameters were determined under optimum conditions with 25 mM formate to investigate the full biodegradative potential of *M. trichosporium* OB3b. The observed K_m of 138 μM is somewhat higher than 35 μM that was determined in vitro with purified sMMO components (Fox et al. 1990). A number of factors might influence the concentration scale of the saturation curve such as the availability of TCE to the enzyme as affected by cell membranes. The experimentally determined V_{max} of 13.2 mmol/h per g cell dry weight transforms to 456 nmol/min per mg of

Table 2. Conditions for expressing optimum TCE biodegradation rates by *M. trichosporium*.

Condition	Optimum range	Comments
pH	6.0–7.5	Steep decline at pH > 8.0
NaCl concentration	0.0–0.5 M	Steep decline at 1.0 M
Cell mass	0.1–5.2 mg dry weight per ml	Increase rate with cell mass
Temperature	28–35° C	Similar initial rate at 40° C but rate decays after 10 min

cell protein using published conversion factors (Ingraham et al. 1983). This value is a significant fraction of the in vitro V_{max} of 682 nmol/min per mg protein (Fox et al. 1990) and may be approaching the in vivo theoretical limit. Regardless, the rates observed with methanotrophs under optimal conditions are extremely high, being at least two orders of magnitude faster than rates reported for organisms containing toluene dioxygenase, toluene monooxygenase, ammonia monooxygenase, or propane monooxygenase (Tsien et al. 1990). Additionally, it is ten times greater than the unoptimized rate previously reported by these laboratories (Tsien et al. 1989), and similar to rates reported by others for formate-stimulated TCE oxidation by *M. trichosporium* OB3b (Oldenhuis et al. 1989).

Conclusions

M. trichosporium OB3b and *M. methanica* 81Z biosynthesize a sMMO that is highly active with TCE and other chlorinated ethylenes. An additional activity with naphthalene was utilized to develop a rapid assay for monitoring the biodegradative potential of methanotrophs that produce sMMO. Parameters that were important in TCE degradation applications include the effects of pH, salt concentration, temperature and cell density. The concentration of TCE present and the requirement for an oxidizable cosubstrate were found to be particularly critical parameters.

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