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Survey of Microbial Oxygenases: Trichloroethylene Degradation by Propane-Oxidizing Bacteria

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Microorganisms that biosynthesize broad-specificity oxygenases to initiate metabolism of linear and branched-chain alkanes, nitroalkanes, cyclic ketones, alkenoic acids, and chromenes were surveyed for the ability to biodegrade trichloroethylene (TCE). The results indicated that TCE oxidation is not a common property of broad-specificity microbial oxygenases. Bacteria that contained nitropropane dioxygenase, cyclohexanone monooxygenase, cytochrome P-450 monooxygenases, 4-methoxybenzoate monooxygenase, and hexane monooxygenase did not degrade TCE. However, one new unique class of microorganisms removed TCE from incubation mixtures. Five *Mycobacterium* strains that were grown on propane as the sole source of carbon and energy degraded TCE. *Mycobacterium vaccae* JOB5 degraded TCE more rapidly and to a greater extent than the four other propane-oxidizing bacteria. At a starting concentration of 20 μM, it removed up to 99% of the TCE in 24 h. *M. vaccae* JOB5 also biodegraded 1,1-dichloroethylene, *trans*-1,2-dichloroethylene, *cis*-1,2-dichloroethylene, and vinyl chloride.

Trichloroethylene (TCE) is an Environmental Protection Agency priority pollutant widely used as an industrial degreaser (32). The contamination of drinking water supplies with TCE is increasing in prevalence and concentration (28). Animal studies indicate that TCE may be carcinogenic (20). A more significant observation, from a human health standpoint, is the biotransformation of TCE to the potent carcinogen vinyl chloride (VC) by consortia of anaerobic bacteria (35). Thus, many water supplies contaminated with TCE will accumulate biologically formed VC, with the potential for serious health consequences. For these reasons, there is great interest in implementing processes to remove TCE from drinking-water supplies.

Bacteria in the environment oxidize many natural products and man-made compounds to carbon dioxide, and this constitutes an important part of the carbon cycle on earth (7). However, many chlorinated organic compounds are known to persist in the environment because of their resistance to microbial attack. For example, TCE was observed to exhibit a half-life of 300 days in one aquifer. Despite this apparent recalcitrance to degradation, recent studies show that TCE can be biodegraded by aerobic bacteria that oxidize toluene (22–24, 36, 37), methane (9, 16, 36), and ammonia (2). Bacteria that grow on hydrocarbons typically initiate oxidation by incorporating oxygen from the atmosphere into organic compounds by the action of enzymes known as oxygenases. Independently codiscovered in 1955 by Hayaishi et al. (11) and Mason et al. (17), oxygenases are generally divided into two groups, monooxygenases and dioxygenases. Both classes of oxygenases are implicated in bacterial TCE degradation. The expression of toluene dioxygenase activity is required for TCE oxidation by *Pseudomonas putida* F1 (24, 36), and gene cloning experiments implicate toluene monooxygenase from *P. mendocina* in TCE degradation (37). Methane monooxygenase in methanotrophs (9, 16, 36) and ammonia monooxygenase in *Nitrosomonas europaea* (2) are also proposed to oxidize TCE. Also, mammalian liver cytochrome P-450 monooxygenase oxidizes TCE (19). Toluene dioxygenase, methane monooxygenase, and cytochrome P-450 monooxygenase all display a relatively broad substrate specificity. Many other catabolic oxygenases produced by bacteria also show a wide substrate tolerance. Gratuitous oxidation of nongrowth substrates by bacteria is termed cooxidation, and this may be important in the decomposition of organic molecules in the environment (3, 26, 27). The nonspecific oxygenases that figure prominently in the process of cooxidation are a great potential resource for uncovering new TCE degradation biocatalysts. In this study, we examined a diverse group of microorganisms known to express catabolic oxygenases that initiate oxidation of such compounds as linear alkanes (6, 24), cyclic ketones (5, 29, 33), camphor (10), nitroalkanes (13, 14), and chromenes (30). The oxygenases elicited to attack these substrates contain flavoprotein, heme iron, nonheme iron, and as yet undefined prosthetic groups. A new class of bacteria that degrade TCE was identified, and propane monooxygenase was implicated in TCE oxidation. Unlike *P. putida* F1, which contains toluene dioxygenase, bacteria expressing propane monooxygenase also degraded VC. The properties of TCE degradation by this class of bacteria were studied as part of a continuing effort to uncover novel bacterial systems that might prove to be useful in TCE and VC bioremediation efforts.

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MATERIALS AND METHODS

Materials. TCE, 1,1-dichloroethylene, *cis*-1,2-dichloroethylene, *trans*-1,2-dichloroethylene, tetrachloroethylene, 1,2-dibromoethane, propane, nitroethane, cyclohexanol, 4-methoxybenzoic acid, and preocene II were purchased from Aldrich Chemical Co., Milwaukee, Wis. VC was obtained from Fluka Chemical Co., Ronkonkoma, N.Y. Hexane and *n*-pentane were from Mallinckrodt Chemical Co., Paris, Ky. Camphor was purchased from Eastman Kodak Co., Roch-

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TABLE 1. Microorganisms surveyed for TCE degradation

Microorganism	Source	Growth conditions [reference(s)]
Hansenula mrakii	Nobuyoshi Esaki, Institute for Chemical Research, Kyoto University, Kyoto, Japan	Inducer was nitroethane (14)
Acinetobacter sp. strain NCIB 9871	Christopher Walsh, Department of Biochemistry & Molecular Pharmacology, Harvard Medical School, Boston, Mass.	29
Bacillus megaterium ATCC 14531	Armand J. Fulco, Department of Biological Chemistry University of California, Los Angeles	8 mM phenobarbital added as inducer (21)
Pseudomonas putida G786	Stephen Sligar, Department of Biochemistry, University of Illinois, Urbana	10
P. putida 3400	Frithjof-Hans Bernhardt, Department of Physiological Chemistry, Rheinisch-Westfälischen Technischen Hochschule, Aachen, Federal Republic of Germany	Grown on mineral salts medium (31) containing 30 mM 4-methoxybenzoic acid
Streptomyces griseus ATCC 13273, *S. griseus* NRRL 8090	John Rosazza, College of Pharmacy, University of Iowa, Iowa City	30
Pseudomonas oleovorans PgG6, *P. aeruginosa* 473	James A. Shapiro, Department of Biochemistry & Microbiology, University of Chicago, Chicago, Ill.	Hexane supplied as a vapor (25)
Mycobacterium convolutum ATCC 29673, *M. rhodochrous* W-21, *M. rhodochrous* W-25, *M. rhodochrous* W-24, *M. vaccae* JOB5	Jerome J. Perry, Department of Microbiology, North Carolina State University, Raleigh	3, 15

ester, N.Y. Phenobarbital was obtained from Elkins-Sinn, Inc., Cherry Hill, N.J.

Organisms and culture conditions. The microorganisms, their sources, and the methods of cultivation are described in Table 1. To ensure the expression of the specific oxygenase activity shown in Table 2, each microorganism was grown on the oxygenase substrate or inducer. Furthermore, resting-cell suspensions were tested for oxygen uptake in the presence of the oxygenase substrate, using an oxygen electrode (Rank Bros., Bottisham, England).

GC. Gas chromatography (GC) was conducted with a Hewlett-Packard 5790A GC as described previously for analysis of pentane extracts containing TCE and other chlorinated olefins (36). Headspace GC analysis was performed with a Hach Carle AGC100 GC equipped with a flame ionization detector and fitted with a Graphpak AT-1000 column (Alltech Associates, Deerfield, Ill.) operated at 150°C with a nitrogen carrier gas flow of 30 ml/min. TCE had a retention time of 2.0 min under these conditions.

Quantitative determination of TCE and other chlorinated olefins. The disappearance of chlorinated solvents was monitored in sealed vials by pentane extraction and GC analysis as described previously (36). As in the previous study, the concentration of TCE is expressed as if all of the TCE was present in the aqueous buffer. TCE biodegradation data are presented in Results as the average of triplicate determinations ± standard error.

The bacteria were grown on the respective oxygenase inducer in basal media as described previously (Table 1). Cells were harvested during exponential growth phase and suspended to an optical density of 1.0 at 600 nm in the respective growth medium lacking the oxygenase substrate. Cell suspensions (2 ml) were transferred to 10-ml glass vials that were crimp-sealed with Teflon-lined rubber septa (American Scientific Products, Bedford, Mass.). The sealed cultures were equilibrated with shaking (180 rpm) at the same temperature used for growth of the culture. A 10-μl sample of a 4 mM TCE stock solution in water was added by gas-tight syringe (Hamilton, Reno, Nev.) to initiate the assay. The TCE stock was prepared in water to preclude possible inhibition of the respective broad-specificity oxygenase by an organic solvent used in the delivery of TCE. Zero-time controls were always conducted to provide a base line for monitoring TCE disappearance over time. In all experiments, heat-killed cells served as an additional control to correct for TCE loss through leakage and to preclude problems due to bioaccumulation. These factors were not problematic as both heat-killed cells and zero-time incubations of viable cells yielded extraction efficiencies of 85 to 90%. These controls served as a background for establishing the extent of biodegradation at 1- and 24-h incubations compared with 0-h controls. Due to cumulative experimental error in the steps of the assay, a decrease in TCE concentration of <8% compared with the heat-killed control was insignificant and hence was considered to be negative for biodegradation.

In the experiment for determining the kinetic course of TCE oxidation by *Mycobacterium vaccae* JOB5 (Fig. 1), headspace GC analysis was conducted. The headspace method was calibrated against the pentane extraction protocol and shown to give similar results. TCE concentrations were determined by injecting 100 μl of the 8-ml gas phase above septum-sealed incubation mixtures (2 ml of cell suspension), using a gas-tight syringe (Hamilton), into a GC (flame ionization detector). Quantitative determination of TCE was made by measuring the peak heights, which were compared with the response obtained with standard injections of TCE. Headspace analysis allowed the continuous measurement of TCE degradation from the same incubation mixture.

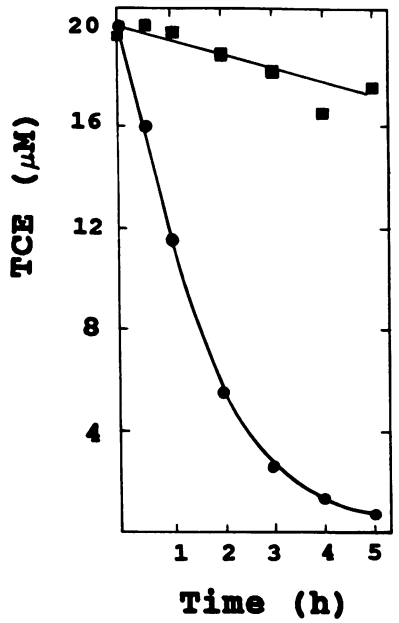

FIG. 1. Time course of TCE degradation by *M. vaccae* JOB5. Symbols: ●, JOB5; ■, heat-killed control. The initial TCE concentration was 20 μM. The concentration is expressed as if all of the TCE was in the liquid phase as all the TCE became available to the bacterium over the course of the assay. Incubations were conducted as described in Materials and Methods.

The experiments to determine biodegradation of various chlorinated alkenes were conducted as described previously (36). In place of *p*-dioxane as the solvent, stock solutions were prepared in methanol, which was added to incubation mixtures at a final concentration of 0.1% (wt/vol). The concentrations of the substrate at 0 and 2 h were determined by pentane extraction and GC. The GC response for the respective chlorinated alkene was compared with that of 1,2-dibromoethane, which served as an internal standard in the pentane extraction.

RESULTS AND DISCUSSION

Nelson et al. (24), Little et al. (16), and Arciero et al. (2) have shown that toluene-, methane-, and ammonia-oxidizing bacteria, respectively, biodegrade the Environmental Protection Agency priority pollutant TCE. In the first example, there is genetic evidence that toluene dioxygenase (24, 36) and toluene monooxygenase (37) are biocatalysts active in TCE degradation. In this context, we surveyed a series of bacteria and one yeast that biosynthesize broad-specificity oxygenases in the hope that one or more might gratuitously oxidize TCE. The growth substrates for the microorganisms, which were in most cases the substrate for the oxygenase to be tested, included alkanes, a nitroalkane, a cycloalkanol, and a bicyclic ketone. Both dioxygenases and monooxygenases were represented, and various oxygenase subgroups included a flavoprotein (cyclohexanone monooxygenase; 5, 8, 29), an iron flavoprotein (nitropropane dioxygenase; 13, 14), heme iron proteins (cytochrome P-450$_{CAM}$, 10; P-450$_{MEG}$; 18, 21), nonheme iron oxygenases (alkane monooxygenase; 25), and an uncharacterized class (propane monooxygenase; 3, 4).

The organisms shown in Table 1 were grown under conditions in which they expressed the catabolic oxygenase to be tested for activity with TCE. Thus, the organisms were grown on the oxygenase inducer or substrate following protocols described in Table 1. The expression of the oxygenase was typically required for growth. Resting-cell suspensions prepared from these cultures were incubated with TCE in the absence of the physiological oxygenase substrate to preclude potential competitive inhibition of TCE oxidation. This protocol for demonstrating TCE biodegradation was shown previously to be effective with toluene- and methane-oxidizing bacteria (36).

TABLE 2. TCE biodegradation by microorganisms in which different catabolic oxygenase activities are expressed

Microorganism	Oxygenase induced[a]	% TCE degraded[b]	
		1 h	24 h
Hansenula mrakii	Nitropropane dioxygenase	<8	<8
Acinetobacter sp. strain NCIB 9871	Cyclohexanone monooxygenase	<8	<8
Bacillus megaterium ATCC 14581	Cytochrome P-450$_{MEG}$	<8	<8
Pseudomonas putida G786	Cytochrome P-450$_{CAM}$	<8	<8
P. putida 3400	4-Methoxybenzoate monooxygenase	<8	<8
Streptomyces griseus ATCC 13273	Preocene monooxygenase	<8	<8
S. griseus NRRL 8090	Preocene monooxygenase	<8	<8
Pseudomonas oleovorans PpG6	Hexane monooxygenase	<8	<8
P. aeruginosa 473	Hexane monooxygenase	<8	<8
Mycobacterium convolutum	Propane monooxygenase	20 ± 2	29 ± 5
M. rhodochrous W-21	Propane monooxygenase	23 ± 4	39 ± 2
M. rhodochrous W-25	Propane monooxygenase	15 ± 3	47 ± 2
M. rhodochrous W-24	Propane monooxygenase	16 ± 3	52 ± 3
M. vaccae JOB5	Propane monooxygenase	41 ± 3	99 ± 4

[a] Addition of growth substrate or gratuitous inducer led to induction of an oxygenase(s) to be examined for TCE-oxidizing ability.
[b] Determined by comparison with a 0-h control as described in Materials and Methods.

The propane-oxidizing bacteria degraded TCE, whereas the other oxygenase-containing microorganisms surveyed did not (Table 2). The analytical procedures used precluded an accurate measurement of TCE biodegradation at a level of <8%. Furthermore, the inability to degrade TCE under the conditions used could be reflective of factors other than oxygenase substrate specificity, such as a failure to take up TCE from the growth medium.

In experiments with the propane oxidizers, TCE oxidation was clearly discernible above the 8% base line. Methanotrophs, which have been demonstrated previously to biodegrade TCE (16, 36), are incapable of utilizing hydrocarbons larger than methane for growth (1). Thus, the *Mycobacterium* strains that grow on C_2 to C_5 linear alkanes (Table 2) represent a newly discovered class of TCE degraders. This observation is rendered more significant by the other data in Table 2, which indicated that TCE biodegradation is not a general property of bacteria that contain broad-specificity catabolic oxygenases.

Several observations with *M. vaccae* JOB5 implicated

propane monooxygenase as playing a role in TCE degradation by this organism. Cells grown on propane or acetone show the ability to oxidize propane, whereas *M. vaccae* JOB5 cultured on a complex medium does not oxidize hydrocarbons (4). The ability of this strain to degrade TCE under different growth conditions was shown to coincide with propane-oxidizing activity. In other experiments, propane was shown to inhibit TCE oxidation when present in the headspace above incubation mixtures. Following consumption of the propane, TCE oxidation was observed to proceed. These results suggested that propane monooxygenase was involved in TCE oxidation by *M. vaccae* JOB5. However, a more rigorous demonstration of the putative role of this oxygenase in TCE degradation will require further studies with a cell-free oxygenase system.

It is of interest that the propane-oxidizing strains did not show identical properties. Four of the propane-oxidizing bacteria removed 29 to 52% of the TCE present in incubation mixtures over 24 h, while *M. vaccae* JOB5 cleared virtually all (99%) of the TCE from reaction vials. Previous studies have indicated that the initial oxygenase attack on propane could occur by either terminal oxidation to yield *n*-propanol or subterminal oxygenation to produce isopropanol (27). Kester and Foster (12) demonstrated that *M. rhodochrous* 7EIC catalyzed terminal oxidation of several alkanes to form *n*-hydroxylated products. In contrast, Vestal and Perry (34) demonstrated that *M. vaccae* JOB5 oxidized propane with subterminal oxygenation to produce isopropanol. It is presently unclear whether this dichotomy in alkane oxygenation modes is relevant to the observed difference in TCE-degrading activity by these propane-utilizing strains.

Since *M. vaccae* JOB5 catalyzed almost complete removal of TCE in the experiment of Table 2, it was important to examine the kinetic course of substrate disappearance. TCE oxidation proceeded linearly for the first hour at a rate of 0.3 nmol/min per unit of cell mass (optical density of 1.0 at 600 nm) (Fig. 1). The observed TCE oxidation rate is comparable to that previously observed with *P. putida* F1, which contains toluene dioxygenase (36). After 1 h, the rate of TCE oxidation by *M. vaccae* JOB5 declined slowly and went 96% to completion in 5 h. This observation contrasted sharply with the previous report concerning *P. putida* F1, which showed that TCE oxidation rates decline rapidly over time and do not proceed more than 68% to completion.

The rate and extent of TCE degradation by *M. vaccae* JOB5 indicated that bacteria of this class have potential application in bioremediation of TCE-contaminated environments. In polluted environments, some anaerobic bacteria biotransform TCE to dichloroethylenes and the potent mutagen and carcinogen VC (35). Since these compounds are often copollutants with TCE, the ability of *M. vaccae* JOB5 to degrade VC and the three isomeric dichloroethylenes was tested.

All of the chlorinated alkenes were observed to be biodegraded at levels above the standard error (Table 3), with the exception of tetrachloroethylene. Heat-killed controls were performed in all cases, which ruled out that the observed decrease in the chlorinated alkenes was due to adsorption by the cells. VC was completely degraded within 2 h, and 1,1-dichloroethylene and *cis*-1,2-dichloroethylene were oxidized to a significant extent. In this experiment, TCE was only marginally degraded, which is likely due to the presence of methanol. Methanol was shown to inhibit TCE oxidation by *M. vaccae* JOB5 in independent experiments. It was necessary to add the chlorinated alkenes in methanol to deliver a reproducible amount of substrate to replicate vials.

TABLE 3. Degradation of chlorinated alkenes by *M. vaccae* JOB5 in a 2-h incubation

Substrate[a]	Initial concn (μM)	Final concn (μM)	% Decrease in 2 h
Tetrachloroethylene	19.8 ± 0.2	19.6 ± 1.1	1 ± 6
TCE	25.2 ± 1.4	22.8 ± 0.4	10 ± 7
cis-1,2-Dichloroethylene	17.1 ± 0.1	7.9 ± 0.6	54 ± 4
trans-1,2-Dichloroethylene	23.4 ± 0.5	21.7 ± 0.4	7 ± 4
1,1-Dichloroethylene	20.8 ± 0.1	10.7 ± 0.8	49 ± 4
VC	14.2 ± 0.6	ND[b]	100 ± 4

[a] Substrates were added from 40 mM stock solutions in methanol.
[b] ND, Not detected.

Despite this inhibition problem, VC was still rapidly oxidized. This result indicates a potential difference in oxygenase specificity for chlorinated ethylenes compared with the toluene dioxygenase system. Previously, *P. putida* F1 was shown to biodegrade dichloroethylenes but not VC (36).

In summary, previous reports of TCE biodegradation by toluene- and methane-oxidizing bacteria inspired the present survey in which microorganisms that produce a diverse collection of catabolic oxygenases were screened for ability to degrade TCE. The results suggested that TCE oxidation is not a common property of broad-specificity microbial oxygenases, but one new unique class was observed to attack TCE. One member of this group, *M. vaccae* JOB5, degraded TCE more rapidly and to a greater extent than the four other propane-oxidizing bacteria examined in this survey. Furthermore, *M. vaccae* JOB5 was also observed to biodegrade the anaerobic biotransformation products of TCE, including VC. Progress in the development of microbial remediation methods for TCE and other chlorinated olefin pollutants may be extended by isolating additional propane-oxidizing microorganisms and by examining the biochemistry underlying TCE degradation by this group of bacteria.

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