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Characterization of Chloroethylene Dehalogenation by Cell Extracts of *Desulfomonile tiedjei* and Its Relationship to Chlorobenzoate Dehalogenation

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We characterized the reductive dehalogenation of tetrachloroethylene in cell extracts of *Desulfomonile tiedjei* and compared it with this organism's 3-chlorobenzoate dehalogenation activity. Tetrachloroethylene was sequentially dehalogenated to trichloro- and dichloroethylene; there was no evidence for dichloroethylene dehalogenation. Like the previously characterized 3-chlorobenzoate dehalogenation activity, tetrachloroethylene dehalogenation was heat sensitive, not oxygen labile, and increased in proportion to the amount of protein in assay mixtures. In addition, both dehalogenation activities were dependent on hydrogen or formate as an electron donor and had an absolute requirement for either methyl viologen or triquat as an electron carrier in vitro. Both activities appear to be catalyzed by integral membrane proteins with similar solubilization characteristics. Dehalogenation of tetrachloroethylene was inhibited by 3-chlorobenzoate but not by the structural isomers 2- and 4-chlorobenzoate. The last two compounds are not substrates for *D. tiedjei*. These findings lead us to suggest that the dehalogenation of tetrachloroethylene in *D. tiedjei* is catalyzed by a dehalogenase previously thought to be specific for *meta*-halobenzoates.

Chlorinated ethylenes are manufactured in massive quantities, and their improper handling and disposal have resulted in the contamination of groundwater reserves (21, 32). Unlike less-halogenated chloroethylenes, tetrachloroethylene (PCE) is not biodegradable under aerobic conditions (2) but can be reductively dehalogenated under anaerobic conditions (3). Reductive dehalogenation is an important fate process for the biodegradation of both chloroaliphatic and chloroaromatic pollutants (for reviews, see references 17 and 20). Because dehalogenation generally results in decreased toxicity, leads to increased biodegradability, and occurs in anaerobic environmental compartments where pollutants often reside, it is considered an attractive strategy for the anaerobic bioremediation of sites contaminated with chloroorganic compounds (1, 23).

The reductive dehalogenation of PCE has been studied in a variety of anaerobic microorganisms. A number of phylogenetically diverse anaerobes are known to cometabolically dehalogenate PCE without an apparent physiological benefit (10, 30). More recently, several isolates which couple growth with the dehalogenation of PCE have been obtained (13, 15, 16, 22); these microorganisms are believed to use PCE as a terminal electron acceptor in a form of anaerobic respiration. A bioenergetic study of PCE dehalogenation by "*Dehalospirillum restrictus*" confirms the respiratory nature of dehalogenation in this isolate (27).

Desulfomonile tiedjei dehalogenates 3-chlorobenzoate (3CBz) as a form of anaerobic respiration and has served as a model organism for study of chloroaromatic compound biodegradation (7, 25). PCE dehalogenation has also been demonstrated in *D. tiedjei* (10), but little is known about the biochemistry of this transformation. PCE dehalogenation by *D. tiedjei* depends on the presence of 3CBz (29) and is coinduced with 3CBz dehalogenation activity (4), suggesting that the two activities share common components. In this study, we characterize the dehalogenation of PCE in cell extracts of *D. tiedjei* and compare it with the previously characterized 3CBz dehalogenation activity (8) in order to determine whether chloroaliphatic and chloroaromatic dehalogenation reactions are biochemically related in this microorganism.

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MATERIALS AND METHODS

Preparation of extracts. *D. tiedjei* was grown as previously described (7) on pyruvate (40 mM) and 3CBz (2 mM). Cells were harvested aerobically, unless otherwise noted, by centrifugation at 15,000 × *g* for 20 min at 4°C. Cells were washed once and resuspended in phosphate buffer (4°C, 50 mM, pH 7.8) at a concentration of 1 g (wet weight)/5 ml of buffer, broken twice by French pressure cell disruption (124 mPa), and centrifuged at 15,000 × *g* for 20 min at 4°C to remove unbroken cells. For oxygen lability experiments, aliquots of cells harvested anaerobically were broken and subsequently stored both aerobically and anaerobically. For localization studies, cell extracts were ultracentrifuged at 150,000 × *g* for 2 h at 4°C to yield a supernatant consisting of cytoplasmic and periplasmic contents and a pellet consisting largely of membrane fragments and vesicles. For solubilization experiments, the membrane pellet was resuspended in phosphate buffer and aliquots were amended with various detergents, incubated at 4°C for 2 h, and ultracentrifuged. The supernatant containing solubilized membrane proteins was assayed for dehalogenation activity.

Dehalogenation assay. Dehalogenation assay mixtures were prepared in 10-ml serum vials inside an anaerobic glove box with anoxic stock solutions. Assay mixtures were 1 ml in volume and contained the following: TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (50 mM, pH 8.0), methyl viologen (5.0 mM), 10 μl of *Clostridium pasteurianum* extracts for hydrogenase activity, and *D. tiedjei* extracts. Vials were sealed with Teflon-lined stoppers, removed from the glove box, and crimp sealed. The headspace gas was replaced with 100% hydrogen at 5-kPa overpressure. Vials were amended with 10 μl of a chloroethylene stock solution prepared in 95% ethanol to yield an aqueous concentration as determined by Henry's constant (14); unless otherwise stated, assay mixtures contained PCE in excess of its solubility (~1 mM). Vials were incubated at 37°C in the dark with shaking at 200 rpm. Abiotic controls contained all of the above except *D. tiedjei* extracts; standards were made identically with 1 ml of nanopure water. Chlorobenzoate dehalogenation assay mixtures contained 3CBz (8 mM) instead of chloroethylene as a halogenated substrate.

Analytical methods. PCE, trichloroethylene (TCE), and dichloroethylene (DCE) were measured by gas chromatographic analysis of the headspace gases of assay mixtures. Fifty microliters of the headspace atmosphere was injected into a model 3300 gas chromatograph (Varian, Walnut Grove, Calif.) equipped with a 1% SP-1000 on 60/80 Carbopack B column (1.8 m by 3.2 mm; Supelco, Inc., Bellefonte, Pa.) and a flame ionization detector, and nitrogen served as the carrier gas. The injector and detector temperature settings were 200 and 250°C, respectively. The column temperature program was 2 min at 140°C followed by

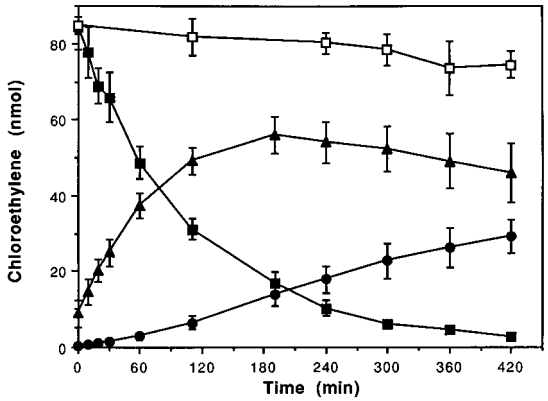

FIG. 1. Reductive dehalogenation of PCE (■) to TCE (▲) and DCE (●) by cell extracts of *D. tiedjei*. In control assays without cell extracts, PCE (□) was not dehalogenated and minimal abiotic loss occurred. Assay mixtures contained 6.68 mg of protein. Data represent the averages and standard deviations of triplicate determinations.

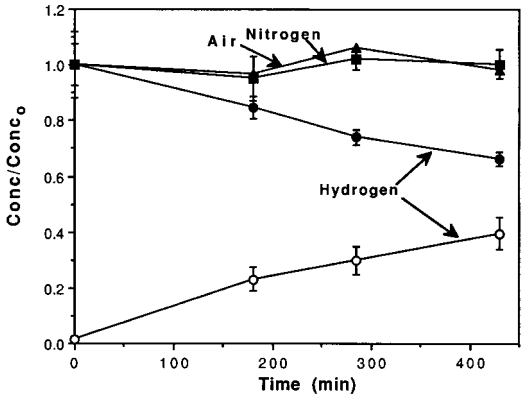

FIG. 2. Effect of headspace gas composition on PCE dehalogenation (closed symbols) and TCE appearance (open symbol) by extracts of *D. tiedjei*. The initial PCE concentration was approximately 200 μM. Assay mixtures contained 3.37 mg of protein. Data represent the averages and standard deviations of triplicate determinations.

a 5°C/min increase until 190°C was achieved and a hold at 190°C for 1 min. In order to identify the DCE isomers, which coeluted in the above analysis, a model 5980 gas chromatograph (Hewlett-Packard, Wilmington, Del.) fitted with a Carbograph Volatile Organic Compound column (30 m by 0.25 mm; Alltech Associates, Deerfield, Ill.) was used. The injector, detector, and column were operated at 200, 250, and 40°C, respectively. Benzoate and 3CBz were quantitated by a solvent extraction procedure and high-pressure liquid chromatography analysis as previously described (8). Protein concentrations were determined colorimetrically by the bicinchoninic acid method (28), using bovine serum albumin as the standard.

Chemicals. Halogenated compounds were obtained from Aldrich Chemical Co., Milwaukee, Wis., in the highest available purity. A Surfact-Pak Detergent Sampler for solubilization assays and bicinchoninic acid protein assay reagents were obtained from Pierce Chemical Co., Rockford, Ill. Triquat was a gift from Stephen Ragsdale. All other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Cell extracts of *D. tiedjei*, previously grown on pyruvate and 3CBz, were incubated with approximately 100 nmol of PCE in an assay optimized for the reductive dehalogenation of 3CBz to benzoate (8). In this assay, which relies on hydrogen as a source of electrons and methyl viologen as an artificial electron carrier, PCE was immediately and rapidly dehalogenated to TCE and subsequently to DCE, as determined by gas chromatographic analysis of headspace samples (Fig. 1). Although DCE isomers were not resolved by this analysis, *cis*-DCE, later determined to be the primary metabolite, was used as a standard for DCE identification and quantification. In the absence of *D. tiedjei* extracts, minimal abiotic loss of PCE was observed (<10%) and, more importantly, no dehalogenated metabolites were detected. The rate of dehalogenation increased with increasing PCE concentrations up to and beyond its limit of solubility (~1 mM), and the dehalogenation rate increased linearly with increasing protein concentrations up to 12 mg/ml (data not shown). Extracts boiled for 5 min lost their ability to dehalogenate PCE, and exposure of extracts to air prior to being assayed did not influence the rate of dehalogenation (data not shown).

We examined the source of reducing equivalents for the reductive dehalogenation of PCE by *D. tiedjei*. No dehalogenation of PCE was observed in assay mixtures with headspace contents of either air or 100% nitrogen, indicating that hydrogen served as the source of electrons in this assay (Fig. 2). Assay mixtures with a nitrogen headspace amended with acetate or pyruvate (with 1 mM coenzyme A) also did not dehalogenate PCE, while those incubated with formate as an electron donor did (data not shown). All subsequent experiments utilized hydrogen as the source of electrons for reductive dehalogenation reactions.

To confirm the dehalogenation pathway, the fate of less-chlorinated intermediates was examined. TCE was dehalogenated predominately to *cis*-DCE, with small amounts of *trans*-DCE and 1,1-DCE formed as well (Fig. 3). All three DCE isomers were tested as potential dehalogenation substrates in extended incubations, but no discernible loss above abiotic controls of any DCE isomer was observed (data not shown). The rates of dehalogenation of 3CBz, PCE, and TCE by fully induced extracts of *D. tiedjei* are 0.627, 0.396, and 0.004 nmol min^{-1} mg^{-1}, respectively.

We examined the electron carrier requirements for PCE dehalogenation. When the artificial electron carrier methyl viologen was omitted, PCE dehalogenation activity was abolished. Methyl viologen could be replaced by another low-potential viologen, triquat, but not by heptyl or benzyl viologen. All artificial electron carriers tested demonstrated similar abilities to promote both PCE and 3CBz dehalogenation in cell extracts of *D. tiedjei* (Table 1). Physiological electron carriers were also evaluated for their ability to function in the alkyl dehalogenation reaction. PCE was not dehalogenated when

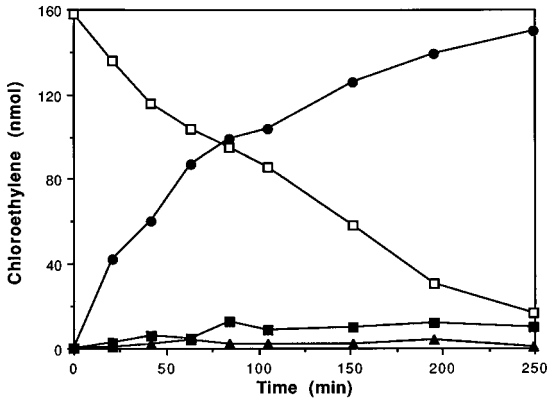

FIG. 3. Reductive dehalogenation of TCE (□) to *cis*-DCE (●), *trans*-DCE (■), and 1,1-DCE (▲) by *D. tiedjei*. The assay mixture contained 8.35 mg of protein.

TABLE 1. Effect of artificial electron carriers on 3CBz and PCE dehalogenation in cell extracts of *D. tiedjei*[a]

Electron carrier	Rate of dehalogenation (nmol min^{-1} mg^{-1}) of:	
	3CBz	PCE
None	0.033 ± 0.010	0.033 ± 0.001
Methyl viologen	0.607 ± 0.063	0.489 ± 0.005
Triquat	0.788 ± 0.066	0.532 ± 0.011
Benzyl viologen	0.038 ± 0.007	0.044 ± 0.004
Heptyl viologen	0.040 ± 0.008	0.053 ± 0.004

[a] Data are means ± standard deviations of triplicate determinations.

NADH, reduced flavin adenine dinucleotide, NADPH, or clostridial ferredoxin was supplied as a potential electron carrier (data not shown).

We compared the cellular location of the PCE and 3CBz dehalogenation activities and the ability of detergents to solubilize membrane-bound dehalogenation activity (Fig. 4). Following ultracentrifugation of cell extracts, we found comparable ratios of the two dehalogenation activities present in the supernatant and pellet fractions of the cell extracts, with the majority of both activities being present in the insoluble membrane fraction. We found that both activities were difficult to release from this fraction, with less than 40% of the activities solubilized by 1% Triton X-100. Furthermore, we tested 12 different detergents for their ability to release both dehalogenation activities from the membrane fraction. Although detergents varied widely in the amount of dehalogenation activities solubilized, in all cases, individual detergents solubilized both dehalogenation activities to similar extents (data not shown).

Finally, we examined the effect of chlorobenzoates as possible competing substrates on PCE dehalogenation. Assay mixtures containing 0.2 mM PCE were challenged with 3CBz at concentrations of 0.2, 2.0, and 20 mM. As shown in Fig. 5A, we observed decreasing PCE dehalogenation rates with increasing concentrations of 3CBz. However, inhibition was not observed when extracts were challenged with either 2- or 4-chlorobenzoate (Fig. 5B). These two isomers are not susceptible to dehalogenation by *D. tiedjei* (8).

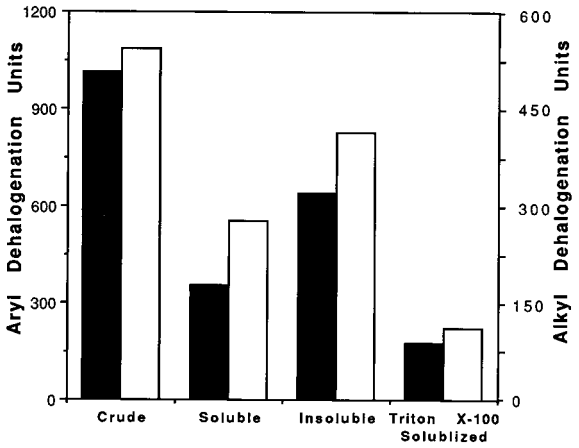

FIG. 4. Comparison of the cellular localization and solubilization of 3CBz dehalogenation (black bars) and PCE dehalogenation (open bars) activities. Units of activity are nanomoles of dehalogenated product per minute.

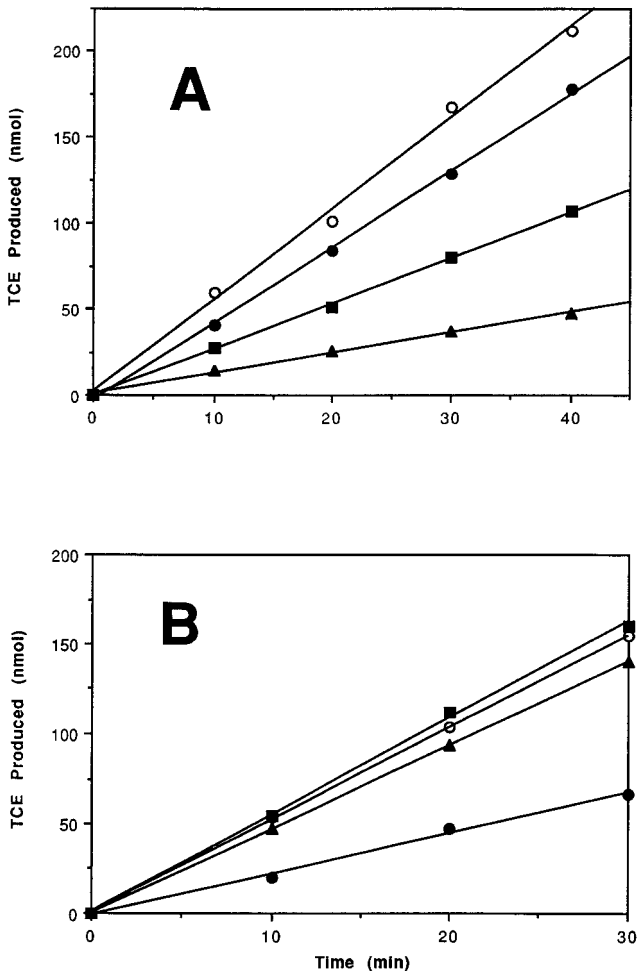

FIG. 5. (A) Effect of increasing concentrations of 3CBz on the rate of PCE dehalogenation to TCE. Assay mixtures were amended with PCE at an aqueous concentration of 0.2 mM and 3CBz at concentrations of 0 (○), 0.2 (●), 2.0 (■), and 20 (▲) mM. (B) Effect of 2 mM 3CBz (●), 2CBz (■), or 4CBz (▲) on PCE dehalogenation by extracts of *D. tiedjei*. All assay mixtures contained 6.8 mg of protein; data are the averages from triplicate assays.

DISCUSSION

Cell extracts of *D. tiedjei* sequentially dehalogenated PCE to TCE and predominately *cis*-DCE. Extracts were unable to dehalogenate *cis*-, *trans*-, or 1,1-DCE, confirming DCE as the terminal metabolite of PCE dehalogenation by *D. tiedjei*. All known PCE-dehalogenating isolates incompletely dehalogenate PCE, yielding either TCE or, more commonly, *cis*-DCE as the end product (15, 16, 22, 24, 30). To date, dehalogenation of PCE to ethene and/or ethane has been demonstrated exclusively in mixed-culture studies (6, 11, 31), suggesting that distinct chloroethylene-dehalogenating populations are required for the complete dehalogenation of PCE to nonhalogenated products.

D. tiedjei's PCE dehalogenation activity shares a number of common characteristics with this microorganism's previously characterized 3CBz dehalogenation activity. As reported elsewhere, both activities are coinduced by *meta*-halobenzoates (4). Additionally, we have found that both activities use either hydrogen or formate, but not acetate or pyruvate, as an electron donor. Both activities are heat sensitive, while neither is oxygen labile. Dehalogenation activities in *D. tiedjei* are mem-

brane associated and, on the basis of their solubilization characteristics, appear to be catalyzed by integral membrane proteins. In cell extracts, both activities are dependent on the presence of specific artificial electron carriers, either methyl viologen or triquat, to transfer low-potential electrons from the electron donor to the dehalogenase. Neither activity is stimulated by a variety of physiological electron carriers; thus, the true physiological electron carrier remains unknown.

Furthermore, 3-chlorobenzoate inhibited the dehalogenation of PCE in a concentration-dependent manner, while no inhibition was apparent when 2- and 4-chlorobenzoate were used as substrates. The inhibition of chloroaliphatic dehalogenation by 3-chlorobenzoate but not by the structural isomers suggests that 3CBz and PCE serve as competing substrates for the cell's aryl dehalogenase. The recent purification of the aryl dehalogenase from *D. tiedjei* (26) may allow more-detailed kinetic analysis of this contention.

The induction of dehalogenation activity by 3CBz (5) and the chemiosmotic coupling of 3CBz dehalogenation to ATP synthesis (19) strongly suggests that the physiological function of this process in *D. tiedjei* is for the anaerobic respiration of *meta*-halogenated benzoates. The reductive dehalogenation of chloroethylenes by what appears to be the same enzyme system constitutes a novel form of cometabolism. Low-molecular-weight chlorinated solvents are often noted as substrates for cometabolic transformations under both aerobic and anaerobic conditions (9, 18). It appears that PCE can be reductively dehalogenated by a diverse group of biological agents, including terminal reductases in PCE-respiring microorganisms (15, 22); low-potential transition-metal complexes involved in a variety of metabolic processes, including acetogenesis and methanogenesis (12, 31); and, as reported here, a 3-chlorobenzoate dehalogenase. Further studies are required to determine if the cometabolic dehalogenation of chloroaliphatic compounds by chloroaromatic-dehalogenating microorganisms is a generalizing phenomenon. Moreover, it may also prove useful to check cells known to catalyze chloroaliphatic dehalogenation for their ability to transform haloaromatic substrates. In this respect, it is interesting to note the recent isolation of a bacterium from a PCE-dechlorinating enrichment which uses PCE and *ortho*-chlorinated phenols as electron acceptors (13).

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