

Reductive Dehalogenation of Chlorobenzene Congeners in Cell Extracts of *Dehalococcoides* sp. Strain CBDB1

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Enzymatic reductive dehalogenation of tri-, tetra-, penta-, and hexachlorobenzenes was demonstrated in cell extracts with low protein concentration (0.5 to 1 µg of protein/ml) derived from the chlorobenzene-respiring anaerobe *Dehalococcoides* sp. strain CBDB1. 1,2,3-trichlorobenzene dehalogenase activity was associated with the membrane fraction. Light-reversible inhibition by alkyl iodides indicated the presence of a corrinoid cofactor.

Chlorinated benzenes are highly persistent pollutants which are ubiquitously distributed in the environment and impose a significant risk for human health (24, 27). Reductive dechlorination of multiple chlorinated benzenes was detected in various anaerobic mixed cultures (3). The isolation of the first anaerobic bacterium capable of reductive dechlorination of chlorinated benzenes, *Dehalococcoides* sp. strain CBDB1, was previously reported (2). Strain CBDB1 is able to grow with trichlorobenzene (TCB), hydrogen, and acetate, indicating that it conserves energy by using TCB as the terminal electron acceptor in a respiratory process (2). Recently, it was shown that strain CBDB1 also dechlorinates chlorinated dioxins (5). Several anaerobic bacteria couple the reductive dehalogenation of chlorinated aliphatic or aromatic compounds to ATP synthesis via an electron transport chain, a process which is referred to as dehalorespiration (8). Reductive dehalogenases of species dehalorespiring with chlorinated ethenes, phenols, or benzoates have been isolated and characterized (6, 10, 12, 16, 18, 20, 21, 25). In the present study, reductive dehalogenation of TCB and highly chlorinated benzenes was demonstrated in cell extracts of strain CBDB1 and an initial characterization of 1,2,3-TCB dehalogenase activity was carried out.

Dehalococcoides strain CBDB1 was cultivated under strictly anaerobic conditions in flasks sealed with Teflon-lined rubber septa in completely defined medium containing vitamins including 50 ng of vitamin B₁₂/liter, 5 mM acetate, 15 µM 1,2,3-TCB, and 15 µM 1,2,4-TCB (1). The headspace was flushed with N₂-CO₂ (80%-20%), and hydrogen was added to 8% of the gas phase. Two weeks after inoculation, cultures were supplied with additional 10 mM 1,2,3-TCB (nominal concentration), added as a hexadecane solution (2, 7). Three weeks after inoculation, cultures reached their maximum dechlorination activities. Total protein concentrations of cultures were very low and amounted to 0.5 to 1 µg/ml, corresponding to cell numbers of 10⁷/ml. Cells were harvested at this stage by centrifugation at 10,000 × g for 20 min at 4°C. The barely visible

sediment was resuspended in 50 mM Tris-HCl, pH 7.5, containing 1.5 mM titanium(III) citrate (28). Five to 10 ml of the resulting cell suspension (0.5 to 1 µg of protein/ml) was subjected to three cycles of French press treatment at 28 MPa, and the lysate was centrifuged at 10,000 × g for 20 min at 4°C. The supernatant (crude extract) contained 0.3 to 0.5 µg of protein/ml.

Dehalogenase activity was routinely measured with reduced methyl viologen (MV) as the artificial electron donor (17). The final assay contained 100 mM Tris-HCl (pH 7.5), 1 mM MV, 2 mM titanium(III) citrate, and chlorobenzene congeners at concentrations of 50 µM (TCBs; 1,2,3,4-tetrachlorobenzene [1,2,3,4-TeCB]; 1,2,3,5-TeCB) or 15 µM (chlorinated benzenes with very low solubility in water: 1,2,4,5-TeCB; pentachlorobenzene [PeCB]; hexachlorobenzene [HCB]). 1,2,3-TCB was used as a model substrate. To test different electron donors, MV in the assay solution was replaced by 1 mM ethyl viologen (EV), 1 mM benzyl viologen (BV), 1 mM anthraquinone 2,6-disulphonic acid (AQDS), or 1 mM NADH. Reactions were started by addition of 200 µl of crude extract corresponding to about 100 ng of protein. After 5 to 120 min of incubation at 25°C (when necessary, incubation was extended to 24 h), reactions were stopped by extracting the reaction mixture with hexane. Extracts were analyzed by gas chromatography and flame ionization detection (2). 1,2,3-TCB dechlorination in whole-cell suspensions was determined with the same assay.

1,2,3-TCB, 1,2,4-TCB, and 1,2,3,4-TeCB were obtained from Merck-Schuchard (Hohenbrunn, Germany). 1,3,5-TCB, 1,2,4,5-TeCB, and PeCB were from Aldrich (Steinheim, Germany). 1,2,3,5-TeCB was purchased from Riedel-de Haën (Seelze, Germany). HCB was from Fluka (Neu-Ulm, Germany). Viologens and AQDS were from Aldrich. Solvents were from Roth (Karlsruhe, Germany) and Merck-Schuchard. Alkyl iodides were obtained from Acros (Nidderau, Germany) and Aldrich. Buffers and other chemicals were from AppliChem (Darmstadt, Germany), Fluka, and Merck-Schuchard.

Although the amount of protein obtained from cultures of strain CBDB1 was extremely low, dechlorination of all tested chlorobenzene congeners except 1,3,5-TCB could be demonstrated in crude extracts (Table 1). Final dechlorination products were 1,3-dichlorobenzene (1,3-DCB), 1,4-DCB, and 1,3,5-

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TABLE 1. Dehalogenase activity in crude extracts with highly chlorinated benzenes as electron acceptors^a

Added chlorobenzene congener	First dechlorination product(s)	Specific activity \pm SD (nkat/mg of protein) ^b
1,2,3-TCB	1,3-DCB	11 \pm 0.7
1,2,4-TCB	1,3-DCB, 1,4-DCB	0.3 \pm 0.0
1,3,5-TCB		<0.05
1,2,3,4-TeCB	1,2,4-TCB	355 \pm 21
1,2,3,5-TeCB	1,3,5-TCB	76 \pm 1.7
1,2,4,5-TeCB	1,2,4-TCB	3 \pm 0.1
PeCB	1,2,3,5-TeCB, 1,2,4,5-TeCB	171 \pm 12
HCB	PeCB	0.4 \pm 0.0

^a Means of results of triplicate assays \pm standard deviation.

^b One nanokatal is defined as 1 nmol of dechlorination products formed per s at 25 °C.

TCB. PeCB dechlorination proceeded via the formation of 1,2,3,5-TeCB and 1,2,4,5-TeCB (Fig. 1). 1,2,3,5-TeCB was further dechlorinated to 1,3,5-TCB, whereas 1,2,4,5-TeCB temporarily accumulated due to its slow dechlorination rate (Table 1). HCB was dechlorinated to 1,3,5-TCB, 1,3-DCB, and 1,4-DCB via PeCB. With heat-inactivated crude extracts, no dechlorination could be detected. To our knowledge, this is the first report of reductive dechlorination of PeCB and HCB by cell extracts of a pure culture. Previously, it was demonstrated that strain CBDB1 in culture reductively dechlorinates 1,2,3-TCB, 1,2,4-TCB, and all TeCB isomers with hydrogen as the electron donor (2). In contrast, PeCB and HCB were not dechlorinated by the pure culture (2). Experiments are in progress to find the reasons for the difference between results obtained with PeCB and HCB *in vitro* and in culture.

Reductive dehalogenases from other dehalorespiring bacteria dechlorinating chlorobenzoate, chlorophenol, or chlorinated ethenes have specific activities between 2.5 and 25 nkat/mg in crude extracts with MV as the electron donor (8). 1,2,3-TCB dehalogenase activity in crude extracts of strain CBDB1 showed a similar specific activity of 11 nkat/mg, but much higher specific activities were obtained for dechlorination of 1,2,3,4-TeCB (355 nkat/mg) and PeCB (171 nkat/mg).

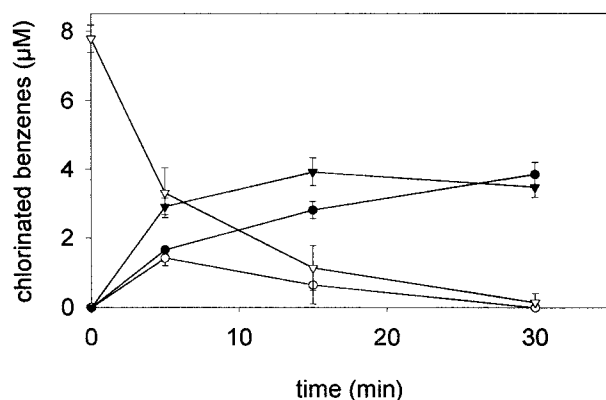


FIG. 1. Product formation from PeCB by cell extracts of strain CBDB1. PeCB, open triangles; 1,2,4,5-TeCB, solid triangles; 1,2,3,5-TeCB, open circles; 1,3,5-TCB, solid circles. Values are means of results of triplicate assays \pm standard deviation.

Whether all detected dechlorination reactions are mediated by one enzyme or several enzymes is not yet known.

In crude extracts of strain CBDB1, 1,2,3-TCB dehalogenase activity was highest with MV ($E_o' = -446$ mV [14]) as the electron donor. With EV ($E_o' = -480$ mV [14]) and BV ($E_o' = -360$ mV [14]), 89 and 12% of the MV-supported activity were measured, respectively. With AQDS ($E_o' = -184$ mV [4]) and NADH ($E_o' = -320$ mV) or in the absence of any added electron donor, no dehalogenation was observed within 24 h. A higher activity with MV as the electron donor than with EV was also described for the tetrachloroethene (PCE) dehalogenases of *Dehalospirillum multivorans* and *Desulfitobacterium* sp. strain PCE-S (14, 15). For *D. multivorans*, a hampered access of the larger molecule EV to the electron-accepting site of the dehalogenase was assumed (14). Whereas no dechlorination with BV was detected in crude extracts of *D. multivorans* and *Desulfitobacterium chlororespirans* Co23 (11, 14), BV supported dehalogenase activity in strain PCE-S (15), *Dehalococcoides ethenogenes* (I. Nijenhuis and S. H. Zinder, Abstr. 100th Gen. Meet. Am. Soc. Microbiol. 2000, abstr. Q-126, p. 569, 2000), and strain CBDB1. The results suggest that the cofactors of the dehalogenases from *D. multivorans* and *D. chlororespirans* Co23 possess more negative redox potentials than the cofactors of the dehalogenases from strain PCE-S, *D. ethenogenes*, and strain CBDB1, although steric effects cannot be excluded. For *D. ethenogenes*, it was suggested that no quinone is involved in the respiratory electron transport (Nijenhuis and Zinder, Abstr. 100th Gen. Meet. Am. Soc. Microbiol. 2000). In crude extracts of strain CBDB1, the quinone analog AQDS did not serve as electron donor for dechlorination, also suggesting that quinones are not involved in the transport of electrons to TCB.

To assess oxygen sensitivity of 1,2,3-TCB dehalogenase, crude extracts of strain CBDB1 were stored at 4°C under (i) reduced anaerobic conditions [$E_h < -200$ mV; anaerobic gas phase; extracts reduced with titanium(III) citrate (28)], (ii) anoxic conditions [$E_h \geq 0$ V; anaerobic gas phase; extracts without titanium(III) citrate], or (iii) aerobic conditions [aerobic gas phase; extracts without titanium(III) citrate]. Crude extracts lost most of the initial TCB dehalogenase activity during 48 h of storage. Anoxic and even more anaerobic storage conditions increased the stability of the enzyme significantly (Fig. 2). The pH optimum of 1,2,3-TCB dehalogenase activity in crude extracts was 6.1. Crude extracts lost dehalogenase activity after 1 h of incubation at 60°C or above.

Reductive dehalogenases of most dehalorespiring species are light-reversibly inhibited by alkyl iodides, indicating the involvement of corrinoids as cofactors (8, 10, 12, 15, 17). In fact, corrinoids have been detected in purified enzymes (6, 10, 16, 19, 21). In whole cells and crude extracts of strain CBDB1, the presence of 10 μ M ethyl iodide or propyl iodide in the dark reduced 1,2,3-TCB dehalogenase activity to 50 to 75% of that of control samples without inhibitor. Activity could be restored to 78 to 96% of control activity by 10 min of exposure to the light of a 250-watt lamp. The results suggest the involvement of a corrinoid cofactor also in dehalogenation of chlorobenzenes by strain CBDB1.

1,2,3-TCB dehalogenase activity of strain CBDB1 was found to be associated with the membrane fraction which was obtained after ultracentrifugation of the crude extract at 120,000

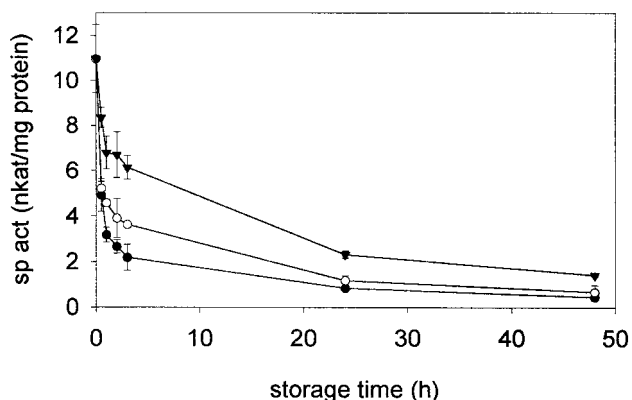


FIG. 2. Aerotolerance and stability of TCB dehalogenase activity. Crude extracts were stored at 4°C under reduced anaerobic conditions ($E_h < -200$ mV) (triangles), anoxic conditions ($E_h \geq 0$ V) (open circles), or aerobic conditions (solid circles). Values are means of results of triplicate assays \pm standard deviation. sp act, specific activity.

\times g for 1 h. Treatment of whole cells with 0.01% Triton X-100 or 10 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) resulted in solubilization of TCB dehalogenase activity. Solubilization by such low concentrations of the nonionic detergent Triton X-100 indicates a peripheral attachment to the membrane (22). This was also suggested for trichloroethene dehalogenase from *D. ethenogenes*, which could be extracted from the membrane with 0.1% Triton X-100 or 1 M NaCl (13).

The orientation of reductive dehalogenases of dehalorespiring bacteria in the cell membrane is still under investigation. For PCE dehalogenase of strain PCE-S, experiments with the hydrophilic electron donor MV indicate a cytoplasmic orientation of the enzyme (15). In contrast to PCE dechlorination in strain PCE-S, dechlorination of 1,2,3-TCB in strain CBDB1 with MV as the electron donor did not increase after cell disruption. Also, permeabilization of whole cells with 0.4% toluene did not enhance dechlorination. MV has been shown to permeate cell membranes only to a very limited extent in *Escherichia coli* (9). Therefore, our findings match with the hypothesis that TCB dehalogenase of strain CBDB1 is oriented to the outside of the membrane, as was also suggested for the reductive dehalogenases of *D. ethenogenes* (13) and *Desulfotobacterium dehalogenans* (23, 26).

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