# Initial Characterization of a Reductive Dehalogenase from *Desulfitobacterium chlororespirans* Co23†

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**Desulfitobacterium chlororespirans Co23 is capable of using 3-chloro-4-hydroxybenzoate as terminal electron acceptor for growth. Membrane preparations from cells grown fermentatively on pyruvate in the presence of 3-chloro-4-hydroxybenzoate dechlorinated this compound at a rate of 3.9 nmol min$^{-1}$ mg of protein$^{-1}$. Fivefold-greater dechlorination rates were measured with reduced methyl viologen as the artificial electron donor. Reduced benzyl viologen, NADH, NADPH, reduced flavin adenine dinucleotide, and reduced flavin mononucleotide could not substitute for reduced methyl viologen. The maximal initial rate of catalysis was achieved at pH 6.5 and 60°C. The membrane-bound dechlorinating enzyme system was not oxygen sensitive and was stable at 57°C for at least 2 h. Sulfite inhibited dechlorination in cell-free assays, whereas sulfate did not. Several chlorophenols were dehalogenated exclusively in the *ortho* position by cell extracts.**

Intensive studies over the last decades have led to considerable knowledge about biological strategies for degrading halogenated compounds under aerobic conditions. However, many contaminated sites are anoxic, and much less is known about the organisms and especially the mechanisms involved in the liberation of halogens in these anaerobic environments. A variety of halogenated compounds, including many of which are not attacked by aerobic organisms, can be completely dehalogenated or at least transformed to less halogenated compounds under anaerobic conditions (15).

Under aerobic conditions, organisms compete for carbon and energy sources while using oxygen as the terminal electron acceptor. In anaerobic environments, organisms often compete not only for a carbon source but also for an electron acceptor. Reductive dechlorination is an electron-consuming process, indicating that halogenated compounds could potentially serve as electron acceptors. Furthermore, reductive dehalogenation could provide enough free energy to allow the formation of biological energy (7). This energy-yielding process, which uses a halogenated compound as a terminal electron acceptor, is known as halorespiration. A few strictly anaerobic bacteria with this capability have been obtained in pure culture. *Desulfomonile tiedjei* DCB-1 (5, 20) and a marine isolate, strain DCB-F (3), dechlorinate 3-chlorobenzoate to benzoate. *Dehalospirillum multivorans* (21) and "*Dehalobacter restrictus*" (9, 22) convert tetrachloroethene to *cis*-dichloroethene. The anaerobic myxobacterial strains 2CP-1, 2CP-C, and 2CP-3 dechlorinate 2-chlorophenol to phenol (1, 18), and *Desulfitobacterium* strain PCE1 (8) dechlorinates tetrachloroethene to trichloroethene and 4-chlorophenylacetate to 4-hydroxyphenylacetate. Finally, *Desulfitobacterium chlororespirans* Co23 was characterized as a new species in the genus *Desulfitobacterium* (19) and dechlorinates 3-chloro-4-hydroxybenzoate (3Cl-4-HBA) to 4-hydroxybenzoate (4-HBA). In these examples, the bacteria can fulfill their energy requirement for growth by utilizing reductive dechlorination. The physiological significance of the reductive dehalogenation reaction in other aryl-dechlorinating organisms available as pure cultures is as yet unclear (13, 23, 26, 27).

Knowledge of the components involved in electron transfer from a donor molecule to an electron-accepting chlorinated compound is very limited. Recently, Louie and Mohn found a unique membrane-bound cytochrome *c* induced in *Desulfomonile tiedjei* DCB-1 by 3-chlorobenzoate (12). Of particular interest are the terminal reductases, the dechlorinases. A membrane-bound dechlorinase was purified from the gram-negative organism *Desulfomonile tiedjei* DCB-1 and subjected to preliminary characterization (10, 17). Furthermore, the tetrachloroethene-dechlorinating enzyme from *Dehalospirillum multivorans* was purified from the soluble cytoplasmic protein fraction (6). This paper describes the detection of cell-free dechlorinating activity and the initial characterization of an aryl reductive dehalogenase involved in chlororespiration from the gram-positive organism *D. chlororespirans* Co23.

## MATERIALS AND METHODS

**Cultivation of cells.** *D. chlororespirans* Co23 was grown under strict anaerobic conditions in 160-ml and 1-liter serum bottles (containing 125 and 900 ml of medium, respectively) closed with butyl rubber stoppers. Anaerobic medium was prepared as described by Cole et al. (1). The medium contained the following (per liter): NaCl, 1.0 g; MgCl$_2$ · 6H$_2$O, 0.5 g; KH$_2$PO$_4$, 0.2 g; NH$_4$Cl, 0.3 g; KCl, 0.3 g; CaCl$_2$ · 2H$_2$O, 0.015 g; resazurin, 1 mg; trace element solution A, 1 ml; trace element solution B, 1 ml; Na$_2$S · 9H$_2$O, 0.048 g; L-cysteine, 0.035 g; NaHCO$_3$, 2.52 g; vitamin solution (19), 10 ml; pyruvate, 20 mmol; and 3 Cl-4-HBA, 1 mmol. Trace element solution A contained the following (per liter): HCl (25% [wt/wt] solution), 10 ml; FeCl$_2$ · 4H$_2$O, 1.5 g; CoCl$_2$ · 6H$_2$O, 0.19 g; MnCl$_2$ · 4H$_2$O, 0.1 g; ZnCl$_2$, 70 mg; H$_3$BO$_3$, 6 mg; Na$_2$MoO$_4$ · 2H$_2$O, 36 mg; NiCl$_2$ · 6H$_2$O, 24 mg; and CuCl$_2$ · 2H$_2$O, 2 mg. Trace element solution B contained 6 mg of Na$_2$SeO$_3$ per liter, 8 mg of Na$_2$WO$_4$ · 2H$_2$O per liter, and 0.5 g of NaOH per liter.

Reductants were added to the medium after it had been boiled and cooled to room temperature. The headspace was subsequently flushed with oxygen-free N$_2$-CO$_2$ (80:20), and the pH was adjusted to 7.2 to 7.3 by varying the flow of CO$_2$. Vitamins, pyruvate, and 3Cl-4-HBA were added from anoxic stock solutions after autoclaving.

The serum bottles were inoculated with 0.5% (vol/vol) of an exponentially growing culture and incubated at 37°C without agitation. After 48 and 72 h, samples were withdrawn and the concentrations of pyruvate and 3Cl-4-HBA were measured by high-pressure liquid chromatography (HPLC) (see below). When all the pyruvate and 3Cl-4-HBA were depleted, another 20 mmol of pyruvate per liter and 1 mmol of 3Cl-4-HBA per liter were added and the pH was adjusted to 7.2 to 7.3 with an anoxic 2 M KOH solution. At the end of the logarithmic growth phase (days 5 to 7), the cells were harvested by centrifugation and washed twice with 50 mM anoxic potassium phosphate buffer (pH 7.5). To obtain noninduced cells, *D. chlororespirans* Co23 was transferred five times in

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medium lacking 3Cl-4-HBA before the cells were harvested for use in the cell-free experiments.

**Preparation of membrane fraction.** Cells (30 g [wet weight]) were suspended in 45 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 1 mM EGTA [ethylene glycol-bis($\beta$-aminoethyl ether)-$N,N,N',N'$-tetraacetic acid]. The suspension was subjected to ultrasonic treatment for 20 min with 1-s bursts from an ultrasonic disintegrator (Heat Systems-Ultrasonics sonicator W-385). Unbroken cells were collected by centrifugation at $11,000 \times g$ and 4°C for 30 min. The pellet was suspended in the same buffer, and sonication was repeated. The combined supernatants were designated crude cell extracts and contained 40 to 45 mg of protein ml$^{-1}$. The turbid supernatant was subjected to ultracentrifugation at $200,000 \times g$ and 4°C for 5 h. The clear supernatants were removed, and the pellets were carefully suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol. The suspension was again fractionated by ultracentrifugation. This washing procedure was repeated until the supernatant after ultracentrifugation contained less than 0.1 mg of protein ml$^{-1}$. For the final washing step, 50 mM potassium phosphate buffer containing 1 mM dithiothreitol (pH 7.5) was used. The pellet containing the membranes was suspended in the same buffer, and the protein concentration was adjusted to 10 mg ml$^{-1}$. Both crude cell extracts and membrane preparations were frozen and stored at $-80$°C.

**Dechlorination assay (standard enzyme assay).** All cell-free assays were done in an anaerobic glove box with a N$_2$-H$_2$ (97:3) atmosphere. Dehalogenation assays were performed in 5-ml crimp-sealed serum vials. The assay mixtures contained, in a total volume of 1 ml, 100 mM HEPES ($N$-2-hydroxyethylpiperazine-$N'$-2-ethanesulfonic acid; pH 7.2), 2 mM methyl viologen (1,1'-dimethyl-4,4'-bipyridinium dichloride), 2 mM titanium(III) citrate, 2 mM 3Cl-4-HBA, and 0.1 ml of protein fraction (0.05 to 0.5 mg of protein). Samples of 50 µl were withdrawn and diluted with 200 µl of H$_2$O-acetonitrile-H$_3$PO$_4$ (1,000:500:1, vol/vol/vol). For experiments outside the glove box, the vials were closed with Teflon-lined butyl rubber stoppers.

Titanium(III) citrate solution was prepared fresh 1 day before use and stored anaerobically at 4°C for no longer than 3 days (28).

For determining the temperature optimum of the maximal initial rate of catalysis, the reaction mixtures (containing 0.1 mg of protein) were prepared in the glove box and kept on ice prior to incubation at the different temperatures. The temperature stability in complete standard assay mixtures (0.1 mg of protein) lacking the substrate 3Cl-4-HBA was determined. After incubation at the different temperatures, the vials were allowed to equilibrate to room temperature and the enzymatic reaction was started by adding 3Cl-4-HBA.

The pH optimum was determined in 100 mM MES [2-($N$-morpholino)ethanesulfonic acid] buffer (pH 5.45 to 6.78), 100 mM MOPS [3-($N$-morpholino)propanesulfonic acid] buffer (pH 6.51 to 7.81), 100 mM HEPES buffer (pH 6.88 to 8.16), 100 mM TES [$N$-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (pH 6.88 to 8.16), 100 mM Tris buffer (pH 7.29 to 8.85), and 100 mM Bis-Tris propane (1,3-bis[tris(hydroxymethyl)methylamino]propane) buffer (pH 6.34 to 9.2). All buffers contained 20% (vol/vol) glycerol. The pH values were verified with a pH electrode immediately after removal of a sample for HPLC analysis.

All data represent the mean of at least five independent experiments. When the standard enzyme assay (described above) was used, the differences in dehalogenation rates among five individual experiments and up to four sampling times were below 20%.

**Analytical methods.** 4-HBA, the product of the reductive dechlorination of 3Cl-4-HBA, was measured by HPLC. A Perkin-Elmer binary LC pump 250 equipped with a Millipore Waters detector (Lambda-Max model 480 LC spectrophotometer) was used. 4-HBA and 3Cl-4-HBA were detected at 254 nm with H$_2$O-methanol-H$_3$PO$_4$ (600:400:1, vol/vol/vol) as the eluent. Dechlorination products were quantified by comparing the peak area response of the samples with that of identically treated standards (five-point calibration, 0.01 to 2 mM). Samples were centrifuged at $13,000 \times g$ for 5 min, and 20-µl samples were injected manually onto a Brownlee RP-18 column (220 by 4.6 mm; particle size, 5 µm; Applied Biosystems). Pyruvate and chlorophenols were analyzed chromatographically as described by Sanford et al. (19).

**Protein determination.** Protein concentration was estimated by a modification of the Lowry assay (24). Buffers identical to those containing the protein samples were used as blanks, and ovalbumin was used as the standard.

## RESULTS

**Growth of _D. chlororespirans_ Co23 and induction of dechlorinating activity.** _D. chlororespirans_ Co23 was routinely grown on pyruvate in the presence of 3Cl-4-HBA. The late exponential growth phase was reached within 1 week, and the mean cell yield was 1.2 to 1.8 g (wet weight) of cells per liter. Cell extracts dechlorinated 3Cl-4-HBA with titanium(III) citrate as the reductant (Table 1). No dechlorinating activity was detected in crude cell extracts from cells grown in the absence of 3Cl-4-

TABLE 1. Reduction of 3Cl-4-HBA to 4-HBA by cell extracts from _D. chlororespirans_ Co23 under different incubation conditions

| Incubation condition | Sp act (nmol min$^{-1}$ mg$^{-1}$)$^a$ of: | |
| --- | --- | --- |
| | Crude extracts | Membranes |
| Extracts from induced cells, no MV | 2.0 | 3.7 |
| Extracts from induced cells + extracts from noninduced cells, no MV$^b$ | 1.1 | 1.75 |
| Extracts from induced cells | 9.0 | 19.3 |
| Extracts from induced cells, no titanium(III) citrate | 0 | 0 |

$^a$ Crude extracts (0.2 mg of protein) and membranes (0.1 mg of protein) were incubated under standard assay conditions of 2 mM titanium(III) citrate, 2 mM MV, 2 mM 3Cl-4-HBA, and an N$_2$-H$_2$ (97:3) atmosphere unless indicated otherwise. Samples were analyzed after 2 and 4 h.

$^b$ Crude extracts (0.2 mg of protein) or membranes (0.1 mg of protein) from both induced and noninduced cells were incubated together under standard assay conditions lacking MV.

HBA, indicating that the dechlorinating enzyme system was inducible.

**Localization and oxygen sensitivity of the dechlorinating enzyme system.** After ultracentrifugation of the crude extracts, no dechlorinating activity was detected in the soluble protein fraction. Between 82 and 90% of the initial activity measured in crude extracts was recovered in the particulate fraction containing the membranes. Similar activities were measured when the membranes were prepared either under strictly anaerobic conditions or in the presence of air. Also, similar activities were detected when air-bubbled membrane preparations (0.1 mg of protein ml$^{-1}$) were compared with argon-bubbled controls. Therefore, the membrane-associated dechlorinating enzyme system was not oxygen sensitive. However, because the electron donor was rapidly oxidized when exposed to air, oxygen had to be removed before dechlorinating activity could be measured.

**Electron donors for reductive dehalogenation in cell-free systems.** In crude extracts derived from induced cells, reductive dechlorination could be detected without adding the artificial electron donor methyl viologen (MV) (Table 1). An unknown component present in these crude extracts appeared to serve as an electron donor when reduced with titanium(III) citrate. Furthermore, the washed membrane fraction dechlorinated 3Cl-4-HBA without MV at rates comparable to those of the crude extracts, indicating that the unknown component was not removed by ultracentrifugation. The addition of MV to the test system increased the dechlorination rate by fivefold (Table 1). The addition of cell extracts from noninduced cells (cells grown with pyruvate in the absence of 3Cl-4-HBA) to the standard assay mixture lacking MV had no effect on the dechlorination activity (Table 1). This indicates that not only the dechlorinating enzyme system but also the unknown electron donor present in cell extracts from induced cells was specifically induced by 3Cl-4-HBA.

NADH, NADPH, reduced flavin adenine dinucleotide, reduced flavin mononucleotide, methylene blue, and benzyl viologen could not serve as electron donors for the reductively dechlorinating enzyme system (data not shown).

The enzymatic reduction of MV due to hydrogenase activity was not detected in cell extracts, even after the headspace gases were replaced with 100% hydrogen.

**Standard enzyme assay.** A linear relationship between product formation, incubation time (up to 6 h), and protein concentration (0.05 and 0.1 mg) was obtained in the standard assay
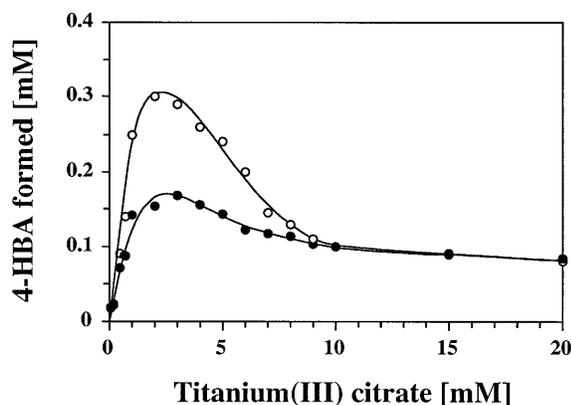
FIG. 1. Titanium(III) citrate dependence of 3Cl-4-HBA reduction. Membrane fractions (0.1 mg of protein) were incubated under standard conditions with various amounts of titanium(III) citrate for 1 h (solid circles) and 2 h (open circles).
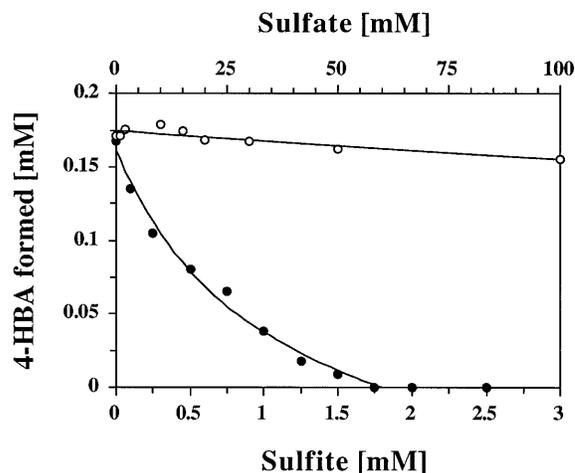


FIG. 2. Effect of sulfite (solid circles) and sulfate (open circles) on the dechlorination of 3Cl-4-HBA to 4-HBA. The assay mixtures containing membrane preparations (0.1 mg of protein) from *D. chlororespirans* Co23 were incubated for 1 h under standard conditions.

system used in this study. After incubation periods longer than 6 h, reduced MV became limiting as indicated by a visible color change. By adding another 2 mM titanium(III) citrate to the reaction mixture, complete conversion of the initial amount of 3Cl-4-HBA was achieved within 24 h.

Cell extracts could be stored for 3 months at $-80°C$ without significant loss of activity. When membrane preparations were stored at 4°C, about 10% of the dechlorinating activity was lost within 36 h.

Dechlorination was never detected in controls without the cell extracts or the membrane preparation or with the addition of heat-denatured protein.

**Influence of reductants on the dechlorinating enzyme system.** Titanium(III) citrate was used in the cell-free assay systems to reduce MV to the intensely blue monocation radical which acts as the artificial electron donor for the dechlorinating reductase. A decrease in dechlorinating activities was measured at titanium(III) citrate concentrations below 1 mM and above 5 mM (Fig. 1). In both cases, the dark blue color of the assay mixtures vanished within minutes, indicating that insufficient amounts of the monocation form of MV were present.

Dithionite was an inappropriate reductant in cell-free assay systems because it showed inhibitory effects on the dechlorinating enzyme system. No dechlorination could be observed in the standard enzyme assay (0.5 mg of protein) at dithionite concentrations above 4 mM (data not shown). Figure 2 shows the strong inhibition by sulfite, a chemical degradation product of dithionite. Sulfate had no significant effect on dechlorination.

**Influence of pH and temperature on enzyme activity and stability.** The maximal initial rate of dechlorination was observed between pH 6.2 and 7.0 (Fig. 3). Fifty percent of the maximal activity was measured at pH 5.4 and pH 8.0. Similar activities were measured in the HEPES, MOPS, TES, Tris, Bis-Tris propane, and potassium phosphate buffer systems (100 mM, 20% glycerol, pH 7.5). Storing the membrane preparations (1 mg of protein ml$^{-1}$ at pH 6.5, 7.5, and 8.5) at 4°C for 16 h did not affect the dechlorination rates compared with those measured by the standard enzyme assay at time zero.

The highest initial dechlorination rates were measured at 60°C (Fig. 4). The activities measured at 60°C exceeded the activities at room temperature by nearly threefold. The enzyme system was stable under the assay conditions for at least 2 h at temperatures below 57°C (Fig. 4). Incubation at higher temperatures caused a rapid loss of enzyme activity.

**Substrate specificity.** In addition to 3Cl-4-HBA, 3,5-dichloro-4-hydroxybenzoate, 3-chloro-4-hydroxyphenylacetate, and several polychlorophenols were dechlorinated, as summarized in Table 2. Dechlorination occurred exclusively in the *ortho* position with respect to the hydroxy group. Compounds lacking the hydroxy group (e.g., 3-chlorobenzoate) and compounds in which the hydroxy group is replaced by a chlorine (e.g., 3,4-dichlorobenzoate), a methyl (e.g., 3-chloro-4-methylbenzoate), a methoxy (e.g., 3-chloro-4-methoxybenzoate) or an amino (e.g., 4-amino-3,5-dichlorobenzoate) group were not dechlorinated. Several substituted aromatic compounds containing the structural element of an *ortho*-chlorinated phenol were dechlorinated at different rates or not at all, indicating an effect of the other ring substituents on the dechlorination activity.

**Effect of metals, reducing agents, chelating agents, and detergents.** Adding $Fe(NH_4)_2(SO_4)_2$, $MnCl_2$, $MgCl_2$, $ZnCl_2$, $NiSO_4$, $CoCl_2$, $Na_2SeO_4$, $NaN_3$, NaCl, KCl, dithiothreitol, or reduced glutathione (1 mM each) to the standard enzyme assay mixture (0.1 mg of protein) had no significant effect on the dechlorination activity. Also, CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) at concentrations up to 20 mM and Triton X-100 (up to 0.5%, wt/vol) were
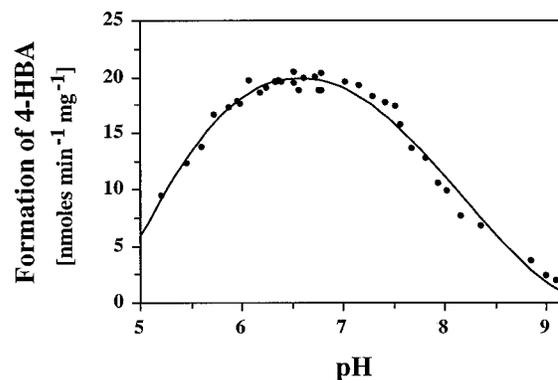


FIG. 3. pH dependence of 3Cl-4-HBA dechlorination. Membrane preparations (0.05 and 0.1 mg of protein) were incubated under standard conditions, and samples were analyzed after 1, 2, and 4 h.
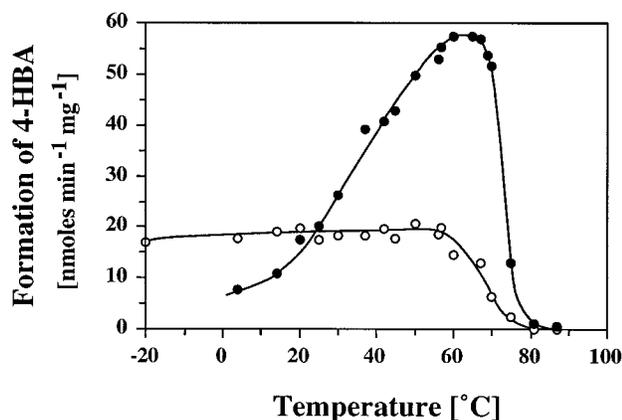
FIG. 4. Temperature dependence of 3Cl-4-HBA reduction by membrane preparations from *D. chlororespirans* Co23. Enzyme assay mixtures (0.1 mg of protein) were prepared in a glove box and kept on ice; they were then subjected to a 1-h incubation at the different temperatures (solid circles). For determining the thermal stability, complete assay mixtures containing 0.1 mg of protein but lacking the substrate 3Cl-4-HBA were incubated at the different temperatures for 2 h. After addition of the substrate 3Cl-4-HBA, the vials were incubated at room temperature for 1 h (open circles).

without effect. Sodium dodecyl sulfate (1 mM) showed a 75% inhibition. The metal-chelating agents EDTA and sodium citrate had no influence on the dechlorinating activity at concentrations up to 200 mM.

TABLE 2. Substrate specificity profile of membranes associated with reductive dehalogenase obtained from *D. chlororespirans* Co23 grown in the presence of 3-Cl-4-HBA

| Substrate[a] | Product(s)[a] | Mean sp act[b] (nmol min$^{-1}$ mg$^{-1}$) ± SD |
|---|---|---|
| 3Cl-4-HBA | 4-HBA | 17.2 ± 2.1 |
| 3,5-Dichloro-4-hydroxybenzoate | 3Cl-4-HBA, 4-HBA | 8.67 ± 1.44 |
| 3-Chloro-4-hydroxyphenylacetate | 4-Hydroxyphenylacetate | 4.75 ± 0.75 |
| 2,3-DCP | 3-CP | 2.62 ± 0.35 |
| 2,4-DCP | 4-CP | 0.127 ± 0.04 |
| 2,6-DCP | 2-CP | 0.43 ± 0.073 |
| 2,3,4-TCP | 3,4-DCP | 7.4 ± 0.45 |
| 2,3,5-TCP | 3,5-DCP | 0.73 ± 0.1 |
| 2,4,6-TCP | 2,4-DCP, 4-CP | 1.5 ± 0.43 |
| 2,3,4,5-TCP | 3,4,5-TCP | 0.538 ± 0.056 |
| 2,3,4,6-TCP | 2,4,5-TCP | 0.1 ± 0.01 |
| 2,3,5,6-TCP | 2,3,5-TCP, 3,5-DCP | 0.72 ± 0.18 |
| PCP | 2,3,4,5-TCP, 3,4,5-TCP | 1.4 ± 0.45 |

[a] CP, monochlorophenol; DCP, dichlorophenol; TCP, trichlorophenol; TeCP, tetrachlorophenol; PCP, pentachlorophenol.
[b] Activities were determined by using the standard assay system with MV as an electron donor and titanium(III) citrate as a reductant. Samples were taken after 1, 2, 4 and 6 h. Each chlorophenol was used at concentrations of 0.5, 1, and 2 mM (except 2,3,4,5-tetrachlorophenol and pentachlorophenol, which were used at 0.5 and 1 mM). The rates shown were calculated for the removal of the first chlorine and represent the average of five independent measurements. No dechlorination was detected with 2-chlorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate, 3,4-dichlorobenzoate, 2,5-dichlorobenzoate, 2-chloro-4-hydroxybenzoate, 4-amino-3,5-dichlorobenzoate, 3-chloro-4-methylbenzoate, 3-chloro-4-methoxybenzoate, 3-chloro-L-tyrosine, 3,4-dichlorophenoxyacetate, tetrachloroethene, 1,2-dichloropropane, 2,5-dichlorophenol, 3,4-dichlorophenol, 3,5-dichlorophenol, 2-monochlorophenol, 3-monochlorophenol, or 4-monochlorophenol.

## DISCUSSION

*D. chlororespirans* Co23 was enriched on the basis of its ability to *ortho* dechlorinate 2,3-dichlorophenol (19). In contrast to most other anaerobic dechlorinators isolated to date, strain Co23 grows rapidly and attains high cell densities on pyruvate in the presence of 3Cl-4-HBA. This has made strain Co23 an ideal model system for studying aryl halorespiration. Other halorespiring organisms like *Desulfomonile tiedjei* DCB-1 and DCB-F and "*Dehalobacter restrictus*" grow much more slowly and do not easily produce the biomass required to assess cell-free activity. In addition to 2,3-dichlorophenol, *D. chlororespirans* Co23 could dechlorinate 3Cl-4-HBA, 3,5-dichloro-4-hydroxybenzoate, 3-chloro-4-hydroxyphenylacetate, and several polychlorophenols. Interestingly, cell-free membrane preparations dechlorinated some polychlorophenols that could not serve as electron acceptors supporting the growth of strain Co23 (19).

As stated above, titanium(III) citrate was used to reduce the artificial electron donor MV for the reductive dechlorinase in cell-free assay systems. The optimal Ti$^{3+}$ concentration in the standard assay system was 2 mM. At lower titanium(III) citrate concentrations, not enough MV$^{2+}$ was reduced to the deep blue monocation radical (MV$^{\cdot+}$). At high titanium(III) citrate concentrations, MV$^{\cdot+}$ was reduced to the uncharged dihydro-bipyridyl (MV$^0$), which does not function as an electron donor (4). Optimal enzymatic activity and linearity of product formation were obtained at equimolar concentrations of MV and titanium(III) citrate (2 mM each).

During the chemical reduction of MV with dithionite (S$_2$O$_4^{2-}$), undesirable by-products such as SO$_3^{2-}$, SO$_4^{2-}$, S$_2$O$_3^{2-}$, and S$^{2-}$ are formed (4). Sulfite, which is known to form complexes with many flavoproteins as well as some heme proteins, strongly inhibited the reductase from *D. chlororespirans* Co23. The reductive dehalogenase from *Desulfomonile tiedjei* DCB-1 was shown to be a heme protein, and the dechlorinating activity was also inhibited by sulfite (5, 10, 17).

In contrast, metal-chelating agents showed no inhibitory effect on the dechlorinating enzyme systems from both organisms (10). The presence of metals tightly bound to the proteins, however, cannot be excluded from consideration.

The only characterized aryl-reductive dehalogenases involved in halorespiration are the membrane-bound enzyme systems derived from *Desulfomonile tiedjei* DCB-1 and *D. chlororespirans* Co23. They revealed different physicochemical and catalytic properties. The optimal temperature for the initial rate of catalysis was around 37°C in cell extracts from *Desulfomonile tiedjei* (5, 10, 17), compared with 60°C observed in extracts from strain Co23. The pH optimum was determined to be 7.2 in extracts from *Desulfomonile tiedjei* (11, 17) and 6.5 in extracts from strain Co23. The specific activities measured in crude cell extracts and membrane preparations from *D. chlororespirans* Co23 (measured at 25°C) exceeded the values obtained for cell-free preparations from *Desulfomonile tiedjei* (measured at the optimal temperature of 37°C) by 50- to 100-fold (10, 17). Considering that the activities measured in extracts from *D. chlororespirans* Co23 were threefold higher at the optimal temperature of 60°C, the difference in dechlorinating activity between extracts from strain Co23 and *Desulfomonile tiedjei* is even greater.

The substrate specificity was different for *D. chlororespirans* Co23 and *Desulfomonile tiedjei* DCB-1 dehalogenases; for example, 3-chlorobenzoate was not dehalogenated by membrane preparations from strain Co23. Unfortunately, no data on the specificity of cell extracts from *Desulfomonile tiedjei* toward chlorinated phenols are available. Studies by Mohn and

Kennedy (14) with whole cells of *Desulfomonile tiedjei* showed that only chlorines on the *meta* position were removed. In contrast, both whole cells (19) and cell extracts from strain Co23 removed chlorines only from the *ortho* position. In both cases, monochlorinated phenols were not dehalogenated. Furthermore, cell extracts from *Desulfomonile tiedjei* were shown to dechlorinate tetrachloroethene and trichloroethene to *cis*-dichloroethene with reduced MV as the electron donor (25). The same dechlorinating activity was detected in 3-chlorobenzoate-induced whole cells of *Desulfomonile tiedjei* (2). Neither whole cells nor cell extracts of strain Co23 showed any dechlorinating activity toward tetrachloroethene (11). However, the reductive dechlorinases from *Desulfomonile tiedjei* DCB-1 and *D. chlororespirans* Co23 might be mechanistically related despite the differences in substrate specificity and catalytic activity.

To date, aryl-reductive dehalogenation in cell extracts derived from anaerobic organisms could be shown with strain DSL-1 (a 2,4,6-tribromophenol-debrominating bacterium) (23), *Desulfomonile tiedjei* DCB-1 (5, 10, 17), and *D. chlororespirans* Co23. In the last two cases, the reductase (dechlorinase) was membrane bound and was capable of using MV as the artificial electron donor. Dechlorination in cell extracts from *Desulfomonile tiedjei* was strictly dependent on MV (5, 17), whereas dechlorination in extracts from strain Co23 could also be detected without the addition of MV. The unidentified electron donor present in cell extracts from induced *D. chlororespirans* Co23 cells was membrane bound and could be reduced by adding titanium(III) citrate. Neither NADH nor NADPH supported reductive dechlorination in extracts from *Desulfomonile tiedjei* or from strain Co23. In contrast, the reductive debromination in crude extracts from strain DSL-1 was stimulated by NADH and NADPH. For the soluble tetrachloroethene and trichloroethene reductive dehalogenase from the anaerobe *Dehalospirillum multivorans*, MV was found to be the only effective electron donor in cell extracts (16).

The mechanism of how enzymes perform aryl-reductive dehalogenation is not yet understood. If the different reductive dehalogenases have common mechanistic features, this will become apparent once detailed information on the enzymes and sequence data are available.

Because of the ease with which *D. chlororespirans* Co23 can be grown and the higher catalytic activities of the reductively dechlorinating enzyme system compared with *Desulfomonile tiedjei* DCB-1, the reductive dechlorinase from strain Co23 is a promising candidate to enable us to gain further insight in this process.

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