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NADPH-Dependent Reductive *ortho* Dehalogenation of 2,4-Dichlorobenzoic Acid in *Corynebacterium sepedonicum* KZ-4 and Coryneform Bacterium Strain NTB-1 via 2,4-Dichlorobenzoyl Coenzyme A

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***Corynebacterium sepedonicum* KZ-4, described earlier as a strain capable of growth on 2,4-dichlorobenzoate (G. M. Zaitsev and Y. N. Karasevich, Mikrobiologiya 54:356–369, 1985), is known to metabolize this substrate via 4-hydroxybenzoate and protocatechuate, and evidence consistent with an initial reductive dechlorination step to form 4-chlorobenzoate was found in another coryneform bacterium, strain NTB-1 (W. J. J. van den Tweel, J. B. Kok, and J. A. M. de Bont, Appl. Environ. Microbiol. 53:810–815, 1987). 2-Chloro-4-fluorobenzoate was found to be converted stoichiometrically to 4-fluorobenzoate by resting cells of strain KZ-4, compatible with a reductive process. Experiments with cell extracts demonstrated that Mg · ATP and coenzyme A (CoA) were required to stimulate reductive dehalogenation, consistent with the intermediacy of 2-chloro-4-fluorobenzoyl-CoA and 2,4-dichlorobenzoyl-CoA thioesters. 2,4-Dichlorobenzoyl-CoA was shown to be converted to 4-chlorobenzoyl-CoA in a novel NADPH-dependent reaction in extracts of both KZ-4 and NTB-1. In addition to the ligase and reductive dehalogenase activities, hydrolytic 4-chlorobenzoyl-CoA dehalogenase and thioesterase activities, 4-hydroxybenzoate 3-monooxygenase, and protocatechuate 3,4-dioxygenase activities were demonstrated to be present in the soluble fraction of KZ-4 extracts following ultracentrifugation. We propose that the pathway for 2,4-dichlorobenzoate catabolism in strains KZ-4 and NTB-1 involves formation of 2,4-dichlorobenzoyl-CoA, NADPH-dependent *ortho* dehalogenation yielding 4-chlorobenzoyl-CoA, hydrolytic removal of chlorine from the *para* position to generate 4-hydroxybenzoyl-CoA, hydrolysis to form 4-hydroxybenzoate, oxidation to yield protocatechuate, and oxidative ring cleavage.**

Chlorobenzoates are known to be common intermediates of polychlorinated biphenyl (PCB) degradation by microorganisms (10). In order to bioremediate PCB-contaminated sites, it is important to effectively integrate microorganisms containing broad-spectrum PCB dioxygenase activities with mechanisms for eliminating the resulting variety of chlorobenzoates that are produced (16). The latter reactions can take place either within the same organism or in appropriate cocultures or consortia. Of particular significance to chlorobenzoate metabolism are those enzymes that catalyze dehalogenation reactions, since the products are generally less toxic and more easily utilized by most microorganisms (8). This paper reports the presence of a novel reductive dehalogenase within the pathway for 2,4-dichlorobenzoate (24DCB) metabolism in *Corynebacterium sepedonicum* KZ-4 and coryneform bacterium strain NTB-1.

C. sepedonicum KZ-4 can grow on 4-chlorobenzoate (4CB) or 24DCB as a sole source of carbon and energy (42), but the metabolic pathway for utilization of these substrates is poorly defined. For example, 4-hydroxybenzoate was reported to be the first intermediate detected in the metabolism of either substrate by strain KZ-4. Work carried out with other strains, however, can provide insights into the likely metabolic pathways in this strain. As an illustration, the mechanism for conversion of 4CB to 4-hydroxybenzoate has been elucidated in *Pseudomonas* sp. strain CBS3. The overall reaction involves four distinct steps: (i) Mg · ATP- and coenzyme A (CoA)-dependent modification of 4CB to form 4CBCoA by 4CBCoA ligase, (ii) attack by an enzymic carboxylate on the C-4 position of the benzoyl ring and elimination of chloride, (iii) hydrolysis of the arylated enzyme to release 4-hydroxybenzoyl-CoA, and (iv) thioesterase-catalyzed hydrolysis of the thioester to yield the product 4-hydroxybenzoate (3, 21, 23, 35, 41). The 4CB CoA dehalogenase that catalyzes steps ii and iii in this pathway leading to hydrolytic dehalogenase activity also has been purified and characterized in other 4CB utilizers such as *Arthrobacter* sp. strain 4-CB1 (4) and has been demonstrated by sequence analysis to be present in *Arthrobacter* sp. strain SU (34) and *Arthrobacter globiformis* KZT1 (38). The same set of reactions also is likely to account for 4CB metabolism by *C. sepedonicum* KZ-4; however, experiments to directly test this hypothesis have not been described. With regard to 24DCB conversion to 4-hydroxybenzoate by strain KZ-4, the only closely related studies are those involving the coryneform bacterium strain NTB-1 (formerly named *Alcaligenes denitrificans* NTB-1). This organism was reported to convert 24DCB to 4CB by a reductive dehalogenation reaction and to subsequently convert 4CB to 4-hydroxybenzoate (12, 39). We set out to establish whether this reductive dehalogenation reaction was utilized for 24DCB metabolism in strain KZ-4 and to characterize some of the properties of this system. We have discovered the presence of a novel reductive dehalogenation reaction that is present in strains KZ-4 and NTB-1.

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Microbial reductive dehalogenation systems have been reviewed elsewhere (8, 15, 18, 27), and only two reasonably well characterized mechanisms for aryl halide reductive dehalogenation have been described. In the first of these mechanisms, *Flavobacterium* sp. strain ATCC 39723 was shown to possess a soluble, 28,263-Da glutathione *S*-transferase that catalyzes reductive elimination of tri- or tetrachloro-*p*-hydroquinone, presumably via two glutathione transfer reactions with the intermediate formation of *S*-glutathionyl adducts (31, 40). In the second example, a reductive dehalogenase that is coupled with energy production from 3-chlorobenzoate in *Desulfomonile tiedjei* DCB-1 has been purified (25, 28). The natural electron donor for the membrane-bound enzyme is not known; however, the activity was able to be monitored by using an artificial electron donor as the reducing agent. Spectroscopic analysis of the two-subunit enzyme is compatible with the presence of a redox-active heme chromophore. The results that we present here demonstrate that 24DCB metabolism by strains KZ-4 and NTB-1 occurs by a mechanism distinct from either of those described above.

MATERIALS AND METHODS

Cultivation and harvesting. *C. sepedonicum* KZ-4 was maintained on solid agar medium as described earlier (42). Routine cultivation was carried out at 30°C in 600 ml of medium by using 2-liter Erlenmeyer flasks with agitation of 240 rpm or in 10 liters of medium by using an MG-114 fermentor (New Brunswick Scientific, New Brunswick, N.J.) at 27°C with agitation at 400 rpm and air supplied at 2 liters/min. Each liter of medium contained 0.7 g of NaH_2PO_4, 3.1 g of K_2HPO_4, 0.15 g of $MgSO_4$, 0.5 g of urea, 50 mg of yeast extract, 0.02 mg of biotin, 0.5 mg of thiamine, 0.5 mg of riboflavin, 0.5 mg of inositol, and trace elements as described for medium 12C (13). 24DCB (1 mM) was added daily for 5 consecutive days. Cultures grew with an approximate doubling time of 36 h. Biomass was harvested by centrifugation (8,000 × *g*), washed three times with 25 mM potassium phosphate buffer (pH 7.25), and stored at −20°C.

Strain NTB-1 was cultivated as described earlier (12) in medium containing 0.5% glucose and 2 mM 24DCB. Under these conditions, the culture exhibits a doubling time of 13 h (39).

Resting cell experiments. Cells were resuspended to a density of 10 U of optical density at 600 nm in 25 mM potassium phosphate buffer (pH 7.25) containing 1 mM benzoate or one of several halobenzoates, incubated on a rotary shaker at 30°C, and sampled (0.5 ml) at 1-h intervals. Cells were removed by centrifugation, and the concentrations of substrate and product in the supernatant solution were determined by high-pressure liquid chromatography (HPLC).

Preparation of cell extracts. Frozen biomass was thawed, suspended in 25 mM potassium phosphate (pH 7.25), and disrupted by five passages through a French pressure cell at 18,000 lb/in^2. Debris was removed by centrifugation at 40,000 or 100,000 × *g* for 1 h to obtain the cell extracts. Membrane and cytosolic fractions were prepared from the 40,000 × *g* cell extracts by ultracentrifugation at 110,000 × *g* for 1 h. Membrane fractions were further purified by resuspension in the potassium phosphate buffer and reultracentrifugation.

HPLC methods. Concentrations of halobenzoates and their CoA derivatives were determined by reverse-phase HPLC on a Lichrosorb C_{18} column (4 by 250 mm; Merck, Rahway, N.J.) with an ammonium acetate (pH 5.0, 100 mM)-acetonitrile eluant (91% aqueous–9% organic phase for halobenzoates and 80% aqueous–20% organic phase for CoA derivatives) at a flow rate of 1.5 ml/min. Ion pair HPLC was used as an alternative system for determination of CoA derivatives. In this method, a BioSil C_{18} column (4 by 250 mm; Bio-Rad) was eluted at 1 ml/min with a mixture of 70% 100 mM ammonium acetate buffer containing 5.71 g of tetrabutyl ammonium chloride (pH 5.0) per liter and 30% acetonitrile. 4-Hydroxybenzoate concentrations were determined by using the same BioSil column eluted at 1 ml/min with 10% acetonitrile in 100 mM ammonium acetate buffer (pH 5.0) containing 1 g of tetrabutyl ammonium chloride per liter. In all methods, eluate was monitored at 254 nm. Concentrations were calculated by comparison with calibration standards.

Synthesis of thioesters of 4CB and 24DCB. The thioesters of 4CB and 24DCB with CoA (4CBCoA and 24DCBCoA, respectively) or glutathione were prepared by modification of a published method (26) as follows. One hundred-microliter aliquots of 4-chloro- or 2,4-dichlorobenzoyl chloride (Aldrich) were added to solutions of 15 mg of CoA or glutathione (Sigma) in ~3 ml of 0.1 M $NaHCO_3$ while flushing with nitrogen gas. The samples were sealed with rubber stoppers, and the vials were shaken at room temperature for 1 h. The reactions were stopped by acidification to pH 2 to 3, and the contaminating chlorobenzoic acids formed as a result of chlorobenzoyl chloride hydrolysis were allowed to precipitate at 4 to 6°C. After filtration (0.2-μm-pore-size nylon Acrodisc filters; Gelman Sciences, Ann Arbor, Mich.), the solutions were applied to a PepRPC 10/10 column (Pharmacia, Uppsala, Sweden) previously equilibrated with 20 mM ammonium acetate buffer (pH 5.0), and the reaction products were eluted by using a 60-ml linear gradient to 60% acetonitrile in the same buffer. The major peaks detected at 254 nm were evaporated to dryness and identified by negative-ion fast atom bombardment mass spectrometry, using procedures described previously (17).

Assays. 4CBCoA ligase and 24DCBCoA ligase activities were measured by monitoring the formation of the corresponding thioesters. Cell extracts were subjected to gel filtration chromatography on a Superose 12 column (1.6 by 50 cm; Pharmacia) in 25 mM potassium phosphate buffer (pH 7.25). Fractions that possessed low levels of thioesterase were combined and concentrated by using a PM10 ultrafiltration membrane. These samples were incubated at 30°C with 5 mM CoA and 5 mM chlorobenzoate in the presence of 7.5 mM ATP and 5 mM $MgCl_2$ (24). Reactions were quenched by acidification and analyzed by HPLC.

24DCBCoA reductase activity was monitored by HPLC for samples incubated at 30°C in 25 mM potassium phosphate buffer (pH 7.25) with 0.5 mM 24DCB CoA and 2 mM NADPH. Total reductase activity was based on the rate of formation of both 4CBCoA and 4CB, the latter arising from hydrolysis of 4CB CoA as a result of contaminating thioesterase activity.

4CBCoA dehalogenase activity was measured by HPLC quantitation of 4-hydroxybenzoate formed in aliquots of samples incubated at 30°C in 25 mM potassium phosphate buffer (pH 7.25) with 0.5 mM 4CBCoA.

4CBCoA and 24DCBCoA thioesterase assays were carried out in 25 mM potassium phosphate buffer (pH 7.25) at 30°C. The reaction mixtures (final volume of 2 ml) contained 1 to 10 mg of protein per ml and 0.5 mM thioester substrate. The reactions were initiated by adding substrate. Samples of 200 μl were taken every 10 min, immediately added to 30 μl of 0.5 M HCl, and left at 4°C overnight. The precipitates formed were removed by centrifugation, and the remaining 24DCBCoA or 4CBCoA levels were assessed by HPLC. Alternatively, the amounts of 4CB or 24DCB were analyzed by HPLC after ethyl acetate extraction as described earlier (32).

The activity of 4-hydroxybenzoate 3-monooxygenase in cell extracts was measured spectrophotometrically at 340 nm by monitoring NADH consumption in the presence of 4-hydroxybenzoate (7). Protocatechuate 3,4-dioxygenase, catechol 1,2-dioxygenase, and catechol 2,3-dioxygenase were assayed by spectrophotometric methods as described earlier (5, 9, 29).

Glutathione *S*-transferase activity was measured as described by Habig et al. (14), using 1,2-dichloro-4-nitrobenzene as a substrate. In addition, glutathione-dependent modification of 24DCBCoA was examined. Samples were incubated in a reaction mixture containing 0.5 mM 24DCBCoA and 5 mM glutathione in 25 mM potassium phosphate buffer (pH 7.25) at 30°C. Reactions were terminated at selected time points, and substrate and product levels were determined as described for the thioesterase assay.

Glutathione reductase activity was measured spectrophotometrically at 340 nm by monitoring the decrease of NADH or NADPH concentration. The reactions were initiated by addition of the oxidized disulfide form of glutathione to reaction mixtures (1 ml) containing 100 μl of cell extract and 0.2 mM NADH or NADPH. Reaction rates were corrected for nonspecific (base-level) NAD(P)H oxidation in the absence of glutathione.

Protein concentrations were estimated by the method of Bradford (2).

Metabolite identification. Cell extracts (4 ml) containing 7 mg of protein per ml and 2 mM NADPH in 25 mM potassium phosphate buffer (pH 7.25) were incubated for 5 min at 30°C, and the reactions were initiated by addition of 1 mM of 24DCBCoA. Reactions were terminated by addition of 0.5 ml of 0.5 N HCl, left overnight at 4 to 6°C, and cleared of precipitate by centrifugation. The supernatant solutions were subjected to PepRPC chromatography as described above for synthetic CoA adducts. A purified sample of the product (0.5 μg) was dissolved in a glycerol-methanol-water mixture containing *N*-octylnicotinium bromide, and the spectrum was acquired by using a JEOL HX-110 mass spectrometer in the negative-ion mode with Xe bombardment (17).

RESULTS

Resting cell experiments. Resting cells of *C. sepedonicum* KZ-4 that were grown on 24DCB revealed a narrow specificity for degradation of halobenzoates (Fig. 1A). The cells rapidly degraded 24DCB and 4CB (the latter was used at a rate approximately three times that for 24DCB; data not shown), slowly degraded 2-chloro-4-fluorobenzoate (2C4FB), utilized benzoate only after an extended lag period, and failed to degrade 2CB, 2-iodobenzoate, or 4-fluorobenzoate (4FB). In contrast to these results, no degradation of 24DCB, 4CB, or 2C4FB was observed in experiments using cells that were grown in medium containing alternative carbon sources, such as 1/10 LB. More detailed examination of 2C4FB utilization by 24DCB-grown cells demonstrated that this substrate was stoichiometrically transformed to 4FB (Fig. 1B).

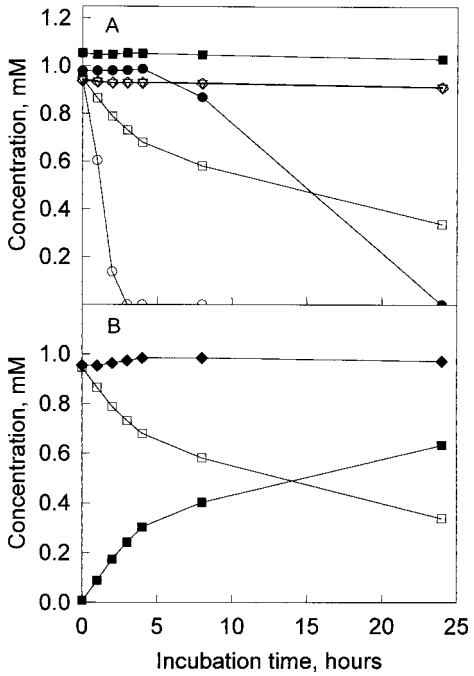

FIG. 1. Degradation of halobenzoates by resting cells of *C. sepedonicum* KZ-4 grown on 24DCB. (A) The effects of incubation time on the concentration of substrate remaining were assessed for 24DCB (○), 2C4FB (□), 2-chlorobenzoate (▽), 2-iodobenzoate (◇), benzoate (●), and 4FB (■). (B) The amount of 2C4FB remaining (□), the amount of 4FB formed (■), and the sum of 4FB and 2C4FB concentrations (◆) were monitored as a function of incubation time.

Enzyme activities present in cell extracts. The activities of key enzymes of aromatic metabolism were measured in cell extracts of *C. sepedonicum* KZ-4. The values shown in Table 1 are representative of one experiment; however, the relative values are consistent between repetitions. No CoA ligase activities were detected in cell extracts, probably because of the presence of high thioesterase activities in assays using either 4CBCoA or 24DCBCoA. When most of the thioesterase activity was chromatographically removed from the extracts, CoA ligase activity was observed for both 4CB and 24DCB substrates. The former substrate was utilized at a rate 12-fold greater than that for the latter substrate. Additionally, the presence of 4CBCoA dehalogenase, 4-hydroxybenzoate 3-monooxygenase, and protocatechuate 3,4-dioxygenase was demonstrated, whereas catechol 1,2- and 2,3-dioxygenases were not detected. The activity values shown represent lower estimates of the actual activity levels, as the assay conditions for each of the enzymes from this strain were not optimized. These data are consistent with the intermediacy of 4CBCoA, 4CB, 4-hydroxybenzoate, and protocatechuate in 24DCB degradation.

When cell extracts were incubated with 24DCBCoA and glutathione, the levels of 24DCBCoA were found to decrease and a new peak that coeluted with 4CBCoA during HPLC on a Lichrosorb C_{18} column was observed. This observation led to the preliminary proposal for the presence of 2,4-dichlorobenzoyl-CoA glutathione *S*-transferase activity reported earlier (33). When the samples were chromatographed on an ion pair HPLC system, the peak representing the new product was clearly resolved from that of authentic 4CBCoA and instead coeluted with the glutathione thioester of 24DCB. The identity of this species as 2,4-dichlorobenzoyl-*S*-glutathione was later confirmed by mass spectrometry (data not shown). This compound was shown to form in a control mixture lacking added protein and may arise by nonenzymatic thiol exchange reactions. Furthermore, glutathione *S*-transferase activity was not observed by using the substrate 1,2-dichloro-4-nitrobenzoate. Finally, glutathione reductase activity was not detected in cell extracts. Thus, no evidence for a glutathione *S*-transferase involvement in 24DCB degradation was obtained.

TABLE 1. Activities of selected enzymes of aromatic metabolism in cell extracts of *C. sepedonicum* KZ-4

Enzyme	Sp act (nmol min^{-1} mg^{-1})[a]
CoA ligase with:	
4CB	0.118
24DCB	0.010
4CBCoA dehalogenase	0.80
Thioesterase with:	
4CBCoA	8.3
24DCBCoA	4.8
4-Hydroxybenzoate 3-monooxygenase	106
Protocatechuate 3,4-dioxygenase	30
Catechol 1,2-dioxygenase	ND
Catechol 2,3-dioxygenase	ND
Glutathione *S*-transferase	ND
Glutathione reductase	ND

[a] CoA ligase activities were determined in thioesterase-depleted cell extracts after chromatography on Superose 12 as described in Materials and Methods. For these activities, the values were calculated from the total amount of protein applied to the column. ND, not detected.

Reductive dehalogenation of 24DCB and 2C4FB by cell extracts. Cell extracts of *C. sepedonicum* KZ-4 were shown to be

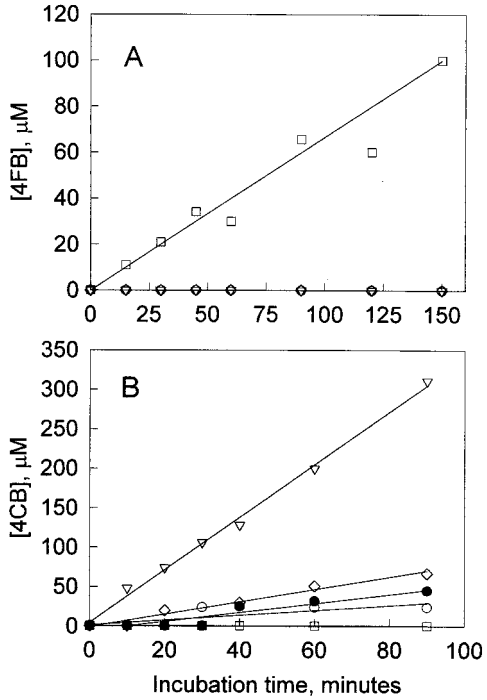

FIG. 2. Transformations of 2C4FB to 4FB and 24DCB to 4CB by cell extracts of *C. sepedonicum* KZ-4. (A) Extracts (8.3 mg/ml) were incubated with 0.5 mM 2C4FB either without additional cofactors (○) or in presence of 5 mM Mg · ATP (▽), Mg · ATP and 1 mM CoA (□), or Mg · ATP and 1 mM glutathione (◇). (B) Samples analyzed included the membrane fraction (□), the extract with no amendments (●), or extracts in the presence of 5 mM Mg · ATP (◇), Mg · ATP and 1 mM CoA (▽), or Mg · ATP and 1 mM glutathione (○).

TABLE 2. Effects of different cofactors on accumulation of 4CB from 24DCB and 24DCBCoA in cell extracts of *C. sepedonicum* KZ-4

Cofactor(s)[a]	Substrate	4CB concn (μM)	% of maximal accumulation
NADPH	24DCBCoA[b]	71	100
NADPH + Fe^{2+} + FAD	24DCBCoA	64	90
NADH + Fe^{2+} + FAD	24DCBCoA	7.4	10
Vitamin B_{12}	24DCBCoA	0	0
Tetrahydrobiopterin	24DCBCoA	0	0
Methyl viologen	24DCBCoA	0	0
Glutathione	24DCBCoA	0	0
β-Mercaptoethanol	24DCBCoA	0	0
NADPH	24DCB	0	0
NADPH + Fe^{2+} + FAD	24DCB	0	0
ATP + Mg^{2+} + CoA	24DCB	0.005	0.01
ATP + Mg^{2+}	24DCB	0	0
ATP + CoA	24DCB	0	0
None	24DCB	0	0

[a] The reaction mixtures (2 ml) containing 1.5 ml of cell extract (3.1 mg/ml) and 0.5 mM aromatic substrate (except where indicated) were supplemented with the cofactors shown at the following final concentrations: NADH or NADPH, 2 mM; $Fe(NH_4)_2(SO_4)_2$, 0.1 mM; flavin adenine dinucleotide (FAD), 2 μM; glutathione, 1 mM; β-mercaptoethanol, 1 mM; vitamin B_{12} (reduced by dithionite), 50 μM; methyl viologen (reduced by dithionite), 0.5 mM; tetrahydrobiopterin, 0.5 mM; ATP, 7.5 mM; $MgCl_2$, 5 mM; CoA, 1 mM; and EDTA, 5 mM. Reactions were initiated by substrate addition and terminated after 15 min by addition of 0.5 N HCl.

[b] The concentration of 24DCBCoA was 0.9 mM in this experiment.

able to dehalogenate 2C4FB to form 4FB in the presence of ATP, Mg^{2+}, and CoA (Fig. 2A). As shown for sample lacking CoA, the absence of any of these three cofactors in the reaction mixture greatly reduced the dehalogenation rate. Glutathione was not able to substitute for CoA in this reaction. These results do not address the source of reductant in the reaction, but they are consistent with the presence of an endogenous reductant in the cell extracts or utilization of reduced CoA. Substitution of NADH or NADPH for Mg · ATP and CoA failed to provide any 4FB product (data not shown).

Analogous to the 2C4FB results, the conversion of 24DCB to 4CB was detected in cell extracts and shown to require Mg · ATP and CoA (Fig. 2B). This activity was localized to the cytosolic fraction, as shown by the absence of activity in membrane preparations.

Reductive dechlorination of 24DCBCoA. 24DCBCoA was reduced by an NADPH-dependent reaction to form 4CBCoA in cell extracts, as shown by using HPLC methods to quantitate the levels of 4CB arising from thioesterase-catalyzed hydrolysis of the immediate product (Table 2). Further addition of Fe^{2+}, flavin adenine dinucleotide, or EDTA (data not shown) had a negligible effect on the activity. NADH could partially substitute for NADPH in this reaction; however, the level of 4CB accumulated in its presence was about 1/10 of that observed with NADPH. In the absence of NADPH or NADH, none of a range of other cofactors (Table 2) were shown to stimulate reductase activity. The immediate product of the enzyme-catalyzed NADPH reduction of 24DCBCoA was shown to be 4CBCoA by using mass spectrometric methods (Fig. 3). The major ion at *m/z* 904 has a related species at *m/z* 906 consistent with the expected ratio of ^{35}Cl and ^{37}Cl isotopes.

Strain NTB-1. Results similar to those described for strain KZ-4 were also observed in strain NTB-1. In particular, NTB-1 cell extracts were found to possess the ability to reductively dehalogenate 24DCBCoA in an NADPH-dependent manner, as shown in Table 3. For comparison, the 24DCBCoA reductase activity in NTB-1 cell extracts (9.8 nmol min^{-1} mg^{-1}) was significantly greater than the activity in KZ-4 cell extracts (0.67 nmol min^{-1} mg^{-1}).

DISCUSSION

We propose that *C. sepedonicum* KZ-4 degrades 24DCB by the pathway shown in Fig. 4. The first important aspect of this pathway is the transformation of 24DCB to 24DCBCoA in a Mg · ATP- and CoA-dependent reaction. 4CBCoA ligase activity also is present in these cells; however, we have not examined whether the same enzyme is utilized for both chlorobenzoate substrates. The higher growth rates for 4CB-grown cells than for cells grown on 24DCB may correlate to the 12-fold-higher activity of 4CBCoA ligase compared to that of 24DCBCoA ligase. The second important feature of the pathway is a novel, reductive dehalogenation reaction in which 24DCBCoA is reduced to 4CBCoA. This reaction is addressed in greater detail below. 4CBCoA is then the substrate for a hydrolytic dehalogenase that yields 4-hydroxybenzoyl-CoA. Corresponding 4CBCoA dehalogenases have been characterized in *Pseudomonas* sp. strain CBS3 (3, 21, 23, 35, 41) and *Arthrobacter* sp. strain 4-CB1 (4) and are likely to be present in numerous other species, but this is the first demonstration of 4CBCoA dehalogenase activity in strain KZ-4. By exploiting the greater bond strength of the C-F bond than of the C-Cl bond, we were able to probe the two initial steps of the path-

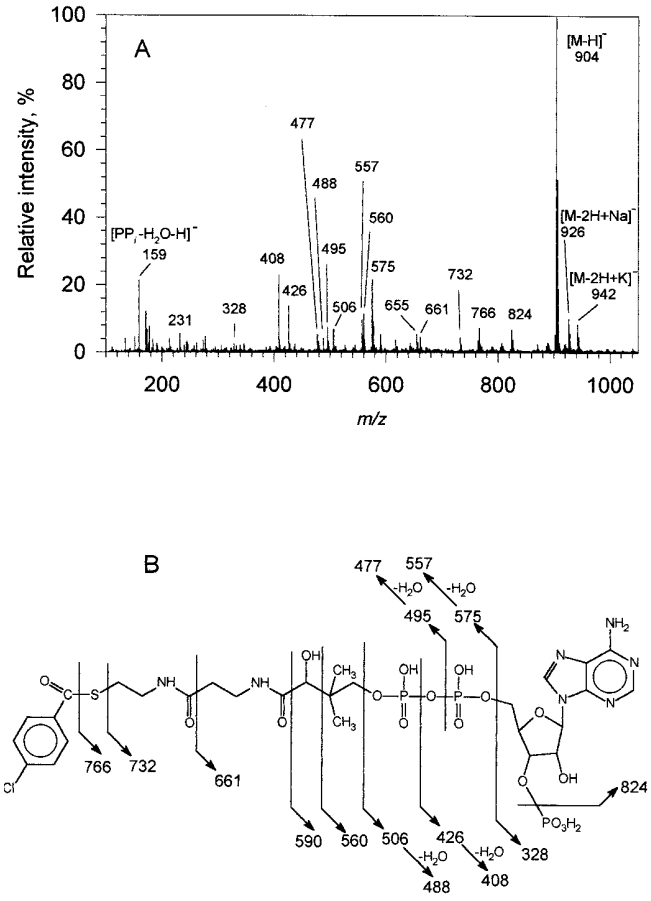

FIG. 3. Mass spectrometric analysis of the product of enzymatic reduction of 24DCBCoA as isolated from cell extracts. (A) Negative-ion mass spectrum of the product resulting from NADPH-dependent reduction of 24DCBCoA. (B) Fragmentation pattern of 4CBCoA.

TABLE 3. Effects of different cofactors on accumulation of 4CB from 24DCBCoA in cell extracts of strain NTB-1

Cofactor(s)[a]	4CB concn (μM)	% of maximal accumulation
NADPH	295	100
NADPH + EDTA	294	100
NADH	5.0	2
Glutathione	42	14
Dithiothreitol	40	13
β-Mercaptoethanol	15	5
Tetrahydrobiopterin	3.0	1
None	28	10

[a] Reaction mixtures of 0.5 ml contained 0.4 ml of cell extract (7.54 mg of protein per ml), 0.5 mM 24DCBCoA, and the indicated cofactors at the following concentrations: NADH or NADPH, 2 mM; glutathione, 2 mM; dithiothreitol, 2 mM; β-mercaptoethanol, 2 mM; tetrahydrobiopterin, 0.5 mM; and EDTA, 10 mM. The reactions were initiated by addition of substrate and terminated after 20 min by addition of 600 μl of 0.5 N HCl.

way while preventing action by the 4CBCoA dehalogenase. 2C4FB was shown to be stoichiometrically transformed to 4FB by whole cells and cell extracts. Of particular importance, we found that this transformation required Mg · ATP. Conversion of 2C4FB to 4FB was not unexpected since coryneform bacterium strain NTB-1 and *Arthrobacter* sp. strain SB8 are known to degrade 4CB but not 4FB (36, 39), cell extracts of strain CBS3 are known to convert 4CB to 4-hydroxybenzoate but fail to act on 4FB (37), and purified 4CBCoA dehalogenases from strains 4-CB1 and CBS3 either use 4FBCoA at a rate that is only 0.23% of that for 4CBCoA (4) or fail to use it at all (23). High levels of thioesterase activity are present in extracts of KZ-4 cells, resulting in hydrolysis of each of the benzoylthioesters. Whereas hydrolyses of the 24DCBCoA and 4CBCoA thioesters can be viewed as nonproductive pathways, the hydrolysis of 4-hydroxybenzoyl-CoA is likely to provide a true intermediate in the pathway. The presence of 4-hydroxybenzoate 3-monooxygenase and protocatechuate 3,4-dioxygenase activities provides compelling evidence that further metabolism occurs by the protocatechuate branch of the *ortho* pathway. Because we find that 24DCBCoA reductase activity also is present in coryneform bacterium strain NTB-1, it is likely that the pathway shown in Fig. 4 also is utilized in this 24DCB-degrading strain. This 24DCBCoA-dependent pathway sharply contrasts with the previous suggestion of a direct reductive dehalogenation of 24DCB in strain NTB-1 (39).

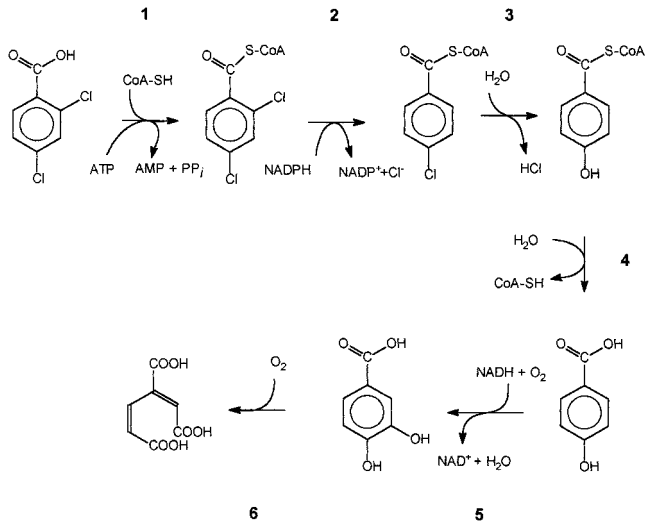

FIG. 4. Proposed pathway of 24DCB metabolism in *C. sepedonicum* KZ-4. Enzymes: 1, 24DCBCoA ligase; 2, 24DCBCoA reductase; 3, 4CBCoA dehalogenase; 4, thioesterase; 5, 4-hydroxybenzoate 3-monooxygenase; 6, protocatechuate 3,4-dioxygenase.

A unique aspect of the 24DCB pathway is the NADPH-dependent reductive dehalogenation of 24DCBCoA. The reaction may involve hydride attack on the *ortho* position of 24DCBCoA with elimination of chloride. In this speculative mechanism, the electrophilicity of C-2 in 24DCBCoA is much greater than that in 24DCB because the charge can be delocalized into the carbonyl. This mechanism is analogous to that proposed for 4CBCoA hydrolytic dehalogenation (35); in that case, C-4 exhibits enhanced electrophilicity in the thioester compared with 4CB. In the reductive dehalogenation reaction, it is presently unclear whether hydride is directly transferred from NADPH or if there is an intermediate reduced species. A bacterial example of reductive dehalogenation via an electron transport chain (although not involving an aromatic substrate) is the dehalogenation of pentachloroethane by cytochrome $P450_{CAM}$. In this reaction, chloride elimination can be driven by NADH or NADPH; however, the reduced nicotinamides donate electrons to a flavoprotein reductase that in turn reduces putidaredoxin, which transfers electrons to the enzyme (20). As possible mammalian precedents, a three-component system that uses thioredoxin as the intermediate electron carrier is involved in NADPH-dependent iodothyronine 5′-deiodinase activity (1), and an NADPH- and ferredoxin-dependent system is likely to be present in iodotyrosine deiodinase activity (11). The absence of glutathione *S*-transferase and glutathione reductase activities makes it unlikely that strain KZ-4 utilizes NADPH to drive a glutathione-dependent reductase system, similar to that involved in chlorohydroquinone dehalogenation (31, 40). Furthermore, the KZ-4 pathway exhibits no relationship to the membrane-associated 3-chlorobenzoate dehalogenation system found in *D. tiedjei* DCB-1 (25, 28). We conclude that the cytoplasmic 24DCBCoA reductive dehalogenase enzyme system is distinct from other known bacterial reductive dehalogenases and offers a unique opportunity to explore novel reductive elimination chemistry with bioremediation potential.

Although reductive dehalogenation of compounds via their CoA thioesters has not been described previously, it is important to note that numerous aromatic compounds are degraded via reductive reactions carried out on the CoA derivatives (reviewed in reference 6). For example, anaerobic degradation of benzoate initiates with formation of benzoyl-CoA followed by reduction of the aromatic ring (19). Furthermore, the metabolism of *o*-phthalate and that of 2-aminobenzoate are proposed to involve formation of the CoA thioesters and transformation to benzoyl-CoA (22, 30). We raise the possibility that 24DCBCoA reductase may be mechanistically, and perhaps structurally, related to these poorly characterized systems.

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