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Characterization of Aromatic Dehalogenases of *Mycobacterium fortuitum* CG-2

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Two different dehalogenation enzymes were found in cell extracts of *Mycobacterium fortuitum* CG-2. The first enzyme was a halophenol *para*-hydroxylase, a membrane-associated monooxygenase that required molecular oxygen and catalyzed the *para*-hydroxylation and dehalogenation of chlorinated, fluorinated, and brominated phenols to the corresponding halogenated hydroquinones. The membrane preparation with this activity was inhibited by cytochrome P-450 inhibitors and also showed an increase in the A_{448} caused by CO. The second enzyme hydroxylated and reductively dehalogenated tetrahalohydroquinones to 1,2,4-trihydroxybenzene. This halohydroquinone-dehalogenating enzyme was soluble, did not require oxygen, and was not inhibited by cytochrome P-450 inhibitors.

Actinomycetes are a large and variable group of microorganisms occurring in many habitats, where they take part in the degradation of organic material. Many microbial strains degrading various aromatic pollutants have been isolated from this group. Enzymes so far characterized have been reported to be monooxygenases, hydroxylases, or dioxygenases (for a review, see reference 31). Actinomycetes, including those degrading xenobiotic compounds, have also been shown to be environmentally tenacious, making them a potential source of microorganisms for bioremediation (6, 18, 29, 30).

Mycobacterium fortuitum CG-2 was isolated from a tetrachloroguaiacol enrichment culture and shown to degrade several chlorinated phenols, guaiacols, and syringols at micromolar levels (15). Pentachlorophenol (PCP) was shown to be mineralized to CO_2, and tetrachloro-*para*-hydroquinone (TeCH) was detected as an initial intermediate. We have shown that the degradation of polychlorophenols, guaiacols, and syringols proceeds through chlorinated *para*-hydroquinones in three different *Rhodococcus chlorophenolicus* strains (1, 3, 12, 13, 15) and is mediated by a membrane-associated cytochrome P-450 enzyme (28). The product of this *para*-hydroxylation, TeCH, is further *ortho*-hydroxylated by a soluble halohydroquinone dehalogenase (4, 14, 28).

In this paper, we report on the enzymatic dehalogenation of chlorinated, brominated, and fluorinated phenols and hydroquinones by *M. fortuitum* CG-2 cell fractions. We show that the halogenated phenols were converted to halogenated *para*-hydroquinones by a membrane-associated cytochrome P-450 enzyme. The halohydroquinones formed by the *para*-hydroxylation were further dehalogenated by a soluble halohydroquinone dehalogenase. The results show that *M. fortuitum* CG-2 dehalogenases are similar to those previously described by us for *R. chlorophenolicus* PCP-1 and CP-2 (3, 4, 14, 15, 28).

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. fortuitum* CG-2 (12, 16) was grown in nutrient broth-yeast extract medium (21) in a gyratory shaker at 28°C. Two-day-old cultures were harvested or induced by the addition of increasing amounts (10 to 50 μM) of PCP at 24-h intervals and then harvested.

Cell fractionation. Cells were harvested by centrifugation (3,000 × *g*, 10 min, 4°C), resuspended (1 g [wet weight] ml^{-1}) in borax buffer (50 mM, pH 8.0) (22), and washed once with the same buffer. Cells were disrupted in a French press at 700 lb/in^2, DNase I (50 μg ml^{-1}; Boehringer Mannheim, Mannheim, Germany) was added to the cell extract, and the mixture was incubated for 1 h at 22°C. The unbroken cells were removed by centrifugation (3,000 × *g*, 10 min, 4°C), and the supernatant was further centrifuged at 150,000 × *g* for 90 min at 4°C. The supernatant contained the soluble proteins, and the pellet contained the membrane-associated proteins. The pellet was washed once with 10 volumes of borax buffer and resuspended in the original volume of the same buffer.

Analyses. For activity assays, 1.0 mg of protein ml^{-1} and 20 to 50 μM substrate (PCP, pentafluorophenol [PFP], pentabromophenol [PBP], TeCH, tetrafluoro-*para*-hydroquinone [TeFH], or tetrabromo-*para*-hydroquinone [TeBH]) were used. Protein was determined by the method of Bradford (5) with a reagent from Bio-Rad Laboratories, Richmond, Calif., and with egg white lysozyme (Sigma, St. Louis, Mo.) for calibration. Assays for halophenol dehalogenation were carried out in borax buffer at pH 8.0. The pH optimum of the reaction was tested with borax buffer (pH range, 5.8 to 9.2). For assays for halohydroquinone dehalogenation, ascorbic acid (final concentration, 1 mg ml^{-1}) was added to prevent abiotic oxidation of halohydroquinones. The enzymatic activity was calculated from the linear period of activity (4 h). A reaction mixture with no enzyme added served as the blank for both types of assays.

Halogenated phenolic compounds were analyzed as acetylated derivatives by gas-liquid chromatography (GLC) by an internal standard method as described earlier (2, 3). To improve the acetylation efficiency of the brominated conge-

ners, we added acetone to the acetylation buffer to 10% (2). Fluorinated congeners were extracted in pentane, and brominated and chlorinated congeners were extracted in heptane. An HP-5 fused silica capillary column (25 m by 0.2 mm; 0.33 μm; Hewlett Packard, Palo Alto, Calif.) was used. The temperature programs used for the analysis were as follows: for TeCH and TeBH, 50°C for 1 min and 10°C/min to 300°C; for TeFH, 20°C for 1 min, 10°C/min to 60°C, and 50°C/min to 180°C. Metabolites emerging were identified by GLC-mass spectrometry (MS) as described by Apajalahti and Salkinoja-Salonen (3, 4). In the analysis of *para*-hydroxylation products with an $^{18}O_2$ label, the following ions were used: 2,3,5-trichloro-*para*-hydroquinone (2,3,5-TCH), *m/e* 212, 214, 216, 218, 220.

Anoxic assays and isotopic labeling. For the anoxic assays, the reaction mixture was enclosed in a gas-tight vial and oxygen was removed by sequential evacuation and purging with argon (99.998%). A similarly deoxygenated substrate was injected into the vial to start the reaction, and acetylation reagents were injected to stop the reaction. In the oxygen labeling experiments, $^{18}O_2$ gas (isotopic purity, 80 to 90%; Msd isotopes, Montreal, Quebec, Canada) was injected into the ampoule to a partial $^{18}O_2$ pressure of 0.1 and the reaction was started by the injection of a deoxygenated substrate (2,3,5-trichlorophenol [2,3,5-TCP]) into the ampoule to a final concentration of 20 μM and stopped by the addition of acetylation reagents after 4 h of incubation at 22°C. Labeling experiments with $H_2{}^{18}O$ were performed like the aerobic ones, except that the solution was made 20% with respect to $H_2{}^{18}O$-labeled water (isotopic purity, 97%; Aldrich Chemical Co., Milwaukee, Wis.). The reaction was started by the addition of an anaerobic substrate solution (2,3,5-TCP; final concentration, 20 μM).

Inhibition assays. The inhibitors tested were Tiron (5 mM; Siegfried S.A.), methylenebisthiocyanate (5 mM; Riedel-de-Haen), metyrapone (5 mM; Sigma), menadione (5 mM; vitamin K_3, prereduced; Sigma), parathion (5 mM; Ehrenstorfer, Augsburg, Germany) and SKF-525A (5 mM; Smith Kline & French Laboratories). Inhibition by carbon monoxide was tested at partial pressures of 1.0, 0.1, and 0.01 in gas-tight vials.

Spectrophotometric analysis. Absorption spectra were measured at room temperature with the single-beam mode of a Shimadzu UV-3000 spectrophotometer. Sodium dithionite (a few grains) was added to the protein solution (2.3 mg in 0.5 ml of borax buffer) to create a reduced environment, and the spectrum (400 to 500 nm) was measured before and after purging with carbon monoxide. The concentration of cytochrome P-450 was calculated from the reduced carbon monoxide differential spectrum with the specific extinction coefficient of the *Pseudomonas putida* enzyme, $A_{450-490}$ = 92.8 cm^{-1} mM^{-1} (11).

RESULTS

Localization of the halophenol-*para*-hydroxylating activity in cell extracts of *M. fortuitum* CG-2. From previous work, we knew that *M. fortuitum* CG-2 was able to degrade (mineralize) PCP via *para*-hydroxylation to TeCH (15). To test whether other pentahalophenols would be attacked, we tested the degradation of PFP and PBP by crude extracts of PCP-induced cells and analyzed the products formed from PFP and PBP by GLC-MS. A new peak emerged in the gas chromatogram of each halophenol after incubation with the crude extract, the retention times (the elution times, in minutes, of the model compounds TeCH, TeBH, and TeFH were 11.3, 16.1, and 8.1, respectively, and those for the metabolites formed from these pentahalogenated phenols were the same) and the mass spectra (Fig. 1) being identical to those of authentic TeCH, TeFH, and TeBH. These results indicate that the pentahalophenols had undergone dehalogenation and *para*-hydroxylation while in contact with the crude extracts of *M. fortuitum* CG-2. The reaction was optimal at pH 7 to 8. Outside the pH range of 6.5 to 9.0, no dehalogenation was observed.

To localize the PCP-, PFP-, and PBP-*para*-hydroxylating activity in *M. fortuitum* CG-2 cells, we prepared cell-free crude extracts from the cells and separated them into a 150,000 × *g* supernatant (soluble proteins) and a 150,000 × *g* pellet (membrane-associated proteins). Dehalogenation of halophenols was tested for each fraction as described in Materials and Methods. Table 1 shows the dehalogenation activities of the extracts prepared from PCP-induced and uninduced cells of *M. fortuitum* CG-2 and the localization of the halophenol-consuming enzyme activity in the cell fractions. The rates of turnover of PCP, PFP, and PBP in crude extracts from PCP-induced cells ranged from 1.7 to 2 nmol h^{-1} mg of $protein^{-1}$ (Table 1). Crude extracts prepared from uninduced cells showed no detectable halophenol-degrading enzyme activity, indicating that this enzyme activity was inducible. Treatment of the crude extracts with proteinase K (1 mg ml^{-1}) destroyed the halophenol-*para*-hydroxylating activity, showing that it was mediated by enzyme protein. The induction factor for the halophenol dehalogenases was ca. 40-fold in *M. fortuitum* CG-2. When the cell extracts were fractionated by centrifugation (150,000 × *g*), 67% of the halophenol-degrading enzyme activity of the crude extracts was recovered in the 150,000 × *g* pellet, which contained 33% of the total cell extract protein (Table 1). No halophenol-*para*-hydroxylating activity was found in the 150,000 × *g* supernatant. Phenol was not degraded (<0.05 nmol h^{-1} mg of $protein^{-1}$) by induced or noninduced cells.

Localization of the halohydroquinone-consuming activity in cell extracts of *M. fortuitum* CG-2. Table 1 shows that the halohydroquinone-consuming activity was mainly localized in the 150,000 × *g* supernatant (soluble proteins). This activity was destroyed by proteinase K (1 mg ml^{-1}) treatment, showing that it was enzymatic. The halohydroquinone-consuming activity was proven to be dehalogenation, since products of dehalogenation of TeCH, TeFH, and TeBH were observed by GLC-MS (Fig. 2). Nonhalogenated compound 1,2,4-trihydroxybenzene (1,2,4-THB) was produced not only from TeCH but also from TeFH and TeBH. This result indicates that reductive dehalogenation took place. Consumption of tetrahalohydroquinones did not occur in the cell extracts prepared from uninduced cells, indicating that the halohydroquinone-consuming activity was inducible. Halohydroquinones were consumed by the cell extracts prepared from induced cells at rates five to seven times higher than were the corresponding halophenols. Turnover rates of 10, 14, and 7 nmol h^{-1} mg of $protein^{-1}$ were measured for TeCH, TeFH, and TeBH (Table 1).

Inhibition of the halophenol-*para*-hydroxylating enzyme activity. To determine the nature of the *para*-hydroxylating enzyme activity, we tested the effect of selected inhibitors on the activity. Table 2 shows the response of the halophenol-consuming enzyme (150,000 × *g* pellet) to inhibitors. The turnover of each of the halophenols was sensitive to inhibition by the cytochrome P-450 inhibitors SKF-525A, metyrapone, menadione, and parathion at 5 mM (Table 2). The product of the *para*-hydroxylation of PCP, TeCH, also

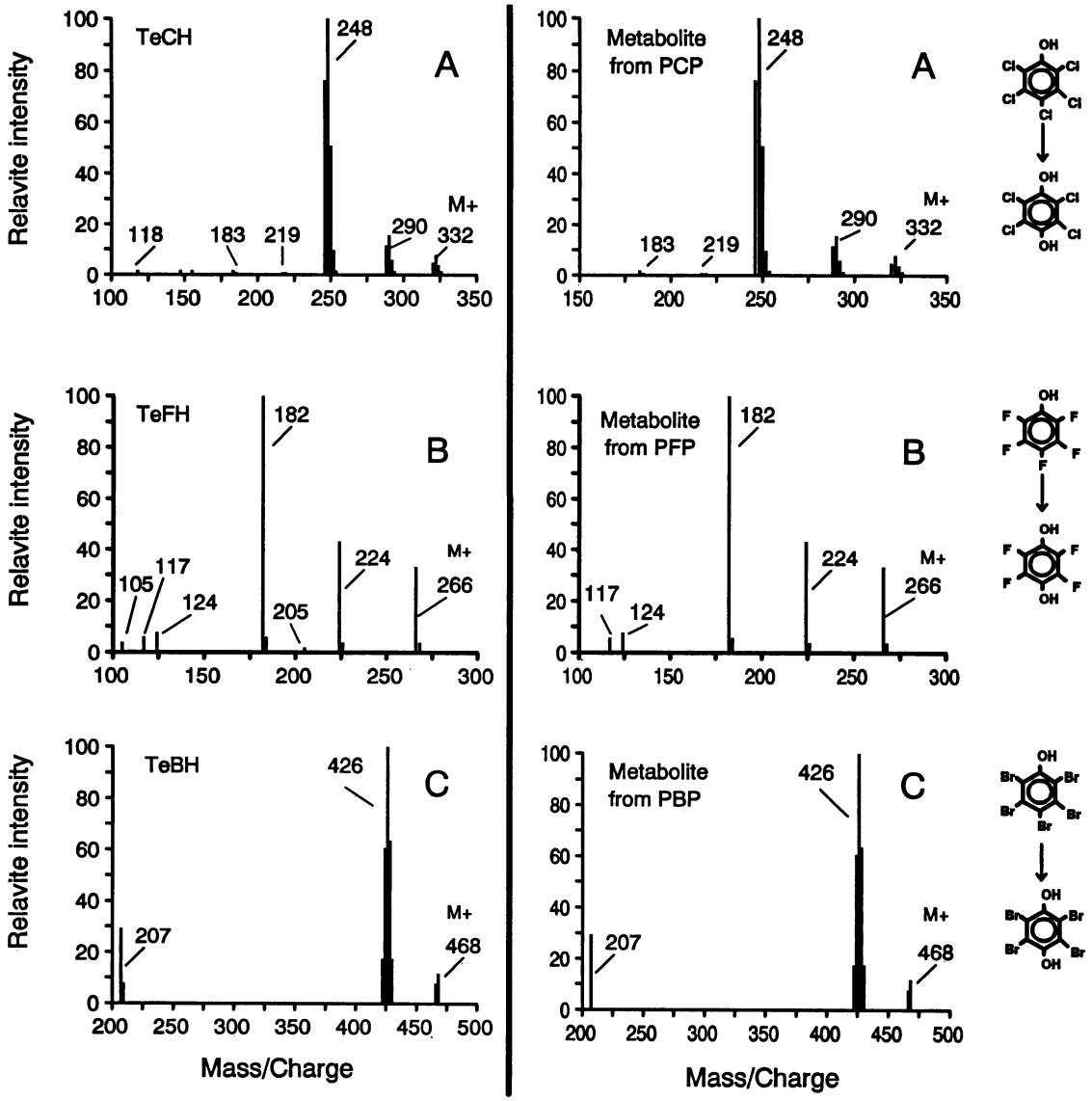

FIG. 1. Mass spectra of the acetylated products formed from each three different pentahalogenated phenols by membrane preparations of *M. fortuitum* CG-2. Left panels, mass spectra of acetylated model compounds. Right panels, mass spectra of acetylated metabolites of pentahalogenated phenols. (A) Product from PCP. (B) Product from PFP. (C) Product from PBP.

inhibited the *para*-hydroxylation not only of its precursor, PCP, but also of the other halophenols at as low a concentration as 50 μM. Under anoxic conditions, *para*-hydroxylation did not occur, but activity was resumed when oxygen was reintroduced into vials after 4 h of anoxic incubation. This result shows that oxygen was needed for the *para*-hydroxylating activity and that the activity was preserved during anaerobic incubation. Reducing conditions (sodium dithionite) did not impair the activity, but when carbon monoxide was introduced, the activity was inhibited by 54% at a partial CO pressure of 0.01 and by 100% at a partial CO pressure of 0.1 (Table 2). Methylenebisthiocyanate, a chelator of ferrous iron, inhibited the activity, but a chelator of ferric iron (Tiron) did not. These results indicate that the pattern of inhibition of the *para*-hydroxylation of PCP, PFP, and PBP was similar to that of cytochrome P-450 enzymes.

Inhibition of the halohydroquinone-dehalogenating activity. We also tested the effect of inhibitors on the halohydroquinone-consuming activity. The results in Table 3 show that the halohydroquinone-dehalogenating activity was not affected by the absence of oxygen or the presence of carbon monoxide in a reduced environment. In addition, the cytochrome P-450 inhibitors metyrapone and parathion had little effect on dehalogenation. SKF-525A (5 mM) and menadione inhibited the halohydroquinone-degrading activity by about 70%. The results show that the halohydroquinone-dehalogenating enzyme differed from the halophenol-*para*-hydroxylating enzyme by not being inhibited by several of the cytochrome P-450 inhibitors. Methylenebisthiocyanate, a chelator of ferrous iron, inhibited the enzyme, but a chelator of ferric iron (Tiron) did not.

Spectrophotometric analysis. To investigate more closely the involvement of cytochrome P-450 in the dehalogenation reactions, we measured the carbon monoxide differential

TABLE 1. Turnover of halogenated phenols and hydroquinones by cell extracts from *M. fortuitum* CG-2

Cells	Substrate	Sp act (nmol of substrate consumed h^{-1} mg of protein^{-1}) in[a]:		
		Cell extract	Supernatant[b]	Pellet[b]
Uninduced	PCP	<0.05	<0.05	<0.05
	PFP	<0.05	<0.05	<0.05
	PBP	<0.05	<0.05	<0.05
	TeCH	<0.05	<0.05	<0.05
	TeFH	<0.05	<0.05	<0.05
	TeBH	<0.05	<0.05	<0.05
PCP induced	PCP	1.8	<0.05	1.2
	PFP	2.0	<0.05	1.1
	PBP	1.7	<0.05	0.9
	TeCH	10.1	8.8	1.0
	TeFH	14.3	12.5	1.4
	TeBH	7.1	6.0	0.5

[a] The data were based on three trials, with a mean error of less than 6%.
[b] From centrifugation for 90 min at 150,000 × *g*.

spectra. Figure 3 shows the spectra of dithionite-reduced membrane fractions of PCP-induced and uninduced cells of *M. fortuitum* CG-2. When dithionite-reduced membrane fractions of PCP-induced cells were purged with carbon monoxide, an increase in the A_{448} ($A_{1\ cm}$ = 0.019) and A_{460} ($A_{1\ cm}$ = 0.024) was observed (Fig. 3A). A smaller increase in the A_{448} ($A_{1\ cm}$ = 0.007) was observed in membrane fractions of uninduced cells (Fig. 3B). The increase in the peak at 460 nm ($A_{1\ cm}$ = 0.024) was similar in membrane preparations from both induced and uninduced cells. The peak at 448 nm in the differential spectrum indicated the presence of an inducible cytochrome P-450 coenzyme in *M. fortuitum* CG-2, whereas the cofactor responsible for the peak at 460 nm was constitutively expressed. A similarly treated 150,000 × *g* supernatant (soluble proteins) showed no change in the A_{400} to A_{500} under these conditions ($A_{1\ cm}$ < 0.002; detection limit).

Source of oxygen in the *para*-hydroxylation reaction. We used $^{18}O_2$-labeled molecular oxygen and water to investigate the origin of oxygen required for the *para*-hydroxylation of halophenols, mediated by the membrane enzymes in *M. fortuitum* CG-2. With a heavier isotope of oxygen, it is possible to observe the incorporation of oxygen in the hydroquinone product as a shift in the mass fragments. 2,3,5-TCP was fed to a concentration of 20 μM to membrane preparations from PCP-induced cells. 2,3,5-TCP was chosen for the substrate because of its chemical stability when hydroxylated into 2,3,5-TCH. Figure 4a shows the selected mass fragments 212, 214, 216, 218, and 220 of the authentic model compound, 2,3,5-TCH. In the presence of the $^{18}O_2$ label, 2,3,5-TCH formed from 2,3,5-TCP showed a shift in the relative intensities of the indicative mass fragments (Fig. 4b), when compared with the authentic model compound (Fig. 4a). The shift in the relative intensities of the mass fragments of 2,3,5-TCH corresponded well with the amount of $^{18}O_2$ in the chemical (80 to 90%). When the $H_2{}^{18}O$ label was used, 2,3,5-TCH formed from 2,3,5-TCP showed no shift in the mass fragments (Fig. 4c). 2,3,5-TCH was chemically stable; exchange of the hydroxyl groups with labeled water was observed neither in the presence nor in the absence of enzyme preparations. The results showed that under oxic conditions, the oxygen for the *para*-hydroxylation of 2,3,5-TCP was derived from molecular oxygen.

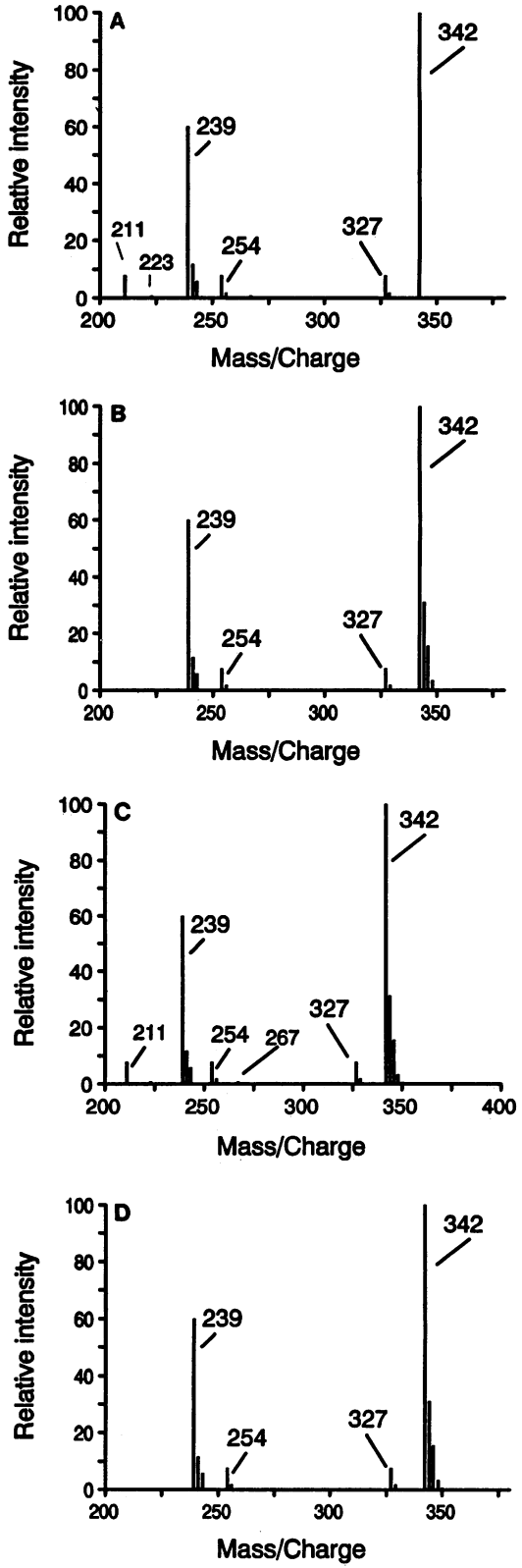

FIG. 2. Mass spectra of the authentic silylated model compound 1,2,4-THB and silylated products formed from each halogenated hydroquinone. (A) Model compound, 1,2,4-THB. (B) Product from TeCH. (C) Product from TeFH. (D) Product from TeBH.

TABLE 2. Effect of cytochrome P-450 inhibitors on the halophenol-*para*-hydroxylating enzyme activity of the 150,000 × *g* pellet from PCP-induced *M. fortuitum* CG-2

Test condition	Turnover (nmol of substrate consumed h^{-1} mg of $protein^{-1}$) of[a]:		
	PCP	PFP	PBP
No inhibitor present	1.2	1.1	0.9
TeCH (50 μM)	0.7	0.6	0.5
SKF-525A (5 mM)	0.1	0.08	0.07
Metyrapone (5 mM)	0.7	0.6	0.3
Menadione (5 mM)	0.2	0.1	0.1
Parathion (5 mM)	0.3	0.3	0.2
Anoxic conditions	<0.05[b]	<0.05[b]	<0.05[b]
Sodium dithionite (5 mM) in:			
Ambient air	1.15	1.0	0.8
pCO[c] = 1.0	<0.05	<0.05	<0.05
pCO = 0.1	0.06	<0.05	<0.05
pCO = 0.01	0.6	0.6	0.5
Tiron (5 mM)	1.2	1.0	0.9
Methylenebisthiocyanate (5 mM)	0.2	0.2	0.1

[a] The data were based on three trials, with a mean error of less than 6%.
[b] The activity totally resumed when air was reintroduced into ampoules after 4 h of incubation.
[c] pCO, partial CO pressure.

DISCUSSION

Cytochrome P-450 is a coenzyme of monooxygenases having multiple catalytic activities, one of them being dehalogenation (7, 8, 17). P-450 enzymes are typical microsomal eukaryotic enzymes involved in both biosynthetic and detoxification reactions. Microbial P-450 enzymes are less well understood, and their catalytic properties are only partially characterized. Microbial P-450 enzymes are known to be involved in the dehalogenation of a haloaromatic compound in at least one bacterium, an actinomycete, *R. chlorophenolicus* PCP-1 (1, 28), in which they catalyze the *para*-hydroxylation of a halophenol, deriving the oxygen required in this reaction from molecular oxygen under aerobic conditions (27).

TABLE 3. Effect of cytochrome P-450 inhibitors on the halohydroquinone-dehalogenating activity of the 150,000 × *g* supernatant from PCP-induced *M. fortuitum* CG-2

Test conditions	Sp act (nmol of substrate consumed h^{-1} mg of $protein^{-1}$) with the following substrate[a]:		
	TeCH	TeFH	TeBH
No inhibitor present	10.1	14.3	7.1
PCP (20 μM)	<0.05	<0.05	<0.05
SKF-525A (5 mM)	3.0	4.1	2.5
Metyrapone (5 mM)	9.7	13.6	6.3
Menadione (5 mM)	3.2	3.5	2.1
Parathion (5 mM)	8.3	12.3	6.2
Anoxic conditions	9.9	14.1	7.0
Sodium dithionite (5 mM) in:			
Ambient air	10.0	14.2	6.9
pCO[b] = 1.0	9.9	14.1	7.0
pCO = 0.01	10.0	14.3	7.0
pCO = 0.01			
Tiron (5 mM)	9.9	14.0	6.5
Methylenebisthiocyanate (5 mM)	0.5	0.7	0.3

[a] In the blank incubation with no enzyme, halohydroquinones were stable under the conditions tested (less than 5% loss of halohydroquinones in 24 h).
[b] pCO, partial CO pressure.

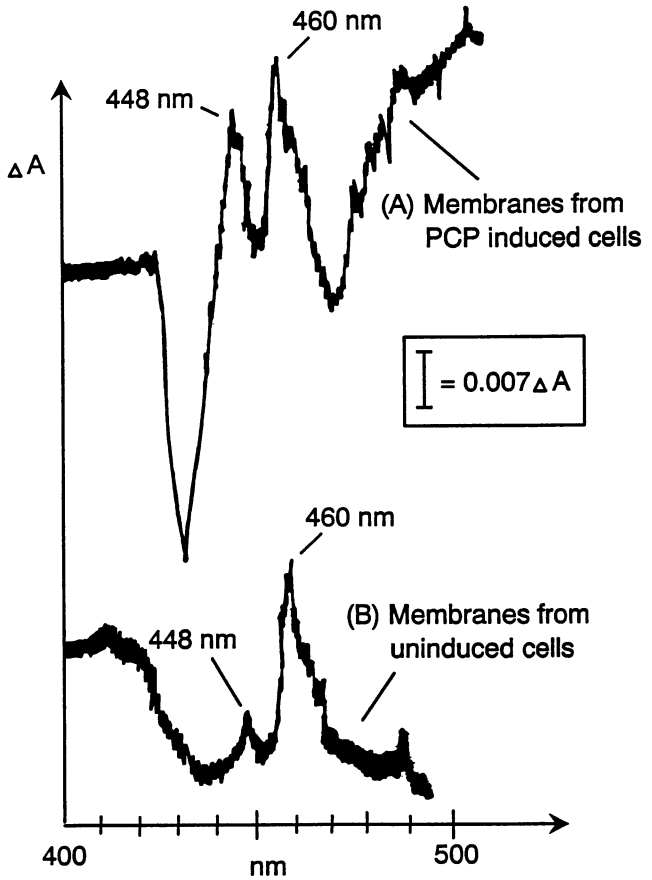

FIG. 3. Differential absorbance spectra (1 cm) of membrane preparations (150,000 × *g*; 4.6 mg of protein ml^{-1}) from *M. fortuitum* CG-2 reduced with dithionite, obtained by purging with CO.

Monooxygenases are an important group of enzymes participating in the hydroxylation of various phenolic compounds (9). These modifications may render recalcitrant molecules susceptible to further degradation by other enzymes. Monooxygenases were shown to be involved in halophenol degradation by an *Arthrobacter* sp. (10), a *Mycobacterium* sp. (24–26), and *R. chlorophenolicus* PCP-1 (3, 4, 28). The present paper demonstrates halophenol *para*-hydroxylation in cell extracts of *M. fortuitum* CG-2. The enzyme was located in the membrane fraction and hydroxylated chlorinated, fluorinated, and brominated compounds. Nonsubstituted phenol was not attacked at all. The halophenol-*para*-hydroxylating activity increased by ca. 40-fold upon induction.

Our evidence that typical P-450 inhibitors blocked *para*-hydroxylation indicates that *M. fortuitum* CG-2 also uses a cytochrome P-450 enzyme in *para*-hydroxylation of halophenols. Spectral evidence revealed the presence of P-450 and P-460 coenzymes in the cells, but only the P-450 coenzyme increased in quantity as a response to induction of the dehalogenating activity. Assuming that the extinction coefficient for the cytochrome P-450 enzyme is $A_{450-490}$ = 98.8 cm^{-1} mM^{-1} (11), there would be 0.042 nmol of cytochrome P-450 mg of membrane $proteins^{-1}$ in PCP-induced cells, a concentration threefold higher than the cytochrome P-450

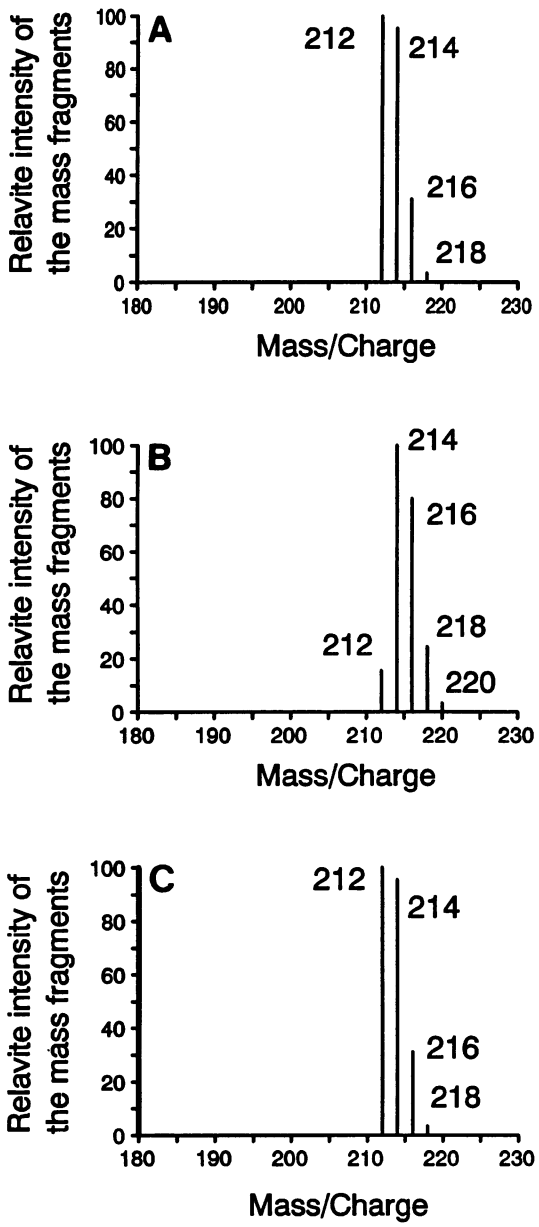

FIG. 4. GLC-MS analysis of the products formed from 2,3,5-TCP by membrane preparations (150,000 × *g*) from *M. fortuitum* CG-2. (A) Mass spectrum of the model compound, 2,3,5-TCH. (B) Mass spectrum of the product formed from 2,3,5-TCP in the presence of $^{18}O_2$. (C) Mass spectrum of the product formed from 2,3,5-TCP in the presence of $H_2{}^{18}O$.

concentration in cells prior to induction with PCP. In *R. chlorophenolicus* PCP-1, the induction of P-450 enzymes was observed to be 10-fold (28). Extracts prepared from uninduced cells of *M. fortuitum* CG-2 had 35% of the P-450 enzyme levels but only 5% of the activity of extracts prepared from induced cells. This result indicates that during induction, other proteins also needed for dehalogenation must be synthesized. The presence of P-450 in uninduced cell extracts suggests the presence of catalytic functions involving P-450 enzymes.

We recently showed that the source of oxygen in the monooxygenase-mediated dehalogenation of PCP by *R. chlorophenolicus* PCP-1 can be either water or molecular oxygen, depending on the environment of the enzyme (27). In the presence of sulfite ions or iodosobenzene, the monooxygenase from *R. chlorophenolicus* PCP-1 was able to bypass the requirement for molecular oxygen and use water as a source of oxygen. The reaction thus proceeded under anoxic conditions in the presence of sulfite or iodosobenzene. The oxygenase in *M. fortuitum* CG-2 used molecular oxygen as a source of the hydroxyl group incorporated into the *para*-position of halophenol under aerobic conditions with no sulfite present. The removal of dissolved oxygen by an excess of dithionite did not block the reaction, indicating that sulfur-containing ion species in *M. fortuitum* CG-2 may also bypass the requirement for molecular oxygen, similar to the situation in *R. chlorophenolicus* PCP-1. In a *Flavobacterium* sp. (23) and an *Arthrobacter* sp. (19), water served as a source of oxygen for the monooxygenase-catalyzed *para*-hydroxylation of PCP.

The subcellular localization of the dehalogenases found in *M. fortuitum* CG-2 was the same as that of the dechlorinases in *R. chlorophenolicus* PCP-1 and CP-2 (4, 14), whereas the halophenol *para*-hydroxylase was located in the membrane fraction and the halohydroquinone dehalogenase was soluble (4, 28). In an *Arthrobacter* sp. and a *Flavobacterium* sp., the monooxygenases involved in the *para*-hydroxylation of PCP were shown to be soluble (19, 20, 32).

Halohydroquinone dehalogenase has been described so far in *R. chlorophenolicus* PCP-1 and CP-2 (4, 14, 28). The activity is soluble, the only requirement being a reductant for the reaction. The first step in dehalogenation, *ortho*-hydroxylation, leads to (3,5,6)trichloro-1,2,4-trihydroxybenzene, which is then stepwise reductively dehalogenated to 1,2,4-THB (4, 14). In this paper, we demonstrate that in *M. fortuitum* CG-2 the dehalogenation of TeCH, TeBH, and TeFH proceeded in a similar way, via *ortho*-hydroxylation followed by reductive dehalogenation, to 1,2,4-THB. Whole cells of *R. chlorophenolicus* and *M. fortuitum* have been shown to convert U-^{14}C-PCP into $^{14}CO_2$, with a yield of 70 to 90% (2, 15). The dehalogenation of hydroquinones proceeded anaerobically in *R. chlorophenolicus* PCP-1 and CP-2 (4, 15) as well in *M. fortuitum* CG-2 (this study). Like *R. chlorophenolicus* PCP-1, *M. fortuitum* CG-2 halohydroquinone dehalogenase was a soluble enzyme. It had a broad substrate specificity, since chlorine, fluorine, and bromine atoms were removed from the respective halohydroquinones. The mycobacterial halohydroquinone enzyme was also not inhibited by P-450 inhibitors.

Our work indicates that pentachlorophenol-degrading *M. fortuitum* CG-2 and *R. chlorophenolicus* PCP-1 possess two different enzymatic mechanisms for dehalogenation: an oxygenase for the *para*-hydroxylation of halophenol and a second enzyme(s) for the removal of halogens from halohydroquinones. In the strains studied, the specific activities of the halohydroquinone dehalogenases were approximately 10 times higher in both whole cells and extracts than those of the corresponding halophenol *para*-hydroxylases, indicating that any intracellular accumulation of halohydroquinone is unlikely to occur. This possibility may be important for the protection of these aerobic dehalogenating bacteria against the toxic effects of halohydroquinones and quinones formed from them by reactions with oxygen.

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