JOURNAL OF BACTERIOLOGY, Jan. 1995, p. 307–311 Vol. 177, No. 2
0021-9193/95/$04.00+0

Metabolism of Polychlorinated Phenols by *Pseudomonas cepacia* AC1100: Determination of the First Two Steps and Specific Inhibitory Effect of Methimazole

I. TOMASI,[1] I. ARTAUD,[1]* Y. BERTHEAU,[2] AND D. MANSUY[1]

Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques Associé an CNRS, URA 400, Université René Descartes, 75270 Paris Cedex 06,[1] and Laboratoire de Pathologie Végétale, Institute National de la Recherche Agronomique-INA-PG, 75231 Paris Cedex 05,[2] France

Received 18 July 1994/Accepted 10 November 1994

Resting cells of 2,4,5-trichlorophenoxyacetic acid-grown *Pseudomonas cepacia* AC1100 metabolize both dichlorophenols, such as 2,4-dichlorophenol, 2,6-dichlorophenol, 3,4-dichlorophenol, and 3,5-dichlorophenol, and more highly substituted phenols, such as 2,4,6-trichlorophenol and pentachlorophenol, to the corresponding chlorohydroquinones. The first hydroxylation occurs in the *para* position of the phenol regardless of whether this position is replaced by a chlorine substituent. The first evidence leading to the characterization of *para*-hydroxylase as a flavin-containing enzyme is provided by the inhibitory effect of methimazole, an alternate substrate for this monooxygenase, on the degradative ability of the strain. In a second step, with tetrachlorohydroquinone, trichlorohydroxyquinone was isolated and completely characterized. Trichlorohydroxyquinone was also obtained from tetrachloroquinone. Incubation of the cells in the presence of an external source of NADPH prevents the further degradation of tetrachlorohydroquinone, suggesting that the quinone derived from the two-electron oxidation of the hydroquinone is more likely the substrate for the second hydroxylation.

Bacterial degradation of chlorinated phenols is presently the focus of extensive work (5, 6). Usually, bacteria that catalyze the aerobic degradation of halophenols are divided into two classes: strains that degrade mono- and dichlorophenols but do not attack more highly halogenated phenols and strains that exclusively degrade pentachlorophenol and other highly chlorinated phenols. Two specific metabolic pathways seem to be associated with halophenol degradation by these two types of strains: polychlorinated hydroquinones and chlorocatechols being, respectively, the first metabolites of highly chlorinated phenols and of mono- or dichlorophenols (5, 6). Some years ago, a mixed culture degrading the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was developed by a method called plasmid-assisted molecular breeding (10). A strain, identified as *Pseudomonas cepacia* AC1100, that utilized this herbicide as a sole source of carbon and energy was isolated and was induced for the degradation of 2,4,5-trichlorophenol (2,4,5-TCP) and other halophenols (9). Interestingly, this strain degraded and dechlorinated a wide range of halophenols, including both di- and trichlorophenols and more highly substituted halophenols. However, very few data are presently available on the general degradative pathways of these halophenols. The metabolism of 2,4,5-T was first established on a mutant, PT88, of the *P. cepacia* strain (15). After the formation of 2,4,5-TCP, two products resulting from two successive hydroxylative dechlorinations were observed, namely 2,5-dichlorohydroquinone (2,5-DCHQ) and 5-chloro-1,2,4-trihydroxybenzene (Fig. 1). Because this strain was able to degrade a wide range of halophenols, our objective was to determine if, in all cases, as with 2,4,5-TCP, when the cells were induced with 2,4,5-T, the first hydroxylation reaction led to the formation of a *p*-hydroquinone or if, particularly with dichlorophenols, some catechols were also obtained. Using the effect of specific inhibitors of cytochrome P-450 and flavin-containing monooxygenases on the degradative ability of the AC1100 resting cells, we provide the first evidence implicating the involvement of a flavoprotein in the first hydroxylation step.

* Corresponding author. Mailing address: Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques (URA 400), Université René Descartes, 45 Rue des Saints-Pères, 75270 Paris Cedex 06, France.

MATERIALS AND METHODS

Materials. All the halophenols and chlorohydroquinone (CHQ), tetrachlorocatechol (TeCC), and tetrachloro-1,4-benzoquinone were purchased from Aldrich or Janssen, and 2,6-dichloroquinone was purchased from Pfaltz and Bauer. $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) used to generate NADPH, tetrachloro-*p*-benzoquinone, and all the inhibitors tested in this work were obtained from Sigma.

Growth media and cultures. *P. cepacia* AC1100 cells, kindly provided by A. M. Chakrabarty, were grown in the basal salts medium previously described by Kilbane et al. (11) and composed of 33 mM K_2HPO_4, 33 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 0.3 mM $MgSO_4 \cdot H_2O$, 0.2 mM $CaCl_2$, 13 μM $Na_2MoO_4 \cdot 2H_2O$, 6.5 μM $FeSO_4$, and 8 μM $MnCl_2 \cdot 4H_2O$. The concentrated stock solution of 2,4,5-T (1.5 mg/ml) was prepared as a potassium salt by dissolving the solid and adjusting the pH to 8 by the dropwise addition of 10 N KOH. After autoclaving and filtration (0.45-μm-pore-size Millipore-type filter), an accurate determination of the 2,4,5-T concentration was obtained by spectrophotometric analysis at 288 nm ($\varepsilon = 23{,}000\ M^{-1}\ cm^{-1}$) with a Kontron Uvicon 810 or 820 apparatus. 2,4,5-T was added to the medium at a final concentration of 3.5 mM. Cultures were inoculated at 5×10^7 to 5×10^8 cells, grown to a mid-exponential phase, and harvested by centrifugation ($8{,}000 \times g$, 10 min) at 4°C. The cell pellets were washed once with a 50 mM phosphate (pH 7)–10 mM $MgSO_4$ buffer and suspended in the same buffer at 10^9 cells per ml, yielding, after disruption of the cells by sonication, a protein content of 110 μg/ml as determined by the Bradford method (3).

Standard incubations and HPLC analysis. Incubations of the resting cells (10 ml) were done in flasks of 50 ml and in a shaking water bath at 28°C with various halophenols at a final concentration of 0.1 mM (mother solutions of halophenols in CH_3CN or methanol, 10 mM). Aliquots were withdrawn (0.7 ml) at various times, and 100 μl of a 1/1 (vol/vol) mixture of CH_3CN and 1 N HCl was added to stop the reaction. After the addition of the internal standard, the sample was centrifuged ($13{,}000 \times g$, 10 min), and 20 μl of the supernatant was then subjected to high-performance liquid chromatography (HPLC) analysis. The HPLC was performed with a Merck Lichrocart 250-4 Lichrospher 100 RP-18 (5-μm-particle-size column [4 by 250 mm]) and a 3 mM H_3PO_4-CH_3CN gradient (CH_3CN content from 30 to 100% in 25 min) at a flow rate of 1 ml/min. The chlorophenols and the hydroquinones were detected by monitoring the A_{230} with a Beckman

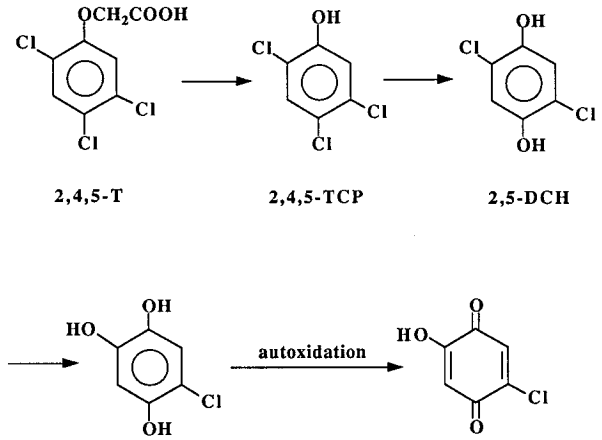

FIG. 1. Proposed mechanism for conversion of 2,4,5-T into 5-chloro-2-hydroxy-1,4-benzoquinone by AC1100 PT88 cells (15).

TABLE 1. Degradation and dechlorination of chlorophenols after 1 h of incubation of AC1100 resting cells

Substrate	Degradation (%)[a]	Dechlorination (%)
2,4,5-TCP	100	100
PCP	40	100
TeCC	85	100
TeCH	100	50
2,6-DCP	100	50
3,5-DCP	30	ND[b]
2,4,6-TCP	83	100
2,6-DCHQ	45	100
2,4-DCP	100	ND
3,4-DCP	55	ND

[a] The resting cell suspensions (OD_{540} of 1) were incubated with the substrates at a concentration of 0.1 mM. The degradation ratios were determined as the average value for two sets of experiments from two independent cultures.
[b] ND, not determined.

165 spectrophotometric detector. 2,4-DCP was selected as the internal standard for pentachlorophenol (PCP) or 3,5,6-trichloro-2-hydroxy-1,4-benzoquinone (TCHQ), and PCP was used as the internal standard for 2,6-DCHQ and all the other halophenols. For the inhibition assays, the cells suspensions (optical density at 540 nm [OD_{540}] of 1) were preincubated for 15 min with the cytochrome P-450 inhibitors and methimazole (MMI) at 0.5 mM before the addition of the substrates at 0.1 mM.

Chloride anion release in the supernatants was determined by direct potential measurements with a chloride-selective electrode (ionic specific electrode [ISE]; Metrohm).

The results discussed in this work are based, for all the experiments, on two independent cultures and on HPLC analysis of two replicate aliquots removed from the supernatants after centrifugation. Moreover, no reaction occurred in the absence of the cells.

Isolation and characterization of 2,6-DCHQ from 2,6-DCP degradation. In order to obtain a sufficient amount of the hydroquinone, a large-scale incubation (120 ml) was carried out under standard conditions with 2,6-DCP (60 μmol) and an NADPH-generating system (120 μmol of $NADP^+$, 1.2 mmol of glucose-6-phosphate, 2 U of glucose-6-phosphate dehydrogenase per ml). After shaking for 90 min at 28°C, 10 ml of 1 N HCl and 110 ml of CH_3CN were added to stop the reaction. After centrifugation (8,000 × *g*, 20 min) at 4°C, the supernatant was extracted with ethylacetate, and the organic layer was dried over $MgSO_4$ and evaporated to dryness in a rotary evaporator. The residue was purified by thin-layer chromatography (TLC) (Merck SiO_2-6O F_{254}, silica gel plates, 1 mm thick, 1 mm eluted with ethylacetate [CH_3COOEt] and characterized by ^{1}H-nuclear magnetic resonance (NMR) analysis in d^6 dimethyl sulfoxide recorded at 250 MHz on a Bruker WM250 spectrometer: chemical shift δ in ppm relative to tetramethylsilane (TMS) 9.58 (OH, s), 9.23(OH, s), 6.75(2H, s).

Isolation and characterization of TCHQ from TeCH degradation. TCHQ was first detected from standard incubations of the resting cell suspensions with tetrachlorohydroquinone (TeCH) by HPLC analysis. The respective retention times of TCHQ and TeCH were 9.1 and 14.6 min, respectively. The UV visible spectrum of the metabolite at 9.1 min, recorded by using diode array detector spectra (Focus, Spectra Physics), showed an absorption maximum at 293 nm. This product was isolated from a large-scale incubation (150 ml) of a cell suspension at a turbidity of 2 at 540 nm with 75 μmol of TeCH for 2 h at 28°C. After the addition of 12 ml of 1 N HCl and 140 ml of CH_3CN and centrifugation (8,000 × *g*, 20 min) at 4°C, the supernatant was evaporated to dryness under vacuum. The raw product, dissolved in CH_3CN, was subjected to a filtration (0.45-μm-pore-size Millipore-type filter) to clarify the sample. The residue was then purified by TLC (Merck SiO_2-60 F_{254} −1 mm; ethylacetate-methanol, 90/10). The UV visible spectrum of the purified product in phosphate buffer (pH 7) showed a strong absorption at 296 nm and a broad one at 530 nm. A sample of TCHQ (16 mg) was then treated for 5 min with 1 ml of acetic anhydride at 100°C in the presence of a small amount of zinc, as a reducing agent, and fused sodium acetate. After cooling and the addition of water, the aqueous layer was extracted twice with ether. The combined etheral layers were washed with aqueous $NaHCO_3$, dried over $MgSO_4$, and evaporated to dryness. The trichlorotriacetoxybenzene was characterized by mass spectrometry and ^{1}H- and ^{13}C-NMR analysis. For mass spectrometry, the theoretical values for *m/e* were 354 ($[M]^+$, 100%), 356 ($[M + 2]^+$, 99%), and 358 ($[M + 4]^+$, 33%) and the experimental values (chemical ionization with NH_3) for *m/e* were 372 ($[M + NH_4]^+$, 95%), 374 ($[M + NH_4 + 2]^+$, 100%), and 376 ($[M + NH_4 + 4]^+$, 40%). For ^{1}H-NMR in $CDCl_3$, δ ppm was 2.39 (3H, s); 2.34 (3H, s); and 2.33 (3H, s). For ^{13}C-NMR in $CDCl_3$, δ ppm was 20.09 (CH_3), 166.45 (CO); and 122.43, 126.25, 126.87, 139.69, 140.16, and 143.31 (C-O and C-Cl).

RESULTS AND DISCUSSION

Metabolism of halophenols by AC1100 resting cells. (i) First hydroxylation step. Two groups of halophenols, each of which was expected to give the same CHQ-type products, were selected: 2,4,6-TCP, 2,6-DCP, and 3,5-DCP on one side and 3,4-DCP and 2,4-DCP on the other side. The resting cell suspensions of AC1100 at a turbidity of 1 at 540 nm were incubated with the various halophenols at a final concentration of 0.1 mM. To avoid any destructive extraction of CHQs or chlorocatechols during the work up, the reactions were quenched by the addition of a 1/1 (vol/vol) mixture of 1 N HCl and acetonitrile, leading to disruption of the cells and precipitation of the proteins. After centrifugation of the samples, the supernatant was directly analyzed by HPLC. We first determined the percentage of degradation of the phenols and of their potential metabolites as 2,6-DCHQ for the first halophenol group and TeCH or TeCC for PCP. The degradation and mineralization of halophenols have been previously reported (9), but not those of hydroquinones or catechols. As shown in Table 1, after a 1-h incubation, most of the halophenols tested are less efficiently degraded by this strain than 2,4,5-TCP. TeCH and TeCC are degraded to 100 and 85% and mineralized to 50 and 100% respectively, while 2,6-DCHQ is only partially degraded but completely dechlorinated. No metabolites have been detected during PCP degradation, but as shown in Fig. 2, 2,4,6-TCP (panel A), 2,6-DCP (panel B), and 3,5-DCP (panel C) are converted into 2,6-DCHQ. This metabolite was identified in all cases by its HPLC retention time and its UV spectrum, which were found to be identical to those of an authentic sample. Moreover, in a large-scale incubation of the cells with 2,6-DCP in the presence of an external source of NADPH, complete conversion of 2,6-DCP to this metabolite was observed without any trace of 3-chlorocatechol formation (Table 2). Interestingly, identical incubations of the cells in the absence of added NADPH led to a 75% conversion of 2,6-DCP and to only a 15% yield in 2,6-DCHQ. The 2,6-DCHQ metabolite was isolated, purified by TLC, and completely characterized by ^{1}H-NMR analysis. In the same way, 3,4-DCP (Fig. 2D) and 2,4-DCP (Fig. 2E) led also to the formation of CHQ, the product derived from a *para* hydroxylation of the phenol ring, but in this case the identity of the metabolite has been proved only by HPLC and UV spectroscopy.

These results indicate that, whatever their degree of halogenation, all the halophenols seem to be metabolized by the same enzyme that catalyzes the *para* hydroxylation of the phenols, regardless of whether that position was originally re-

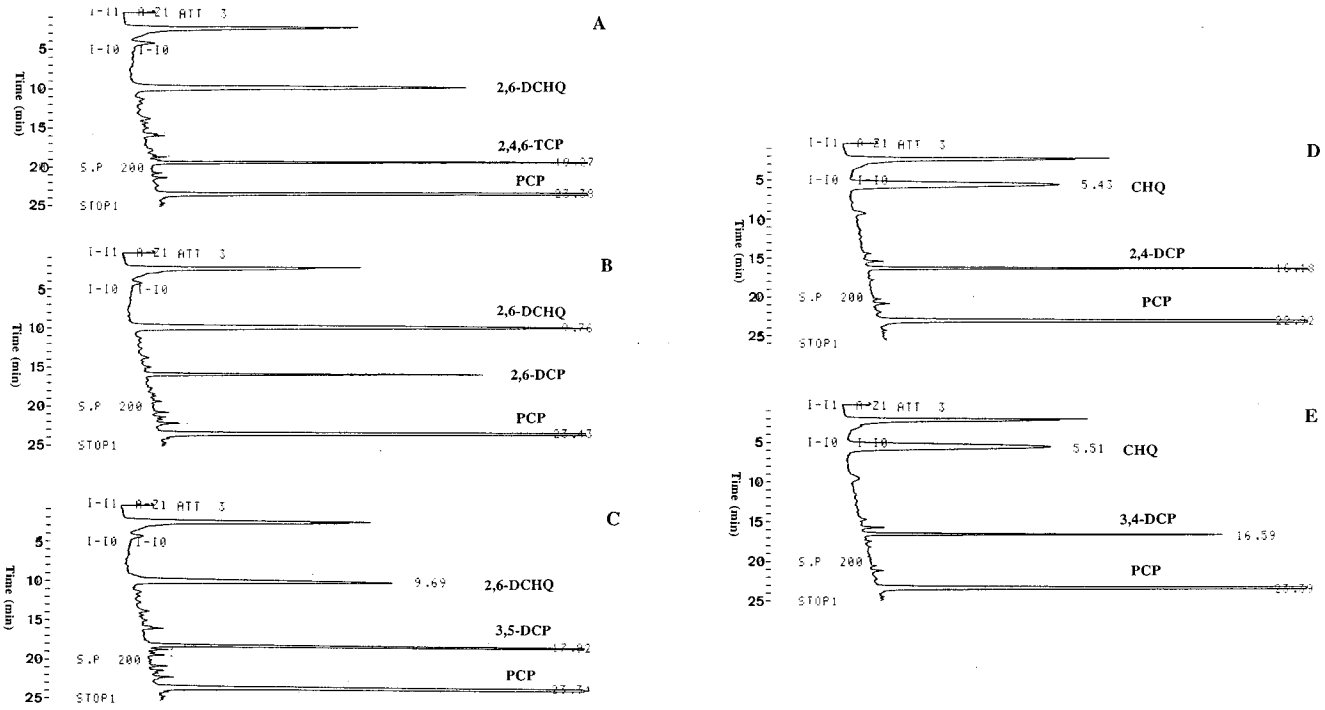

FIG. 2. HPLC spectra derived from cells incubation with 2,4,6-TCP (A), 2,6-DCP (B), 3,5-DCP (C), 3,4-DCP (D), 2,4-DCP (E) and identification of hydroquinones, 2,6-DCHQ (A, B, and C) and CHQ (D and E).

placed by a chlorine in the starting product. The same selectivity has been previously reported for a *Rhodococcus* sp. which degrades both PCP and 2,3,5,6-tetrachlorophenol via TeCH formation (1) and for *Pseudomonas picketti* which, when induced with 2,4,6-TCP (12), catalyzes, as *Streptomyces rochei* 303 (4), the degradation of a wide range of halophenols through the corresponding *para*-hydroquinone.

(ii) Second hydroxylation step. The cell suspensions (OD_{540} of 2) were incubated with TeCH (0.5 mM), the product derived from the first dehalogenative hydroxylation of PCP. Very rapidly, the solution developed a deep purple color, as previously observed during the formation of the hydroxyquinone derived from 2,4,5-TCP (15). A new product was detected by HPLC whose UV-visible spectrum in phosphate buffer at pH 7 exhibited a strong absorption at 293 nm and a broad one at 530 nm. This spectrum is identical to that of an authentic sample of TCHQ obtained from tetrachloro-1,4-benzoquinone under highly basic conditions (8). This product was then isolated from a large-scale incubation and purified by TLC, but the hydroxyquinone could not be characterized by mass spectrometry because of its instability during this analysis. Therefore, it was reduced and then acetylated with acetic anhydride, and the corresponding trichloro-triacetoxybenzene was completely characterized by mass spectrometry and ^{1}H- and ^{13}C-NMR analyses. It is noteworthy that TeCC, which is also degraded by this strain (Table 1), does not yield the same metabolite as that identified from TeCH (data not shown). The yield of this metabolite was insufficient for isolation and further characterization.

When the cells (OD_{540} of 1) were incubated with TeCH or TeCQ at a concentration of 0.1 mM, the starting products were completely consumed within 1 or 2 h and the metabolite was detected only as a trace by HPLC analysis. Increasing the concentration of TeCH to 0.4 mM and the cell suspension turbidity to 2, (OD_{540} of 2) led to an almost complete conversion of this product to TCHQ (85% yield). In the same conditions, tetrachloroquinone was also but partially metabolized to TCHQ (50% yield). Therefore, to determine whether the quinone or the hydroquinone was the substrate for the second hydroxylation catalyzed by the bacterial strain, the cells (OD_{540} of 2) were incubated in the presence of TeCH (0.4 mM) with, or without, the external addition of NADPH produced by an NADPH-generating system, in order not to be limited in the reducing agent during the overall time course of the experiment. From the results listed in Table 2, it appears that TeCH is metabolized to only TCHQ in the absence of added reducing agent. In fact, TeCH is detected, albeit in low yield (6%), as the first metabolite of PCP only upon the addition of an external source of NADPH. By this procedure, as aforementioned, 2,6-DCP is completely converted to 2,6-DCHQ, imply-

TABLE 2. Effect of external source of NADPH on metabolism of various chlorophenols by AC1100 resting cells

Substrate[a]	Degradation (%)	% Metabolite[b]	NADPH
2,6-DCP	75	15	−
	100	100	+
PCP	32	0	−
	34	6	+
TeCH	84	85	−
	12	0	+

[a] The cell suspensions (OD_{540} of 2) were incubated for 60 min at 28°C with the substrates at a concentration of 0.4 mM, with or without an NADPH-generating system ($NADP^{+}$, 1 mM; glucose-6-phosphate, 10 mM; glucose-6-phosphate dehydrogenase, 2 U/ml).

[b] 2,6-DCHQ, tetrachlorohydroquinone and trichloro-hydroxy-1,4-benzoquinone were the metabolites derived from 2,6-DCP, PCP, and TeCH, respectively.

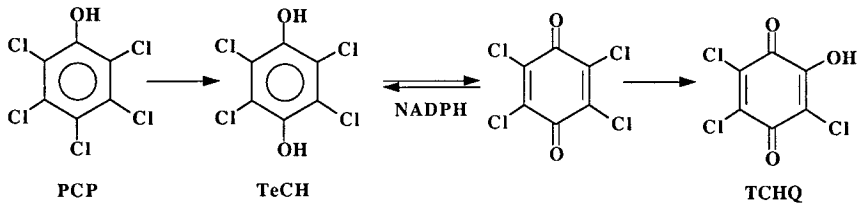

FIG. 3. Proposed pathway for metabolism of PCP to TCHQ by AC1100 resting cells.

ing that the further metabolization of this hydroquinone is also inhibited in these conditions. On the other hand, a 10-fold excess of potassium ferricyanide oxidized around 30% of TeCH to TeCQ in the mixing time, but the two substrates disappeared and TCHQ formation was not inhibited. It is to be noticed that, at a lower concentration (0.1 mM), TeCH was slowly and spontaneously oxidized to TeCQ in the buffer without the cells.

These results strongly suggest that, in the case of PCP, the chloroquinone derived from a two-electron oxidation of the hydroquinone is the substrate for the second hydroxylation reaction (Fig. 3) rather than the CHQ. The second dehalogenative hydroxylation observed in the absence of previous reduction of a chlorine substituent has been described so far with this strain only with 2,5-DCH (15) or TeCH. The degradation of TeCH by a *Rhodococcus* sp. (2, 7) is supposed to follow the same mechanistic pathway, but, at present, the direct formation of 5,6-dichloro-1,2,4-trihydroxybenzene resulting from both reductive and hydroxylative dechlorinations (2) has been detected. With a *Flavobacterium* sp., TeCH is metabolized by two reduction reactions to 2,6-DCHQ (20, 21).

Characterization of *para*-hydroxylating enzyme. From previous work, it was known that oxygen consumption was associated with halophenol degradation and halogen release (11). Furthermore, the requirement for an excess of NADPH to completely convert halophenols into the corresponding hydroquinones indicated that the *para*-hydroxylating enzyme might be a monooxygenase. Recently, several kinds of bacterial monooxygenases involved in the hydroxylation of halophenols have been characterized. Two cytochrome P-450 membrane-bound enzymes have been identified from *Rhodococcus chlorophenolicus* (19) and *Mycobacterium fortuitum* (18). Two soluble flavin-containing monooxygenases have also been isolated and purified: (i) a 2,4-dichlorophenol hydroxylase which catalyzes the *ortho* hydroxylation of 2,4-DCP (14) and (ii) a pentachlorophenol hydroxylase from a *Flavobacterium* sp. (22) which catalyzes the *para* hydroxylation of several halophenols. Assuming the involvement of such a cytochrome P-450 or flavin-containing monooxygenase in *P. cepacia* AC1100, we tried to determine the nature of the enzymatic system involved in the *para* hydroxylation of halophenols by testing the effect of selected inhibitors on the degradative ability of the bacteria. Such experiments have been routinely performed on purified enzymes, cell extracts, and microsomes, but to our knowledge, no data have yet been published about the study of inhibitory effects on whole cells.

α- and β-Naphthoflavone, miconazole, metyrapone, piperonylbutoxide, and CO were chosen as specific cytochrome P-450 inhibitors (16). These inhibitors have little effect on 2,4,6-TCP degradation. Even CO, a small molecule which could easily diffuse inside the cell membranes and which is one of the most efficient inhibitor of cytochrome P-450 (16), does not produce any effect. These data rule out the involvement of such a cytochrome P-450 monooxygenase in the first hydroxylation of the halophenols.

MMI (2-mercapto-1-methylimidazole) was assayed as a classical competitive inhibitor of flavin monooxygenases. This compound is known to have a very high affinity for mammalian flavin-containing monooxygenase and to be selectively oxidized by this enzymatic system in the presence of cytochrome P-450 (23, 24). It has thus been used as an alternate substrate in hepatic microsomes that contain both types of monooxygenases to discriminate between reactions catalyzed by flavin-containing monooxygenase and cytochrome P-450 (13, 17). Even though the substrate specificity of bacterial flavin-containing monooxygenase is not well defined, this enzyme is known to oxidize soft nucleophiles such as amines and alkyl or aryl sulfides and phenolic compounds (24). The effect of MMI was tested on the degradative ability of AC1100 on several chlorophenols, including TeCC and TeCH. The cells were preincubated with the inhibitor for 15 min before the addition of the substrate. After 2 and 4 h of incubation, aliquots were withdrawn and quenched as previously described, and the remaining substrate was quantitated by HPLC analysis. As shown in Table 3, the degradation of all the chlorophenols studied was strongly inhibited, the inhibitory effect increased as the chlorophenols were less efficiently degraded by the strain. The inhibition ratio increased after 2 h of incubation from 70 to 100%, while the degradation yield decreased from 100 to 50% in the order 2,4,5-TCP, 2,6-DCP, 2,4-DCP, PCP, and 2,4,6-TCP. Such a competitive effect of MMI is consistent with the hypothesis that a flavin monooxygenase catalyzes the first hydroxylation of halophenols. However, MMI (Table 3) and cytochrome P-450 inhibitors (data not shown) have no effect, or slightly enhance, the rate of TeCH or TeCC degradation. Therefore, it seems that the second hydroxylation catalyzed by AC1100 strain does not involve such a monooxygenase en-

TABLE 3. Effect of MMI, a competitive inhibitor of flavin-containing monooxygenase, on the degradation of chlorophenols by AC1100 resting cells

Substrate[a]	Degradation (%)				Inhibition (%)[b]	
	Without MMI		With MMI			
	2 h	4 h	2 h	4 h	2 h	4 h
2,4,5-TCP	100	100	31	45	69	55
2,6-DCP	100	100	31	33	69	67
2,4-DCP	83	100	13	13	84	87
PCP	53	67	3	2	94	97
2,4,6-TCP	50	100	0	0	100	100
TeCC	73	79	100	100	−34[c]	−26[c]
TeCH	100	100	100	100	0	0

[a] The cell suspensions (OD_{540} of 1) were incubated first with MMI (0.5 mM) at 28°C for 15 min before the addition of the substrate (0.1 mM).

[b] The inhibition was determined as the ratio of the difference between the degradation without and with MMI relative to that without MMI.

[c] In this case, an activation of TeCC degradation of 34 and 26% was observed after 2 and 4 h, respectively.

zyme, as previously reported for the *Rhodococcus* and *Mycobacterium* strains (18, 19).

In conclusion, our work shows that *P. cepacia* AC1100 catalyzes the degradation of many halophenols via two hydroxylation steps. The first one introduces an OH group in the *para* position of the starting phenol, whether this position has a chlorine substituent. The use of specific inhibitors of either cytochrome P-450 or flavin monooxygenase favors the involvement of the latter enzyme in the first step. However, to definitively established this point, supplementary experiments, including the purification of the enzyme, which is in progress, are required. The hydroquinone metabolite derived from the first step (Fig. 3) should be spontaneously oxidized into the corresponding quinone. In the case of PCP degradation, this chlorinated quinone is clearly the substrate for a second hydroxylating dechlorination, which leads to a polyhalo-hydroxyquinone. This step does not seem to be catalyzed by either a cytochrome P-450 or a flavin-containing monooxygenase. Interestingly, it is possible to isolate the hydroquinone derived from the first step more easily by adding NADPH to the incubated resting cells, which shifts the redox equilibrium between hydroquinone and quinone towards the hydroquinone compound, which is not further metabolized.

ACKNOWLEDGMENTS

This work was supported by a grant from Rhône-Poulenc Company to Isabelle Tomasi.

We thank Dominique Pétré for his interest in this work and for helpful discussions.

REFERENCES

1. **Apajalahti, J. H. A., and M. S. Salkinoja-Salonen.** 1987. Dechlorination and *para*-hydroxylation of polychlorinated phenols by *Rhodococcus chlorophenolicus*. J. Bacteriol. **169:**675–681.
2. **Apajalahti, J. H. A., and M. S. Salkinoja-Salonen.** 1987. Complete dechlorination of tetrachlorohydroquinone by cell extracts of pentachlorophenol-induced *Rhodococcus chlorophenolicus*. J. Bacteriol. **169:**5125–5130.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Golovla, L. A., O. Zaborina, R. Pertsova, B. Baskunov, Y. Schuruklin, and S. Kuzmin.** 1992. Degradation of polychlorinated phenols by *Streptomyces* rochei 303. Biodegradation **2:**201–208.
5. **Häggblom, M. M.** 1990. Mechanism of bacterial degradation and transformation of chlorinated monoaromatic compounds. J. Basic Microbiol. **30:** 115–141.
6. **Häggblom, M. M.** 1992. Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiol. Rev. **103:**29–72.
7. **Häggblom, M. M., D. Janke, and M. S. Salkinoja-Salonen.** 1989. Hydroxylation and dechlorination of tetrachlorohydroquinone by *Rhodococcus* sp. strain CP-2 cell extracts. Appl. Environ. Microbiol. **55:**516–519.
8. **Hancock, J. W., C. E. Morrell, and D. Rhum.** 1962. Trichlorohydroxyquinone. Tetrahedron Lett. **22:**987–988.
9. **Karns, J. S., J. J. Kilbane, S. Duttagupta, and A. M. Chakrabarty.** 1983. Metabolism of halophenols by 2,4,5-trichlorophenoxyacetic acid-degrading *Pseudomonas cepacia*. Appl. Environ. Microbiol. **46:**1176–1181.
10. **Kellogg, S. T., D. K. Chatterjee, and A. M. Chakrabarty.** 1981. Plasmid-assisted molecular breeding: new technique for enhanced biodegradation of persistent toxic chemicals. Science **214:**1133–1135.
11. **Kilbane, J. J., D. K. Chatterjee, J. S. Karns, S. T. Kellogg, and A. M. Chakrabarty.** 1982. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Pseudomonas cepacia*. Appl. Environ. Microbiol. **44:**72–78.
12. **Kiyohara, H., T. Hatta, Y. Ogawa, T. Kakuda, H. Yokoyama, and N. Takizawa.** 1992. Isolation of *Pseudomonas picketti* strains that degrade 2,4,6-trichlorophenol and their dechlorination of chlorophenols. Appl. Environ. Microbiol. **58:**1276–1283.
13. **Poulsen, L. L., R. M. Hyslop, and D. M. Yiegler.** 1979. S oxygenation of N-substituted thioureas catalyzed by the pig liver microsomal FAD-containing monooxygenase. Arch. Biochem. Biophys. **198:**78–88.
14. **Radjendirane, V., M. A. Bhat, and C. S. Vaidyanathan.** 1991. Affinity purification and characterization of 2,4-dichlorophenol hydroxylase from *Pseudomonas cepacia*. Arch. Biochem. Biophys. **288:**169–176.
15. **Sangodkar, U. M. X., P. J. Chapman, and A. M. Chakrabarty.** 1988. Cloning, physical mapping and expression of chromosomal genes specifying degradation of the herbicide 2,4,5-T by *Pseudomonas cepacia* AC1100. Gene **71:**266–277.
16. **Testa, B., and P. Jenner.** 1981. Inhibitors of cytochrome P-450s and their mechanism of action. Drug Metab. Rev. **12:**1–117.
17. **Tynes, R. E., and E. Hogdson.** 1983. Oxidation of thiobenzamide by the FAD-containing and cytochrome P-450 dependent monooxygenases of liver and lung microsomes. Biochem. Pharmacol. **32:**3419–3428.
18. **Uotila, J. S., V. H. Kitunen, T. Saastamoinen, T. Coote, M. M. Häggblom, and M. S. Salkinoja-Salonen.** 1992. Characterization of aromatic dehalogenases of *Mycobacterium fortuitum* CG-2. J. Bacteriol. **174:**5669–5676.
19. **Uotila, J. S., M. S. Salkinoja-Salonen, and J. H. A. Apajalahti.** 1991. Degradation of pentachlorophenol by membrane bound enzymes from *Rhodococcus chlorophenolicus* PCP-1. Biodegradation **2:**25–31.
20. **Xun, L., and C. S. Orser.** 1991. Purification and properties of pentachlorophenol hydroxylase, a flavoprotein from *Flavobacterium* sp. strain ATCC 39723. J. Bacteriol. **173:**4447–4453.
21. **Xun, L., E. Topp, and C. S. Orser.** 1992. Glutathione is the reducing agent for the reductive dehalogenation of tetrachloro-p-hydroquinone by extracts from a *Flavobacterium* sp. Biochem. Biophys. Res. Commun. **182:**361–366.
22. **Xun, L., E. Topp, and C. S. Orser.** 1992. Purification and characterization of a tetrachloro-*p*-hydroquinone reductive dehalogenase from a *Flavobacterium* sp. J. Bacteriol. **174:**8003–8007.
23. **Ziegler, D. M.** 1980. Microsomal flavin-containing monooxygenase: oxygenation of nucleophilic nitrogen and sulfur compounds, p. 201–227. *In* W. B. Jakoby (ed.), Enzymatic basis of detoxification, vol. 2. Academic Press, New York.
24. **Ziegler, D. M.** 1993. Recent studies on the structure and function of multisubstrate flavin-containing monooxygenases. Annu. Rev. Pharmacol. Toxicol. **33:**179–199.