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Microbial Degradation of Chloroaromatics: Use of the *meta*-Cleavage Pathway for Mineralization of Chlorobenzene

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***Pseudomonas putida* GJ31 is able to simultaneously grow on toluene and chlorobenzene. When cultures of this strain were inhibited with 3-fluorocatechol while growing on toluene or chlorobenzene, 3-methylcatechol or 3-chlorocatechol, respectively, accumulated in the medium. To establish the catabolic routes for these catechols, activities of enzymes of the (modified) *ortho*- and *meta*-cleavage pathways were measured in crude extracts of cells of *P. putida* GJ31 grown on various aromatic substrates, including chlorobenzene. The enzymes of the modified *ortho*-cleavage pathway were never present, while the enzymes of the *meta*-cleavage pathway were detected in all cultures. This indicated that chloroaromatics and methylaromatics are both converted via the *meta*-cleavage pathway. *Meta* cleavage of 3-chlorocatechol usually leads to the formation of a reactive acylchloride, which inactivates the catechol 2,3-dioxygenase and blocks further degradation of catechols. However, partially purified catechol 2,3-dioxygenase of *P. putida* GJ31 converted 3-chlorocatechol to 2-hydroxy-*cis*,*cis*-muconic acid. Apparently, *P. putida* GJ31 has a *meta*-cleavage enzyme which is resistant to inactivation by the acylchloride, providing this strain with the exceptional ability to degrade both toluene and chlorobenzene via the *meta*-cleavage pathway.**

Various bacterial cultures which can use chloroaromatics as the single source of carbon and energy for growth, resulting in the formation of carbon dioxide, chloride, and biomass, have been described. These organisms can be differentiated on the basis of the catabolic pathways dealing with the substituents. Chlorosubstituents can be removed by initial oxygenolytic, reductive, or hydrolytic reactions. Further mineralization can then occur via classical pathways such as the 3-oxoadipate and the *meta*-cleavage pathways. However, the majority of the organisms able to mineralize chlorinated aromatics do not possess enzyme systems capable of initial dechlorination. They transform chlorinated aromatics to chlorocatechols, which are further metabolized by the enzymes of the modified *ortho*-cleavage pathway, and dechlorination occurs after ring cleavage (32).

It is generally accepted that degradation of chloroaromatics does not proceed via the *meta*-cleavage pathway (20, 26, 29). An explanation for this has been found in the production of an acylchloride from 3-chlorocatechol by the catechol 2,3-dioxygenase of the *meta*-cleavage pathway, which leads to rapid suicide inactivation of the enzyme (3). Therefore, *meta* cleavage is considered to be unsuitable for the mineralization of haloaromatics that are degraded via halocatechols.

Whereas chlorocatechols are mineralized via *ortho*-cleavage pathways, methylaromatics are commonly mineralized via *meta*-cleavage routes. Simultaneous metabolism of chloro- and methylcatechols often creates biochemical anarchy. *meta* cleavage leads to substrate misrouting in the case of 4-chlorocatechol or formation of a suicide product in the case of 3-chlorocatechol. Formation of dead-end methyllactones can occur when the *ortho*-cleavage pathway is dealing with methylcatechols.

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Consequently, only a few strains which can grow on mixtures of methylated and chlorinated aromatics are known (9, 26, 37). They all use a modified *ortho*-cleavage pathway for the conversion of the chlorinated substrate. *Pseudomonas putida* GJ31 (24) and *Pseudomonas* sp. strains JS6 (26) and JS150 (9) are the only strains known to grow on a mixture of chlorobenzene and toluene. In *Pseudomonas* sp. strain JS6, the presence of toluene slightly decreased the yield on chlorobenzene. The organism degraded both substrates primarily via a modified *ortho*-cleavage pathway, which is less efficient for methylated substrates than the *meta*-cleavage pathway. In *P. putida* GJ31, the presence of toluene enhanced the degradation of chlorobenzene (18). Here, we show that this organism grows on chlorobenzene via a *meta*-cleavage pathway which allows the simultaneous utilization of toluene. In addition, 3-chlorocatechol was found to be the ring cleavage substrate formed from chlorobenzene, which was dehalogenated during ring cleavage to produce 2-hydroxymuconic acid.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. putida* GJ31 was isolated from sediment from the river Rhine, with chlorobenzene as the sole carbon and energy source (24). The organism was grown in the mineral medium described by Dorn et al. (5). Cells were grown in 500-ml cultures in 3-liter sealed Erlenmeyer flasks with baffles; the flasks were shaken on a rotary shaker at 100 rpm at 30°C. Volatile substrates (1 ml) were added via an open reservoir connected to the screw cap in the top of the Erlenmeyer flask. This allowed the hydrocarbons to evaporate and enter the liquid medium via the headspace.

P. putida PaW1 (ATCC 33015) harbors the TOL plasmid pWW0 (2) and was grown on mineral medium containing 5 mM 4-methylbenzoate.

Preparation of cell extracts. Cells were harvested by centrifugation (15 min, 8,000 × *g*, 4°C), washed twice with 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1 mM 1,4-dithiothreitol, and disrupted by one passage through a French pressure cell (Aminco, Silver Spring, Md.; 140 MPa, 0°C) before centrifugation (60 min, 100,000 × *g*, 4°C) to remove cell debris. The clear supernatant solution was used as a source of crude cell extract.

Enzyme assays. All enzyme assays were done at 25°C. Purified enzymes of *Pseudomonas* sp. strain B13 were taken as a positive control of the assays of the modified *ortho*-cleavage pathway.

Benzene dihydrodiol dehydrogenase was measured by determining NAD reduction at 340 nm (ε_{NADH} = 6,300 liters mol^{-1} cm^{-1}). Reaction mixtures

contained (in 1 ml) 20 μmol of Tris-HCl (pH 8.0), 2 μmol of NAD, and 0.4 μmol of *cis*-1,2-dihydroxycyclohexa-3,5-diene. The reaction was started by adding crude extract (0.02 to 0.2 mg of protein).

Benzoate dihydrodiol dehydrogenase was measured by determining NAD reduction at 340 nm. Reaction mixtures contained (in 1 ml) 20 μmol of Tris-HCl (pH 8.0), 2 μmol of NAD, and 0.4 μmol of 3-chloro-*cis*-1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate. The reaction was started by adding crude extract (0.02 to 0.2 mg of protein).

Catechol 2,3-dioxygenase was measured by determining product formation (2-hydroxy-6-oxohepta-2,4-dienoate [HOMS]) at 375 nm (ε_{HOMS} = 36,000 liters mol^{-1} cm^{-1}) (23). Reaction mixtures contained (in 1 ml) 45 μmol of phosphate buffer (pH 7.4) and 0.5 μmol of catechol. The reaction was started by adding crude extract (0.02 to 0.2 mg of protein).

2-Hydroxymuconic semialdehyde hydrolase was measured by determining the NAD-independent decrease in concentration of the yellow ring cleavage product from catechol at 375 nm. Reaction mixtures contained (in 1 ml) 45 μmol of phosphate buffer (pH 7.4) and about 60 nmol of freshly prepared 2-hydroxy-6-oxohepta-2,4-dienoate. The reaction was started by adding crude extract (0.02 to 0.2 mg of protein).

2-Hydroxymuconic semialdehyde dehydrogenase was measured by determining the NAD-dependent decrease in concentration of the yellow ring cleavage product from catechol at 375 nm. Reaction mixtures contained (in 1 ml) 45 μmol of phosphate buffer (pH 7.4), about 60 nmol of freshly prepared 2-hydroxy-6-oxohepta-2,4-dienoate, and 0.5 μmol of NAD. The reaction was started by adding crude extract (0.02 to 0.2 mg of protein). The 2-hydroxymuconic semialdehyde hydrolase activity was subtracted from the rate of 2-hydroxy-6-oxohepta-2,4-dienoate disappearance to obtain the 2-hydroxymuconic semialdehyde dehydrogenase activity.

Catechol 1,2-dioxygenase was measured by determining the formation of *cis*,*cis*-muconate at 260 nm ($\varepsilon_{cis,cis\text{-muconate}}$ = 16,800 liters mol^{-1} cm^{-1}) as described previously by Dorn and Knackmuss (6). Reaction mixtures contained (in 1 ml) 33 μmol of Tris-HCl (pH 8.0), 1.3 μmol of Na_2EDTA, 2.6 μmol of H_2O_2, and crude extract (0.02 to 0.2 mg of protein). After the assay mixture was incubated for 10 min to destroy all catechol 2,3-dioxygenase activity (22), the reaction was started by adding 0.5 μmol of catechol.

Chlorocatechol 1,2-dioxygenase was measured by a test similar to that for catechol 1,2-dioxygenase but with 3-chlorocatechol as the assay substrate ($\varepsilon_{2\text{-chloro-}cis,cis\text{-muconate}}$ = 17,100 liters mol^{-1} cm^{-1}).

Muconate cycloisomerase was measured by determining the decrease in substrate concentration at 260 nm. Reaction mixtures contained (in 1 ml) 30 μmol of Tris-HCl (pH 8.0), 1 μmol of $MnCl_2$, and 0.1 μmol of *cis*,*cis*-muconic acid. The reaction was started by adding crude extract (0.02 to 0.2 mg of protein).

Chloromuconate cycloisomerase was measured by a test similar to that for muconate cycloisomerase but with 2-chloro-*cis*,*cis*-muconic acid as the assay substrate.

Dienelactone hydrolase was measured at 280 nm by determining the decrease of substrate concentration ($\varepsilon_{cis\text{-dienelactone}}$ = 17,000 liters mol^{-1} cm^{-1}). Reaction mixtures contained (in 1 ml) 10 μmol of histidine-HCl (pH 6.5) and 0.1 μmol of *cis*-dienelactone. The reaction was started by adding crude extract (0.02 to 0.2 mg of protein).

Maleylacetate reductase was measured by determining maleylacetate-dependent NADH oxidation at 340 nm. Reaction mixtures contained (in 1 ml) 50 μmol of Tris-HCl (pH 7.5), 0.4 μmol of NADH, and crude extract (0.02 to 0.2 mg of protein). After the unspecific reduction rate was determined, the reaction was started by adding 0.4 μmol of freshly prepared maleylacetate.

3-Oxoadipate:succinyl-coenzyme A (CoA) transferase was measured by determining the succinyl-CoA-dependent formation of the 3-oxoadipyl-CoA-magnesium complex at 305 nm ($\varepsilon_{oxoadipyl\text{-}CoA\text{-}magnesium}$ = 25,000 liters mol^{-1} cm^{-1}). Reaction mixtures contained (in 1 ml) 35 μmol of Tris-HCl (pH 8.0), 7 μmol of $MgCl_2$, 3.5 μmol of disodium 3-oxoadipic acid, and crude extract (0.02 to 0.2 mg of protein). The reaction was started by adding 0.15 μmol of succinyl-CoA.

Resting-cell transformations. Metabolites that accumulated in the culture fluid during turnover of chlorobenzene, benzene, or toluene were measured by high-performance liquid chromatography (HPLC) directly after inactivation and removal of the cells by acidification with 0.4% H_3PO_4 and centrifugation.

Conversion of several catechols. The capacity of crude extract of toluene-grown cells of *P. putida* GJ31 to degrade several catechols was tested. For this, 0.26 mg of crude extract was incubated per ml of 33 mM Tris-HCl (pH 8.0) to which a 0.5 mM catechol solution was added. After the assay mixtures were incubated for 30 min at 25°C, samples were analyzed for the presence of catechols by HPLC.

To study the effect of inhibition of the catechol 2,3-dioxygenase, 1.3 mM Na_2EDTA and 2.6 mM H_2O_2 were added to the assay mixture containing crude extract and 33 mM Tris-HCl (pH 8.0). After 10 min of incubation at 25°C, 0.5 mM catechol solution was added, and samples were analyzed by HPLC for the presence of catechols after 30 min of incubation at 25°C.

Production of pyruvate from catechol and 3-chlorocatechol. The amount of pyruvate formed from catechol or 3-chlorocatechol by crude extract of chlorobenzene-grown cells of *P. putida* GJ31 was measured by adding 0.4 mM NADH and 5 μg of lactate dehydrogenase per ml to an assay mixture and measuring the amount of NADH consumed at 340 nm. The assay mixture contained 45 mM phosphate buffer (pH 7.4), 0.3 mM catechol or 3-chlorocatechol, and 0.13 mg of crude extract per ml. The values were corrected for endogenous NADH oxidation of the assay mixture.

Partial purification of catechol 2,3-dioxygenase of *P. putida* GJ31. Crude extract from *P. putida* GJ31 grown on chlorobenzene (3 ml; 14.5 U of catechol 2,3-dioxygenase activity) was applied on a monoQ column preequilibrated with 50 mM Tris-HCl buffer (pH 7.0). After unbound protein was removed by washing the column with 50 ml of the same buffer, a gradient of 0 to 1 M NaCl in a total volume of 20 ml was used for elution. Catechol 2,3-dioxygenase eluted at approximately 150 mM NaCl, whereas oxalocrotonate decarboxylase activity was found in the fraction containing 300 mM NaCl. The activity of oxalocrotonate decarboxylase was detected by a decrease of the absorption of the keto form of freshly prepared oxalocrotonate (0.2 mM) at 235 nm in 45 mM phosphate buffer (pH 7.4) containing 1 mM $MgCl_2$. After chromatography the pooled catechol 2,3-dioxygenase fractions were reactivated by adding 0.1 mM $(NH_4)_2Fe(SO_4)_2$ to give 10.1 U in total.

Analytic methods. The protein content of extracts was determined with Coomassie brilliant blue, with bovine serum albumin as a standard.

HPLC of substrates and metabolites was conducted as described previously with a detector of constant wavelength or by diode array detection, which allows a determination of the UV spectrum of the respective substrate or metabolite (15).

Chemicals. 3-Chloro-*cis*-1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate was prepared by the method of Reineke et al. (27). 4-Chlorocatechol was prepared by the method of Willstätter and Müller (40). 3-Chlorocatechol was prepared as described by Schreiber et al. (34). 2-Hydroxy-6-oxohepta-2,4-dienoate was prepared in situ by incubation of a solution containing 0.1 mM catechol in 45 mM phosphate buffer (pH 7.4) with heat-treated crude extract of *P. putida* PaW1. The resulting product was diluted to give an absorption of about 2 in the enzyme assays. 2-Pyrone-6-carboxylic acid was synthesized according to the method of Wiley and Hart (39). A 4-oxalocrotonate solution was prepared by alkaline hydrolysis of 2-pyrone-6-carboxylic acid (5 mg ml^{-1}) by heating in a boiling water bath for 1 to 2 min with a fivefold molar excess of NaOH. 2-Hydroxy-*cis*,*cis*-muconic acid was prepared by acidifying oxalocrotonate. *cis*,*cis*-Muconic acid and 2-chloro-*cis*,*cis*-muconic acid were chemically synthesized by peracetic acid oxidation of catechol and 3-chlorocatechol, respectively (16). *cis*-Dienelactone was prepared by the method of Kaschabek (14). 2-Methyl- and 4-methyllactone were prepared as described by Hartmann et al. (10). Maleylacetate was prepared by alkaline hydrolysis of dienelactone (7). All other chemicals, including *cis*-benzene dihydrodiol, 4-methylbenzoate, catechol, 3-fluoro-, 3-methyl-, and 4-methylcatechol, 3-oxoadipic acid, and succinyl-CoA, were obtained and are available from several commercial firms.

RESULTS

Enzymes used in the degradation of monoaromatic compounds. To investigate which degradation pathway is used by *P. putida* GJ31 for growth on aromatic compounds, the presence of enzymes involved in the *meta*-, *ortho*-, and modified *ortho*-cleavage pathways was determined in crude extracts prepared from cells grown on toluene, chlorobenzene, benzene, benzoate, a mixture of toluene and chlorobenzene, succinate, or acetate (Table 1). When measuring catechol 1,2-dioxygenase enzymes of the *ortho*- and modified *ortho*-cleavage pathways, putative interfering catechol 2,3-dioxygenase activity in the extract was inhibited with hydrogen peroxide. Catechol 2,3-dioxygenases usually contain ferrous iron as a cofactor, which makes them sensitive to hydrogen peroxide, in contrast to catechol 1,2-dioxygenases, which use ferric iron (22).

Surprisingly, activities of the enzymes of the modified *ortho*-cleavage pathway, which are usually involved in the degradation of chlorinated aromatics, were not detectable in crude extracts of cells grown on chlorobenzene or the other substrates.

Some activity of a catechol 1,2-dioxygenase towards catechol was found in extracts made of cells pregrown on benzene and benzoate, showing that this enzyme indeed resisted the hydrogen peroxide treatment. A muconate cycloisomerase, which is the second enzyme of the *ortho*-cleavage pathway, was also active in these extracts, indicating that *P. putida* GJ31 can induce an *ortho*-cleavage pathway to degrade catechol.

For all the extracts made of cells grown on the aromatic substrates including chlorobenzene, the activities of the enzymes of the *meta*-cleavage pathway were high, with a maximal specific activity of 934 U g of protein^{-1} for the catechol 2,3-

TABLE 1. Specific activities of enzymes in crude extracts of *P. putida* GJ31 pregrown on various substrates

Enzyme	Sp act (U g of protein^{-1}) of enzyme in crude extracts of *P. putida* GJ31 pregrown on:						
	Acetate	Succinate	Chlorobenzene	Toluene	Chlorobenzene + toluene	Benzene	Benzoate
Benzene dihydrodiol dehydrogenase	0	n.d.[a]	89	150	179	141	0
Benzoate dihydrodiol dehydrogenase	n.d.	n.d.	0	1	7	0	118
meta-Cleavage enzymes							
Catechol 2,3-dioxygenase	203	429	343	268	934	452	164
2-Hydroxymuconic semialdehyde dehydrogenase	197	365	452	313	n.d.	297	n.d.
2-Hydroxymuconic semialdehyde hydrolase	11	8	222	210	180	264	50
ortho-Cleavage enzymes							
Catechol 1,2-dioxygenase	8	n.d.	67	4	4	111	115
Muconate cycloisomerase	2	n.d.	26	1	2	167	435
3-Oxoadipate:succinyl-CoA transferase	0	n.d.	0	0	n.d.	21	n.d.
Modified *ortho*-cleavage enzymes							
Chlorocatechol 1,2-dioxygenase	n.d.	n.d.	2	2	1	1	3
Chloromuconate cycloisomerase	0	n.d.	0	0	0	0	0
Dienelactone hydrolase	0	n.d.	0	0	n.d.	0	n.d.
Maleylacetate reductase	0	n.d.	9	1	n.d.	1	n.d.

[a] n.d., not determined.

dioxygenase in crude extracts of cells which were grown on a mixture of toluene and chlorobenzene. This indicates that both methylaromatics and chloroaromatics were converted by a *meta*-cleavage pathway. Some enzymes of the *meta*-cleavage pathway were also active in extracts made of cells grown on succinate or acetate, indicating that they are constitutively expressed.

The key enzyme in the *meta*-cleavage pathway is a catechol 2,3-dioxygenase, which converts catechol to 2-hydroxymuconic semialdehyde. The activity of this enzyme was unstable in crude extracts but could be partially restored by addition of a Fe^{2+} solution (~50 μM). After incubation with hydrogen peroxide (0.009%), the conversion of catechol was inhibited and no yellow product was formed (Fig. 1A). These data indicate that the catechol 2,3-dioxygenase uses Fe^{2+} for its activity. Also, 3-chlorocatechol was no longer converted after hydrogen peroxide treatment (Fig. 1A), indicating that the same enzyme was responsible for the conversion of both compounds.

A putative product of the conversion of 3-chlorocatechol by a catechol 2,3-dioxygenase is 2-pyrone-6-carboxylate. However, spectrophotometric analysis of absorbance spectra from 250 to 400 nm did not reveal any detectable turnover of 2-pyrone-6-carboxylate (20 μM) in 50 mM Tris-HCl buffer (pH 7.5) by crude extract (0.4 mg ml^{-1}) of cells of *P. putida* GJ31 grown on chlorobenzene.

Conversion of toluene by chlorobenzene-grown cells. When chlorobenzene-grown bacteria cooxidize toluene, methyllactones are formed if degradation proceeds via the modified *ortho*-cleavage pathway (28). Accumulation of 2- or 4-methyllactone would indicate that chlorobenzene degradation proceeds via 3- or 4-chlorocatechol, respectively, as ring cleavage substrates. In experiments with cells of *P. putida* GJ31, no formation of 2-methyl- or 4-methyllactone was observed in HPLC chromatograms obtained at the highest sensitivity setting of the instrument. This confirms the enzyme data, suggesting that chlorobenzene is degraded via a *meta*-cleavage pathway.

Conversion of several catechols. Crude extracts prepared from toluene-grown cells were tested for their capacity to degrade substituted catechols. HPLC measurements showed that the extract (0.26 mg ml^{-1}) converted 0.5 mM catechol and 3-chloro-, 4-chloro-, 3-methyl-, and 4-methylcatechol within 30 min. These activities were lost after hydrogen peroxide treatment, indicating that a catechol 2,3-dioxygenase was responsible for the conversion of all the catechols. A yellow compound which could be detected at 375 nm accumulated when the extract was converting catechol, 4-methylcatechol, or 4-chlorocatechol. However, very little product could be detected when the extract was converting 3-methylcatechol. The same was observed at 388 nm, at which wavelength the *meta*-cleavage product of 3-methylcatechol possessed maximum absorption (30). This indicates that enzymes further downstream in the

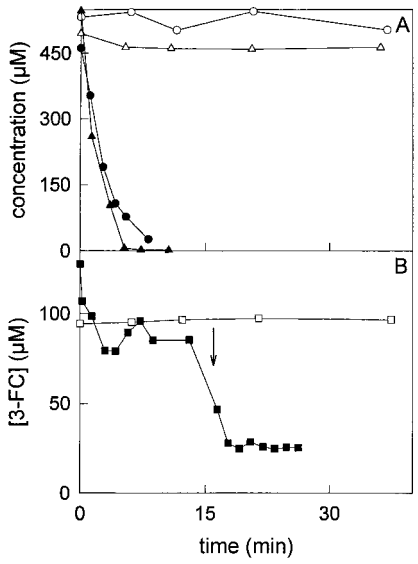

FIG. 1. Effect of hydrogen peroxide on the conversion of several catechols by crude extract (0.2 mg ml^{-1}) of *P. putida* GJ31 grown on chlorobenzene. The conversion of catechol (A; circles), chlorocatechol (A; triangles), and 3-fluorocatechol (3-FC) (B; squares) was monitored over time. Solid symbols, no H_2O_2 was added; open symbols, conversion was inhibited with 0.009% H_2O_2. ↑, time at which extra crude extract (0.2 mg ml^{-1}) was added to the incubation mixture containing 3-fluorocatechol.

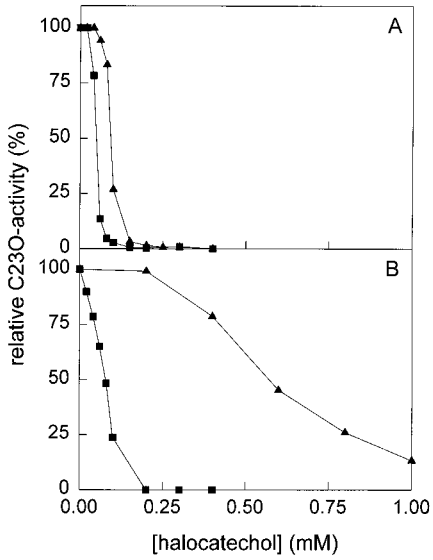

FIG. 2. Inactivation of catechol 2,3-dioxygenase in whole cells of *P. putida* PaW1 (A) and *P. putida* GJ31 (B). Fresh cultures (optical density of 2 at 546 nm) were washed with 45 mM phosphate buffer (pH 7.4) and incubated for 10 min in the presence of various concentrations of 3-chlorocatechol and 3-fluorocatechol. Then, 4-chlorocatechol (0.2 mM) was added, and the cell suspension was incubated for an additional period of 10 min. Cells were separated by centrifugation, and the supernatant was spectrophotometrically analyzed for 5-chloro-2-hydroxymuconic semialdehyde formation at 375 nm as a measure of the residual activity of the catechol 2,3-dioxygenase. Symbols: ▲, relative activity of the catechol 2,3-dioxygenase in a culture inhibited with 3-chlorocatechol; ■, relative activity of the same enzyme with 3-fluorocatechol as the inhibitor.

pathway converted the degradation product of 3-methylcatechol at a rate which does not allow significant accumulation of the yellow product. During conversion of 3-chlorocatechol, a small increase and subsequent decrease of the absorption at 260 nm were observed. This could indicate the formation of a (substituted) muconic acid. When the extract was inactivated with hydrogen peroxide, this pattern was no longer observed, indicating that it was not due to some catechol 1,2-dioxygenase activity.

Only a small amount of 3-fluorocatechol was converted by the extract of chlorobenzene-grown cells; after conversion, the activity stopped. This small amount of activity was lost after treatment with hydrogen peroxide (Fig. 1B).

Inhibition of catechol 2,3-dioxygenase in whole cells of *P. putida* GJ31 and PaW1. The catechol 2,3-dioxygenase of *P. putida* PaW1 is rapidly inhibited by several 3-halocatechols (3). To study the putative inhibition of the catechol 2,3-dioxygenase of *P. putida* GJ31 by these compounds, chlorobenzene-grown cells were incubated for 10 min in the presence of various concentrations of 3-chlorocatechol and 3-fluorocatechol. The relative activity of the catechol 2,3-dioxygenase was calculated from the amounts of 5-chloro-2-hydroxymuconic semialdehyde formed after 10 min of incubation of the cells with 4-chlorocatechol and compared with that produced by similarly treated 4-methylbenzoate-grown cells of *P. putida* PaW1 (Fig. 2). The enzyme of *P. putida* PaW1 was much more sensitive to 3-chlorocatechol than the enzyme of strain *P. putida* GJ31. 3-Fluorocatechol was a stronger inhibitor than 3-chlorocatechol in both strains.

Accumulation of catechols from chlorobenzene and toluene. To identify which catechols are formed when *P. putida* GJ31 is growing on chlorobenzene or toluene, 0.1 mM 3-fluorocatechol was added to cultures of *P. putida* GJ31 growing on these aromatic substrates. 3-Fluorocatechol inhibits the conversion of several catechols by the catechol 2,3-dioxygenase. Culture samples were inactivated with H_3PO_4 and centrifuged, after which putative accumulating metabolites were identified in the supernatant by HPLC. The retention times of the compounds which accumulated were compared with the retention times of standard solutions of catechol and 3-chloro-, 4-chloro-, 3-fluoro-, 3-methyl-, and 4-methylcatechol. The culture growing on toluene accumulated 3-methylcatechol (Fig. 3A), and the culture growing on chlorobenzene accumulated 3-chlorocatechol (Fig. 3B). No other products could be identified by HPLC.

These results showed that 3-chlorocatechol and 3-methylcatechol are intermediates in the degradation of chlorobenzene and toluene, respectively. The enzyme which initially oxidizes these aromatics is probably a dioxygenase, because high activities of a benzene dihydrodiol dehydrogenase were detected in crude extracts made of cells pregrown on toluene or chlorobenzene (Table 1).

Since 3-chlorocatechol is the sole metabolite from chlorobenzene, initial dechlorination, for example by formation of an unstable *gem*-alcohol, can be ruled out as the strategy to degrade chlorobenzene.

Growth of *P. putida* GJ31 and PaW1 with 3-chlorocatechol, 4-chlorocatechol, and catechol. Since catechols and their autoxidation products are known to be toxic at high concentrations, which makes it difficult to use them as growth substrates in liquid cultures, growth on catechols was tested on solid medium by using a simple gradient plate technique. Suspensions of strains PaW1 and GJ31 were spread on the surfaces of mineral medium plates. Different catechols were dissolved in acetone and applied on paper disks. After evaporation of the solvent the disks were applied on the plates. Figure 4 shows plates with *P. putida* PaW1 and GJ31 after an incubation period of 5 days. Since dark-brown coloration originated from the autoxidation of the catechols, the plates were sprayed with a solution of fluorescein diacetate in *N*,*N*-dimethylformamide

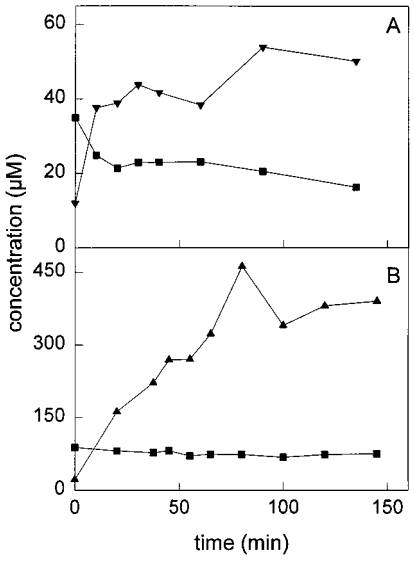

FIG. 3. Accumulation of metabolites in the supernatants of 3-fluorocatechol-inhibited cultures of *P. putida* GJ31 as a function of time. The cultures were growing on toluene (A) or chlorobenzene (B) when 3-fluorocatechol (0.1 mM) was added. Symbols: ■, 3-fluorocatechol; ▼, 3-methylcatechol; ▲, 3-chlorocatechol.

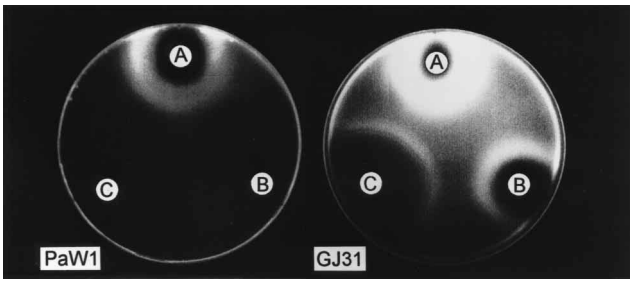

FIG. 4. Growth of *P. putida* strains PaW1 (left) and GJ31 (right) with catechol, 3-chlorocatechol, and 4-chlorocatechol. Freshly grown cells of strains PaW1 and GJ31 were spread on the surfaces of mineral agar plates (300-μl samples with an optical density of 0.8 at 546 nm). Small paper disks containing 3 mg of catechol (A), 3-chlorocatechol (B), or 4-chlorocatechol (C) were applied to the plates. After an incubation of 5 days at 30°C the plates were sprayed with fluorescein diacetate in *N*,*N*-dimethylformamide (1 mg ml^{-1}). The formation of fluorescein due to the esterase activity of the cells allowed the sensitive detection of living cells at 366 nm after an incubation period of 1 h by means of a video scan system.

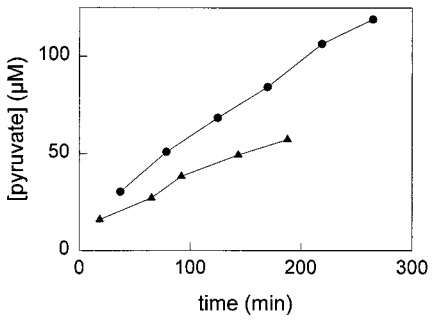

FIG. 5. Formation of pyruvate from catechol (●) and 3-chlorocatechol (▲) as a function of time by crude extract (0.13 mg ml^{-1}) of *P. putida* GJ31 pregrown on chlorobenzene. The concentration of pyruvate in the assay mixture was determined from the amount of NADH consumption by lactate dehydrogenase after addition of NADH (0.4 mM) and lactate dehydrogenase (5 μg ml^{-1}).

for better visualization of active cells, which are able to hydrolyze fluorescein diacetate to fluorescein. Cells which were spread on mineral medium plates and incubated for 5 days without catechols as the potential growth substrate did not give a significant coloration after the plates were sprayed with fluorescein diacetate, showing that growth is necessary for a positive response. *P. putida* PaW1 was shown to grow only with catechol. Even after 14 days no growth was observed in the presence of 3-chloro- and 4-chlorocatechol. Instead, diffusion of 3-chloro- and 4-chlorocatechol led to an inhibition of the growth of strain PaW1 with catechol so that the growth halo around the disk with catechol was incomplete. In contrast, *P. putida* GJ31 was able to use catechol and 3-chloro- and 4-chlorocatechol as the sole source of carbon and energy. The fastest growth was observed with catechol, resulting in only a small clear zone around the disk without living cells. The zone of growth inhibition was larger with 3-chlorocatechol and reached a maximum with 4-chlorocatechol. A yellow halo was observed during growth of *P. putida* GJ31 with 4-chlorocatechol in the first 24 h, indicating the accumulation of the *meta*-cleavage product. This yellow coloration disappeared during the following days.

Production of pyruvate from catechol and 3-chlorocatechol. To prove that 3-chlorocatechol was degraded by the *meta*-cleavage pathway, the amount of pyruvate, which is one of the products of the *meta*-cleavage pathway, was determined in assay mixtures where crude extract of chlorobenzene-grown cells was converting 3-chlorocatechol or catechol. For this, NADH and lactate dehydrogenase were added to a sample of the assay mixture, and the decrease in the concentration of NADH was determined spectrophotometrically as a measure of the concentration of pyruvate. During conversion of catechol or 3-chlorocatechol by the crude extract, an increasing amount of NADH was consumed after addition of lactate dehydrogenase, showing that pyruvate was formed (Fig. 5). The rate at which pyruvate was formed was much lower than the rate at which 3-chlorocatechol was degraded, indicating accumulation of intermediates.

Conversion of 3-chlorocatechol to 2-hydroxymuconate by a catechol 2,3-dioxygenase preparation free of oxalocrotonate decarboxylase. Although 3-chlorocatechol is an intermediate in the degradation of chlorobenzene, it was not possible to identify the product of conversion of 3-chlorocatechol by the catechol 2,3-dioxygenase. Enzymes further downstream in the catabolic pathway are probably converting the conversion product of 3-chlorocatechol at a rate which does not allow significant accumulation of it. The same holds for the conversion product of 3-methylcatechol. One of the possible enzymes which could be responsible for this is a decarboxylase involved in the degradation of 4-oxalocrotonate to 3-oxopentenoate. This enzyme could be detected in crude extract of chlorobenzene-grown cells by measuring the decrease of the keto form of oxalocrotonate at 235 nm.

To determine the product of 3-chlorocatechol conversion by the catechol 2,3-dioxygenase, the decarboxylase was separated from the catechol 2,3-dioxygenase by anionic exchange chromatography. When the fraction containing the catechol 2,3-dioxygenase was incubated with 3-chlorocatechol, a product accumulated; this product was identified as 2-hydroxy-*cis*,*cis*-muconic acid by HPLC with standards of 2-chloro-*cis*,*cis*-muconic acid, *cis*,*cis*-muconic acid, 2-hydroxy-*cis*,*cis*-muconic acid, and 2-pyrone-6-carboxylic acid (Fig. 6). After approximately 80 μM 3-chlorocatechol was converted to 2-hydroxy-*cis*,*cis*-muconic acid, the conversion stopped. After the addition of new protein, the 3-chlorocatechol conversion resumed and then stopped again (Fig. 6).

DISCUSSION

All pure cultures of *Alcaligenes*, *Pseudomonas*, *Xanthobacter*, and other bacteria which can use chlorobenzenes as their sole source of carbon and energy degrade them via the modified *ortho*-cleavage pathway (4, 8, 25, 28, 31, 33, 35, 36, 38), and it

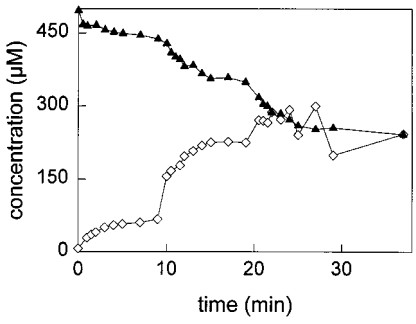

FIG. 6. Conversion of 3-chlorocatechol (▲) to 2-hydroxy-*cis*,*cis*-muconate (◇) by partially purified catechol 2,3-dioxygenase of *P. putida* GJ31 (13.5 mg ml^{-1}). Extra enzyme (13.5 mg $liter^{-1}$) was added to the assay mixture at $t = 10$ min and at $t = 20$ min.

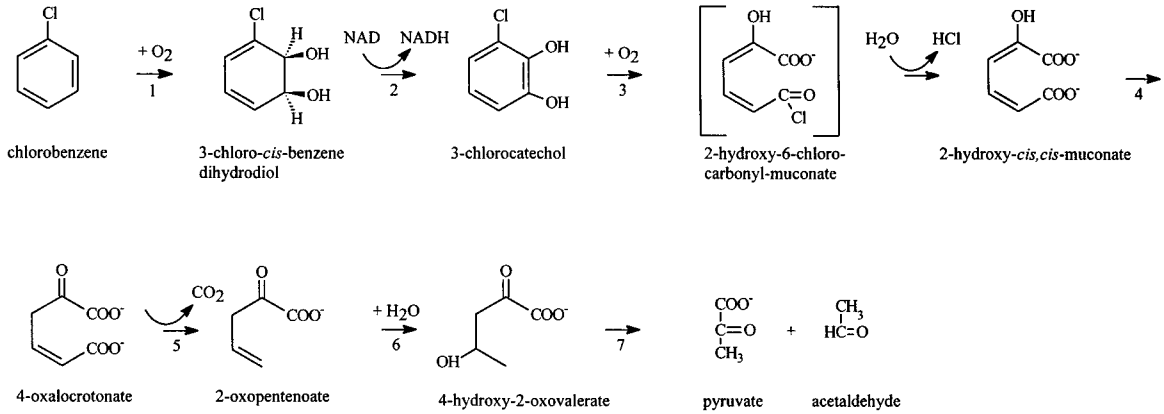

FIG. 7. Proposed catabolic pathway of chlorobenzene by *P. putida* GJ31 by analogy to the known *meta*-cleavage pathway. Enzymes: 1, chlorobenzene dioxygenase; 2, chlorobenzene dihydrodiol dehydrogenase; 3, catechol 2,3-dioxygenase; 4, oxalocrotonate isomerase; 5, oxalocrotonate decarboxylase; 6, 2-oxopent-4-enoate hydratase; 7, 4-hydroxy-2-oxovalerate aldolase. Enzymes 2, 3, and 5 have been detected, while 3-chlorocatechol and pyruvate were determined to be intermediates.

is generally accepted that the *meta*-cleavage pathway is not suitable for the degradation of haloaromatic compounds. In fact, halocatechols usually are strong inhibitors of the *meta*-cleavage pathway. 3-Halocatechols can be suicide substrates for the *meta*-fission enzyme catechol 2,3-dioxygenase of *P. putida* PaW1 (3) or can inactivate the enzyme by chelating the ferrous iron, as in *P. putida* F1 (19). In the first case, an acylhalide is formed which probably acylates the protein and destroys its enzymatic activity. 4-Halocatechols usually yield dead-end products, although there are indications that some organisms are capable of mineralizing catechols chlorinated at the 4 position via the *meta*-cleavage pathway (1, 11–13, 21). However, information about the type of dechlorination is missing, while in some cases, dechlorination occurred very slowly.

Since toluene is usually metabolized via the *meta*-cleavage pathway and since chloroaromatics inhibit this route, we decided to study the type of ring cleavage in *P. putida* GJ31. This organism is capable of growth both on chlorobenzene and on toluene, either separately or added in mixtures (24).

Surprisingly, no activity of enzymes of the modified *ortho*-cleavage pathway could be detected in cells of strain GJ31 grown on chlorobenzene or on any other aromatic substrate. However, a high level of catechol 2,3-dioxygenase was detected in extracts of cells grown on aromatic substrates, including chlorobenzene. This suggested that *P. putida* GJ31 degrades chlorobenzene by a *meta*-cleavage pathway.

Direct evidence for the degradation of chlorobenzene via *meta* cleavage of 3-chlorocatechol was obtained by testing the induction of several enzymes of the *meta*-cleavage pathway in crude extract and by demonstrating the accumulation of catechols in 3-fluorocatechol-inhibited cells. Hydrogen peroxide inhibited the conversion of 3-chlorocatechol in crude extract, which also indicates that a catechol 2,3-dioxygenase is converting 3-chlorocatechol. The product of the *meta* cleavage was identified to be 2-hydroxy-*cis*,*cis*-muconic acid, which is an intermediate of the *meta*-cleavage pathway, and complete conversion of 3-chlorocatechol to pyruvate was shown to occur in the crude extract. Apparently, *P. putida* GJ31 contains a special catechol 2,3-dioxygenase which is able to convert 3-chlorocatechol to 2-hydroxy-*cis*,*cis*-muconic acid and which resists rapid inactivation by the acylchloride, although some inactivation occurs. This allows the organism to use the more efficient *meta*-cleavage pathway for toluene degradation (26) while chlorobenzene is present. Figure 7 summarizes the data on the pathway used by *P. putida* GJ31 for the degradation of chlorobenzene.

Besides the *meta*-cleavage pathway, *P. putida* GJ31 probably also possesses an *ortho*-cleavage pathway for the degradation of catechol, since some activity of enzymes of this route was found in crude extracts of cells grown on benzene or benzoate. The combination of a *meta*- and an *ortho*-cleavage pathway has also been observed in *P. putida* mt-2. In this strain, the genes of the *meta*-cleavage pathway are located on a 117-kb TOL plasmid (pWW0), while the *ortho*-cleavage pathway is encoded on the chromosome (2). *P. putida* GJ31 also possesses a large plasmid, and mutants of this strain which are unable to grow on chlorobenzene and toluene but which could still grow on benzoate and benzene were isolated (unpublished data). This phenotype could be explained by the loss of *meta*-cleavage genes, which are possibly encoded on the plasmid.

3-Fluorocatechol was found to be a much stronger inhibitor of the catechol 2,3-dioxygenase of *P. putida* GJ31 than 3-chlorocatechol, although acylfluorides are expected to be less reactive than acylchlorides. Bartels et al. (3) also observed that 3-fluorocatechol is a stronger inhibitor than 3-chlorocatechol for the catechol 2,3-dioxygenase of *P. putida* PaW1. Their results indicated that reactive oxygen species causing additional inactivation are produced during conversion of 3-fluorocatechol, but not during 3-chlorocatechol conversion.

Although it has long been thought that simultaneous conversion of halo- and methylcatechols is not possible and can only be obtained in constructed strains (29), *P. putida* GJ31 is not unique in its ability to simultaneously metabolize chlorobenzene and toluene. *Pseudomonas* sp. strain JS6 has the same capacity but degrades toluene and chlorobenzene via the modified *ortho*-cleavage pathway (26). The presence of toluene reduced the efficiency of chlorobenzene degradation because of induction of a catechol 2,3-dioxygenase. In contrast, toluene stimulated chlorobenzene degradation by *P. putida* GJ31 in continuous culture, and it was suggested that toluene might be a better inducer of catabolic enzymes (18). The specific activities of the enzymes of the *meta*-cleavage pathway in crude extracts of batch-grown cultures on chlorobenzene and toluene are comparable, which does not indicate that there are differences in induction levels of these enzymes. However, when *P. putida* GJ31 was pregrown on a mixture of chlorobenzene and toluene, the specific activity of the catechol 2,3-dioxygenase increased approximately threefold, suggesting that a combina-

tion of both substrates leads to a higher induction of the extradiol cleavage enzyme.

P. putida GJ31 appears to dechlorinate 3-chlorocatechol during the extradiol cleavage reaction without cyclization. An alternative would be the formation of a cyclic product from the acylchloride, yielding 2-pyrone-6-carboxylate. However, since we did not detect any activity towards 2-pyrone-6-carboxylate in crude extract of chlorobenzene-grown cells and since the only metabolite which was detected after conversion of 3-chlorocatechol by partially purified catechol 2,3-dioxygenase was 2-hydroxy-*cis*,*cis*-muconic acid, this cyclization probably did not occur. This is in contrast to what was found for an organism described by Kersten et al. (17), who examined bacteria which could grow on 5-chlorovanillate by using a *meta*-cleavage pathway. After conversion of 5-chlorovanillate to 5-chloroprotocatechuate, the latter compound was converted to 2-pyrone-4,6-dicarboxylate by extradiol-cleaving protocatechuate 4,5-dioxygenase. This indicates that cyclization of the generated acylhalide can provide an effective alternative to the enzyme suicide which occurs when a nucleophilic group of the enzyme undergoes acylation. Degradation then continued via a 2-pyrone-4,6-dicarboxylate hydrolase, finally resulting in pyruvate and oxaloacetate.

In conclusion, we have shown that *P. putida* GJ31 degrades chlorobenzene via 3-chlorocatechol by using a *meta*-cleavage pathway which contains a catechol 2,3-dioxygenase that is able to convert 3-chlorocatechol to 2-hydroxy-*cis*,*cis*-muconic acid. It has been assumed for a long time that *meta* cleavage of halocatechols is not a feasible route (20). Although this may be true for most of the isolates that have been studied, the work described here illustrates that the diversity of organisms and routes makes it difficult to develop such biochemical generalizations based on the study of individual pathways and organisms. Currently, research focuses on differences between the catechol 2,3-dioxygenase of *P. putida* PaW1 and that of *P. putida* GJ31 which enables the latter to prevent suicide inactivation by the acylchloride that is expected to be formed from 3-chlorocatechol.

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