Metabolism of Chlorotoluenes by *Burkholderia* sp. Strain PS12 and Toluene Dioxygenase of *Pseudomonas putida* F1: Evidence for Monooxygenation by Toluene and Chlorobenzene Dioxygenases

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The degradation of toluene by *Pseudomonas putida* F1 and of chlorobenzenes by *Burkholderia* sp. strain PS12 is initiated by incorporation of dioxygen into the aromatic nucleus to form *cis*-dihydrodihydroxybenzenes. Toluene-grown cells of *P. putida* F1 and 3-chlorobenzoate-grown cells of *Burkholderia* sp. strain PS12 were found to monooxygenate the side chain of 2- and 3-chlorotoluene to the corresponding chlorobenzyl alcohols. Further metabolism of these products was slow, and the corresponding chlorobenzoates were usually observed as end products, whereas the 3-chlorobenzoate produced from 3-chlorotoluene in *Burkholderia* sp. strain PS12 was metabolized further. *Escherichia coli* cells containing the toluene dioxygenase genes from *P. putida* F1 oxidized 2- and 3-chlorotoluene to the corresponding chlorobenzyl alcohols as major products, demonstrating that this enzyme is responsible for the observed side chain monooxygenation. Two methyl- and chloro-substituted 1,2-dihydroxycyclohexadienes were formed as minor products from 2- and 3-chlorotoluene, whereas a chloro- and methyl-substituted cyclohexadiene was the only product formed from 4-chlorotoluene. The toluene dioxygenase of *P. putida* F1 and chlorobenzene dioxygenase from *Burkholderia* sp. strain PS12 are the first enzymes described that efficiently catalyze the oxidation of 2-chlorotoluene.

Little is known about the degradation of chlorotoluenes. Vandenberg et al. (27) briefly reported that bacteria were able to degrade some mono- and dichlorinated toluenes. However, no indications of the metabolic pathway were given. Two different metabolic routes have been described for the degradation of 4-chlorotoluene (Fig. 1). Initial dioxygenation and dehydrogenation to form 3-chloro-6-methylcatechol, followed by mineralization via the *ortho*-cleavage pathway, was shown to occur in *Pseudomonas* sp. strain JS21 (12). A pathway based on broad substrate specificity enzymes of the TOL plasmid-encoded pathway for xylene mineralization was constructed by Brinkmann and Reineke (2). Xylene oxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase of this pathway were able to transform 4-chlorotoluene to 4-chlorobenzoate, which is in turn transformed by toluate dioxygenase. Conjugal transfer of the TOL plasmid to *Pseudomonas* sp. strain B13 harboring enzymes of the modified *ortho*-cleavage pathway for the metabolism of chlorocatechol resulted in strains mineralizing 4-chlorotoluene via 4-chlorobenzoate as an intermediate. Similarly, 3-chlorotoluene was mineralized by these derivatives via 3-chlorobenzoate as an intermediate. Until now, no metabolic route for the mineralization of 2-chlorotoluene has been reported. In the case of the pathway constructed by Brinkmann and Reineke (2), this reflects the lack of transformation of 2-chlorotoluene by xylene monooxygenase, since transfer of the TOL plasmid to strain JH230 able to grow with 2-chlorobenzoate resulted in derivatives capable of 2-chlorobenzyl alcohol mineralization, thereby indicating that the ac-

tivities of benzyl alcohol and benzaldehyde dehydrogenases for the 2-chloro-substituted derivatives are high enough to catalyze effective transformation to 2-chlorobenzoate.

The 1,2,4-trichlorobenzene-degrading *Burkholderia* sp. strain PS12 was reported to mineralize not only a variety of chloro-substituted benzenes, but also 4-chloro- and some dichlorotoluenes (21). However, 2- and 3-chlorotoluene are not growth substrates. As chlorobenzenes were reported to be subject to an initial dioxygenation by chlorobenzene dioxygenase, a pathway similar to that reported by Haigler and Spain (12) can be assumed for 4-chlorotoluene metabolism by *Burkholderia* sp. strain PS12. However, the reason why this strain cannot use 2- and 3-chlorotoluene as growth substrates is not evident. In the present study, we analyzed the metabolism of 2-chloro- and 3-chlorotoluene by PS12 and *P. putida* F1, a strain which degrades toluene by a dioxygenolytic pathway (8), to determine why a dioxygenolytic pathway cannot deal with these compounds.

MATERIALS AND METHODS

Organisms and culture conditions. *Burkholderia* sp. strain PS12 (21) and *P. putida* F1 (9) have been described previously. *Escherichia coli* JM109(pDT601) and *E. coli* JM109(pDT602) containing the *todC1C2BA* and *todC1C2BAD* genes from *P. putida* F1, respectively, have been described by Zylstra and Gibson (30) and were kindly supplied by D. T. Gibson. Growth of PS12 and F1 in liquid culture was in mineral medium (5) containing 5 mM 3-chlorobenzoate or liquid toluene (corresponding to a final concentration of 5 mM) which was fed over the gas phase by evaporation from a test tube placed in the Erlenmeyer flasks. Flasks were incubated at 30°C on a rotary shaker at 150 rpm. *E. coli* strains were grown at 37°C in Luria broth medium.

Transformation of chlorotoluenes. PS12 or F1 bacteria in late exponential growth were harvested by centrifugation and resuspended to an A_{546} of 1 to 10 in 10 ml of phosphate buffer (50 mM, pH 7.4). Chlorotoluenes were added from 100 mM stock solutions in dimethyl sulfoxide to give a final concentration of 0.3 mM. Flasks were incubated at 30°C on a rotary shaker. For quantification of water-soluble compounds, cell-free supernatant fluids were directly analyzed by

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FIG. 1. Bacterial metabolism of 4-chlorotoluene via 4-chlorobenzoate (A), as described by Brinkman and Reineke (2), and via 3-chloro-6-methylcatechol (B), as described by Haigler and Spain (12).

reverse phase high-performance liquid chromatography (HPLC). Chlorotoluenes were extracted by 1 volume of chloroform, and the extract was analyzed by HPLC. For quantification of chloride elimination, additional 0.3 mM 3-chlorotoluene was added after consumption of the substrate.

Cultures of *E. coli* JM109(pDTG601), *E. coli* JM109(pDTG602), and *E. coli* JM109(pKK223-3) were supplemented with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) when they reached an A_{546} of 0.5. After 1 h, cells were harvested by centrifugation, washed with 50 mM phosphate buffer (pH 7.4), and resuspended to an A_{546} of 1 to 10 in the same buffer containing 0.3 mM chlorotoluene. Incubation and quantification of substrate and metabolites were carried out as described for PS12 and F1.

Oxygen uptake. The determination of oxygen uptake rates of washed resting cell suspensions was performed polarographically by using a Clark-type electrode as described previously (17). Protein was determined by the method of Schmidt et al. (22).

Extraction and derivatization of metabolites. For the extraction of dihydrodiol intermediates, 2 ml of cell-free supernatant was extracted with an equal volume of ethyl acetate, dried over $MgSO_4$, and evaporated under a stream of nitrogen, and the residue redissolved in 80 μ l of acetone. For the extraction of acids, the cell-free supernatant was acidified to pH 2 and subjected to extraction by ethyl acetate as described above. The dried residue was redissolved in 50 μ l of diethyl ether.

Dihydrodiol intermediates were derivatized with *n*-butylboronic acid (BBA) by the addition of 20 μ l of BBA reagent (50 mg of BBA/ml of acetone) to the acetone sample (13). The mixture was incubated for 10 min at 50°C, dried under nitrogen, and redissolved in 30 μ l of *n*-hexane. For the derivatization of benzoic acids, 100 μ l of trimethylsulfonium hydroxide reagent was added to 50 μ l of the diethyl ether sample and boiled for 10 min (3). All reactions were carried out in sample vials sealed with Teflon caps.

Analytical methods. Bioconversion of chlorotoluenes and accumulation of metabolites were determined with a Shimadzu HPLC system (LC-10AD liquid chromatograph, DGU-3A degasser, SPD-M10A diode array detector, and FCV-10AL solvent mixer) equipped with an SC125/Lichrospher 5- μ m (Bischoff, Leonberg, Germany) column. The solvent system (flow rate, 1 ml/min) contained 1 ml of H_3PO_4 (85%) and 450 ml (for characterization of metabolites), 540 ml (for quantification of metabolites), or 720 ml (for determination of chlorotoluenes) of methanol per liter of solvent. The alcohol and acid metabolites were quantified by comparison with authentic standards. Typical retention volumes (in a solvent system containing 45% of methanol) were as follows: 2-chlorobenzyl alcohol, 5.0 ml; 2-chlorobenzoate, 4.4 ml; 3-chlorobenzyl alcohol, 5.6 ml; and 3-chlorobenzoate, 10.4 ml. For analysis of metabolites, samples of culture fluid (2 to 10 μ l) were injected after cells had been removed by centrifugation. For quantification of water-insoluble chlorotoluenes, the whole reaction mixture was extracted with an equal volume of chloroform, and 5 μ l of the chloroform extract was analyzed by HPLC.

Gas chromatography-mass spectrometry (GC-MS) analyses were conducted on a Shimadzu (Kyoto, Japan) GC-17A gas chromatograph equipped with an XTI-5 column (30 m by 0.25 mm; film thickness, 0.5 μ m; Resteck, Bellefonte,

Pa.) and coupled to a QP-5000 quadrupole mass spectrometer injector. The mass spectrometer was operated in the electron impact mode at 70 eV with an ion source temperature of 320°C. Helium was used as carrier gas with a flow rate of 1 ml/min. The oven temperature was maintained at 60°C for 2 min and then increased to 130°C at a rate of 20°C/min followed by an increase to 240°C at a rate of 5°C/min and to 320°C at a rate of 10°C/min. Samples (1 μ l) were injected into the GC, operating in the splitless mode with an injector temperature of 270°C.

Chloride ion concentration was measured with an ion selective combination electrode (model 96/17; Orion Research Inc., Cambridge, Mass.).

Chemicals. BBA was obtained from Acros organics (Geel, Belgium), and trimethylsulfonium hydroxide was obtained from Macherey-Nagel (Dueren, Germany). All other chemicals were purchased from Aldrich Chemie (Steinheim, Germany), Fluka AG (Buchs, Switzerland), or Merck AG (Darmstadt, Germany).

RESULTS

Metabolism of chlorotoluenes by *Burkholderia* sp. strain PS12. Of the three monochlorotoluenes, only 4-chlorotoluene served as a growth substrate for *Burkholderia* sp. strain PS12 (21). Oxygen uptake rates by 3-chlorobenzoate-grown cells, which have been reported to be induced for the metabolism of chlorobenzenes when cultured under these conditions (21), were similar with all three chlorotoluenes (Table 1), which suggests that both 2- and 3-chlorotoluene were oxidized.

To analyze the critical steps in the metabolism of 2- and 3-chlorotoluene, they were subjected to transformation by 3-chlorobenzoate-grown cells. Two metabolites which exhibited retention behavior on HPLC identical to authentic 2-chlorobenzyl alcohol and 2-chlorobenzoate and which showed identical UV absorption spectra were observed during 2-chlorotoluene turnover. Formation of 2-chlorobenzyl alcohol occurred at a rate of 28 μ mol/(min $\times$ g of protein) (28 U/g of protein) (Fig. 2), which is about the rate of oxygen uptake with this compound. Further metabolism of the alcohol to the benzoate was at a much slower rate of 4 U/g of protein. Up to 70% of the 2-chlorotoluene added could be recovered as 2-chlorobenzyl alcohol. At the end of the experiment, up to 80% was recovered as 2-chlorobenzoate, indicating that dioxygenation of the aromatic ring occurred, if at all, only to a minor extent. Only one major metabolite, identified by its HPLC properties as 3-chlorobenzyl alcohol, was produced during transformation of 3-chlorotoluene. This alcohol was produced at the reasonably high rate of 37 U/g of protein and was further metabolized at a rate of 5 U/g of protein. Taking into account the further metabolism of the alcohol, recoveries of up to 80% indicate that dioxygenation of the aromatic ring is of minor importance. In contrast to the formation of 2-chlorobenzoate from 2-chlorotoluene, there was no accumulation of 3-chlorobenzoate from 3-chlorotoluene, because PS12 is able to grow with 3-

TABLE 1. Oxygen uptake rates with substituted benzenes by washed-cell suspensions of *P. putida* F1 and *Burkholderia* sp. strain PS12^a

Substrate	<i>P. putida</i> F1	<i>Burkholderia</i> sp. strain PS12
Toluene	890	ND ^b
2-Chlorotoluene	40	35
3-Chlorotoluene	35	25
4-Chlorotoluene	35	35
1,4-Dichlorobenzene	35	80
1,2,4-Trichlorobenzene	40	130

^a F1 was pregrown with toluene and PS12 was pregrown with 3-chlorobenzoate. Oxygen uptake rates are expressed as specific activities (micromoles of O_2 consumption per minute per gram of protein) and corrected for endogenous respiration.

^b ND, not determined.

FIG. 2. Transformation products of 2-chlorotoluene (● and ▲) and 3-chlorotoluene (■) by 3-chlorobenzoate-grown cells of PS12. Cell suspensions contained 0.3 mg of protein per ml. Substrates were added to a final concentration of 0.3 mM.

chloro- but not 2-chlorobenzoate. Approximately 490 μmol of chloride was eliminated from 600 μmol of 3-chlorotoluene, which suggests that the metabolic pathway for 3-chlorotoluene is mineralization.

Metabolism of chlorotoluenes by *P. putida* F1. *P. putida* F1 degrades toluene by a pathway similar to that for chlorobenzene degradation by PS12, i.e., dioxygenation of the aromatic ring (29) followed by dehydrogenation to form the corresponding catechol (20). The genes and enzymes of this so-called TOD pathway have been intensively characterized (7–9, 14, 30, 31). Whereas chlorocatechols formed from chlorobenzenes are subject to intradiol cleavage in PS12 (21), methylcatechol formed from toluene by F1 is subject to extradiol cleavage (30). In order to investigate whether side chain oxidation of 2- and 3-chlorotoluene is also a property of enzymes of the TOD pathway, metabolism of the three chlorotoluenes by *P. putida* F1 was analyzed. As previously reported (10), 4-chlorotoluene was metabolized by toluene-grown cells to one dead-end product. GC-MS data are consistent with the reported structure 3-chloro-6-methylcatechol. The spectrum of the butylboronate reveals a molecular ion at m/z 224 and major fragments due to the loss of $-\text{C}_4\text{H}_8$ (m/z , 168) and the subsequent loss of chloride (m/z , 133). No further metabolism of this compound was observed. Metabolism of 2-chlorotoluene was similar to that observed with PS12 with fast transformation to the corresponding alcohol, followed by slow oxidation to the benzoate. Similarly, 3-chlorotoluene was converted via 3-chlorobenzyl alcohol to 3-chlorobenzoate but not further, in contrast to the situation with PS12. Mass spectra of the final metabolites formed from 2- and 3-chlorotoluene confirmed their identity with 2- and 3-chlorobenzoate. The spectra of the methyl esters reveals molecular ions at m/z 170 and major fragments due to the loss of $-\text{OCH}_3$ (m/z , 139) and $-\text{COOCH}_3$ (m/z , 111). Accumulation of 2- and 3-chlorobenzoate (up to ca. 80% of the applied chlorotoluene) was nearly quantitative, again indicating dioxygenation of the aromatic ring to be of minor importance. However, the appearance of yellow coloration in the flasks amended with 3-chlorotoluene indicates that a small part of the substrate actually is dioxygenated (see below) and the resulting catechols are subjected to *meta* ring-cleavage.

Metabolism of chlorotoluenes by *E. coli* JM109(pDTG601) and JM109(pDTG602). The oxidation of the methyl group of 2- and 3-chlorotoluene by PS12 and F1 leads to the assumption that chlorobenzene and toluene dioxygenase are responsible for this reaction. This was tested by using *E. coli* JM109 strains containing the genes for the toluene dioxygenase (*todC1C2BA* [pDTG601]) or the dioxygenase plus the dehydrogenase (*todC1C2BAD* [pDTG601]) (30) in the expression vector pKK223-3 under the control of the *tac* promoter. Incubation of IPTG-induced cells with 2-, 3-, and 4-chlorotoluene (300 μM each) resulted in the formation of up to 230 and 240 μM 2- and 3-chlorobenzyl alcohol from 2- and 3-chlorotoluene at rates (10 to 20 U/g of protein) similar to those observed with the parent strain with no further metabolism of the benzyl alcohols. 4-Chlorotoluene was converted to 1-chloro-2,3-dihydro-2,3-dihydroxy-4-methylbenzene by *E. coli* JM109(pDTG601) (see Fig. 3A) and to 3-chloro-6-methylcatechol by JM109(pDTG602) (data not shown) as evidenced by GC-MS analysis.

GC-MS analysis of the supernatants after transformation of 2- and 3-chlorotoluene by *E. coli* JM109(pDTG601) showed monooxygenation not to be the only reaction. In each case, the accumulation of minor quantities of two chloro- and methyl-substituted 2,3-dihydro-2,3-dihydroxybenzenes was observed.

All five dihydrodiols (Fig. 3) showed the expected fragmentation pattern of butylboronate derivatives with a molecular ion at m/z 226. The major fragments observed are formed by the loss of CH_3 (m/z , 211); $-\text{Cl}$ (m/z , 191); $-\text{C}_4\text{H}_9$ (m/z , 166); $-\text{OBC}_4\text{H}_9$ (m/z , 142); $-\text{Cl}$, $-\text{C}_4\text{H}_9$ (m/z , 135); $-\text{OBC}_4\text{H}_9$, $-\text{CO}$ (m/z , 114); $-\text{OBC}_4\text{H}_9$, $-\text{Cl}$ (m/z , 107); and $-\text{OBC}_4\text{H}_9$, $-\text{CO}$, $-\text{Cl}$ (m/z , 79).

DISCUSSION

The initial reaction in chlorobenzene degradation is usually catalyzed by a dioxygenase and yields the corresponding *cis*-dihydrodihydroxybenzene as the product (4, 11, 15, 16, 21, 23, 24, 27a), which is then dehydrogenated to the corresponding catechol. Chlorocatechols in turn are transformed by enzymes

FIG. 3. Mass spectra (m/z) of dihydrodihydroxybenzenes (as their butylboronates) formed from 2-chlorotoluene (A, B), 3-chlorotoluene (C, D), and 4-chlorotoluene (E) by IPTG-induced cells of *E. coli* JM109(pDTG601). Three different dihydrodihydroxybenzenes can theoretically be formed from 2-chlorotoluene, whereas two dihydrodihydroxybenzenes can be formed from 3-chlorotoluene. The final assignment to the different structures as shown in panels A to D is therefore tentative. The formation of 3,4-dihydro-3,4-dihydroxy-5-chlorotoluene from 2-chlorotoluene was regarded to be improbable as dioxygenation usually occurs at the 2,3 positions regarding chloro or methyl substituents (10).

of the chlorocatechol pathway to Krebs cycle intermediates by strains mineralizing chlorobenzenes. Similar reactions of dioxygenation and dehydrogenation were reported for the degradation of toluene by *P. putida* F1 (9) and of benzene by *P. putida* ML2 (26). By analogy, chlorobenzene dioxygenase of PS12 and toluene dioxygenase of F1 would be expected to

produce dihydrodiols from 2-, 3-, and 4-chlorotoluene. Whereas 4-chlorotoluene was in fact subject to dioxygenation by toluene dioxygenase, monooxygenations of the side chain to produce the corresponding benzyl alcohols were the major reactions with 2- and 3-chlorotoluene (Fig. 4). Since these benzyl alcohols are also formed by PS12 induced for chlorobenzene metabolism, it is clear that chlorobenzene dioxygenase from this organism is also able to catalyze monooxygenation. The dioxygenase genes of PS12 have now been cloned, and chlorotoluene transformation by their products is currently being analyzed.

A number of studies have shown that toluene dioxygenase of F1 catalyzes not only dioxygenation but also the benzylic monooxygenation of indan (1, 28), indanone (18), and phenols (25) and the oxidation of methyl substituents of nitrotoluenes (19). Interestingly, 2- and 3-nitrotoluene were subject to mono-

FIG. 4. Proposed transformation of 2- and 3-chlorotoluene by toluene and chlorobenzene dioxygenase. The formed benzyl alcohols are slowly transformed further by *P. putida* F1 and *Burkholderia* sp. strain PS12.

oxygenation, whereas 4-nitrotoluene was dioxygenated. The same pattern was observed here with chlorotoluenes, indicating mono- versus dioxygenation to be dependent on regio-chemistry.

It is perhaps worth mentioning here that toluene and chlorobenzene dioxygenases are distinct from xylene monooxygenase in their monooxygenation capabilities, since xylene monooxygenase is able to transform 3- and 4- but not 2-chlorotoluene (2), and hence they are particularly interesting for the construction of chlorotoluene-degrading bacteria.

Whereas *E. coli* cells containing cloned TOD genes did not transform the benzyl alcohols further, oxidation to the corresponding benzoates was slow by F1 and PS12. These activities can be attributed to low specificity dehydrogenases or, in the case of F1, to dehydrogenases of the cymene pathway (6). Given the reasonably high activity of chlorobenzene dioxygenase for 2- and 3-chlorotoluene and the fact that 3-chlorobenzoate is a growth substrate for PS12 (21), the only pathway barriers preventing growth with 3-chlorotoluene seem to be the dehydrogenase activities.

Whereas 3- and 4-chlorotoluene can be degraded by spontaneous mutants of wild-type strains (12) or constructed microorganisms (2), no organisms able to degrade 2-chlorotoluene have so far been described. To our knowledge this is the first report of an effective 2-chlorotoluene-transforming activity. Since an organism able to mineralize 2-chlorobenzyl alcohol is available (2), this opens up the possibility of designing

organisms by the assemblage of different catabolic segments to mineralize this recalcitrant compound.

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