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-dihydrodiolxybenzenes. Toluene-grown cells of *P. putida* F1 and 3-chlorobenzoate-grown cells of *Burkholderia* sp. strain PS12 were found to transform the toluene side chain to 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 tolucne 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. Vandenbergh 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-methylcyclohexadienol, 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 monoxygenase, 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 activities 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 *P. putida* F1 and *Burkholderia* sp. strain PS12, 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 toIC/C2BA and toIC/C2BAD genes from *P. putida* F1, respectively, have been described by Zystra 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 A540 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
reverse phase high-performance liquid chromatography (HPLC). Chlorotolu- enes were extracted by 1 volume of chloroform, and the extract was analyzed by HPLC. For quantification of chloride elimination, additional 0.3 mM 3-chloro- toluene was added after consumption of the substrate.

Cultures of *E. coli* JM109(pDTG601), *E. coli* JM109(pDTG602), and *E. coli* JM109(pKK23-3) were supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when they reached an *A*₅₆₀ 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*₅₆₀ 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. (12).

![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).](http://aem.asm.org/)

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

<table>
<thead>
<tr>
<th>Substrate</th>
<th><em>P. putida</em> F1</th>
<th><em>Burkholderia</em> sp. strain PS12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>890</td>
<td>ND</td>
</tr>
<tr>
<td>2-Chlorotoluene</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>3-Chlorotoluene</td>
<td>35</td>
<td>25</td>
</tr>
<tr>
<td>4-Chlorotoluene</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>1,4-Dichlorobenzene</td>
<td>35</td>
<td>80</td>
</tr>
<tr>
<td>1,2,4-Trichlorobenzene</td>
<td>40</td>
<td>130</td>
</tr>
</tbody>
</table>

F1 was pregrown with toluene and PS12 was pregrown with 3-chlorobenzoate. Oxygen uptake rates are expressed as specific activities (micromoles of O₂ consumption per minute per gram of protein) and corrected for endogenous respiration.

ND, not determined.
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 –C₆H₄ (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 from 2- and 3-chlorotoluene (300 mol of 3-chlorotoluene, m/z 226. The major fragments observed are formed by the loss of CH₃ (m/z, 211), –Cl (m/z, 191), –C₆H₅ (m/z, 166), –OBC₄H₉ (m/z, 142); –Cl, –C₆H₅ (m/z, 135); –OBC₄H₉, –CO (m/z, 114); –OBC₄H₉, –Cl (m/z, 107), and –OBC₄H₉, –CO, –Cl (m/z, 79).

DISCUSSION

The initial reaction in chlorobenzene degradation is usually catalyzed by a dioxygenase and yields the corresponding cis-dihydrodihydrobenzene 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...
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-
oxygenation, whereas 4-nitrotoluene was dioxygenated. The same pattern was observed here with chlorotoluenes, indicating mono- versus dioxygenation to be dependent on regiochemistry.

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-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.

ACKNOWLEDGMENT
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REFERENCES
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