Degradation of Fluorobenzene by *Rhizobiales* Strain F11 via *ortho* Cleavage of 4-Fluorocatechol and Catechol

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Received 19 May 2006/Accepted 5 September 2006

The aerobic metabolism of fluorobenzene by *Rhizobiales* sp. strain F11 was investigated. Liquid chromatography-mass spectrometry analysis showed that 4-fluorocatechol and catechol were formed as intermediates during fluorobenzene degradation by cell suspensions. Both these compounds, unlike 3-fluorocatechol, supported growth and oxygen uptake. Cells grown on fluorobenzene contained enzymes for the *ortho* pathway but not for *meta* ring cleavage of catechols. The results suggest that fluorobenzene is predominantly degraded via 4-fluorocatechol with subsequent *ortho* cleavage and also partially via catechol.

During the last decades, environmental contamination by fluorinated organic compounds has received increasing attention because of their use as herbicides, fungicides, surfactants, refrigerants, intermediates in organic synthesis, solvents, and pharmaceuticals (11). Whereas the biodegradation of chlorinated compounds has been studied quite extensively (19), little is known about the bacterial metabolism of fluororomatic compounds, even though there have been several reports on the degradation of fluorobenzoic acids (5, 6, 7, 16). With chloroaromatics, most degradation routes involve dioxygenase- and dehydrogenase-mediated conversion to the corresponding chlorocatechols, which are further metabolized by a dioxygenase that cleaves the aromatic ring. Dehalogenation occurs during metabolism of the ring-cleavage products (19). Most described strains degrade chlorocatechols via the *ortho*-cleavage pathway (14, 18, 19, 20), but *meta* cleavage of 3-chlorocatechol can also occur (13), even though the *meta*-cleavage route is often unproductive due to the formation of toxic or dead-end products (1, 19). Dehalogenation may in some cases occur prior to ring cleavage. For example, mutants of *Pseudomonas* sp. strain B13 and *Alcaligenes eutrophus* B9 that grow on 2-fluorobenzoate use a dioxygenase to convert it to catechol, with concomitant decarboxylation and defluorination (5). *Pseudomonas putida* strain CLB 250, which can use three different 2-halobenzoates, also converts these substrates by initial dehalogenating dioxygenation (6), and a defluorinating 4-fluorobenzoate monoxygenase has been reported as well (16).

The present paper describes a metabolic pathway for fluorobenzene (FB). Information about the bacterial metabolism of this compound is scarce, despite studies on its chlorinated analogue (13, 18). Lynch et al. (12) described the oxidation of FB to 3-fluorocatechol by a strain of *Pseudomonas putida*, but in this study FB was not used as a carbon source. Recently, FB was reported to be completely degraded by a bacterial consortium (2) and by a pure bacterial culture that utilized it as a sole carbon and energy source (3). This gram-negative bacterium, phylogenetically classified within the order *Rhizobiales*, was named strain F11 and was used here to investigate the metabolism of FB.

**Intermediates produced during FB degradation.** In order to obtain information about the degradation pathway of FB, we tested which intermediates accumulated upon incubation of concentrated cell suspensions of strain F11 with FB. First, cells were grown in sealed flasks on FB in mineral medium as described previously (3), harvested by centrifugation (10,000 × g for 15 min at 4°C), washed twice with mineral medium, and resuspended in the same medium to give an optical density at 600 nm of 0.3. Glucose (1 mM) was added, since it was found in preliminary experiments that this enhanced degradation of FB and stimulated accumulation of intermediates. The suspensions were incubated in closed flasks with FB, and samples were taken at appropriate times, centrifuged, and subjected to high-performance liquid chromatography (HPLC), gas chromatography, and fluoride measurements. Fluoride was measured with a Dionex Dx-120 ion chromatograph equipped with an AllSep A-2 anion column from Alltech, and the eluent was a mixture of NaHCO₃ and Na₂CO₃ in deionized water. For FB analysis, culture samples were extracted with diethylether and analyzed by gas chromatography as described previously (3). It was observed that whole cells of strain F11 completely removed 1.1 mM FB in 13 h, but stoichiometric fluoride release was seen only after 29 h (Fig. 1). This indicates that fluorinated intermediates did temporarily accumulate but that there was no formation of high levels of fluorinated dead-end metabolites.

Samples (20 μl) from the same culture fluid were also analyzed by HPLC and liquid chromatography-mass spectrometry (LC-MS). HPLC analysis was carried out on a Lichrospher 100 RP8 reversed-phase column in connection with Jasco PU-980 pumps, a Jasco MD-910 diode array detector, and a Jasco UV-2075 detector. Compounds were isocratically eluted at a...
flow rate of 1 ml/min with a solution of water-acetonitrile (80:20) and 10 mM formic acid. LC-MS was carried out with a Micromass ZMD detector equipped with a Xterra MS, a SymmetryShield C8 column (4.6 mm by 150 mm), a 996 photodiode array detector, and a 2690 separations module, all from Waters. While degradation proceeded, five metabolites (compounds I to V) appeared in the culture medium (Table 1). Four of these (compounds I, II, IV, and V) were completely consumed during prolonged incubation. One minor metabolite (compound III) remained in the culture supernatant even after incubation for 48 h.

Metabolite I, which appeared early, coeluted with catechol and had a molecular mass (negative-mode MS) of m/z = 109 (M-H⁻). Metabolite II was identified as 4-fluorocatechol on the basis of cochromatography with a standard in HPLC analysis and its negative-mode mass spectrum with m/z = 126.91 (M-H⁻). The dead-end metabolite III was identified as cis-dienelactone by cochromatography and mass spectrometry (positive ionization, m/z = 140.04 [M+H⁺]). Metabolites IV and V could not be identified, since no ionization was obtained with LC-MS.

The occurrence of the two catechols during the initial 20 h of FB degradation (Fig. 1) suggests that strain F11 converts FB partially to catechol and partially to 4-fluorocatechol during the first metabolic step. Approximately 0.6 mM of FB that was converted transiently appeared as 4-fluorocatechol, and about 0.3 mM was detected as catechol. This is in agreement with the observation that significant fluoride release is taking place already during the initial period of FB degradation, i.e., when catechol is formed but that fluoride release is only complete when the intermediate 4-fluorocatechol and possibly other fluorinated metabolites have been degraded. The formation of both catechol and 4-fluorocatechol from FB is also consistent with the ability of strain F11 to grow on both of these catechols. The fact that catechol remained in the medium for quite a long time even though it is a better growth substrate than 4-fluorocatechol suggests inhibition of the catechol pathway by the presence of 4-fluorocatechol.

Substrate-dependent oxygen consumption by whole cells. In order to test the inducibility of FB transformation activity, oxygen uptake measurements were done. Cells of strain F11 were grown on FB, benzene, or citrate, harvested by centrifugation, washed, resuspended to a density of 0.43 mg of cellular protein per ml, and transferred to a stirred vessel that was equipped with a fiber optic oxygen sensor (MOPS-1; ProSense BV, Hanover, Germany). The rate of O₂ consumption was measured at room temperature in the presence of different substrates (Table 2).

Cells grown on FB oxidized fluorobenzene and cis-1,2-dihydrobenzenediol as well as 4-fluorocatechol and were also highly induced for the oxidation of catechol. Rapid oxidation of catechol, 4-fluorocatechol, and cis-1,2-dihydrobenzenediol was also obtained with cells that were pregrown on benzene. With fluorobenzene and benzene, these cells showed even higher oxygen uptake rates than cells grown on FB. With cells grown on citrate, the aromatic substrates were not oxidized, suggesting that the formation of the first catabolic enzyme was induced during growth on the aromatic substrates and repressed on citrate. The oxygen uptake rates with 3-fluorocatechol were very low, independent of whether the cells were grown on FB, benzene, or citrate. The patterns of oxygen consumption were measured with an oxygen sensor as described in Materials and Methods. All substrates were used at a concentration of 1 mM. Results represent the means of the results of at least three independently performed experiments. Oxygen uptake experiment results are corrected for endogenous respiration; replicates showed less than 10% variation.
TABLE 3. Enzyme activities in cell extracts of strain F11
grown on FB

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay substrate</th>
<th>Sp actb (U - mg of protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol 1,2-dioxygenase</td>
<td>Catechol</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>3-Fluorocatechol</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>4-Fluorocatechol</td>
<td>0.16</td>
</tr>
<tr>
<td>Catechol 2,3-dioxygenase</td>
<td>Catechol</td>
<td>0.002</td>
</tr>
<tr>
<td>Muconate cycloisomerase</td>
<td>cis,cis-Muconic acid</td>
<td>0.200</td>
</tr>
<tr>
<td>Diene lactone hydrolase</td>
<td>cis-Diene lactone</td>
<td>0.002</td>
</tr>
<tr>
<td>Maleylacetate reductase</td>
<td>Maleylacetate</td>
<td>0.78</td>
</tr>
<tr>
<td>3-Oxoadipate:succinyl-CoA</td>
<td>3-Oxoadipic acid</td>
<td>0.12</td>
</tr>
</tbody>
</table>

activity. Conversion of the other product, 1-fluoro-
metal cycloisomerization (23), although it is risky to generalize
such a finding to other organisms. Furthermore, in mutants of
Alcaligenes eutrophus B9 and Pseudomonas sp. strain B13 that
use 2-fluorobenzoate for growth, the formation of toxic 3-fluo-
rocatechol is prevented by loss of dihydrodihydroxybenzoate
dehydrogenase activity, allowing growth on catechol that can
be formed by initial dioxygenation of the aromatic ring (5). The
absence of a 3-haloacetate intermediate clearly distinguishes
the fluorobenzene pathway of strain F11 from chlorobenzene
catabolic pathways, which proceed via 3-chlorocatechol (13, 17,
18). This could explain the lack of growth of strain F11 on
chlorobenzene.

Pathway of FB degradation. We propose the pathway for FB
metabolism shown in Fig. 2. The initial attack of FB by dioxy-
genase activity yields two different fluorinated dihydrodilols.
The 4-fluoro-cis-benzene-1,2-dihydrodilol that is produced is
transformed into 4-fluorocatechol by a dihydrodilol dehydro-
genase. Conversion of the other product, 1-fluoro-cis-benzene-
1,2-dihydrodilol, to catechol can proceed without involvement
of dehydrogenase that reduces a cofactor, since the electrons
are transferred to the fluoride that is being released (Fig. 2).
Simultaneous conversion of a fluorinated compound to cate-
chol and a fluorinated catechol was described earlier for the
degradation of 2-fluorobenzoate by Pseudomonas sp. strain B13 and
strain FLB300 (5, 7).

The capability of strain F11 cells to use 4-fluorocatechol as
a growth substrate, its transient accumulation in cell suspen-
sions to higher levels than catechol, and the fact that 4-fluo-
rocatechol stimulated oxygen uptake by whole cells lead to the
conclusion that 4-fluorocatechol is the predominant interme-
diate. The occurrence of 4-fluorocatechol as an intermediate
has also been described for the aforementioned α-proteobac-
terium strain FLB300, which degrades both benzoate and
all monofluorosubstituted benzoates (7). The metabolism of
4-fluorocatechol is proposed to proceed through ortho cleav-
age by a (fluoro)catechol 1,2-dioxygenase that yields 3-fluoro-
cis,cis-muconate. This product could be transformed with
concomitant defluorination into maleylacetate via either 4-flu-
oro muconolactone or another lactone derivative. ortho cleav-

consumption show that oxidation of catechol, 4-fluorocatechol,
and cis-1,2-dihydrobenzenediol was induced by FB and benz-
ene, whereas 3-fluorocatechol was never oxidized. When batch
cultures of strain F11 growing with 1 mM of FB were
supplemented with 0.1 mM of 3-fluorocatechol, FB was no
longer converted by the cells, and fluoride, measured with a
fluoride-selective electrode (2), was not released.

Enzymes involved in the degradation of FB. To investigate
whether degradation of FB proceeds via meta or ortho cleav-
age, the presence of several enzymes involved in these routes
was tested (Table 3). Late exponential FB-grown cells were
harvested by centrifugation, washed twice with 0.1 M Tris-HCl
buffer (pH 7.5) containing 0.1 mM 1,4-dithiothreitol, and dis-
rupted by sonication in the same buffer. After centrifugation
(90,000 × g for 60 min at 4°C) the clear supernatant was used
as the cell extract for enzyme assays. Its protein content was
determined with Coomassie brilliant blue using bovine serum
albumin as the standard.

Catechol 2,3-dioxygenase was measured by determining the
formation of 2-hydroxymuconic semialdehyde (ε = 44,000
M⁻¹ cm⁻¹) at 375 nm, according to the method of Nozaki (15).
(Fluoro) catechol 1,2-dioxygenase activity was measured simi-
larly, as described by Dorn and Knackmuss (4) (ε₃,₁₂-muconate
= 16,800 M⁻¹, ε₂,₃-fluoro-cis,cis-muconate = 14,900 M⁻¹ cm⁻¹, ε₃,
fluoro-cis,cis-muconate = 14,900 M⁻¹ cm⁻¹). Muconate cyclo-
somerase activity was measured by following the consump-
tion of cis,cis-muconate in an assay mixture containing 30 mM
Tris-HCl (pH 8.0), 1 mM MnCl₂, and 0.1 mM cis,cis-muconate.
Diene lactone hydroxylation activity was determined by following
at 280 nm (ε = 17,000 M⁻¹ cm⁻¹) the decrease in the level of 0.1
mM cis-diene lactone that was incubated with enzyme in 10
mM histidine-HCl (pH 6.5). Maleylacetate was prepared on the
do of its use by alkaline hydrolysis of cis-diene lactone (8),
and the reductase was measured by following maleylacetate
(0.1 mM)-dependent NADH (0.2 mM) oxidation at 340 nm in
50 mM Tris-HCl (pH 7.5). Activities were corrected for sub-
strate-independent NADH oxidation. 3-Oxoadipate:succinyl-
coenzyme A transferase was measured as described by Mars et
al. (13). One unity of activity was defined as the amount of
enzyme required to convert 1 μmol of substrate per min at
25°C.

Activities of the ortho pathway enzymes catechol 1,2-dioxy-
genase, muconate cycloisomerase, maleylacetate reductase,
and 3-oxoadipate:succinyl-coenzyme A transferase were found
in cell extracts of strain F11 grown on FB. Catechol 2,3-dioxy-
genase activity was not detected, indicating that strain F11
does not use a meta-cleavage pathway to degrade FB. A 1,2-
dioxygenase activity was detected with both catechol and
4-fluorocatechol but was hardly detected with 3-fluorocatechol.
Instead, the catechol 1,2-dioxygenase activity was reduced by
70% and 90% in the presence of 0.1 and 0.5 mM 3-fluorocate-
chol, respectively. A muconate cycloisomerase activity with
respect to cis,cis-muconic acid was also detected. No activity
was found for cis-diene lactone hydroxylation in extracts of strain
F11. These observed enzyme activities suggest that the cate-
chols in the FB degradation pathway undergo ortho cleavage.

We judge it highly unlikely that 3-fluorocatechol is an inter-
mediate in FB degradation pathway, since it was not used as a
growth substrate and it strongly inhibited FB degradation and
growth and since no dioxygenase activity with 3-fluorocatechol
was detected in cell extracts of strain F11 grown on FB. This
conclusion is in line with the previously described resistance of
the expected product 2-fluoro-cis,cis-muconic acid to enzy-
matic cycloisomerization (23), although it is risky to generalize
such a finding to other organisms. Furthermore, in mutants of
Alcaligenes eutrophus B9 and Pseudomonas sp. strain B13 that
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FIG. 2. Proposed pathway for fluorobenzene metabolism by strain F11. The enzyme activities are denoted as follows: 1, fluorobenzene dioxygenase; 2, fluorobenzene dihydrodiol dehydrogenase; 3, fluorocatechol 1,2-dioxygenase; 4, fluoromuconate cycloisomerase; 5 and 6, catechol 1,2-dioxygenase; 2, fluorobenzene dihydrodiol dehydrogenase; 3, fluoromuconate cycloisomerase; 4, fluoromuconate cycloisomerase; 5 and 6, fluorobenzene dioxygenase; 7, fluorobenzene dioxygenase.

Maleylacetate can be channeled into the tricarboxylic acid cycle via 3-oxoadipate. Catechol, the minor product of the initial dioxygenation reaction, is proposed to be metabolized to cis,cis-muconate, converted to the lactone derivative, and then also channeled into the 3-oxoadipate route (Fig. 2).

M.F.C. acknowledges a research grant from Fundação para a Ciência e Tecnologia (FCT), Portugal (BD/21839/99), and Fundo Social Europeu (III Quadro Comunitário de Apoio). This work was supported in part by the European Community’s Human Potential Programme under contract HPRN-CT-2002-00213 [BIOSAP] and by a grant from the European Science Foundation.

We thank Paolo De Marco for help in the preparation of cell extracts and Filip Kaminski for valuable discussions and a kind gift of protoanemonin. Both 4-fluorocatechol and cis,cis-muconate (activity 5) or by slow spontaneous conversion (activity 6); 7, fluorobenzene dioxygenase; 8, maleylacetate reductase; 9, fluorobenzene dioxygenase; 10, nonenzymatic defluorination; 11, catechol 1,2-dioxygenase; 12, muconate cycloisomerase; 13, muconolactone isomerase; 14, 3-oxoadipate enol-lactone hydrolase.

REFERENCES