# Identification of Fluoropyrogallols as New Intermediates in Biotransformation of Monofluorophenols in *Rhodococcus opacus* 1cp

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The transformation of monofluorophenols by whole cells of *Rhodococcus opacus* 1cp was investigated, with special emphasis on the nature of hydroxylated intermediates formed. Thin-layer chromatography, mass spectrum analysis, and <sup>19</sup>F nuclear magnetic resonance demonstrated the formation of fluorocatechol and trihydroxyfluorobenzene derivatives from each of three monofluorophenols. The <sup>19</sup>F chemical shifts and proton-coupled splitting patterns of the fluorine resonances of the trihydroxyfluorobenzene products established that the trihydroxylated aromatic metabolites contained hydroxyl substituents on three adjacent carbon atoms. Thus, formation of 1,2,3-trihydroxy-4-fluorobenzene (4-fluoropyrogallol) from 2-fluorophenol and formation of 1,2,3-trihydroxy-5-fluorobenzene (5-fluoropyrogallol) from 3-fluorophenol and 4-fluorophenol were observed. These results indicate the involvement of fluoropyrogallols as previously unidentified metabolites in the biotransformation of monofluorophenols in *R. opacus* 1cp.

Halophenols and their derivatives are priority pollutants of mainly anthropogenic origin. Over several decades, these compounds have been widely used as building blocks in chemical and pharmaceutical syntheses and as herbicides and pesticides, and they have caused serious local contamination of the environment. Soil microorganisms have developed the capacity of utilizing halophenols for their growth by a diverse set of biodegradation pathways (8). Aerobic soil microorganisms generally degrade mono- and dihalophenols through the initial action of (chloro)phenol ortho-hydroxylases, leading to the formation of halocatechols (1, 7, 9, 10, 12). In the framework of a project devoted to the biodegradation of halophenols by gram-positive bacteria, we investigated the formation of hydroxylated intermediates formed upon the conversion of halophenols by various Rhodococcus species and previously demonstrated the formation of (halo)catechols as initial intermediates in the biodegradation pathways (3). However, identification of the subsequent biodegradation pathways of the chlorocatechols appeared hampered by the fact that unequivocal identification of the site of introduction of a third hydroxyl group is difficult because <sup>1</sup>H nuclear magnetic resonance (NMR) splitting patterns combined with <sup>1</sup>H chemical shift data of the protons present in these metabolites can be compatible with more than one substitution pattern (13). Therefore, in this paper, we have studied the possible formation of trihydroxyfluorobenzene metabolites from fluorophenols by whole cells of Rhodococcus opacus 1cp in detail. The fluorine substituent provides the possibility to detect and quantify the possible hydroxyfluorobenzene intermediates by <sup>19</sup>F NMR, allowing the identification of the exact substitution pattern. Using this technique we unambiguously demonstrate the formation of fluoropyrogallols (1,2,3-trihydroxyfluorobenzenes) as new intermediates in the biotransformation of monofluorophenols by *R. opacus* 1cp.

### MATERIALS AND METHODS

**Chemicals.** Phenol was purchased from Merck (Darmstadt, Germany). 2-Fluorophenol, 3-fluorophenol, and 4-fluorophenol were purchased from Janssen Chimica (Beerse, Belgium). Fluorocatechols were prepared from the corresponding fluorophenols using purified phenol hydroxylase from *Trichosporon cutaneum* (14). Fluoromuconates were prepared and identified as described previously (2) by incubating the fluorocatechols with catechol 1,2-dioxygenase from *Pseudomonas arvilla* C-1.

**Growth of** *R. opacus* **1cp.** The strain *R. opacus* **1cp** was isolated and maintained as described previously (6). The strain can grow on phenol as the sole source of carbon. For cultivation, a mineral synthetic medium containing, per liter, 1 g of NH<sub>4</sub>NO<sub>3</sub>, 1 g of K<sub>2</sub>HPO<sub>4</sub>, 1 g of KH<sub>2</sub>PO<sub>4</sub>, 0.2 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.02 g of CaCl<sub>2</sub>, and 2 drops of a saturated solution of FeCl<sub>3</sub> (pH 7.2) was used. Phenol was used as the source of carbon and was initially added at a 200-mg/liter final concentration. *R. opacus* **1cp** did not grow on either of the three monofluorophenols as the sole source of carbon, and, therefore, fluorophenols were added as inducers in addition to phenol. After 6 h an additional portion of phenol (200 mg/liter) was added together with 2 mg of the fluorophenol to be tested/liter. Cultures were incubated at 28°C on an orbital shaker (2,000 rpm). After 20 to 22 h cell density was 0.24 to 0.36 at 540 nm. After 20 to 22 h of cultivation the cells were harvested by centrifugation (20 min, 5,000 × g) and washed twice in potassium phosphate, pH 7.0.

**Incubation conditions.** Measurements using cell extracts of *R. opacus* 1cp appeared to be hampered by the fact that the phenol hydroxylases from the *Rhodococcus* species appear to be highly labile (4, 11, 15, 18, 23). Thus, bio-transformation of the fluorophenols was investigated in incubations with whole cells. To test the biotransformation of fluorophenols, fresh, washed *R. opacus* 1cp cells, harvested from five to seven flasks with 200 ml of culture liquids, were resuspended in mineral medium without phenol (optical density, 2 to 3 at 540 nm). To 20 ml of this cell suspension the fluorophenol under investigation was added to a final concentration of 1 mM, and the cultures were incubated at 28°C on an orbital shaker. Each experiment was conducted in triplicate, and control experiments were performed with medium without cells or with medium without the corresponding phenol. To monitor the conversion of substrates, every 0.5 h the metabolite profile in one of the flasks was analyzed. To this end, the incubation mixture was acidified with 1 N HCl to pH 2.0, followed by extraction with

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Substrate	Intermediate	Bruto formula (mass)	Main characteristic peaks in mass spectrum, m/z (%)	
2-Fluorophenol	3-Fluorocatechol	C <sub>6</sub> H <sub>5</sub> O <sub>2</sub> F (128.0273)	M <sup>+</sup> 128(100), 110(3.3), 108(9.2), 99(8.0), 82(19.2) 81(39.4), 52(57.8), 51(36.1)	
2-Fluorophenol	1,2,3-Trihydroxy-4-fluorobenzene	$C_6H_5O_3F$ (144.0223)	M <sup>+</sup> 144(12.6), 126(100), 108(26.4), 97(9.7), 80(31.9), 79(10.5), 52(43.9), 51(23.5)	
3-Fluorophenol 4-Fluorophenol	4-Fluorocatechol	$C_6H_5O_2F$ (128.0274)	M <sup>+</sup> 128(100), 110(6.5), 99(16.4), 82(47.9), 81(13.2), 72(6.2), 63(11.3), 51(23.2)	
3-Fluorophenol 4-Fluorophenol	1,2,3-Trihydroxy-5-fluorobenzene	C <sub>6</sub> H <sub>5</sub> O <sub>3</sub> F (144.0223)	M <sup>+</sup> 144(100), 128(1.3), 126(9.2), 115(13.0), 98(37.7), 97(11.8), 70(45.6), 69(15.2)	

TABLE 1. MS analysis of the intermediates of monofluorophenol degradation by *R. opacus* 1cp

evaporation under vacuum, and the samples thus obtained were analyzed by thin-layer chromatography (TLC), mass spectrometry (MS), and/or  $^{19}{\rm F}$  NMR.

TLC, HPLC, and MS analysis. Qualitative analysis of intermediates was performed by TLC on DC-AlufolienKieselgel 60 F254 plates (Merck) developed with benzene-dioxane-acetic acid (90:10:2). After the processing of the plates, metabolites were detected under UV light, with diazotized benzidine and AgNO<sub>3</sub> in acetone solution. After visualization, compounds with corresponding  $R_f$  values were eluted from the plates with methanol. Methanol extracts were evaporated, and the purity of the compounds was checked by TLC. Pure compounds were analyzed on a Finnigan MAT 8430 mass spectrometer operated at an ionization energy of 70 eV with direct evaporation of samples. For high-pressure liquid chromatography (HPLC) analysis the culture liquid was acidified to pH 2.0 and extracted three times with ethyl acetate. HPLC analysis was conducted using a reversed-phase column (4.0 by 250 mm; Spherisorb ODS-2,2134 LKB). Elution was carried out isocratically at a flow rate of 0.8 ml min<sup>-1</sup> with 1.5 mK KH<sub>2</sub>PO<sub>4</sub> containing 30% methanol. Detection was at 280 nm. Metabolite peaks were identified and quantified in comparison with standard compounds.

<sup>19</sup>F NMR measurements. <sup>19</sup>F NMR measurements were performed on Bruker AMX 300 and Bruker DPX 400 NMR spectrometers as described previously (2, 2000) (2 21). The temperature was 7°C unless indicated otherwise. A dedicated 10-mm <sup>19</sup>F NMR probe head was used for both instruments. The spectral width for the  $^{19}\text{F}$  NMR measurements was between 20,000 and 50,000 Hz depending on the sample of interest. The number of data points used for data acquisition was 65,536. Pulse angles of 30° were used. Between 2,000 and 60,000 scans were recorded, depending on the concentrations of the fluorine-containing compounds and the signal-to-noise ratio required. The detection limit of an overnight run (60,000 scans) was 1 µM. The sample volume was 1.74 ml, containing 20 µl of the concentrated ethyl acetate extract as the sample, 1.6 ml of 0.1 M potassium phosphate (pH 7.6), 100 µl of <sup>2</sup>H<sub>2</sub>O, used as the deuterium lock, and 20 µl of 8.4 mM 4-fluorobenzoate added as an internal standard. Concentrations of the various metabolites were calculated by comparison of the integrals of the 19F NMR resonances of the metabolites to the integral of the 4-fluorobenzoate resonance. Chemical shifts are reported relative to that for CFCl3. <sup>19</sup>F NMR chemical shift values for the various fluorine-containing compounds were identified using authentic reference compounds for fluoride anions and all fluorophenols. The resonances of the different fluorocatechol and fluoromuconate metabolites have been identified and reported previously (2, 14). <sup>1</sup>H decoupling was achieved with a Waltz16 decoupling sequence.

# RESULTS

**Conversion of monofluorophenols by** *R. opacus* **1cp.** Table 1 presents the characteristic mass spectrum peaks of the various hydroxylated compounds identified in the ethyl acetate extracts from the incubations of *R. opacus* **1cp** with the different monofluorophenols. Based on time-dependent TLC and HPLC patterns and MS analysis of the compounds, the formation of catechol-type intermediates, resulting from initial *ortho* hydroxylation of the phenols, could be identified (Table 2). Conversion of 2-fluorophenol mainly resulted in the formation of 3-fluorocatechol. In incubations with 3-fluorophenol, formation of 4-fluorocatechol could be detected. Conversion of 4-fluorocatechol.

Interestingly, unknown metabolites were detected in the incubation mixtures of *R. opacus* 1cp with each of the three monofluorophenols. These intermediates were only observed in incubations with *R. opacus* 1cp cells grown on phenol in the presence of a fluorophenol inducer (Table 2). Further studies were conducted to identify this new type of metabolites. These intermediates were formed in addition to the fluoromuconates, which are formed by the cleavage of fluorocatechols by intradiol dioxygenases known to be present in *R. opacus* 1cp (3). Based on the MS characteristics (Table 1) and the exact mass of 144.0223, which is consistent with a  $C_6H_5O_3F$  composition, these compounds were identified as trihydroxyfluorobenzene derivatives.

Figure 1 presents the possible trihydroxyfluorobenzene derivatives that may be formed from the different fluorocatechols. It is important to note that formation of 1,2,4-trihydroxybenzene-type intermediates would require the action of an aromatic *para*-hydroxylase, different from the *ortho*-hydroxylating phenol hydroxylase converting the fluorophenols to their corresponding catechols. It is possible that 1,2,3-trihydroxyfluorobenzenes (fluoropyrogallols) are formed from fluorocatechols by the action of the same *ortho*-hydroxylating phenol hydroxylase that transforms fluorophenols to fluorocatechols. This formation of 1,2,3-trihydroxyhalobenzenes in the biotransformation of fluorophenols has not been reported before in prokaryotes.

**Identification of the substituent pattern in the trihydroxylated fluorobenzene compounds.** In order to establish the nature of the trihydroxyfluorobenzene derivatives in more detail, the ethyl acetate extracts of the incubations with 2-fluorophenol and 3-fluorophenol, each containing one of the two different trihydroxyfluorobenzene derivatives, were analyzed by <sup>19</sup>F NMR.

Figure 2a presents the <sup>19</sup>F NMR spectrum of the ethyl acetate extract of the mixture containing *R. opacus* 1cp incubated for 2 h with 2-fluorophenol. From this spectrum it can be concluded that the parent 2-fluorophenol (at -141.9 ppm) has disappeared and 3-fluorocatechol is the major fluorine-con-

 

 TABLE 2. Fluorophenol degradation and intermediate formation in 1-h incubations of *R. opacus* 1cp grown in the presence of different (fluoro)phenol inducers<sup>a</sup>

Inducer	Substrate	% Fluoro- phenol	% Fluoro- catechol	% Fluoro- pyrogallol
4-Fluorophenol	2-Fluorophenol	40–45	38–42	0
	3-Fluorophenol	2–4	20–22	3–5
	4-Fluorophenol	0	36–50	8–14
Phenol	4-Fluorophenol	10–30	16–21	0
2-Fluorophenol	2-Fluorophenol	6–20	1–3	0-1
3-Fluorophenol	3-Fluorophenol	2–8	38–40	0-3

<sup>a</sup> Data are ranges of results obtained in four to six experiments.



FIG. 1. Aromatic hydroxylation of fluorophenols providing possible pathways for formation of trihydroxy-fluorobenzene derivatives formed from fluorocatechols. Solid arrows, pathways detected and observed in the present study.

taining compound present. This is completely consistent with what could be derived from the TLC patterns at this time of the incubation. The <sup>19</sup>F NMR spectrum reveals the presence of a small amount of 2-fluoromuconate resulting from *ortho* cleavage of 3-fluorocatechol. In addition, a <sup>19</sup>F NMR signal at -149.2 ppm is observed, probably representing the trihydroxy-fluorobenzene metabolite detected by TLC and MS (Table 1).

The exact substituent pattern of the trihydroxyfluorobenzene was determined based on the chemical shift and the proton-coupled <sup>19</sup>F NMR spectrum of this compound. In theory, the formation of three different trihydroxyfluorobenzene isomers can be foreseen, taking into account formation of 3fluorocatechol as the major initial step in 2-fluorophenol conversion (Fig. 1). Two of these metabolites, 1,2,4-trihydroxy-3-fluorobenzene and 1,2,4-trihydroxy-6-fluorobenzene, are hydroxyhydroquinone-type metabolites, whereas the other, 1,2,3-trihydroxy-4-fluorobenzene, is an *ortho*-hydroxylated catechol-type metabolite.

Based on the literature (14) it can be concluded that incorporation of a hydroxyl moiety *ortho*, *meta*, or *para* with respect to a fluorine in a benzene derivative will result in a change in the chemical shift of that fluorine substituent by  $-23.1 \pm 0.3$  ppm,  $+1.3 \pm 0.4$  ppm, and  $-11.2 \pm 0.7$  ppm, respectively. The

chemical shift value of 3-fluorocatechol, which has been identified as -140.4 ppm (14), and that of 4-fluorocatechol, identified as -126.7 ppm (14), can be used to calculate the chemical shift values expected for the three possible trihydroxyfluorobenzenes. Figure 2a indicates these chemical shift positions. This analysis reveals that formation of 1,2,4trihydroxy-6-fluorobenzene (predicted to be around -139.1 ppm and recently observed at -138.4 ppm) (19) and of 1,2,4trihydroxy-3-fluorobenzene (predicted at -163.5 and recently observed at -161.7) (19) is not observed. The resonance at -149.2 ppm, however, is in the parts per million range expected for 1,2,3-trihydroxy-4-fluorobenzene, which is predicted to be centered on -150.7 ppm. Final proof for the assignment of the derivative with its resonance at -149.2 ppm as 1,2,3trihydroxy-4-fluorobenzene comes from the splitting pattern observed for this signal in proton-coupled <sup>19</sup>F NMR measurements, revealing a double doublet with <sup>3</sup>J<sub>HF</sub> = 10.0 Hz and <sup>4</sup>J<sub>HF</sub> = 5.2 Hz (Fig. 2a, inset). This confirms the presence of a proton ortho as well as meta with respect to the fluorine substituent in this intermediate. This clearly identifies the trihydroxylated metabolite as 1,2,3-trihydroxy-4-fluorobenzene.

Figure 2b presents the <sup>19</sup>F NMR spectrum of the ethyl acetate extract of the reaction mixture of *R. opacus* 1cp incu-



FIG. 2.  $^{19}$ F NMR spectra of ethyl acetate extracts of 2-h incubations of *R. opacus* 1cp with 2-fluorophenol (a) and 3-fluorophenol (b). IS, resonance from the internal standard 4-fluorobenzoate; arrows, predicted resonance positions of possible hydroxylated catechol and hydroxyhydroquinone metabolites that can be formed from 3-fluorocatechol (a) and 3-fluoro- and 4-fluorocatechol (b), the initial hydroxylated products formed from these phenols. Insets, <sup>1</sup>H-coupled <sup>19</sup>F NMR splitting patterns of the peaks of the trihydroxyfluorobenzene metabolites, as well as of the peak tentatively identified as 2-pyrone-4-fluoro-6-carboxylic acid. For further details see text and Table 1.

bated for 2 h with 3-fluorophenol. The spectrum reveals complete transformation of the parent substrate, reflected by the absence of a <sup>19</sup>F resonance peak at -116.5 ppm. Accumulation of fluorocatechols is observed at this time of the incubation in amounts that were almost below the detection limit of the <sup>19</sup>F NMR measurement (1  $\mu$ M). Formation of 2-fluoro- and 3-fluoromuconate, resulting from *ortho* cleavage of 3-fluorocatechol and 4-fluorocatechol, respectively, is also observed in trace amounts. The main fluorine-containing peaks detected at this time of the incubation reflect the formation of a large amount of fluoride anions (at -123.0 ppm) and the formation of unknown intermediates with <sup>19</sup>F chemical shift values of -90.3, -119.0, -119.1, -125.9, -141.8, and -154.3 ppm. Based on TLC and MS analysis (Table 1), the presence of a trihydroxyfluorobenzene metabolite can be expected.

By using the chemical shift values of 3-fluorocatechol and 4-fluorocatechol (see above), chemical shift values expected for the five possible trihydroxyfluorobenzenes that can be formed from either 3-fluorocatechol or 4-fluorocatechol (Fig. 1) can be calculated. Figure 2b indicates these chemical shift positions. Notably, resonances corresponding to 1,2,4-trihydroxy-6-fluorobenzene (-138.4 ppm [19]), 1,2,4-trihydroxy-3-fluorobenzene (-161.7 ppm [19]), 1,2,3-trihydroxy-4-fluorobenzene (-149.2 ppm; see above), and 1,2,4-trihydroxy-5-fluorobenzene (-149.8 ppm [19]) were not observed. However, the chemical shift of the resonance at -125.9 ppm corresponds to that of 1,2,3-trihydroxy-5-fluorobenzene, which is predicted to be -125.4 ppm. The proton-coupled splitting pattern of the <sup>19</sup>F NMR resonance at -125.9 shows a clear triplet with two <sup>3</sup>J<sub>HF</sub> values of 10.1 Hz each (Fig. 2b, inset). This unequivocally shows the presence of two protons, one at each position *ortho* with respect to the fluorine substituent. Together this identifies the trihydroxylated fluorobenzene derivative as 1,2,3-trihydroxy-5-fluorobenzene.

Figure 2b also presents the <sup>1</sup>H-coupled <sup>19</sup>F NMR splitting pattern of the resonance at -90.3 ppm, the other major unidentified metabolite formed. Identification of this metabolite may give a clue to the possible conversion of 1,2,3-trihydroxy-5-fluorobenzene by ring-cleaving dioxygenases. The cleavage of 1,2,3-trihydroxybenzene by both intra- and extradiol dioxygenases has been described as resulting in the formation of either a 3-hydroxymuconate or a 2-pyrone-6-carboxylate or both (17). By analogy, conversion of 1,2,3-trihydroxy-5-fluorobenzene may result in the formation of 2-hydroxy-4-fluoromuconate or, alternatively, 2-pyrone-4-fluoro-6-carboxylate (Fig. 3). The <sup>1</sup>H-coupled <sup>19</sup>F NMR splitting pattern of the resonance at -90.3 ppm clearly shows a triplet with two coupling constants of 7.9 Hz (Fig. 2b, inset). The size of these coupling constants is consistent with  ${}^{3}J_{\rm HF}$  values generally observed in planar aromatic geometries and in fluorinated pyrones (5, 22) but is not consistent with <sup>3</sup>J<sub>HF</sub> values previously reported for fluoromuconates, which vary between 10 and 40 ppm (2, 22). This demonstrates that this resonance is not indicative of 2-hydroxy-4-fluoromuconate. In contrast, the two J<sub>HF</sub> coupling



FIG. 3. Possible oxygenolytic cleavage of 1,2,3-trihydroxy-5-fluorobenzene (5-fluoropyrogallol) in analogy to what has been observed for the conversion of 1,2,3-trihydroxybenzene by different intradiol and extradiol dioxygenases (17). Solid arrow, pathway proposed in the present study.

constants of 7.9 Hz are consistent with what would be expected for the two <sup>3</sup>J<sub>HF</sub> coupling constants in 2-pyrone-4-fluoro-6carboxylate. The reported chemical shifts of <sup>19</sup>F for substituted 4-fluoropyrones are -85.7 and -86.5 ppm, while those of the C-4-fluoro substituent of 3,4-difluoropyrones are between -118 and -123 ppm (5, 22). Taking into account a correction for the approximately -25-ppm change in chemical shift value upon introduction of an aromatic ortho-fluorine substituent (14, 16), the chemical shift of 2-pyrone-4-fluoro-6-carboxylate is expected to be between -85 and -98 ppm, consistent with the observed value of -90.3 ppm. Based on this observation, the TLC extracts of the incubations of R. opacus 1cp with 3-fluorophenol and 4-fluorophenol were analyzed for the presence of 2-pyrone-4-fluoro-6-carboxylate. Although the compound could not be completely purified from the TLC plates, a fraction containing a compound with a molecular ion m/z in the mass spectrum at 158 could be observed, eliminating a COOH fragment to give a fragment peak at m/z 113, followed by CO elimination to give a fragment peak at m/z 85. This fragmentation pattern together with the <sup>19</sup>F NMR data tentatively indicates the formation of 2-pyrone-4-fluoro-6carboxylate in incubations of R. opacus 1cp with 3-fluorophenol and 4-fluorophenol and suggests the conversion of 1,2,3-trihydroxy-5-fluorobenzene to 2-pyrone-4-fluoro-6-carboxylate.

Furthermore, the <sup>19</sup>F NMR spectra presented in Fig. 2 also give quantitative information. The conversion of 2-fluorophenol results in the formation of small amounts of fluoride anions and 2-fluoromuconate and 1,2,3-trihydroxy-4-fluorobenzene, which amount to, respectively, 6.4, 4.6, and 5.9% of the total amount of fluorine-containing metabolites. This implies that formation of 4-fluoropyrogallol efficiently competes with formation of 2-fluoromuconate from 3-fluorocatechol.

In the incubations with 3-fluorophenol, 1,2,3-hydroxy-5-fluorobenzene and the peak tentatively assigned to 2-pyrone-4fluoro-6-carboxylate make up 9.7 and 9.2%, respectively, of the total amount of fluorine-containing intermediates detected. Eventually in all incubations the trihydroxylated derivatives as well as the 2-pyrone-4-fluoro-6-carboxylate disappear from the medium, indicating their further degradation, which, for 1,2,3trihydroxy-5-fluorobenzene in particular, seems to be accompanied by efficient defluorination, reflected by a large percentage of fluoride anions in the incubation medium amounting to 54.0% of all fluorine-containing metabolites in the <sup>19</sup>F NMR spectrum of Fig. 2b.

Finally, Fig. 4 presents the time course for biotransformation of 4-fluorophenol by whole cells of R. opacus 1cp coinduced with 4-fluorophenol. Degradation of 4-fluorophenol and transient formation of 4-fluorocatechol and 5-fluoropyrogallol can be observed. After 4 to 6 h of incubation, 4-fluorophenol, 4-fluorocatechol, and 5-fluoropyrogallol disappeared from the reaction mixture. Similar results were obtained for the other two isomers (Table 2). The data in Table 2 indicate that 2-fluorophenol and 3-fluorophenol, when incubated with cells coinduced with the corresponding monofluorophenol or with 4-fluorophenol, were converted at lower rates than 4-fluorophenol. This indicates that, at least in the 4-fluorophenol-induced cells, their lower rates of conversion are due to a lower rate of conversion by the phenol hydroxylase present. Table 2 also presents data on the degradation of 4-fluorophenol by cells from R. opacus 1cp induced solely with phenol. Degradation of 4fluorophenol by these cells is significantly slower, suggesting the induction of phenol hydroxylase activity by 4-fluorophenol. Moreover, the fact that in the incubations with phenol-induced cells, no formation of fluoropyrogallols was observed suggests



FIG. 4. Time course of 4-fluorophenol degradation by whole cells of *R. opacus* 1cp coinduced with 4-fluorophenol as determined by HPLC.  $\bullet$ , 4-fluorophenol;  $\blacksquare$ , 4-fluorocatechol;  $\blacktriangle$ , 5-fluoropyrogallol.

that the phenol hydroxylase induced by 4-fluorophenol is different from the one induced upon induction by phenol.

## DISCUSSION

In the present study, the transformation of the isomeric monofluorophenols by whole cells of *R. opacus* 1cp was investigated, with particular emphasis on the nature of the hydroxy-lated intermediates formed. The results obtained indicate that, upon conversion of the fluorophenols, transient formation of trihydroxyfluorobenzene metabolites occurs. The chemical shifts and splitting patterns of the <sup>19</sup>F NMR resonances in <sup>1</sup>H-coupled <sup>19</sup>F NMR measurements were used to establish that 1,2,3-trihydroxyfluorobenzenes (fluoropyrogallols) occur as new intermediates in the transformation of fluorophenols by *R. opacus* 1cp.

The formation of the 1,2,3-trihydroxyfluorobenzenes from the fluorophenols could result from two successive ortho hydroxylation steps by the ortho-phenol hydroxylase of R. opacus 1cp. Interestingly, a sequential ortho hydroxylation of fluorinated substrates was recently also reported for para-hydroxybenzoate hydroxylase from Pseudomonas fluorescens (20). Fluoropyrogallol accumulation was only observed in incubations with cells grown on phenol in the presence of a fluorophenol inducer. This suggests that the phenol hydroxylase responsible for formation of the fluoropyrogallols is different from the phenol hydroxylase responsible for conversion of phenol in cells grown on phenol without a fluorophenol inducer. Further characterization of the phenol hydroxylases was hampered by their high lability. This is a common phenomenon among Rhodococcus species, and therefore these enzymes are difficult to detect and/or purify from cell extracts (4, 11, 18, 23). Possible reasons for these difficulties in studying phenol hydroxylase activities from Rhodococcus strains are (i) that the enzymes in question require special in vitro additions to remain in an active status or (ii) that the enzymes are of a multicomponent nature (15) and readily dissociate and/or contain one or more components which rapidly inactivate (11, 18, 23).

After several hours of incubation all fluorocatechols and fluoropyrogallols disappeared from the reaction mixtures. This suggests that ring-cleaving enzymes may be capable of converting not only the fluorocatechols but also their *ortho*-hydroxylated derivatives. Additional results of the present study tentatively indicated the formation of 2-pyrone-4-fluoro-6-carboxylate from 1,2,3-trihydroxy-5-fluorobenzene in incubations with *R. opacus* 1cp. This would imply that the dioxygenase(s) present in this species would behave similarly to protocatechuate-3,4-dioxygenase from *Pseudomonas aeruginosa*, preferentially catalyzing the conversion of the 1,2,3-trihydroxybenzene derivative to a 2-pyrone-6-carboxylate derivative instead of to a 2-hydroxymuconate (17). This also discriminates the dioxygenase(s) from *R. opacus* 1cp from the extradiol dioxygenase from *P. arvilla* (ATCC 23973), which catalyzes preferential ring cleavage of 1,2,3-trihydroxybenzene to give 2-hydroxymuconate, and from the intradiol catechol dioxygenase from *P. arvilla* C1 (ATCC 23974), which catalyzes the formation of a mixture of about equimolar amounts of both products (17). The isolation and characterization of the enzyme from *R. opacus* 1cp that transforms the 1,2,3-trihydroxyhalobenzene derivatives are under way.

In analogy to the experiments reported in the present study for fluorophenols, preliminary studies using chlorophenols provided evidence for formation of chloropyrogallols. Especially for 2-chlorophenol and 2,3-dichlorophenol, formation of the corresponding chloro- and dichloropyrogallol was observed in TLC and MS experiments. For 3-chloro- and 4-chlorophenol, no accumulation of pyrogallol metabolites occurred (Finkelstein et al., unpublished results). These data support the link between the two types of halophenols. Formation of 1,2,3-trihydroxychlorobenzene has been reported in the transformation of isomeric monochlorophenols by *Penicillium simplicissimum* SK9117 (13), but the present study demonstrates for the first time the formation of this type of hydroxylated metabolites in the transformation of halophenols by a prokaryote.

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