# AEROBIC BIOTRANSFORMATION OF FLUOROAROMATIC COMPOUNDS BY PSEUDONOCARDIA BENZENIVORANS

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**Abstract**

Widespread usage of fluoroaromatic compounds as agrochemicals and pharmaceuticals has lead to an increased appearance of fluorine-containing pollutants. Therefore it is essential to understand its fate and accumulation potential in the environment. Compared with chlorinated and brominated compounds, sparse information about metabolism of fluorinated compounds by microorganisms is available. In this study, we found that *Pseudonocardia benzenivorans* which was previously isolated from an enrichment based on tetrachlorobenzene has broad metabolic diversity for monofluoroaromatic compounds. *P. benzenivorans* mineralized 4-fluorophenol (4-FP) as a sole source of carbon and energy. Besides, this strain could also transform other mono-fluorophenols (mono-FPs) *via* monooxygenase activity. The metabolic route of FP isomers was investigated using enzyme assay and isolation of intermediates by high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). Two forms of fluorocatechol were revealed as the key degradation products during mono-FPs biotransformation. This is first comprehensive report about metabolic process of all mono-FPs by a single bacterium.

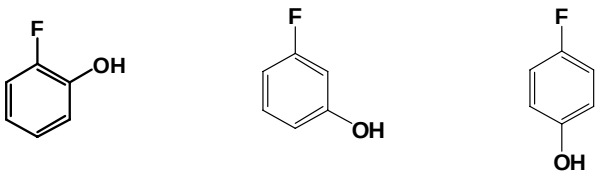
**Introduction**

Haloaromatic compounds have been synthesized industrially on a large scale for several decades, becoming common environmental pollutants. In recent years, one or two fluorine attached aromatics are increasingly used in commercial purposes for manufacture of agrochemicals, pharmaceuticals and dyes[1]. Unlike chlorinated aromatics, fluorinated aromatics were rarely the objects of microbial degradation studies[2,3]. Although hydrogen substitution by fluorine is accompanied with only a small increase of molecular size, negative effects on enzymatic turnover has been observed. The exceptional recalcitrance of these fluoroaromatics can be related with high electronegativity of fluorine. Its strong electron-withdrawing characteristic of fluorine lowered the electron density of the aromatic nucleus so hampered the electrophilic attack by bacterial monooxygenase[4].

Regardless of resistance of most fluoroaromatic compounds to biodegradation, several bacterial strains have been reported that can degrade or transform these compounds under aerobic conditions. Examples of biodegradation of fluorinated compounds most commonly found in the literature involve fluorobenzoic acid[5-7], but few bacterial cultures can convert FPs and FB[8-15]. The oxidation of FB to form fluorocatechol by *Pseudomonas putida* F6[14] and *Pseudomonas mendocina* KR1[15] has been studied. Until now, only *Rhizobiales* strain F11 is known to utilize FB as the growth substrate[8-10]. Research on bacterial FPs degradation was generally restricted to cometabolism by whole cells, crude cell extracts, and purified enzymes from *Rhodococcus* species grown on chlorophenol as a carbon source[12,13]. Recently, *Arthrobacter* sp., which can completely degrade 4-FP to carbon dioxide was isolated[11].

*Pseudonocardia benzenivorans* was isolated from an enrichment culture that contained 1,2,3,5-tetrachlorobenzene as the carbon source[16]. The aim of this study was to evaluate the ability of *P. benzenivorans* to degrade mono-fluoroaromatics, especially mono-FPs. In the present paper, we checked that *P. benzenivorans* was able to grow in 4-FP and transform 2-FP and 3-FP. Based on the identification of a number of intermediates, metabolic routes for each compound were proposed. Our findings strongly indicate that this actinomycete strain provides good possibilities for degradation of fluorinated compounds.

**Figure 1**. Chemical structure of mono-FPs



## Materials and Methods

■ *Chemicals*

2-Fluorophenol (2-FP), 3-fluorophenol (3-FP), 4-fluorophenol (4-FP) and 3-fluorocatechol were purchased from Sigma-Aldrich (St. Louis, MO). 4-fluorocatechol was got from TCI America (Tokyo, Japan). Ethyl acetate, acetonitrile, 85% *ortho*-phosphoric acid and nutrient broth (NB) were purchased from Merck (Darmstadt, Germany). All chemicals and solvents were of the highest grade available.

■ *Bacterial strain and culture conditions*

*Pseudonocardia benzenivorans* was purchased from Deusche Sammlung von Mikroorganismenund Zellkulturen GmbH (DSMZ). The cells were grown in 100 ml Erlenmeyer flasks containing 20 ml mineral salts medium[17] or nutrient broth medium with phenol. Cultures were grown in shaking incubator (160 rpm) at 30°C.

■ *Growth of this strain on mono-FPs*

To estimate the ability of *P. benzenivorans* to use mono-FPs as a sole carbon substrate, we observed the growth of the strain in the presence of 2-FP, 3-FP and 4-FP with 1 mM. Several compounds were also checked, such as catechol and 4-fluorocatechol. Pre-grown cells on phenol were inoculated in 25 ml mineral media in 100 ml baffled Erlenmeyer flasks with screw caps. All cultures were incubated at 30°C in rotary shaker. In this study, to evaluate the growth of *P. benzenivorans*, total weight of dried biomass was selected instead of UV/Vis spectrophotometer due to the aggregation characteristic of proliferated cells. Samples were filtered through the dried membrane filter.

■ *Degradation experiments*

Cell suspension, which was pre-grown in NB medium or mineral medium with 4-FP was harvested by centrifugation at 15,000×g for 15 min at 4°C and washed three times using 20 mM phosphate buffer (pH 7.2). Then, cells were resuspended in phosphate buffer and transferred 10ml of cell suspension to 100 ml baffled Erlenmeyer flasks including 2.5 mM 2-FP, 3-FP and 4-FP. Heat-killed (70°C, 40 min) and poisoned (with 10 mM sodium azide) cells were also prepared as controls.

■ *Preparation of cell extracts and enzyme assay*

Pre-grown cells in 4-FP were harvested by centrifugation at 14,000×g for 20 min at 4°C, washed three times with 50 mM Tris-HCl buffer (pH 7.5), and resuspeded in 10 ml of 33 mM Tris-HCl buffer (pH 8.0). Suspended cells were incubated with lysozyme (5 mg/ml) for 1 h at 30°C, and then disrupted by a chilled homogenizing sonicator. The cell extracts were centrifuged at 15,000×g for 30min at 4°C. The separated supernatant was used for further experiments. Protein concentration was measured by Bradford assay. All enzyme assays were determined at 25°C by UV/Vis spectrophotometer. Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities were measured at 260 and 375 nm, respectively. The reaction samples contained 0.1 mM catechol, 33 mM Tris-HCl buffer (pH 8.0), and cell extracts (0.05 mg protein). 3-fluorocatechol and 4-fluorocatechol dioxygenase were measured with 3-fluorocatechol and 4-fluorocatechol in place of catechol.

■ *Analytical procedure*

Quantification of mono-FPs was analyzed *via* HPLC connected to a diode array detector system. 1 ml culture from the all samples was withdrawn and filtered through a 0.45 μm syringe filter. The solvent mixture used was a binary mixture of 0.1% (*w/v*) phosphoric acid and 20% acetonitrile. Detection was performed at 210 nm. Analysis of other polar intermediates was carried out using GC-MS. Before GC-MS analysis, all samples were extracted four times with an equal volume of chilled ethyl acetate and concentrated under reduced pressure. Metabolites were identified by comparison of retention time and mass spectra in GC-MS of those standards. The measurement of free fluoride in the aqueous phase was determined using an ion selective electrode.

## Results and Discussion

■ Growth of P. *benzenivorans* in various aromatic compounds

The capability of *P. benzenivorans* to use various aromatics as growth substrate was tested. The growth was monitored for two weeks. During the growth, the cells tend to aggregate together. This strain was able to grow with 4-FP, catechol and 4-fluorocatechol (Table 1). It seems that 4-FP is converted partially to catechol and in part to 4-fluorocatechol. Other FP analogues, such as 2-FP and 3-FP did not serve as carbon substrates for *P.*

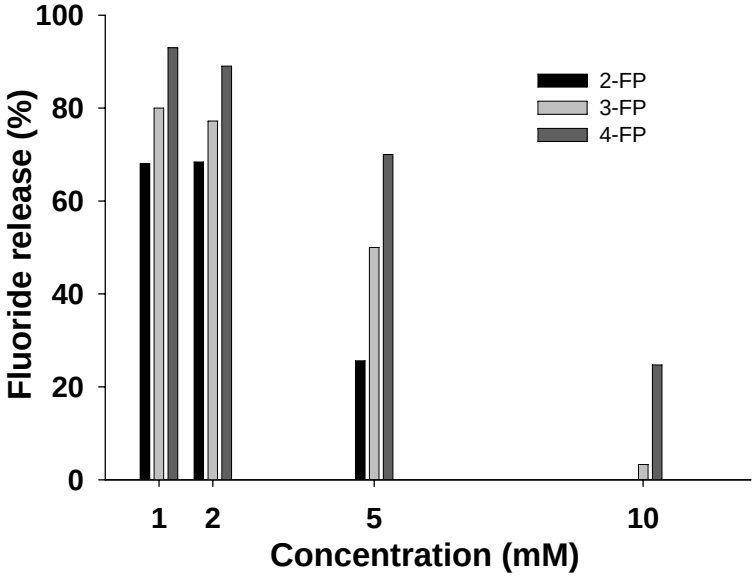
*benzenivorans*.

**Table 1**. Utilization of various compounds at 1mM concentration by *P. benzenivorans*

| Compounds | Growth in substrate |
|---|---|
| Benzene | + |
| Fluorobenzene | — |
| Phenol | + |
| 2-fluorophenol | — |
| 3-fluorophenol | — |
| 4-fluorophenol | + |
| Catechol | + |
| 4-fluorocatechol | + |

To check the transformation efficiency of other mono-fluoroaromatics by *P. benzenivorans*, experiments were performed with 2-FP, 3-FP and 4-FP at concentrations from 1 to 10 mM. Fluoride liberation was nearly stoichiometric at 1 and 2 mM 4-FP, while fluoride release gradually decreased at higher concentrations. Although bacterial growth was not shown with 2-FP and 3-FP, at 1 and 2 mM concentrations, over 6o percent of total fluoride ions was released. This indicates that *P. benzenivorans* can use only 4-FP as carbon source but transform 2-FP and 3-FP.

**Figure 2**. Fluoride release on mono-FPs at different concentrations



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