Original paper (regular paper)

Fluorine Elimination from 4-Fluorobenzyl Alcohol by *Pseudomonas* spp.

Toshinari Maeda, Daisuke Okamura, Masaya Yokoo, Eisaku Yamashita and Hiroaki I. Ogawa*

Department of Biological Functions and Engineering, Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2–4 Hibikino, Wakamatsu-ku, Kitakyushu 808–0196, Japan
* TEL: +81–(0)93–695–6059 FAX: +81–(0)93–695–6012
* E-mail: ogawahi@life.kyutech.ac.jp

Pseudomonas aeruginosa strain IE653 can use 4-fluorobenzyl alcohol (4FBA) as a sole source of carbon and energy, whereas *P. aeruginosa* strain PAO1 and *P. putida* strain mt-2 cannot utilize it. This strain converted 4FBA into 4-fluorobenzoic acid (4FBZ), 4-fluorophenol (4FP) and 4-fluorocatechol (4FCAT), and further into 3-fluoromuconate or fluoro-2-hydroxyl-muconic semialdehyde via ortho or meta cleavage. Strain mt-2 also converted 4FBA into the same metabolites as those detected in IE653 except 4FP. Contrary to an abundant accumulation of 4FBZ in strains PAO1 and mt-2, IE653 transiently accumulated and gradually biotransformed 4FBZ. The fluoride ions from 4FBA by *P. aeruginosa* strain IE653 were released after 8-hour cultivation, indicating that this strain was able to efficiently eliminate them from 4FBA metabolites much better than strains *P. aeruginosa* PAO1 and *P. putida* mt-2 that have less effective on eliminating fluoride ions. An elimination of fluoride ions in strain IE653 was initiated after metabolism of 4FCAT, judging from the results that this strain immediately released from 4FCAT. It was implied that ortho cleavage pathway of strain IE653 is major pathway for the release of fluoride ions, unlike meta cleavage pathway, which had only a slight effect. The efficient release of fluoride ions by *P. aergunosa* strain IE653 could contribute to develop the bioremediation technology for elimination of fluorine and detoxification of fluoroaromatics.

Key words: fluoroaromatics, elimination of fluorine, pseudomonad strains, metabolic pathway, ortho pathway

1. Introduction

The advances in organic synthesis have led to the introduction of numerous new organic compounds into the environment, whose susceptibilities to biotreatment processes are mostly unknown. Fluoroaromatics are being increasingly used in a wide range of agrochemical and pharmaceutical products (herbicides and fungicides), due to the need to find environmentally acceptable alternatives to chlorinated compounds[24,41,47]. The diversity of structures and the chemical inertness of many halogenated organic compounds pose particular problems and challenges for microbial degradation[13]. Some scientists proposed that the recalcitrance of a halogenated organic compound usually becomes greater with the increase of the electronegativity of the substituents; thus, the recalcitrance of F-C is greater than that of Cl-C, Br-C and I-C[12]. Also, the exceptional biological activities of fluorinated aromatic compounds can be explained by the dichotomic chemical behavior of the fluorine substituent with its +M and –I effects together with its hydrogen-resembling size, reflected by the van der Waals radii of 120 pm (for H) and 135 pm (for F)[35]. This relationship shows that monofluorobenzoates undergo dioxygenation, forming fluorocatechols, or become subject to anaerobic degradation by benzoate-degrading bacteria[40]. On the other hand, the exchange of a hydrogen atom by a fluorine atom can create analogs that precede analog normal metabolic pathways until the fluorine substituent produces a specific enzyme inhibitor. Such a metabolic transformation is termed lethal synthesis[36]. This effect was found with fluoroacetate and fluorocitrate as dead-end products[1,11]. The critical step in the degradation of fluorohydrocarobons is the fission of the carbon-fluorine bond. For the cleavage of this bond in fluoroacetic acid, a rather specific enzyme has been found[15,45]. In monofluorobenzoates, fluoride release can occur after oxidative decarboxylation and ring fission of fluorocatechol[16,18,42]. Alternatively, the fluorine is replaced in a dioxygenase-catalyzed reaction, as in the degradation of 2-fluorobenzoate, in which unsubstituted catechol and 3-fluorocatechol are the products of metabolic transformation[6,28,47].

The biodegradation of a vast range of halogenated aromatic compounds, especially chlorinated compounds, has been described[17,37], but scant information is available on the metabolic and cometabolic fate of fluorinated aromatic compounds in bacteria. Examples of biodegradation of fluorinated compounds most commonly found in the literature involve fluorobenzoic acids[9,18,28,33,43] and fluorophenols[3,4,38,40]. These literatures have reported on classic pathways, in which brilliant bacteria convert fluorinated compounds and

ultimately eliminate fluoride ions from them, and biodegradability. However, to our knowledge, relationship between the release of fluoride ions and metabolic pathway has not yet been reported. As an example, though *P. aeruginosa* strain PAO1, in which genome project has finished, has probable haloacid dehalogenase in range 887583 to 888284 of genome map at the Pseudomonas Genome Project (http://www.pseudomonas.com), this strain was not able to release the fluoride ions from fluoroaromatics (results in this study), indicating that their efficient elimination may depend to metabolic pathway of fluoroaromatics.

Benzyl alcohol (BA) has been reported to form glutathione conjugates[7,26] and DNA adducts[48], to cause perturbations in river function and decreased clearance[7,27], has demonstrated potential carcinogenicity in experimental animals[14,27]. There is currently very little literature available on toxicity of 4-fluorobenzyl chloride (4FBCl), a structural analogue of BA. However, 4FBCl may be more risky than BA because F-C bond is the recalcitrance[12]. Also, 4FBCl is spontaneously converted to 4-fluorobenzyl alcohol (4FBA) in water although at a slower rate; however, 4FBA is still a recalcitrant compound, and forms N-acetylcysteinyl conjugates[2]. We screened 4FBA-biodegradaing bacteria from industrial effluents in Kitakyushu, Japan, and found 1 strain. One strain was designated as *P. aeruginosa* strain IE653, judging from the results of characterization with NF-18 and API20NE systems, and homology search of 16S ribosomal RNA gene. This paper describes that biotransformation and detoxification of 4FBA by *P. aeruginosa* strain IE653 were much higher than those by strains *P. aeruginosa* strain PAO1 and *P. putida* strain mt-2 (a toluene degrader), and that strain IE653 could efficiently eliminate the fluoride ions from fluoroaromatics.

2. Materials and Methods

2.1. Chemicals

We used 4-fluorobenzyl alcohol (4FBA) (Merck KGaA, Germany), 4-fluorobenzoic acid (4FBZ), 4-fluorophenol (4FP), 4-fluorocatechol (4FCAT) (Tokyo Chemical Industry Co. Ltd., Japan), benzyl alcohol (BA), catechol (CAT) (Wako Pure Chemical Industries Ltd., Japan), benzoic acid (BZ) and *cis,cis*-muconic acid (Kanto Kagaku Co. Ltd., Japan) as chemical standards. All chemicals were of the highest purity commercially available.

2.2. Bacterial strains and culture conditions

Bacteria used in this study were *P. aeruginosa* strains IE653 and PAO1, and *P. putida* strain mt-2. These bacteria were aerobically grown until the late logarithmic growth phase in M9 minimal medium[41] containing benzyl alcohol (1 mM) and succinic acid (1 mM) as carbon source at 30°C in the dark with shaking (120 rpm). Cells were washed twice with 0.1 M sterilized phosphate buffer (pH 7.5) and resuspended in the same buffer. Cell suspensions were mixed into reaction solution, and then the mixture was aerobically

incubated at 30°C in the dark with shaking (120 rpm).

2.3. Determination of viable cells

M9 minimal medium (100 mL) with and without a carbon source (1 mM succinic acid or 1 mM 4FBA) was mixed with cell suspensions (a final concentration; about 1×10^6 cells/mL), and then the mixture was aerobically incubated at 30°C in the dark with shaking. Periodically, serial dilutions of the reaction solution were spread on Luria-Bertani (LB) agar plate (10 g tryptone, 5 g yeast extract, 5 g sodium chloride and 15 g agar/L), and then these plates were incubated at 30°C for 18 h. We counted the colonies to calculate colony forming units (cfu).

2.4. Identification of 4FBA metabolites

The cells (about 5×10^8 cells/mL) were aerobically grown for different periods at 30°C and were removed by centrifugation at $5000\times g$ for 10 min. The culture supernatant (adjustment of pH with 6 N HCl; pH 3.0) was extracted 3 times with 100 mL ethyl ether, then the extracts were dried over anhydrous sodium sulfate, and excess solvent was removed by rotary evaporation at 30°C. When required, the derivatization of samples (trimethyl silylation or methylation) was carried out. Gas chromatography-mass spectrometry (GC-MS) analyses of these samples were performed with a HP6890 Series GC system/5973 Mass Selective Detector (Hewlett Packard, USA). 4FBA metabolites were identified by comparison of retention times and mass spectra between samples and standard chemicals. Also, the metabolites (after complete elimination of excess solvent) were dissolved in 600 μL of 0.1 M phosphate buffer (pH 7.0). Then samples were transferred to NMR tubes, and D_2O (100 μL) was added to provide a lock signal. The mixtures were analyzed by ^{19}F nuclear magnetic resonance spectroscopy (^{19}F NMR) with a Burker Avance 400S (400 MHz) spectrometer (Germany).

2.5. Measurement of 4FBA, its metabolites and fluoride ion

4FBA and its metabolites were determined by high-performance liquid chromatography (HPLC). HPLC analyses were performed on an Inertsil ODS-2 column (GL Sciences Inc., Japan) with acetonitrile-water (25 : 75) as the mobile phase, with a flow rate of 0.4 mL/min. All compounds were detected at 254 nm with a Shimadzu SPD-10A$_{VP}$ UV-VIS detector (Japan). Fluoride ion concentrations were measured with a TOA Ion Meter IM-40S (Japan) containing a fluoride-selective lanthanum fluoride electrode as previously described[34]. Concentrations of 2-hyroxymuconic semialdehyde and fluoro-2-hydroxymuconic semialdehyde were calculated from the molar absorptivity[25,31].

2.6. Preparation of extracts

The cells (*P. aeruginosa* IE653) were grown at 30°C with shaking for 18 h in 4FBY medium (M9 with 0.2% 4FBA and 0.05% yeast extract). Cultures were harvested by

centrifugation (5000×*g*, 10 min), and the cells were washed twice with 0.1 M phosphate buffer (pH 7.5). Cells were suspended in this buffer at a density of 7×10^9 to 10^{10} cells/mL and disintegrated for 5 min in an ice bath with a Branson ultrasonics, model Sonifier 450 (Central Scientific Commerce Co. Ltd., Japan). The preparation was centrifuged at 15000×*g* for 20 min at 0°C to remove whole cells and large debris. Protein was measured by the method of Bradford using bovine serum albumin as a standard[5].

2.7. 4FCAT biotransformation by cell extracts.

Enzyme assays of catechol-1,2-dioxygenase and catechol-2,3-dioxygenase were performed at 30°C with a UV-VIS Spectrophotometer V-530 (JASCO, Japan). Catechol-1,2-dioxygenase and catechol-2,3-dioxygenase activities were assayed by measuring the formation of *cis,cis*-muconate and 2-hydoxymuconic semialdehyde, respectively. The molar absorption coefficient was 16800 at 260 nm for *cis,cis*-muconate[25] and 42000 $M^{-1} cm^{-1}$ at 375 nm for 2-hydroxymuconic semialdehyde[8]. One unit of these enzyme activities was defined as the formation of 1 μmol of the product per min. Specific activity was calculated as the formation of 1 μmol of the product/min/mg-protein. All measurements were made in cuvettes with a 1-cm light path in a total volume of 3.0 mL.

The enzyme activity of catechol-2,3-dioxygenase is inactivated by H_2O_2[30]. We used cell extracts to research which pathways (ortho and meta cleavage pathways) are essential for the elimination of fluorine from 4FCAT in biotransformation. Catechol-2,3-dioxygenase in cell extracts was inactivated as follows. H_2O_2 at a final concentration of 80 mM was added into the extracts (6.8 mg-protein/mL) and incubated at 30°C for 30 min. Then bovine liver catalase was added (100 enzyme units/mL) followed by incubated for another 10 min to destroy excess H_2O_2.

The enzyme activities in H_2O_2-treated or untreated extracts were assayed in 200 mL of 0.1 M phosphate buffer (pH 7.5) containing 0.33 mM 4FCAT. Fluoride ion concentrations were measured to determine a fluorine-releasing activity.

3. Results

3.1. Growth curve of bacterial strains in various conditions

We isolated *P. aeruginosa* strain IE653, which was able to biodegrade 4FBA, and tested whether this strain can utilize it as a sole carbon source. Two strains, *P. aeruginosa* PAO1 that is a delegate among various pseudomonad strains and *P. putida* mt-2 known as a toluene degrader, were used as control strains in this study. We used these two strains because the whole genome sequences of these bacteria have been completely determined and the metabolic pathway by *P. putida* mt-2 has been intensively investigated. Strain IE653 grew in the medium supplemented with 4FBA as a sole carbon source, whereas PAO1 could not grow and strain mt-2 showed only slight growth (Fig. 1). These results indicate that strain IE653 can utilize 4FBA as a sole carbon source.

3.2. 4FBA metabolism pathways by pseudomonad strains

GC-MS analyses identified 4-fluorobenzoic acid (4FBZ) as a major metabolite, and simultaneously 4-fluorophenol (4FP), 4-fluorocatechol (4FCAT) and fluoro-2-hydroxymuconic semialdehyde (FHMS) as minor products in the culture supernatant of strain IE653 (Fig. 2). In addition, ^{19}F-NMR analyses demonstrated that *P. aeruginosa* IE653 produced three metabolites (4FBZ, 4FP and 4FCAT [Fig. 3-A]) and 3-fluoromuconate (3FM) as shown in Fig. 3-B. On the other hand, *P. putida* mt-2 converted 4FBA into the same metabolites except 4FP as those detected in the culture supernatant of strain IE653. Enzyme assays using cell extracts of IE653 showed that this strain has two ring cleavage pathways, in which catechol and 4FCAT were broken down by catechol 1,2-dioxygenase and catechol 2,3-dioxygenase, producing *cis,cis*-muconate and 3FM, and 2-hydroxyl muconate semialdehyde and FHMS, respectively (Table 1); these results are consist with the structural results using GC-MS and ^{19}F-NMR. On the other hand, we implied that there is the possibility that IE653 produces 4-fluoro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid
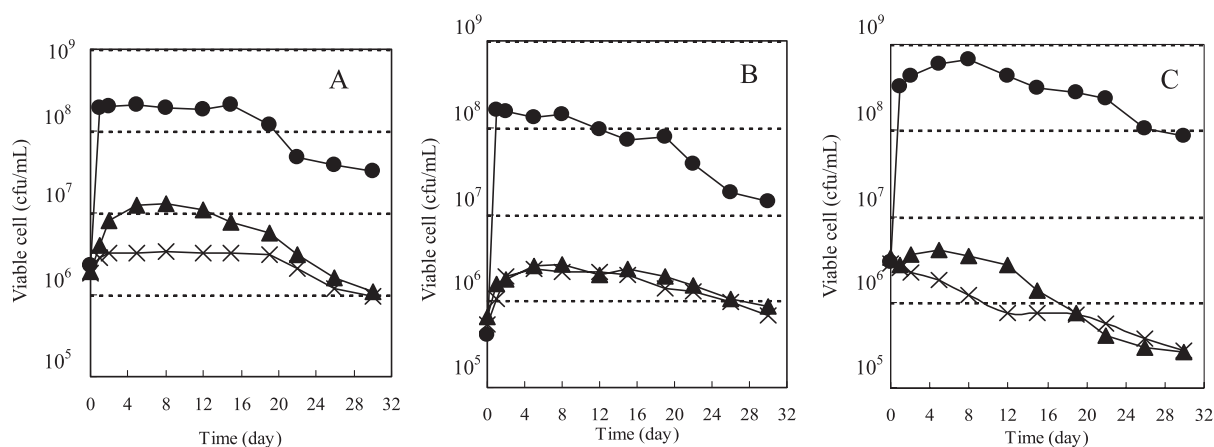

Fig. 1. Growth curves of pseudomonad strains (A, *P. aeruginosa* strain IE653; B, *P. aeruginosa* strain PAO1; C, *P. putida* strain mt-2) in M9 with 1 mM succinic acid (●) and 1 mM 4-fluorobenzyl alcohol (▲), and without a carbon source (×).
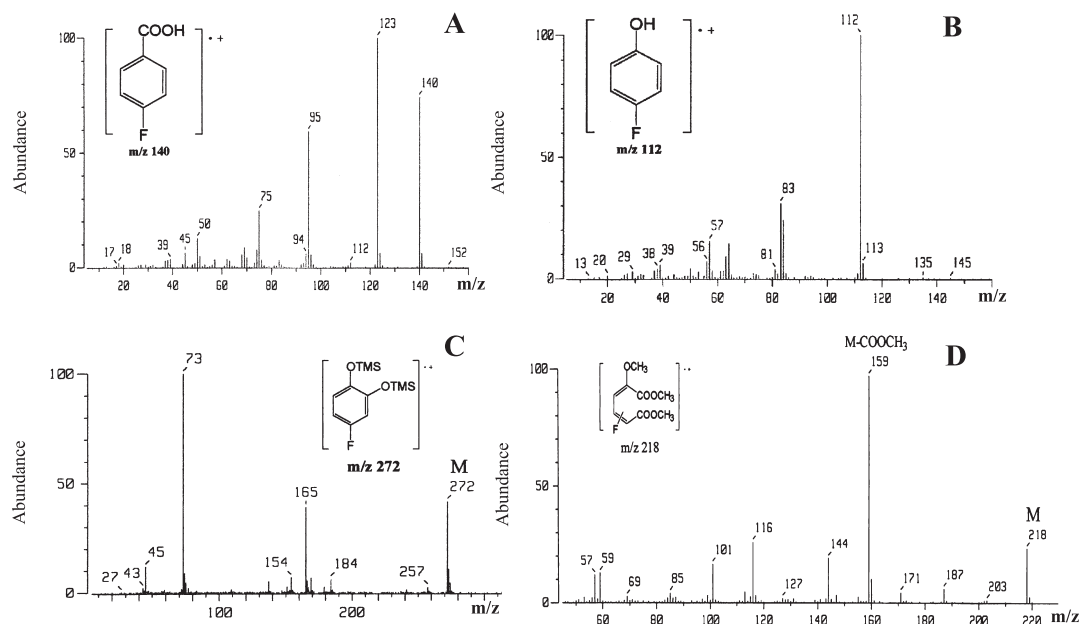
Fig. 2. GC-MS spectra of 4-fluorobenzyl alcohol metabolites produced by *P. aeruginosa* IE653. (A) 4-fluorobenzoic acid, (B) 4-fluorophenol, (C) 4-fluorocatechol, and (D) fluoro-2-hydroxyl-muconic semialdehyde.
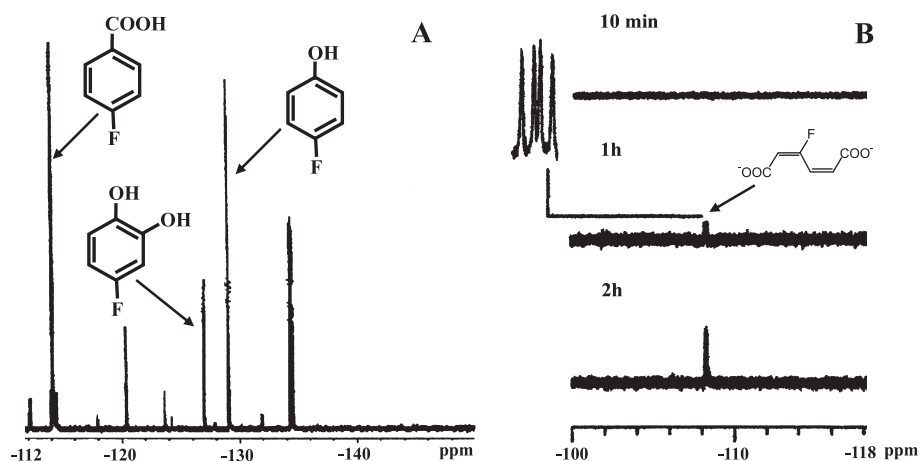

Fig. 3. ^{19}F-NMR spectra of 4-fluorobenzyl alcohol metabolites produced by *P. aeruginosa* IE653. (A) Metabolites from 4-fluorobenzyl alcohol; arrows indicate 4-fluorobenzoic acid, 4-fluorophenol and 4-fluorocatechol. (B) Metabolite from 4-fluorocatechol; arrow indicates 3-fluoromuconate.

(4FCHDC), which is a intermediate product during conversion from 4FBZ to 4FCAT, judging from the comparison of mass fragment patterns in mass spectra between 4-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid and 4FCHDC (it is possible to analyze the mass fragment patterns in chlorinated compounds because Cl has stable isotopes (^{35}Cl and ^{37}Cl; 3 : 1 of ratio)) as shown in Fig. 4. This result implies that *P. aeruginosa* IE653 may have two pathways (4FBZ→4FCHDC→4FCAT and 4FBZ→4FCHDC→4FP→4FCAT) whereas strain mt-2 has a single pathway (just 4FBZ→4FCHDC→4FCAT) in metabolism from 4FBZ to 4FCAT.

3.3. 4FBA biotransformation and 4FBZ accumulation

Figure 5 indicates the patterns of 4FBA biotransformation and 4FBZ accumulation in three pseudomonad strains.

Strain mt-2 biotransformed 4FBA much better than strains IE653 and PAO1. Strains IE653 and PAO1 showed the same level of biodegradation rate. When *P. aeruginosa* IE653 was cultivated with 4FBA, 4FBZ was transiently accumulated and was then gradually decreasing with time. In contrast, strains PAO1 and mt-2 accumulated a high concentration of 4FBZ. However, they did not greatly accumulate BZ when cultivated with BA (Table 2). These results indicate that fluoride substitution in benzoic acid leads to an increased resistance to biodegradation by strains PAO1 and mt-2, while strain IE653 efficiently transforms fluoroaromatics. Also, the accumulations of meta cleavage products (yellow products), FHMS and HMS, which have an absorption maximum at 382 and 375 nm, respectively, were observed in strains IE653 and mt-2 (Table 2).
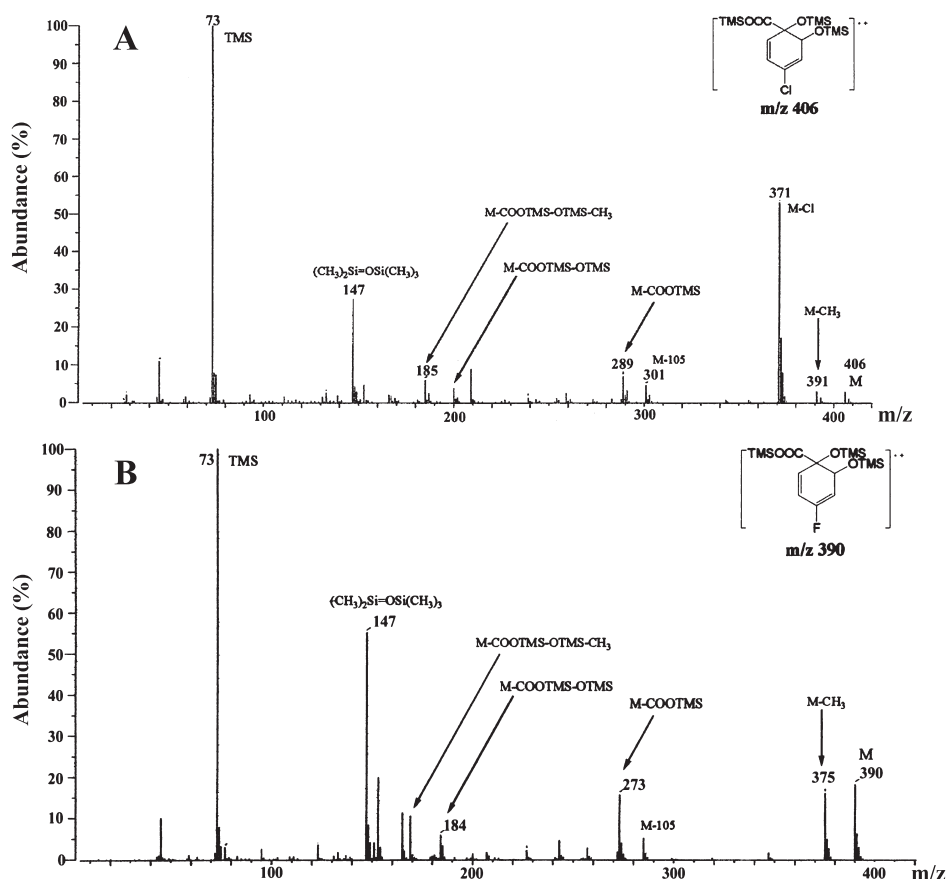
Fig. 4. Production of putative intermediates, 4-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (A) and 4-fluoro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (B) by *P. aeruginosa* IE653. These mass spectra were obtained by GC-MS analyses.

Table 1. Enzyme activity for catechol 1,2-dioxygenase and catechol 2,3-dioxygenase and fluorine elimination by crude extracts of strain IE653.

Extracts	Fluoride ion concentration (mg/L)			Specific activity (units/mg protein)[a]	
	1 h	3 h	7 h	ortho[b]	meta[c]
untreated	0.7±0.1	1.41±0.05	2.0±0.2	0.23±0.05[d]	0.05±0.02
H_2O_2-treated	1.8±0.2	2.7±0.1	2.75±0.05	0.32±0.02	0.001±0.000

Values are the means ± the standard deviations of two independent measurements.

[a] For definition of specific enzyme activity see Materials and Methods.

[b] catechol 1,2-dioxygenase

[c] catechol 2,3-dioxygenase

[d] Initial velocity was determined, since the reaction reached the plateau in approximately 15 sec.

3.4. Fluorine elimination from 4FBA metabolites by pseudomonad strains

Complete detoxification of fluoroaromatics will require an elimination of fluoride ion from such compounds. We tested whether *P. aeruginosa* strains IE653 and PAO1, and *P. putida* strain mt-2 can release the fluoride ions from 4FBA or its metabolites to reveal a relationship between metabolic pathways and fluorine elimination. The high accumulation of fluoride ion was observed in the reaction mixture containing strain IE653 after 8-hour cultivation (Fig. 6-A), although biodegradation of 4FBA was initiated by IE653 within 8 h (Table 2 and Fig. 5). These results indicate that the fluorine is eliminated from 4FBA metabolites not 4FBA itself. Other two strains were only slightly effective for fluorine elimination. On the other hand, fluoride ions from 4FCAT were immediately detected in strain IE653 (Fig. 6-B), indicating that this strain eliminates them after the conversion to 4FCAT. Strains PAO1 and mt-2 released fluoride ions at a much slower rate. *P. aeruginosa* IE653 could not eliminate the fluoride ions from fluoroacetate (data not shown).

Table 2. Mass balance of 4FBA metabolites and BA metabolites by pseudomonad strains.

	IE653							
Time	4FBA biodegradation				BA biodegradation			
	Concentration of chemicals[a] (μM)				Concentration of chemicals (μM)			
(hour)	4FBA	4FBZ	FMS	FHMS	BA	BZ	MS	HMS
4	580±4[b]	260±5	NDeterm[c]	15±3	712±9	120±2	40±5	20±5
8	410±7	122±8	NDeterm	54±5	365±5	60±8	26±2	15±3
24	211±4	81±1	NDeterm	19±3	104±1	75±2	15±3	7±1

	mt-2							
Time	4FBA biodegradation				BA biodegradation			
	Concentration of chemicals (μM)				Concentration of chemicals (μM)			
(hour)	4FBA	4FBZ	FMS	FHMS	BA	BZ	MS	HMS
4	450±7	221±3	NDeterm	28±5	420±3	220±2	21±5	21±1
8	101±7	320±2	NDeterm	75±2	50±2	151±9	9±7	11±1
24	NDetect[d]	561±6	NDeterm	27±2	NDetect	72±4	6±1	7±3

	PAO1							
Time	4FBA biodegradation				BA biodegradation			
	Concentration of chemicals (μM)				Concentration of chemicals (μM)			
(hour)	4FBA	4FBZ	FMS	FHMS	BA	BZ	MS	HMS
4	611±6	289±2	NDeterm	NA[e]	550±6	292±2	NDeterm	NA
8	432±3	519±2	NDeterm	NA	410±2	271±3	NDeterm	NA
24	232±5	430±5	NDeterm	NA	202±1	150±3	NDeterm	NA

[a] 4FBA, 4-fluorobenzyl alcohol. 4FBZ, 4-fluorobenzoic acid. FMS, fluoromuconate. FHMS, fluoro-2-hydroxymuconic semialdehyde. BA, benzyl alcohol. BZ, benzoic acid. MS, muconic acid. HMS, 2-hydroxymuconic semialdehyde.

[b] Values are given ± standard deviations.

[c] Not determined.

[d] Not detected.

[e] Not applicable.

3.5. Fluorine elimination pathway

P. aeruginosa strain IE653 has ortho and meta cleavage enzymes, which cleavage carbon-carbon bonds of catechol in position of C-1,2 and C-2,3, respectively (Table 1). We tested whether the fluorine-elimination pathway is ortho pathway or meta pathway, or both. The active form of iron in catechol 2,3-dioxygenase is in a ferrous state and the enzyme activity is inactivated by H_2O_2 due to the conversion of the active ferrous form to the inactive ferric form[32]. In contrast, the active form of iron in catechol 1,2-dioxygenase is a ferric iron and thus resistance to the oxidant[29]. Therefore, we used this phenomenon for examining an elimination of fluorine from fluorinated compounds. The activity of catechol 2,3-dioxygenase in crude extracts of strain IE653 was completely inactivated by H_2O_2 although catechol 1,2-dioxygenase was active after H_2O_2 treatment (Table 1). Furthermore, the inactivation of meta cleavage enzyme resulted in an increased activity of ortho cleavage enzyme. It is probably due to increased amonts of substrate (catechol) available for catechol 1,2-dioxygenase because of inactivation of the competitor, catechol 2,3-dioxygenase. The fluoride ions were detected from the solution with untreated and H_2O_2-treated cell extracts (Table 1). However, a higher concentration of fluoride ion was produced in the reaction mixture with H_2O_2-treated crude extracts than that with untreated extracts. These results suggest that ortho pathway may be a major pathway for fluorine elimination.

4. Discussion

The utilization of 4-fluorobenzoate as a sole source of carbon and energy has been described earlier for several bacterial genera including *Pseudomonas*[18,33,42,44,46], *Paracoccus*[23], *Acinetobacter*[49], *Corynebacterium*[21,22], *Alcaligenes*[33] and *Aureobacterium*[33]. We isolated *P. aeruginosa* strain IE653, which can utilize 4FBA as a sole energy (Fig. 1), from industrial effluents in Kitakyushu, Japan. This strain IE653 converted 4FBA to 4FBZ, 4FP, 4FCAT, 3FM and FHMS. ^{19}F NMR spectra of 3FM (for chemical shift [–108 ppm] and coupling constant [22 and 32 Hz] were consistent with the results of Boersma *et al.*[3]. Also, *P. putida* mt-2 produced the same metabolites as strain IE653 but not 4FP, in agreement with the results of the previous studies[19,20]. BZ or 4FBZ oxidation pathway described in this paper resembles the toluene dioxygenase pathway. In the toluene dioxygenase pathway, toluene is oxidized to toluene *cis* glycol, which is further oxidized to 3-methylcatechol. It is known that the first intermediate, toluene *cis* glycol, is non-enzymatically dehydrated to cresol[10,39]. Therefore, it is possible that 4FP is produced from 4FCDHC through non-enzymatic or enzymatic dehydration and decarboxylation.
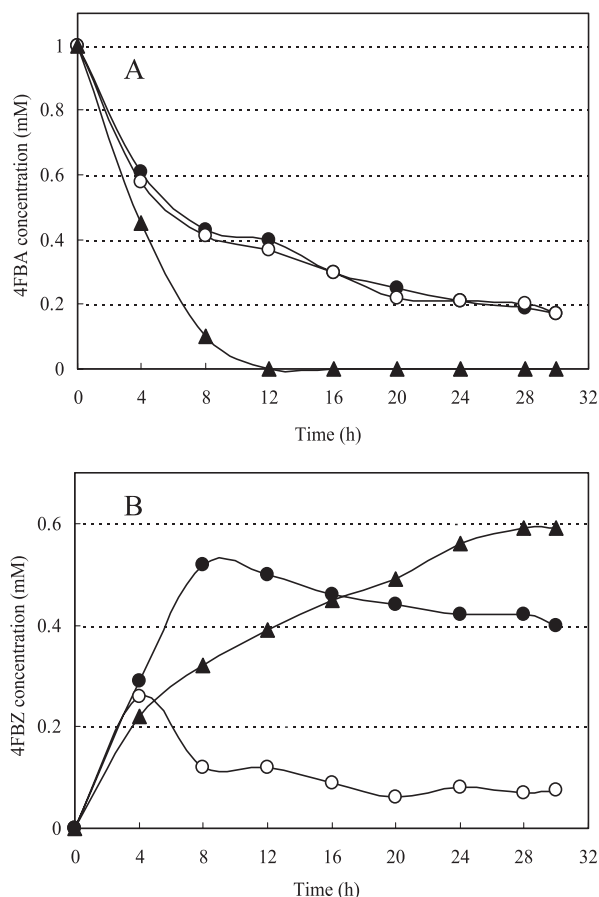
Fig. 5. Biotransformation of 4FBA by *P. aeruginosa* strains IE653 (○) and PAO1 (●), and *P. putida* strain mt-2 (▲). (A) biodegradation of 4FBA. (B) accumulation of 4FBZ.

Fig. 6. Release of fluoride ions from 4FBA (A) or 4FCAT (B) by *P. aeruginosa* strains IE653 (○) and PAO1 (●), and *P. putida* strain mt-2 (▲).

Further investigation is required to elucidate effects of 4FP formation on efficiency of 4FBZ degradation in *P. aeruginosa* IE653.

4FBZ, which is a major product of 4FBA, was resistance to degradation by *P. aeruginosa* PAO1 and *P. putida* mt-2 whereas *P. aeruginosa* IE653 was able to gradually biodegrade 4FBZ. Interestingly, strain IE653 accumulated a high concentration of 4-chlorobenzoate (4CBZ) when it was incubated with 4-chlorobenzyl alcohol (data not shown). These results suggest that enzymes involved in 4FBZ oxidation in IE653 cannot oxidize 4CBZ. The metabolisms of 4FBZ, 4CBZ and BZ may be key steps for efficient biotransformation of cognatic aromatics. Strains PAO1 and mt-2 accumulated 4FBZ from 4FBA but not BZ from BA, suggesting that fluorine substitution in benzoate hampers further degradation of 4FBZ in strains PAO1 and mt-2. We will need to reveal why *P. aeruginosa* IE653 can biodegrade 4FBZ effectively and whether the second pathway (producing 4FP) in IE653 is important for this insight, and investigate the difference of structure or specificity of enzymes involved in degrading 4FBZ.

Results in Fig. 6 suggest that fluorine is eliminated from 4FCAT or its metabolites but not from 4FBA. However, we do not know which compound(s) is a substrate of fluorine elimination. Schreiber *et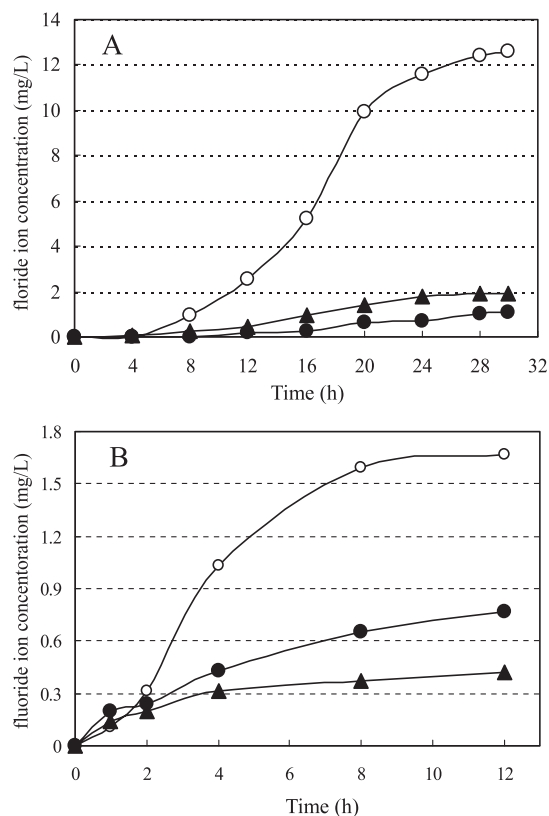 al.*[44] reported that 3FM produced by ortho cleavage of 4FCAT was further converted to maleylacetate via *cis*-dienelactone. They suggested that a nonenzymatic fluorine elimination from 4-fluoromuconolactone occurred in the reaction. Further investigations are required to eludidate whether removal of fluorine from metabolites of 4FBA by *P. aeruginosa* IE653 is enzymatic or chemical reaction.

P. aeruginosa strain IE653 can efficiently biotransform 4FBA and can detoxify it via an elimination of fluorine from it. Our strain may be valuable to develop the bioremediation technology for elimination of fluorine and detoxification of fluoroaromatics.

References

1) Behrman, E.J., and R.Y. Stanier. 1957. Observations on the oxidation of halogenated nicotinic acid. J. Biol. Chem. 228: 947–953.
2) Blackledge, C.A., J.K. Nicholson, and I.D. Wilson. 2003. An NMR study of the metabolic fate of 2-, 3- and 4-fluorobenzyl alcohols in the rat: detection of N-acetylcysteinyl conjugates as minor metabolites in urine. J. Pharm. Biomed. Anal. 32: 133–140.
3) Boersma, M.G., T.Y. Dinarieva, W.J. Middelhoven, W.J.W. Van Berkel, J. Doran, J. Vervoort, and I.M.C.M. Rietjens. 1998. ^{19}F nuclear magnetic resonance as a tool to investigate microbial degradation of fluorophenols to fluorocatechols and fluoromuconates. Appl. Environ. Microbiol. 64: 1256–1263.
4) Bondar, V.S., M.G. Boersma, E.L. Golovlev, J. Vervoort, W.J.H. Van Berkel, Z.I. Finkelstein, I.P. Solyanikova, L.A. Golovleva,

and L.M.C.M. Rietjens. 1998. ¹⁹F NMR study on the biodegradation of fluorophenols by various *Rhodococcus* species. Biodegradation 9: 475–486.

5) Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

6) Clarke, K.F., A.G. Cally, A. Livingstone, and C.A. Fewson. 1975. Metabolism of monofluorobenzoates by *Acinetobacteri calcoaceticus* N.C.I.B. 8250. Formation of monofluorocatechols. Biochim. Biophys. Acta 404: 16–179.

7) Dehab, G.M., S.E. Gerges, and M.S. Abdel-Rahman. 1986. Effect of benzyl chloride on rat liver functions. J. Toxicol. Environ. Health 18: 431–439.

8) Eichelberg, K., and J.E. Galan. 2000. The flagellar sigma factor FliA (σ²⁸) regulates the expression of *Salmonella* genes associated with the centisome 63 type III secretion system. Infect. Immunity 68: 2735–2743.

9) Engesser, K.H., E. Schmidt, and H.-J. Knackmuss. 1980. Adaptation of *Alcaligenes eutrophus* B9 and *Pseudomonas* sp. B13 to 2-fluorobenzoate as growth substrate. Appl. Environ. Microbiol. 39: 68–73.

10) Faizal, I., K. Dozon, C.S. Hong, A. Kuroda, N. Takiguchi, H. Ohtake, K. Takeda, H. Tsunekawa, and J. Kato. 2005. Isolation and characterization of solvent-tolerant *Pseudomonas putida* strain T-57, and its application to biotransformation of toluene to cresol in a two-phase (organic-aqueous) system. J. Ind. Microbiol. Biotechnol. 32: 542–547.

11) Fansheir, D.W., L.K. Gottwald, and E. Kun. 1964. Studies on specific enzyme inhibitors. VI. Characterization and mechanism of action of the enzyme-inhibitory isomer of monofluorocitrate. J. Biol. Chem. 239: 425–434.

12) Fetznar, S., and F. Lingens. 1994. Bacterial dehalogenases: biochemistry, genetics, and biotechnological applications. Microbiol. Rev. 58: 641–685.

13) Fewson, C.A. 1981. Biodegradation of aromatics with industrial relevance, pp. 141–179. In T. Leisinger and A.M. Cook (ed.), Microbial degradation of xenobiotics and recalcitrant compounds. Academic Press, London, United Kingdom.

14) Fukuda, K., H. Matsushita, H. Sakabe, and K. Takemoto. 1981. Carcinogenicity of benzyl chloride, benzal chloride, benzotrichloride and benzoyl chloride in mice by skin application. Gann 72: 655–664.

15) Goldman, P. 1965. The enzymatic cleavage of the carbon-fluorine bond in fluoroacetate. J. Biol. Chem. 240: 3434–3438.

16) Goldman, P., G.W.A. Milne, and M.T. Pignataro. 1967. Fluorine containing metabolites formed from 2-fluorobenzoic acid by *Pseudomonas* species. Arch. Biochem. Biophys. 118: 178–184.

17) Haggblom, M.M. 1992. Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiol. Rev. 103: 29–72.

18) Haper, D.B., and E.R. Blakley. 1971. The metabolism of p-fluorobenzoic acid by a *Pseudomonas* sp. Can. J. Microbiol. 17: 1015–1023.

19) Harayama, S., M. Rekik, M. Wubbolts, K. Rose, R.A. Leppik, and K.N. Timmis. 1989. Characterization of five genes in the upper-pathway operon of TOL plsmid pWW0 from *Pseudomonas putida* and identification of the gene products. J. Bacteriol. 171: 5048–5055.

20) Harayama, S., and M. Rekik. 1990. The meta cleavage operon of TOL degradative plasmid pWW0 comprises 13 genes. Mol. Gen. Genet. 221: 113–120.

21) Ivoilov, V.S., Y.N. Karasevich, and E.G. Surovtseva. 1985. Eine Mutante von Corynebacterium fascians, die die Fahigkeit verloren hat, Benzoesaure zu verwerten. Mikrobiologiya 54: 502–504. (German translation from the Russian original by Universitatsbibliothek Stuttgart, no. U/340.)

22) Ivoilov, V.S., Y.N. Karasevich, and E.G. Surovtseva. 1987. Conversion and utilization of 4-fluorobenzoic acid by Coryne-

23) Karasevich, Y.N., and N.Y. Tatarinova. 1979. Utilization of monofluorobenzoic acids by bacteria *Paracoccus denitrificans*. Microbiology 48: 798–801.

24) Key, B.D., R.D. Howell, and C.S. Criddle. 1997. Fluorinated organics in the biosphere. Environ. Sci. Technol. 31: 2445–2454.

25) Kim, D., M.J. Park, S.-C. Koh, J.-S. So, and E. Kim. 2002. Three separate pathways for the intial oxidation of limonene, biphenyl, and phenol by *Rhodococcus* sp. strain T104. J. Microbiol. 40: 86–89.

26) Masukawa, T., M. Sai, and Y. Tochino. 1989. Methods for depleting brain glutathione. Life Sci. 44: 417–424.

27) McCann, J., N.E. Spingarn, J. Kobori, and B.N. Ames. 1975. Detection of carcinogen as mutagens: bacterial tester strains with R factor plasmids. Proc. Natl. Acad. Sci. USA. 72: 979–983.

28) Milne, G.W.A., P. Golman, and J.L. Holtzman. 1968. The metabolism of 2-fluorobenzoic acid. II. Studies with 18O2. J. Biol. Chem. 243: 5374–5376.

29) Nakazawa, T., M. Nozaki, O. Hayaishi, and T. Yamano. 1969. Studies on pyrocatechase. II. Electron spin resonance and other properties of iron in the active center. J. Biol. Chem. 244: 119–125.

30) Nakazawa, T., and T. Yokota. 1973. Benzoate metabolism in *Pseudomonas putida* (*arvilla*) mt-2: Demonstration of two benzoate pathways. J. Bacteriol. 115: 262–267.

31) Nozaki, M. 1970. Metapyrocatechase (*Pseudomonas*), p. 524. In H. Tabor and C.W. Tabor (ed.), Methods in enzymology, vol. 17A, Academic Press Inc., New York.

32) Nozaki, M., K. Ono, T. Nakazawa, S. Kotani, and O. Hayashi. 1968. Metapyrocatechase. II. The role of iron and sulfhydryl groups. J. Biol. Chem. 243: 2682–2690.

33) Oltmanns, R.H., R. Muller, M.K. Otto, and F. Lingens. 1989. Evidence for a new pathway in the bacterial degradation of 4-fluorobenzoate. Appl. Environ. Microbiol. 55: 2499–2504.

34) Otto, M.K., F. Morsberger, K.D. Kulbe, R. Muller, T. Schenk, and J. Thiele. 1987. Simplified detection of biological dehalogenation with a halide sensor, pp. 437–439. In D. Behren (ed.), DECHEMA biotechnology conferences, vol. 1. Technology of biological processes-safety in biotechnology-applied genetic engineering. VCH Verlags-Gesellschaft, Weinheim, Federal Republic of Germany.

35) Pauling, L. 1948. The nature of the chemical bond, 2nd ed. Cornell University Press, New York.

36) Peters, R.A. 1952. Lethal synthesis. Proc. R. Soc. Lond. B. Biol. Sci. 139: 143–170.

37) Reineke, W., and H.J. Knackmuss. 1989. Microbial degradation of haloaromatics. Annu. Rev. Microbiol. 42: 263–287.

38) Reinscheid, U.M., H. Zuilhof, R. Muller, and J. Vervoort. 1998. Biological, thermal and photochemical transformation of 2-trifluoromethylphenol. Biodegradation. 9: 487–499.

39) Robertson, J.B., J.C. Spain, J.D. Haddock, and D.T. Gibson. 1992. Oxidation of nitrotoluenes by toluene dioxygenase: evidence for a monooxygenase reaction. Appl. Environ. Microbiol. 58: 2643–2648.

40) Ryan, D.A., J.K. Sanders, G.C. Curtis, and H. Hughes. 1995. NMR study of whole rat bile: the biliary excretion of 4-cyano-N,N-dimethyl aniline by an isolated perfused rat liver and a liver in situ. J. Pharm. Biomed. Anal. 13: 735–745.

41) Sambrook, J., E.F. Fritsch, and T. Maniatis (ed.). 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

42) Schennen, U., K. Braun, and H.-J. Knackmuss. 1985. Anaerobic degradation of 2-fluorobenzoate by benzoate-degrading, denitrifying bacteria. J. Bacteriol. 161: 321–325.

43) Schlomann, M., P. Fischer, E. Schmidt, and H.-J. Knackmuss. 1990. Enzymatic formation, stability, and spontaneous reactions of 4-fluoromuconolactone, a metabolite of the bacterial

degradation of 4-fluorobenzoate. J. Bacteriol. 172: 5119–5129.

44) Schreiber, A., M. Hellwig, E. Dorn, W. Reineke, and H.-J. Knackmuss. 1980. Critical reactions in fluorobenzoic acid degradation by *Pseudomonas* sp. B13. Appl. Environ. Microbiol. 39: 58–67.

45) Sota, M., M. Endo, K. Nitta, H. Kawasaki, and M. Tsuda. 2002. Characterization of a class II defective transposon carrying two haloacetate dehalogenase genes from *Delftia acidovorans* plasmid pUO1. Appl. Environ. Microbiol. 68: 2307–2315.

46) Surovtseva, E.G., and Y.N. Karasevich. 1976. para-Aminobenzoic acid as the sole source of carbon and energy for *Pseudo-*

monas desmoliticum. Microbiology 45: 565–568.

47) Tonomura, K., F. Futai, O. Tanabe, and T. Yamaoka. 1965. Defluorination of monofluoroacetate by bacteria. Part I. Isolation of bacteria and their activity of defluorination. Agric. Biol. Chem. 29: 124–128.

48) Uziel, M., N.B. Munro, D.S. Katz, T. Vo-Dinh, E.A. Zeighami, M.D. Waters, and J.D. Griffith. 1992. DNA adduct formation by 12 chemicals with populations potentially suitable for molecular epidemiological studies. Mutat. Res. 277: 35–90.

49) Zaitsev, G.M., and B.P. Baskunov. 1985. Utilization of 3-chlorobenzoic acid by *Acinetobacter calcoaceticus*. Microbiology 54: 158–164.