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Critical Reactions in Fluorobenzoic Acid Degradation by *Pseudomonas* sp. B13

A. SCHREIBER, M. HELLWIG, E. DORN, W. REINEKE, AND H.-J. KNACKMUSS*

Institut für Mikrobiologie der Gesellschaft für Strahlen- und Umweltforschung und der Universität, D-3400 Göttingen, Federal Republic of Germany

3-Chlorobenzoate-grown cells of *Pseudomonas* sp. B13 readily cometabolized monofluorobenzoates. A catabolic pathway for the isomeric fluorobenzoates is proposed on the basis of key metabolites isolated. Only 4-fluorobenzoate was utilized and totally degraded after a short period of adaptation. The isoenzymes for total degradation of chlorocatechols, being found during growth with 3-chlorobenzoate or 4-chlorophenol, were not induced in the presence of fluorobenzoates. Correspondingly, only the ordinary enzymes of the benzoate pathway were detected in 4-fluorobenzoate-grown cells. Ring cleavage of 3-fluorocatechol was recognized as a critical step in 3-fluorobenzoate degradation. 2-Fluoro-*cis,cis*-muconic acid was identified as a dead-end metabolite from 2- and 3-fluorobenzoate catabolism. During 2-fluorobenzoate cometabolism, fluoride is eliminated by the initial dioxygenation.

The fluoro-substituted aromatic nucleus is frequently used as a structural constituent and useful aid in modern preparative organic chemistry. The number of new preparations of pesticides and pharmaceutical agents with the fluorinated aromatic ring is rapidly increasing. The exceptional biological activity of aromatic fluorine compounds can be explained by the dichotomic behavior of fluorine as a substituent together with its hydrogen-resembling size. On the one hand, the π electrons of the aromatic ring are polarized under the influence of fluorine as a substituent; on the other hand, a Coulombic repulsion exists between the electron pair on fluorine and the π electrons. Scant information is available on the metabolic and cometabolic fate of fluorinated aromatic compounds in bacteria. Until now, only a few bacterial strains have been described which utilize fluorobenzoates as the sole source of carbon and energy (13-15, 27).

Pseudomonas sp. B13 (DSM Göttingen 624) utilizes benzoate and 3-chloro- and 3-bromobenzoate (7), but not 3-fluorobenzoate, as sole sources of carbon and energy. The chloro- and bromo-substituted aromatic compounds, including chlorophenols, are completely degraded by this organism via the ortho-cleavage pathway (18). Preliminary experiments have shown that all isomeric fluorobenzoates are also readily cometabolized. In this paper we present analytical data about the metabolic fate and critical steps of the catabolism of fluorobenzoates. These compounds appear to be good models for investigations of the mechanism of the biological persistence of halogenated aromatics. Since fluorine, in contrast to the chlorine and bromine, is only 20% larger than hydrogen, the electronic influences, particularly the inductive effects of fluorine as a substitutent, would largely be unaffected by steric effects.

MATERIALS AND METHODS

Growth of *Pseudomonas* sp. B13. *Pseudomonas* B13 was grown on an appropriate carbon source in a mineral salts medium as described by Dorn et al. (7). For 1-liter cultures, a Multigen F 2000 fermentor, New Brunswick Scientific Co., New Brunswick, N.J., was used. Larger-scale growth was carried out at 30°C in 5- or 10-liter Biostat fermentors from Braun, Melsungen, Germany. Growth was monitored spectrophotometrically by measuring the turbidity at 546 nm. For cometabolism experiments, cells were harvested by centrifugation and washed in 0.05 M phosphate buffer, pH 7.4. Viable counts were made on nutrient broth agar plates.

Analytical methods. Fluoride concentrations in the culture fluid were determined with an ion-selective electrode, model 9606 from Orion Research Inc., Cambridge, Mass. The calibration curve was measured by using freshly prepared standard solutions of sodium fluoride (10^{-6} to 10^{-1} M) in 50 mM phosphate buffer. Concentrations of substrates and metabolites in the culture fluid were determined by reverse-phase high-pressure liquid chromatography (HPLC) as described by Knackmuss et al. (17). Peaks were assigned to authentic compounds by measuring the ultraviolet spectra when the flow was stopped during maximum absorbance, using a variable-wavelength spectrometric detector model 635, Varian-Techtron, Springvale, Australia.

Metabolites were characterized and identified spectrometrically by use of an ultraviolet-visible recording

spectrophotometer model DMR 10 from Zeiss, Oberkochen, Germany, an infrared spectrophotometer model SP 1000 from Pye-Unicam Ltd., Cambridge, England, a nuclear magnetic resonance (NMR) spectrometer model HA-100 from Varian, Palo Alto, Calif., and a mass spectrometer model 21-492 from the Du Pont Co., Wilmington, Del. Melting points, uncorrected, were determined by an apparatus of Tottoli from Büchi, Flawil, Switzerland.

Cell-free extracts were prepared, and enzyme activities were determined as described by Dorn and Knackmuss (8). Dioxygenation of benzoates was measured by use of the HPLC method (23). Protein content of whole cells was assayed by a modified form of the La Riviere method (26). In cell extracts, the method of Warburg and Christian was used (29).

Extraction of metabolites. The culture fluids, which had been separated from bacterial cells by centrifugation, were evaporated at 20°C to 1/15 the original volume. After being cooled to 0°C, the solution was acidified to pH 2 by the addition of concentrated phosphoric acid. To avoid artificial products, the ice-cold solution was extracted as fast as possible with cold ethyl acetate until HPLC analysis indicated complete extraction. The organic phases were combined, dried over $MgSO_4$, and evaporated to a small volume.

Chromatography of metabolites. Metabolites, or their methyl ester derivatives which had been prepared with a slight excess of diazomethane in ether, were separated on preparative silica gel layers (0.2-cm Silica gel, 60 PF 254; E. Merck AG, Darmstadt, Germany). A solvent system of diisopropyl ether/formic acid/water (100:3.5:1.5, vol/vol/vol) was used for free acids, whereas hexane/dichloromethane (60:40, vol/vol) or diisopropyl ether was used for the separation of the methylated metabolites. Bands were located under ultraviolet light and eluted by diisopropyl ether. In the case of the acidic metabolites, the diisopropyl ether solvent contained 5% formic acid. The solvent was evaporated, and the compounds were recrystallized from appropriate solvents.

Chemicals. The 2- and 4-fluorobenzoates were purchased from Fluka, Buchs, Switzerland, and 3-fluorobenzoic acid was from Sigma Chemical Co., St. Louis, Mo. 3,5-Cyclohexadiene-1,2-diol-1-carboxylic acid and 6-fluoro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid were prepared by the method of Reineke et al. (24). Their contents were determined by the amounts of reduced nicotinamide adenine dinucleotide formed by enzymatic dehydrogenation. *cis,cis*-Muconic acid was synthesized by use of the method of Wacek and Fiedler (28). 3-Chlorocatechol was prepared from 3-aminoveratrole (see below) by a Sandmeyer reaction (16) followed by an ether cleavage with $AlCl_3$ (19). Monofluorocatechols were synthesized from the corresponding aminoveratroles by the method of Corse and Ingraham (6). 4-Aminoveratrole was bought from Ega-Chemie, Steinheim, Germany. 3-Aminoveratrole was prepared from 2,3-dimethoxybenzoic acid (Ega-Chemie). The latter compound was converted into the acid chloride by thionyl chloride (21). Aminolysis (20) yielded 2,3-dimethoxybenzoic acid amide (~90% yield referred to 2,3-dimethoxybenzoic acid). 3-Aminoveratrole was obtained in 80% yield from the acid amide by means of a Hofmann reaction (2). Authenticity and purity of fluorocatechols were ascertained by NMR, infrared, and ultraviolet spectra, and by melting points and chromatographic data. All other chemicals used for mineral salts and buffer solutions were of analytical-grade quality and were purchased from Merck.

RESULTS

Fluorobenzoates as growth substrates. None of the isomeric monofluorobenzoates could substitute the growth substrate 3-chlorobenzoate when being added to exponentially growing cells of *Pseudomonas* sp. B13. No significant increase in cell numbers was observed during 6 h when 3-chlorobenzoate-grown cells were transferred into mineral salts medium with fluorobenzoates (Fig. 1). When 3-fluorobenzoate was added to the medium, the number of living cells decreased rapidly after 1 h of incubation.

Growth with 4-fluorobenzoate. After an adaptation period of about 3 days, a 3-chlorobenzoate-grown culture could utilize 4-fluorobenzoate as sole carbon source (Fig. 2). During growth, no major metabolite was detected by the use of HPLC. All of the organically bound fluorine was eliminated as fluoride after exhaustion of 4-fluorobenzoate, indicating complete degradation of the carbon source. The mean doubling time was found to be 6.5 h, compared with 2.25 h during growth on 3-chlorobenzoate.

Specific activities of catabolic enzymes in 4-fluorobenzoate-grown cells resemble those found

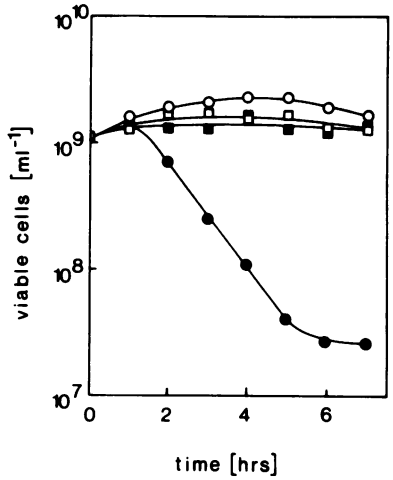

FIG. 1. *Viable count of 3-chlorobenzoate-grown cells in the presence of monofluorobenzoates. 3-Chlorobenzoate-grown cells were harvested and suspended in fresh mineral medium supplemented with 5 mM 2-fluorobenzoate* (○), *3-fluorobenzoate* (●), *or 4-fluorobenzoate* (■). *Mineral medium without substrate was used as a blank* (□). *Samples were taken at hourly intervals and diluted and plated on nutrient broth agar.*

in benzoate-grown cells (Table 1). The isoenzyme pyrocatechase II, indicated by the activity with 3-chlorocatechol as substrate, was induced only to a small extent. Dihydrodihydroxybenzoate dehydrogenase activity was almost three times higher than in benzoate-grown cells.

2- and 3-Fluorobenzoates as potential growth substrates. In batch culture *Pseudomonas* sp. B13 could not be adapted to the utilization of 2- and 3-fluorobenzoate. When the organism was grown in continuous culture on 3-chlorobenzoate, this substrate could gradually be replaced by 2-fluorobenzoate. After an adaption period of about 4 months, strains could be isolated which had adopted the ability to utilize 2-fluorobenzoate as sole source of carbon and energy (11).

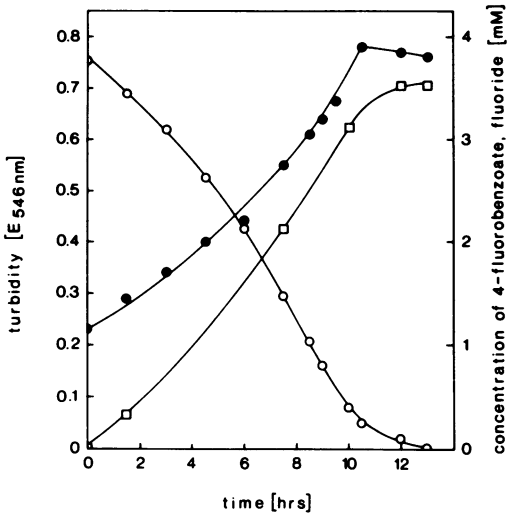

FIG. 2. *Growth of Pseudomonas sp. B13 with 4-fluorobenzoate as sole carbon source. Increase in cell density (●) was determined photometrically at 546 nm. Concentration of substrate (○) was measured by HPLC, and fluoride concentration (□) was measured by an ion-selective electrode.*

Several attempts have been made to replace the growth substrate 3-chlorobenzoate by its 3-fluoro-analog as sole carbon source. A continuous culture is extremely labile in the presence of a relatively low concentration of 3-fluorobenzoate in the culture fluid. With 20 mM 3-chlorobenzoate, the addition of 1 mM 3-fluorobenzoate to the reservoir always resulted in an accumulation of both substrates and a number of metabolites (including fluorocatechols) and subsequent death of the culture.

Cometabolism of fluorobenzoates by 3-chlorobenzoate-grown cells. Comparative kinetic studies of the cometabolism of substituted benzoates by 3-chlorobenzoate-grown cells of *Pseudomonas* sp. B13 indicated that monofluorobenzoates were better substrates than the chloro- or bromo-analogs (23). The turnover of benzoate was inhibited by the presence of equimolar concentration of the fluorobenzoates (2 mM). Inhibition was found to be 80% for 2-fluorobenzoate, 57% for 3-fluorobenzoate, and 84% for 4-fluorobenzoate. This indicates that monofluorobenzoates are bound more strongly to the enzyme than the natural substrate benzoate. To accumulate maximum amounts of metabolites, portions of fluorobenzoates were added to the incubation mixture until the activity of the cells decreased. Considerable differences were found for the amounts of isomeric substrates that could be metabolized by resting cells (Fig. 3). Only for 4-fluorobenzoate, high turnover rates were maintained for more than 6 h. To avoid an excess of fluorobenzoates, which interfered with the chromatographic separation of metabolites, the concentration of fluorobenzoates was carefully controlled by HPLC during the course of the cometabolic turnover.

Metabolites from 2-fluorobenzoate. When 2-fluorobenzoate was cooxidized by 2-chlorobenzoate-grown cells, two major metabolites were

TABLE 1. *Specific activities of characteristic catabolic enzymes from benzoate-, 4-fluorobenzoate-, and 3-chlorobenzoate-grown cells of Pseudomonas sp. B13*[a]

Enzyme activity	Assay substrate	Sp act with the following growth substrate:		
		Benzoate	4-Fluorobenzoate	3-Chlorobenzoate
Benzoate dioxygenase[b]	Benzoate	0.083	0.071	0.161
Dihydrodihydroxybenzoate dehydrogenase	Dihydrodihydroxybenzoate	0.370	1.027	0.600
Pyrocatechase	Catechol	0.177	1.260	0.640
	3-Chlorocatechol	0.001	0.017	0.121
Muconate cycloisomerase	*cis,cis*-Muconic acid	1.400	0.601	0.050
	3-Methylmuconic acid	0.241	0.154	0.360

[a] Cells were harvested during exponential growth. Enzyme activities were determined as described in the text and are expressed in units per milligram of protein.

[b] Has been measured with whole cells by using the HPLC method (23).

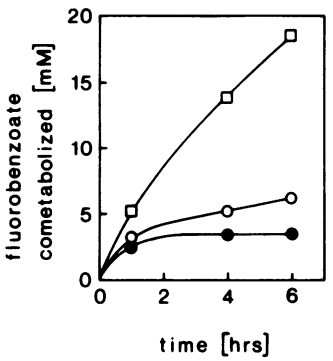

FIG. 3. *Amounts of 2-fluorobenzoate (●), 3-fluorobenzoate (○), and 4-fluorobenzoate (□) that can be cometabolized by resting cells of Pseudomonas sp. B13. Cells were grown on 3-chlorobenzoate in mineral medium, harvested, washed, and suspended in phosphate buffer. The cell density corresponded to a turbidity of E_{546} = 3.5 − 4.5. Substrates were added in small portions so that the concentration of 2- and 4-fluorobenzoate did not exceed 10 mM, and that of 3-fluorobenzoate was kept below 2 mM.*

detected by HPLC. These were extracted from the acidified (pH 2.0) culture fluid by diethyl ether. Part of the etheral solution was methylated with diazomethane for further identification of the metabolites.

Metabolite I was identified as 2-fluoromuconic acid by spectroscopic data. The free acid was obtained as a white crystalline solid, mp 183 to 184°C. The molecular formula of the compound was established as $C_6H_5O_4F$ (160.0172) by high-resolution mass spectrometry and showed a molecular ion with a mass of 160.0179. The ultraviolet absorption spectrum in 0.1 N NaOH had a maximum at 263 nm (log ϵ = 4.3). These findings together with the infrared data (nujol mull, 1,710, 1,700 cm^{-1} for νC=O, 1,640, 1,600 cm^{-1} for νC=C, 1,430, 1,245 cm^{-1} for COOH, and 1,230 cm^{-1} for C-F) agreed with the spectra given by Goldman et al. (14). These authors reported an NMR spectrum (in acetone-d_6) which is similar to that found for metabolite I. Two overlapping multiplets (seven lines) between δ = 7.54 and 8.03 and a doublet (δ = 6.01) result from spin couplings between protons and the fluorine nucleus. Comparison with the data reported for unsubstituted muconic acid esters (10) (see Fig. 4) suggests that the doublet at δ = 6.01 originates from coupling between H_c and H_d. The coupling constant $J_{c,d}$ = 11 Hz favors the *cis* configuration. Furthermore, 2-fluoromuconic acid was characterized as its dimethyl ester, which could be crystallized from aqueous ethanol (colorless needles, mp 49°C). Its ultraviolet spectrum in water exhibited an absorption maximum at 267 nm (log ϵ = 4.3). In the mass spectrum, a molecular ion *m/e* 188.0497 was found, which agrees with a composition of $C_8H_9O_4F$ (188.0485).

Metabolite II from 2-fluorobenzoate cometabolism was formed in very small amounts. A peak in the mass spectrum at *m/e* 170 deviated by 4 ppm from the theoretical value of the molecular ion of unsubstituted muconic acid. In the ultraviolet spectrum (methanol), absorbance maxima were found at 258 and 263 nm which correlated with those of *cis,cis*-muconic acid (I) at λmax 259 and 265 nm (9).

Figure 5 shows the relative yield of 2-fluoromuconic acid and fluoride ion in the culture fluid during cometabolism of 2-fluorobenzoate. Approximately 20% of the cometabolized substrate was converted to 2-fluoromuconic acid, and 80% of the organically bound fluorine appeared as fluoride, which demonstrates that no other fluorine-containing metabolite was formed in larger amounts.

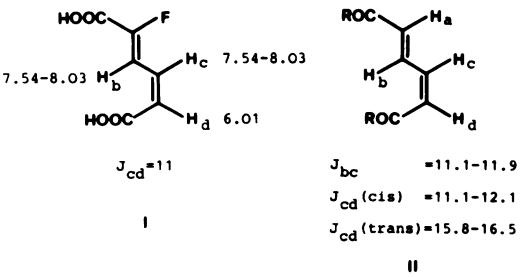

FIG. 4. *NMR data of 2-fluoromuconic acid (metabolite I) from 2-fluorobenzoate. δ values are listed in the formula (ppm, δ_{TMS} = 0, coupling constants are given in hertz). For comparison the range of coupling constants from muconic acid esters II (10) are given.*

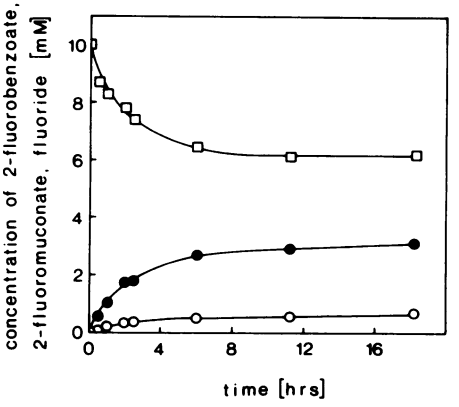

FIG. 5. *Formation of fluoride and 2-fluoromuconic acid from 2-fluorobenzoate by resting cells of Pseudomonas sp. B13 grown with 10 mM 3-chlorobenzoate. 2-Fluorobenzoate (□) and 2-fluoromuconic acid (○) in the culture fluid were determined by HPLC. Fluoride (●) was followed by an ion-selective electrode.*

A mutant of *Pseudomonas* sp. B13, strain 27B, which is defective in the dihydrodihydroxybenzoate dehydrogenase (23), could also cometabolize 2-fluorobenzoate after being induced by 3-chlorobenzoate. A number of metabolites were detected by the use of HPLC. In addition to fluoride, 6-fluoro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid was identified with an authentic sample (retention time and in situ scanning of its ultraviolet spectrum) as a major reaction product. To verify and generalize the elimination of fluoride during dioxygenation of 2-fluorobenzoate, cometabolism was carried out by the corresponding mutant *Alcaligenes eutrophus* B9. About 77% of 2-fluorobenzoate had been converted to 6-fluoro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid, and 23% of the organically bound fluorine had been eliminated as fluoride.

Cometabolism of 3-fluorobenzoate. 3-Chlorobenzoate-grown cells of *Pseudomonas* sp. B13 readily cometabolize 3-fluorobenzoate after prolonged incubation. A single metabolite could be detected and isolated. It was identified with authentic 2-fluoromuconic acid (I) from cometabolism of 2-fluorobenzoic acid. About 30% of 3-fluorobenzoate had been converted to 2-fluoromuconate, whereas 50% of the organically bound fluorine had been eliminated as fluoride. The isolated 2-fluoromuconic acid could not be further degraded by whole cells or crude extracts (11). A faint violet coloration of the medium, indicating fluorocatechol formation, was noticed during independent experiments with 3-fluorobenzoate concentrations exceeding 2 mM. 3-Chlorobenzoate-grown cells were harvested in the late-exponential growth phase and suspended in mineral medium ($E_{546} = 2.5$) containing 5 mM 3-fluorobenzoate. The concentration of 3-fluorobenzoate decreased to 2.4 mM within 30 min. 3- and 4-Fluorocatechols were detected in the culture fluid with maximum concentrations of 1.14 and 0.33 mM. The metabolites were identified by HPLC with authentic compounds (retention time and measuring of their ultraviolet spectra after stopped flow).

Turnover of 4-fluorobenzoate. Cooxidation of 4-fluorobenzoate by 3-chlorobenzoate-grown cells yielded three major metabolites which were separated on preparative silica layers. Compound III, 5-oxo-$^{3(5H),\alpha}$-furan-2-ylacetic acid, was crystallized from dichloroethane (mp 167°C). The ultraviolet spectrum, λ_{max} (water) 277 nm, log $\epsilon = 4.2$, was the same as described by Evans et al. (12) for a metabolite from 4-chlorophenoxyacetate metabolism. Upon addition of alkali, the absorbance maximum was irreversibly shifted to 243 nm, which is typical for 3-hydroxymuconic acid; upon reacidification, only end absorbance was observed. The properties described above were also found for the isomeric metabolite IV, which was available only in very small amounts. The maximum of the ultraviolet spectrum (water) was slightly shifted to shorter wavelengths (274 nm). The two isomers could be separated by reverse-phase HPLC. Also, slight differences were found in the infrared spectra of the methyl esters of III and IV (Table 2).

Significant differences were revealed by the ^{1}H NMR spectra of the methyl esters, so that III could be assigned the *cis-* and IV could be assigned the *trans-* isomer of 5-oxo-$^{3(5H),\alpha}$-furan-2-ylacetic acid. In both spectra, four signals were found for six protons that were consistent with the assignments given in Fig. 6. In the methyl ester of compound III, the low field absorption of H_b results from deshielding by the methoxycarbonyl group attached to a *cis*-configurated exocyclic double bond. The assignment of the *cis-* configuration to compound III is supported by the long-range coupling of the olefinic protons, H_a and H_d, which are only observed for the III isomer. Exclusively in the *cis,cis-* form, five bonds, which separate H_a and H_d, are arranged in a zig-zag configuration. The data found for the methyl esters of III and IV correspond well with the chemical shifts of the δ lactones V and VI along with the long-range coupling for H_a and H_d in the *cis,cis-* isomer (Fig. 6).

Compound VII was isolated in small amounts as a metabolite from 4-fluorobenzoate. After being methylated it was identified as the dimethyl ester of 3-fluoromuconic acid on the basis of its ultraviolet spectrum (methanol, λ_{max} 272 nm), mass spectrum (molecular ion under high-resolution 188.0484, $C_8H_9O_4F$ requires 188.0484), and its ^{1}H NMR spectrum (CCl_4). The NMR data are consistent with the assignment given in Fig. 7, which is supported by comparison with the spectra of muconic esters (II) (10), 3-carboxymuconic acid (1), and 2-fluoromuconic acid (I). The AMX-type spectrum shows a double doublet at $\delta = 7.28$ originating from couplings of H_c with H_d and the fluorine nucleus. When NMR spectra of other fluorine-substituted olefines are compared, the $J_{a,F}$ and $J_{c,d}$ coupling constants suggest the *cis,trans-* configuration of 3-fluoromuconic acid. At least part of the accumulated 3-fluoromuconic acid was lost during extraction and purification because of spontaneous cycloisomerization under acidic conditions (Schmidt and Knackmuss, unpublished data).

The organically bound fluorine of 4-fluorobenzoate was completely eliminated as fluoride upon prolonged incubation. The yield of 3-fluoromuconate never exceeded 5%.

TABLE 2. *Assignments of infrared data of methyl esters of compounds III and IV*[a]

Compound	ν C—H			ν C=O			ν C=C		δ C—H		ν C—O	
	=CH		—CH_3				Exocyclic	Endocyclic				
IV (CCl_4)	3,150 (vw)	3,080 (vw)	2,950 (w)	2,920 (w)	2,850 (vw)	1,805 (s)	1,720 (s)		1,670 (m)	1,365 (m)	1,350 (m)	+	1,150 (s)
III (CCl_4)	3,120 (vw)	3,080 (vw)	3,000 (w)	2,950 (w)	2,850 (vw)	1,805 (s)	1,725 (s)		1,660 (s)	1,375 (s)			1,155 (s)
III (Nujol)	3,150 (w)	3,080 (w)	3,000 (w)	++	++	1,790 (s)	1,710 (s)	1,660 (s)	1,650 (s)	++		1,270 (s)	1,165 (s)

[a] Spectra (in centimeters^{-1}) were measured as CCl_4 solutions or nujol null; areas shaded by the solvents are indicated as + or ++. Relative intensities of bands are marked as: s, strong; m, medium; w, weak; vw, very weak.

Induction of catabolic enzymes. The inability of fluorobenzoates to substitute 3-chlorobenzoate as growth substrate could indicate incomplete induction of certain catabolic enzymes in *Pseudomonas* sp. B13. Therefore, cells were grown on succinate, and the respective fluorobenzoates were added as inducers to the exponentially growing culture, when small amounts of the growth substrate were still present in the medium. The turnover of the fluorobenzoates and specific activities of catabolic enzymes during incubation with the fluorobenzoates are described in Fig. 8.

The rate of decrease in concentration of the fluorobenzoates can be taken as an approximate measure for the benzoate dioxygenase activity of the cells at certain stages of induction. A strong decline in the activity was observed with 3-fluorobenzoate after 5 h of incubation, so that the substrate concentration in the culture fluid kept constant after 7 h. Although enzyme activities of dihydrodihydroxybenzoate dehydrogenase and pyrocatechase I increased rapidly in the presence of 3-fluorobenzoate, only low enzyme levels were attained. It is noteworthy that within 21 h the isoenzyme pyrocatechase II, which was found to be indispensable for the utilization of 3-chlorobenzoate and 4-chlorophenol, was not induced by incubation with fluorobenzoates. Surprisingly, no muconate cycloisomerase activity of benzoate catabolism was detectable.

During incubation with higher concentrations of 3-fluorobenzoate (5 mM), a pinkish color of the culture fluid rapidly developed, again indicating the accumulation of fluorocatechols. These were separated and identified by means of HPLC, using direct injection of the cell-free culture fluid. Relative retention times and in situ-scanned ultraviolet spectra were compared with authentic compounds. The maximum concentration in the culture fluid was found after 1.5 h to be 0.6 mM for 3-fluorocatechol and approximately 0.03 mM for 4-fluorocatechol.

The difficulties encountered in the utilization of fluorobenzoates by bacteria could arise from the accumulation of fluorocatechols as toxic intermediates. Therefore, viability of succinate-grown cells was tested in the presence of catechols.

At catechol concentrations (see Table 3) below 1 mM, 4-substituted catechols exhibited a pronounced higher toxicity than the 3-substituted isomers and unsubstituted catechol. 3-Chlorobenzoate-grown cells were found to be equally sensitive to exogenous catechols.

DISCUSSION

Pseudomonas sp. B13 can readily grow on

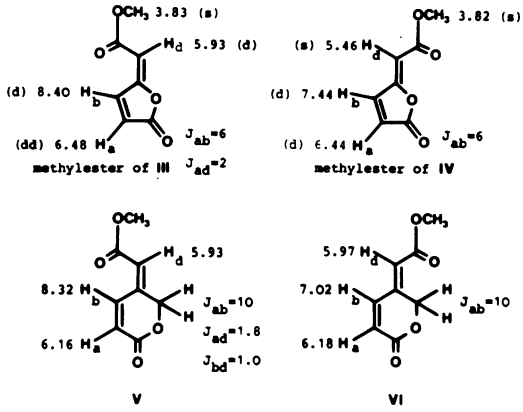

FIG. 6. *NMR data of the methyl esters of metabolite III and IV (deuterochloroform). Assignments were compared with homologous compounds V and VI (1). δ values are listed in the formula (ppm, δ_{TMS} = 0, coupling constants J are given in hertz).*

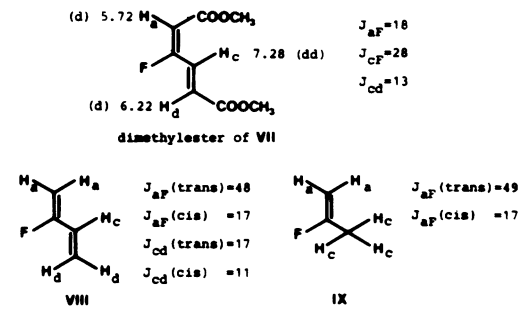

FIG. 7. *NMR data of the dimethyl ester of metabolite VII (CCl_4) (2, 3). δ values are listed in the formula (ppm, δ_{TMS} = 0, coupling constants J are given in hertz).*

benzoate and on 3-chloro- and 3-bromobenzoate. The corresponding 2- and 4-substituted benzoates are not utilized, mainly because of the stereospecificity of the initial enzyme (23). Since fluorine is only 20% larger than hydrogen, all three monofluorobenzoates must be considered as potential growth substrates. Actually, only 4-fluorobenzoate is utilized as the sole source of carbon after a short period of adaptation. The same catabolic enzymes of the 3-oxoadipate pathway are found in *Pseudomonas* sp. B13 when grown on 4-fluorobenzoate or benzoate. In 3-chlorobenzoate-utilizing cells, an approximately twofold-overproduced specific activity of the initial enzymes, benzoate 1,2-dioxygenase

TABLE 3. *Sensitivity of succinate-grown cells against catechols*[a]

Catechol	Survival (%) at the following catechol concn (mM):		
	0.25	0.5	2.0
Catechol	67	12	5
3-Fluorocatechol	98	34	3.5
4-Fluorocatechol	33	5.5	1.5
3-Chlorocatechol	83	11	0.5
4-Chlorocatechol	55	5.5	2

[a] *Pseudomonas* sp. B13 was grown on succinate. Exponentially growing cells were harvested by centrifugation and suspended in 50 mM phosphate buffer, pH 7.4, to 10^8 cells ml^{-1}. Cultures were incubated with various concentrations of the catechols (freshly sublimated before use). The suspensions were aerated by shaking in fluted Erlenmeyer flasks at 30°C. After 2 h, samples were diluted and plated on nutrient broth agar plates. Survival given is related to the control without catechol. The catechol concentrations were constant during incubation, as verified by HPLC analysis.

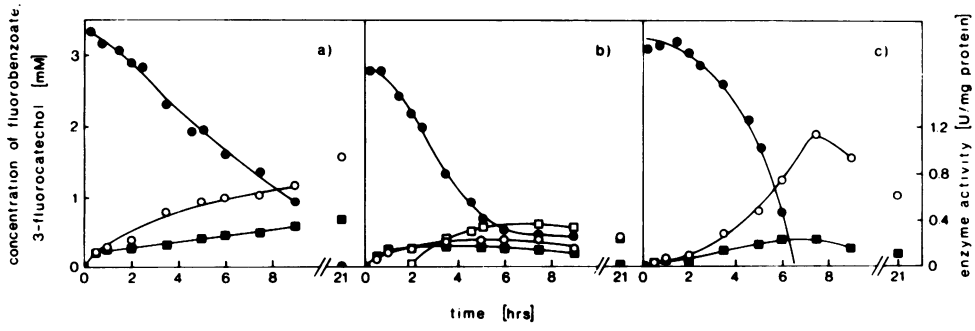

FIG. 8. *Incubation of noninduced cells of Pseudomonas sp. B13 with (a) 2-fluorobenzoate, (b) 3-fluorobenzoate, and (c) 4-fluorobenzoate. Cells were grown overnight in 3.3 liters of mineral medium with a twofold phosphate buffer concentration (pH 6.8) containing 10 mM succinate. After the pH was readjusted to pH 7.0 (4 M H_3PO_4), an additional 70 ml of 1 M sodium succinate was added, and the growing culture was divided into three batches of 1 liter each (multigen F 2000 fermentors). When the culture had grown up to a cell density of about E_{546} = 2.25, fluorobenzoates (3.5 mM) were added. Samples were taken at intervals for determination of fluorobenzoate (●), 3-fluorocatechol (□) in the culture fluid, and for enzyme activities of dihydrodihydroxybenzoate dehydrogenase (■) and pyrocatechase I (○) in cell-free extracts (see text).*

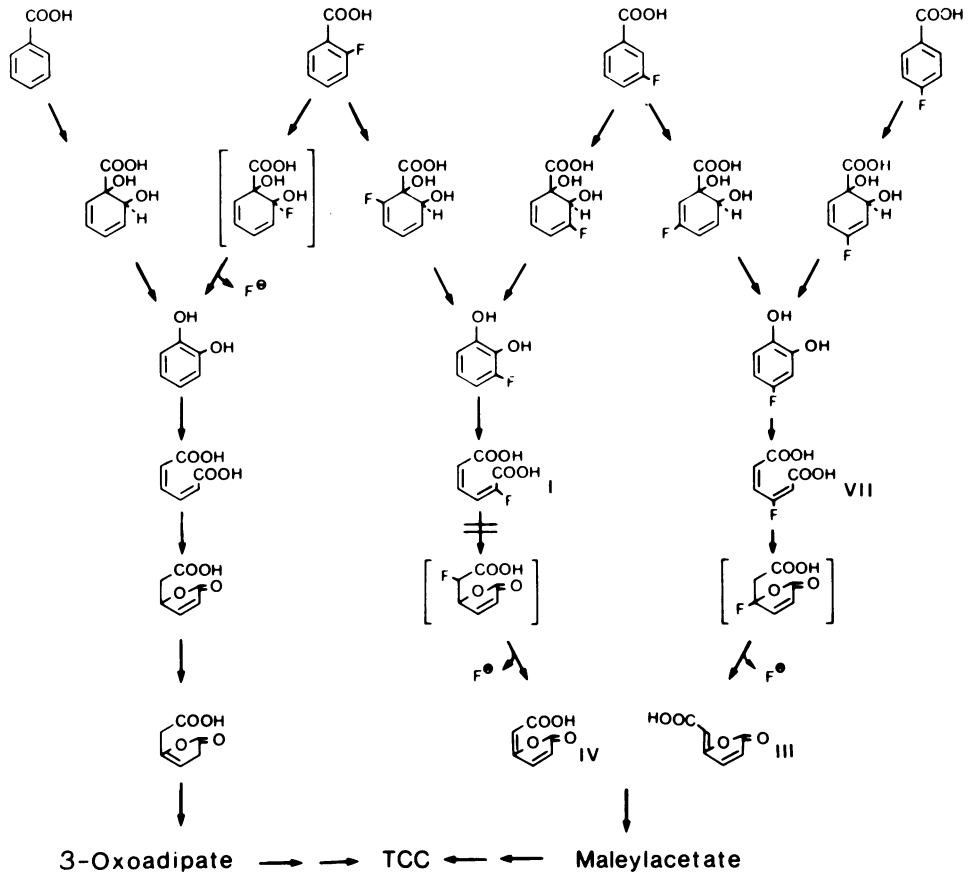

FIG. 9. *Proposed catabolic pathway of fluorobenzoates by Pseudomonas sp. B13.*

and dihydrodihydroxybenzoate dehydrogenase, was found to counterbalance the reduced turnover rates of the chlorinated substrates. Different doubling times during growth of *Pseudomonas* sp. B13 with 3-chloro- or 4-fluorobenzoate can be rationalized when the specific activities of the benzoate 1,2-dioxygenase with the respective substrates from Table 1 and the relative activities published by Reineke and Knackmuss (23) are considered. In 4-fluorobenzoate-grown cells, the activity for this substrate was 0.018 U per mg of protein, whereas 0.05 U per mg of protein was found in 3-chlorobenzoate-grown cells for the latter substrate. This corresponds to the doubling times of 6.5 and 2.25 h with 4-fluoro- and 3-chlorobenzoate, respectively, as growth substrates. In 4-fluorobenzoate-grown cells, the ordinary pyrocatechase I is present in sevenfold-higher specific activity compared with that of benzoate-grown cells. Since 4-fluorocatechol is readily cleaved by this enzyme (8), enzyme overproduction prevented accumulation of this highly toxic intermediate.

Like 4-fluorobenzoate, the 2- and 3-substituted isomers were not recognized as halo-substituted benzoates by *Pseudomonas* sp. B13 (see Fig. 8). Only ordinary enzymes of the benzoate pathway are induced with reasonable activity. In contrast, pyrocatechase II, which was found to be indispensable during growth with 3-chlorobenzoate or 4-chlorophenol, could not be detected during incubations with 2- or 3-fluorobenzoate. The latter two substrates generate 3-fluorocatechol in addition to catechol or 4-fluorocatechol. 3-Fluorocatechol cannot be cleaved and detoxified by ordinary pyrocatechases as being shown for catechol 1,2-dioxygenases from benzoate-grown cells of *Pseudomonas* sp. B13 or *A. eutrophus*. These exhibit less than 0.5% of the reaction rate found with catechol (8). In the course of the induction experiment, no muconate cycloisomerase activity has been induced, not even with 4-fluorobenzoate, which was accepted as a growth substrate after an adaptation period of 2 days. This corresponds to the observation that 3-chlorobenzoate-grown cells could be con-

verted to the utilization of benzoate only after a long lag period. During this period, large amounts of muconate were accumulated (25).

A 3-chlorobenzoate-utilizing continuous culture of *Pseudomonas* sp. B13 was found to be very unstable in the presence of small amounts (10%) of 3-fluorobenzoate in the reservoir. At higher concentrations of the fluoro- compound, accumulation of fluoro-catechols was followed by a rapid decrease in cell density. Lethal catabolism to fluorinated intermediates of the tricarboxylic acid cycle, as suggested for *Acinetobacter calcoaceticus* by Clark et al. (5), is not likely, since *Pseudomonas* sp. B13 is insensitive to fluoroacetate (2.5 mM) even during growth on acetate (2.5 mM). The sensitivity against 3-fluorobenzoate in contrast to that against the 2-substituted isomer can only be explained by the considerably faster turnover of 3-fluorobenzoate so that larger amounts of 3-fluorocatechol could be accumulated.

Characteristic enzyme activities in cell-free extracts suggest that fluorobenzoates follow the catabolic sequence of 3-chloro- or 3-bromobenzoate as shown in Fig. 9 (7). When excess amounts of fluorobenzoates were cometabolized by 3-chlorobenzoate-grown cells, accumulation of metabolites of the proposed pathway occurred. Both fluorocatechols were accumulated in the culture at considerable concentrations when uninduced cells were incubated with relatively high concentrations of 3-fluorobenzoate. 3-Fluorocatechol is the predominant metabolite because its ring cleavage proceeds slowly and 3-fluorobenzoate is preferentially hydroxylated in proximate position to the substituent (23). The identification of the isomeric lactones III and IV, one of which had already been suggested in 4-chlorophenoxyacetate catabolism (12), supports the proposed course of halide elimination (17).

During cooxidation of 2-fluorobenzoate, about 80% of the organic fluorine was found to be eliminated as a consequence of the initial dioxygenation. This is demonstrated by the fluoride-eliminating ability of the dihydrodihydroxybenzoate dehydrogenase-defective mutants *Pseudomonas* sp. B13 strain 27B and *A. eutrophus* B9. The implication of Clarke et al. that subsequent nicotinamide adenine dinucleotide-dependent dehydrogenation of 2-fluoro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid effects fluoride transfer to nicotinamide adenine dinucleotide instead of hydride (5) is inconsistent with this observation. Probably the dihydrodiol or its hypothetical dioxetane precursor, as originally postulated by Milne et al. (22), eliminates fluoride spontaneously. On the basis of this observation, adaptation of *Pseudomonas sp.* B13 and *A. eutrophus* B9 to 2-fluorobenzoate as growth substrate was initiated (11).

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