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3-Fluorobenzoate enriched bacterial strain FLB 300 degrades benzoate and all three isomeric monofluorobenzoates

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Abstract. The bacterial strain FLB300 was enriched with 3-fluorobenzoate as sole carbon source. Besides benzoate all isomeric monofluorobenzoates were utilized. Regioselective 1,2-dioxygenation rather than 1,6-dioxygenation yielded 4-fluorocatechol and minimized the production of toxic 3-fluorocatechol. Degradation of 4-fluorocatechol was mediated by reactions of ortho cleavage pathway activities. Chemotaxonomic and r-RNA data excluded strain FLB300 from a phylogenetically defined genus *Pseudomonas* and suggested its allocation to the alpha-2 subclass of Proteobacteria in a new genus of the *Agrobacterium-Rhizobium* branch.

Key words: 3-Fluorobenzoate – 4-Fluorocatechol – Orthopathway – Chemotaxonomy – rRNA analysis – Quinone pattern analysis – Polyamine pattern analysis – Agrobacterium – Rhizobium branch

Fluorosubstituted aromatic compounds are produced by the plastics, agricultural and pharmaceutical industries (Banks 1979, 1982; Filler 1979; Welch 1987; Gajewski et al. 1988). Despite their widespread use general knowledge of the biochemistry and physiology of these compounds is rather limited when compared with chlorinated chemicals.

Fluorobenzoates have frequently been used as model compounds for bacterial metabolism of fluorinated aromatics (Engesser et al. 1980; Harper and Blakley 1971 a – c; Schreiber et al. 1980; Karasevich and Zaitsev 1984; Hug et al. 1988; Engesser and Schulte 1989).

Although the metabolism of 2- and 4-fluorobenzoate has been investigated in greater detail, little information is available on the microbial degradation of 3-fluorobenzoate. This may be due to the fact that the metabolism of 3-fluorobenzoate by benzoate utilizing bacteria gives rise to the accumulation of fluorocatechols as toxic intermediates (Schreiber et al. 1980; see also Discussion). Karasevich and Tatarinova (1979) reported on a slowly growing *Paracoccus denitrificans* strain degrading 2-, 3- and 4-fluorobenzoate but detailed information on the catabolic pathways were not given. To our knowledge this is the first report which describes the physiology and biochemistry of 3-fluorobenzoate degradation in greater detail.

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Abbreviations: PYES, peptone yeast extract soy medium; TLC, thin layer chromatography; NTA, nitrilotriacetate; SDS-PAGE, sodium dodecylsulphate-polyacrylsulphate gel electrophoresis; FB, fluorobenzoate; DHB, 1,2-dihydro-1,2-dihydroxybenzoate; NB, nutrient broth

Materials and methods

Isolation of strain FLB300. Soil from different places of Nepal was suspended in mineral medium (Dorn et al. 1974) for enrichment with 3-fluorobenzoate (3 mM) as a sole source of carbon and energy. After 3 days of incubation at 30°C in fluted Erlenmeyer flasks on an rotary shaker 0.1 ml of the suspension was plated on mineral medium agar plates containing 3-fluorobenzoate (2.5 mM). One of the fastest growing colonies was picked and purified on the same agar medium. Purity was checked on nutrient agar plates.

Characterization. For taxonomic investigations the strain FLB300 was grown in PYES medium (pH 7.0) containing 0.3% peptone from meat, 0.3% yeast extract, and 0.1% succinic acid. Cells for analysis of polyamines were harvested from exponentially growing cultures, lyophilized, extracted and analyzed according to Scherer and Kneifel (1983). The concentrations of polyamines were calculated as described by Busse and Auling (1988). Extraction and analysis of quinones, isolation of DNA, determination of GC content and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed the procedures of Auling et al. (1986). Serological methods have been described by Auling et al. (1978). Reverse transcriptase sequencing of rRNA basically followed the method developed by Lane et al. (1985). The primer 1392-1406 used (2.5 µg/ml) was a gift from Applied Biosystems.

Growth in liquid culture. Throughout the growth experiments phosphate buffer (50 mM, pH 7.4) was used. Batch cultivation was performed in fluted Erlenmeyer flasks incubated by 30°C on a rotary shaker (150 rpm). For the conditions of continuous cultivation see Knackmuss and Hellwig (1978) with the exception that the culture fluid was pumped of by means of a peristaltic pump (LKB, Bromma, Sweden).

Estimation of doubling times in continuous culture. After determination of the maximal dilution rate td was estimated.

Due to increasing accumulation of autoxidable polyhydric phenols this state was very labile. Repeated measurements had to be done from which the mean values of td were calculated.

For preparation of cell extracts and measurement of enzyme activities with whole cells as well as in crude extracts see Pieper et al. (1988), Schmidt and Knackmuss (1980), Dorn and Knackmuss (1978) and Engesser et al. (1988). Maleylacetate reductase was determined as follows: 500 µl Tris/HCl 100 mM; pH 7.5; 420 µl H_2O; 10 µl crude extract; 20 µl NADH (10 mM); 50 µl Maleylacetate (2 mM). The HPLC solvent system consisted, if not otherwise noted, of water/methanol (85/15 v,v) acidified with H_3PO_4 (1 g/l). The columns were of the reversed phase type (see Pieper et al. 1988). Fluoride was determined with an ion sensitive electrode (Engesser et al. 1980).

Chemicals. Substituted 3,5-cyclohexadiene-1,2-diol-1-carboxylic acids and 2-fluoro-*cis,cis*-muconate were gifts from W. Reineke and E. Schmidt, BUGH Wuppertal, Wuppertal, FRG with the exception of unsubstituted and 6-fluoro-substituted 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid, which were prepared as previously described (Engesser et al. 1980). Highly unstable 3-fluoro-*cis,cis*-muconate was prepared *in situ* by the action of purified catechol-1,2-dioxygenase, a gift from Michael Schlömann (Univ. Stuttgart, Stuttgart, FRG), who also supplied maleylacetate. Substituted catechols were a gift from M. Hellwig (Univ. of Göttingen, Göttingen, FRG).

All other chemicals were of the highest commercially available grade.

Results

Isolation of strain FLB300

Soil from different areas of Nepal was incubated with mineral medium and 3-fluorobenzoate as sole source of carbon and energy. Single colonies were purified on nutrient broth and checked for growth on fluorobenzoates in liquid medium. The fastest growing isolate was designated FLB300. The strain utilized benzoate, the isomeric fluorobenzoates and 4-hydroxybenzoate. After streaking on 3-hydroxybenzoate containing agar plates only few single colonies were observed. No growth could be detected with 2-hydroxy- and 2,3-dihydroxybenzoate.

Taxonomic characterization of strain FLB300

Examination of the Gram-negative strain FLB300 by the multiple-test system API 20 NE yielded the code 1667344 and the isolate was identified by the API profile index as *Agrobacterium radiobacter* with a probability of 99.9%. The GC content of FLB300 was determined as 61.9%. The result would be consistent with an allocation of strain FLB300 to the genus *Agrobacterium*.

Chemotaxonomy has been employed for identification of Gram-negative, aerobic isolates at the genus or even lower level as an approach used alternatively to studies of the ribosomal RNA (Auling et al. 1988a; Busse et al. 1989). For grouping of any isolate within the class *Proteobacteria* which covers the majority of the aerobic, Gram-negative bacteria (Stackebrandt et al. 1988) combined analysis of either quinones and fatty acids (Oyaizu and Komagata 1983) or quinones and polyamines (Busse and Auling 1988) has been recommended. Upon TLC analysis the Gram-negative isolate FLB300 displayed a ubiquinone with 10 isoprenoid units in the side chain (Q-10) as main quinone which allowed its allocation to the alpha-subclass of *Proteobacteria*. Quantitative analysis of polyamines by HPLC yielded the following results (data given in µmoles/g dry weight): putrescine = 5.2, spermidine = 11.2, *sym*-homospermidine = 26.3, and spermine = 0.3. This pattern enabled allocation of strain FLB300 to the alpha-2 subclass of *Proteobacteria* (Busse and Auling 1988).

The analysis of the 16*S* rRNA fragment 1220-1380 (*Escherichia coli* nomenclature) confirmed that FLB300 is a member of the alpha-2 subclass of *Proteobacteria*. The deletion at position 1290 which is typical for members of the alpha subclass (Oyaizu, pers. commun. J. Busse, unpublished results) was detected in the 16*S* rRNA of FLB300. The partial sequences obtained for strain FLB300, *Rhizobium meliloti* strain TE9 which is a nitrilotriacetate (NTA) utilizer (Egli et al. 1988), *Pseudomonas aminovorans* (NCIB 9039, *Agrobacterium tumefaciens* C58, *Agrobacterium spec.* HK4 (Nobile and Deshusses 1986), and *Agrobacterium radiobacter* M2/1 (Oyaizu, unpublished results) displayed at least 98.75% sequence homology.

The strains found to be nearest neighbours of FLB300 as judged by rapid sequencing of rRNA were submitted to SDS-PAGE of soluble proteins together with FLB300 in order to detect any similarity on the subgeneric level. The protein fingerprinting (not shown) indicated a strong similarity of FLB300 to strain HK4. As polyclonal antibodies specific for the NTA utilizing strain TE9 (El-Banna 1989; unpublished results) were available, these were tested for cross-reactivity to FLB300 in Ouchterlony-type double diffusions with bacterial extracts. However, only a week cross-reactivity was detected.

Growth in batch culture

Strain FLB300 was grown in succinate mineral medium as a sole source of carbon and energy. The doubling time was calculated to be 1.2 h at a substrate concentration of 10 mM.

Addition of benzoate to succinate (10 mM) metabolizing cultures influenced growth. The lag phase increased from 1.2 h (2 mM benzoate added) to 2 h (5 mM), 2.5 h (7 mM) and exceeded 3.5 h at a benzoate concentration of 10 mM. This showed the inhibiting influence of benzoate on growing cells of strain FLB300, an effect which was even more pronounced when fluorobenzoates were added. Therefore and due to accumulation of polyhydric phenolic compounds during metabolism of the latter all experiments with fluorobenzoates were done in continuous culture in order to keep substrate concentration low. In batch culture, logarithmic growth could only be detected with benzoate as a substrate (td = 2.1 h; [s] = 5 mM); no constant doubling time was found for fluorobenzoate growth. In the latter case, the cultures were extremely unstable showing a more linear correlation of decrease of substrate concentration and increase in OD. Furthermore a brownish-black coloration was observed indicating intoxication of the cells due to accumulation of fluorosubstituted catechols.

Growth in continuous culture

The steady state optical densities for 2-fluoro-, 3-fluoro- and 4-fluorobenzoate as substrate (influent concentration

Table 1. Relative amounts of fluoride and 2-fluoromuconate after growth of FLB300 with fluorobenzoates. Cells were grown in continuous culture as described in Materials and methods. For 3FB as the most interesting substrate results of three independent experiments are shown. For determination of fluorobenzoates (FB) at the beginning (t_0) or the end of the experiments (t_1) and 2-fluoromuconate (FM) see text; determination of fluoride (F^-) was done as described in Materials and methods

	Compounds and ions [mM] after growth with				
	2FB	3FB			4FB
Growth substrate					
– influent	7.3	4.8	4.9	4.0	5.85
– culture fluid	≤0.1	≤0.1	≤0.1	≤0.1	≤0.1
Fluoride					
– influent	≤0.1	≤0.1	≤0.1	≤0.1	≤0.1
– culture fluid	5.8	4.2	4.4	3.1	4.0
2-Fluoromuconate					
– culture fluid	1.0	0.4	0.6	0.6	–
$\frac{[F^-]\cdot 100}{[F^-]+[FM]}$	85	91	88	84	–
$\frac{[F^-]+[FM]\cdot 100}{[FB]t_0-[FB]t_1}$	97	96	102	93	68

Table 2. Turnover rates of benzoates by FLB300 cells after growth with benzoates. Relative turnover rates of cells of FLB300 were determined with HPLC (see Engesser et al. 1988) using an eluent mixture of water/methanol (80/20 v/v). Rates were calculated taking the value of benzoate as 100%

Test substrate	Relative turnover rates of benzoates after growth with			
	Benzoate	2-Fluoro-benzoate	3-Fluoro-benzoate	4-Fluoro-benzoate
Benzoate	100	100	100	100
2-fluoro-	15	60	60	10
3-fluoro-	35	90	80	60
4-fluoro-	55	75	100	60
3-chloro-	25	40	55	35
4-chloro-	15	15	35	20
2-methyl-	≥5	10	10	5
3-methyl-	20	30	80	35
4-methyl-	20	45	60	30
3,5-dimethyl-	≥5	≥5	10	≥5

10 mM) were measured to be 1.2, 1.35 and 2.1 respectively. The corresponding dilution rates were D = 0.053 h^{-1}, 0.063 h^{-1} and 0.0385 h^{-1} respectively. When benzoate was the substrate (10 mM; estimated only in batch culture) a value of OD = 2.8 was found. When cells of a 3-fluorobenzoate degrading continuous culture were plated on NB, benzoate or fluorobenzoate containing agar plates, cell numbers were calculated in all cases to be approximately the same ($2.5 \cdot 10^9$ cells/ml). This showed that all 3-fluorobenzoate utilizing cells had the capacity to grow with benzoate, 2-fluoro- and 4-fluorobenzoate.

The doubling times in continuous cultures were 13 h (3FB), 11 h (4 FB) and 18 h (2 FB), calculated at the dilution rates mentioned above. The concentration of the respective substrate in the reservoir was 10 mM. The sensitivity of strain FLB300 even in continuous culture with respect to high concentrations of fluorobenzoates was demonstrated after lowering the concentration of 2 FB (i.e. from 10 mM to 5 mM). The corresponding doubling time then was found to be 11 h instead of 18 h.

Table 3. Rates of oxygen uptake by 3-fluorobenzoate grown cells of strain FLB300. Cells were grown in continuous culture with 3-fluorobenzoate as sole carbon source (OD = 0.7). After concentration to $OD_{546\,nm}$ = 9 in phosphate buffer pH 7.4. 1 ml of the cell suspension was diluted in 3 ml phosphate buffer for oxygen measurements adjusting a final OD = 2.25. Substrate were added to final concentrations of 0.25 mM (catechols) and 2.5 mM (acids). Activities are expressed as nmoles $O_2 \cdot min^{-1} \cdot ml^{-1}$ cell suspension. Values in parenthesis refer to 3-fluorobenzoate = 100%

Substrate	Oxygen uptake
Catechol	188 (750)
3-Fluorobenzoate[a]	25 (100)
3-Fluorocatechol	5 (20)
4-Fluorocatechol	45 (180)
2-Hydroxybenzoate	5 (20)
3-Hydroxybenzoate	< 2 (< 10)
4-Hydroxybenzoate	3 (10)
3-Chlorobenzoate	18 (70)
3,4-Dihydroxybenzoate	5 (20)
2,5-Dihydroxybenzoate	< 2 (< 10)
2,3-Dihydroxybenzoate	10 (40)

[a] For comparative purposes the values for benzoate, 2-fluoro- and 4-fluorobenzoate are given as 19, 3 and 16 nmoles $\cdot$ $min^{-1} \cdot ml^{-1}$ cell suspension respectively

Excretion of metabolites

During growth of FLB300 with 2- and 3-fluorobenzoate in continuous culture fluoride and a polar metabolite were excreted into the growth medium. The latter was identified by comparing HPLC retention volumina (Rv) and UV spectra to be 2-fluoro-*cis,cis*-muconate (Rv = 3.25 ml compared to 3.37 ml for authentic 2-fluoro-*cis,cis*-muconate; λ_{max} in both cases 264 nm; λ_{min} at 212 nm). Fluoro-substituted analogues of 1,2-dihydro-1,2-dihydroxybenzoate (3,5-cyclohexadiene-1,2-diol-1-carboxylic acid) could be ruled out by comparison with authentic compounds (HPLC data not shown).

Stoichiometry of accumulated metabolites

During steady state growth on fluorobenzoates in continuous culture the relative amounts of fluoride and 2-fluoromuconate formed were determined (Table 1). With the exception of the 4-fluorobenzoate culture, the sum of excreted metabolites nearly equalled the amount of fluorobenzoates transformed. 2-Fluoromuconate is a dead end product in this strain as no turnover of this compound can be observed neither in crude extracts nor with whole cells.

Enzymatic activities in whole cells

As enzyme activity for benzoate and fluorobenzoates could not be detected in crude extracts, turnover of these substrates

Table 4. Enzymes in cell extracts of strain FLB300. The absolute activities are given in parentheses as U/g protein. The relative activities are referred to the respective unsubstituted parent compounds taken to be 100%. For determination of all enzyme expect maleylacetate reductase see Engesser et al. (1988), Schmidt and Knackmuss (1980) and Dorn and Knackmuss (1978). Maleylacetate reductase was determined as described in Materials and methods. No hydrolizing activity was found for the cis- and trans-isomere of 4-carboxymethylenebut-2-en-4-olide

Enzyme/Substrate	Benzoate	Growth substrate		
		2-Fluorobenzoate	3-Fluorobenzoate	4-Fluorobenzoate
DHB Dehydrogenase				
DHB[a]	100 (4,170)	100 (6,915)	100 (3,000)	100 (2,825)
3-Fluoro-DHB	86 (3,580)	n.d.	72 (2,150)	n.d.[b]
4-Fluoro-DHB	64 (2,660)	90 (6,225)	90 (2,700)	94 (2,650)
5-Fluoro-DHB	9 (380)	6 (415)	9 (270)	13 (355)
6-Fluoro-DHB	82 (3,410)	75 (5,190)	90 (2,700)	108 (3,060)
Catechol 1,2-Dioxygenase				
Catechol	100 (1,340)	100 (700)	100 (860)	100 (585)
3-Fluorocatechol	1 (19)	7 (50)	9 (1)	2 (10)
4-Fluorocatechol	11 (150)	21 (145)	12 (105)	16 (55)
3-Chlorocatechol	1 (14)	4 (30)	1 (6)	3 (15)
4-Chlorocatechol	2 (30)	6 (40)	3 (24)	3 (15)
3-Methylcatechol	12 (160)	8 (55)	15 (130)	8 (45)
4-Methylcatechol	22 (300)	19 (131)	16 (140)	11 (65)
Muconate cycloisomerase				
cis,cis-Muconate	100 (800)	100 (650)	100 (240)	100 (210)
2-Fluoro-*cis,cis*-muconate	1 (8)	1 (6)	≤1 (≤3)	≤1 (≤3)
3-Fluoro-*cis,cis*-muconate	n.d.	25 (160)	21 (50)	33 (70)
Maleylacetate reductase				
Maleylacetate	<3	<3	230	295

[a] DHB: 1,2-Dihydro-1,2-dihydroxybenzoate
[b] n.d. not determined

was determined with whole cells (Table 2). The rates of turnover of the organic substrates were estimated using HPLC because oxygen uptake rates were falsified by other O_2-consuming reactions like ring cleavage of catechols.

Oxygen uptake rates with whole cells were measured in order to investigate the relative rates of metabolism of catechols and benzoates respectively (Table 3). High turnover rates for catechols and low to very low rates for other potential ring cleavage substrates suggested catechols to be intermediates of 3-fluorobenzoate degradation.

Enzymatic activities in crude extracts

After growth of FLB300 with benzoate or the isomeric fluorobenzoates respectively activities of 1,2-dihydro-1,2-dihydroxybenzoate dehydrogenase (DHB-dehydrogenase), catechol-1,2-dioxygenase, muconate cycloisomerase, 4-carboxymethylenebut-2-en-4-olide hydrolase and maleylacetate reductase were determined (Table 4). In contrast to an enzyme preparation of 3-chlorobenzoate grown *Pseudomonas sp.* B13 neither the cis- nor the trans-isomere of 4-carboxymethylenebut-2-en-4-olide was hydrolyzed.

Discussion

The productive metabolism of monofluorobenzoates by bacteria has been the subject of many investigations. Accordingly, also the cometabolism of these xenobiotics by soil bacteria was followed and shown to proceed via the normal routes of benzoate degradation with fluorinated catechols as intermediates (Fig. 1).

These were detected in the growth medium (Hughes 1964; Clarke et al. 1975; Horvath and Flathman 1976) which is explained by the fact that the fluorine substituent being only slightly different in size from hydrogen can exert a strong polarizing effect due to its pronounced electronegative character. Accordingly fluorinated analogues can be metabolized to a metabolic stage at which the electronic influence of the fluorine substituent prevents or at least strongly retards further metabolism (Engesser et al. 1988). Catechol 1,2-dioxygenases from normal benzoate pathways have been reported to exhibit considerably low activity with 4-fluorocatechol compared to catechol and almost no turnover activity with 3-fluorocatechol (Dorn and Knackmuss 1978; Engesser et al. 1988). This inertness of 3-fluorocatechol towards ortho-cleavage enzymes is one of the reasons why benzoate degrading bacteria normally are not able to grow with 2- and 3-fluorobenzoate. In addition the intermediarily formed 3-fluorocatechol tends to accumulate and by autoxidation intoxicates the cells (Schreiber et al. 1980).

In the case of 2-fluorobenzoate, *Pseudomonas* sp. B13 after a long adaptation period utilized this substrate as a carbon source. From Fig. 1 one can deduce that the regioselectivity of dioxygenation of 2-fluorobenzoate determines the ratio of the two products formed: While 1,2-dioxygenation yields an unstable geminal fluoro-hydroxy substituted compound which chemically decomposes to inorganic fluoride and well metabolizable catechol, the 1,6-dioxygenation leads to toxic 3-fluorocatechol. Thus a change

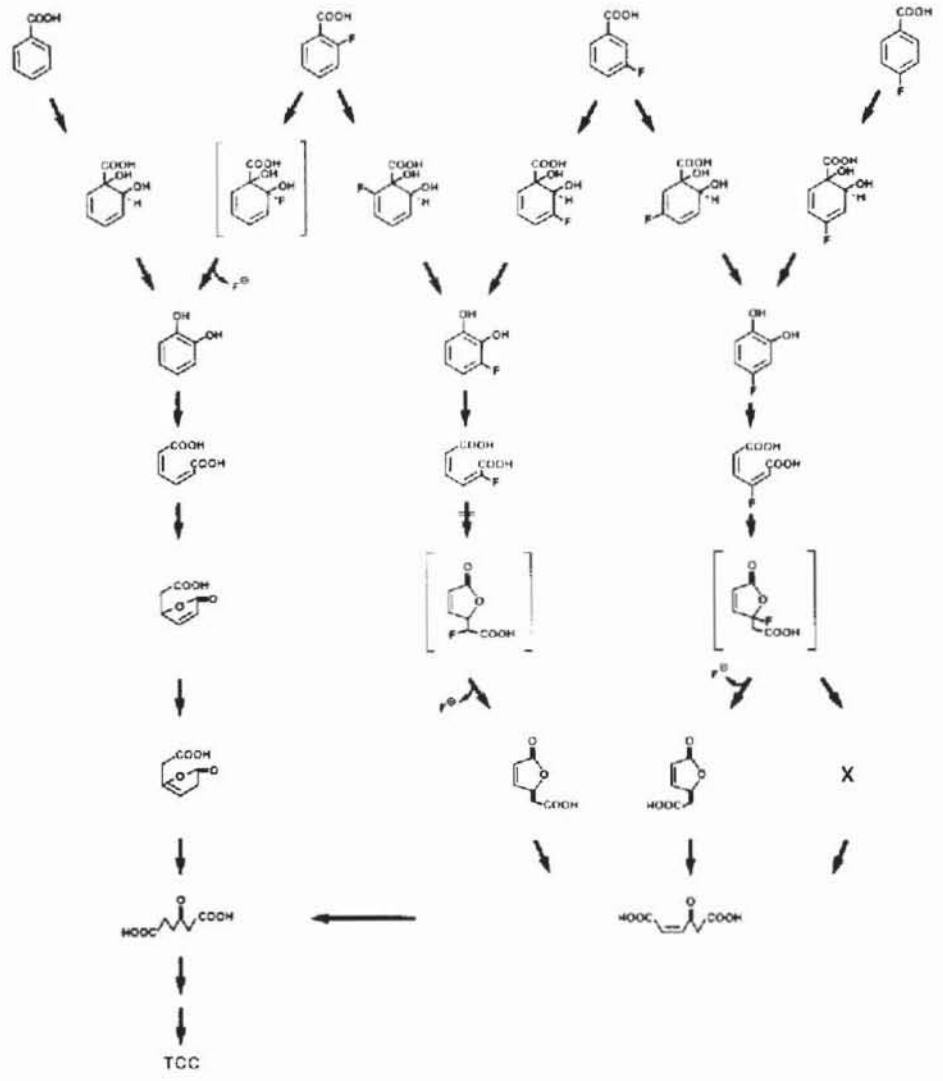

Fig. 1
Pathways of degradation of benzoate and fluorosubstituted benzoates in bacteria (compounds are listed from the left to the right): 1st row: benzoate, 2-, 3- and 4-fluorobenzoate; 2nd row: DHB (1,2-dihydro-1,2-dihydroxybenzoate) and fluorinated derivatives; 3rd row: catechol, 3- and 4-fluorocatechol; 4th row: *cis,cis*-muconate, 2- and 3-fluoromuconate; 5th row: 4-carboxymethylbut-2-en-4-olide (muconolactone) and fluorosubstituted derivatives; 6th row: 4-carboxymethylbut-3-en-4-olide and two stereoisomers of 4-carboxymethylenebut-2-en-4-olide; 7th row: 3-oxoadipate and maleylacetate

in regioselectivity from unfavourable 1,6-dioxygenation to 1,2-attack enabled strain B13 to utilize 2-fluorobenzoate as a carbon source (Engesser et al. 1980). FLB300 seems to have followed the same adaptive strategy.

Degradation of 4-fluorobenzoate has been described earlier (Harper and Blakley 1971c; Schreiber et al. 1980). In this case only 4-fluorocatechol is produced, which was suggested to be metabolized via 3-fluoro-*cis,cis*-muconate (see Table 4) and 4-carboxymethyl-4-fluorobut-2-en-4-olide as intermediates. The latter, after elimination of HF, could lead to formation of 4-carboxymethylenebut-2-en-4-olide (Schreiber et al. 1980). A hydrolase then would produce maleylacetate which after reduction to 3-oxoadipate is degraded via reactions of the normal 3-oxoadipate pathway.

Alternatively a direct ring reaction was proposed opening the lactone ring directly (Harper and Blakley 1971c) to a product carrying a chemically unstable *gem* fluorohydroxy-substituted carbon atom. After spontaneous release of HF maleylacetate could be formed, which could be metabolized as described above.

In FLB300 4-fluorobenzoate seems to be degraded via the latter pathway. Accordingly no activity for both stereoisomeres of 4-carboxymethylenebut-2-en-4-olide was detectable in cells grown on fluorobenzoates although a possible instability of this enzyme in crude extracts could not be ruled out. The next enzyme of the pathway, maleylacetate reductase, was specifically induced in 4- and 3-fluorobenzoate grown cells but not in benzoate and 2-fluorobenzoate grown cells.

The most interesting feature of FLB300, however, is its ability to utilize 3-fluorobenzoate as a carbon source. From Table 3 it is clear that neither 3-hydroxybenzoate nor protocatechuate can be pathway intermediates. This holds also for 2,3-dihydroxybenzoate which cannot be utilized as a carbon source although it is oxygenated at a relatively high rate (Table 3). Instead, fluorosubstituted catechols must be produced from 3-fluorobenzoate. As there is no metabolism of 2-fluoromuconate in crude extracts of 3-fluorobenzoate grown FLB300, ring cleavage of 3-fluorocatechol is unproductive. Therefore, as in the case of 4-fluorobenzoate degradation, only *ortho*-cleavage of 4-fluorocatechol can account for growth on 3-fluorobenzoate. Furthermore, since 3-fluorocatechol is only insufficiently cleaved to untoxic 2-fluoromuconate and tends to intoxicate the cells, the regioselectivity of 3-fluorobenzoate dioxygenation (1,2- or 1,6-position) should strongly influence the growth rate with 3-fluorobenzoate.

Table 5 shows that FLB300 is unique in converting the major part of 3-fluorobenzoate to 4-fluorocatechol thereby allowing convergence with the 4FB pathway. Nevertheless,

Table 5. Regioselectivity of benzoate dioxygenases from different bacterial sources. The regioselectivity of the benzoate dioxygenases was calculated from the ratios of concentrations of fluoride and 2-fluoromuconate after transformation of 3-fluorobenzoate. For determination of these metabolites see Materials and methods. Fluoride was used as an indicator for the 4-fluorocatechol branch (i.e. 1,6-dioxygenation), 2-fluoromuconate for the 3-fluorocatechol branch (1,2-dioxygenation)

Organism	Relative velocities of dioxygenation of 3-fluorobenzoate in		Literature reference
	1,2-position (3-fluorocatechol branch)	1,6-position (4-fluorocatechol branch)	
FLB300	14	86	this study
Alcaligenes eutrophus B9	50	50	Engesser et al. 1980
Pseudomonas sp. B13	87	13	Schmidt and Knackmuss 1984
Alcaligenes sp. A7-2	87	13	Schmidt and Knackmuss 1984
Acinetobacter calcoaceticus	66	34	Clarke et al. 1975

some 3-fluorocatechol is produced which intoxicates the cells and probably is responsible for the rather low growth rate on 3-fluorobenzoate. This problem of cleavage of fluorocatechols could be circumvented by employing highly specialized chlorocatechol-cleaving pyrocatechases. These enzymes are known to be involved in degradation of chloroaromatics and show at least moderate relative rates for cleavage of 3-fluorocatechol and excellent rates for turnover of 4-fluorocatechol. As in most cases the enzymes for degradation of chloroaromatics are not induced by fluorine substituted aromatics, constitutive mutants are of special interest in order to achieve an improved degradation of fluorinated aromatics. Such experiments are currently undertaken with *Alcaligenes eutrophus* JMP 134-1 a constitutive mutant of its 2,4-D (2,4-dichlorophenoxyacetic acid) degrading wildtype (Pieper et al. 1985, 1989).

The results of the taxonomic investigations presented here clearly exclude strain FLB300 from a phylogenetically defined genus *Pseudomonas* (De Vos and De Ley 1983; Woese et al. 1984a). Chemotaxonomic and rRNA data suggest allocation of FLB300 to the alpha-2 subclass of *Proteobacteria* (Woese et al. 1984b; Stackebrandt et al. 1988). Although a phylogenetical relationship of FLB300 to the *Agrobacterium-Rhizobium* branch (De Ley et al. 1987) is indicated, designation by API 20 NE as *Agrobacterium radiobacter* cannot be accepted due to the polyamine pattern found in strain FLB300: Whereas only traces of *sym*-homospermidine are to be expected in *Agrobacterium radiobacter* M2/1 (Busse and Auling 1988), this triamine is the main polyamine in strain FLB300, *Agrobacterium* sp. HK4, strain TE9 and *P. aminovorans* NCIB 9093 – all of them having a very similar 16S rRNA fragment. We do not consider it prudent to make formal taxonomic proposals for isolate FLB300 at present, because further studies for comparison with the phylogenetically related strains emerging are necessary. Facing a similar situation with *P. aminovorans*, Green and Gillis (1989) came to the same decision of avoiding a premature classification.

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