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Degradation of Fluorene by *Brevibacterium* sp. Strain DPO 1361: a Novel C-C Bond Cleavage Mechanism via 1,10-Dihydro-1,10-Dihydroxyfluoren-9-One

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Angular dioxygenation has been established as the crucial step in dibenzofuran degradation by *Brevibacterium* sp. strain DPO 1361 (V. Strubel, K. H. Engesser, P. Fischer, and H.-J. Knackmuss, J. Bacteriol. 173:1932–1937, 1991). The same strain utilizes biphenyl and fluorene as sole sources of carbon and energy. The fluorene degradation sequence is proposed to be initiated by oxidation of the fluorene methylene group to 9-fluorenol. Cells grown on fluorene exhibit pronounced 9-fluorenol dehydrogenase activity. Angular dioxygenation of the 9-fluorenone thus formed yields 1,10-dihydro-1,10-dihydroxyfluoren-9-one (DDF). A mechanistic model is presented for the subsequent C-C bond cleavage by an NAD^+-dependent DDF dehydrogenase, acting on the angular dihydrodiol. This enzyme was purified and characterized as a tetramer of four identical 40-kDa subunits. The following K_m values were determined: 13 μM for DDF and 65 μM for 2,3-dihydro-2,3-dihydroxybiphenyl. The enzyme also catalyzes the production of 3-(2′-carboxyphenyl)catechol, which was isolated, and structurally characterized, in the form of the corresponding lactone, 4-hydroxydibenzo-(b,d)-pyran-6-one. Stoichiometry analysis unequivocally demonstrates that angular dioxygenation constitutes the principal pathway in *Brevibacterium* sp. strain DPO 1361.

Polynuclear aromatic hydrocarbons contribute significantly to industrial soil contamination. In this respect, bioremediation has gained increasing importance. A recent review (28) contends that bacterial degradation of polynuclear aromatic hydrocarbons is without exception initiated by dioxygenation in either the 1,2, 2,3, or 3,4 position of the condensed ring system.

Various pure bacterial cultures have been reported to utilize fluorene as a sole source of carbon and energy. Fluorene degradation via initial 3,4 dioxygenation has been suggested for *Arthrobacter* sp. strain F101. Since this organism utilizes neither 9-fluorenol nor 9-fluorenone, a pathway via oxidation of the methylene bridge seems unlikely (14). There is some indication that degradation of fluorene by a *Pseudomonas vesicularis* strain is also initiated by 3,4 dioxygenation (37, 38). For a *Staphylococcus auriculans* strain reported to grow with dibenzofuran (DBF) and fluorene as sole sources of carbon and energy (18), 1,10-dihydro-1,10-dihydroxyfluoren-9-one (DDF) has been isolated from the culture fluid as one of several metabolites. Since the strain seems to lack the potential to further metabolize this secondary substrate, the authors postulate that degradation is initiated by 3,4 dioxygenation (18).

The present report constitutes, to the best of our knowledge, the first in-depth analysis of a bacterial catabolic pathway for fluorene. *Brevibacterium* sp. strain DPO 1361 was enriched with DBF as the sole source of carbon and energy. The crucial degradation step for this substrate is initial angular dioxygenation (7, 9, 11, 12) by which the biaryl ether structure is transformed into a (chemically unstable) hemiacetal. Thus, 3-(2′-hydroxyphenyl)catechol represents the first metabolite which may be characterized for this pathway. Further degradation proceeds as established for unsubstituted biphenyl (31). A comparable mechanism has been suggested for dibenzo-*p*-dioxin degradation by a *Sphingomonas* strain (41); for the catabolism of 3- and 4-carboxybiphenyl ether, it has been demonstrated definitively (7, 40).

Resting cells of strain DPO 1361, after growth on DBF, transformed fluorene to five distinct metabolite structures (9), one of which was identified as DDF. The presence of this metabolite indicated that dioxygenation of fluorene by DBF-grown cells of *Brevibacterium* sp. strain DPO 1361 unexpectedly takes place also in the angular position. The DDF concentration decreases upon prolonged incubation (5), while it stays constant in the cell-free supernatant. This finding clearly indicates that DDF is subject to further biological metabolization.

Additional investigations have shown DPO 1361 to likewise grow with fluorene as the sole source of carbon and energy (6). It appeared very likely, therefore, that DBF catabolism and fluorene catabolism follow analogous pathways. This hypothesis has now been confirmed. At the same time, we present data by which the initial steps of fluorene degradation by strain DPO 1361 are established unequivocally.

MATERIALS AND METHODS

Organisms. Strain DPO 1361 was classified as a *Brevibacterium* sp. (31) by the Deutsche Sammlung für Mikroorganismen (Braunschweig, Germany). *Pseudomonas stutzeri* AN 10, *Escherichia coli* γδ 14, and *E. coli* γδ 2 were kindly provided by J. F. Amengual (University of Baleares, Palma de Mallorca, Spain).

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Acinetobacter sp. strain P6, *Arthrobacter* sp. strain B1B, and *Pseudomonas* sp. strain FH 23 were kindly supplied by D. Focht (University of California, Riverside). *Pseudomonas* sp. strain NCIB 10643 was obtained from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland).

Growth conditions. Strains *Acinetobacter* sp. strain P6, *Arthrobacter* sp. strain B1B, *Pseudomonas* sp. strain FH 23, *Pseudomonas* sp. strain NCIB 10643, and *Brevibacterium* sp. strain DPO 1361 were cultivated with fluorene or biphenyl as the sole carbon source, applying growth conditions as described previously (32). Yeast extract (0.005% [wt/vol]) was added to supply vitamins. *P. stutzeri* AN 10 was grown in M9 medium (16) containing 10 mM salicylate as the sole carbon source. *E. coli* γδ 2 and γδ 14 were grown in NB (16) containing additional ampicillin (200 μg/ml) and rifampin (50 μg/ml).

Preparation of metabolites. 2,3-Dihydro-2,3-dihydroxybiphenyl (DDB) was isolated and purified as described by Strubel (30). DDF was prepared as described by Engesser et al. (9). 1,2-Dihydro-1,2-dihydroxynaphthalene (DDN) was kindly provided by V. Strubel. Details of characterization will be published separately (6).

To estimate the concentrations of the metabolites DDF and 4-hydroxydibenzo-(b,d)-pyran-6-one (4-HDBP), DDF (approximately 1 mM) and NAD^+ (2.7 mM) were incubated in phosphate buffer (50 mM, pH 7.5) with a crude extract of strain DPO 1361 in the presence of 3-chlorocatechol (1 mM). The concentration of NADH after total conversion of DDF was determined by high-pressure liquid chromatography (HPLC) (30:60 [vol/vol] methanol-water, 1 ml of ion pair reagent Pic A [Waters, Eschborn, Germany] per liter). In the same sample, concentrations of DDF and 4-HDBP were analyzed by HPLC (acetonitrile-water-H_3PO_4, 40:59.9:0.1 [vol/vol/vol]). No pyrocatechase activity or unspecific NADH oxidase activity was present in the crude extract under these conditions, as confirmed by control experiments.

Enzyme assays. One unit of enzyme activity was defined as the amount of enzyme converting 1 μmol of substrate per min. Methods for cell extract preparation and for measuring protein content were as described previously (22, 23), with the modification that cell suspensions were incubated in the presence of 5 mg of lysozyme per ml for 30 min at 37°C prior to disruption by a French pressure cell. Cell debris was removed by centrifugation (60 min at 100,000 × *g*).

9-Fluorenol dehydrogenase activity was measured by monitoring the increase of A_{340} in an assay mixture containing Tris-HCl (100 mM, pH 8.5), 9-fluorenol (0.5 mM), and NAD^+ (2.7 mM) in a final volume of 1 ml. Triton X-100 (0.3% [vol/vol]) was added for substrate solubilization. Under these conditions, the reaction rate was proportional to the enzyme concentration and, within the range studied, independent of substrate concentration. Fluorene monooxygenation activity was investigated under identical conditions except that NADH or NADPH was added as a cofactor.

DDF dehydrogenase activity was monitored by measuring the decrease of A_{313} due to substrate turnover. The reaction vessel contained Tris-HCl (100 mM, pH 8.5), DDF (8.5 μM), and NAD^+ (2.7 mM). In crude extracts and Q Sepharose fractions, 3-chlorocatechol (1 mM) was added to inhibit pyrocatechase activity. The reaction coefficient was calculated as 6.28 $cm^2 \cdot \mu mol^{-1}$ from the difference of the extinction coefficients of DDF (ε_{313} = 10.1 $cm^2 \cdot \mu mol^{-1}$) and NADH (ε_{313} = 3.82 $cm^2 \cdot \mu mol^{-1}$).

DDB dehydrogenase activity was monitored under the same conditions by measuring NADH production at 365 nm. In this case, 60 μM substrate was used.

DDN dehydrogenase activity was monitored spectrophotometrically at 340 nm as described by Patel and Gibson (20). Assays were performed in a final volume of 1 ml.

HPLC. Analyses were performed on a Merck HPLC system (Merck, Darmstadt, Germany) with an RP-8 Lichrosorb column (125 mm; 4.6-mm internal diameter; Bischoff, Leonberg, Germany) and with water-methanol or water-acetonitrile, containing 0.1% (vol/vol) H_3PO_4, as the mobile phase. Individual compounds were detected spectrophotometrically at 210 nm.

For preparative HPLC, an RP-8 Lichrosorb column (25 cm; internal diameter of 2.5 cm; Bischoff) was used, with methanol-water-trifluoroacetic acid (55:49.9:0.1 [vol/vol/vol]; flow rate, 5.5 ml/min) as the mobile phase.

Enzyme purification. Purification of DDF dehydrogenase was carried out at ambient temperature, using a fast-performance liquid chromatography system which consisted of an LCC 500 controller, pump 500, UV-1 monitor, REC-482 recorder, and FR 100 autosampler (all from Pharmacia, Uppsala, Sweden). A crude extract of fluorene-grown cells (12 ml/94.8 mg of protein) was prepared in Tris-HCl buffer (50 mM, pH 7.5), passed over a 0.2-μm-pore-size filter, and applied to a Q Sepharose anion-exchange column (25 cm; inside diameter, 16 mm). Samples were eluted by a nonlinear gradient of 0 to 500 mM NaCl in Tris-HCl buffer (50 mM, pH 7.5) containing 1 mM dithiothreitol at a flow rate of 4 ml/min. Fractions (10 ml) eluting at 0.36 M NaCl contained the dehydrogenase activity; they were pooled and concentrated by ultrafiltration. The concentration of $(NH_4)_2SO_4$ was adjusted to 1 M, and the sample was filtered (0.2-μm-pore-size filter) and applied to a phenyl-Superose HR 10/10 column (Pharmacia). A nonlinear gradient of 1.0 to 0.0 M $(NH_4)_2SO_4$ in Tris-HCl (50 mM, pH 7.5)–1 mM dithiothreitol was used for the mobile phase (flow rate, 1.2 ml/min). Fractions (2 ml) eluting around 0.29 M $(NH_4)_2SO_4$ contained the dehydrogenase activity; they were pooled and purified further on a phenyl-Superose HR 5/5 column (Pharmacia), with the flow rate adjusted to 0.3 ml/min. Fractions (0.5 ml) containing DDF dehydrogenase activity were likewise pooled and concentrated by ultrafiltration to a final volume of 180 μl. The sample was applied to a Superose 6 gel filtration column (HR 10/30 [Pharmacia]; mobile phase, Tris-HCl [50 mM, pH 7.5]–NaCl [100 mM]–dithiothreitol [1 mM]; flow rate, 0.5 ml/min). Fractions (0.5 ml) containing dehydrogenating activity were pooled and analyzed for purity and activity by both native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE. The purified enzyme was stored at −20°C.

SDS-PAGE. SDS-PAGE and native PAGE were carried out on a Bio-Rad Mini Protean II system (development and electrophoresis chambers). Gels were stained with Coomassie blue after electrophoresis. For activity staining, after electrophoresis, native gels were incubated in 10 ml of a solution containing Tris-HCl (100 mM, pH 8.5), NAD^+ (0.27 mM), DDF or DDB (2 mM), *p*-iodonitrotetrazolium violet (0.14 mM), and 8-dimethylamino-2,3-benzophenoxazine (Meldola's blue; 0.12 mM) for at least 1 h.

Chemicals. Chemicals were of the highest purity commercially available (Merck; Serva, Heidelberg, Germany; Fluka, Neu-Ulm, Germany; Aldrich, Heidenheim, Germany). 3-Chlorocatechol was obtained by chlorination of catechol as described by Willstätter and Müller (39).

Spectroscopy. Nuclear magnetic resonance (NMR) spectra were recorded as described by Strubel et al. (31).

TABLE 1. DDF dehydrogenase activity in crude extracts of strain DPO 1361 after growth on different substrates

Substrate	Sp act[a] (U/mg of protein [mean ± SE, n = 3]) after growth on:			
	Gluconate	DBF	Biphenyl	Fluorene
DDF	0.004 ± 0.003	0.023 ± 0.005	0.135 ± 0.024	0.306 ± 0.048
DDB	0.010 ± 0.002	0.022 ± 0.007	0.119 ± 0.011	0.271 ± 0.042

[a] Determined as described in Materials and Methods.

RESULTS

Turnover of DDF by cell extracts. Cell extracts of strain DPO 1361, grown on fluorene, showed a pronounced enzymatic activity which transformed DDF by an NAD^+-dependent reaction. NAD^+ could not be substituted in this reaction by $NADP^+$; anaerobic conditions, on the other hand, did not reduce activity levels. No activity was observed when the cell extract was heat inactivated prior to activity measurements.

The NAD^+ dependence of the reaction suggests that metabolization of DDF is effected by a dehydrogenase. DDF is oxidized very effectively by crude extracts of both biphenyl- and fluorene-grown cells (Table 1). Only low levels of this activity were observed, on the other hand, after growth on gluconate or DBF. The DDB oxidizing activity, therefore, is characterized by the same induction pattern. Thus, it can be reasonably assumed that the same enzyme catalyzes both dehydrogenation reactions. No activity was detected with DDN as the substrate.

In the course of the enzymatic turnover of DDF with crude extracts, two metabolites were formed. One of these was identified as phthalic acid by comparison with authentic material (by HPLC). The second product metabolite, designated metabolite X, also accumulated when fluorene-grown resting cells of strain DPO 1361 were incubated with fluorene in the presence of an established meta-pyrocatechase inhibitor, e.g., 3-chlorocatechol (3, 8, 15). We obtained 2 mg of pure metabolite X for detailed spectroscopic analyses by this procedure, i.e., after two extractions of the cell-free supernatant with chloroform and subsequent purification by preparative HPLC.

Structure of metabolite X. The 300-MHz ^1H NMR spectrum of metabolite X shows signals for seven protons in the aromatic region ($\delta > 7.0$ ppm; Fig. 1), apart from a broad, exchangeable resonance around 6 ppm (phenolic OH group). The aromatic proton resonances can be divided into two subsets: a four-proton one at low field and a three-proton one at rather high field. The low-field proton of this set is linked to the high-field resonance of the other set by a 0.36-Hz long-range coupling. Both chemical shift and H,H coupling data (Table 2) are in excellent agreement with the proposed structure, 4-HDBP, for metabolite X. The 0.6-ppm shift difference between protons H-9/H-7 and H-8/H-1 is consistent with *peri* interactions operative in positions 7 and 9. The same effect is responsible for the substantial low-field shift of H-1 in the dioxyphenyl ring.

Stoichiometry of 4-HDBP formation. Resting cells of strain DPO 1361 (A_{546} = 7) grown on fluorene were incubated with a mixture of fluorene (2.8 mM), supplied as finely dispersed crystals, and 3-chlorocatechol (1 mM). After 210 min, the transformation was stopped by centrifugation. Water-soluble metabolites were analyzed directly by HPLC (water-acetonitrile-H_3PO_4, 59.9:40:0.1 [vol/vol/vol]). The water-insoluble substrate was dissolved by adding four times the volume of 1,4-dioxane to the incubation medium. The concentrations of unchanged fluorene, of 4-HDBP, and of phthalic acid were thus determined as 1.3, 1.3, and 0.2 mM, respectively. Hence, 86% of transformed substrate had accumulated as 4-HDBP; this clearly demonstrates the essential role of angular dioxygenation in this fluorene degradation pathway. Another 10% of metabolized substrate accumulated as phthalic acid, probably as a result of insufficient inhibition of the pyrocatechase activity. Conversion of 9-fluorenone by resting cells was investigated in a similar experiment; after 60 min, 72% of the 9-fluorenone metabolized had accumulated as 4-HDBP. This result clearly shows that fluorene-grown cells of strain DPO

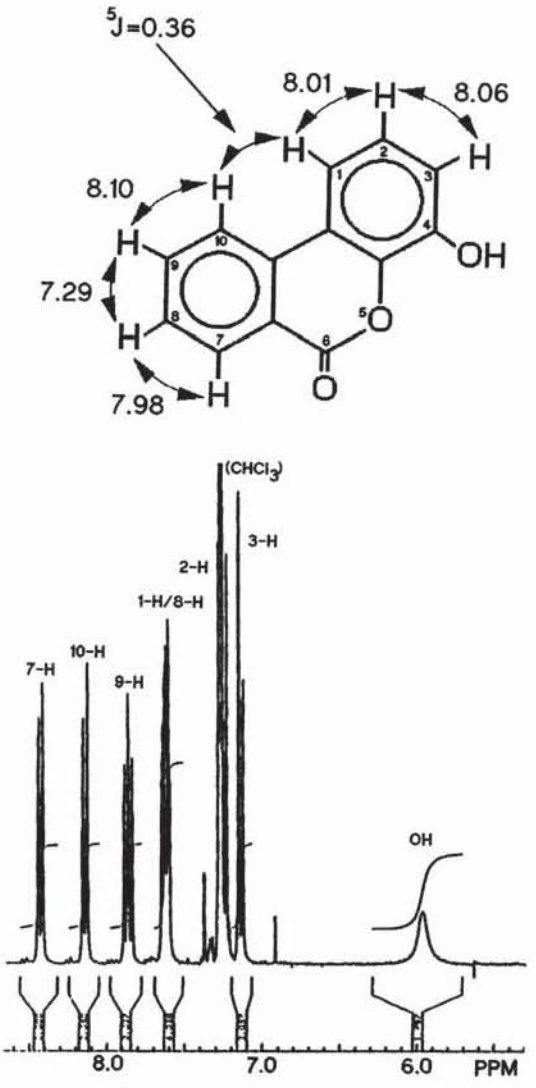

FIG. 1. ^1H NMR spectrum (300 MHz) of metabolite X (4-HDBP). All ^3J (*ortho*) couplings, as well as the ^5J long-range coupling between H-1 and H-10, are indicated.

TABLE 2. ^{1}H NMR data for 4-HDBP[a]

Proton assignment	δ (ppm)[a]	Coupling constant (Hz)		
		^{3}J (*ortho*)[b]	^{4}J (*meta*)[b]	^{5}J (long range)[b]
1	7.615	8.01	1.40 (H-1,H-3)	0.36 (H-1,H-10)
2	7.250	8.01/8.06		
3	7.133	8.06	1.40 (H-1,H-3)	
7	8.424	7.97	1.45 (H-7,H-9)	0.60 (H-7,H-10)
8	7.617	7.29/7.98	1.13 (H-8,H-10)	
9	7.859	8.10/7.31	1.45 (H-7,H-9)	
10	8.135	8.10	1.13 (H-8,H-10)	0.36 (H-1,H-10) 0.60 (H-7,H-10)

[a] Nominal frequency of 300.13 MHz, 0.02 M in $CDCl_3$, internal standard tetramethylsilane.

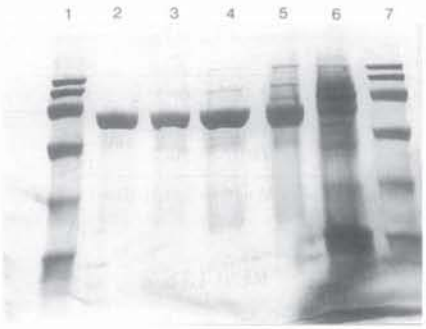

FIG. 2. Purification of DDF dehydrogenase. Protein samples obtained during purification of DDF dehydrogenase were resolved on an SDS–10% polyacrylamide gel and stained with Coomassie blue. Lanes 1 and 7 contain molecular weight markers (94,000, 66,000, 45,000, 36,000, 29,000, 24,000, 20,000, and 14,200). Samples were taken following gel filtration (lane 2; 5 μg), phenyl-Superose HR 5/5 chromatography (lane 3; 10 μg), phenyl-Superose HR 10/10 chromatography (lane 4; 15 μg), Q Sepharose chromatography (lane 5; 20 μg), and crude extract preparation (lane 6; 37 μg).

1361 efficiently dioxygenate 9-fluorenone also in the angular position.

Stoichiometric conversion of DDF to phthalic acid by crude extracts. Cell extracts of strain DPO 1361 metabolized DDF to phthalic acid in stoichiometric amounts. DDF (0.45 mM) was incubated in 5 ml of phosphate buffer (50 mM, pH 7.5) containing NAD^+ (2.7 mM) and crude extract of fluorene-grown cells (1.3 mg of protein). The solution was stirred vigorously during the entire experiment, and aliquots were taken at given intervals. The reaction was stopped after 2 h by addition of 10% (vol/vol) perchloric acid (6% [wt/vol]). The metabolite concentration in each sample was determined by HPLC. DDF turnover was completed within 20 min; the phthalic acid concentration reached a maximum (0.37 mM) after 40 min of incubation. Phthalic acid thus is clearly established as a metabolic intermediate in fluorene degradation by strain DPO 1361. The DDF concentration remained unchanged, on the other hand, when heat-inactivated cell extract was added.

Purification of DDF dehydrogenase. The sequence for purifying DDF dehydrogenase, resulting in a 40-fold enrichment, is summarized in Table 3. Since the first hydrophobic interaction chromatography (at phenyl-Sepharose HR 5/5 material) resulted in an excellent purification, a second hydrophobic cleaning step (stage 3) was included in the overall sequence; however, this step gave only another 3% increase in purity. DDF- and DDB-converting activities could not be separated in the course of this purification procedure; thus, both activities may safely be assumed to be catalyzed by the same enzyme. The molecular mass of the native enzyme was estimated at 160 kDa from gel filtration (Superose 6 column). SDS-PAGE revealed one single band with a molecular mass of 41 kDa, indicating four identical subunit constituents for the native enzyme (Fig. 2). Native PAGE and subsequent activity staining yielded a single band, regardless of whether DDF or DDB was used as a substrate (data not shown).

4-HDBP is definitely ruled out as a true metabolite since, with purified dehydrogenase, no stoichiometric amount of this 3-(2′-carboxyphenyl)catechol (CPC) lactone was detected after total conversion of 0.5 mM DDF (HPLC analysis with a neutral mobile phase, methanol-water [10:90, vol/vol]). Instead, another metabolite (retention volume, 3.65 ml) accumulated, which was shown by HPLC with in situ UV detection to have absorption maxima at 218 and 264 nm. Upon acidification, this metabolite disappeared from the reaction mixture, 4-HDBP (0.48 mM) being formed instead in a close to stoichiometric amount. This result clearly demonstrates 4-HDBP to be an artifact arising from the acidic conditions during isolation of CPC. Accordingly, no 4-HDBP turnover was observed with cell extract; i.e., 4-HDBP is stable toward the enzymes involved at all practical pH values.

The K_m value for DDF, calculated as 13 ± 2 μM, is somewhat lower than that found for DDB (65 ± 5 μM; both values are the means of two independent experiments). Since substrate inhibition was observed in both cases, enzyme assays were performed at the optimum substrate concentrations as given in Materials and Methods. Substrate inhibition constants were not determined.

Dihydrodiol dehydrogenase activities in biphenyl-degrading organisms. Of four biphenyl-degrading strains tested for their fluorene-degrading potential, none could utilize fluorene as

TABLE 3. Purification of DDF dehydrogenase from *Brevibacterium* sp. strain DPO 1361[a]

Purification step	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Recovery (%)	Purification factor	Relative activity[b] *for DDB*
Crude extract	12	94.8	29.0	0.3	100	1	84
Q Sepharose eluate	110	13.0	22.1	1.7	76	6	98
Phenyl-Superose HR 10/10 eluate	20	2.0	19.8	9.9	68	32	103
Phenyl-Superose HR 5/5 eluate	4	1.15	11.8	10.2	40	33	103
Superose 6 eluate	2	0.53	6.51	12.3	22	40	101

[a] Experimental details are given in Materials and Methods.
[b] Relative to the value for DDF, which was taken as 100%.

TABLE 4. Dihydrodiol dehydrogenase activity in biphenyl-degrading strains grown on biphenyl

Strain	Sp act[a] (U/mg of protein [mean ± SE, n = 2]) with:	
	DDN	DDB
Acinetobacter sp. strain P6	0.115 ± 0.012	0.058 ± 0.017
Arthrobacter sp. strain B1B	0.154 ± 0.030	0.200 ± 0.038
Pseudomonas sp. strain FH 23	0.101 ± 0.028	0.050 ± 0.017
Pseudomonas sp. strain NCIB 10643	0.130 ± 0.045	0.187 ± 0.041

[a] Enzyme activities were determined as described in Materials and Methods. In all cases, the value with DDF was 0.

the sole source of carbon and energy. After growth with biphenyl, cell extracts were examined for dihydrodiol dehydrogenase activity (Table 4). No DDF transformation was observed in any case even though all extracts contained dehydrogenase activities for the oxidation of DDB and DDN.

Oxidation of fluorene to 9-fluorenol. No enzymatic activity is detected in crude extracts of strain DPO 1361 which would catalyze monooxygenation of fluorene to 9-fluorenol under the conditions given in Materials and Methods. Catalysis by a membrane-bound dioxygenase would present an alternative mechanistic possibility. A cloned naphthalene dioxygenase gene (*nahA*) of *P. stutzeri* AN 10 was used to test this hypothesis (1). Resting cells of strain γδ 14 (optical density at 546 nm of 80) were incubated with fluorene (3 mM). After 120 min, 0.13 mM 9-fluorenol was detected in the supernatant. In a control experiment, *E. coli* γδ 2, which has a transposon insertion in *nahA*, was incubated under the same conditions. Since the *nahA* gene is no longer operative in this mutant, no naphthalene 1,2-dioxygenase is expressed by this strain (1). Accordingly, no 9-fluorenol could be detected in the medium. Wild-type *P. stutzeri* AN 10, on the other hand, transforms fluorene to 9-fluorenol.

9-Fluorenol dehydrogenase. Cell extracts of fluorene-grown cells of strain DPO 1361 likewise converted 9-fluorenol to 9-fluorenone; both 9-fluorenone formation and 9-fluorenol consumption were monitored by HPLC (mobile phase, methanol-water-H_3PO_4 [60:39.9:0.1, vol/vol/vol]). This NAD^+-dependent enzymatic activity was induced during growth on fluorene (specific activity, 1.05 U/mg of protein). In sharp contrast, extracts of gluconate-, biphenyl-, or DBF-grown cells contained only a minor level of 9-fluorenol dehydrogenase activity (approximately 0.030 U/mg of protein). The 9-fluorenol dehydrogenase activity and DDF dehydrogenase, therefore, clearly belong to distinct enzymatic entities, since 9-fluorenol is not a substrate for purified DDF dehydrogenase.

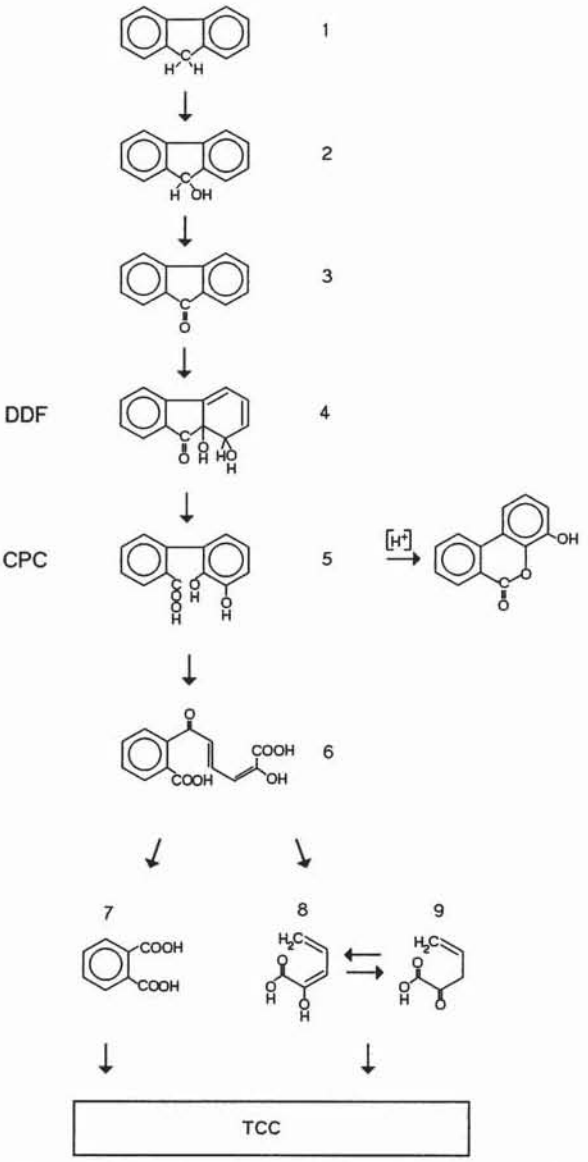

FIG. 3. Proposed degradation pathway for fluorene by strain DPO 1361. 1, fluorene; 2, 9-fluorenol; 3, 9-fluorenone; 4, DDF; 5, CPC; 6, 2-hydroxy-6-(2-carboxyphenyl)-6-oxo-2,4-hexadienoic acid; 7, phthalic acid; 8, 2-hydroxy-4-pentenoate; 9, 2-oxo-4-pentenoate. TCC, tricarboxylic acid cycle.

DISCUSSION

All experimental data presented so far can be accommodated by the degradation pathway outlined in Fig. 3. Thus, fluorene catabolism by *Brevibacterium* sp. strain DPO 1361, after transformation to 9-fluorenone, would follow the catabolic routes established for DBF and biphenyl in all essential steps but one. The pivot of this sequence is the angular dioxygenation step. Contrary to straightforward mechanistic considerations, this sequence does not lead fluorene degradation into a dead-end pathway; actually, *Brevibacterium* sp. strain DPO 1361 utilizes fluorene as a sole source of carbon and energy rather than as a cometabolic substrate (6). DDF turnover was also observed when DBF-grown cells were incubated with fluorene for a prolonged time. Taken together, these results suggest that fluorene degradation operates on the basis of the angular dioxygenation mechanism.

Other than in the case of DBF, the angular dihydrodiol of fluorene, oxidized additionally at C-9 (i.e., DDF), is chemically stable (6). DDF is oxidized, in the ensuing step, by an NAD^+-dependent dehydrogenase (Fig. 4). It seems plausible to assume the primary product of this reaction to immediately add water, yielding CPC (structure B in Fig. 4). CPC is the structural analog of 3-(2′-hydroxyphenyl)catechol and of 3-phenylcatechol, which represent the effective ring fission substrates for the metapyrocatechases involved in DBF and biphenyl degradation (4, 31).

The more detailed mechanism in Fig. 4 illustrates how the dehydrogenase reaction results in the actual C-C bond cleav-

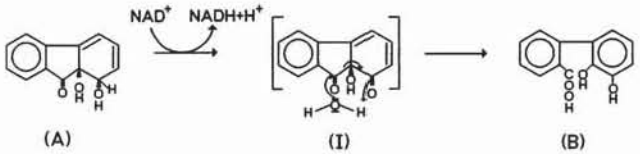

FIG. 4. Proposed mechanism for the DDF dehydrogenase reaction (A, DDF; B, CPC; I, postulated reaction intermediate). For further explanations, see text.

age. This interpretation is consistent with the established fact that dehydrogenases follow a simultaneous NAD^+ reduction-ketonization mechanism (24). An alternative route would require synchronous dehydration at C-1 and OH^- addition to C-9, resulting in an immediate cleavage of the C-9/C-10 bond and rearomatization of the diene ring. For an unequivocal differentiation between these two mechanistic possibilities, it would be necessary to chemically synthesize intermediate I in Fig. 4, e.g., by oxidation of DDF (13), and test it for its structural stability.

The product of the DDF dehydrogenase-catalyzed reaction, i.e., CPC, was isolated in the form of the respective lactone, 4-HDBP. From a chemical point of view, intramolecular esterification of CPC to 4-HDBP under the acidic workup and chromatographic conditions appears straightforward. DDF dehydrogenase, purified to homogeneity, transforms isolated DDF; 4-HDBP is identified as the product formed upon acidification. This enzyme shows high specificity for the substrates DDF and DDB; DDN, on the other hand, is not transformed at all.

These data indicate that DDF dehydrogenase is distinct from other dihydrodiol dehydrogenases described in the literature (16, 20, 21, 26). These enzymes as a rule are characterized by low substrate specificity, and they transform both DDB and DDN (20, 21, 26). We have found ordinary biphenyl-degrading strains to express a dihydrodiol dehydrogenase which, in crude extracts, converts DDB and DDN but not DDF (Table 4). This may be the rationale for the inability of these strains to grow with fluorene as the sole source of carbon and energy. The four-subunit structure is a typical feature of all diol dehydrogenases characterized so far (2, 19, 20, 24, 26); the 160-kDa molecular mass of DDF dehydrogenase, on the other hand, corresponds to a 40-kDa subunit which is definitely larger than in the case of other dehydrogenases.

Fluorene is monooxygenated in the C-9 position by the naphthalene-1,2-dioxygenase of *P. stutzeri* AN 10 (see data presented above). No 9-fluorenol is detected when crude extracts of fluorene-grown DPO 1361 are incubated with fluorene. This clearly indicates the absence of a genuine monooxygenase, especially a 9-fluorene monooxygenase. Therefore, the actual formation of fluorenol is probably due to a monooxygenation mode of a dioxygenase, as described above for the cloned dioxygenase of *P. stutzeri* AN 10. This finding is also consistent with several reports on monooxygenation reactions which are catalyzed by dioxygenases (25, 29, 33, 34, 36). 9-Fluorenol thus formed is then converted to 9-fluorenone by a dehydrogenase that is strongly induced during growth on fluorene but not after growth with DBF or biphenyl.

Despite the similarities between the DBF, biphenyl, and fluorene catabolic pathways, degradation of fluorene seems to recruit a different set of enzymes. In a cell extract of a *Beijerinckia* sp., an analogous 9-fluorenol-dehydrogenating activity was detected by Schocken and Gibson (27); the physiological function of this activity is not known. It is constitutive, though, in contrast to the activity in DPO 1361 extracts. The authors (27) also found that this organism can oxidize 1-acenaphthenol. We have likewise found that cell extracts of DPO 1361, after growth with fluorene, show 1-acenaphthenol-oxidizing activity (35).

Fluorene-grown cells convert both fluorene and 9-fluorenone, in stoichiometric amounts, to CPC in the presence of 3-chlorocatechol. Since this established metapyrocatechase inhibitor does not affect the initial steps of fluorene degradation, angular dioxygenation must constitute the main catabolic route. The effective substrate for the angular dioxygenase seems to be 9-fluorenone; *Sphingomonas* sp. strain RW1, for instance, has been reported to dioxygenate 9-fluorenone, though not fluorene itself, in the angular position (41).

Phthalic acid has been established unequivocally as a metabolic intermediate in fluorene degradation; with crude extracts, it is formed from DDF in the presence of O_2 and NAD^+. Fluorene degradation by DPO 1361 thus is initiated by angular dioxygenation; further down, it follows a pathway analogous to that described for DBF and biphenyl (4, 31). The results presented here indicate that DBF, biphenyl, and fluorene comprise a class of substrates which may substitute for each other as a carbon source for certain strains.

Weissenfels suggested a classical, naphthalene-type degradation for fluorene metabolization (37). Monna et al., in contrast, describe the angular dihydrodiol which we recently have isolated and characterized spectroscopically (18). We therefore believe that an angular dioxygenation mechanism is also operative in *S. auriculans* DBF63.

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