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Actions of a Versatile Fluorene-Degrading Bacterial Isolate on Polycyclic Aromatic Compounds†

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***Pseudomonas cepacia* F297 grew with fluorene as a sole source of carbon and energy; its growth yield corresponded to an assimilation of about 40% of fluorene carbon. The accumulation of a ring *meta*-cleavage product during growth and the identification of 1-indanone in growth media and washed-cell suspensions suggest that strain F297 metabolizes fluorene by mechanisms analogous to those of naphthalene degradation. In addition to fluorene, strain F297 utilized for growth a wide variety of polycyclic aromatic compounds (PACs), including naphthalene, 2,3-dimethylnaphthalene, phenanthrene, anthracene, and dibenzothiophene. Fluorene-induced cells of the strain also transformed 2,6-dimethylnaphthalene, biphenyl, dibenzofuran, acenaphthene, and acenaphthylene. The identification of products formed from those substrates (by gas chromatography-mass spectrometry) in washed-cell suspensions indicates that *P. cepacia* F297 carries out the following reactions: (i) aromatic ring oxidation and cleavage, apparently using the pyruvate released for growth, (ii) methyl group oxidations, (iii) methylenic oxidations, and (iv) S oxidations of aromatic sulfur heterocycles. Strain F297 grew with a creosote-PAC mixture, producing an almost complete removal of all aromatic compounds containing 2 to 3 rings in 14 days, as demonstrated by gas chromatography analysis of the remaining PACs recovered from cultures. The identification of key chemicals confirmed that not only are certain compounds depleted but also the anticipated reaction products are found.**

Polycyclic aromatic compounds (PACs), including polycyclic aromatic hydrocarbons (PAHs), are pollutants of concern because of their toxic and carcinogenic potentials (25). Combustion of organic materials is mainly responsible for their ubiquitous distribution in the atmosphere, surface waters, and sediments (13, 21, 26). PACs are also constituents of crude oils and materials derived from coal (e.g., creosote and coal tar). Accidental spillage and improper disposal during processing, transportation, and commercial use of these mixtures have contaminated various environments, presenting serious health and ecological risks (e.g., at creosote sites, waste oil recovery stations, coal gasification plants, and oil spills). Biological technologies are considered to have potential in the remediation of contaminated sites (36, 37). Their successful application, however, demands a broader understanding of the biochemical pathways by which PACs are degraded, both individually and in mixtures.

An extensive body of literature describing the degradation of individual PACs by microorganisms able to utilize them as sole sources of carbon and energy exists (8, 18). These studies have yielded fundamental information about the biodegradability of individual compounds, but little is known of the fate of those compounds in mixtures. It has been recognized that a number of aromatic hydrocarbon degraders have the ability to transform other aromatic substrates to various extents (9, 14, 15, 30, 31, 33, 44, 45, 47, 52, 53), suggesting that biotransformations may play important roles in the degradation of environmental mixtures. A number of studies document the degradation of PAC mixtures. The removal of creosote components by mixed cultures has been well demonstrated both in the laboratory and in bioremediation field applications (36, 37). The selective removal of certain aromatic compounds from crude oil has also been extensively reported (2). Most of these studies report a depletion of target components, but few attempts have been made to describe the biochemical processes involved and the possibility that such co-oxidation processes actually give rise to dead-end products remains.

Creosote is a complex mixture rich in PAHs (about 85% by weight) that is used extensively as a lumber preservative and, as a result, is found at more than 700 contaminated sites in the United States. Because of the high concentrations of individual PACs in this material in a relatively simple mixture (compared with crude oils), creosote-PACs have been chosen as one model for the study of the biodegradation of other environmental mixtures (such as oil spills, oil wastes, and coal gasification products).

Fluorene, a naphthenoaromatic hydrocarbon possessing two aromatic rings with a benzylic methylenic group in a central five-membered ring, is a major constituent of coal derivatives (about 7.6% of creosote PACs [this study]). Recent studies indicate that microorganisms oxidize fluorene by a variety of mechanisms (19, 20, 40, 45, 46, 51). On the basis of 3,4-dihydrocoumarin identification, Grifoll et al. (19) proposed that the route of utilization of fluorene by an *Arthrobacter* sp. was initiated by dioxygenation at C-3 and C-4, followed by *meta* cleavage and a Baeyer-Villiger reaction, while alternative oxidation at C-9 gave rise to 9-fluorenol and then 9-fluorenone,

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which accumulated as a dead-end product. Recently, a new fluorene catabolic pathway involving productive mono-oxygenation at C-9 to give first 9-fluorenol, then 9-fluorenone, and thereafter dioxygenation at an angular site, with cleavage of the five-membered ring to generate a substituted biphenyl before further metabolism by reactions akin to those of biphenyl utilization, has been described (20, 51).

As a continuation of these studies, we have investigated the degradation of fluorene by another fluorene-utilizing strain, *Pseudomonas cepacia* F297. A preliminary characterization revealed that this strain was able to utilize a variety of PACs, suggesting that a study of the biochemical mechanisms involved could provide useful information about the biotransformation processes leading to an understanding of how PAC mixtures are degraded. In this paper, evidence is presented to show that the utilization of fluorene by *P. cepacia* F297 is based on a set of enzymes analogous to those used in naphthalene degradation. The actions of this strain on a variety of individual PACs are described, accounting for the ability of this organism to remove PACs with 2 to 3 aromatic rings when incubated with creosote PACs.

(Part of this work has been presented previously [20a].)

MATERIALS AND METHODS

Chemicals. Single PACs and derivatives used in this study were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Acenaphthenone was synthesized according to the method of Schocken and Gibson (44). Diazomethane was generated by the alkaline decomposition of Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) (4). Bacteriological media and reagents were purchased from Difco Laboratories, Detroit, Mich. Solvents were from J. T. Baker, Inc. All chemicals and solvents were of the highest purity available.

Creosote-PAC mixture. PACs were purified from P2 creosote (Aristech Chemical Corp., Clairton, Pa.) by chromatography on neutral alumina by modifying a procedure described by Schiller and Mathiason (43). Five grams of creosote in CH_2Cl_2 was applied to a column of activated (300°C) alumina (200 g, Fisher A590). Elution with 500 ml of methylene chloride, followed by careful concentration under reduced pressure (35°C), gave about 4.3 g (86%) of a viscous yellow mixture of PACs from which phenols, dark brown impurities, and the majority of N heterocycles had been removed. Compositional analysis of each batch was by gas chromatography-flame ionization detection (GC-FID), which showed that all hydrocarbons and O and S heterocycles were present in concentrations closely matching those found in unfractionated creosote dissolved in *n*-hexane. The identification of many PACs was facilitated by comparison with a standard reference material (SRM 1597; National Bureau of Standards, Gaithersburg, Md.) (54) with confirmation by GC-mass spectrometry (GC-MS), which also supplied data for components not identified in the reference mixture. For example, peak 15 (see Table 4 and Fig. 4A) is now identified as 2-phenylnaphthalene (not anthraquinone as previously reported [37]).

Media and supply of aromatic compounds. The mineral salts medium (23) with pH adjusted to 7.2 was sterilized prior to the addition of organic substrates. Solid medium was prepared by the addition of 1.5% Noble agar. Water-soluble substrates were added at a final concentration of 5 mM. For single PACs and their analogs in which this and other concentrations exceeded their aqueous solubility, crystals were added to liquid media or phosphate buffer and the mixtures were sonicated for 20 min prior to inoculation. In experiments demonstrating growth at the expense of fluorene, this compound was added to flasks of sterile liquid medium as an acetone solution (2%) to give a final concentration of 5 mM. Flasks were shaken at 200 rpm at 30°C for 24 h before inoculation to allow acetone removal. The creosote-PAC mixture was added (0.1%, wt/vol) to sterile mineral salts medium containing 0.2% dimethyl sulfoxide to enhance the solubility of high-molecular-weight compounds.

Taxonomic characterization of fluorene-degrading strain F297. Strain F297 was isolated from enrichment cultures established in fluorene-mineral salts medium inoculated with soil highly contaminated with creosote (American Creosote Works, Pensacola, Fla.). The isolate grew on solid mineral medium with fluorene as a sole source of carbon and energy, accumulating a diffusible yellow metabolite. Bacteriological and biochemical tests were performed by standard methods (16). Growth substrates were employed at a 5 mM concentration in liquid mineral medium, except for penicillin G, which was tested on mineral medium plates containing 10 g of this compound per liter. Oxidation of protocatechuate by cell extracts was examined according to the methods described by Gibson (17) to establish the ring cleavage mechanism. Flagella were observed by transmission electron microscopy.

Analysis of the fatty acid methyl esters obtained from the lipids of this strain was performed by MIDI Laboratories (Newark, Del.) with cells grown for 24 h on Trypticase soy broth solidified with agar. The isolate was also examined by the API identification system (Biomerieux, Marcy l'Etoile, France).

Utilization of fluorene as a sole source of carbon and energy. Growth at the expense of fluorene was verified by demonstrating an increase in bacterial protein with a concomitant decrease in the concentration of fluorene, as described previously (20). Fluorene utilization and accumulating metabolites were demonstrated by changes in the UV-visible spectra of supernatants and by the examination of centrifuged and filtered culture medium by high-performance liquid chromatography (HPLC). Spectral changes of filtered (pore size, 0.2 μm) culture medium were determined by measurement on a Perkin-Elmer Lambda 6 spectrophotometer at 200 to 700 nm.

Utilization of individual PACs. Growth on and transformation of PACs were first tested on solid mineral media. 9-Fluorenone, anthracene, phenanthrene, dibenzothiophene (DBT), acenaphthene, acenaphthylene, dibenzofuran, carbazole, fluoranthene, and pyrene were supplied by spraying the surface of inoculated mineral medium plates with a solution of each substrate in ethanol-free diethyl ether (5%) (28). Naphthalene, 2,3-dimethylnaphthalene (2,3-DMN), 2,6-dimethyl-naphthalene (2,6-DMN), and biphenyl were supplied as volatile substrates by incubating plates under bell jars containing crystals in separate open containers. Plates were incubated at 23°C for up to 30 days. Growth was evidenced by a significant increase in bacterial mass on test plates compared with that on control plates incubated in the absence of substrate. In the case of sprayed plates, the utilization or transformation of substrates was noted as a clearing of the insoluble substrate adjacent to the inoculated cell mass, occasionally accompanied by the development of diffusible colored products.

The utilization of substrates for growth was confirmed by inoculating strain F297 to replicate 125-ml Erlenmeyer flasks containing 25 ml of mineral medium with individual compounds. Cultures were analyzed for increases in protein content at 0, 7, 14, and 30 days by the bicinchoninic acid method (Pierce Chemical Co., Rockford, Ill.). The transformation of single PACs by fluorene-induced cells was corroborated by conducting washed-cell incubations with test substrates, by monitoring changes in UV-visible spectra of culture filtrates, and, where appropriate, by HPLC and GC-MS analyses of organic extracts.

Washed-cell incubations with single PACs. Cells were grown in 2-liter Erlenmeyer flasks containing, in 400 ml of mineral salts medium, glutamine (5 mM) and fluorene crystals (1 g/liter). Incubation was carried out on a rotary shaker at 23°C and 200 rpm. When the late exponential phase was reached (after approximately 20 h, $A_{650} = 0.65$), fluorene crystals were removed by filtration through sterile glass wool, and cultures were incubated for an additional 30-min period in order to consume any remaining fluorene. Cells were harvested by centrifugation, washed twice with 50 mM Na-K phosphate buffer, pH 7.2, and resuspended in a small volume of the same buffer to give a cell density approximately 40 times that of the harvested culture.

PACs and analogs were added (1 g/liter) to 250-ml Erlenmeyer flasks containing 50 ml of the same buffer, and the mixtures were sonicated as above. Fluorene-induced cells were then added to give a final cell suspension with an A_{650} of approximately 2.5. Flasks were placed in a rotatory shaker at 23°C and 200 rpm and incubated for 36 h. UV-visible spectra of the filtrates were recorded (as described above) at 0, 5, 18, and 36 h of incubation. Controls without cells were included to assess possible abiotic degradation.

Extraction and identification of metabolites. Washed-cell suspensions were centrifuged to remove cells and any undissolved PACs. The supernatants were first extracted (at a neutral pH) three times with 0.5 volume of ethyl acetate and then acidified to pH 2.5 with 6 N HCl and extracted again in the same manner. Extracts were dried over anhydrous Na_2SO_4 and concentrated to a small volume in a rotatory evaporator. Portions of the extracts were treated with an excess of ethereal diazomethane before the solvent removal. For the detection and identification of metabolites, residual neutral, methylated neutrals, and methylated acidic extracts were redissolved in methylene chloride and analyzed by GC-FID and GC-MS.

Chemical structures of metabolites were suggested on the basis of their mass spectra, an instrument library search, and literature data. Where authentic samples were available, metabolites were identified by comparing mass spectra and GC retention times (R_t) with those observed for authentic compounds and their methylated derivatives and by coelution by GC. The identification of certain metabolites not previously described was confirmed by proton nuclear magnetic resonance (NMR).

Biodegradation of the creosote-PAC mixture. The action of strain F297 on components of a creosote-PAC mixture was determined by GC analysis of PACs remaining in growing cultures.

A culture grown for 24 h in glutamine (5 mM) mineral medium was used to inoculate (5×10^4 cells per ml) replicate 125-ml Erlenmeyer flasks containing 25 ml of mineral medium, dimethyl sulfoxide (0.2%), and creosote PACs (1 g/liter). Cultures were incubated on a rotatory shaker at 23°C and 200 rpm. Sterile uninoculated flasks were included as controls. The creosote-PAC concentration and the accumulation of neutral metabolites in cultures and controls were determined after 0, 2, 4, and 6 weeks of incubation. The growth of strain F297 was determined by plate counts on Luria-Bertani agar at 24-h intervals during the first week and then at weekly intervals for up to 6 weeks.

To assess creosote-PAC removal and the accumulation of neutral metabolites, entire flask contents were extracted four times with 10 ml of methylene chloride. Extracts were dried over Na_2SO_4 before being concentrated to a 1-ml volume

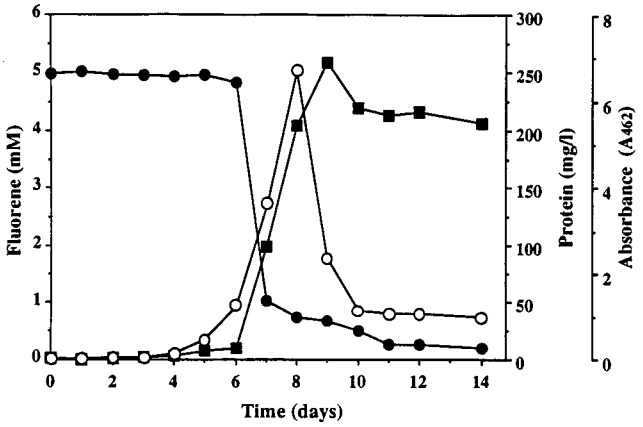

FIG. 1. Utilization of fluorene by *P. cepacia* F297 and accumulation of a yellow metabolite (absorption maximum at 462 nm), during growth in liquid mineral medium with fluorene as the sole source of carbon and energy at 23°C and 200 rpm. Growth is shown as an increase in cell protein (■). Fluorene concentration (●) was determined by HPLC analyses of organic extracts. The appearance and disappearance of the yellow metabolite (○) were monitored by UV-visible spectrophotometric analysis of supernatants.

under dry nitrogen. Duplicate samples, corresponding to concentrations 1 and 5 times those of the original cultures, were analyzed by GC-FID. Each remaining extract was concentrated to dryness and weighed. By this method, 80% of the weight of the creosote-PAC mixture was recovered from nonincubated controls. Extraction followed by GC analysis showed that 64% of the weight of the starting mixture could be accounted for as specific identifiable PACs (shown below). Where new chromatographic peaks appeared, samples were analyzed by GC-MS. Compounds corresponding to new peaks were identified by comparing mass spectra and GC R_t with those obtained with authentic samples and by coelution by GC-FID.

After 6 weeks of incubation, cultures were also extracted with ethyl acetate following acidification to pH 2.5. Acidic extracts were treated with diazomethane and subsequently analyzed by GC-MS. Acidic metabolites were identified on the basis of their mass spectra and GC R_t matching those of authentic compounds similarly methylated.

Analytical methods. Reversed-phase HPLC, GC-MS, and NMR analyses were performed as described elsewhere (20). GC analyses were conducted with a Hewlett-Packard 5890 series II gas chromatograph equipped with an autosampler, splitless injector, and flame ionization detectors coupled to a Hewlett-Packard Chem Station data processing system. Helium was used as the carrier gas (linear velocity, 20 cm/s). Nitrogen (flow rate, 35 ml/min) was used as the makeup gas for the flame ionization detectors. Creosote-PACs were separated on a Hewlett-Packard 5 capillary column (25 m by 0.32 mm, inside diameter, with a 0.17-μm thickness). The column temperature was set at 50°C for 1 min and then increased linearly at 10°C/min until a final temperature of 310°C was reached. Injector and detector temperatures were set at 290 and 315°C, respectively. The amounts of targeted analytes present were calculated for duplicate samples from the peak areas obtained by 1-μl injections, by using standard calibration curves for each chemical. The limit of detection for PACs was set at 100 ppb.

RESULTS

Taxonomic characterization of strain F297. Strain F297 grew on solidified minimal medium, with fluorene supplied as crystals, in the lids of petri plates over 7 days at 23°C, accumulating a diffusible yellow metabolite in the culture medium. The isolate was a rod-shaped gram-negative bacterium, aerobic, motile by a single polar flagellum, nonfermentative, and oxidase positive. Growth at 41°C was not observed. Fluorescent pigments were not produced on Bacto *Pseudomonas* agar F medium (Difco Laboratories), and the extracts of cells grown with *p*-hydroxy-benzoate effected *ortho* cleavage of protocatechuate. Other enzyme activities detected were catalase, esculin hydrolysis, and β-galactosidase, while nitrate reduction, arginine dehydrolase, urease, and gelatinase were not found. Strain F297 showed growth on D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, D-gluconate, caprate, adipate, L-malate, citrate, phenylacetate, phthalate, and penicillin G (at a concentration of 1.0%). On the basis of these characteristics, F297 was identified as a strain of *P. cepacia*. The GC profiles obtained by fatty acid methyl ester analysis of the total lipids and analysis by the API system were both consistent with this identification.

Utilization of fluorene and identification of fluorene metabolites. The utilization of fluorene as a sole source of carbon and energy by *P. cepacia* F297 was confirmed by an increase in bacterial protein with a concomitant loss of fluorene (Fig. 1). During exponential growth (with an estimated generation time of 11 h), approximately 710 μg of fluorene was converted to 500 μg (dry weight) of cells (assuming protein to be about 50% of cell dry weight) (38). Given that cell dry weight is approximately 50% carbon, there appears to be a cellular assimilation of fluorene with an efficiency of about 40%. During growth, a yellow metabolite (absorption maximum at 462 nm, pH 7.0) was accumulated transiently in the medium, reaching a maximum during late exponential phase (A_{462} = 5). After acidification (Fig. 2) the absorption maximum shifted to 353 nm, suggesting the keto-enolic tautomerism characteristic of *meta*-cleavage products of aromatic ring systems. This product was not observed with 9-fluorenone either during growth or in washed-cell incubations. An absorption maximum at 460 has also been reported by Foght and Westlake (14) in supernatants of washed-cell incubations of *Pseudomonas* sp. strain HL7b with fluorene. Our attempts to isolate this metabolite were unsuccessful, evidently because of its chemical instability.

HPLC analyses of culture filtrates failed to reveal this yellow product; however, six other products were detected. Because of the poor chromatographic resolution of these compounds, it was not possible to determine the kinetics of their formation and disappearance. In sterile controls no abiotic degradation of fluorene was observed. The analyses of supernatants from washed-cell suspensions incubated with fluorene revealed a number of products (Fig. 3), some of which had previously been observed in growing cultures. A compound eluting at 7.15 min exhibited an R_t and UV-visible absorbance spectrum (absorption maxima [λ_{max}] at 215 nm, 246 nm, and 295 nm)

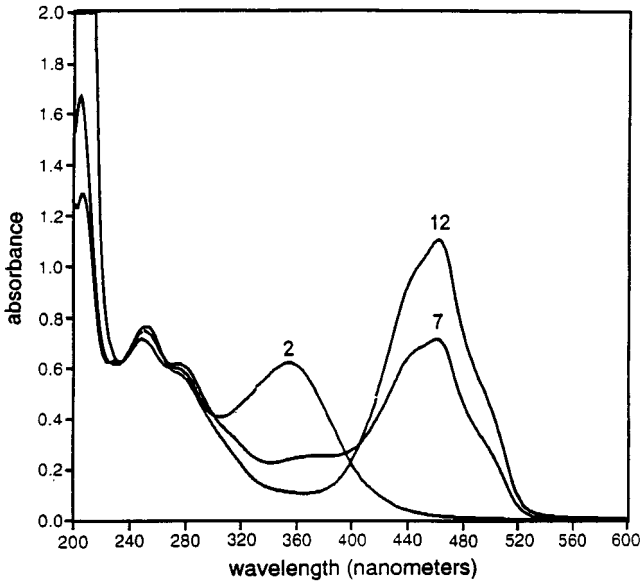

FIG. 2. UV-visible spectra of the yellow metabolite produced from fluorene by *P. cepacia* F297. Spectra were recorded from supernatants from washed-cell incubations at pHs 7.0, 2.0, and 12.0.

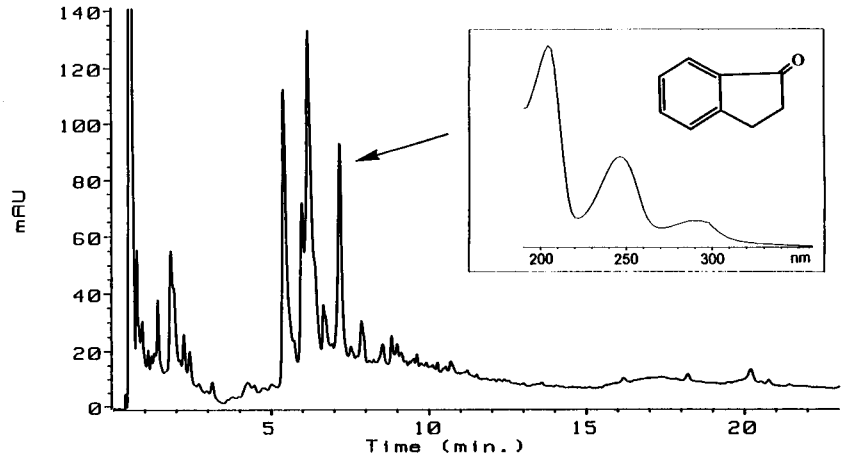

FIG. 3. HPLC elution profile of the supernatant from a washed-cell incubation of strain F297 with fluorene after 18 h. One of the major metabolites showed an R_t and UV-visible spectrum (inset) indistinguishable from those obtained for authentic 1-indanone. mAU, milli-absorbance units.

identical to those observed for authentic 1-indanone. GC-MS analysis of neutral extracts showed four major compounds. One of them (20% of total ion chromatogram) possessed an R_t (10.9 min), mass spectrum (M^+ at *m/z* 132 [100%]), and fragment ions (at *m/z* 104 [M^+-CO, 91%], 78 [37%], 63 [9%], and 51 [28%]) identical to those observed for authentic 1-indanone. Coinjection of the neutral extract with authentic 1-indanone in HPLC and GC analyses resulted in an increase in the single peak at the mentioned R_ts, establishing the identity of 1-indanone as a product of biodegradation of fluorene by *P. cepacia* F297.

Utilization of other PACs. *P. cepacia* F297 grew on naphthalene, 2,3-DMN, anthracene, phenanthrene, and DBT (Table 1). In addition, washed-cell suspensions of fluorene-induced cells transformed 2,6-DMN, biphenyl, acenaphthene, acenaphthylene, dibenzofuran, and carbazole. No activity towards fluoranthene or pyrene was observed. The visible absorption maxima observed for yellow-orange metabolites accumulated from anthracene (435 nm), dibenzothiophene (460 nm), biphenyl (430 nm), and dibenzofuran (460 nm) are identical to those reported in the literature for the *meta*-cleavage products accumulated during the metabolism of these PACs by different bacteria (7, 12, 29, 47).

TABLE 1. Activities of *P. cepacia* F297 on single PAHs[a]

Substrate	Growth	Transformation	Color	UV-visible λ_{max} of accumulated products (nm)
Naphthalene	+	+	Yellow	376
2,3-DMN	+	+	Yellow	Shoulder at 344
Anthracene	+	+	Yellow	435
Phenanthrene	+	+	Yellow	334 and 344
DBT	+	+	Orange	Shoulder at 300, 402, and 470
2,6-DMN	−	+	Pink	ND[b]
Biphenyl	−	+	Yellow	430
Acenaphthene	−	+	Brown	ND
Acenaphthylene	−	+	ND	ND
Dibenzofuran	−	+	Yellow-orange	444 and 460
Carbazole	−	+	Grey	ND
Fluoranthene	−	−	ND	ND
Pyrene	−	−	ND	ND

[a] Transformation was demonstrated by changes in the UV-visible spectra (between 300 and 700 nm) in washed-cell incubations with single PAHs.
[b] ND, none detected.

Identification of metabolites from single PAC incubations. In order to identify metabolites from the degradation or transformation of these PACs, fluorene-grown washed cells were incubated with single compounds. Mass spectral characteristics of the major metabolites are shown in Tables 2 and 3.

Incubations with naphthalene yielded an acidic metabolite (57%; R_t, 14.56 min) showing an R_t and mass spectrum identical to those of the methyl ester of authentic gentisic acid. When incubated with 2,3-DMN, strain F297 carried out ring cleavage as well as methyl group oxidation. The only neutral metabolite detected was tentatively identified as 2-hydroxymethyl-3-methylnaphthalene on the basis of molecular weight (molecular ion at *m/z* = 172), loss of water to give a base peak (*m/z* at 154), and the presence of a naphthalene ion (*m/z* at 128). Two major metabolites were detected in acidic extracts after methylation. The most abundant (66%) possessed a molecular ion at an *m/z* of 180 and a fragmentation pattern consistent with the methyl ester of 4,5-dimethylsalicylic acid. This compound was isolated by extracting supernatants of washed-cell suspensions first at pH 6.5 and then at pH 2.5. This last extraction yielded 4 mg of the product with 95% purity (total ion chromatogram) which was analyzed by ^{1}H NMR as its methyl ester derivative. The ^{1}H NMR spectrum revealed the presence of one phenolic proton (δ = 10.5 ppm; s, 1H), three protons of a methyl ester (3.93 ppm; s, 3H), two isolated (noncoupled to other protons) aromatic protons (H-3 at 6.78 ppm; s, 1H; and H-6 at 7.57 ppm; s, 1H), and protons of two nonequivalent methyl aromatic-ring substituents, −2.19 ppm (s, 3H) and 2.26 ppm (s, 3H). The *para* relationship of the two isolated aromatic protons was assigned definitively because of (i) the absence of H-H couplings and (ii) an *ortho* effect in the mass spectrum (M^+-CH_3OH [M^+-32]) indicative of the *ortho* relationship of $COOCH_3$ and OH groups. According to these properties, this metabolite was identified as 4,5-dimethylsalicylic acid. The mass spectrum of the other methylated component of the acidic extract (R_t, 16.97 min; 4%) presented a molecular ion at an *m/z* of 200, with losses of methyl, methoxy, methanol, and carbonyl groups, giving a naphthalene ion (*m/z* at 128). On the basis of these data, this metabolite was identified as the methyl ester of 3-methyl-2-naphthoic acid.

Anthracene and phenanthrene were each transformed by F297 to different acidic metabolites, which gave, respectively,

TABLE 2. GC R_t and electron impact mass spectral properties of major compounds formed from PACs which support growth by fluorene-grown cells of *P. cepacia* F297

Substrate	Extract[a]	R_t (min)	Abundance (%)	*m/z* of fragment ions (% relative intensity)	Identification[b]	Product no.
Naphthalene	AM	14.56	70	168 (M^+, 39), 136 (M^+-CH_3OH, 100), 198 (M^+-CH_3OH-CO, 22), 80 (14), 69 (3), 53 (9)	Gentisic acid (ME)[c]	1
2,3-DMN	N	16.88	100	172 (M^+, 68), 154 (M^+-H_2O, 100), 143 (38), 128 (54), 115 (22), 89 (8), 76 (22)	2-Hydroxymethyl-3-methylnaphthalene[d]	2
	AM	13.57	66	180 (M^+, 39), 148 (M^+-CH_3OH, 100), 120 (M^+-CH_3OH-CO, 21), 105 (M^+-CH_3OH-CO-CH_3, 2.4), 91 (25), 77 (6)	4,5-Dimethylsalicylic acid (ME)[c]	3
	AM	16.97	4	200 (M^+, 100), 185 (M^+-CH_3, 6.0), 168 (M^+-CH_3OH, 89), 154 (M^+-CH_3-OCH_3, 16), 141 (76), 140 (M^+-CH_3OH-CO, 74), 128 (12), 115 (7), 100 (5), 84 (10), 70 (10)	3-Methyl-2-naphthoic acid (ME)[d]	4
Anthracene	AM	17.60	100	202 (M^+, 52), 170 (M^+-CH_3OH, 100), 142 (M^+-CH_3OH-CO, 71), 114 (M^+-CH_3OH-CO-CO, 33), 89 (8), 85 (6), 71 (9)	2-Hydroxy-3-naphthoic acid (ME)[c]	5
Phenanthrene	AM	17.25	92	202 (M^+, 35), 170 (M^+-CH_3OH, 100), 142 (M^+-CH_3OH-CO, 5), 114 (M^+-CH_3OH-CO-CO, 40), 85 (4), 63 (4), 57 (2.3)	1-Hydroxy-2-naphthoic acid (ME)[c]	6
DBT	N	15.00	65	178 (M^+, 100), 160 (M^+-H_2O, 4), 132 (M^+-H_2O-CO, 2), 121 (25), 109 (2.6), 89 (3), 77 (13)	3-Hydroxy-2-formylbenzothiophene[e]	7
	N	21.43	35	216 (M^+, 100), 187 (35), 168 (29), 160 (22), 139 (24), 136 (24), 115 (12), 104 (11), 79 (12), 63 (10)	Dibenzothiophene sulfone[c]	8
	AM	16.01	28	210 (M^+, 23), 192 (M^+-H_2O, 0.4), 151 (M^+-$COOCH_3$, 100), 134 (M^+-$COOCH_3$-OH, 1.7), 133 (M^+-$COOCH_3$-H_2O, 1.9), 123 (8), 105 (1.4), 89 (3), 77 (0.2)	3-Hydroxy-2,3-dihydrobenzothiophene-2-carboxylic acid (ME)[d]	9

[a] N, neutral extract; AM, acidic extract treated with diazomethane.
[b] ME, methyl ester.
[c] Identified by comparison (of R_t and mass spectra) with authentic material or by NMR.
[d] Suggested structure.
[e] The identification is supported by other studies.

TABLE 3. GC R_t and electron impact mass spectral properties of major compounds formed from PACs which do not support growth by fluorene-grown cells of *P. cepacia* F297

Substrate	Extract[a]	R_t (min)	Abundance (%)	*m/z* of fragment ions (% relative intensity)	Identification[b]	Product no.
2,6-DMN	N	16.36	61	172 (M^+, 100), 157 (M^+-CH_3, 16), 155 (M^+-OH, 3), 154 (M^+-H_2O, 3), 141 (8), 128 (23), 115 (7), 95 (3), 77 (5.3)	2-Hydroxymethyl-6-methylnaphthalene[c]	10
	AM	17.40	84	200 (M^+, 77), 169 (M-OCH_3, 100), 141 (M^+-$COOCH_3$, 42), 126 (M^+-$COOCH_3$-CH_3, 2), 115 (21), 99 (3), 84 (6), 70 (4)	6-Methyl-2-naphthoic acid (ME)[d]	11
Biphenyl	AM	16.29	47	206 (M^+, 5), 175 (M^+-OCH_3, 10), 147 (M^+-$COOCH_3$, 7), 120 (M^+-$COOCH_3$-CH_2-CH_2 + H, 14), 105 (M^+-$COOCH_3$-CH_2-CH_2-CH_2, 100), 77 (105-CO, 36)	5-Oxo-5-phenylpentanoic acid (ME)[c]	12
	AM	18.69	36	234 (M^+, 0.8), 216 (M^+-H_2O, 0.6), 175 (M^+-$COOCH_3$, 65), 147 (M^+-$COOCH_3$-CO, 39), 120 (2.8), 105 (100), 77 (35)	2-Hydroxy-6-oxo-6-phenyl-4-hexenoic acid (ME)[d]	13
Acenaphthene	N	16.23	68	168 (M^+, 100), 140 (M^+-CO, 84), 113 (6), 98 (2), 89 (4), 87 (7), 84 (4), 74 (3), 70 (12), 63 (8), 50 (5)	Acenaphthenone[e]	14
	N	18.68	28	182 (M^+, 71), 154 (M^+-CO, 100), 126 (M^+-CO-CO, 88), 98 (8), 87 (7), 74 (14), 63 (20), 50 (7)	Acenaphthoquinone[e]	15
	N	20.35	2	198 (M^+, 89), 170 (M^+-CO, 14), 154 (M^+-CO-O, 100), 126 (M^+-CO-O-CO, 97), 98 (7), 87 (10), 74 (23), 63 (24), 50 (10)	Naphthalic anhydride[e]	16
	AM	19.95	70	244 (M^+, 34), 213 (M^+-OCH_3, 100), 185 (M^+-$COOCH_3$, 28.8), 170 (M^+-$COOCH_3$-CH_3, 35), 154 (M^+-$COOCH_3$-OCH_3, 8), 142 (2.6), 133 (7), 126 (M^+-$COOCH_3$-$COOCH_3$, 13), 114 (18), 77 (7)	1,8-Naphthalene dicarboxylic acid (DiME)[e]	17
Acenaphthylene	N	16.09	17	168 (M^+, 100), 140 (M^+-CO, 84), 113 (6), 98 (2.4), 89 (4), 87 (7), 84 (4), 74 (3), 70 (12), 63 (8)	Acenaphthenone[e]	14
	N	18.62	69	182 (M^+, 71), 154 (M^+-CO, 100), 126 (M^+-CO-CO, 88), 98 (8), 87 (7), 74 (14), 63 (20)	Acenaphthoquinone[e]	15
	N	20.38	9	198 (M^+, 87), 154 (M^+-CO-O, 100), 126 (M^+-CO-O-CO, 97), 98 (7), 87 (10), 74 (23), 63 (24), 50 (10)	Naphthalic anhydride[e]	16
	AM	19.96	27	244 (M^+, 34), 213 (M^+-OCH_3, 100), 185 (M^+-$COOCH_3$, 29), 170 (M^+-$COOCH_3$-CH_3, 35), 154 (M^+-$COOCH_3$-OCH_3, 8), 142 (2.6), 133 (7), 126 (M^+-$COOCH_3$-$COOCH_3$, 13), 114 (18), 77 (7), 63 (12)	1,8-Naphthalene dicarboxylic acid (DiME)[e]	17
Dibenzofuran	AM	22.04	20	260 (M^+, 13), 201 (M^+-$COOCH_3$, 100), 186 (M^+-$COOCH_3$-CH_3, 30), 170 (M^+-$COOCH_3$-OCH_3, 3.3), 158 (13), 145 (1.4), 130 (3.2), 102 (9), 76 (7)	4-(3′-Methoxy-2′-benzofuranyl)-2-oxo-3-butenoic acid (ME)[d]	18
Carbazole	AM	22.39	66	259 (M^+, 50), 228 (M^+-OCH_3, 10), 200 (M^+-$COOCH_3$, 100), 196 (26), 185 (18), 168 (14), 140 (16), 129 (5), 113 (8), 98 (4), 83 (5), 70 (5)	4-(3′-Methoxy-2′-indolyl)-2-oxo-3-butenoic acid (ME)[c]	19
	AM	20.56	34	247 (M^+, 57), 219 (M^+-CO, 12), 188 (M^+-$COOCH_3$, 23), 159 (M^+-CO-$COOCH_3$ + 1, 48), 161 (M^+-CO-$COOCH_3$-1, 43), 133 (M^+-CO-$COOCH_3$ + 1-CO, 30), 130 (40), 93 (100), 77 (20)	4-(3′-Oxo-2′-indolinyl)-2-oxo-3-butenoic acid (ME)[c]	20

[a] N, neutral extract; AM, acidic extract methylated.
[b] ME, methyl ester; DiME, dimethyl ester.
[c] Suggested structure.
[d] The identification is supported by other studies.
[e] Identified by comparison (of R_t and mass spectra) with authentic material.

R_t and fragmentation patterns identical to those of the methyl esters of authentic 2-hydroxy-3-naphthoic and 1-hydroxy-2-naphthoic acids. These data are consistent with data reported in the literature (53).

Incubations of DBT with strain F297 yielded products indicative of both aromatic-ring fission and sulfur oxidation. The major metabolite detected in neutral extracts (64.7%) possessed a molecular ion at an *m/z* of 178, showing elimination of water and a carbonyl group. These data are consistent with those reported in the literature for 3-hydroxy-2-formylbenzothiophene (29). A methoxy derivative (*m/z* at 192) of this compound was also found in neutral extracts treated with diazomethane, adding support to this identification. Another neutral compound was observed with an R_t of 21.43 min and a mass spectrum indistinguishable from that of authentic dibenzothiophene sulfone (*m/z* = 216). A major compound detected in acidic extracts gave a molecular ion at an *m/z* of 210, which, together with the elimination of methylated carboxy and hydroxyl groups and water, suggested that this product is the methyl ester of 3-hydroxy-2,3-dihydrobenzothiophene-2-carboxylic acid. This assignment further supports the identification of 3-hydroxy-2-formylbenzothiophene.

As mentioned above, UV-visible spectrophotometric analyses of filtrates from fluorene-induced washed-cell incubations demonstrated that strain F297 can also transform a broad range of compounds which fail to serve as growth substrates for this microorganism. These results were corroborated by GC-MS analysis of organic extracts from supernatants (Table 3). Strain F297 oxidizes one of the methyl groups of 2,6-DMN, apparently producing 2-hydroxymethyl-6-methylnaphthalene. This compound has been tentatively identified as the most abundant component in the neutral extract (61%), on the basis of its molecular weight (with a molecular ion at an *m/z* of 172); the elimination of methyl (*m/z* = 157), hydroxyl (*m/z* = 154), and water (*m/z* = 155); and the presence of a naphthalene ion (*m/z* = 128). The major component (84.2%; R_t, 17.4 min) of acidic extracts exhibited a molecular ion at an *m/z* of 200, with the subsequent elimination of methylated carboxyl and methyl groups, to give a bis-deprotonated naphthalene ion (*m/z* = 126). From these data, this acidic metabolite was identified as the methyl ester of 6-methyl-2-naphthoic acid.

Strain F297 transforms biphenyl via dioxygenation and *meta* cleavage of one of the aromatic rings. The mass spectra of two methylated acidic compounds gave molecular weights of 206 and 234, with each eliminating methylated carboxylic groups and possessing a C_6H_5CO group (with a parent peak at an *m/z* of 105). On the basis of these data the methylated acidic compounds were tentatively identified as the methyl esters of 5-oxo-5-phenylpentanoic acid and 2-hydroxy-6-oxo-6-phenyl-4-hexenoic acid, respectively. The formation of these products is consistent with *meta* cleavage of 2,3-dihydroxy-biphenyl (7) and could result from the reduction and decarboxylation of the 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid previously detected by UV-visible spectrometry (λ_{max} at 430 nm). In fact, both reduction (apparently by broad-specificity reductases) and decarboxylation of *meta*-cleavage products from biphenyl and related compounds have been previously reported (39).

Neutral extracts of incubations with both acenaphthene and acenaphthylene yielded three compounds exhibiting R_t and mass spectra indistinguishable from those found for authentic acenaphthenone, acenaphthoquinone, and naphthalic anhydride. In acidic extracts the major components identified were naphthalic anhydride and the methyl ester of 1,8-naphthalene dicarboxylic acid.

Methylated acidic extracts from dibenzofuran incubations showed a peak at an R_t of 22.04 min (20%), which, on the basis of molecular weight (*m/z* = 260) and the elimination of methylated carboxyl and methoxy groups, was tentatively identified as the dimethyl derivative (methyl ester, *O*-methoxy) of a *meta*-cleavage product from a dihydroxydibenzofuran. This mass spectrum and the UV-visible spectrum obtained from supernatants (λ_{max} at 444 and 460 nm) are consistent with literature data for 4-(3′-hydroxy-2′-benzofuranyl)-2-oxo-3-butenoic acid (47). Carbazole seems to undergo similar reactions, since the major component in acidic methylated extracts (R_t = 22.39 min; 66%) shows molecular weights (at an *m/z* of 259) and a fragmentation pattern consistent with a methyl ester of a methoxy derivative of a *meta*-cleavage product from dihydroxycarbazole. No metabolites were detected with fluoranthene or pyrene.

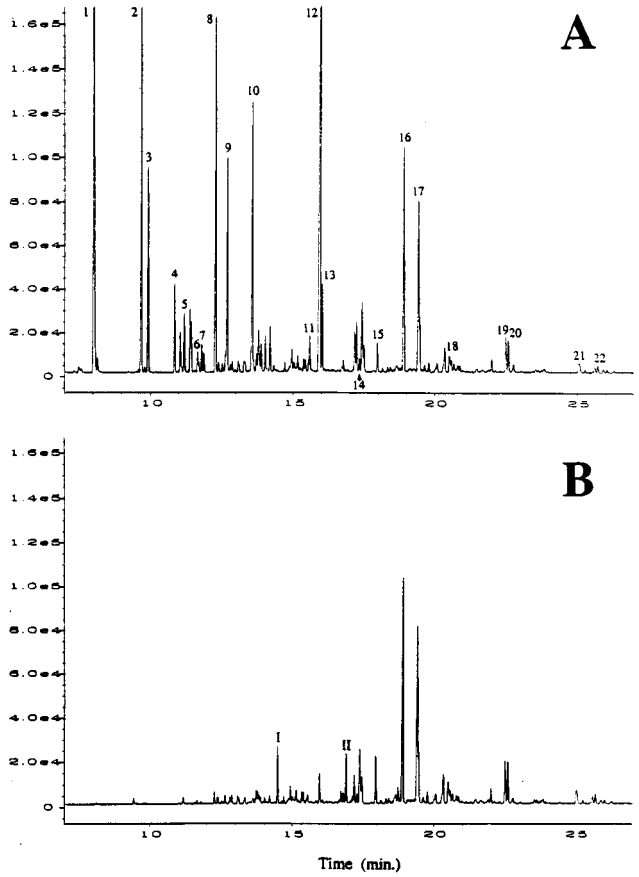

FIG. 4. GC-FID profiles of neutral extracts from a 14-day culture (B) and control (A) of *P. cepacia* F297 in mineral medium and creosote PAHs. The identity of compounds is shown in Table 5. The chromatogram (B) shows the accumulation of two neutral metabolites identified as acenaphthenone (I) and acenaphthoquinone (II). Peak numbers are identified in Table 4.

Biodegradation of creosote PACs. *P. cepacia* F297 was able to grow (from 5×10^4 to 5×10^7 cells per ml) in mineral medium with creosote PACs producing an almost complete depletion (94% with respect to uninoculated controls) of compounds containing 2 to 3 aromatic rings in 14 days (Fig. 4 and Table 4). Compounds depleted included naphthalene and methyl derivatives, naphthenoaromatic compounds, O and S heterocycles, and aromatic hydrocarbons with three rings. 2-Methylanthracene is also transformed (36% in 14 days). 2-Phenylnaphthalene does not seem to be modified. High-molecular-weight PACs (four and five aromatic rings) were attacked to a lesser extent; they were depleted by only 5% over

TABLE 4. Concentration of selected PACs and analogs in controls and cultures of *P. cepacia* F297 in mineral medium with creosote PACs

Peak no.	Substrate	Recovery of PAHs (μg/ml)[a] from:			
		Culture at:			Control at day 28
		Time zero	Day 14	Day 28	
1	Naphthalene	108.51	ND[b]	ND	2.38
2	2-Methylnaphthalene	65.20	ND	ND	13.73
3	1-Methylnaphthalene	29.48	ND	ND	7.56
4	Biphenyl	9.20	ND	ND	6.54
5	2,6-DMN	9.82	1.32	ND	6.96
6	2,3-DMN	3.50	ND	ND	1.18
7	Acenaphthylene	4.15	ND	ND	2.48
8	Acenaphthene	71.81	1.95	1.27	49.29
9	DBF	40.72	1.37	1.34	30.06
10	Fluorene	42.95	1.72	1.87	34.66
11	DBT	9.31	2.40	2.06	9.08
12	Phenanthrene	121.49	ND	ND	110.94
13	Anthracene	12.28	6.24	3.59	7.45
14	2-Methylanthracene	3.19	2.05	6.59	1.67
15	2-Phenylnaphthalene[c]	7.69	9.43	9.67	11.19
16	Fluoranthene	39.14	35.98	31.80	35.73
17	Pyrene	32.33	32.53	27.97	32.99
18	Benzo[*b*]fluorene	4.53	1.80	1.53	1.90
19	Benzo[*a*]anthracene	6.03	5.92	5.50	5.90
20	Chrysene	7.87	7.97	7.94	7.93
21	Benzo[*b*]fluoranthene	3.31	4.38	4.10	4.53
22	Benzo[*a*]pyrene	2.52	2.38	1.68	2.87
Total		635.03	117.44	106.90	387.06
Weight recovered		800.00	300.00	288.00	516.00
Amt accounted for by GC analysis (%)		79	39	37	75

[a] Quantified by GC-FID analysis. Data reported are averages of duplicate samples.
[b] ND, not detected.
[c] This compound was previously incorrectly identified as anthraquinone (36).

14 days, and by 12% over 28 days, compared with the values for controls. No further degradation was observed in 6-week cultures. The most recalcitrant component appears to be chrysene, which exhibited no significant changes throughout the incubations.

GC profiles of neutral organic extracts of 14-day cultures (Fig. 4) showed the occurrence of two new chromatographic peaks with respect to controls. In GC-MS analyses these new peaks exhibited R_t and mass spectra indistinguishable from those observed for authentic acenaphthenone and acenaphthoquinone, previously identified as metabolites of the transformation of acenaphthene and acenaphthylene by *P. cepacia* F297 (Table 3). This identification was confirmed by coelution with authentic materials in GC analysis (Fig. 5). An increase in the peak corresponding to 2-methylanthracene is also thought to be due to the accumulation of a metabolite coeluting with this compound.

In order to identify acidic metabolites indicative of dead-end products and metabolic pathways, an acidic extract (about 2 mg) was obtained from a 6-week culture. GC-MS analysis of the methylated extract (Fig. 6) showed the occurrence of five major compounds exhibiting R_t and mass spectra identical to those found for compounds previously identified in washed-cell incubations with single PACs (Tables 2 and 3). These compounds were the methyl esters of 1-hydroxy-2-naphthoic and 2-hydroxy-3-naphthoic acids, identified as metabolites of phenanthrene and anthracene, respectively; the methyl ester of 5-oxo-5-phenyl-pentanoic acid, identified in incubations with biphenyl; and 1,8-naphthalic anhydride and the dimethyl ester of 1,8-naphthalene dicarboxylic acid, products of oxidation of acenaphthene and acenaphthylene.

DISCUSSION

P. cepacia F297 grew in liquid mineral medium with fluorene as the sole source of carbon and energy, its growth yield suggesting an assimilation of about 40% of fluorene carbon. During growth, a yellow metabolite (with an absorption maximum at 462 nm) with the UV-visible spectral characteristics of an aromatic *meta*-cleavage product was transiently accumulated. 1-Indanone was identified (by HPLC, UV-visible spectrophotometry, and GC-MS) in growth media and in washed-cell incubations. These results suggest that utilization of fluorene by strain F297 is based on mechanisms analogous to those of naphthalene degradation, involving aromatic-ring dioxygenation, ring cleavage, and the release of a molecule of pyruvate (Fig. 7). The formation of 1-indanone is here accounted for by reductive dioxygenation of fluorene at C-3 and C-4 to give the corresponding *cis*-dihydrodiol, which undergoes dehydrogenation to 3,4-dihydroxyfluorene. An extradiol dioxygenase attack

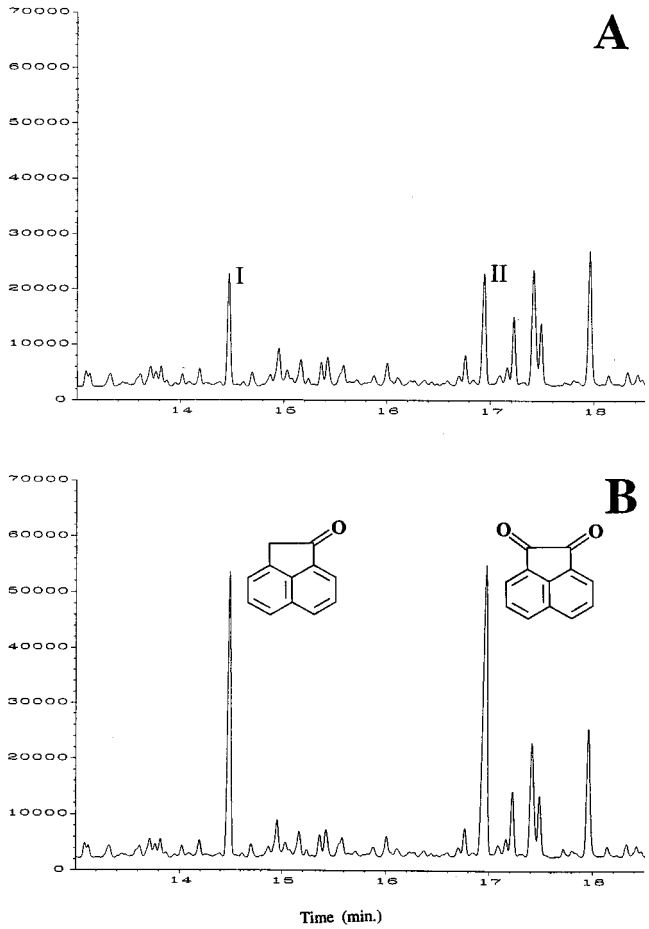

FIG. 5. Expanded GC-FID profiles of (A) the neutral extract from a 14-day culture of strain F297 in mineral medium with creosote PACs showing the accumulation of metabolites I (acenaphthoquinone) and II (acenaphthenone); and (B) the same extract spiked with authentic acenaphthenone and acenaphthoquinone.

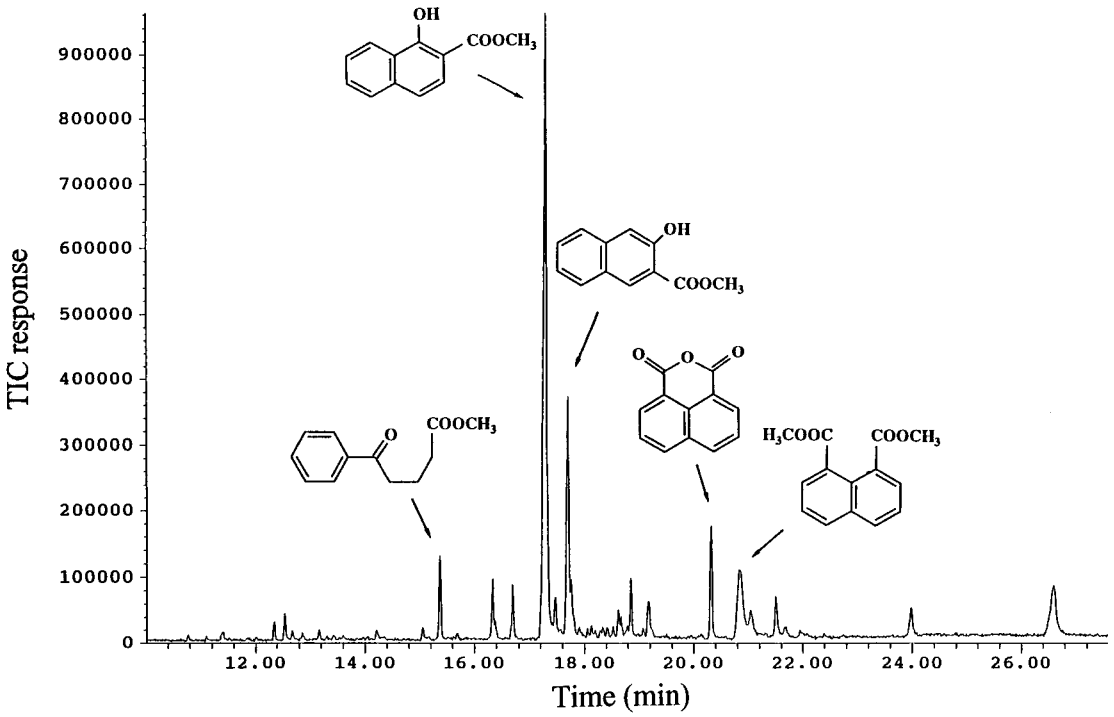

FIG. 6. Total ion chromatogram (TIC) of the acidic extract (treated with diazomethane) from a 6-week culture of *P. cepacia* F297 in mineral medium with creosote PAHs, showing the occurrence of metabolites previously identified in washed-cell incubations with single PAHs. Mass spectral characteristics of the identified peaks are shown in Tables 2 and 3.

would produce a *meta*-cleavage product. After spontaneous and/or enzymatic isomerization, a hydratase-aldolase reaction would yield 2-formyl-1-indanone by loss of a molecule of pyruvate. The removal of the formyl group might then occur by decarboxylation of the β-keto acid resulting from oxidation of the formyl group to a carboxyl. As of now, there is no evidence of further utilization of 1-indanone.

In previous work (19) the occurrence of 3,4-dihydrocoumarin in culture fluids of a species of the genus *Arthrobacter* growing with fluorene was explained as a result of a biological Baeyer-Villiger oxidation of 1-indanone, for which a similar pathway was proposed; 1-indanone itself was not detected. The *Arthrobacter* sp. also mono-oxygenated fluorene at C-9 to give first 9-fluorenol and then 9-fluorenone, which apparently accumulated as a dead-end product. 9-Fluorenol, 9-fluorenone, and 1-indanone were also observed (by HPLC) when fluorene was oxidized by a pyrene-degrading strain of the genus *Mycobacterium* (5) that was unable to use this hydrocarbon as a carbon source.

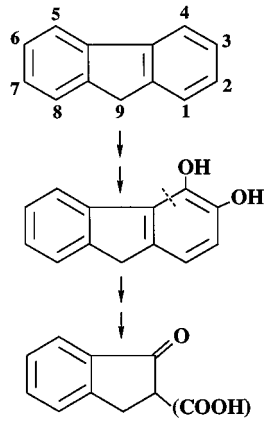

FIG. 7. Proposed metabolic pathway for the utilization of fluorene by *P. cepacia* F297.

Neither 9-fluorenone nor the angular dioxygenation products indicative of the pathway recently described by Grifoll et al. (20) and Trenz et al. (51) were detected during growth or in washed-cell incubations. However, *P. cepacia* F297 utilizes 9-fluorenone as a sole carbon source and, as discussed below, is capable of mono-oxygenation of benzylic methylenic groups. Available evidence supports 3,4-dioxygenation, but neither 1,2-dioxygenation nor mono-oxygenation to give 9-fluorenone and its further utilization can be excluded. The growth yield observed suggests a higher efficiency than that corresponding to the utilization of merely a single molecule of pyruvate per molecule of fluorene. Another aromatic ketone, 9-xanthenone, undergoes productive dioxygenation and extensive catabolism by a strain of the genus *Arthrobacter* (50).

Strain F297 showed broad versatility in its action on polycyclic aromatic substrates. In addition to fluorene, it utilizes naphthalene, 2,3-DMN, phenanthrene, anthracene, and DBT as substrates for growth. The identification of metabolites (by GC-MS) and the detection of *meta*-cleavage products in incubations of fluorene-grown cells with individual PACs are consistent with the above-mentioned conclusion that this organism possesses enzymes akin to those of the naphthalene pathway to salicylate (Fig. 8). The formations of 4,5-dimethylsalicylic acid from 2,3-DMN, 1-hydroxy-2-naphthoic acid from phenanthrene, 2-hydroxy-3-naphthoic acid from anthracene, and 3-hydroxy-2-formylbenzothiophene from DBT are all consistent with aromatic-ring dioxygenation and *meta* cleavage, followed by the release of a molecule of pyruvate (8, 11, 18).

Fluorene-grown cells of strain F297 formed gentisic acid from naphthalene. In most naphthalene-degrading bacteria in-

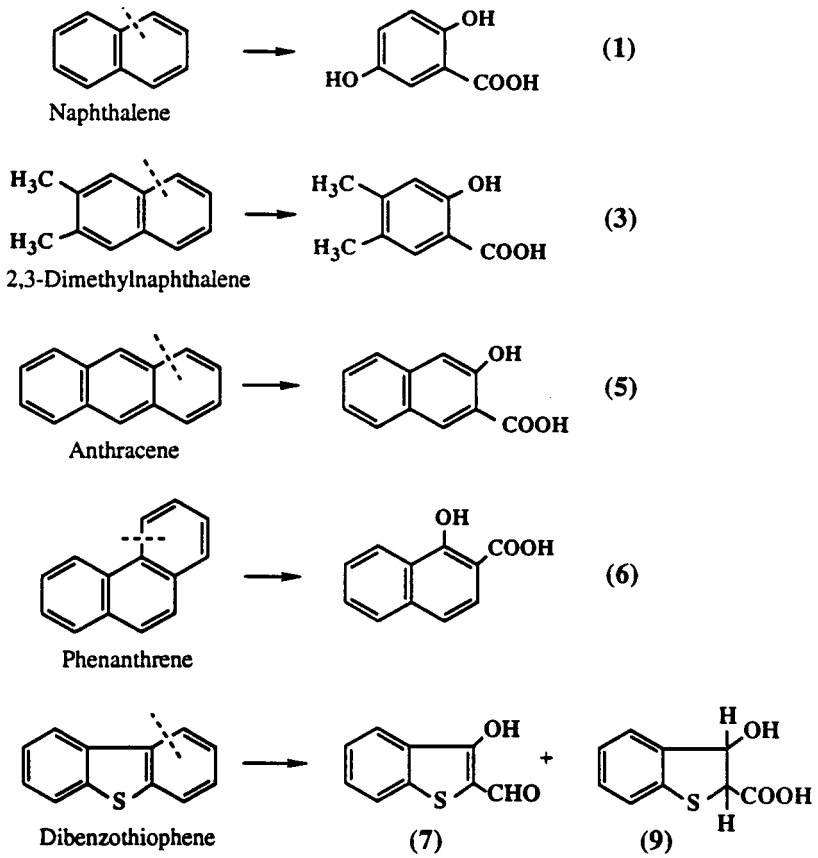

FIG. 8. Metabolites identified in washed-cell suspensions of *P. cepacia* F297 incubated with growth substrates. The formation of these metabolites is explained by aromatic-ring dioxygenation and cleavage (dotted lines).

vestigated (8, 11, 18) salicylate undergoes oxidative decarboxylation to catechol. However, in a number of cases gentisate has been established as an alternative metabolite of salicylate and, by extension, of naphthalene catabolism (22, 34, 49).

1-Hydroxy-2-naphthoic and 2-hydroxy-3-naphthoic acids are key metabolites in the pathways proposed for the utilization of phenanthrene and anthracene in all bacteria studied (8, 18, 27, 33). The metabolism of DBT via a naphthalene-type pathway to give 3-hydroxy-2-formylbenzothiophene as a dead-end product has been reported for a number of microorganisms (24, 29, 31, 32, 35). Bacteria using this route have also been shown to oxidize the S heteroatom to give dibenzothiophene-5-oxide (31) or, ultimately, dibenzothiophene sulfone as dead-end products (35). As discussed below, the sulfone is also formed from DBT by *P. cepacia* F297.

Recent work has pointed out that these same transformations of phenanthrene, anthracene, and DBT can be carried out by naphthalene-degrading systems. Menn et al. (33) provided biochemical evidence that NAH7 and NAH7-like plasmids are responsible for the transformation of anthracene and phenanthrene to the corresponding hydroxy-naphthoic acids. Denome et al. (9) reported that a single genetic system is responsible for the metabolism of DBT to 3-hydroxy-2-formylbenzothiophene, naphthalene to salicylate, and phenanthrene to 1-hydroxy-2-naphthoic acid in *Pseudomonas* strain C18 and that there is a high degree of similarity between its DNA sequence and the sequence encoding enzymes converting naphthalene to salicylate.

Fluorene-grown cells transformed a number of PACs which failed to support growth of the strain: biphenyl, dibenzofuran, 2,6-DMN, acenaphthene, acenaphthylene, and carbazole. The identification of products from these compounds (by GC-MS), together with results discussed above, allows the reactions carried out by F297 to be grouped into four discrete types: (i) aromatic-ring dioxygenation and cleavage, which furnish a molecule of pyruvate from compounds that clearly support growth and which apparently lead to the accumulation of *meta*-cleavage products from those compounds that cannot support growth, (ii) methyl group oxidations, (iii) methylenic oxidations, and (iv) S oxidations in sulfur aromatic heterocycles (Fig. 9).

Biphenyl, dibenzofuran, and carbazole underwent ring oxidation and *meta* cleavage to produce polar products which accumulated in supernatants. Evidently, the release of a molecule of pyruvate by hydration and aldolase cleavage is not possible for these ring cleavage products. Once again, the conversions are consistent with the actions of a naphthalene catabolic system referred to above. Dioxygenation and ring cleavage of dibenzofuran by naphthalene-degrading systems has been described previously. Selifonov et al. (47) implicated the NAH7 plasmid in the conversion of dibenzofuran to a dead-end product, 4-[2′-(3′-hydroxy)benzofuranyl]-2-keto-3-butenoic acid, through actions of sequence from naphthalene dioxygenase to 2-hydroxychromene-2-carboxylase isomerase. Eaton and Chapman (11) reported the formation of the *meta*-cleavage product *trans*-4-(3′-hydroxy-2′-benzofuranyl)-2-oxo-but-3-enoate by biotransformation of dibenzofuran by *Pseudomonas aeruginosa* PAO1, carrying the corresponding NAH7

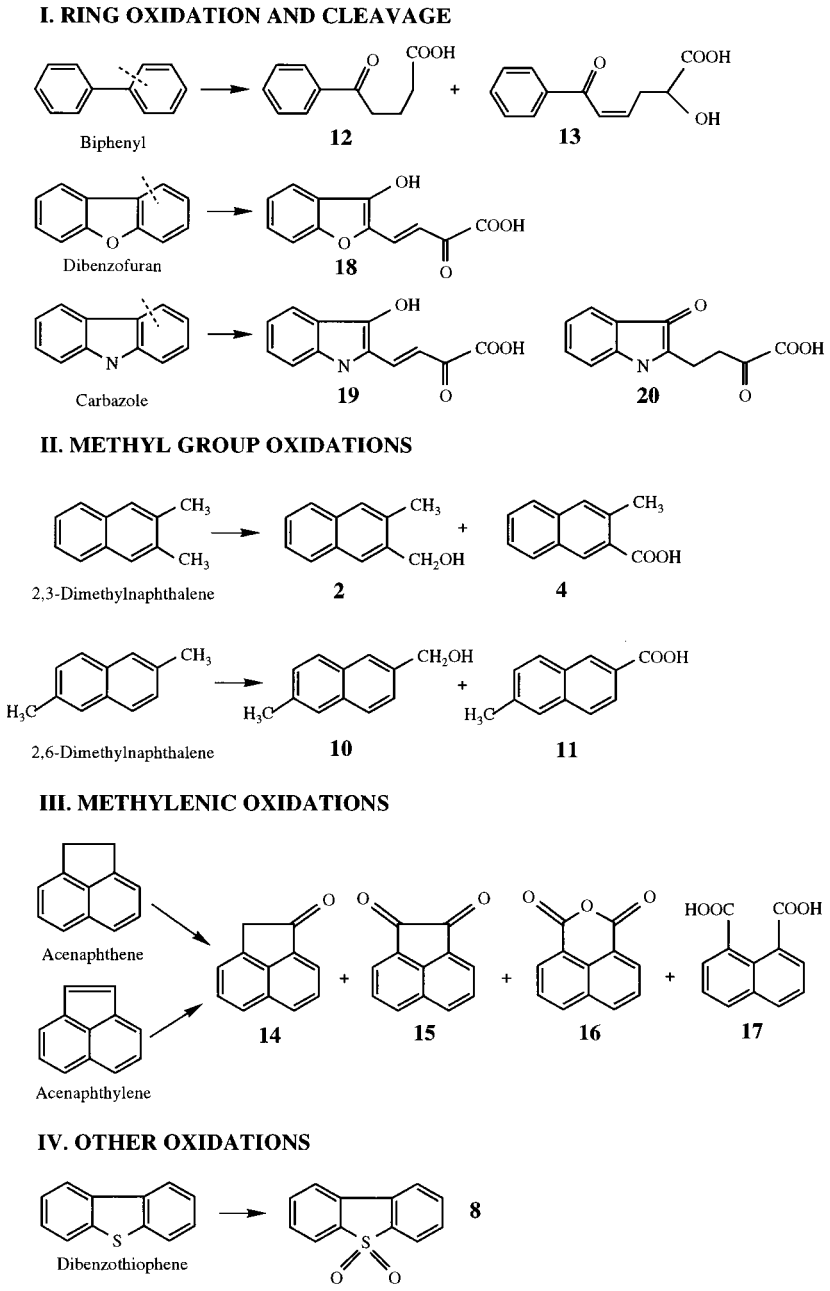

FIG. 9. Metabolites identified in washed-cell incubations of *P. cepacia* F297 with PACs which fail to support growth. Reactions are grouped by type.

genes. *meta*-cleavage products formed by strain F297 from carbazole have not been reported previously. However, 3-hydroxycarbazole has been identified as a product of the oxidation of carbazole by both naphthalene 1,2-dioxygenase and biphenyl 2,3-dioxygenase (42), apparently as a result of the ready dehydration of an unstable *cis*-diol.

P. cepacia F297 performed dioxygenation and cleavage of the unsubstituted aromatic ring of 2,3-DMN to give 4,5-dimethylsalicylic acid. Alternatively, one of the methyl groups of both 2,3- and 2,6-DMN is mono-oxygenated to give the corresponding methyl-naphthoic acids, which apparently accumulate as a dead-end products. The biotransformation of dimethylnaphthalenes to methyl-substituted naphthalene carboxylic acids has been reported previously for a numbers of organisms (10, 41, 48). However, 2-methyl-6-naphthoic acid is not an end product but an intermediate in the utilization of 2,6-DMN by flavobacteria (3).

The formation of products of oxidation of the methylenic and methyne groups of acenaphthene and the oxygenated methyne group of acenaphthylene by strain F297 is not unexpected. A number of studies have shown that certain arene dioxygenases act as monooxygenases and hydroxylate the benzylic functions of naphthenoaromatic compounds. The resultant secondary alcohols serve as substrates for dehydrogenases to yield the corresponding ketones (6, 45, 52). Schocken and Gibson (44) reported similar oxidations of acenaphthene and acenaphthylene by a diol-accumulating mutant (B8/36) of a biphenyl-degrading strain of the genus *Beijerinckia*, suggesting

acenaphthoquinone as a dead-end product. In a recent work (45) *P. aeruginosa* PAO1, carrying the naphthalene dioxygenase genes cloned from the naphthalene plasmid NAH7, mono-oxygenated acenaphthene to 1-acenaphthenol and then to a mixture of *cis*- and *trans*-acenaphthene diols, which were transformed to acenaphtenone and acenaphtoquinone by the action of nonspecific dehydrogenases. Acenaphthylene was dioxygenated, giving *cis*-acenaphthene-1,2-diol and acenaphthoquinone. With both substrates the quinone was further oxidized to naphthalene 1,8-dicarboxylic acid, which was recovered as its anhydride. The same system converted fluorene to 9-hydroxyfluorene and 9-fluorenone. In accord with these and other results discussed above, the arene dioxygenase of strain F297 appears to catalyze similar reactions, accounting for the products observed from acenaphthene and acenaphthylene.

Strain F297 also oxidized the S heteroatom in dibenzothiophene to give the sulfone. It appears that all bacterial strains reported to dioxygenate and cleave one of the aromatic rings of dibenzothiophene can also catalyze the formation of S-oxidation products (29, 31); in some of the cases the further oxidation of DBT-5-oxide to the DBT sulfone was also observed. It is tempting to implicate the arene dioxygenase(s) of strain F297 in these sulfoxygenation reactions (1, 45); however, there is no direct evidence supporting that conclusion at the present time.

According to the results presented here and the reports cited above, it appears that *P. cepacia* F297 possesses a set of enzymes akin to those that degrade naphthalene to salicylate and that these enzymes have a breadth of substrate specificity that permits oxidation and ring cleavage of a number of PACs containing 2 and 3 aromatic rings. The arene-dioxygenase system may also be responsible for the mono-oxygenation of benzylic methyl and methylenic groups.

The wide substrate specificity that strain F297 shows with individual aromatic substrates is also observed with mixed creosote PACs, as evidenced by growth (a 10^3-fold increase in the number of cells) and an extensive depletion of all compounds with 2 and 3 rings. This extent of removal of PACs is similar to that reported for indigenous mixed cultures from creosote-contaminated soil incubated with contaminated groundwater (37). Generally, measures of the biodegradation of chemical mixtures are based on the extent of loss of parent compounds; seldom is evidence available to indicate the processes involved or whether products are accumulated. For example, Foght and Westlake (14) reported an extensive depletion of compounds present in an aromatic fraction of crude oil by a *Pseudomonas* species without accounting for products formed by its metabolic actions. Here the identification of key chemicals confirms not only that certain compounds are depleted but also that they are transformed into anticipated reaction products. The formation of acenaphthenone and acenaphthoquinone, observed in neutral extracts, and naphthalene 1,8-dicarboxylic acid, observed in acidic extracts, results from the oxidation of acenaphthene and acenaphthylene. Acenaphthenone has been proposed as a metabolite in the degradation of fluoranthene (53), but strain F297 was not shown to act on this compound, and no significant depletion was observed. The presence of 5-oxo-5-phenyl-pentanoic acid is explained by the dioxygenation and cleavage of biphenyl present in creosote PACs. Apparently, these transformations are fortuitous and occur as strain F297 grows on compounds such as naphthalene, fluorene, 2,3-DMN, DBT, phenanthrene, and anthracene. The most abundant acidic metabolites found in creosote-PAC cultures are 1-hydroxy-2-naphthoic and 2-hydroxy-3-naphthoic acids, whose formation can be firmly attributed to utilization of phenanthrene and anthracene, respectively.

It can be concluded from this study that PACs, singly and in mixtures, are depleted both by productive pathways specific for certain substrates and by fortuitous biotransformations. Biotransformation is due to the broad specificity of enzymes and regulatory mechanisms of the pathways for the degradation of aromatic compounds. The advantages of organisms with wide substrate specificity to the development of bioremediation systems for PACs are evident. It is important to recognize, however, the potential for the accumulation of products whose toxicity and subsequent biodegradation require assessment.

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