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Characterization of fluoranthene- and pyrene-degrading bacteria isolated from PAH-contaminated soils and sediments

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Sixteen environmental samples, from the United States, Germany and Norway, with histories of previous exposure to either creosote, diesel fuel or coal tar materials, were screened for bacteria which could degrade high molecular weight (HMW) polycyclic aromatic hydrocarbons (PAHs). A modified version of the spray plate technique was used for the isolations. Using fluoranthene (FLA) and pyrene (PYR) as model HMW PAHs, we isolated 28 strains on FLA and 21 strains on PYR. FLA degraders were defined as able to grow on FLA but not PYR. PYR degraders grew on both PAHs. All PYR degraders were found to be Gram-positive and all FLA degraders were Gram-negative. GC-FAME analysis showed that many of the PYR degraders were *Mycobacterium* spp and many of the FLA degraders were *Sphingomonas* spp. Comparison of the metabolic characteristics of the strains using the spray plate technique and direct growth studies revealed that more than half of the FLA degraders (59%) were able to cometabolize PYR (ie, they produced clearing zones or colored metabolites on spray plates but did not grow on the PAH) and the ability of many of these strains to cometabolize fluorene, anthracene, benzo[b]fluorene, benzo[a]anthracene and benzo[a]pyrene was significantly affected by pre-exposure to phenanthrene. Studies on the metabolic products produced from PYR cometabolism by strain EPA 505 suggested the possibility of attack at two different sites on the PYR molecule. However, the inability to derive degradable carbon from initial opening of one of the PYR rings probably accounted for the lack of growth on this PAH by the FLA-degrading strains. The PYR degraders on the other hand, were less able to cometabolize HMW PAHs, even following pre-exposure to PHE. Characterization of the FLA degradation pathway for several of the *Sphingomonas* isolates indicated oxidation and ring opening through to acenaphthenone as the principle metabolite. Strain CO6, however, also oxidized FLA through fluorenone, suggesting a dual attack on the FLA molecule, similar to that observed by others in *Mycobacterium* spp. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 100–112.

Keywords: biodegradation; cometabolism; fluoranthene; pyrene; *Sphingomonas*; *Mycobacterium*

Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent a large group of organic pollutants which have contaminated the environment through improper disposal of materials such as creosote, coal tar, and hydrocarbon fuels [30,31,35]. The high molecular weight (HMW) PAHs (four or more fused rings) are of particular environmental concern because of their potential mutagenicity and carcinogenicity in mammalian test organisms [1,15] and in a number of critical aquatic species [5,20]. Several different bacterial genera are capable of degrading PAHs including species of *Pseudomonas*, *Alcaligenes*, *Mycobacterium*, *Rhodococcus*, *Sphingomonas* [3,31] and *Cycloclasticus* [9]. This is a relative small range of genera considering the prevalence of PAHs in the environment. Numerous fungal species also degrade PAHs, both low and high molecular weight [31]. For the bacterial isolates, most have been enriched based on their ability to grow on low molecular PAHs (naphthalene, phenanthrene, fluorene, indan, acenaphthene, and anthracene). But several studies have shown that bacteria are able to grow on the four-ring PAHs, specifically, fluoranthene [2,7,21,28,32,36,44] and pyrene [2,4,8,11,14,16,19,21,28,37,40]. *Mycobacterium*, *Rhodococcus*, *Alcaligenes*, and *Sphingomonas* are the genera encountered in this regard. Moreover, there are a number of other LMW PAH degraders that are known to cometabolize HMW PAHs [7,29,33,40,41]. We define cometabolism here as the ability to transform (oxidize) a particular PAH but without growth on that PAH; presumably partial degradation products are generated but they can not be further metabolized to produce carbon and energy.

There have been several reports of PAH-degrading *Sphingomonas* species [7,10,21,32], and one of these strains, *Sphingomonas paucimobilis* strain EPA 505 has a substantial cometabolic capability for high molecular weight PAHs [33,46]. *Sphingomonas* strain B1 (formally *Beijerinckia* B1), a strain that was isolated originally for its ability to grow on biphenyl, has been shown to cometabolize benzo(a)anthracene to acid metabolites [29]. Whether sphingomonads are commonly associated with PAH degradation is yet to be assessed, but a study examining the diversity of bacteria able to degrade PAHs [33], showed that *Sphingomonas* species tended to be the isolates capable of degrading fluoranthene, whereas the bacteria able to degrade phenanthrene were more commonly associated with *Pseudomonas* strains. Dagher *et al* [7] compared three PAH-degrading *Pseudomonas* sp with a *Sphingomonas* sp and found the latter the most efficient PAH

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degrader, with the ability to possibly grow on FLA (they reported growth on pyrene but not clearing on spray plates). With these previous studies as a basis, we have continued to isolate other fluoranthene degraders to compare their taxonomic and metabolic characteristics. Little is known on a geographic scale of what bacterial genera may be responsible for microbial degradation of HMW PAHs in PAH-contaminated sites throughout the world. The results reported here suggest that, in fact, *Sphingomonas* spp are commonly isolated from PAH-contaminated soils and sediments and their metabolic diversity may vary considerably.

Materials and methods

Chemicals and supplies

All PAHs were purchased from Sigma (St Louis, MO, USA) or Aldrich (Milwaukee, WI, USA) using the highest purity grade available. Bacterial culture media and reagents were purchased from Difco Laboratories, Detroit, MI, USA. All solvents were purchased from Fisher Scientific (Fair Lawn, NJ, USA) of the highest grade. Solid phase extraction columns (1 or 3 ml) packed with C_{18} resin were purchased from Supelco, Inc, USA. Distilled water was organic free and deionized using a Corning Mega-Pure System.

Analytical procedures

For the measurement of growth rate, the optical density of the bacterial liquid culture was measured at 600 nm with a Spectronic 21 spectrophotometer from Milton Roy, Inc (Rochester, NY, USA). Reversed-phase high pressure liquid chromatographic (HPLC) analysis was performed with a Hewlett Packard (Palo Alto, CA, USA) HPLC Model-1090 equipped with an on-line 1040A diode array UV detector. A 5-mm Supelcosil spherical C_{18} octadecyl column (2.1 mm $\times$ 15 cm) was used for the separation of pyrene and fluoranthene metabolites. The mobile phase was programmed from 10% methanol with water to 100% methanol in 30 min or from 10% to 80% acetonitrile with 50 mM phosphate buffer (pH 3.5) in 20 min. A linear gradient was maintained at a flow rate of 0.5 ml min^{-1}. The acidic buffer system was used for better separation of acidic metabolites. UV absorbance was measured at 254 nm for the methanol mobile phase and 207 nm for the acetonitrile mobile phase. The UV-visible absorption spectrum from 200 to 600 nm was recorded for each individual peak. Bacterial isolates for GC-FAME analysis were incubated at 30°C on trypticase soy broth (TSB). Cells were harvested after 24 h and subjected to an extraction and derivitization procedure (MIDI, Inc, Newark, DE, USA, 1993). Identification of bacterial species or genus used the MIDI™ Microbial Identification System. Similarity coefficients for species identification were interpreted using MIDI™ Aerobe (TSBA & CLIN) database version 3.90.

To produce coatings of PAHs on agar plates, we used a thin layer chromatography aerosol spray system (pressurized air) in a cabinet equipped with a vacuum motor designed to create a negative pressure inside the cabinet.

Soil samples

A detailed list of the soil or sediment samples and the isolates obtained is shown in Table 1. Many of these samples were the same as those studied previously [32]. Soil samples 11–16 were collected from four different sites in Norway. Soil at these sites had been exposed to creosote for as long as 50 years. Soil sample 3 was obtained at a relatively recent diesel fuel spill site in Berlin, Germany. Soil sample 6 was collected from an abandoned wood-preserving facility in Pensacola, Florida, USA which had 80 years exposure to creosote. Soil samples 1, 2, 4, 5, and 7 were collected from several wood preserving sites with long-term exposure to creosote. Soil number 8 was collected from Tyndall Air Force Base in Panama City, FL. The soil had a history of exposure to diesel fuel and jet fuel. Soils 9 and 10 were collected from sediment and surface soil at Port Newark, NJ. Both sampling sites contained residual polycyclic aromatic hydrocarbons from historical exposure to fuel oil. All soil and sediment samples were stored at 4°C.

Media and culture conditions

Sterilized Bushnell–Haas (B–H) medium (per liter: magnesium sulfate, 0.2 g; calcium chloride, 0.02 g; mono-potassium phosphate, 1 g; dibasic ammonium phosphate, 1 g; potassium nitrate, 1 g; and ferric chloride, 0.05 g; final pH 7.0 $\pm$ 0.02 after autoclaving) was used as a spray solution to wash cells. For the PAH spray plate assay, PYGT medium (ingredients per liter: peptone, 0.06 g: yeast extract, 0.1 g; glucose, 0.1 g; tryptone, 0.05 g) was used with 1.5% agar. Also, 10 $\times$ and 20 $\times$ PYGT media were used for more bacterial growth and for purity check on agar plates.

The spray plate method of Kiyohara *et al* [25] was used. Fluorene (FLU), phenanthrene (PHE), fluoranthene (FLA), and pyrene (PYR) were prepared as 5 mg ml^{-1} stock solutions in acetone. Anthracene (ANT), benzo[b]fluorene (B(b)FLU), benzo[a]anthracene (B(a)ANT), chrysene (CHR) and benzo[a]pyrene (B(a)PYR) were prepared in 3:1 acetone/methylene chloride at 5 mg ml^{-1}. PAHs were sprayed on the surface of PYGT plates using a thin layer chromatographic spray unit.

PAHs used for studies of growth and isolation of metabolic products in liquid culture were dissolved in acetone to produce a concentration of 20 mg ml^{-1} and then the desired amount of acetone was added to an empty sterile flask and allowed to evaporate before B–H medium was added.

Enrichments and isolations

Ten grams of each soil sample were placed in individual 50-ml sterile centrifuge tubes filled with 30 ml phosphate buffer solution (pH 7.0). The soil-buffer mixture was vortexed for 1 min and the solution was allowed to settle for 20 min. One milliliter of the supernatant was transferred into a fresh sterile test tube containing 6 ml of B–H medium plus 4 mg of either FLA or PYR. Two test tubes were used for each individual soil or sediment sample. Tubes were shaken at 180 rpm in a 30°C incubator for 2 weeks and at that point, an aliquot from each tube was diluted $1{:}10^{-2}$ and $1{:}10^{-4}$ and 100 microliters of each dilution were spread on a PYGT plate. These plates were incubated at 30°C for 1 day and were sprayed with either FLA or PYR. The plates

Table 1 Location of the PAH-contaminated soil sites and the names of PAH degraders isolated from these soils

Sample location	Source of contaminant	FLA degraders	PYR degraders
(1) Gainesville, FL, USA	Creosote	FLA 1–1	PYR 1–1
(2) Nashua, NH, USA	Creosote	None	PYR 2–1
(3) Berlin, Germany	Diesel fuels	FLA 3–1, FLA 3–2, FLA 3–3	PYR 3–1, PYR 3–2
(4) Hocomonco Pond, MA, USA	Cresote	FLA 4–1, FLA 4–2	None
(5) Tupper ware	Creosote	FLA 5–1, FLA 5–3	PYR 5–1, PYR 5–3
(6) Pensacola (ACW), FL, USA	Creosote	FLA 6–1, FLA 6–2	None
(7) Fence Post, FL, USA	Creosote	FLA 7–1	PYR 7–1
(8) Tyndall AFB, FL, USA	Diesel & jet fuel	FLA 8–1	None
(9) Port Newark, NJ, USA (sediment)	Fuel	FLA 9–1	PYR 9–1
(10) Port Newark, NJ, USA	Fuel	FLA 10–1, FLA 10–3	PYR 10–1
(11) NSR (No. 1), Rade, Norway	Creosote	FLA 11–1	PYR 11–1, PYR 11–2, PYR 11–3
(12) NSR (No. 2), Rade, Norway	Creosote	FLA 12–1, FLA 12–2	PYR 12–2
(13) NSR (No. 3), Lillestrom, Norway	Creosote	FLA 13–1	PYR 13–1, PYR 13–2
(14) NSR (No. 4), Lillestrom, Norway	Creosote	FLA 14–1	PYR 14–1
(15) NSR (No. 6), Drammen, Norway	Creosote	FLA 15–1, FLA 15–2, FLA 15–3	PYR 15–1, PYR 15–2
(16) NSR (No. 7), Hommelvik, Norway	Creosote	None	PYR 16–1, PYR 16–2
(17) Boston, MA, USA (sediment)	Fuel	UMB[a]	
(18) Boston, MA, USA (sediment)	Fuel	CO6[a]	
(19) Pensacola, FL, USA	Creosote	EPA505	
(20) Germany	Diesel fuel	UN1F1, UN1F2	

[a]UMB and CO6 were provided by Michael Shiaris, University Massachusetts, Boston.

were incubated at 30°C and periodically examined for clearing zones surrounding the bacterial colonies.

Colonies showing clearing zones (generally after 2–3 weeks) were then streaked on 10 × PYGT agar plates. If the isolated strain showed impurity, a mixture of strains, then the streak and spray procedure was applied again until a single strain was obtained. If no colonies appeared within a clearing zone, enrichment tubes were incubated for an additional 6 weeks and the isolation procedure was repeated. In the present work, no serial transferring of bacterial cultures was performed to avoid bias from selective enrichment.

Growth and degradation of PAH

Tubes for growth experiments were prepared by adding aliquots of the test PAH in acetone at a designated concentration and allowing the acetone to evaporate. Sterile Bushnell–Hass medium was then added and the tubes were sonicated for 2 min to break up crystals. For the growth study, each test tube contained 2 mg of the designated PAH and 6.5 ml of Bushnell–Hass medium. Bacterial strains were harvested from the 10 × PYGT medium, washed twice with Bushnell–Haas medium and transferred into the growth tube to give an initial optical density of 0.05 at 600 nm. Changes in optical density were measured daily over a 7-day period. Visible color change in the culture medium and a tripling of optical density was considered to represent growth. For induction experiments, bacterial strains were grown on PYGT agar plates and sprayed with phenanthrene. After 48 h incubation, clearing was observed and a second spraying was performed with one of the HMW PAHs. Subsequent incubation and monitoring were performed as described above.

Preparation of pyrene metabolites

For the pyrene metabolite study, stationary phase cells were harvested from growth in 10× PYGT and were washed twice with Bushnell–Haas (B–H) medium. The washed cells were transferred into a 250-ml Erlenmeyer flask with 20 mg of PYR and 150 ml B–H medium. For the bacterial isolates that could not grow on PYR, B–H medium was supplemented with sodium pyruvate at 2 g L^{-1}. Cell density was adjusted to 0.8 absorbance units (600 nm) and the flasks were incubated at 30°C for 7 days. The culture medium was then centrifuged at 12 400 × *g* for 15 min and the supernatant was then passed through a 1-ml LC-18 SPE cartridge using a Visi-prep solid-phase extraction vacuum manifold (Supelco Inc, Bellefonte, PA, USA) that filtered at the rate of two drops per second. Metabolites sorbed on the LC-18 columns were eluted with 0.4 ml of acetonitrile or methanol. The effluent from the column was centrifuged at 9000 × *g* for 5 min. The supernatant was transferred into a 1-ml glass vial with a 200-μl glass insert. The vial was sealed with a Teflon-lined cap and stored at 4°C.

Results

PAH-degrading bacteria

A total of 28 FLA-degraders and 21 PYR degraders were isolated (Tables 2–5). A prefix of FLA or PYR and a number was assigned to indicate the selection substrate and the soil source. A second number was assigned to designate different isolates from the same soil. Generally, it took 2 weeks for the FLA isolates to show as colonies with a clearing zone and about 3 weeks for PYR isolates. However, subsequent culturing of FLA or PYR degraders on fresh PYGT plates showed clearing of FLA and PYR in 36–48 h. Based on the pigments they produced and the colony mor-

Table 2 Ability of fluoranthene-degrading colonies grown on PYGT medium to produce clearing zones and color changes when sprayed with different PAHs

FLA strains	FLU	PHE	FLA	PYR	ANT	B[b]FLU	B[a]ANT	CHR	B[a]PYR
1–1[a]	+	++	+ (2)	–/gb	++	–	–	–	–
3–1	–	+ (2)	+ (2)	–	–	–	–	–	–
3–2	–	+ (2)	+	–	–	–	–	–	–
3–3	+	++	+ (2)	–	–	–	–	–	–
4–1[a]	–	+ (2)	+ (2)	–	–	–	–	–	–
4–2	+	++	++ (2)	–/gb	++	–	+ (6)	–	–
5–1	+/b	++	++	–/gb	++	–	+ (6)	–	–
5–3[a]	+/y	+	++	–/gb	++	–	–	–	–
6–1[a]	+/b	+	++	–/gb	++	+	–	–	–
6–2	+/b	++	++	–/gb	++	–	+ (6)	–	–
7–1	+	++	++	–	–	–	–	–	–
8–1	+	++	++	–	–	–	–	–	–
9–1[a]	+/y	++	++	–/g	++	–	+ (6)	–	–
10–1[a]	+/b	++	++	–/gb	++	–	+ (6)	–	–
10–3	++/b	++	++	–/gb	++	–	+ (6)	–	–
11–1	–	+	++	–	++	–	–	–	–
12–1	–	++	+	–	–	–	–	–	–
12–2	–	++	+	–	–	–	–	–	–
13–1[a]	+/b	++	++	–/gb	++	–	+ (6)	–	–
14–1	–	++	++	–	–	–	–	–	–
15–1[a]	–	++	++	–/gb	++	–	+ (6)	–	–
15–2	+	++	+	–	++	–	–	–	–
15–3[a]	–	++	++	++	–	–	–	–	–
EPA505[a]	++/y	++	++	–/gb	++	–	–	–	–
CO6[a]	++/y	++	+ (2)	–/gb	++	–	–	–	–
UMB[a]	++/b	++	+ (2)	–/gb	++	–	+ (6)	–	–
UN1F1	–	++	+ (2)	–	++	–	–	–	–
UNIF2	+	++	+ (2)	–	++	–	–	–	–

FLU = fluorene; PHE = phenanthrene; FLA = fluoranthene; PYR = pyrene; ANT = anthracene; B[b]FLU = benzo(b)fluorene; B[a]ANT = benzo(a)anthracene; CHR = chrysene; B[a]PYR = benzo(a)pyrene.
Examinations for clearing zones were performed after 1, 2, and 6 weeks of incubation, the latter two being indicated by number in parentheses.
Letters refer to appearance of colored metabolites (b = brown, y = yellow, gb = green brown). [a]Strains identified as *Sphingomonas* sp.
The symbols were defined as: ++, clearing zone equal to or wider than 2 mm; +, clearing zone less than 2 mm; –, no clearing zone formed.

Table 3 Ability of pyrene-degrading colonies grown on PYGT medium to produce clearing zones when sprayed with different PAHs

PYR strains	FLU	PHE	FLA	PYR	ANT	B[b]FLU	B[a]ANT	CHR	B[a]PYR
1–1	–	++	++	++	–	–	–	–	–
2–1	–	++	++	++	–	–	–	–	–
3–1	–	++	++	++	–	–	–	–	–
3–2	–	++	++	++	–	–	–	–	–
5–1	–	++	++	++	++	++	++	+	–
5–3	–	++	++	++	–	–	–	–	–
7–1	–	++	++	++	–	–	–	–	–
9–1	++	++	++	++	++	++	++	+	–
10–1	–	++	++	++	–	–	–	–	–
11–1	–	++	++	++	–	–	–	–	–
11–2	–	++	+	++	–	–	–	–	–
11–3	–	++	+	++	–	–	–	–	–
12–1	++	++	+	++	–	–	–	–	–
12–2	–	++	++	++	–	–	–	–	–
13–1	++	++	++	++	–	–	–	–	–
13–2	–	++	++	++	–	–	–	–	–
14–1	–	++	++	++	–	–	–	–	–
15–1	–	++	++	++	–	–	–	–	–
15–2	–	++	++	++	–	–	–	–	–
16–1	–	++	++	++	–	–	–	–	–
16–2	–	++	++	++	–	–	–	–	–

FLU = fluorene; PHE = phenanthrene; FLA = fluoranthene; PYR = pyrene; ANT = anthracene; B[b]FLU = benzo(b)fluorene; B[a]ANT = benzo(a)anthracene; CHR = chrysene; B[a]PYR = benzo(a)pyrene.
Examinations for clearing zones were performed after 1, 2, and 6 weeks of incubation, the latter two being indicated by number in parentheses.
The symbols were defined as: ++, clearing zone equal to or wider than 2 mm; +, clearing zone less than 2 mm; –, no clearing zone formed.

Table 4 Ability of fluoranthene-degrading colonies grown on PYGT medium to produce clearing zones when sprayed with different PAHs following preexposure to PHE

FLA strains	ANT	B[b]FLU	B[a]ANT	CHR	B[a]PYR
1–1[a]	++	–	–	–	–
3–1	–	–	–	–	–
3–2	–	–	–	–	–
3–3	–	–	++ (6)	–	–
4–1[a]	–	–	–	–	–
4–2	++	++ (6)	++ (6)	–	–
5–1	++	++ (6)	++ (6)	–	+ (6)
5–3[a]	++	+ (6)	++ (6)	–	–
6–1[a]	++	+ (6)	++ (6)	–	–
6–2	++	++ (6)	++ (6)	–	–
7–1	–	–	–	–	–
8–1	–	–	–	–	–
9–1[a]	++	++ (6)	++ (6)	–	+ (6)
10–1[a]	++	++ (6)	++ (6)	–	–
10–3	++	++ (6)	++ (6)	–	–
11–1	–	–		–	–
12–1	++	–	++ (6)	–	–
12–2	++	–	++ (6)	–	–
13–1[a]	++	+ (6)	+ (6)	–	–
14–1	–	–	–	–	–
15–1[a]	++	++ (6)	++ (6)	–	–
15–2	++	–	++ (6)	–	–
15–3[a]	–	–	–	–	–
EPA505[a]	++	++ (3)	++ (6)	+ (6)	+ (6)
CO6[a]	++	+ (6)	++ (6)	–	–
UMB[a]	++	++ (3)	++ (6)	–	–
UN1F1	++	–	++ (6)	–	–
UN1F2	++	–	–	–	–

FLU = fluorene; PHE = phenanthrene; FLA = fluoranthene; PYR = pyrene; ANT = anthracene; B[b]FLU = benzo(b)fluorene; B[a]ANT = benzo(a)anthracene; CHR = chrysene; B[a]PYR = benzo(a)pyrene.
Examinations for clearing zones were performed after 1, 2, and 6 weeks of incubation, the latter two indicated in parentheses. [a]Strains identified as *Sphingomonas* sp.
The symbols were defined as: ++, clearing zone equal to or wider than 2 mm; +, clearing zone less than 2 mm; –, no clearing zone formed.

Table 5 Ability of pyrene-degrading colonies grown on PYGT medium to produce clearing zones when sprayed with different PAHs following preexposure to PHE

PYR strains	ANT	B[b]FLU	B[a]ANT	CHR	B[a]PYR
1–1	++ (6)	–	–	–	–
2–1	++	–	++ (6)	–	–
3–1	++ (6)	–	++ (6)	–	–
3–2	++	–	++ (6)	–	–
5–1	++	++		+	–
5–3	++	–	++ (6)	–	–
7–1	++	–	++ (6)	–	–
9–1	++	++		+	–
10–1	++	–	++ (6)	–	–
11–1	++ (6)	–	++ (6)	–	–
11–2	–	–	++ (6)	–	–
11–3	–	–	++ (6)	–	–
12–1	++	–	++ (6)	–	–
12–2	++	–	++ (6)	–	–
13–1	++	–	++ (6)	–	–
13–2	–	–	–	–	–
14–1	–	–	–	–	–
15–1	++	–	++ (6)	–	–
15–2	++	–	++ (6)	–	–
16–1	–	–	–	–	–
16–2	++	–	++ (6)	–	–

FLU = fluorene; PHE = phenanthrene; FLA = fluoranthene; PYR = pyrene; ANT = anthracene; B[b]FLU = benzo(b)fluorene; B[a]ANT = benzo(a)anthracene; CHR = chrysene; B[a]PYR = benzo(a)pyrene.
Examinations for clearing zones were performed after 1, 2, and 6 weeks of incubation, the latter two indicated by number in parentheses.
The symbols were defined as: ++, clearing zone equal to or wider than 2 mm; +, clearing zone less than 2 mm; –, no clearing zone formed.

phology on 20 × PYGT plates, colonies with no visible difference were reconsidered the same isolate from each specific soil sample.

No PYR isolates were obtained from soil samples 4, 6, and 8. Bacterial strains isolated within the PYR group were all Gram-positive except PYR 5-1 and PYR 9-1, which were Gram-variable. No Gram-negative bacteria were found that grew on PYR. GC-FAME results revealed that isolates PYR 2–1, PYR 3–1, PYR 3–2, PYR 5–3, PYR 12–2, PYR 15–1 and PYR 15–2 were likely *Mycobacterium* species. Several other isolates with no match to the GC-FAME data base, had colony and cell morphologies that resembled *Mycobacterium*. Isolates PYR 5–1 and PYR 9–1 had notably different colony morphologies forming dry, small, white, slow-growing colonies. Microscopically, cells of these isolates were short rods, often forming long chains of cells when grown on agar plates.

Several soils had more than one morphologically different FLA degrader. UMB and CO6 were isolated on phenanthrene in earlier work from sediment samples [18,33] but they also grew on FLA. UN1F1 and EPA505 were isolated on fluoranthene by Mueller *et al* [32–34] and were included in this study. All of the FLA isolates, as designated by their ability to grow on FLA but their inability to grow on PYR, were Gram-negative, and GC-FAME analysis indicated that isolates FLA 1–1, FLA 4–1, FLA 5–3, FLA 6–1, FLA 9–1, FLA 10–1, FLA 13–1, FLA 15–1, FLA 15–3, EPA 505, CO6, and UMB were likely *Sphingomonas* spp. All of these isolates produced yellow colonies on 10 × PYGT. Several other isolates that had no match in the GC-FAME database also grew as yellow colonies. These are being tested further for taxonomic identification.

PAH biodegradation spectrums

PAH transformation ability was determined by the production of clearing zones on agar plates following incubation with the respective PAH. Clearing zones were generally slowest to form with ANT, B(b)FLU, FLU, B(a)ANT, B(a)PYR and CHR. Results are shown in Tables 2–5 along with the effect of induction on PHE. Production of color was very prominent for some PAHs and some isolates.

Most FLA degraders (78%), under non-induced conditions, showed correspondence between the ability to produce clearing zones with FLA (Table 2) and the ability to grow in liquid medium with FLA as the sole carbon source (Table 6). However, seven isolates (FLA 3–1, 3–3, 7–1, 8–1, 11–1, 12–1, and 14–1) were unable to grow, or grew poorly, on FLA but still produced clearing zones. Each of these isolates grew well on PHE, except FLA 8–1 and 14–1. In general, growth of FLA degraders on PHE corre-

Table 6 Growth of fluoranthene-degrading isolates on phenanthrene and fluoranthene

Isolates	Phenanthrene	Fluoranthene
FLA 1–1[a]	+++	+
FLA 3–1	++	+/–
FLA 3–2	+	++
FLA 3–3	+	+/–
FLA 4–1[a]	–	++
FLA 4–2	++	+
FLA 5–1	++	+
FLA 5–3[a]	++	+++
FLA 6–1[a]	++	++
FLA 6–2	++	++
FLA 7–1	+	+/–
FLA 8–1	–	–
FLA 9–1[a]	++	+
FLA 10–1[a]	+	++
FLA 10–3	+	++
FLA 11–1	+	–
FLA 12–1	++	–
FLA 12–2	++	+
FLA 13–1[a]	+	+++
FLA 14–1	–	+/–
FLA 15–1[a]	++	++
FLA 15–2	+	+
FLA 15–3[a]	–	+
EPA 505[a]	+++	++
CO6[a]	+	+
UMB[a]	++	++
UN1F1	++	+
UN1F2	++	+

Isolates were grown in Bushnell–Hass medium with the respective PAH. [a]Identified as *Sphingomonas* sp. Growth designations; – no growth; +/– slight growth; + growth between optical density (600 nm) 0.06 and 0.15 in 7 days; ++ optical density between 0.15 and 0.25; +++ optical density above 0.25.

sponded with their ability to cause clearing zones with PHE (Tables 2 and 6). They generally grew faster and to a higher optical density on PHE than on FLA. Isolates FLA 4–1 and FLA 15–3 were unique in that they could grow on FLA but not on PHE, yet they readily cometabolized PHE as evidenced by the formation of clearing zones. Isolate FLA 15–3 did not grow on either FLA or PHE. In addition, many of the isolates (14/28) produced clearing zones with naphthalene (data not shown) although growth of the colonies was noticeably less, probably due to the toxicity of naphthalene (NAP). Since NAP volatilized from the agar plates quite quickly (a sprayed film of NAP lasted 48–64 h), only those isolates that rapidly metabolized NAP showed clearing zones around the colonies.

Approximately 70% of the FLA degraders caused clearing zones with FLU. Accumulation of colored metabolites from the transformation of FLU was widely observed in FLA isolates suggesting that FLU was cometabolized. Similar results were obtained with PYR; 59% of the FLA degraders produced colored products with PYR, again indicating cometabolism of this PAH. We have also surveyed the ability of FLA degraders to grow on acenaphthene (spray plates were not possible with this PAH because it did not form a contiguous film on the agar surface) and 39% of the isolates were positive (FLA 1–1, 4–2, 5–1, 6–2, 10–1, 12–1, UN1F1, UN1F2, CO6, EPA 505). Finally, since the ability to grow on FLA and PHE may be the result of obtaining pyruvate from the opening of an aromatic ring on FLA, we checked the isolates for growth on pyruvate. Surprisingly, nine isolates (FLA 4–2, 5–1, 5–3, 6–1, 6–2, 9–1, 10–3, 13–1 and 15–1) were unable to grow on pyruvate.

The ability of the FLA degraders to cometabolize higher molecular weight PAHs (no growth was observed on these PAHs) was restricted to ANT (60% of the isolates) and B(a)ANT (44% of the isolates), the latter taking 6 weeks to produce clearing zones (Table 2). The ability to cometabolize ANT was a prerequisite for cometabolism of B(a)ANT, but not all ANT-metabolizers were able to metabolize B(a)ANT.

All PYR degraders, under non-induced conditions, were able to grow on and cause clearing zones with PYR, FLA and PHE (Table 3). However, only three isolates, PYR 9–1, PYR 12–1, and PYR 13–1, were able to cause clearing zones with FLU. Each of these isolates was also able to grow on FLU. No colored products were observed with any of these isolates. The ability of PYR degraders to metabolize high molecular weight PAHs was far more restricted than with the FLA degraders. Only isolates PYR 5–1 and PYR 9–1 showed any ability to degrade ANT, B(b)FLU, B(a)ANT and CHR. The metabolism of chrysene is particularly notable since FLA degrader EPA 505 was the only other isolate that degraded CHR. Isolates PYR 5–1 and PYR 9–1, however, were very slow-growing organisms.

Pregrowth of the isolates on PHE had a significant effect on their ability to cometabolize the high molecular weight PAHs (the effect of PHE induction was not tested on the low molecular weight PAHs) (Tables 4 and 5). Whereas none of the non-induced FLA degraders were capable of cometabolizing B(b)FLU, 13 of 25 showed this activity, albeit slowly, following PHE induction. Isolates UMB and EPA 505 were especially responsive, producing clearing zones in 3 weeks. Although PHE induction had little effect on metabolism of ANT (ie, most strains metabolized it in the first place), it had a significant effect on metabolism of B(a)ANT, causing an additional six isolates to show clearing zones, but also causing all isolates to produce larger clearing zones, presumably an indication of enhanced cell activity. Of particular note was the effect of PHE induction on metabolism of B(a)PYR by three isolates, FLA 5–1, FLA 9–1, and EPA 505 and metabolism of chrysene by EPA 505. EPA 505 was the only FLA degrader to show any activity on chrysene.

PHE induction also affected the PYR degraders in a somewhat similar manner. Relatively few of the isolates could cometabolize ANT and B(a)ANT under non-induced conditions, but approximately 76% of the isolates metabolized these PAHs under induced conditions. There was no effect of induction on metabolism of B(B)FLU, CHR, or B(a)PYR.

Metabolic products from pyrene

To verify the apparent cometabolism seen on agar spray plates (production of colored products), studies were conducted with the FLA degrader EPA 505 in the presence of PYR in B–H medium. Following 7 days of exposure, two major metabolites were detected in the culture supernatant

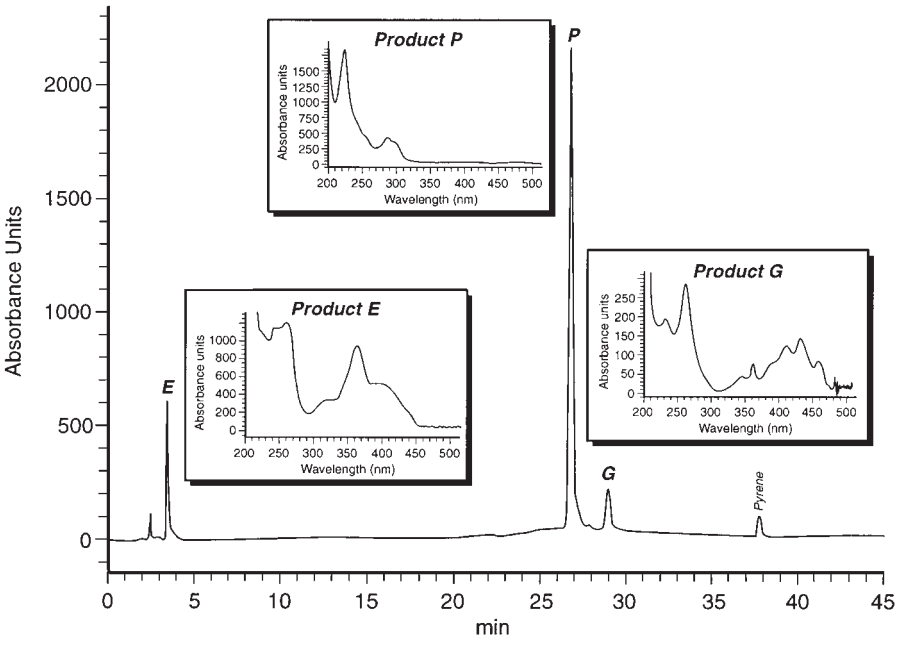

Figure 1 HPLC profile (254 μm) of extract of culture fluid following exposure of strain EPA 505 (grown in 10 × PYGT medium) to pyrene (20 mg in 150 ml of medium) for 7 days in BH medium.

by HPLC, peaks G and P (Figure 1). Peak G was purified as a bright fluorescing green product that had an adsorption spectrum with maxima at 204, 232, 260, 360, 410, 434, and 460 μm. However, by keeping the culture supernatant acidic (pH 4) during extraction, only trace amounts of product G could be detected but a new product (Ga) was present that had a unique spectrum (Figure 2). This product, however, was unstable and rapidly converted to product G, especially under alkaline conditions. Purified product Ga produced a compound in GCMS analysis with a principle mass ion of 194 and a m/z spectrum that matched (system library) hydroxy-phenanthrene which we believe is a thermal decomposition product of 10-hydroxy-1-phenanthroic acid. This decomposition seems reasonable since 1-hydroxy-2-naphthoic acid (the common ring opening product from metabolism of PHE) also underwent removal of the

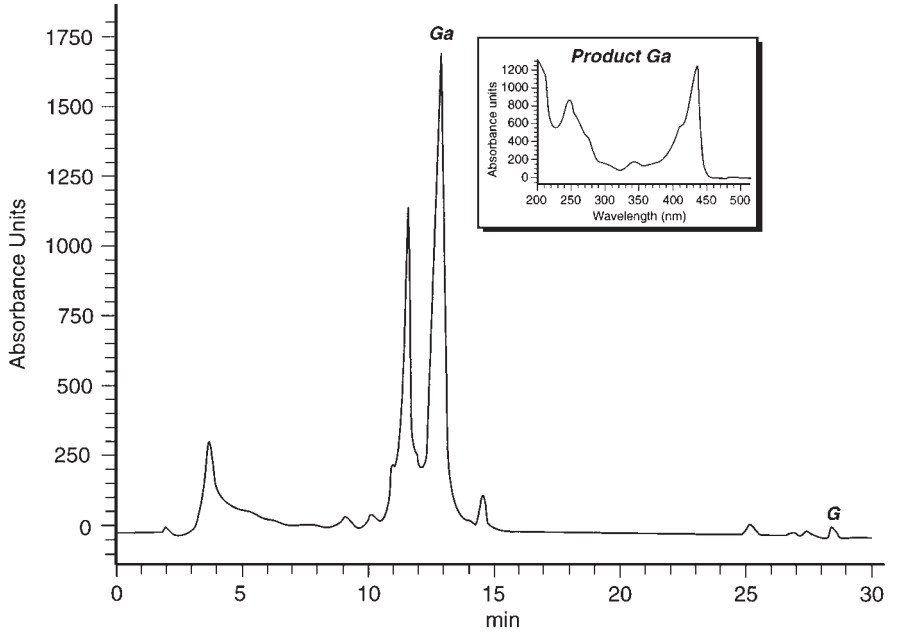

Figure 2 HPLC profile (254 μm) of acidic extract of culture fluid following exposure of strain EPA 505 (grown in 10× PYGT medium) to pyrene (20 mg in 150 ml of medium) for 3 h.

carboxyl group during GC analysis. In addition, Kropp *et al* [26] observed the similar thermal decomposition of hydroxybenzothiophene carboxylic acid, a product produced by the microbial oxidation of naphthothiophene. Based on this information, we hypothesize that EPA 505 can attack PYR by initial dioxygenation at the 4,5-position, followed by an ortho cleavage to give the phenanthrene dicarboxyclic acid (Figure 3). Rehmann *et al* [37] observed the same product with a *Mycobacterium* strain. Subsequent removal of a carboxyl group would produce 10-hydroxy-1-phenanthroic

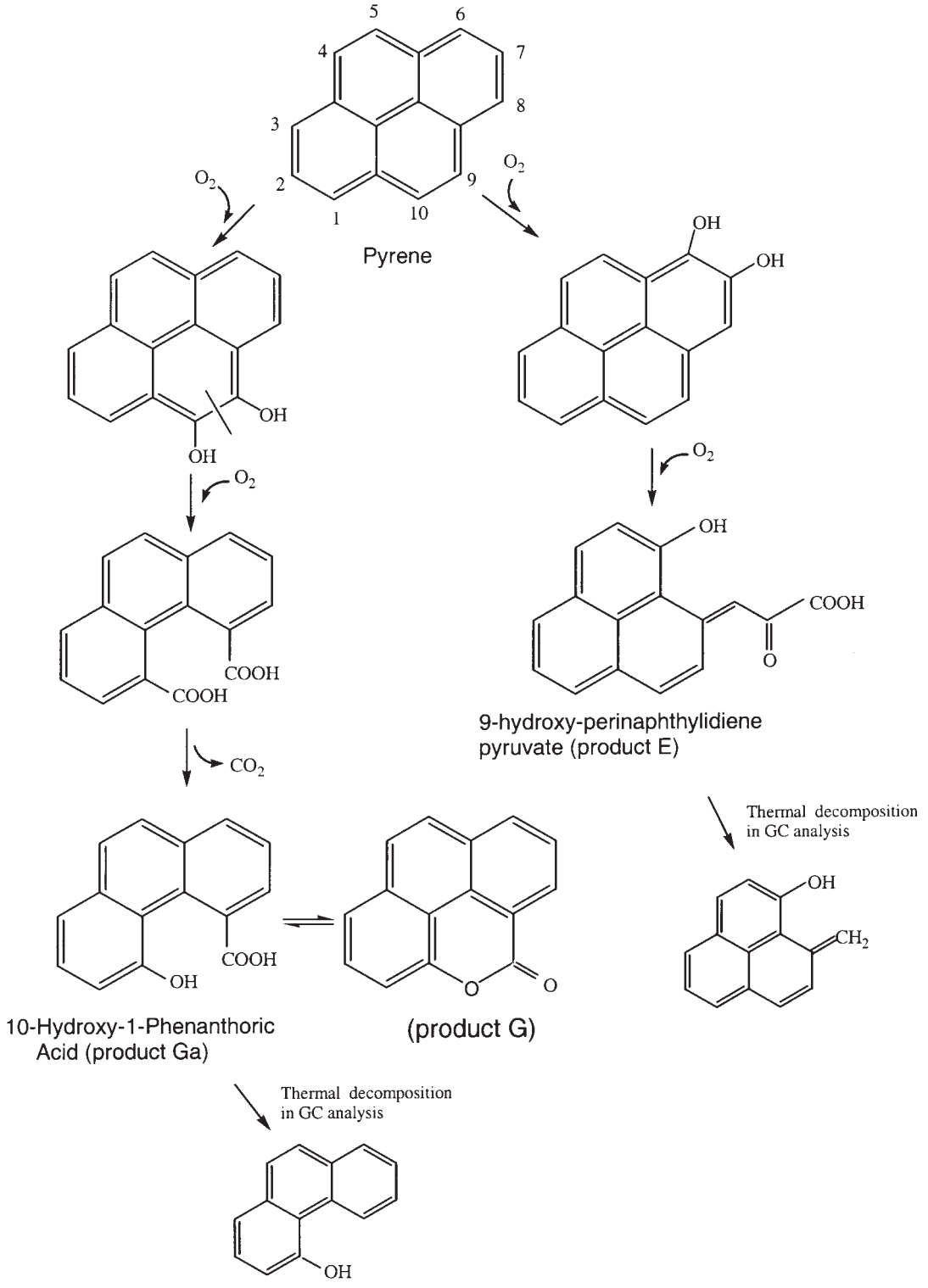

Figure 3 Proposed pathway for the cometabolism of pyrene by strain EPA 505. Structures in brackets are hypothesized. Smaller structures are those observed from GCMS analysis.

acid (product Ga) which readily forms the more stable ketal, product G. No further metabolism of these compounds occurred.

Product P has not yet been identified but it has a main mass ion at 208 based on GCMS analysis. Initial examination of products produced from several of the PYR degraders growing on PYR showed the presence of a distinct peak with the same adsorption spectrum as peak P but at much lower concentrations. It is possible that the cometabolism of PYR by EPA 505 follows the same degradation pathway as that used by the Gram-positive PYR degraders. That is, both produced product P, but EPA 505 lacks the enzymatic capability to further metabolize this product and consequently accumulates product P without growth on PYR.

We have also detected a metabolite that elutes early (product E) and has an absorption spectrum with maxima at 244, 260, 321, 362, and 410 μm. Purification of this product resulted in a compound with a mass ion of 182 by GCMS analysis, corresponding to 1-hydroxy-9-methylene perinaphthalene. We hypothesize that this compound may be a thermal decomposition product of hydroxy-perinaphylidienepyruvate (Figure 3). We did not detect a mass ion corresponding to 266, or the *cis*-2-hydroxy-3-(perinaphthenone-9-yl)-acetic acid observed by Walter *et al* [42]. Our preliminary data, therefore, suggest that EPA 505 also dioxygenates at the 1,2-position of PYR and produces a substituted perinaphthalene compound by meta cleavage. Walter *et al* [42] demonstrated a similar degradation sequence of PYR with a *Rhodococcus* sp. The inability of strain EPA 505 to remove a 3-carbon fragment following this meta cleavage, may account for its lack of growth on PYR.

Metabolic characteristics of FLA degraders

Previous work with *Sphingomonas paucimobilis* strain EPA 505 showed that it is one of the most versatile isolates for cometabolizing high molecular weight PAHs [33]. It is of interest then, to examine the metabolic diversity of some of the other *Sphingomonas* spp that we have isolated. Studies with FLA degrader strain CO6 revealed that it is capable of simultaneously attacking FLA at the single benzyl ring and at the naphthyl ring, producing acenaphthenone and fluorenone as the respective major products [18]. The pathway is diagrammed in Figure 4. A similar dual attack on FLA has been reported with *Mycobacterium* [22,23]. Our results are the first report of this capability in a Gram-negative strain of a *Sphingomonas* sp. Of the other *Sphingomonas* strains that we have examined so far, CO6 is the only one that can attack both ends of the FLA molecule. The others, including EPA 505 and FLA 1–1, appear to attack only the benzyl ring of FLA with acenaphthenone as the principal product, which corresponded with their ability to grow on acenaphthene and acenaphthylene. From this initial degradation pathway information, one might suggest that all FLA degraders should be acenaphthylene degraders. In fact, only 11 FLA degraders were able to grow on acenaphthylene.

In addition, strain CO6 grew on fluorene, biphenyl and indan (all connected to the fluorenone degradation pathway), along with acenaphthene [18], whereas strains EPA 505 and FLA 1–1 grew only on acenaphthene (Figure 4). The strains may also appear to differ in their cell membrane characteristics. Several of the strains produced a bright red product when grown on FLA, but strain EPA 505 only produced the red product when exposed to surfactants (Triton X-100 or Tween 80). Perhaps the surfactant permeablizes the cell membrane of strain EPA 505, making it similar to the other strains. Whether this is related to the metabolic diversity of the *Sphingomonas* strains is not yet clear.

Discussion

The modified enrichment procedure and plate-screening technique used in this study to isolate PAH degraders from contaminated soils and sediments readily provided strains with the ability to utilize FLA or PYR as growth substrates. Many of these isolates were also able to transform other nongrowth PAHs. Both fast-growing and slow-growing bacterial strains had an equal chance of being isolated with our technique since no serial transfer or dilution was performed. The spray plate method has been used previously [7,21,25,28], but generally with mineral salts medium and with the PAH as the only carbon and energy source. We used PYGT medium to provide supplemental growth substrates along with the PAH, assuming that PAH degradation would be enhanced rather than inhibited. Initiating oxidation of a PAH requires an adequate energy reserve and we believe PYGT medium functioned in providing that reserve. Most of our isolates, in fact, degraded PAHs faster when grown on PYGT, suggesting that PAH degradation capability was not affected by alternative growth substrates. Interestingly, several of the FLA degraders (FLA 3–1, 3–3, 7–1, 8–1, and 11–1) were unable to grow, or grew very poorly, in liquid culture with FLA, although they consistently produced clearing zones with FLA on PYGT plates. Since five of these isolates were phenanthrene degraders, we can argue that our enrichment procedure was capable of detecting FLA-cometabolizing, PHE-degrading strains. The low incidence of FLA-cometabolizing/PHE degrader strains was not surprising given that there are no reports of PHE degraders in pure culture with the ability to cometabolize FLA.

Sphingomonas and *Mycobacterium* species are well represented among the FLA degraders. Their incidence in our study attests to the importance of further detailed examination of these genera for their role in PAH degradation in the environment and in bioremediation. Knowledge about the metabolism of PAHs by *Mycobacterium* spp is currently more extensive than for *Sphingomonas* spp. Degradation pathways for the metabolism of NAP, PYR, FLA, B(a)ANT, and B(a)PYR in *Mycobacterium* spp have been reported [8,14,16,17,22,23,35,36,39,41]. *Mycobacterium* spp are also reported to mineralize PHE [2,16] and ANT [6], but could only cometabolize FLU [2]. In addition, three studies have examined the taxonomic similarity of two PAH-degrading *Mycobacterium* spp (PYR-1 and PAH 135) and found that the strains were distinctly different, both phylogenetically and metabolically [13,28,43].

The importance of *Sphingomonas* spp as degraders of HMW PAHs was first recognized by Mueller *et al* [32] who

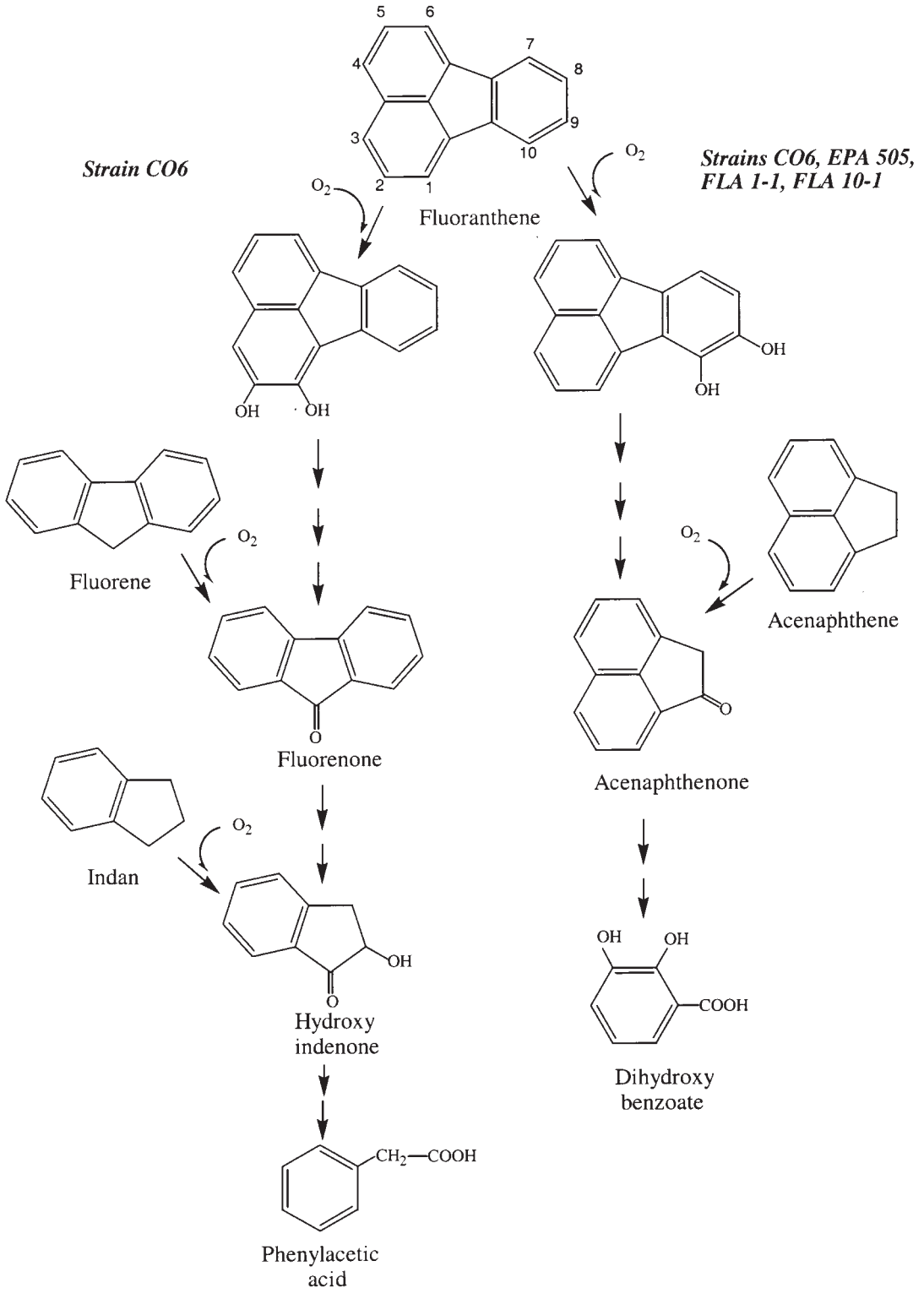

Figure 4 Proposed pathway for the degradation of fluoranthene and other PAHs by three *Sphingomonas* sp strains, CO6, EPA 505, and FLA 1–1.

suggested that the ability to degrade FLA was associated with *Sphingomonas* species commonly isolated from contaminated soils and that these organisms were equally as capable of degrading high molecular weight PAHs as the *Mycobacteria* spp. This work was stimulated by studies on *S. paucimobilis* strain EPA 505, an isolate that was able to grow on FLA and cometabolize several HMW PAHs, including PYR, CHR, B(b)FLA, and B(a)PYR [32,46]. Few cases of PAH-degrading *Sphingomonas* spp have been reported, despite recognition of this genus as a common degrader of aromatic compounds [38]. This may be the result of the recent recognition of *Sphingomonas* as an

accepted genus [45]. Indeed, *Pseudomonas paucimobilis* Q1 [27] and *Beijerinckia* sp strain B1 [12] are now recognized as sphingomonads [24]. Both of these strains were isolated for their ability to grow on biphenyl and naphthalene. The initial steps in the oxidation of PAHs by strain B1 have been studied extensively [29,41] and this organism is able to grow on PHE and acenaphthene and to cometabolize ANT, B(a)ANT, and B(a)PYR. Kastner *et al* [21] isolated a *S. paucimobilis* strain BA2 that was able to degrade NAP, PHE, and ANT which paled in comparison with the metabolic capabilities of the *Mycobacterium* and *Rhodococcus* species they also isolated and characterized. Dagher *et al* [7] reported on the ability of *Sphingomonas* sp strain 107 to grow on PHE and FLU and apparently to cometabolize FLA, ANT, PYR, and B(a)ANT, but not B(a)PYR. This is an interesting strain because it is one of the few PHE degraders reported to have the ability to cometabolize PYR. All of our PHE degraders (FLA 3–1, 3–3, 7–1, 8–1, and 11–1) were unable to attack PYR. Recently, five *Sphingomonas* spp (F199, BO478, BO522, BO695, and BO712), isolated from deep subsurface sediment samples, were shown to mineralize both naphthalene and toluene (a rare combination) and apparently to cometabolize FLU [10]. Sequencing of the entire 180-kbp plasmid (pNL1) in strain F199, designated a *Sphingomonas aromaticivorans*, showed unusual co-clustering of the genes for the different aromatic pathways for NAP, biphenyl, *m*-xylene and *p*-cresol, suggesting that *Sphingomonas* species may represent novel evolutionary histories [38]. The increasing number of *Sphingomonas* isolates will allow further comparisons to be conducted.

In comparing the metabolic differences between the FLA and PYR degraders, and to a similar extent the *Mycobacterium* and *Sphingomonas* isolates, two characteristics stand out. First, the ability to grow on PYR may be dependent on a metabolic characteristic that is unique to *Mycobacterium*, or perhaps to Gram-positive bacteria in general. None of our Gram-negative isolates grew on PYR and we are not aware of any definitive reports showing PYR degradation by Gram-negative organisms. This metabolic difference appears to be due in part to the absence of an appropriate enzyme or enzymes in the Gram-negative FLA degraders that is required for further metabolism of the ring-opened products generated by an initial *nah*-type dioxygenation and subsequent meta cleavage. A product, we designated as peak P, was detected during PYR metabolism by both PYR and FLA degraders (*Sphingomonas* and *Mycobacterium* spp as well), based on the presence of peaks with identical UV/vis spectra, but this product accumulates with the FLA degraders and is only transient in the PYR degraders, suggesting that it may be a dead-end product for FLA degraders.

Second, the cometabolic capabilities of the PYR degraders appear to be more limited than those of the FLA degraders. This is reflected in the surprising inability of PYR isolates, as a whole, to metabolize B(b)FLU (they all grow well on FLA), even when pregrown on PHE. In addition, the ability of the PYR degraders to cometabolize B(a)ANT is almost completely dependent on pre-exposure to PHE, whereas with the FLA degraders, many cometabolized B(a)ANT without PHE induction. The only exceptions appear to be isolates PRY 5–1 and PRY 9–1. Clearly, then, there are considerable differences in substrate specificity of the enzymes in these isolates for the degradation and transformation of PAHs and further study is required to verify that some of these differences may, in fact, be phylogenetically linked. We previously suggested such a linkage with other PAH degraders [33].

The mechanism of cometabolism in these strains probably involves a loose specificity of the initial dioxygenases used in metabolism of fluoranthene and other PAHs. Cloned dioxygenase genes from the naphthalene pathway, for example, have been shown to catalyze a variety of oxidations (mono- and dioxygenation) of many different PAHs [39]. In the case of *S. paucimobilis* strain EPA 505, the presumed loose specificity permits oxidation of several high molecular weight PAHs that are not growth substrates. Using the degradation of PYR (not a growth substrate) as a model for this cometabolism, it appears that strain EPA 505 attacks at two different sites on the PYR molecule. Our preliminary evidence suggests that dioxygenation at the 1,2-position and subsequent meta cleavage proceeded in a manner similar to that reported for Gram-positive *Mycobacterium* and *Rhodococcus* spp [17,42] that are able to grow on PYR. However, strain EPA 505 was apparently unable to convert the ring-opening products into growth substrates. This metabolic block may be caused by the narrow substrate specificity of an hydratase/aldolase-type removal of the enoic-α-keto acid side chain produced from the meta fission. Strain EPA 505 also attacked PYR at the 4,5-position but in contrast to results reported by Walter *et al* [41], opened the hydroxlyated ring using an ortho cleavage mechanism. Rehmann *et al* [37] suggested a similar attack on PYR based on studies with *Mycobacterium* sp strain KR2 which grew on PYR. Attack at the 4,5-position of PYR by strain EPA 505 was unproductive in terms of obtaining carbon and energy for growth.

Other than initial oxidation reactions of PAHs, there are few published reports of the metabolic pathways used by *Sphingomonas* spp for PAHs. Most of the information on degradation of FLA is with *Mycobacterium* [17,22,23,28,36] and *Alcaligenes denitrificans* [44]. But we have recently demonstrated that FLA can be attacked at two different sites by *Sphingomonas* strain CO6 [18], much in the same manner as demonstrated for mycobacteria. The evolutionary aspects of this dual pathway are intriguing. Since strain CO6 is able to grow on naphthalene, fluorene, indan, and acenaphthene, it is possible that it recruited degradation pathways for all these PAHs and 'married' them together for maximal ability to degrade FLA. The advantage imparted to the organisms is not clear but it seems excessive since other *Sphingomonas* spp that use only the acenaphthenone side of the pathway grow just as well on FLA. When strain CO6 is grown with both fluorene and acenaphthalene, they appear to be used simultaneously [18]. Thus the cell must maintain as many as 10 separate dioxygenase activities if we assume that the same dioxygenase(s) is not used more than once in the pathway. Further studies are underway to study this aspect, with additional emphasis on the regulatory strategies used for the PAH degradation pathways among the *Sphingomonas* spp and their genetic makeup.

It is hoped that this information on FLA and PYR degraders will provide a general guideline for the future design of biological treatment systems that incorporate bioaugmentation as a means to improve the degradation of high molecular weight PAHs. Comparisons of PAH-degrading strains will not only improve the potential of bioaugmentation but will also provide further information on how microbial communities degrade mixtures of PAHs.

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