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Competitive Metabolism of Naphthalene, Methylnaphthalenes, and Fluorene by Phenanthrene-Degrading Pseudomonads

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Polynuclear aromatic hydrocarbons (PAHs) typically exist as complex mixtures in contaminated soils, yet little is known about the biodegradation of PAHs in mixtures. We have isolated two physiologically diverse bacteria, *Pseudomonas stutzeri* P-16 and *P. saccharophila* P-15, from a creosote-contaminated soil by enrichment on phenanthrene as the sole carbon source and studied their ability to metabolize several other two- and three-ring PAHs. Naphthalene, 1-methylnaphthalene, and 2-methylnaphthalene served as growth substrates for both organisms, while fluorene was only cometabolized. We also studied the effects of these compounds on initial rates of phenanthrene uptake in binary mixtures. Lineweaver-Burk analysis of kinetic measurements was used to demonstrate competitive inhibition of phenanthrene uptake by all four compounds, suggesting that multiple PAHs are being transformed by a common enzyme pathway in whole cells. Estimates of the inhibition coefficient, K_i, are reported for each compound. The occurrence of competitive metabolic processes in physiologically diverse organisms suggests that competitive metabolism may be a common phenomenon among PAH-degrading organisms.

Polynuclear aromatic hydrocarbons (PAHs) form an important class of environmental pollutants produced as a consequence of fossil fuel utilization, combustion processes, and chemical manufacture (10, 13, 21). PAHs also occur naturally, as the result of fires and geologic processes, and are carbon compounds of significant energy potential (1, 4). Many PAHs are subject to bacterial degradation (7, 8, 31).

There is current interest in the utilization of bacterial processes for the treatment of contaminated soils and groundwaters (18, 38, 39), including hazardous-waste sites contaminated with mixtures of PAHs (14, 27, 44). However, biological remediation of PAH-contaminated sites is not a proven technology and has rarely been completely effective (44).

Little is known about the biodegradation of mixtures of PAHs, especially the effect of one PAH component on the biodegradability of another (7). Previous studies of PAH degradation by mixed and pure cultures presented evidence that there are interactions between PAHs in mixtures that influence biodegradation. For example, exposure of marine sediments to one PAH was observed to enhance degradation of other, subsequently added PAHs (3). Mixed microbial populations from marine waters sequentially mineralized naphthalene, phenanthrene, and anthracene, suggesting preferential utilization of substrates by a general PAH-degrading population (15). Sequential removal of PAHs has also been demonstrated in batch incubations where recalcitrant PAHs were removed only after more-labile PAHs were degraded (28). Park et al. (29) noted that higher-molecular-weight PAHs were more resistant to biotransformation when present as pure compounds in soil than when present in complex waste mixtures in soil, whereas lower-molecular-weight PAHs were transformed more rapidly as pure compounds.

Although substantial evidence exists that PAH-degrading bacteria often metabolize a range of PAH substrates (2, 5, 16, 17, 22, 25, 26, 32–34, 36, 43), little is known about the specificity of the enzymes involved in PAH degradation. It has been demonstrated that *cis*-naphthalene dihydrodiol dehydrogenase oxidizes *cis*-dihydrodiols of other polycyclic aromatic hydrocarbons (30, 31). Sanseverino et al. (35) showed that NAH7 and NAH7-like plasmids, which encode genes responsible for naphthalene metabolism, can also mediate the mineralization of phenanthrene and anthracene. Metabolites from anthracene and phenanthrene catabolism by NAH7-like plasmid encoded enzymes were identified as 2-hydroxy-3-naphthoic acid and 1-hydroxy-2-naphthoic acid, respectively (24), which are metabolites of previously described bacterial degradation pathways (8). Denome et al. (11) recently presented molecular evidence that dibenzothiophene, naphthalene, and phenanthrene are metabolized by a single set of upper-pathway enzymes in a soil pseudomonad.

In the present work, we use kinetic analyses to evaluate whether bacteria grown on phenanthrene oxidize other PAHs by the enzyme pathway responsible for phenanthrene degradation. Concentration-dependent, competitive interactions of binary mixtures of phenanthrene and other low-molecular-weight PAHs (naphthalene, methylnaphthalenes, and fluorene) provide evidence that these compounds share common enzymes along the degradation pathway.

MATERIALS AND METHODS

Chemicals. Phenanthrene (99% purity) was purchased from Kodak, Rochester, N.Y. Naphthalene (reagent grade) was purchased from Baxter Chemicals, Phillipsburg, N.J. 1-Methylnaphthalene (98%), 2-methylnaphthalene (98%), fluorene (98%), indan (97%), and 9-hydroxyfluorene (≥97%) were purchased from Aldrich Chemicals, Milwaukee, Wis. Bacto Peptone was purchased from Difco, Detroit, Mich.

Bacterial strains. *Pseudomonas stutzeri* P-16 and *P. saccharophila* P-15 were isolated from a creosote-contaminated soil by enrichment on phenanthrene as the sole carbon source. Isolation, purification, and characterization of the bacteria have been described previously (40, 41).

Cultivation of bacteria for spectrophotometric and respirometric resting-cell assays. Phenanthrene-degrading cultures were grown in liquid medium containing 5 g of peptone per liter supplemented with 0.5 g of phenanthrene per liter (PEP-PAT medium). PEP-PAT medium was made as previously described (40). Isolate P-15 does not express significant phenanthrene-degrading capability when grown on peptone alone, while P-16 does (41).

Growth on PAHs as sole carbon sources. Bacteria were tested for growth on

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PAHs other than phenanthrene as the sole source of carbon and energy. Media were prepared by dissolving 50 mg of each compound in 10 ml of methylene chloride and adding 1 ml of each solution to a 225-ml, solvent-washed sterile bottle. After the methylene chloride had evaporated, 50 ml of sterile tap water buffer (TWB) was added to each bottle and the bottles were inoculated with cells washed from a slant with sterile TWB. Controls consisted of phenanthrene (positive control) and bottles receiving only methylene chloride (negative control). The bottles were sealed and incubated in the dark for 9 days and were shaken once daily. TWB was made as previously described (40). Growth was evaluated as a significant increase in optical density relative to the controls. Negative controls exhibited no significant change in optical density during incubation.

Measurement of PAH oxidation rates. The oxidation of phenanthrene and other compounds was measured by an initial rate assay with resting cells in a stirred, 1.7-ml water-jacketed respirometer cell (Gilson Medical Electronics, Middleton, Wis.) equipped with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio) and maintained at a constant temperature in a circulating-water bath. Oxidation rates were measured at 25 ± 0.1°C.

Cultures were grown at 25 ± 0.5°C on PEP-PAT medium and harvested in the late exponential phase. The cultures were centrifuged, washed in dechlorinated tap water, centrifuged again, and resuspended in TWB. Washed cells were placed in the respirometer cell, an initial endogenous oxygen uptake rate was measured, 10 μl of a methanol solution containing the substrate of interest or a combination of substrates was injected into the cell, and oxygen uptake rate was measured again. PAH oxidation by resting cells was tested at substrate concentrations which were approximately 90% of the substrate's aqueous solubility limits. Oxygen consumption in the presence of substrate was corrected for endogenous respiration. Results are expressed in terms of specific oxygen uptake rate and reported as milligrams of oxygen consumed per minute per gram of bacteria.

Measurement of phenanthrene uptake by spectrophotometric rate assay. Resting cells were prepared as described above and diluted in TWB to an A_{420} of approximately 0.1. A 2-ml volume of this suspension was placed in a 3-ml quartz cuvette in a spectrophotometer. The cuvette was mixed with a Teflon stir bar and a micromagnetic stirrer. Then 10 μl of a methanol solution of phenanthrene or phenanthrene in combination with another PAH was injected into the cuvette, and the decrease in A_{250} was measured over time. The A_{250} was measured every 10 s between 30 and 90 s after injection (for a total of seven measurements), and the linear decrease in A_{250} was used to calculate the initial phenanthrene uptake rate. No significant uptake occurred in the presence of a suspension of killed cells (40).

The A_{250} value was converted to phenanthrene concentration by using an extinction coefficient of 6.46×10^4 liters mol^{-1} cm^{-1} (42). At 250 nm, phenanthrene has an extinction coefficient 4 times higher than that of fluorene and 25 to 30 times higher than those of the naphthalenes. Results are expressed as specific phenanthrene degradation rate in milligrams of phenanthrene consumed per minute per gram of bacteria. When necessary, phenanthrene degradation rates were corrected for slight changes in A_{250} caused by the simultaneous consumption of coinjected substrates, by subtracting the A_{250} decrease resulting from the cosubstrate injected alone. This correction method overcompensates slightly for the consumption of the cosubstrate in the presence of phenanthrene. However, even at the highest cosubstrate concentrations, corrections were typically 10% or less of the rates measured for 5 μM phenanthrene.

Estimation of kinetic coefficients. Kinetic coefficients for the uptake of oxygen in the presence of naphthalene were estimated by nonlinear regression with the Michaelis-Menten equation. Michaelis-Menten parameters for phenanthrene oxidation (by the respirometric method) and degradation (by the spectrophotometric assay) were reported previously (40).

Competitive inhibition of phenanthrene by other low-molecular-weight PAHs was demonstrated by Lineweaver-Burk transformations of specific phenanthrene degradation rates in the presence and absence of a fixed concentration of potentially competitive compounds. Once competitive inhibition had been demonstrated, the inhibition coefficient (K_i) was estimated by measuring the specific phenanthrene degradation rate at constant phenanthrene concentration ([S]) in the presence of different inhibitor concentrations ([I]) and at constant inhibitor concentration in the presence of different phenanthrene concentrations. A fractional activity equation for simple competitive inhibition (37) was then fitted to the data:

$$\frac{V_i}{V_o} = \frac{K_s + [S]}{K_s\left\{1 + \left(\frac{[I]}{K_i}\right)\right\} + [S]} \quad (1)$$

where v_0 is the specific phenanthrene degradation rate in the absence of an inhibitor, v_i is the specific phenanthrene degradation rate in the presence of an inhibitor, and K_s is the independently measured Michaelis-Menten half-saturation coefficient for phenanthrene (40). Nonlinear estimation was performed by the Quasi-Newton method (SYSTAT Inc., Evanston, Ill.).

Analysis of fluorene metabolites. Experiments to confirm the metabolism of fluorene by P-16 were conducted as follows. A 0.5-ml portion of a filter-sterilized fluorene-hexane solution (2 g/100 ml) was added to sterile, methanol-washed 50-ml flasks. Duplicate flasks were inoculated with *P. stutzeri* P-16 which were washed from a slant with sterile dechlorinated tap water. Control flasks contained sterile fluorene-peptone medium and inoculated peptone without fluorene.

After 3 days of incubation at 25 ± 0.5°C in the dark, media were harvested and filtered through 0.2-μm-pore-size polycarbonate filters. Hydroxylated fluorene metabolites were identified by high-pressure liquid chromatography (HPLC) coupled to electrospray ionization mass spectrometry. HPLC was performed with a Beckman model 126 solvent delivery system and 168-photodiode-array UV detector. The column was an Ultracarb 5-μm octyldecyl silane column, 150 by 2 mm (Phenomenex). The mobile phase was 70% methanol in water at a flow rate of 200 μl/min and was split postcolumn with a Valco stainless steel tee; 2 to 5 μl/min was diverted to the electrospray ionization source via fused-silica capillary tubing (approximately 1.5 m by 50 μm [inner diameter]; Polymicro Corp.). The remainder of the flow was directed into the diode array detector cell. Samples were injected via a 20-μl sample loop (Rheodyne model 7725 injector). The HPLC was controlled and the data were collected by the Beckman System Gold software package.

Electrospray ionization mass spectra were obtained on a Finnigan 4000 quadrupole mass analyzer fitted with an electrospray source (Analytica of Branford, Inc.). Nitrogen was used as the drying gas and was heated to 200°C. Spectra were collected in the positive-ion mode. The inlet capillary was held at ground, while the cylindrical, backing, and capillary electrodes were held at −2,600, −3,400, and −3,900 V, respectively. The capillary skimmer voltage was tuned for minimum fragmentation of adenosine. The mass analyzer was scanned from 10 to 500 *m/z* twice per second, and the data were collected on a Technivent Vector 2 data system.

TABLE 1. Oxidation of low-molecular-weight PAHs

Compound	Concn (μM)	Mean specific oxygen uptake rate[a] ± SD for:	
		P-15	P-16
Phenanthrene	5	1.47[b] ± 0.06	1.98[b] ± 0.41
Naphthalene	200	1.89[b] ± 0.17	1.55[b] ± 0.11
2-Methylnaphthalene	150	1.60[b] ± 0.10	2.03[b] ± 0.19
1-Methylnaphthalene	150	1.29[b] ± 0.14	1.45[b] ± 0.13
Fluorene	10	0.63[b] ± 0.05	0.06 ± 0.04
Indan	250	0.10 ± 0.06	0.12 ± 0.01
Control (methanol)		0.07 ± 0.01	0.01 ± 0.04

[a] Expressed as milligrams of oxygen per minute per gram of cells ($n = 3$).
[b] Significantly different from the control at $\alpha = 0.01$.

RESULTS

Growth and oxidation assays. *P. stutzeri* P-16 and *P. saccharophila* P-15 were able to grow on phenanthrene, naphthalene, 1-methylnaphthalene, and 2-methylnaphthalene as the sole source of carbon and energy. Fluorene and indan did not serve as growth substrates for either organism. Experiments were then conducted to determine if resting cells could oxidize the nongrowth substrates as well as the growth substrates.

P. stutzeri P-16 and *P. saccharophila* P-15 were tested for their ability to oxidize other low-molecular-weight PAHs after growth in the presence of phenanthrene by the respirometric resting-cell assay (Table 1). Both organisms were able to oxidize naphthalene, 2-methylnaphthalene, and 1-methylnaphthalene ($\alpha < 0.01$ in comparison with methanol alone). P-15 exhibited significant levels ($\alpha < 0.01$) of fluorene oxidation, whereas P-16 did not ($\alpha > 0.05$). Oxygen uptake in the presence of indan was not significantly greater ($\alpha > 0.05$) than in controls for either P-15 or P-16.

Kinetics of naphthalene oxidation. The initial specific oxygen uptake rate was determined as a function of naphthalene concentration for P-15 and P-16. In both organisms, naphthalene oxidation followed Michaelis-Menten kinetics. Estimates for Michaelis-Menten kinetic coefficients and 95% confidence intervals are as follows. The maximum specific levels of oxidation were almost twice as high in P-16 (2.4 mg of O_2 min^{-1} g of $cells^{-1}$, with a confidence interval of 2.0 to 2.6 mg of O_2 min^{-1} g of $cells^{-1}$) as in P-15 (1.4 mg of O_2 min^{-1} g of $cells^{-1}$,

TABLE 2. Effect of naphthalene and fluorene on phenanthrene degradation and oxidation

Culture	Second substrate (10 μM)	Mean oxygen uptake ± SD[a] (% activity)	Mean phenanthrene degradation ± SD[a] (% activity)
P-16	None	100 ± 3.3	100 ± 3.3
	Naphthalene	102 ± 3.7	82.2[b] ± 3.5
	Fluorene	82.3[b] ± 5.9	81.2[b] ± 6.7
P-15	None	100 ± 3.9	100 ± 12.6
	Naphthalene	120[b] ± 9.7	79.4[c] ± 6.7
	Fluorene	110 ± 8.1	79.3[c] ± 8.9

[a] Mean and standard deviation of three determinations. Activity is expressed as a percentage of the control value, which is set at 100%.
[b] Significantly different from phenanthrene alone at $\alpha = 0.05$.
[c] Significantly different from phenanthrene alone at $\alpha = 0.10$.

with a confidence interval of 1.3 to 1.5 mg of O_2 min^{-1} g of $cells^{-1}$). P-15 also had a lower apparent affinity for naphthalene, as indicated by a higher K_s than that for P-16 (K_s of P-15 is 17 μM, with a confidence interval of 10 to 24 μM; K_s of P-16 is 3.0 μM, with a confidence interval of 1.9 to 4.1 μM).

Phenanthrene uptake and oxidation in binary mixtures with other PAHs. The influence of co-occurring compounds on the metabolism of phenanthrene was investigated by measuring the degradation of phenanthrene and the uptake of oxygen in the presence of phenanthrene alone (5 μM) and in the presence of phenanthrene in a binary mixture with either naphthalene, 1- or 2-methylnaphthalene, indan, or fluorene.

As shown in Table 2, 10 μM naphthalene interfered with the degradation of phenanthrene but did not inhibit the uptake of oxygen under the same conditions. In fact, the amount of oxygen uptake by P-15 in the presence of phenanthrene plus naphthalene was significantly greater than for phenanthrene alone, indicating that the two compounds were oxidized simultaneously. Fluorene (10 μM) also inhibited degradation of phenanthrene, but not oxygen uptake, in resting-cell assays with P-15. In P-16, which had not demonstrated the ability to oxidize fluorene in respirometric assays (Table 1), fluorene interfered with both oxygen uptake and phenanthrene degradation.

The influence of co-occurring PAHs on phenanthrene degradation was further investigated with other compounds at 30 μM (Table 3). Results from experiments with 1-methylnaphthalene, 2-methylnaphthalene, and indan demonstrated that inhibition of phenanthrene uptake in the presence of other PAHs was a general phenomenon in these organisms. Naphthalene, 1-methylnaphthalene, and 2-methylnaphthalene appear to be stronger inhibitors of phenanthrene degradation

TABLE 3. Effect of methylnaphthalenes and indan on phenanthrene degradation

Second substrate (30 μM)	Mean % of uninhibited initial degradation rate ± SD[a] for:	
	P-15	P-16
None	100 ± 4.0	100 ± 2.1
1-Methylnaphthalene	42.9 ± 22.3	58.6 ± 5.7
2-Methylnaphthalene	46.1 ± 4.8	45.0 ± 1.1
Indan	83.4[b] ± 13.9	87.0 ± 2.0

[a] Mean and standard deviation of three determinations.
[b] Not significantly different from control (phenanthrene alone) at $\alpha = 0.05$. All other treatments are significantly different ($\alpha = 0.05$) from phenanthrene alone.

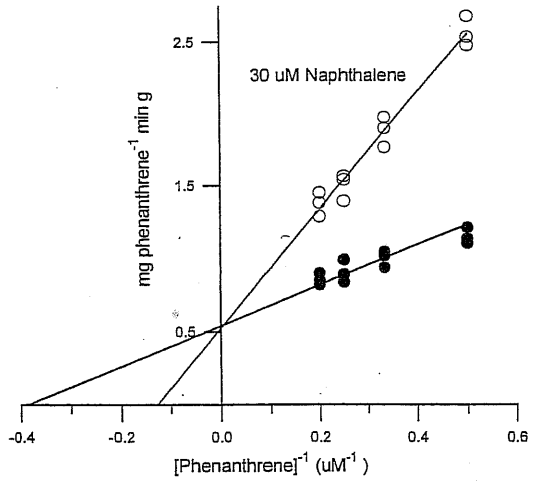

FIG. 1. Lineweaver-Burk plot of initial phenanthrene degradation rate by *P. stutzeri* P-16 as a function of phenanthrene concentration alone (●) and in the presence of 30 μM naphthalene (○).

than is indan, as indicated by the amount of inhibition that occurred at 30 μM each PAH.

Demonstration that PAHs are competitive inhibitors of phenanthrene degradation. Results shown in Tables 2 and 3 clearly illustrate that other low-molecular-weight PAHs can interfere with phenanthrene metabolism but are not sufficient to determine a mechanism by which this inhibition takes place. Lineweaver-Burk analysis of phenanthrene uptake kinetics was used as a means of distinguishing between competitive, uncompetitive, and noncompetitive forms of inhibition (37). Experiments were conducted to measure the uptake of phenanthrene in the presence and absence of the "inhibitors" naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, and indan at 30 μM or fluorene at 10 μM. The competitive influence of naphthalene on phenanthrene degradation by P-16 is shown by a double-reciprocal plot in Fig. 1; similar results were obtained for P-15 (data not shown). Fluorene, 1-methylnaphthalene, and 2-methylnaphthalene were also competitive inhibitors of phenanthrene metabolism (data not shown). Indan was a weak inhibitor, and results with this compound were inconclusive.

Determination of the inhibition coefficient, K_i, for naphthalene and other PAHs. The effect of varying naphthalene concentration on the relative specific uptake of 5 μM phenanthrene by P-16 is plotted in Fig. 2. These data were fit to equation 1 to estimate K_i by using a half-saturation coefficient (K_s) for phenanthrene of 1.34 μM (40). Results of nonlinear regression analysis gave an estimate of K_i to be 10 μM with a 95% confidence interval of between 8.5 and 12 μM.

The fractional phenanthrene degradation rate in the presence and absence of 30 μM naphthalene was measured as a function of phenanthrene concentration for P-16, and equation 1 was fit to the data (Fig. 3). The estimate of K_i for naphthalene obtained by using this experimental design with P-16 (Table 4) agreed closely with the estimate from experiments with fixed phenanthrene concentrations (reported above). Estimates of K_i for the other substrates and for P-15 were also made by using this experimental design (Table 4). For P-15, equation 1 was fit to the data by using a K_s value of 1.12 μM for phenanthrene (40).

Oxidation of fluorene by *P. stutzeri* P-16. Resting-cell respirometry assays had not detected fluorene oxidation by P-16 (Table 1), but kinetic evidence (Table 4) suggested that fluorene might be co-oxidized by P-16. We therefore suspected

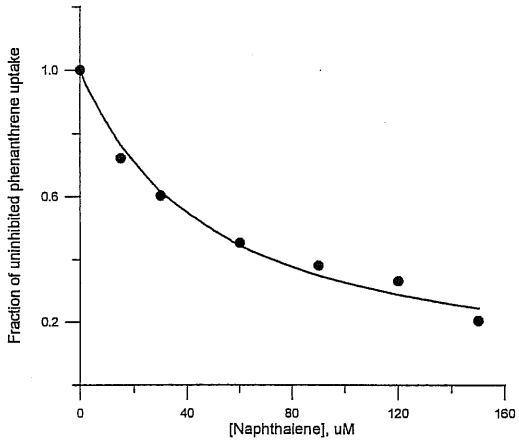

FIG. 2. Initial phenanthrene degradation rate by *P. stutzeri* P-16 as a function of increasing naphthalene concentration in a binary mixture with 5 μM phenanthrene. The initial rate is expressed as a fraction of the initial uninhibited specific phenanthrene degradation rate. The curve represents a nonlinear fit to equation 1.

that the oxygen uptake assay may not have been sensitive enough to detect fluorene oxidation by this isolate. Since it had been demonstrated that P-16 is constitutive for phenanthrene oxidation when grown on peptone (41), an experiment was conducted to determine if metabolites of fluorene oxidation could be detected in P-16 cultures grown on peptone supplemented with fluorene.

After 3 days of incubation, the culture fluid from P-16 grown on peptone supplemented with 500 mg of fluorene per liter was bright yellow. Controls (sterile media and peptone inoculated with P-16) were not bright yellow. Filtered (pore size, 0.2 μm) samples of the media were analyzed by HPLC and electrospray ionization mass spectrometry. The two major peaks observed in the medium supernatant were identified as 9-hydroxyfluorene and 9-fluorenone by retention time and mass spectra, which were identical to those of standards. 9-Hydroxyfluorene and 9-fluorenone have been previously identified as metabolites of fluorene oxidation by bacteria (26).

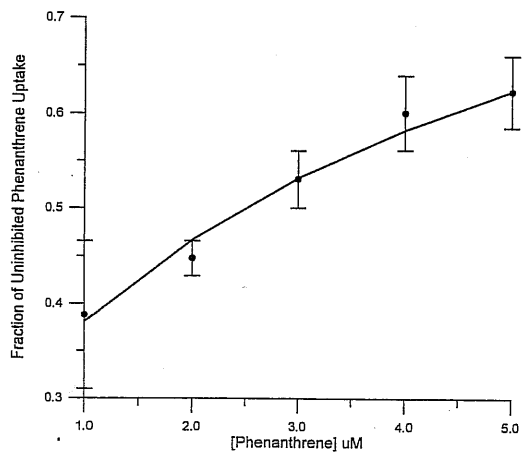

FIG. 3. Initial phenanthrene degradation rate by *P. stutzeri* P-16 as a function of increasing phenanthrene concentration in a binary mixture with 30 μM naphthalene, expressed as a fraction of the initial specific phenanthrene degradation rate at the appropriate concentration of phenanthrene alone. The curve represents a nonlinear fit to equation 1.

TABLE 4. Estimates of inhibition coefficients

Compound	K_i (μM) (95% CI)[a] for:	
	P-16	P-15
Naphthalene	11 (9.6, 12)	14 (9.6, 18)
Fluorene	5.1 (4.3, 6.0)	4.6 (3.9, 5.4)
1-Methylnaphthalene	10 (8.7, 12)	12 (6.0, 18)
2-Methylnaphthalene	6.2 (5.3, 7.1)	12 (7.8, 17)

[a] 95% CI, 95% confidence interval.

DISCUSSION

Bioremediation of PAH-contaminated sites requires the microbial degradation of complex waste mixtures. For example, the bioremediation of creosote-contaminated sites is expected to require treatment of at least 17 PAHs that also occur in mixtures with phenolic and heterocyclic compounds (27). A survey of soils beneath unlined waste pits associated with natural gas production found over 30 PAHs and alkylated PAHs, at total PAH concentrations as high as 683 mg/kg (12). How such mixtures of aromatic hydrocarbons are biodegraded is not well understood. The objective of this work was to investigate the range of low-molecular-weight PAHs that could be metabolized by two phenanthrene-degrading bacteria and to determine if multiple PAHs were being degraded by the same enzymes in these bacteria.

The two species of bacteria investigated in this report, *P. stutzeri* P-16 and *P. saccharophila* P-15, were isolated from a creosote-contaminated soil by their ability to grow on phenanthrene as a sole carbon and energy source (40, 41). Naphthalene and the methylnaphthalenes also served as growth substrates for both organisms, but fluorene and indan did not. As expected, these organisms were able to oxidize naphthalene and 1- and 2-methylnaphthalene in respirometric resting-cell assays (Table 1). In addition, P-15 was shown to oxidize fluorene in this assay, indicating that fluorene is cometabolized by this organism.

The degradation of naphthalene by phenanthrene degraders is not uncommon, but it has been shown that not all bacteria able to grow on phenanthrene will grow on naphthalene (2, 17, 23). The metabolism of methylnaphthalenes has been less well studied, but it appears that metabolism of methylnaphthalenes also varies among organisms. In one study, a *Pseudomonas* strain grown on naphthalene could grow on and transform 2-methylnaphthalene but not 1-methylnaphthalene (43); however, in another study, naphthalene and phenanthrene degraders grew on both 1-methylnaphthalene and 2-methylnaphthalene (33). Fluorene can serve as a primary growth substrate for some microorganisms (19, 26) and is cometabolized by others (5).

Although many studies have demonstrated that bacteria grown on one aromatic substrate can transform other aromatic substrates as well (2, 5, 16, 17, 22, 25, 26, 33, 34, 36, 43), it is usually not known whether the same enzyme system is involved in multiple-substrate transformation. It is evident, from physiological, biochemical, and genetic studies, that more than one PAH degradation pathway exists in different species (2, 6–8, 20); therefore, it is possible that individual bacteria able to degrade more than one aromatic substrate will have more than one pathway for their metabolism.

The results of this study support a hypothesis that enzymes involved in the transformation of one PAH can interact with other PAHs. Kinetic analyses were used to demonstrate competitive inhibition of phenanthrene degradation by naphtha-

lene, 1-methylnaphthalene, 2-methylnaphthalene, and fluorene. This finding suggests that multiple PAHs are being transformed by a common enzyme system in whole cells. Whether the competition is for oxidases, transport enzymes, or both is not clear from the available evidence.

It has been demonstrated previously that genes encoding naphthalene metabolism were linked to the metabolism of phenanthrene and anthracene (24, 35) and that *cis*-naphthalene dihydrodiol dehydrogenase oxidizes *cis*-dihydrodiols of several other polycyclic aromatic hydrocarbons (30, 31). A *Pseudomonas* strain has also been shown to metabolize naphthalene, phenanthrene, and dibenzothiophene by a common set of upper-pathway enzymes (11). To our knowledge, previous studies have not demonstrated that PAHs are actually competitive substrates for PAH-degrading bacteria. Only recently has the competitive metabolism of monoaromatic substrates been demonstrated (9).

Once competitive metabolism between naphthalene and phenanthrene was demonstrated, estimates of the inhibition coefficient (K_i) for naphthalene could be made from the fractional-velocity equation (equation 1) derived by Segel (37). Two measurements of K_i made for P-16, with different experimental designs, showed close agreement with each other but were higher than measurements of the K_s for naphthalene made by the oxygen uptake assay. The reason for this discrepancy is not clear; however, it is possible that phenanthrene uptake and naphthalene oxidation have different rate-limiting steps in this organism. In contrast, measurements of K_i and K_s for naphthalene are in close agreement for P-15.

Inhibition coefficients were also estimated for the other competitively metabolized PAHs (Table 4). In *P. stutzeri* P-16, the K_i for 2-methylnaphthalene was lower than the K_i for 1-methylnaphthalene and naphthalene. The K_i for fluorene was lower than the K_i for naphthalene in both organisms, suggesting that fluorene is a slightly more potent competitive inhibitor.

The finding that fluorene was a competitive substrate with phenanthrene for P-16 was somewhat unexpected, because fluorene oxidation by this organism was not detected by the oxygen uptake assay (Table 1). However, we demonstrated that fluorene was in fact oxidized by P-16 by measuring metabolites produced during growth in peptone supplemented with fluorene. The primary metabolites were identified as 9-hydroxyfluorene and 9-fluorenone by comparison with authentic standards in HPLC and mass spectrometry. 9-Hydroxyfluorene and 9-fluorenone have been identified as metabolites of bacterial fluorene metabolism in other studies (5, 19, 26). These results demonstrate that the oxygen uptake assay, although convenient and economical, is less sensitive than metabolite analysis and may give falsely negative results for compounds that are only partially oxidized in a metabolic pathway.

Results from our experiments, combined with recent molecular evidence, present an emerging view that enzymes produced by bacteria for the degradation of PAHs can have a broad substrate range. Furthermore, the occurrence of competitive metabolic processes in physiologically diverse organisms suggests that competitive metabolism may be a common phenomenon among PAH-degrading organisms. An understanding of the competitive mechanisms involved in bacterial PAH metabolism can contribute to the development of better approaches for modeling and controlling PAH biodegradation within complex mixtures, such as occur in contaminated soils.

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