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Salicylate Stimulates the Degradation of High-Molecular Weight Polycyclic Aromatic Hydrocarbons by *Pseudomonas saccharophila* P15

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Pseudomonas saccharophila P15 was isolated from soil contaminated with polycyclic aromatic hydrocarbons (PAH) and previously was reported to degrade a variety of low- and high-molecular weight PAH. Strain P15 grows on phenanthrene by a known pathway in which salicylate is an intermediate. Preincubation with phenanthrene and downstream intermediates through salicylate stimulated PAH dioxygenase activity and initial rates of phenanthrene removal, suggesting that salicylate is the inducer of these activities. Salicylate also greatly enhanced initial rates of removal of fluoranthene, pyrene, benz[*a*]anthracene, chrysene, and benzo[*a*]pyrene, high-molecular weight substrates that strain P15 does not use for growth. The specific rate of removal of benzo[*a*]pyrene was at least 2 orders of magnitude lower than that of the four-ring compounds and nearly 5 orders of magnitude lower than that of phenanthrene. The mineralization of phenanthrene, benz[*a*]anthracene, chrysene, and benzo[*a*]pyrene was stimulated by preincubation with phenanthrene or salicylate, although significant mineralization of phenanthrene, benz-[*a*]anthracene, and chrysene occurred in uninduced cultures. Further experiments with chrysene indicated that chrysene does not appear to induce its own mineralization. Our results suggest that *P. saccharophila* P15 expresses a low level of constitutive PAH metabolism which is inducible to much higher levels and that high-molecular weight PAH metabolism by this organism is induced by the low-molecular weight substrate phenanthrene and by salicylate.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are common pollutants in contaminated soils and usually occur as a complex mixture of low- and high-molecular weight (HMW) compounds. These compounds are of concern due to their acute toxicity, mutagenicity, or carcinogenicity. Prior laboratory and larger scale work on biodegradation of PAH has indicated that the removal of compounds with four or more rings (defined here as high-molecular weight PAH) is often less extensive than the removal of lower molecular weight compounds (*1*, *2*).

One of the strategies proposed to enhance the degradation of specific PAH is to offer bacteria one or more known inducers to stimulate both selective growth of PAH degraders and induction of PAH metabolism (*3*−*8*). However, little work has been done on the regulation of PAH metabolism by bacteria for compounds other than naphthalene. The transformation of benz[*a*]anthracene was reported to be inducible by salicylate (*9*) in a strain that has recently been identified as *Sphingomonas yanoikuyae* (*10*), but little else is known about the regulation of metabolism for HMW PAH.

Many bacteria with PAH-transforming capabilities have a relatively broad substrate range (*11*−*17*), and preexposure of an individual bacterium or a microbial community to one PAH can result in enhanced degradation of other PAH (18, 19). Such observations suggest that these organisms might possess one or more broad-specificity enzymes for PAH metabolism. Naphthalene dioxygenase, the enzyme responsible for the initial oxidation of naphthalene, has a relaxed substrate specificity that permits the *cis*-dihydroxylation of several aromatic compounds (*20*, *21*) and consequently has been referred to as PAH dioxygenase (*22*). Molecular evidence also indicates that an individual bacterium may transform multiple PAH through a common upper pathway in several bacterial strains (*23*−*27*).

If PAH-degrading microorganisms use broad-specificity enzymes or common pathways to transform multiple PAH, then inducers for the metabolism of one PAH substrate might coinduce the transformation of a range of PAH. Preliminary evidence indicated that the transformation of naphthalene, phenanthrene, fluoranthene, and pyrene by *P. saccharophila* P15 was stimulated by salicylate (*8*), a known inducer of naphthalene metabolism in pseudomonads (*28*). We now report in more detail the inducing effects of salicylate on the transformation of various HMW PAH by *P. saccharophila* P15, including initial rates of transformation and the mineralization of benz[*a*]anthracene, chrysene, and benzo-[*a*]pyrene.

Materials and Methods

Chemicals. Sodium salicylate was purchased from Mallinckrodt Chemicals (Paris, KY). Naphthalene was obtained from Baxter Chemicals (Phillipsburg, NJ). Phenanthrene, 1-hydroxy-2-naphthoic acid (1H2N), indole, indigo, chloramphenicol, [9-^{14}C]phenanthrene (8.3 mCi/mmol), [3-^{14}C]-fluoranthene (45 mCi/mmol), [4,5,9,10-^{14}C]pyrene (32.3 mCi/mmol), and [7-^{14}C]benzo[*a*]pyrene (26.6 mCi/mmol) were purchased from Sigma Chemicals (Milwaukee, WI). [5,6,11,12-^{14}C]Chrysene (47.6 mCi/mmol) and [5,6-^{14}C]benz[*a*]anthracene (54.6 mCi/mmol) were obtained from Chemsyn Science Laboratories (Lenexa, KS). Catechol, salicylaldehyde, protocatechuate, phthalate, 2-carboxybenzaldehyde, pyrene, fluoranthene, chrysene, and benzo[*a*]pyrene were purchased from Aldrich Chemicals (Milwaukee, WI). The purity of all PAH, both labeled and unlabeled, was reported by the vendors to be greater than 98%. All other chemicals were ACS reagent grade or purer. For purposes of introducing unlabeled or labeled PAH into incubation vessels, concentrated stock solutions were prepared in methanol.

Organism and Growth Conditions. *P. saccharophila* P15 was isolated from a creosote-contaminated soil by enrichment on phenanthrene as the sole carbon source (*19*). This organism grows on naphthalene, methylnaphthalenes and phenanthrene, while it metabolizes but does not grow on fluorene, acenaphthene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, and benzo[*a*]pyrene (*17*, *19*, *29*).

Cultures of *P. saccharophila* P15 were preserved in cryostat vials with 10% DMSO at −70 °C. To recover a frozen culture,

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a sterilized toothpick was stabbed into the cryostat vial and inoculated into 5 mL of LB broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter of reagent water). Inocula for all experiments were prepared by diluting the recovered cultures into desired media (1:100). Unless noted otherwise, cultures were grown in M9 salt-based mineral medium (*30*) supplemented with either solid phenanthrene, salicylate (3.6 mM), or succinate (4.3 mM).

Enzyme Assays. Cell-free extracts were prepared by harvesting cultures at midexponential phase and rapidly washing with 0.1 M NaCl. The cell paste was resuspended in 50 mM Tris-HCl buffer, pH 7.8, containing 10% (v/v) glycerol, 10% (v/v) ethanol, and 0.5 mM dithiothreitol. A 2 mL cell suspension was subjected to disruption with an ultrasonic oscillator (Lab-Line Instruments, Melrose Park, IL) at 50 W in an ice bath for six 30-s periods. Each period of disruption was followed by 1 min during which the disrupted cells and oscillator probe were cooled in ice water. Particulate matter was removed from the extract by centrifugation at 12 000 g for 1 h at 4 °C. Protein concentrations were determined by the Bradford method (Bio-Rad protein assay kit; Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard. Published spectrophotometric procedures were used to assay 1H2N-degrading enzyme (*31*), protocatechuate 2,3-dioxygenase (*32*), protocatechuate 3,4-dioxygenase (*33*), catechol 1,2-dioxygenase (*34*), and catechol 2,3-dioxygenase (*35*).

PAH dioxygenase activity and the initial rate of phenanthrene removal were measured with whole cells grown on succinate, which were then transferred to M9 medium containing 0.43 mM succinate and 5 μM of a potential inducer of the respective activity. Succinate was added as a primary energy source to all samples and controls in these assays to exclude the possibility that low activity in uninduced controls might be caused by endogenous energy losses over the incubation period. Although succinate has been observed to be a catabolite repressor at high concentrations in some pseudomonads (*36*), at a concentration of 0.43 mM succinate exerted neither positive nor negative effects on initial rates of phenanthrene removal when phenanthrene, salicylate, or catechol was used as a potential inducer. A concentration of 5 μM was chosen for potential inducers because this is slightly lower than the aqueous solubility of phenanthrene, so that all of the potential inducers could be compared in soluble form at the same concentration. After 3 h of incubation with a potential inducer the cells were washed and resuspended in M9 medium. For PAH dioxygenase activity, the M9 medium was supplemented with 4.3 mM succinate and 2 mM indole. The cultures were placed on a rotary shaker at 25 ± 0.5 °C for 3 h and then extracted twice with an equal volume of ethyl acetate. The extracts were combined, and the indigo concentration determined spectrophotometrically (600 nm) by comparison to a standard curve for reagent-grade indigo dissolved in ethyl acetate. The initial rate of phenanthrene removal by washed cells was measured spectrophotometrically as described previously (*19*).

Salicylate as an Intermediate of Phenanthrene Metabolism. Cells grown on solid phenanthrene were washed and resuspended in M9 medium containing solid phenanthrene at a concentration equivalent to 500 μM. The cultures were shaken at 25 ± 0.5 °C in the dark for 21 h, then the reaction was terminated with 0.5% H_3PO_4, and the samples supplemented with an equal volume of methanol. Salicylate was identified in these samples by high-pressure liquid chromatography (HPLC) analysis with a Waters 600E system and Model 996 photodiode array detector, based on identical retention time and absorbance spectrum (254−450 nm) as that of a salicylate standard.

Initial Rates of High-Molecular Weight PAH Removal. Cultures grown in LB medium to midexponential phase were supplemented with 0.25 mM salicylate. After 3 h of incubation, cells were harvested, washed, and resuspended in M9 medium ($OD_{420} \sim 0.03$ for phenanthrene, fluoranthene, benz[*a*]anthracene, or chrysene and ~0.3 for pyrene or benzo[*a*]pyrene; different initial cell concentrations were required because of the very wide range of PAH removal rates observed). PAH removal was initiated by injecting a methanol solution containing the substrate of interest into these washed culture suspensions. The final concentration of each compound was approximately 80% of the reported aqueous solubility (*37*) (1 μM for fluoranthene, 0.6 μM for pyrene, 20 nM for benz[*a*]anthracene, 8.8 nM for chrysene, and 10 nM for benzo[*a*]pyrene). Uninduced controls were not preincubated with salicylate, and killed controls were acidified with 0.5% H_3PO_4 to pH 2.0. All vials were then shaken at 25 ± 0.5 °C in the dark. The reaction was stopped with 0.5% H_3PO_4 at selected time intervals within the first 30 min for phenanthrene, fluoranthene, benz[*a*]anthracene, or chrysene and within 3 h for pyrene or benzo[*a*]pyrene. The samples were then supplemented with an equal volume of acetonitrile to desorb any sorbed PAH. The initial linear decrease in concentration was used to calculate the initial specific removal rate, except in those cases in which the decrease in concentration beyond the first sampling event was not linear; in these cases the initial rate was estimated as the difference in concentration at the first sampling event divided by the incubation time. Samples were analyzed by HPLC with fluorescence detection as described previously (*17*).

Mineralization of ^{14}C-Labeled PAH. Each PAH was added individually to 10 mL M9 medium in 20 mL glass vials at a concentration approximately 80% of its reported aqueous solubility. Chrysene and benzo[*a*]pyrene were added entirely in radiolabeled form, whereas the remaining compounds were added as mixtures of radiolabeled and unlabeled compounds. The net specific radioactivity added was 0.2 mCi/mmol for phenanthrene, 1.0 mCi/mmol for fluoranthene, 1.3 mCi/mmol for pyrene, 13.6 mCi/mmol for benz[*a*]anthracene, 47.6 mCi/mmol for chrysene, and 26.6 mCi/mmol for benzo[*a*]pyrene. A small glass tube containing a piece of Whatman no. 1 filter paper soaked with 100 μL of 2 N KOH solution was placed in each vial as a CO_2 trap.

The vials were inoculated to an $OD_{420} \sim 0.05$ with cells that were preincubated with a potential inducer and washed as described above for the PAH dioxygenase and phenanthrene removal rate assays. Each vial was then covered by a piece of aluminum foil, a Teflon septum, and a screw cap. Uninoculated and killed controls were prepared as described above. Vials were shaken at 25 ± 0.5 °C in the dark for a period determined from preliminary experiments to be sufficient to observe substantial differences in mineralization between induced and uninduced cells for a given target compound. The solution in each vial was then acidified by injecting 250 μL of 20% H_3PO_4, the vials were shaken for an additional 24 h, and the radioactivity trapped on the filter paper traps was counted as described previously (*17*). In general, the recovery of radiolabel in the $^{14}CO_2$ traps was low in uninoculated and killed controls for all compounds. A small fraction of the added ^{14}C was recovered in $^{14}CO_2$ traps in controls for phenanthrene, which was probably due to volatilization of this compound over the incubation period.

A separate experiment was conducted to determine whether the mineralization of chrysene observed in uninduced control cultures was induced by chrysene itself. Cells were prepared as described above for the mineralization experiment and then were incubated with 5 μM phenanthrene, salicylate, or chrysene. After 3 h the cultures were washed, resuspended in M9 medium, and inoculated into vials containing ^{14}C-chrysene amended with 10 μg/mL chloramphenicol to inhibit further protein synthesis. Mineralization of chrysene was determined as described above.

TABLE 1. Effects of Potential Inducers on the Initial Rate of Phenanthrene Removal in *P. saccharophila* P15

preincubation substrate[a]	SPRR[b] (μmol h^{-1} [mg protein]$^{-1}$)
naphthalene	0.78 ± 0.06
phenanthrene	1.92 ± 0.24
1-hydroxy-2-naphthoate	1.56 ± 0.30
salicylaldehyde	1.26 ± 0.24
salicylate	1.86 ± 0.18
catechol	<0.19
none	<0.19

[a] Cells grown on succinate were preincubated with 0.43 mM succinate and a potential inducer at a concentration of 5 μM. [b] SPRR, specific phenanthrene removal rate. Data represent means and standard deviations of triplicates.

TABLE 2. Initial Specific Rate of High-Molecular Weight PAH Removal by *P. saccharophila* P15 Preincubated with or without Salicylate

PAH (initial concn, nM)	initial removal rate (nmol h^{-1} [mg protein]$^{-1}$)[a] salicylate-induced	uninduced	killed control
fluoranthene (1000)	419 ± 14	<0.75	<0.75
pyrene (600)	18.4 ± 0.04[b]	<0.01	<0.01
benz[*a*]anthrancene (20)	15.5 ± 0.23	<0.15	<0.15
chrysene (8.8)	11.5 ± 1.62[b]	<0.07	<0.07
benzo[*a*]pyrene (10)	0.043 ± 0.001	<0.001	<0.001

[a] Data represent means and standard deviations of triplicates. [b] Initial rate was determined as the change in concentration at the first sample time divided by the time interval.

Results

Role of Salicylate in Phenanthrene Metabolism. Aerobic bacteria are known to oxidize phenanthrene to 1-hydroxy-2-naphthoate (1H2N), from which two divergent pathways have been observed in different bacteria (*38*). One pathway proceeds through salicylate and catechol, while the other proceeds through phthalate and protocatechuate. The inducer of phenanthrene metabolism has not been identified for either pathway in any organism.

Previous respirometric evidence indicated that *P. saccharophila* P15 oxidizes intermediates of the salicylate pathway for phenanthrene metabolism and is unable to oxidize intermediates of the phthalate pathway downstream of 1H2N (*19*). Use of the salicylate pathway by this organism was confirmed by identifying salicylate (~70 μM) in cultures incubated with solid phenanthrene at an initial concentration equivalent to 500 μM. Furthermore, catechol 2,3-dioxygenase was present in cultures grown on phenanthrene but not in cultures grown on succinate. There were no detectable activities of 1H2N-degrading enzyme (which converts 1H2N to its ring-cleavage product in the phthalate pathway) or protocatechuate dioxygenases in cells grown on phenanthrene.

Preincubation of *P. saccharophila* P15 with naphthalene, phenanthrene, 1H2N, salicylaldehyde, or salicylate greatly increased the initial rate of phenanthrene removal (Table 1). No significant phenanthrene removal occurred in samples preincubated with catechol or in controls that were not preincubated with a potential inducer. The effects of potential inducing substrates on PAH dioxygenase activity in strain P15 were similar to the effects on initial phenanthrene removal rate (not shown). Preincubation with salicylate at concentrations above 0.3 mM led to decreased expression of PAH dioxygenase activity and increased inhibition of phenanthrene removal (not shown).

High-Molecular Weight PAH Removal. Salicylate was tested for its ability to induce the removal of several HMW PAH which *P. saccharophila* P15 could not use for growth. Preincubation of *P. saccharophila* P15 with salicylate greatly increased initial rates of removal of all the PAH tested (Table 2). No significant PAH removal was observed over the assay period in uninduced or killed controls. Although the removal of benzo[*a*]pyrene was very slow relative to the other PAH, results from a parallel experiment indicated that benzo[*a*]-pyrene was removed to below detectable levels in salicylate-induced cultures after 72 h of incubation, whereas about 100% was recovered in uninduced and killed controls. The initial specific rate of benzo[*a*]pyrene removal was nearly 5 orders of magnitude lower than that of phenanthrene (as reported in Table 1).

Mineralization of PAH. Salicylate and phenanthrene were evaluated for their ability to enhance the mineralization of phenanthrene and the HMW PAH over longer incubation periods than used in the initial rate experiment described above. The $^{14}CO_2$ generated from ^{14}C-labeled phenanthrene, benz[*a*]anthracene, chrysene, and benzo[*a*]pyrene by cultures preincubated with salicylate or phenanthrene was substantially greater than in uninduced controls (Table 3). However, significant mineralization of ^{14}C-labeled phenanthrene, benz-[*a*]anthracene, and chrysene was also observed in uninduced controls in comparison to uninoculated and killed controls (*t*-test, $p \leq 0.05$). Fluoranthene and pyrene were not mineralized by strain P15 under any conditions, although pyrene transformation by this organism has been shown to be accompanied by the accumulation of at least one major metabolite (*17*).

A followup experiment was conducted to test the effects of preincubation with chrysene on its own mineralization. Chloramphenicol (10 μg/mL) was added to the vials containing cells preincubated with potential inducers and to uninduced controls to preclude further protein synthesis once the cells were incubated with ^{14}C-chrysene. At this concentration, chloramphenicol did not inhibit phenanthrene removal in initial rate assays but completely blocked induction of phenanthrene removal by salicylate (not shown). In the presence of chloramphenicol, the mineralization of chrysene in phenanthrene- or salicylate-induced cultures and in uninduced cultures (Table 4) was similar to levels found in incubations without chloramphenicol (Table 3). Mineralization in cultures preincubated with chrysene was significantly lower than in phenanthrene- or salicylate-induced cultures and not greater than in uninduced cultures. Preincubation with chrysene also did not stimulate PAH dioxygenase activity or initial rates of phenanthrene removal in strain P15 (not shown).

Discussion

Although naphthalene metabolism in pseudomonads has been known for a long time to be induced by salicylate (*28*), almost nothing is known about induction of the metabolism of other PAH. We began by investigating the induction of phenanthrene metabolism in *P. saccharophila* P15, since phenanthrene is a growth substrate for this organism, and then investigated whether the induction of phenanthrene degradation would coinduce the degradation of HMW PAH. We had already determined that strain P15 could degrade a broad range of PAH substrates it could not use for growth after it was grown on phenanthrene (*17*).

Since *P. saccharophila* P15 uses the salicylate pathway for phenanthrene metabolism, we questioned whether salicylate might be the inducer of this pathway by analogy to naphthalene metabolism. Intermediates of phenanthrene metabolism upstream of catechol stimulated PAH dioxygenase activity and initial rates of phenanthrene removal by *P. saccharophila* P15 (Table 1), suggesting that salicylate is the

TABLE 3. Mineralization of PAH by *P. saccharophila* P15 Preincubated with Phenanthrene, Salicylate, or Neither Compound

PAH (initial concentrated, nM)	$^{14}CO_2$ recovery[a] (%)				
	phenanthrene-induced	salicylate-induced	uninduced	killed control	uninoculated control
phenanthrene (5000)	48.3 ± 0.5	46.6 ± 1.2	8.7 ± 3.0	4.2 ± 0.3	5.0 ± 0.3
benz[*a*]anthracene (20)	36.2 ± 3.5	41.3 ± 1.5	10.4 ± 2.5	0.9 ± 0.4	1.3 ± 0.7
chrysene (8.8)	45.3 ± 2.8	46.7 ± 0.7	14.6 ± 4.9	0.9 ± 0.6	1.5 ± 0.5
benzo[*a*]pyrene (10)	30.0 ±1.6	19.8 ± 2.6	0.9 ± 0.3	0.6 ± 0.2	0.6 ± 0.1

[a] $^{14}CO_2$ recovery was determined after incubation for 5, 24, 24, or 48 h with phenanthrene, benz[*a*]anthracene, chrysene, or benzo[*a*]pyrene, respectively. Total ^{14}C recovery in samples and controls was 84−95% for phenanthrene, 83−95% for benz[*a*]anthracene, 85−100% for chrysene, and 93−102% for benzo[*a*]pyrene. Data represent means and standard deviations of triplicates.

TABLE 4. Mineralization of Chrysene (8.8 nM) in the Presence of Chloramphenicol by *P. saccharophila* P15 Preincubated with Potential Inducers

preincubation substrate	$^{14}CO_2$ recovery[a] (%)
phenanthrene	42.0 ± 2.6
salicylate	47.1 ± 1.4
chrysene	9.8 ± 0.9
none	15.0 ± 1.1
killed control	0.9 ± 0.3

[a] $^{14}CO_2$ recovery was determined after incubation of strain P15 with [5,6,11,12-^{14}C]chrysene for 24 h. Total ^{14}C recovery in samples and controls after 24 h incubation was 81−95%. Data represent means and standard deviations of triplicates.

natural inducer for at least the initial steps of phenanthrene metabolism. The mineralization of phenanthrene was greatly enhanced when strain P15 was preincubated with salicylate compared to uninduced controls (Table 3), and salicylate-grown cultures expressed both catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities (not shown), suggesting that salicylate induces the entire phenanthrene degradation pathway in this organism. It has been suggested that naphthalene induces its own metabolism by synthesis of salicylate through a low-level constitutive expression of the catabolic enzymes, and it is possible that phenanthrene removal is induced through a similar mechanism in strain P15; a small but significant extent of phenanthrene mineralization was observed in uninduced controls over a 5-h incubation period (Table 3).

Salicylate also stimulated the initial removal rate (Table 2) and, in some cases, the mineralization (Table 3) of HMW PAH that are not growth substrates for *P. saccharophila* P15. This finding suggests that there may be a link between low- and high-molecular weight PAH degradation in this organism, at least at the level of regulation of enzyme synthesis and perhaps at the level of enzyme activity. We have observed that several low-molecular weight PAH are metabolized competitively in this organism, suggesting that there is some level at which common enzymes are used in the degradation of these compounds (*29*). However, there is not yet enough information on *P. saccharophila* P15 to know the extent to which the enzymes of the phenanthrene degradation pathway exhibit activity toward other PAH and their metabolites or more generally whether a common suite of enzymes is involved in the metabolism of multiple PAH. In some organisms, broad substrate specificity applies to the upper pathway only, whereas lower pathways diverge for different PAH (*23*, *25*).

P. saccharophila P15 mineralized benz[*a*]anthracene and chrysene under uninduced conditions, although the extent of mineralization over the incubation period was significantly lower than in cultures induced with phenanthrene or salicylate (Table 3). Preincubation of cultures with chrysene did not enhance chrysene mineralization, and significant chrysene mineralization was observed in uninduced cultures even in the presence of chloramphenicol (Table 4), indicating that chrysene does not induce its own mineralization; it also does not induce PAH dioxygenase activity or phenanthrene removal. Strain P15 therefore appears to express low-level constitutive metabolism of chrysene and benz[*a*]anthracene. The fact that benzo[*a*]pyrene was not mineralized in uninduced controls (Table 3) was likely a result of its slow rate of degradation relative to the other compounds. The initial rate of benzo[*a*]pyrene removal was more than 2 orders of magnitude lower than that of chrysene (Table 2), which has similar aqueous solubility, and the other four-ring compounds.

The biodegradation of PAH in contaminated soils and sediments is often thought to be limited by bioavailability (*39*). However, Cornelissen et al. (*40*) recently reported that the biodegradation of HMW PAH in a contaminated sediment was limited by biological factors, not by bioavailability. It was not possible to elucidate from their data which factor(s) may have limited biodegradation. Clearly, however, biodegradation requires the presence of the appropriate enzymes at significant levels, and the inducibility of PAH degradation by different bacteria appears to be the rule rather than the exception (*17*). Induction of enzyme synthesis may be particularly important for the degradation of compounds that do not serve as growth substrates for the degrading microorganisms and which do not induce their own degradation.

Although we do not know the extent to which the phenomena we have observed for *P. saccharophila* P15 are applicable to other PAH-degrading bacteria, salicylate has been used successfully to sustain or select for naphthalene- or salicylate-degrading organisms in soil microbial communities (*4*, *7*, *41*). Since salicylate is water soluble, its addition to PAH-contaminated systems is a potentially attractive option for stimulating or sustaining the biodegradation of HMW PAH once the low molecular weight growth substrates are depleted. Other substrates that can induce alternative pathways of PAH degradation might serve a similar role. However, both the cost and potential effects of adding carbon sources to soil systems must be considered. For example, salicylate exerted concentration-dependent effects on soil microbial communities (*7*), and we found that the expression of PAH dioxygenase activity and the initial rate of phenanthrene removal were inhibited by salicylate at concentrations higher than 0.3 mM. Additional carbon sources can increase the oxygen demand in soil and thus may influence aerobic degradation processes, and the long-term introduction of inducing substrates to a microbial community might also result in selective growth of heterotrophic microorganisms other than PAH degraders. Such potential effects remain to be examined.

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