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Effect of Root-Derived Substrates on the Expression of *nah-lux* Genes in *Pseudomonas fluorescens* HK44: Implications for PAH Biodegradation in the Rhizosphere

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The bioluminescent reporter strain *Pseudomonas fluorescens* HK44 with a *nah-lux* fusion, was used to investigate the effect of root material (from hybrid poplars, willow, kou, milo, Osage orange, mulberry, and switch grass) and potential root-derived substrates (e.g., sugars, carboxylic acids, amino acids, and phenolics) on the expression of *nahG*, one of the genes responsible for naphthalene dioxygenase transcription. Whereas *nahG* was induced by some phenolic substrates that could be released by plants (i.e., salicylate, methyl salicylate, and acetyl salicylate), no induction by root extracts was observed. Rather, increasing root extract concentrations (50 to 275 mg L^{-1} as total organic carbon) inhibited *nahG* expression in assays with cells concurrently exposed to naphthalene. Root extracts also decreased *nahG* expression at the individual cell level during naphthalene degradation assays. However, treatments with root extracts exhibited significantly higher microbial growth and overall bioluminescence, indicating a higher level of *nahG* expression by the resulting larger microbial population. This generally resulted in faster naphthalene degradation rates, suggesting that plant-promoted proliferation of competent genotypes could compensate for the interference that labile substrates exert on the expression of genes that code for the degradation of polynuclear aromatic hydrocarbons (PAHs). This could explain the faster PAH degradation commonly reported in planted than in unplanted soils.

Introduction

Phytoremediation appears to have great potential for the treatment of soils contaminated with residual levels of polynuclear aromatic hydrocarbons (PAHs) (*1*−*7*), particularly for areas with shallow (i.e., root-accessible) contamination. Research examining the fate of PAHs in planted soil has shown that PAH removal is most likely due to enhanced microbial degradation in the rhizosphere (*5*−*9*). PAH biodegradation is often attributed to cooxidation processes that depend on the presence of lower molecular weight aromatic compounds that trigger enzyme induction (*10, 11*). Nevertheless, microbial−plant interactions that influence the expression of microbial genes responsible for PAH catabolism have not been thoroughly investigated, and there is a need for improved understanding of the mechanisms that enhance PAH biodegradation in the rhizosphere.

A major driving force for the rhizosphere effect appears to be the continuous throughput of plant-derived substrates in the form of root exudates and (mainly) root turnover (*12*). Rhizodeposition, which includes exudation of soluble plant products plus root turnover from sloughing and cell death, accounts for 7−27% of the total plant mass annually (*13*). The amount of organic carbon released varies between plants, but is generally estimated to be between 10 and 100 mg of C (g of root material)$^{-1}$ (*14*) and includes many organic molecules that stimulate microbial growth (Table 1). This higher availability of organic matter in the rhizosphere has been reported to increase the size of the heterotrophic microbial community (4- to 100-fold) (*5*−*7, 9*) and the concentration of PAH degrader populations (4.5- to 15-fold) (*5*−*7*) compared to bulk soils. Furthermore, some phenolic compounds released by plants have been postulated to enhance microbial degradation of polychlorinated biphenyls (PCBs), by serving as growth substrates for PCB-degrading bacteria (e.g., naringin and cathechin) (*12, 20*) and/or as potential inducers of microbial oxygenase enzymes that initiate PCB degradation (e.g., flavonoids, *l*-carvone, and *p*-cymene) (*17*). Therefore, it is plausible that plants that release phenolics could also enhance PAH bioremediation in the rhizosphere by sustaining the induction of similar oxygenase enzymes that initiate aerobic PAH degradation and by serving as primary substrates for PAH cooxidation as reported for trichloroethylene (*21*). However, the concurrent release of easily degradable compounds at a much greater concentration than the phenolics could offset stimulating effects by repressing the expression of pertinent catabolic genes.

In general, root material is composed of sugars (15−65% of total organic carbon, TOC), organic acids (9−33% of TOC), amino acids (2−31% of TOC) (*6, 22, 23*), and phenolics (0.3−4 mg of C (g of root material)$^{-1}$) (*24, 25*). Among the most common components of root turnover, sugars such as glucose and organic acids such as acetate and succinate are known to inhibit the degradation of aromatic compounds such as benzene (*26*), catechol (*26*), and toluene (*27*) by *Pseudomonas* sp. by affecting global regulatory controls.

Evaluating the net effect of microbial exposure to root-derived substrates on catabolic gene expression is of critical importance in understanding the mechanisms that enhance PAH bioremediation in the rhizosphere. However, there are no reports in the literature addressing the role of plant-derived phenolics or other substrates as inducers of PAH catabolic genes. This paper is the first to describe the effect of root-derived material on the expression of a PAH catabolic gene in a common soil bacterium. Specifically, the expression of *nahG*, one of the genes responsible for naphthalene dioxygenase transcription, was studied in *Pseudomonas fluorescens (P. flourescens)* HK44 exposed to different plant-derived substrates. Root extracts from various plants (which represent root turnover in the rhizosphere) and numerous substrates were screened to identify potential inducers and repressors and to quantify their overall effect on *nahG* expression and naphthalene biodegradation. Tropical plants were included in this study because climatic conditions and economic constraints often make phytoremediation a particularly attractive alternative for many tropical regions of the world.

Methodology

***Pseudomonas fluorescens* HK44**. The reporter strain *Pseudomonas fluorescens* HK44 (*28*), which has a *nah-lux* fusion

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TABLE 1. Compounds Detected in Root Exudates

compounds	example of compound	ref
carbohydrates	glucose, fructose, sucrose, maltose, galactose, xylose, oligosaccharides	*13, 14*
amino acids	glycine, glutamic acid, aspargine, serine, alanine, lysine, arginine, threonine, homoserine	*13, 14*
aromatics	phenols, *l*-carvone, *p*-cymene, limonene, isoprene, salicylate, flavones, xanthones, naphthaquinones	*11, 15*
organic acids	acetic acid, propionic acid, citric acid, butyric acid, valeric acid, malic acid, lactic acid	*14*
volatile compounds	ethanol, methanol, formaldehyde, acetone, acetaldehyde, propionaldehyde, methyl sulfide, propyl sulfide, allyl sulfide	*14*
vitamins	thiamine, biotin, niacin, riboflavin, pyridoxine, pantothenic acid	*14*
enzymes	phosphatase, dehydrogenase, peroxidase, dehalogenase, nitroreductase, laccase, nitrilase	*14, 16, 17*

(courtesy: Gary Sayler, University of Tennessee at Knoxville, TN) was used to identify potential inducers and repressors in root extracts and their overall effect on the expression of a PAH catabolic gene. Naphthalene dioxygenase was selected as a model PAH-degrading enzyme system, not only because it is widely spread among naphthalene-degrading soil bacteria but also because its relaxed substrate specificity allows cooxidation of numerous aromatic hydrocarbons such as phenanthrene, anthracene, biphenyl, toluene, and fluorene (*29*). Moreover, *P. fluorescens* is capable of thriving in rhizosphere soils (*30*). Therefore, HK44 is a good model to study the potential response of bacteria in rhizosphere systems.

Plasmid pUTK21 in HK44 was constructed with genes coding for naphthalene degradation homologous to the archetypical naphthalene catabolic plasmid, NAH7. The upper pathway encodes for transformation of naphthalene to salicylate and is positively regulated by a LysR type protein, NahR, which also regulates the lower pathway. The *lux* transposon Tn4431 was inserted into the lower pathway, which encodes for salicylate degradation. Despite this, HK44 is a Nah^+Sal^+ phenotype since it is able to degrade salicylate constitutively. This helped alleviate any potential toxicity problems that could have occurred due to accumulation of salicylate during naphthalene degradation.

Physiological limitations of HK44 to quantify induction have been reported earlier (*31*). Oxygen and aldehyde availability can affect the bioluminescence emitted by HK44. However, adequate air supply and the short duration of our assays preclude potential oxygen limitation. Also, aldehyde was not limiting since addition of decanal prior to sample measurement did not enhance bioluminescence readings. Therefore, the assays described herein were appropriate to discern relative effects of potentially cooccurring substrates on *nahG* induction.

Plant Selection. Plants were selected for their resistance to organic pollutants and their ability to pump relatively large amounts of soil pore water, thus facilitating soil aeration. Milo (*32*) and switch grass (*1*) were selected for their apparent ability to enhance total petroleum hydrocarbon (TPH) and PAH removal from contaminated soils. Some plants such as Osage orange and mulberry from the *Moraceae* family (*33*) were selected because of their ability to release phenolics that could induce PAH-degrading enzymes. Willow (*Salicaceae* family) was included since it is known to produce large quantities of salicylates in the bark (*34*). Poplars were included because of their widespread usage as phytoremediation tools and their wide range of adaptation, high growth rate, and ease of establishment. Further, it has been shown that poplar rhizospheres might enhance aromatic hydrocarbon degradation by fostering a larger population of specific degraders (along with the total heterotrophic population) without exerting any selective pressure (*35*).

None of these plant root extracts have been previously investigated for their potential to enhance PAH bioremediation in the rhizosphere. The plants used in this study were between 6 months and 1 year old, which is representative of plants introduced to contaminated sites undergoing phytoremediation. Kou (*Cordia subcordata*) and milo plants (*Thespesia populanea*) were obtained from Future Forests Nursery (Holualoa, HI). Osage orange (*Maclura pomifera*) was obtained from Green Plant Market, Inc. (Senoia, GA). Mulberry (*Morus rubra*) and hybrid willow (*Salix alba* × *matsudana*) were obtained from Forest Farm (Williams, OR), and Hramor Nursery (Maistee, MI), respectively. Switch grass (*Panicum virgatum*) and hybrid poplars (*Populus nigra* × *deltoides*) were grown under laboratory conditions for 3 months.

Collection and Characterization of Root Extracts. Plant roots were collected during the spring season. To be representative of root turnover in the rhizosphere, mostly dark colored fine roots (<1 mm) were used as the source of root extracts (*12*). Once collected, plant roots were homogenized in distilled water using a pestle and mortar and a hand homogenizer (Biospec Products Inc, Bartlesville, OK), centrifuged at 13 000 rpm and filter-sterilized (0.22 μm). Prior to storage at 4 °C, basic characterization, including measuring TOC (milligrams of C per gram of FW (fresh weight) root material) and determining yield coefficient (grams of cells per gram of TOC fed) (Y), pH, and phenolic and glucose contents, were carried out. All root extract samples were stored at the working concentration of 50 mg of C L^{-1} which is representative of TOC values published for oat seedling root exudates (*6*, personal communication).

Induction Assays. Two types of assays were used to investigate the potential of root extracts and various substrates to induce and/or repress the nah system. To identify inducers, resting HK44 cells (initially grown on YEPSS medium (*28*), transferred for 1 h to Hutner's mineral medium (*36*), and then washed with phosphate buffer) were resuspended in 0.1 mM phosphate buffer (pH 6.9) and exposed to 50 mg of C L^{-1} of a given test substrate. Although the YEPSS medium contains salicylate which can induce cells, washing and resting the cells after growth resulted in a relatively low induction status at the beginning of the induction assay.

Tested substrates were considered as potential inducers if the resulting bioluminescence was significantly greater (at the 95% confidence level) than that of controls without the substrate. To identify substrates that repress *nah*G induction, resting HK44 cells were concurrently exposed to the test substrate (40 mg of C L^{-1}) and an inducer, naphthalene (2.7 mg L^{-1}). Substrates that significantly reduced bioluminescence in the presence of naphthalene relative to controls with naphthalene alone ($p \leq 0.05$) were considered to be repressors. This experiment also served to verify results from the previous assay since concurrent exposure to an inducer and naphthalene leads to greater bioluminescence than exposure to naphthalene or the test substrate alone.

The experiment was started when 2 mL of the bacterial suspension was added to the vials containing the test substrates. Measurement of bioluminescence was made between 60 and 75 min after inoculation when HK44 typically reached a peak in bioluminescence. The intensity of light production or bioluminescence (relative luminescence units, RLU) was normalized to the biomass concentration to

calculate the specific bioluminescence (RLU mg^{-1} L) as a measure of specific levels of enzyme induction. Biomass concentration (W, mg L^{-1}) was calculated by the following correlation to optical density, measured at 600 nm (OD_{600}) (*37*):

$$W = 364.74OD_{600} + 6.7OD_{600}^{2}$$

The results were then presented as relative specific bioluminescence, which is the specific bioluminescence emitted in a treatment reactor relative to the specific bioluminescence emitted by the positive control that was exposed to naphthalene only.

No significant growth occurred during induction assays in phosphate buffer due to the absence of nutrients such as a nitrogen source or growth factors; thus, any effect due to growth and metabolism that could potentially confound *nahG* expression results was avoided.

Naphthalene Degradation Assays. The effect of root extracts on naphthalene degradation by HK44 was studied in 100-mL batch reactors containing 30 mL of mineral medium, 20−30 mg of TOC L^{-1} of different root extracts and 3−4 mg of naphthalene L^{-1}. Naphthalene biodegradation patterns were characterized by determining lag periods and subsequent biodegradation rates. The lag period, which reflects the acclimation phase, was determined as the time during which naphthalene concentrations remained constant or did not significantly decrease relative to the sterile controls. The degradation rate coefficient (r_d) was determined by linear regression of concentration (C) versus time (t) data obtained after the lag period. Whether the degradation rates in the presence of root extracts were significantly different from the control was determined by the student t-test at the 95% significance level (*38*).

Analytical Methods. TOC was measured using a DR/2500 spectrophotometer (Hach Co., Loveland, CO). The pH was measured using a Beckman Φ45 pH meter. Cell growth in root extracts (100 mg of TOC L^{-1}) was determined using a Genesys 5 spectrophotometer (Thermo Spectronic, Rochester, NY) at 600 nm. Bioluminescence (RLU) was measured using a TD-20e luminometer (Turner Designs, Sunnyvale, CA). Naphthalene concentration was measured using HPLC with a fluorescence detector (Agilent 1100) using the following protocol: solvent, 85:15 AcN:water (v/v); excitation wavelength, 272 nm; emission wavelength, 330 nm; retention time, 1.99 min. Phenolic content was measured using the Folin Ciocalteau procedure (*39*) with salicylate as a standard. Glucose was measured using the Glucose (HK) Assay Kit (Sigma, St. Louis, MO).

Test Substrates. In addition to root extracts, tested substrates included known components of root extracts such as amino acids (glutamate, aromatic−phenylalanine, tryptophan), sugars (glucose, lactose), carboxylic acids (acetate, adipate, citrate, lactate, malate, oxalate, pyruvate, succinate), and phenolic acids (acetyl salicylate, *l*-carvone, *p*-cymene, indole acetic acid, methyl salicylate, *o*-coumaric, *p*-aminobenzoate, salicylic acid). All chemicals used in these experiments were bought from Sigma Aldrich.

Results and Discussion

Characterizing the net effect of microbial exposure to root-derived substrates on catabolic gene expression is of paramount importance in understanding and optimizing PAH degradation in the rhizosphere. *Pseudomonas fluorescens* HK44 with a *nah-lux* fusion proved to be a good reporter organism to quantify the effect of such potential cosubstrates on the induction of the *nah* operon, using bioluminescence as an indicator. Induction of *nahG* in HK44, which was measured as specific bioluminescence (RLU (mg of biomass)$^{-1}$ L), increased with naphthalene concentration up to a certain level that was apparently inhibitory (ca. 6 mg L^{-1}) (Figure 1). This suggests that induction of the *nah* operon might be maximal near the source of release of an inducer and that induction would taper off as the inducer concentration decreases with increasing distance from the source. Figure 1 also shows that, in the absence of an inducer, a basal level of induction of the *nah-lux* gene occurs in the cell. However, this level of transcription is significantly lower than that observed during the induced phase.

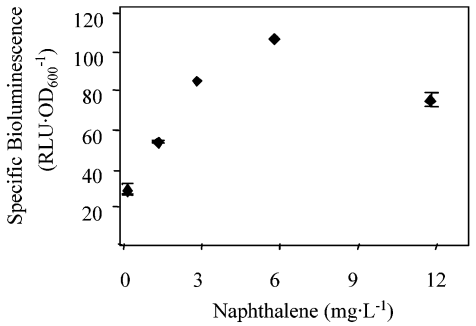

FIGURE 1. Effect of naphthalene concentration on specific bioluminescence of the bioreporter microorganism *Pseudomonas fluorescens* HK44. Error bars represent the range of data from duplicate reactors.

TABLE 2. Relative Specific Bioluminescence of Resting HK44 Cells Concurrently Exposed to Substrates Found in Rhizodeposition (40 mg of TOC L^{-1}) and Naphthalene (2.7 mg L^{-1})[a]

cosubstrates	relative specific biolumine scence
Controls	
buffer only	11.4 ± 3.0
naphthalene	100 ± 6.0
Sugars	
D-glucose	115 ± 10.5
α-lactose	102 ± 9.8
Amino Acids	
L-(+)-glutamate	85.1 ± 10.3
L-phenylalanine	90.4 ± 5.1
tryptophan	92.4 ± 6.9
Aromatic Substrates	
acetylsalicylate*	153 ± 12.0
l-carvone	99.0 ± 17.9
p-cymene	114 ± 12.1
indole acetic acid	80.3 ± 2.74
methylsalicylate*	128 ± 4.80
o-coumaric acid	106 ± 11.9
p-aminobenzoate	92.0 ± 6.60
salicylate*	140 ± 15.9
Carboxylic Acids	
acetate	64.6 ± 7.0
adipate	91.4 ± 13.5
citrate	109 ± 18.0
lactate	93.7 ± 6.4
malate	107 ± 17.7
oxalic	103 ± 14.6
pyruvate	100 ± 5.85
succinate	84.8 ± 6.7

[a] Results are averages of three trials ± one standard deviation. The asterisk (*) identifies compounds that induced *nahG* in the absence of naphthalene.

Induction Assays with Substrates Potentially Present in Root Extracts. Screening tests were conducted to identify plant-derived substrates that could affect specific and global regulation of the *nah* operon. Of the 21 sugars, carboxylic acids, amino acids, and aromatic compounds tested (Table 2), only salicylic acid, 4-methylsalicylic acid, and acetylsalicylic acid induced the *nah* operon in resting HK44 cells.

TABLE 3. Characteristics of Root Extracts Collected from Different Plant Species

common name	pH	TOC [(mg of C extracted) (g of FW root material)$^{-1}$]	glucose content (% glucose C in extracted TOC)	phenolic content [(mg of salicylate equivalent) (g of FW root material)$^{-1}$]	Y [(mg of biomass) (mg of TOC fed)$^{-1}$]
kou[a]	6.1	16.5	5.35	3.95	0.57 ± 0.05
milo[a]	6.2	3.70	5.60	0.41	0.65 ± 0.04
mulberry	6.1	15.9	0.34	8.53	0.33 ± 0.01
Osage orange	6.1	10.3	24.3	2.62	0.82 ± 0.09
poplar	6.1	2.20	9.40	0.90	0.73 ± 0.08
switch grass	6.9	2.10	2.86	0.42	0.32 ± 0.02
willow	7.2	5.00	1.99	0.24	0.71 ± 0.02

[a] Tropical plants.

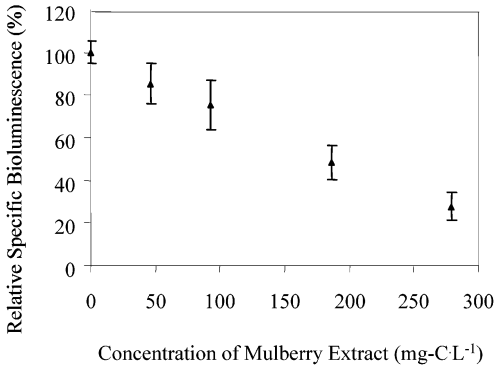

FIGURE 2. Relative specific bioluminescence of resting cells of HK44 concurrently exposed to mulberry root extracts (50−275 mg of TOC L^{-1}) and naphthalene (2.7 mg L^{-1}). Results are averages of duplicates from two trials ± the range.

Salicylic acid and 4-methylsalicylic acid are known inducers of *nahG* (*40*). Other aromatic substrates, such as *l*-carvone and *p*-cymene, which are inducers of PCB degradation (*17*), had no effect on the specific bioluminescence emitted at the concentrations tested.

When resting HK44 cells were concurrently exposed to naphthalene (2.7 mg L^{-1}) and the test substrates (40 mg of C L^{-1}), only the previously identified inducers increased the specific bioluminescence relative to treatments with naphthalene alone. With the exception of acetate, most compounds tested did not appear to significantly interfere with induction by naphthalene at the transcription level when present at 40 mg of C L^{-1}.

Characterization of Root Extracts. Analysis of the root extracts (Table 3) indicated some variability in TOC but were within the range of 0.4−27.7 mg of C (g of root material)$^{-1}$ reported in the literature (*22, 23*). The phenolic content of the root extracts ranged from 0.24 to 8.6 mg as salicylate equivalent (g of FW root material)$^{-1}$ and was highest in mulberry and Osage orange as reported by Fletcher et al. (*33*). The glucose content ranged from 0.3 to 25% of the extracted TOC. All root extracts proved to be good growth substrates for HK44 with relatively high yield coefficients (Table 3).

Induction Assays with Root Extracts. None of the tested root extracts (i.e., from poplar, milo, kou, Osage orange, willow, mulberry, and switch grass) induced *nahG* in HK44 (data not shown). Furthermore, when HK44 was concurrently exposed to naphthalene (2.7 mg L^{-1}) and root extracts (50−275 mg of TOC L^{-1}), the specific bioluminescence decreased with increasing TOC for all plants, as shown for mulberry root extract dilutions (Figure 2). At high concentrations, the specific bioluminescence was 50−80% lower than that of controls amended with naphthalene alone. Apparently, the negative effect that common, easily degradable substrates present in root extract exerted on *nahG* expression overshadowed any positive effect of potential inducers, which would have been present at much lower concentrations, possibly below the induction threshold.

TABLE 4. Naphthalene Degradation Patterns by HK44 in Batch Reactors Amended with Different Root Extracts (Initial OD_{600} = 0.001, Naphthalene = 3.56 ± 0.15 mg L^{-1})[a]

cosubstrate	rd
none	1.00 ± 0.08
kou (30 mg of C L^{-1})	1.12 ± 0.14
milo (20 mg of C L^{-1})	1.43 ± 0.41
mulberry (30 mg of C L^{-1})	1.77 ± 0.28*
Osage orange (30 mg of C L^{-1})	1.73 ± 0.10*
poplar (30 mg of C L^{-1})	1.36 ± 0.29
switch grass (20 mg of C L^{-1})	1.97 ± 0.37*
willow (20 mg of C L^{-1})	1.41 ± 0.10*

[a] The dimensionless naphthalene degradation rate (r_d) is the rate of degradation after the lag phase (mg of naphthalene day^{-1}) for treatments with root extracts normalized to the control rate (without root extracts). Results were calculated using data from duplicate reactors repeated over three trials. The asterisk (*) indicates rates significantly faster than controls without root extracts ($p \leq 0.05$).

Naphthalene Degradation Assays. Naphthalene degradation was faster in reactors amended with naphthalene (3.56 ± 0.15 mg of TOC L^{-1}) plus root extracts (20−30 mg of TOC L^{-1}) than in controls amended with naphthalene alone, especially for reactors with root extracts from mulberry, Osage orange, switch grass, and willow (Table 4). Figure 3 shows representative data from batch reactors containing naphthalene and mulberry root extracts. Degradation of naphthalene showed similar trends in all reactors, with an initial lag phase during which very little naphthalene degradation occurred (Figure 3A). This lag phase partly reflects the time required for the microbial concentration to increase to a critical level capable of exerting measurable degradation rates (*41*). Following the lag phase, naphthalene removal was relatively fast and could be approximated by a linear trend (Table 4). No obvious correlations between naphthalene degradation rates and root extract characteristics from different plants (Table 3) were discernible.

A rapid increase in bioluminescence was observed in reactors with root extract in the late exponential (declining growth) phase (Figure 3B). Apparently, induction of *nahG* was inhibited during the initial stages of growth when the availability of relatively labile substrates was higher. Depletion of the easily degradable fraction of root extracts and the presence of an inducer (naphthalene) probably enhanced induction during the declining growth phase. It is also plausible that the decreased availability of σ factors during fast exponential growth limited catabolic gene expression. This phenomenon, termed exponential silencing, has been shown to decrease σ^{54}-dependent *Pu* promoter activity and down-regulate toluene degradation by the TOL pathway (*42*).

Down-regulation of genes responsible for aromatic hydrocarbon degradation in the presence of alternate C-sources

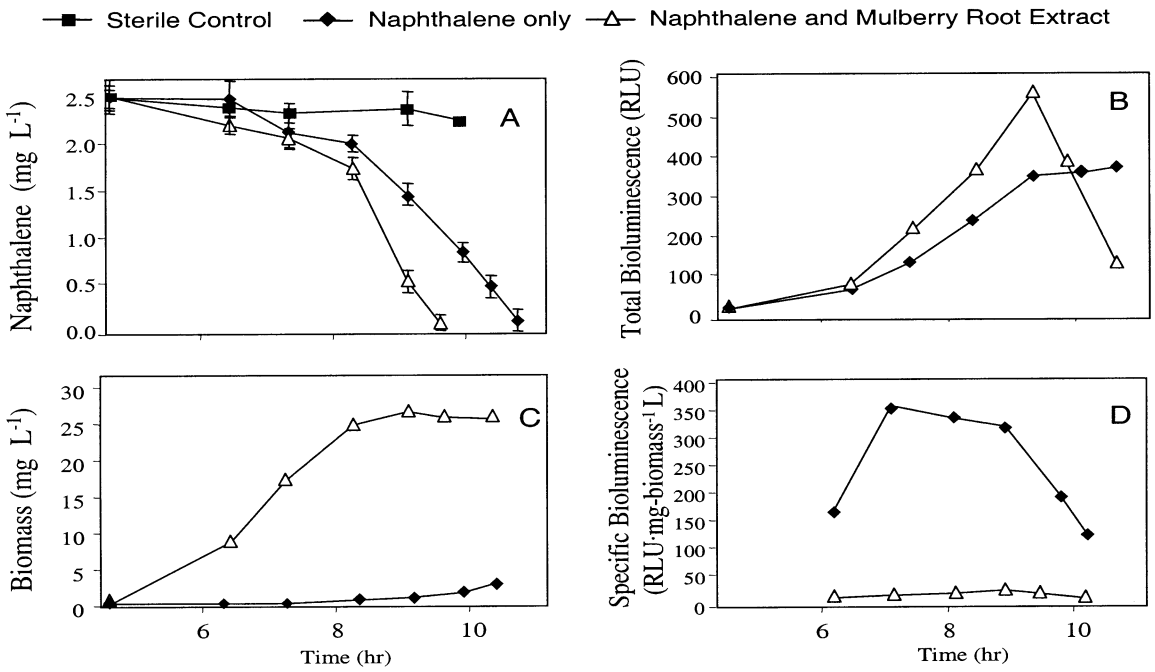

FIGURE 3. Degradation of naphthalene (2.7 mg L^{-1}) by *Pseudomonas fluorescens* HK44 in triplicate reactors in the presence and absence of 30 mg of TOC L^{-1} mulberry root extracts. Panels depict (A) the removal of naphthalene, (B) total bioluminescence readings, (C) biomass growth, and (D) specific bioluminescence trends over a 10-h incubation time.

has been previously reported (*43*). Global regulatory control by organic acids in pseudomonads is well-documented. It has been found to be compound-specific and concentration-dependent. For example, at high concentrations, succinate has been observed to inhibit degradation of aromatic compounds such as benzene (*26*), catechol (*26*), and toluene (*27*). Organic acids have been shown to repress phenol catabolism at the transcription level in *Ralstonia eutropha* (*44*). Under C-rich conditions, *Pseudomonas* species growing simultaneously on toluene and succinate in chemostats (*27*) exhibited repression of the *xylS* gene. However, under C-limiting conditions (i.e., low succinate concentrations), the same genes were easily inducible. In the presence of acetate and succinate, consumption of aromatic compounds such as *p*-hydroxybenzoic acid by *Acinetobacter* sp. strain ADP1, a closely related species to *Pseudomonas*, was delayed until the labile substrates had been depleted (*43*). Furthermore, the repression effect was reported to be greater when acetate and succinate were added simultaneously than when fed at the same total concentration separately. Similar to bioluminescence results reported in this paper, the repression effect was more pronounced under early exponential growth phase as corroborated by dot blot analysis (*43*). However, the molecular mechanism responsible for these observations is not fully understood.

Enhanced microbial growth in reactors with root extracts and naphthalene (Figure 3C) resulted in a higher level of total *nahG* expression (Figure 3B) and overall biodegradation activity (Figure 3A), even though *nahG* induction was lower on a per cell basis than in control reactors with naphthalene alone (Figure 3D). These results suggest that plant-promoted proliferation of competent genotypes can compensate for the interference that labile substrates exert on the expression of catabolic genes. Biomass concentrations in the treatment reactors containing both root extracts and naphthalene reached levels that were between 2 and 50 times higher than in the control reactors (Figure 3C), well within the range of the 4- to 100-fold increase reported for the rhizosphere effect (*5*−*7*, *9*).

These results have numerous implications for PAH degradation in the rhizosphere. First, exposure to the C- and nutrient-rich environment in the rhizosphere can lead to enhanced rates of degradation of naphthalene unrelated to induction of naphthalene dioxygenases by root extracts. Whereas some plants release compounds that induce oxygenase enzymes that degrade PAHs, the release of labile substrates that repress catabolic enzyme induction is likely to have an overall negative effect on PAH biodegradation activity per unit cell. However, this negative effect is likely to be offset by a positive effect associated with the proliferation of desirable geno(pheno)types, which is conducive to faster biodegradation rates.

Regarding plant selection criteria for PAH phytoremediation, the ability to release phenolics might be a bonus but is not essential. Other plant characteristics might be more important such as hardiness, ability to release substrates that stimulate microbial growth, and high water uptake rate to enhance soil re-aeration.

A practical limitation to extrapolating these results to field observations is that HK44 only reports induction by compounds that can be transformed to salicylate. Compounds such as phenanthrene, which can be cooxidized to phthalic acid but is not transformed to salicylate, do not induce the *nah* or *sal* operons. Nevertheless, the high levels of bioluminescence observed in reactors with root extracts and naphthalene (Figure 3B) suggest that a higher abundance of PAH catabolic enzymes that might fortuitously lead to enhanced cooxidation of other PAHs in the rhizosphere. It should also be recognized that PAH degradation in the rhizosphere might be mediated by numerous organisms including bacteria and fungi using different degradation pathways. Our experimental model cannot infer the effect of plant-derived substrates on such microorganisms nor consider the contribution of PAH cooxidizers and commensal bacteria that consume labile substrates that would otherwise interfere with catabolic gene expression.

Results from the characterization of the root extracts showed that their TOC concentration (*6*) and composition were representative of substrates released by plants (*22*−*25*). Nevertheless, the composition and quantity of root-derived material released into the rhizosphere varies within a plant depending on its age (*24*), season (*12*, *24*), distance

from the root tip (*45*), and health of the plant (*46*). Older plants have been shown to release more phenolics (*24*), and it is possible that these transient compounds found during senescence might aid in induction. Several environmental stresses associated with water, light, oxygen and nutrient availability (*23, 47*), or extreme temperatures and pathogens are also likely to influence rhizodeposition, especially its phenolic content. Plants under stress synthesize more salicylate to induce systemic resistance (*46*) and release more phenolics into the rhizosphere under nutritional deficiency (*47*). Therefore, whereas rhizodeposition is likely to have an overall positive effect on PAH biodegradation (primarily due to proliferation of specific degraders), the variability in concentration and composition of root-derived substrates makes it difficult to predict the extent of this beneficial effect.

In conclusion, the rhizosphere effect is a very complex phenomenon and quantifying the specific contribution of different mechanisms toward the enhancement of PAH degradation in contaminated soils is a difficult task. This paper has taken a first step toward discerning the effect of root substrates on catabolic gene induction versus proliferation of competent strains. Whereas microbial catabolic gene induction by plant-derived substrates in the rhizosphere might occur, concurrent exposure to labile substrates is likely to repress PAH catabolic genes to a greater extent. However, the presence of a large heterotrophic community is conducive to faster degradation rates, and this might aid in reducing the concentration of labile C-substrate, thus, facilitating the induction and PAH degradation processes.

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