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Analyses of polycyclic aromatic hydrocarbon-degrading bacteria isolated from contaminated soils

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Abstract

Polycyclic aromatic hydrocarbon (PAH)-degrading bacteria isolated from PAH-contaminated soils were analyzed genotypically and phenotypically for their capacity for metabolism of naphthalene and other PAH substrates. The methods used for the analyses were DNA hybridization using NAH7-derived gene probes, PAH spray plate assays, ^{14}C-PAH mineralization assays, and dioxygenase activity assays. The results of the analyses showed a dominant number of PAH-degrading bacteria with a NAH7-like genotype. The results support the continued use of the *nahA* probe for contaminated soils to monitor the genetic potential of indigenous microorganisms to degrade PAHs. However, the finding of non-*nahA*-hybridizing PAH-degrading bacteria show the limitation of NAH7-derived gene probes. Fifteen percent (13/89) of PAH-degrading bacteria isolated were not detected with the *nahA* gene probe. Four isolates (designated A5PH1, A8AN3, B1PH2, and B10AN1) did not hybridize with any of the NAH7-derived gene probes (*nahA*, *nahG*, *nahH*, and *nahR)* used in this study. Considering the numerous unculturable microorganisms in nature and their potential genotypes, NAH7-derived gene probes may underestimate the microbial potential to catabolize PAHs. This necessitates development of new gene probes for enumeration and isolation of PAH-degrading bacteria to better understand the *in situ* microbial potential to degrade PAHs.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants produced from incomplete combustion of organic materials (Harvey 1991). Major sources of PAHs include fossil fuel combustion, manufactured gas production, wood treatment facilities, automobile exhaust, and waste incineration. Environmental concern exists about the persistence and toxicity of PAHs, especially high-molecular-weight (HMW) PAHs (four or more aromatic rings). These compounds exhibit limited solubility in water (MacKay & Shiu 1977) and absorb onto soils and particulates influencing their bioavailability and biodegradation (Means et al. 1980; Heitkamp & Cerniglia 1987; Shiaris 1989; Weissenfels et al. 1995).

Naphthalene biodegradation has been the best studied of the PAHs because it is the simplest and the most soluble PAH, and naphthalene-degrading bacteria are relatively easy to isolate. Since naphthalene-degrading bacteria were first reported by Tausson (1927), the ubiquitous presence of naphthalene-degraders has been shown (Kiyohara et al. 1992; Kastner et al. 1994). The bacterial degradation of naphthalene encoded by the plasmid NAH7 of *Pseudomonas putida* PpG7 (Dunn & Gunsalus 1973) has been well characterized biochemically (Davies & Evans 1964; Yen & Gunsalus 1982; Eaton & Chapman 1992) and genetically (Grund & Gunsalus 1983). Based on this information, NAH7-derived gene probes have been used to determine the *in situ* microbial potential to degrade naphthalene (Fleming et al. 1993; Sanseverino et al. 1993b).

In addition to naphthalene, recent studies demonstrated that the NAH system can mediate degradation of phenanthrene, anthracene, dibenzothiophene, fluorene, and methylated naphthalenes (Sanseverino et al. 1993a; Denome et al. 1993; Yang et al. 1994;

Leblond et al. 1995; Selifonov et al. 1996). Such studies suggest that NAH7-derived gene probes can be used to determine the *in situ* microbial potential to degrade not only 2-ring PAHs but also 3-ring PAHs. However, some PAH-degrading bacteria are not detected by NAH7-derived gene probes (Foght & Westlake 1988; Fredrickson et al. 1991; Goyal & Zylstra 1996) and NAH7-derived gene probes may not assure detection of HMW PAH-degrading bacteria. Therefore, biochemical and genetic information of HMW PAH degradation is necessary for the development of new information which can provide a better understanding of microbial activity in the environment.

In studies on the molecular ecology of NAH genotypes in PAH-contaminated manufactured gas plant soils, *nahA* gene abundance was shown to directly correlate with soil PAH contamination levels (Sanseverino et al. 1993b). *NahA* estimated cell types ranged from 10^6 to 10^{10} organisms per gram soil depending on the use of colony hybridization or slot blot DNA hybridization, respectively. In addition, *nahA* mRNA has been isolated from the soil suggesting not only gene enrichment but expression in such PAH-contaminated soils (Fleming et al. 1993; Sanseverino et al. 1993b). It is of interest to determine if this enrichment of *nahA* genotypes is reflected in the bacterial populations recovered from these and similar soils and whether non-*nahA* PAH phenotypes co-occur with the *nahA* genotypes.

In this regard, a bacterial culture collection originally derived from several PAH-contaminated soils was analyzed with phenotypic and genotypic tests. The analyses were designed 1) to screen for HMW PAH degrading bacteria, and 2) to screen non-NAH7-hybridizing PAH-degrading bacteria for the future development of new gene probes.

Materials and methods

Bacterial strains and culture conditions. A bacterial culture collection was previously isolated from PAH-contaminated soils (King et al. 1991; Sanseverino et al. 1993b). Each isolate was chosen based on its ability to hybridize with the *nahA* DNA probe or by its ability to clear a phenanthrene, anthracene, or pyrene spray plate. The soil samples were from 6 manufactured gas plant site soils and one creosote-contaminated site soil. The physical and chemical characterization of the soils was described in detail elsewhere (Cushey & Morgan 1990, Sanseverino et al. 1993b). Total PAH concentration ranged from 230 ± 150 to $49{,}000 \pm 2{,}800$ mg/kg soil. Benz(a)pyrene (BaP) and naphthalene concentrations ranged from 3 ± 1 to 330 ± 40 and 11 ± 7 to $4{,}100 \pm 300$ mg/kg soil, respectively. Individual bacterial isolates were stored in glycerol at -70 °C.

Pseudomonas putida strains PpG7 (NAH7) (Dunn & Gunsalus 1973) and 2440 (Bagdasarian et al. 1981; Bagdasarian et al. 1983) were employed for PAH-degradation tests as positive and negative controls, respectively. *P. putida* 2440 is a derivative of the strain mt-2, defective in host-specific restriction. The strains were also used as controls in the NAH7-derived gene probing and dioxygenase activity assays.

All strains were grown on Yeast Extract-Polypeptone-Glucose (YEPG) medium (per liter: 0.25 g glucose, 0.5 g polypeptone, 0.05 g yeast extract, 0.05 g ammonium nitrate, pH 7.0; Sanseverino et al. 1993a) at 28 °C. For plates, 15 g/l of agar was incorporated into the medium. Broth cultures were aerated by shaking at 150 rpm.

Chemicals. All chemicals were purchased from Mallinckrodt Chemical, Inc. (Paris, KY) unless otherwise specified. BaP (purity 99%) was obtained from Eastman Kodak Co. (Rochester, NY). The other PAHs were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). All solvents and chemicals were the highest purity available. Except for ^{14}C-BaP, ^{14}C-labeled compounds were purchased from Sigma Chemical Company (St. Louis, MO). Radiochemical purities and specific activities of the compounds were as follows: [1-^{14}C]naphthalene (10.1 mCi/mmol; purity, >98%), [9-^{14}C]phenanthrene (10.4 mCi/mmol; purity, >99%), [U-^{14}C]anthracene (15.0 mCi/mmol; purity, >98%), and [4,5,9,10-^{14}C]pyrene (10.4 mCi/mmol; purity, >98%). [7,10-^{14}C]BaP (60 mCi/mmol; purity, >98%) was obtained from Amersham (Arlington Heights, IL).

PAH spray plate assay. The PAH spray plate assay was employed to test the PAH degradability of isolates (Kiyohara et al. 1982). The PAHs used in the spray plate methods were phenanthrene, fluorene, anthracene, pyrene, and BaP. Bacterial colonies were pre-grown on YEPG agar plates and then sprayed with a 1% (w/v) PAH dissolved in acetone. After evaporating the acetone by placing the plates in a fume hood, the plates were placed in plastic bags and incubated at 28 °C for up to 3 weeks. The plates were observed daily. Colonies producing clear zones were scored positive.

^{14}C-PAH mineralization assay. The assay was performed to determine if $^{14}CO_2$ may be produced from ^{14}C-PAH. Cells were grown while shaking (150 rpm) in 50 ml of YEPG medium to an OD_{600} of 0.8. Cells were washed once and resuspended in YEPG broth to an OD_{600} of 0.4. Four ml of the suspension was added to a 25-ml EPA vial (Pierce, Rockford, IL). A CO_2 trap (8-ml vial) containing 0.5 ml of 0.5 M NaOH was placed inside the EPA vial. Before the EPA vial was sealed with a Teflon-lined silicone septum, an appropriate amount of ^{14}C-PAH dissolved in acetone (toluene in the case of BaP) was added: BaP, 65,000 dpm; pyrene, 14,000 dpm; naphthalene, 100,000 dpm; phenanthrene, 16,000 dpm; anthracene, 65,000 dpm. The assays were performed in triplicate. A negative control consisted of cells killed with 0.5 ml of 1 M H_2SO_4. The vials were incubated at 28 °C with shaking at 150 rpm for 7 days for BaP and pyrene and 3 days for the other PAHs. The assays were stopped by injecting 0.5 ml of 1 M H_2SO_4 through the septa. The vials were shaken for 1 h to drive $^{14}CO_2$ into the NaOH traps. Mass balances were achieved as described previously (Sanseverino et al. 1993b) by measuring radioactivity from $^{14}CO_2$, the aqueous portion, and the organic-extractable portion of the mineralization assay. The ^{14}C-radioactivity was measured with a Beckman model LC5000 scintillation counter.

Dioxygenase test. Aromatic ring dioxygenase activity was examined using indole because the formation of indigo from indole is presumptive for aromatic ring dioxygenases (Ensley et al. 1983). Bacterial colonies were pre-grown on YEPG agar plates, and then indole crystals were placed in the lids of the petri dishes. After 1 day of incubation at 20 °C, colonies producing a blue color were scored positive.

DNA isolation. Total DNA was prepared by the procedure of Ausubel et al. (1989). Large-scale plasmid DNA was prepared by an alkaline lysis method (Sanseverino et al. 1993a). DNA was further purified by ethidium bromide-cesium chloride gradient ultracentrifugation, resuspended in TE buffer (Sambrook et al. 1989) and stored at −20 °C.

Gene probe preparation. PCR (polymerase chain reaction) amplification was employed to generate gene probes. A *GeneAmp*®PCR reagent kit (Perkin-Elmer Cetus) with *AmpliTaq*® DNA polymerase was used for the amplification. Plasmids NAH7 and pFH360 were used as templates to prepare NAH7-derived and the 23S rDNA gene probes, respectively. Oligonucleotide primers were synthesized by Genosys, Inc. (Woodlands, TX). The nucleotide sequences of primers are as follows: *nahA* probe, 5′-CCCTAGCGCGTAACTACCCC-3′ and 5′-GGTCCAGACCTCGGTGGTG-3′ (Simon et al. 1993); *nahG* probe, 5′-GCGCATCGGTATCGTCGGCGGCGG-3′ and 5′-CGTGGCCGGGGCGCATTACACC-3′ (You et al. 1991); *nahH* probe, 5′-GTATCGAGCGTTACTAGTAGCCG-3′ and 5′-CGTCCTAGGTCATAACGGTCATG-3′ (Ghosal et al. 1987; You et al. 1991); *nahR* probe, 5′-ATGGAACTGCGTGACCTG-3′ and 5′-TCAATTCTCTCTATCCTGCG-3′ (You et al. 1988); 23S rDNA probe, 5′-AAGCTTGCTGGAGGTATC-3′ and 5′-TTGGAAGCATGGCATCAA-3′ (Festl & Schleifer 1986).

PCR conditions consisted of 30 cycles: *nahA*, *nahH*, and *nahR* probes, 94 °C for 1 min, 37 °C for 30 sec, and 72 °C for 1 min; *nahG* probe, 94 °C for 1 min, 55 °C for 30 sec, and 72 °C for 3 min; 23S rDNA, 94 °C for 30 sec, 42 °C for 30 sec, and 72 °C for 30 sec. Before the 30 cycles, preheating at 94 °C (5 min) was employed. After the 30 cycles, final chain elongation was conducted at 72 °C for 5 min. The amplified products were detected by 1% (w/v) agarose gel electrophoresis. The sizes of *nahA*, *nahH*, and *nahR* probes were approximately 1 kb each. The sizes of *nahG* and 23S rDNA probes were 2.1 kb and 360 bp, respectively.

Double-stranded PCR products were used as a template for re-amplification under the same amplification conditions. Re-amplified PCR products were used as templates to make single-stranded radiolabeled probes. [α-^{32}P]dCTP (ICN Biomedical Inc., Irvine, CA) was used for radiolabeling. Radiolabeled single-stranded DNA probes were obtained by asymmetric amplification using only one primer. The labeled probes were purified using a NucTrap Push Column according to manufacturer's instructions (Stratagene, La Jolla, CA).

DNA hybridization. Gene probing was used to determine if the isolates contained NAH7-like PAH-catabolic genes. All DNA hybridizations were performed as described previously (Sanseverino et al. 1993b). One buffer (0.5 M NaH_2PO_4, 1 mM EDTA, and 7% (w/v) sodium dodecyl sulfate [SDS]; pH 7.2) was used for prehybridization and hybridization at 65 °C (Church & Gilbert 1984).

Table 1. Comparative analysis of PAH-degrading strains used in this study

Tests	Number of strains		Number of *nahA* strains[a]	
	Tested	Positive	Positive (%)	Negative (%)
Genotypic test				
Gene *nahA* probing	141	96		
Phenotypic tests				
Dioxygenase test[b]	141	109	95 (87)	14 (13)
Spray plate				
Fluorene	141	35	31 (89)	4 (11)
Phenanthrene	141	83	70 (84)	13 (16)
Anthracene	141	13	10 (77)	3 (23)
Pyrene	141	3	2 (67)	1 (33)
Benzo[a]pyrene	141	0	0	0
^{14}C-Naphthalene mineralization	59	27	21 (78)	6 (22)
Total PAH-degraders[c]		89	76 (85)	13 (15)

[a] *nahA* probing results from strains showed (+) from phenotypic test.
[b] Dioxygenase activity as determined by indigo formation from indole (Ensley et al. 1983).
[c] As determined by the spray plate method and ^{14}C-naphthalene mineralization in this study.

Colony hybridization was performed using NAH7-derived gene probes: *nahA*, *nahG*, *nahH*, and *nahR*. Probe *nahA* was used in the initial colony hybridization assay. Non-*nahA*-hybridizing strains were further analyzed with the other probes. For strains that did not hybridize with any of NAH7-derived gene probes, total DNA isolated from the bacteria was subjected to slot blot hybridization using the same probes. Total DNA samples were blotted onto a nylon membrane by using the slot blotting system (BioDot SF Microfiltration Apparatus, BioRad, Richmond, CA) in accordance with manufacturer's protocol. The blotted DNA on the membranes was subject to DNA hybridization. For re-hybridization, probes on the membrane were stripped by boiling in 0.1% (w/v) SDS according to manufacturer's protocol (ICN Biomedical, Inc., Aurora, OH). The stripped membranes were monitored for complete removal of radiolabeled probes by extended autoradiography before re-hybridization with different probes. To prove the presence of the blotted DNA on the membranes, the membranes were re-hybridized with a 23S rDNA probe under low-stringency conditions: prehybridization, hybridization, and washing were performed at 45 °C.

Results and discussion

Phenotypic characterization. A total of 141 PAH-degrading bacteria isolated from several PAH-contaminated soils were analyzed phenotypically for their ability to degrade PAH and genotypically to determine if they contain NAH7-like genes. The analyses were designed to screen HMW PAH-degrading strains as potential sources for new gene probes and to better understand biodegradation of these compounds. In this study, pyrene and BaP were used as HMW PAHs. Low molecular weight (LMW; two- or three-ring) PAHs were also used in the analyses to determine the capability of current NAH7-derived gene probes to detect various PAH-degrading bacteria.

All strains were subjected to the PAH spray plate method, ^{14}C-PAH mineralization assays, and dioxygenase activity tests. Fifty-nine randomly selected strains were subjected to the ^{14}C-naphthalene mineralization test. From 141 bacterial strains, 109 were dioxygenase positive (Ind^+; Table 1) and 89 showed PAH degradation ability as determined by the spray plate method and the ^{14}C-naphthalene mineralization assay. Since not all of the 141 strains were subjected to the naphthalene mineralization test, the remaining 52 strains could be naphthalene degraders. Among the 89 PAH-degrading strains, 87 showed dioxygenase activity. However, 2 phenanthrene-degrading strains did not show dioxygenase activity, suggesting no or low (less than the detection limit) dioxygenase activity under the conditions used.

The dioxygenase test suggested a strong relationship between PAH-degrading bacteria and their dioxygenase activity: 98% (87/89) of the screened strains showed dioxygenase activity. Although toluene-4-

Table 2. Multiple phenotypes of isolated PAH-degrading bacteria

Phenotype[a]	Number of strains tested		
	Total	Dioxygenase positive[b]	*nahA* positive
Phn^+	43	41	37
Flu^+	6	6	6
Nah^+ Phn^+	6	6	3
Phn^+ Flu^+	8	8	7
Phn^+ Ant^+	4	4	4
Nah^+ Phn^+ Flu^+	13	13	13
Nah^+ Phn^+ Ant^+	1	1	1
Phn^+ Flu^+ Ant^+	1	1	
Nah^+ Phn^+ Flu^+ Ant^+	4	4	2
Nah^+ Phn^+ Flu^+ Ant^+ Pyr^+	3	3	2
Total	89	87	76

Abbreviations: Nah, naphthalene; Flu, fluorene; Phn, phenanthrene; Pyr, pyrene; Ant, anthracene.

[a] As determined by ^{14}C-naphthalene mineralization and the spray plate method. Since only randomly selected 59 strains were used for ^{14}C-naphthalene mineralization, estimation of Nah^+ phenotype is incomplete among 141 strains used in this study. Therefore, Phn^+ strains may also be nah^+.

[b] Dioxygenase activity as determined by indigo formation from indole.

monooxygenase was also reported to produce indigo from indole (Yen et al. 1991), indole-conversion to indigo is considered representative of dioxygenase activity (Ensley et al. 1983). The indole conversion was shown by aromatic ring dioxygenases from not only PAH degraders (Ensley et al. 1983; Goyal & Zylstra 1996) but also monocyclic aromatic hydrocarbon degraders (Ensley et al. 1983; Eaton & Timmis 1986; Wubbolts et al. 1994). The high percentage of Ind^+ PAH degraders shown in this study suggests that the majority of bacteria may initiate degradation via dioxygenases.

The results of the phenotypic tests suggested that the PAH-degrading bacteria showed multiple phenotypes in terms of PAH degradation (Table 2). All of the naphthalene-degrading strains were able to degrade 3-ring PAHs as determined by the spray plate method and ^{14}C-PAH mineralization assays. All naphthalene-degrading strains (27), anthracene-degrading strains (13), and pyrene-degrading strains (3) formed clear zones on the phenanthrene spray plates. In the spray plate assays, the time required for the formation of clear zones was as follows: 1 day for fluorene, 2–3 days for phenanthrene, 3–4 days for anthracene, and 3 weeks for pyrene. No strain produced clear zones on the BaP spray plates.

HMW PAH degraders. Three pyrene-degrading strains (A8AN3, B1PH6, and N1-4PH) were found using the pyrene spray plate method. These 3 microorganisms degraded a broad range of PAHs (i.e., pyrene, anthracene, fluorene, phenanthrene, and naphthalene) as determined by the spray plate method or ^{14}C-PAH mineralization assay. These 3 strains produced unknown pyrene-metabolite(s) (data not shown). Metabolite production was suggested when the strains were grown in YEPG broth containing pyrene. The cultures became gray or black in color after 1 week of incubation at 28 °C. Abiotic (no cells) and biotic (no pyrene) controls did not produce the color change.

Although the pyrene degraders produced clear zones on pyrene spray plates (data not shown), they did not produce $^{14}CO_2$ from [4,5,9,10-^{14}C]pyrene after 7 days, suggesting no ring-cleavage or unlabeled-ring cleavage in the incubation time. Initial ring-cleavage of pyrene can occur in either the 1,2- or 4,5-positions, based on reported ring-cleavage products (Heitkamp et al. 1988; Walter et al 1991; Schneider et al. 1996). Strains B1PH6 and N1-4PH did not produce $^{14}CO_2$ from [9-^{14}C]phenanthrene in 3 days (Table 4), although they formed clear zones on phenanthrene spray plates.

Strain A8AN3 was originally isolated from a MGP soil because of its ability to form clear zones on anthracene spray plates. Strain A8AN3 produced $^{14}CO_2$ from [7,10-^{14}C]BaP although the strain did not produce observable clear zones on BaP spray plates (Table 4). Considering the positions of $^{14}CO_2$ labels,

Table 3. Phenotypes and genotypes of selected PAH-degrading strains

Strain	Dioxygenase test	*nah* Probes[a]				^{14}C-PAH mineralization				Spray plate		Growth on PIA[b]
		A	G	H	R	Nah	Phn	Ant	BaP	Flu	Pyr	
Non-*nahA*-hybridizing HMW PAH degrader												
A8AN3	+	−	−	−	−	+	+	+	+	+	+	−
Non-*nahA*-hybridizing PAH degraders												
A5PH1	+	−	−	−	−	+	+	−	−	−	−	−
B1PH2	+	−	−	−	−	+	+	+	−	+	−	−
B10AN1	+	−	−	−	−	+	+	−	−	−	−	−
HMW PAH degraders												
B1PH6	+	+	*	*	*	+	−	+	−	+	+	*
N1-4PH	+	+	+	*	*	+	−	+	−	+	+	*

[a]NAH7-derived gene probes.
[b]*Pseudomonas* isolation agar.
*Not determined.
Abbreviations: Nah, naphthalene; BaP, benzo[a]pyrene; Phn, phenanthrene; Ant, anthracene; Flu, fluorene; Pyr, pyrene.

the mineralization result indicated that at least one ring of BaP can be cleaved by A8AN3. Attempts to identify BaP metabolites from A8AN3 were unsuccessful. Initial oxygenated-metabolites and ring cleavage products of BaP were reported from the cultures of *Beijerinckia* sp. strain B836 (Gibson et al. 1975) and *Mycobacterium* sp. strain RJGII-135 (Scheider et al 1996). However, a complete BaP degradation pathway has not been proposed to date. Bacteria capable of growing with BaP as the sole carbon and energy source or forming clear zones on BaP spray plates have not been reported. A8AN3 degraded a broad range of PAHs (Tables 3 and 4); naphthalene, fluorene, phenanthrene, anthracene, pyrene, and BaP. However, the strain could grow only on either naphthalene or phenanthrene as sole carbon and energy sources among the PAHs (growth on fluorene was not examined). The strain showed dioxygenase activity as determined by ability to convert indole to indigo.

Genotypic characterization. All strains from the culture collection were screened for NAH7-like genes. Specifically, each strain was tested for *nahA*. Of the 141 strains examined, 96 strains tested positive for the *nahA* probe (Table 1). From the 89 PAH-degrading strains, 76 strains (85%) were detected by the *nahA* probe. The *nahA* probe detected 78% of the ^{14}C-naphthalene-degrading strains. The *nahA* probe also detected bacteria that degraded 3- and 4-ring PAHs: 89% of the fluorene-degraders; 84% of the phenanthrene-degraders; 77% of the anthracene-degraders and 67% of the pyrene-degraders. However, the BaP-degrading strain was not detected by the *nahA* probe. Almost all of the strains (99%, 95/96) detected by the *nahA* probe showed dioxygenase activity as determined by the ability to convert indole to indigo.

Thirteen percent (14/109) of Ind$^+$ strains were not detected by the *nahA* gene probe (Table 1). Approximately, 22% of naphthalene-degraders, 11% of fluorene-degraders, 16% of phenanthrene-degraders, 23% of anthracene-degraders, and 33% of pyrene-degraders were not detected by the *nahA* probe. Overall, 15% (13/89) of the PAH-degrading strains did not hybridize with the *nahA* probe. Four of these 13 strains, (A5PH1, A8AN3, B1PH2, and B10AN1) did not hybridize with any of the NAH7-derived gene probes used (Table 3; *nahG*, *nahH*, and *nahR*) under high-stringency conditions. The strains did not hybridize with the *nahA* probe under low (55 °C)-stringency conditions, suggesting dissimilar PAH-degradative genes in these 4 strains.

Table 3 summarizes genotypes and phenotypes of selected *nahA*$^-$ and HMW PAH-degrading strains. All of the 6 strains showed aromatic ring dioxygenase activities (Ind$^+$) as judged by indole conversion to indigo. The 4 *nahA*$^-$ strains did not grow on *Pseudomonas* isolation agar plates. Among the 6 PAH-degraders, A8AN3 was a HMW PAH degrader that did not hybridize with any of the NAH7-derived gene probes. ^{14}C-PAH mineralization data by these strains is summarized in Table 4.

Table 4. Mineralization of ^{14}C-PAHs by selected strains

Strain	Percent $^{14}CO_2$ recovered from ^{14}C-labeled:				
	Nah	Phn	Ant	Pyr	BaP
A5PH1	60.6±3.3[a] (96.1±2.1)[b]	11.2±0.7 (98±3.2)	0	0	0
A8AN3	74.0±6.2 (93z±5.7)	53.3±15 (119±7.3)	17.0±2.7 (102±1.2)	0	45.0±2 (91.0±0.6)
B1PH2	61.2±1.2 (97.2±3.6)	57.0±0.6 (104±1.2)	13.1±0.3 (95±2.1)	0	0
B1PH6	56±0.8 (83.4±2.6)	0	14.8±2.4 (87.7±3.2)	0	0
B10AN1	46.1±1.7 (87±2.6)	9.0±0.3 (97.1±0.5)	0	0	0
N1-4PH	60±2.1 (95.1 ± 2.7)	0	12.3±+0.9 (97.1 ± 2.3)	0	0
PB2440[c]	0	0	0	0	0

[a]Data are given as mean ± standard deviation (n = 3).
[b]Total ^{14}C recovery (expressed as percentages).
[c]*P. putida* PB2440.
Abbreviations: Nah, naphthalene; BaP, benzo[*a*]pyrene; Phn, phenanthrene; Ant, anthracene; Flu, fluorene; Pyr, pyrene.

Summary

The genotypic tests showed that the NAH7-like degradative genes were dominant among PAH-degrading bacteria in the culture collection (Table 1) and suggested long-term selection or enrichment of strains containing NAH7-like genes at PAH-contaminated sites. Because plasmid NAH7 is self-transmissible (Dunn & Gunsalus 1973), the horizontal transfer of NAH7 or NAH7-like plasmids might have occurred among bacterial strains in the soils, contributing to the dominance of the NAH7-like genotype among PAH-degraders. Most PAH-degrading bacteria in this strain collection contained cryptic and NAH7-like plasmids (King et al. 1991; Sanseverino et al. 1991). Using the *nahA* gene probe, this study confirmed the previous work of Sanseverino et al. (1993a) that NAH7-derived gene probes could be used to determine the microbial potential to degrade not only naphthalene but also 3- or 4-ring PAHs. The probe was especially effective for detecting phenanthrene degraders or fluorene degraders among 3 or more ring PAH-degraders. The results of the genotypic tests support the continued use of the NAH7-derived probes to monitor genetic potential of *in situ* microorganisms to degrade PAHs in contaminated soils.

Further, the result of this study reinforces the distribution and prevalence of the *nahA* genotype associated with PAH-contaminated soils as previously suggested by colony hybridization and DNA extraction and hybridization results (Sanseverino et al. 1993b). However, these results and apparent correlation must be taken in context with the likelihood that culturing and isolation procedures may bias culture collections to typical organisms of the γ division proteobacteria such as *Pseudomonas*. PAH-degrading *Pseudomonas* species are frequently associated with cultures obtained from PAH-contaminated soils (Mueller et al. 1994).

However, the occurrence of non-*nahA* genotypes in culturable bacteria recovered from such sites, such as strain A8AN3, indicates that other PAH-degrading strains, while evident in contaminated soils, may not be numerically dominant. Yet, the fact that these strains demonstrate multiple PAH phenotypes, makes these strains important for assessing the overall capacity for significant biodegradation of a broad spectrum of PAH. Therefore, new gene probe development is necessary to better understand the significance of these non-*nahA* PAH-degrading bacteria and the role they play in remediation of contaminated soils.

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