APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1993, p. 1938–1942
0099-2240/93/061938-05$02.00/0
Vol. 59, No. 6

NOTES

NAH Plasmid-Mediated Catabolism of Anthracene and Phenanthrene to Naphthoic Acids

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Received 2 October 1992/Accepted 8 March 1993

***Pseudomonas fluorescens* 5R contains an NAH7-like plasmid (pKA1), and *P. fluorescens* 5R mutant 5RL contains a bioluminescent reporter plasmid (pUTK21) which was constructed by transposon mutagenesis. Polymerase chain reaction mapping confirmed the localization of *lux* transposon Tn*4431* 300 bp downstream from the start of the *nahG* gene. Two degradation products, 2-hydroxy-3-naphthoic acid and 1-hydroxy-2-naphthoic acid, were recovered and identified from *P. fluorescens* 5RL as biochemical metabolites from the biotransformation of anthracene and phenanthrene, respectively. This is the first report which provides direct biochemical evidence that the naphthalene plasmid degradative enzyme system is involved in the degradation of higher-molecular-weight polycyclic aromatic hydrocarbons other than naphthalene.**

The degradation of naphthalene by *Pseudomonas putida* PpG7 is a well-established model for the study of dicyclic aromatic hydrocarbons (22, 32). Strain PpG7 contains the archetypal NAH7 plasmid that encodes the genes for the degradative pathway of naphthalene. The NAH7 gene order and the organization and direction of the genes' transcription have been well-characterized (30, 31). In plasmid NAH7, the naphthalene catabolic genes are organized into two operons, *nah* and *sal*. The two catabolic operons are controlled by a positive regulator gene, *nahR*, that is located immediately upstream of the *nahG* gene. Induction of the operons is controlled not by naphthalene but by its metabolite, salicylate (30, 31).

Bacteria with the ability to metabolize phenanthrene and anthracene can be readily isolated from soil (7, 10, 17, 18, 28). Substantial information exists demonstrating the biodegradation of anthracene and phenanthrene in sediments (2–4, 20, 26, 27, 29). *Pseudomonas* spp. degrade phenanthrene to 1-hydroxy-2-naphthoic acid through a *meta*-cleavage pathway. The 1-hydroxy-2-naphthoic acid is subsequently decarboxylated to 1,2-dihydroxynaphthalene, and then the compound is further metabolized through salicylate and catechol by the enzymes of the naphthalene catabolism pathway (5, 7, 17).

The reactions involved in the bacterial oxidation of anthracene have not been completely elucidated. The initial reactions in the bacterial degradation of anthracene involve oxygenation at the 1,2 positions with the formation of *cis*-1,2-dihydroxy-1,2-dihydroanthracene, which is then oxidized to 1,2-dihydroxyanthracene prior to ring fission (1, 7). The 1,2-dihydroxyanthracene is further metabolized to 2-hydroxy-3-naphthoic acid by soil pseudomonads (7, 18). The further oxidation of 2-hydroxy-3-naphthoic acid and the role of this oxidation in anthracene catabolism remain to be elucidated.

In a recent paper Selifonov et al. (24) implicated the NAH7 plasmid in conversion of dibenzofuran to a dead-end product, 4-[2′-(3′-hydroxy)benzofuranyl]-2-keto-3-butenoic acid, via naphthalene dioxygenase to 2-hydroxychromene-2-carboxylate isomerase. More significantly, Sanseverino et al. (19) found that *Pseudomonas fluorescens* 5R isolated from a manufactured gas plant soil contained an NAH7-like plasmid (pKA1) specifically responsible for the mineralization of anthracene and phenanthrene.

Studies were undertaken to directly confirm the metabolism of anthracene and phenanthrene by plasmid pKA1 and to demonstrate the role of the NAH upper-pathway operon by using mutant strain 5RL containing a *lux* reporter gene transposon (Tn*4431*) in the *sal* operon of pKA1.

Localization of *lux* transposon Tn*4431* in pUTK21. The results of a previous study suggested that the insertion site of Tn*4431* is located in the *nahG* gene (11). Three oligonucleotide primers were synthesized by Genosys, Inc., Woodlands, Tex., and were used in polymerase chain reaction (PCR) amplification experiments. Primer 1 (GCG CAT CGG TAT CGT CGG CGG CGG) was determined from the previously published partial nucleotide sequence of the *nahG* gene (21). Primer 2 (CGT GGC CGG GGC GCA TTA CAC C) was chosen from the nucleotide sequence of the *nahH* gene (8). Primer 3 (ACG ATT TTT TCC GAA TTC TGC GG) was determined from the nucleotide sequence of the insertion site of Tn*1721*, which is the parental transposon of Tn*4431* (23). Primer 3 can be used for amplification in both directions, because the insertion sites of the transposon are inverted repeats.

Plasmid NAH7 from *P. putida* PpG7 and plasmid pUTK21 from strain 5RL were used as template DNAs for PCR amplification. PCR amplifications were carried out by using a DNA thermal cycler and an AmpliTaq kit (Perkin Elmer Cetus, Norwalk, Conn.). The template DNA was initially denatured at 94°C for 5 min. A total of 25 cycles were performed under the following conditions: primer annealing at 55°C for 1 min, DNA extension at 72°C for 2 min, and denaturation at 94°C for 1 min. PCR-amplified DNAs were detected by using gel electrophoresis (1% agarose gel).

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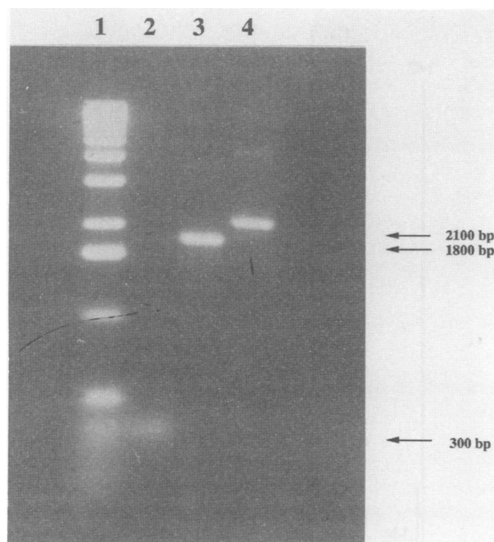

FIG. 1. Agarose (1%) gel of PCR products. Lane 1, 1-kb ladder; lane 2, 300-bp DNA fragment which was generated from primers 1 (*nahG*) and 3 (Tn*4431*); lane 3, 1,800-bp DNA fragment which was generated from primers 2 (*nahH*) and 3 (Tn*4431*); lane 4, 2,100-bp DNA fragment which was generated from primers 1 and 2.

Parent plasmid pKA1 (11) and highly characterized plasmid NAH7 were subjected to PCR by using primers 1 and 2, generating a 2,100-bp fragment (Fig. 1, lane 4). The results confirmed that these two plasmids share a high degree of complementary gene sequence and gene order for the lower pathway.

It had been proposed previously that Tn*4431* was inserted into the *nahG* gene of pUTK21 (11). No amplified fragment was generated by the PCR when pUTK21 was used as the template DNA with primers 1 and 2. The 2,100-bp fragment, which was amplified by using parent plasmid pKA1 as the template DNA, was absent because insertion of the 15-kb Tn*4431* exceeded the approximately 3,000-bp limit for the PCR under the amplification conditions used. Amplification with the PCR by using primers 1 and 3 and primers 2 and 3 generated DNA fragments that were 300 and 1,800 bp long, respectively (Fig. 1, lanes 2 and 3). Using the same primer pairs with parent plasmid pKA1 did not produce any amplified products. These results show that the insertion site of *lux* transposon Tn*4431* (25) is located 300 bp from the start of the *nahG* gene. These data also show that there is no sequence complementary to primer 3 on pKA1 that would generate the results with pUTK21 as the template DNA.

Metabolism of anthracene and phenanthrene. *P. fluorescens* 5RL was grown in a yeast extract-peptone-salicylate-succinate (YEPSS) medium containing (per liter) 0.2 g of yeast extract, 2 g of peptone, 0.5 g of sodium salicylate, 2.7 g of sodium succinate, and 0.2 g of NH_4NO_3. The medium was adjusted to pH 7.0 with 10 N NaOH. Before use as inocula, the bacteria were transferred from a YEPSS agar plate to 100 ml of YEPSS medium in a 500-ml flask. Cultures were incubated at 27°C in an environmental growth chamber at 200 rpm. After 24 h, 25-ml quantities of the culture were used to inoculate six 2.8-liter Erlenmeyer flasks, each of which contained 500 ml of YEPSS medium; cells were harvested by centrifugation in the exponential phase of growth. The cells were washed three times with 50 mM KH_2PO_4 buffer (pH 7.5).

Polycyclicaromatic hydrocarbon (PAH) biotransformation experiments were performed by suspending cells to a final concentration of 2.0 g (wet weight) per 100 ml of phosphate buffer in a 500-ml Erlenmeyer flask. Substrates were added (20 mg dissolved in 0.5 ml of *N,N*-dimethylformamide), and the cultures were incubated at 27°C as described above for 24 h. Incubation of the substrates in the YEPSS medium without microorganisms was used as a substrate control. Phenanthrene was purified by sublimation before use. All biotransformation experiments were repeated three times.

After centrifugation, the cell-free suspensions were extracted with ethyl acetate (neutral extract; three 100-ml volumes). The aqueous layer was then acidified (pH <2.0) with concentrated H_2SO_4 and extracted with ethyl acetate (acid extract) as described above. Both ethyl acetate extracts (neutral and acid extracts) were dried over anhydrous sodium sulfate, and the solvent was removed in vacuo at 30°C, leaving dark yellow residues. The residues were dissolved in 1.0 ml of chloroform or methanol and analyzed for metabolites by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography-mass spectrometry (GC-MS). Compounds corresponding to anthracene or phenanthrene-related metabolites were not detected in the substrate controls.

Analysis and identification of metabolites. Ethyl acetate extraction residues were separated and identified by TLC and HPLC. All TLC analyses were performed with Silica Gel 60 (F_{254}) on a precoated plastic sheet (EM Separations, Gibbstown, N.J.). The TLC preparations were developed in either a chloroform-methanol (98:2, vol/vol) solvent system or a benzene-acetate-water (125:74:1, vol/vol/vol) (16) solvent system. Metabolites were visualized under UV light. The chloroform-methanol system separated undegraded substrates and other less polar compounds from more polar metabolites. The benzene-acetate-water system separated highly polar, possibly acidic metabolites from substrates that remained at or near the origin of the TLC plates with the chloroform-methanol solvent system.

Normal-phase HPLC analyses were performed with a Waters system (Millipore Corp., Milford, Mass.) consisting of three model 501 pumps, a model 486 tunable absorbance detector operated at 254 nm, and a model 420 fluorescence detector equipped with an excitation-emission filter (338 and 425 nm). The metabolites of anthracene and phenanthrene were separated on a 5-μm Adsorbosphere CN column (4.6 by 250 mm; Alltech, Deerfield, Ill.) with an absolute ethanol–*n*-hexane linear gradient solvent system (2 to 4% [vol/vol] ethanol, 15 min) at a flow rate of 1 ml/min. All samples were prefiltered through a 0.45-μm-pore-size PTFE filter (Gelman) prior to analysis by HPLC.

The metabolites were identified by GC-MS by using a Hewlett-Packard model 5995A apparatus equipped with a GC-MS capillary interface (model 18964A) and a flame ionization detector (model 18965A). Analyses were carried out by using the electron impact mode at an ionization voltage of 70 eV. A BP5 capillary column (0.22 mm by 12 m; SGE, Inc., Austin, Tex.) was used for the gas chromatography analysis. Samples were injected into the gas chromatograph at 50°C and held isothermally for 2 min, and the oven temperature was programmed to increase to 250°C at a rate of 10°C/min.

Preliminary results from TLC indicated that acidic extracts of both anthracene and phenanthrene produced tailing spots that resembled the spots of the standard compounds, 2-hydroxy-3-naphthoic acid (ICN Biomedicals, Inc., Costa

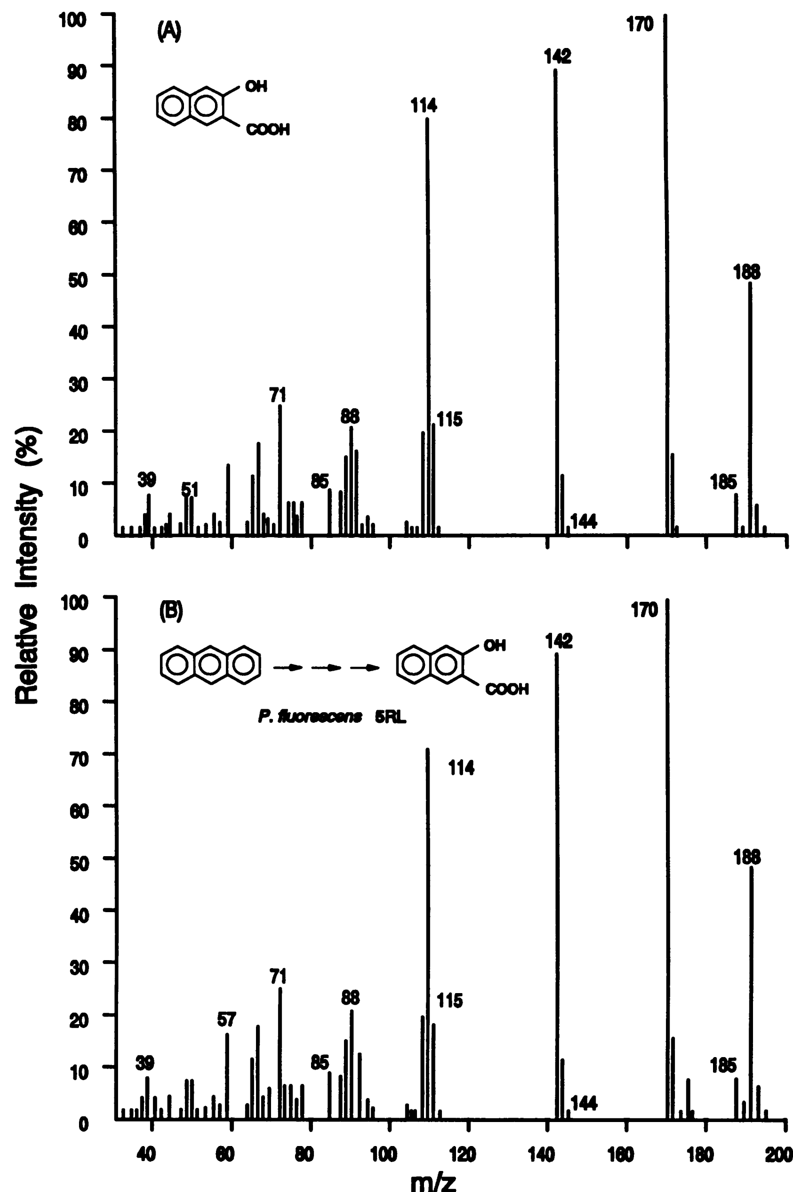

FIG. 2. Mass spectrum of the metabolite formed from the degradation of anthracene. (A) Authentic 2-hydroxy-3-naphthoic acid. (B) Metabolite extracted from ethyl acetate and purified by preparative TLC. This metabolite had a GC-MS retention time of 14.08 min.

Mesa, Calif.) and 1-hydroxy-2-naphthoic acid (Aldrich Chemical Co., Milwaukee, Wis.), respectively.

HPLC analysis of the metabolite produced from anthracene by *P. fluorescens* 5RL showed that a compound eluted from the column at 11.8 min, which was consistent with the retention time obtained for the authentic standard 2-hydroxy-3-naphthoic acid. HPLC analysis showed that the acidic sample formed from phenanthrene, a compound with a retention time of 9.8 min, was similar to the standard compound, 1-hydroxy-2-naphthoic acid.

The metabolite formed from anthracene produced a mass spectrum (Fig. 2B) that had an M^+ at *m/z* 188 and major fragment ions at *m/z* 170 (base peak, M^+-H_2O), 142 (M^+-H_2O-CO), and 114 (142-CO). The spectrum was consistent with the spectrum of authentic 2-hydroxy-3-naphthoic acid (Fig. 2A). The intermediate detected in the biotransformation of phenanthrene gave a mass spectrum (Fig. 3B) that had a molecular ion (*m/z* 188) and fragmentation pattern (*m/z* 170 [M^+-H_2O, base peak] and 114) consistent with the spectrum of the authentic compound, 1-hydroxy-2-naphthoic acid (Fig. 3A). The results described above from HPLC and GC-MS analyses indicate that the polycyclic aromatic acids which we found are intermediates in the degradation pathways of anthracene and phenanthrene by *P. fluorescens* 5RL.

Accumulation of the two metabolites (2-hydroxy-3-naphthoic acid and 1-hydroxy-2-naphthoic acid formed from anthracene and phenanthrene) was detected only when blocked mutant strain 5RL was used in the transformation study. These two compounds are structurally analogous to salicylate, which cannot be further degraded by strain 5RL in the naphthalene pathway because of blockage of the *nahG*

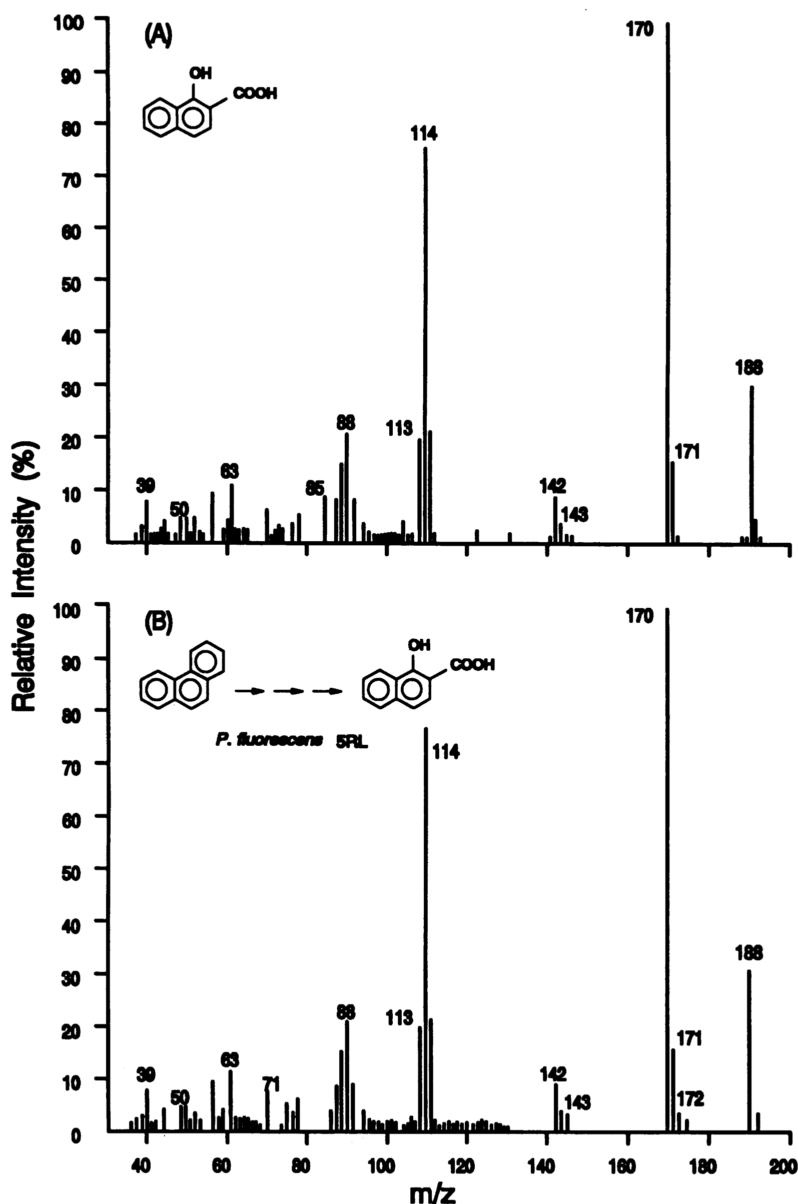

FIG. 3. Mass spectrum of the intermediate produced from the oxidation of phenanthrene. (A) Standard 1-hydroxy-2-naphthoic acid. (B) Intermediate extracted from ethyl acetate and analyzed by GC-MS. The retention time of the intermediate was 14.32 min.

gene. A similar result was observed in spray plate assays, in which strain 5RL could not clear anthracene and phenanthrene on a plate, but parental strain 5R could produce a clear zone (12).

It is not yet clear whether the two intermediates described above can be further degraded by salicylate hydroxylase and then reenter the upper or lower pathway of naphthalene catabolism or whether they utilize a different pathway. It is not clear that these two acid metabolites are further utilized by salicylate hydroxylase (*nahG*) to form 2,3- and 1,2-dihydroxynaphthalenes, natural intermediates in the naphthalene pathway formed by soil pseudomonads (9). The later metabolite formed from phenanthrene should reenter the upper naphthalene pathway for further complete degradation.

However, phenanthrene might be metabolized by an alternative mechanism which has been reported in an *Aeromonas* sp. (13–15). In the alternative pathway, 1-hydroxy-2-naphthoic acid is oxidized to 2-carboxybenzaldehyde, *o*-phthalic acid, and protocatechuic acid, which undergoes either *ortho* or *meta* cleavage depending on the organism. The 2,3-dihydroxynaphthalene formed from anthracene might be further cleaved through a *meta* pathway to form salicylaldehyde by releasing pyruvate. In other words, the acid metabolite formed from anthracene might go through the lower naphthalene pathway twice if there is no other pathway present in strain 5RL.

Microbial degradation of PAHs has been demonstrated for a wide variety of microorganisms (6, 9). However, no information about the association between pathways has

been reported, nor is it known whether a common degradative pathway exists in the bacteria for the degradation of PAHs. The character of the naphthalene degradative pathway in the biodegradation of polycyclic compounds must be determined because it has been shown that the NAH plasmid mediates the degradation of anthracene and phenanthrene (this study) and is implicated in dibenzofuran degradation (24). In order to characterize the catabolic pathway relationships between the higher-molecular-weight PAHs (more than two rings) and naphthalene and to elucidate the catabolic pathways of these PAHs, genetically engineered mutants such as strain 5RL that are blocked in different stages of the naphthalene degradation pathway are useful. The role of the NAH lower naphthalene pathway (salicylate to catechol) in the degradation of higher-molecular-weight PAHs also will require further investigation.

We thank A. Heitzer for discussions during manuscript preparation and K. Harp for editing the manuscript.

This investigation was supported by the Gas Research Institute (contract 5089-226-1933) and by the U.S. Air Force Office of Scientific Research (grant F49620-92-J-0147).

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