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EXPERIMENTAL ARTICLES

Degradation of Phenanthrene by Mutant Naphthalene-Degrading *Pseudomonas putida* Strains

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Abstract—Five naphthalene- and salicylate-utilizing *Pseudomonas putida* strains cultivated for a long time on phenanthrene produced mutants capable of growing on this substrate and 1-hydroxy-2-naphthoate as the sole sources of carbon and energy. The mutants catabolize phenanthrene with the formation of 1-hydroxy-2-naphthoate, 2-hydroxy-1-naphthoate, salicylate, and catechol. The latter products are further metabolized by the *meta*- and *ortho*-cleavage pathways. In all five mutants, naphthalene and phenanthrene are utilized with the involvement of plasmid-born genes. The acquired ability of naphthalene-degrading strains to grow on phenanthrene is explained by the fact that the inducible character of the synthesis of naphthalene dioxygenase, the key enzyme of naphthalene and phenanthrene degradation, becomes constitutive.

Key words: phenanthrene, naphthalene, biodegradation, *Pseudomonas putida*, naphthalene dioxygenase

Polycyclic aromatic hydrocarbons (PAHs) are abundant pollutants of the environment. The microbial degradation of PAHs is the main process leading to the self-cleaning of PAH-contaminated soils and water bodies. Of great potential in this respect are pseudomonads, which are able to utilize or partially transform naphthalene, phenanthrene, fluorene, and other PAHs [1]. The biodegradation of naphthalene has been studied in great detail [2]. At the same time, little is known about phenanthrene-degrading microorganisms, since phenanthrene is a recalcitrant compound poorly soluble in water. The similarity of the structure of naphthalene and phenanthrene and the enzyme systems involved in their catabolism in naphthalene-degrading microorganisms suggests that some modification of these systems may lead to the ability of the microorganisms to utilize phenanthrene. The obtaining of bacterial strains with such ease, as well as strains with the ability to utilize several PAHs, is of great interest from the standpoint of understanding the main principles of PAH utilization and their use in bioremediation technologies. The acquisition of novel degradative properties from microorganisms cultivated under laboratory conditions simulates the respective processes occurring in nature. Therefore, studies of the underlying genetic events leading to changes in the range of utilizable substrates can provide better insight into the evolution of the systems responsible for the degradation of various pollutants by microorganisms.

The aim of the present work is to study the biochemical pathways and respective genetic systems involved in the degradation of naphthalene and phenanthrene by *P. putida* strains, as well as to study the mutations that make naphthalene-degrading microorganisms capable of utilizing phenanthrene.

MATERIALS AND METHODS

The microorganisms used in this study are listed in Table 1. Bacteria were grown in mineral Evans medium [3] at 28°C. Salicylate and 1-hydroxy-2-naphthoate were added to the medium at a concentration of 1 g/l, and phenanthrene was added at a concentration of 0.2 g/l. When the microorganisms were grown on agar media, naphthalene was placed onto the lids of inverted petri dishes. The ability of the strains to transform phenanthrene was tested by the Kiyohara method [4].

Phenanthrene metabolites were separated by HPLC using a (60 × 250 mm) reversed-phase Octadecyl Si-100 (particle size 30 μm) column safeguarded with a (4.6 × 75 mm) Octadecyl Si-60 (particle size 20–40 μm) precolumn (Serva, Germany). The metabolites were eluted with a gradient of 0–15% B. Solvent A was 45% methanol containing 25 mM H_3PO_4, and solvent B was 100% methanol containing the same amount of H_3PO_4. The column was kept at 50°C. The absorbance of the eluate was detected at 254 nm and interfaced, using a Nelson Analytical 900 Series, to an Olivetty M-24 PC. The retention times of 2-naphthol, 1-hydroxy-2-naph-

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Table 1. *P. putida* strains and plasmids used in the work

Strain	Plasmid	Relevant phenotype*	Source
BS202	NPL-1	$Nah^+Sal^+Phn^-Hna^-$	Laboratory collection
BS202-P1	pBS1180	$Nah^+Sal^+Phn^+Hna^+$	The same
BS202-P2	pBS1180-1	$Nah^+Sal^+Phn^+Hna^+$	The present work
BS3701-N	pBS1141 pBS1142	Nah^+Sal^+	Laboratory collection
BS3701-P	pBS1141-1 pBS1142	$Nah^+Sal^+Phn^+Hna^+$	The same
BS590	pBS216	$Nah^+Sal^+Cys^-$	"
BS590-P	pBS216-1	$Nah^+Sal^+Phn^+Hna^+Cys^-$	"
BS3749	pBS1181	Nah^+Sal^+	"
BS3750	pBS1181-1	$Nah^+Sal^+Phn^+Hna^+$	"
BS575	pBS101	$Nah^+Sal^+Cys^-$	"
BS575-P	pBS101-1	$Nah^+Sal^+Phn^+Hna^+Cys^-$	The present work
BS394	–	$Nah^-Sal^-Cys^-Nal^RSm^R$	Laboratory collection

* Nah^+, Sal^+, Hna^+, and Phn^+ stand for the ability to grow on naphthalene, salicylate, 1-hydroxy-2-naphthoate, and phenanthrene, respectively. Cys^- stands for cysteine auxotrophy. Nal^R and Sm^R indicate resistance to nalidixic acid and streptomycin, respectively.

thoic acid (1H2N), 2-hydroxy-1-naphthoic acid (2H1N), dihydroxy-2-naphthoic acid, and phenanthrene were 8.83, 9.65, 10.06, 11.23, and 12.80 min, respectively.

Cell-free extracts were obtained by disintegrating the frozen biomass in an IBPM press (Russia). Cell debris was removed by centrifugation at 15000 rpm for 60 min. Salicylate hydroxylase and 1-hydroxy-2-naphthoate hydroxylase were assayed using the methods described by Shamsuzzaman and Barnsley [5]. The activities of catechol-1,2-dioxygenase and catechol-1,2-dioxygenase were measured using the methods of Hegeman [6] and Feist and Hegeman [7], respectively. Membrane-bound proteins were isolated according to the descriptions of Saemory *et al.* [8]. The activities of naphthalene and phenanthrene dioxygenases were measured using the method of Dua and Meera [9], and those of gentisate-1,2-dioxygenase using the method of Crawford and Frick [10].

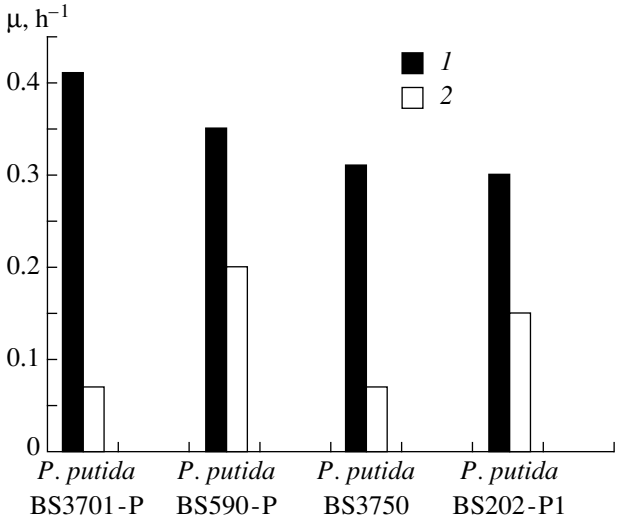

Fig. 1. Specific growth rates of PAH-degrading *P. putida* strains batch cultivated on (*1*) naphthalene and (*2*) phenanthrene as the sole sources of carbon and energy.

Protein concentration was determined spectrophotometrically [11].

Plasmid DNA was isolated by the rapid alkaline extraction procedure [12]. Plasmids were eliminated according to the methods of Rheinwald *et al.* [13]. The conjugative transfer of plasmids was carried out on an agar medium [14]. DNA electrophoresis was performed by the standard procedure [15]. Plasmid DNA was digested by restriction endonucleases, as recommended by their producer (Amersham, United Kingdom), using buffer solutions from the same company.

RESULTS

Isolation and Characterization of Phenanthrene-degrading Strains

The analysis of 40 naphthalene-degrading strains from the collection of microorganisms of the Laboratory of Plasmid Biology, Skryabin Institute of Biochemistry and Physiology of Microorganisms at Pushchino, showed that all of the strains were able to transform phenanthrene, but only three of them were able to grow on it, i.e., to utilize this substrate as the sole source of carbon and energy [16]. The cultivation of the 40 strains mentioned in a mineral medium with

Table 2. Specific activities of the naphthalene and phenanthrene catabolism enzymes in *P. putida* strains

Strain	Carbon source	Specific activity, μmol/(min mg protein)					
		NO	PO	SH	1H2NH	C1, 2O	C2, 3O
BS202	Succinate	<0.001	ND	0.001	0.003	0.003	0.003
	Salicylate	0.016	ND	0.266	0.047	0.110	<0.001
BS202-P1	Succinate	0.038	0.018	<0.001	<0.001	0.006	0.042
	Salicylate	0.023	0.015	0.163	0.032	0.006	0.016
BS202-P2	Succinate	0.010	0.014	<0.001	<0.001	0.005	0.015
	Salicylate	0.010	0.010	0.264	0.040	0.183	0.004
BS3701-N	Succinate	<0.001	<0.001	<0.001	<0.001	<0.001	0.014
	Salicylate	0.012	0.010	0.090	0.040	0.120	0.015
BS3701-P	Succinate	0.010	0.011	<0.001	<0.001	<0.001	0.012
	Salicylate	0.015	0.014	0.094	0.074	0.174	0.014
BS590	Succinate	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Salicylate	0.024	0.021	0.118	0.014	0.014	<0.001
BS590-P	Succinate	0.008	0.009	<0.001	<0.001	0.003	<0.001
	Salicylate	0.036	0.011	0.123	0.019	0.075	0.023

Note: NO is naphthalene dioxygenase; PO is phenanthrene dioxygenase; SH is salicylate hydroxylase; 1H2NH is 1-hydroxy-2-naphthoate hydroxylase; C1,2O is catechol-1,2-dioxygenase; and C2,3O is catechol-2,3-dioxygenase. "ND" stands for "not determined."

phenanthrene for 1.5 months resulted in the acquired ability of the additional five *Pseudomonas putida* strains, BS202, BS3701-N, BS590, BS3749, and BS575, to grow on phenanthrene. The two similar phenanthrene-utilizing mutants derived from strain BS202 were designated BS202-P1 and BS202-P2. The phenanthrene-utilizing mutants derived from strains BS3701-N, BS590, BS3749, and BS575 were designated BS3701-P, BS590-P, BS3750, and BS575-P, respectively (Table 1).

All the phenanthrene-utilizing mutants were able to grow on 1-hydroxy-2-naphthoate (1 g/l), an intermediate of phenanthrene catabolism, while the parent strains of these mutants were unable to grow on this substrate. Strain BS3701-P exhibited the highest specific growth rate on the naphthalene, while BS590-P was the fastest-growing strain on the phenanthrene (Fig. 1).

Identification of Phenanthrene Intermediates

Phenanthrene is catabolized by bacteria with the formation of 1-hydroxy-2-naphthoic acid, which is further utilized either via salicylate and catechol or via *ortho*-phthalate and protocatechuate [17].

Analysis of the culture liquid of strain BS3701-P in the process of its growth on phenanthrene showed that this strain initially produces 1H2N, the concentration of which then drops, and two new products are formed. Mass spectrometry revealed that one of these products is 2-hydroxy-1-naphthoate and the other is dihydroxy-2-naphthoate. The culture liquid was also found to contain trace amounts of 2-naphthol, which, as further experiments showed, is the product of 2H1N autoxidation. Therefore, the phenanthrene utilization pathway in strain BS3701-P differs from those described earlier for other bacteria [17]. When strain BS3701-P was grown on 1H2N, neither 2H1N nor dihydroxy-2-naphthoate was detected in the culture liquid, although mass spectrometry showed the presence of trace amounts of salicylate formed from 1H2N. Salicylate was further metabolized with the formation of the Krebs cycle products.

Analysis of the other four phenanthrene-utilizing mutants showed that they produce 1H2N and 2H1N, while no dihydroxy-2-naphthoate was detected in any of these strains.

Assay of Enzymes Involved in the Biodegradation of Naphthalene and Phenanthrene

All of the phenanthrene-degrading strains grown on salicylate (Table 2) or phenanthrene (data not shown) exhibited activities of the key enzymes of naphthalene and phenanthrene degradation, salicylate hydroxylase, catechol-1,2-dioxygenase, and catechol-2,3-dioxygenase (Table 2). These data suggest that phenanthrene is utilized through 1-hydroxy-2-naphthoate, salicylate, and catechol, with the cleavage of the last intermediate occurring in the ortho- and meta-pathways. Based on the data from the enzyme assays and the mass spectrometry of products present in culture liquids, we proposed a putative scheme of phenanthrene metabolism in the strains studied (Fig. 2). The data presented in Table 2 show that all of the strains, both parent and mutant, possess 1-hydroxy-2-naphthoate hydroxylase, and that salicylate hydroxylase, and 1-hydroxy-2-naphthoate hydroxylase are induced by salicylate.

The other key enzymes of naphthalene and phenanthrene catabolism, naphthalene dioxygenase and phenanthrene dioxygenase, were also detected in both

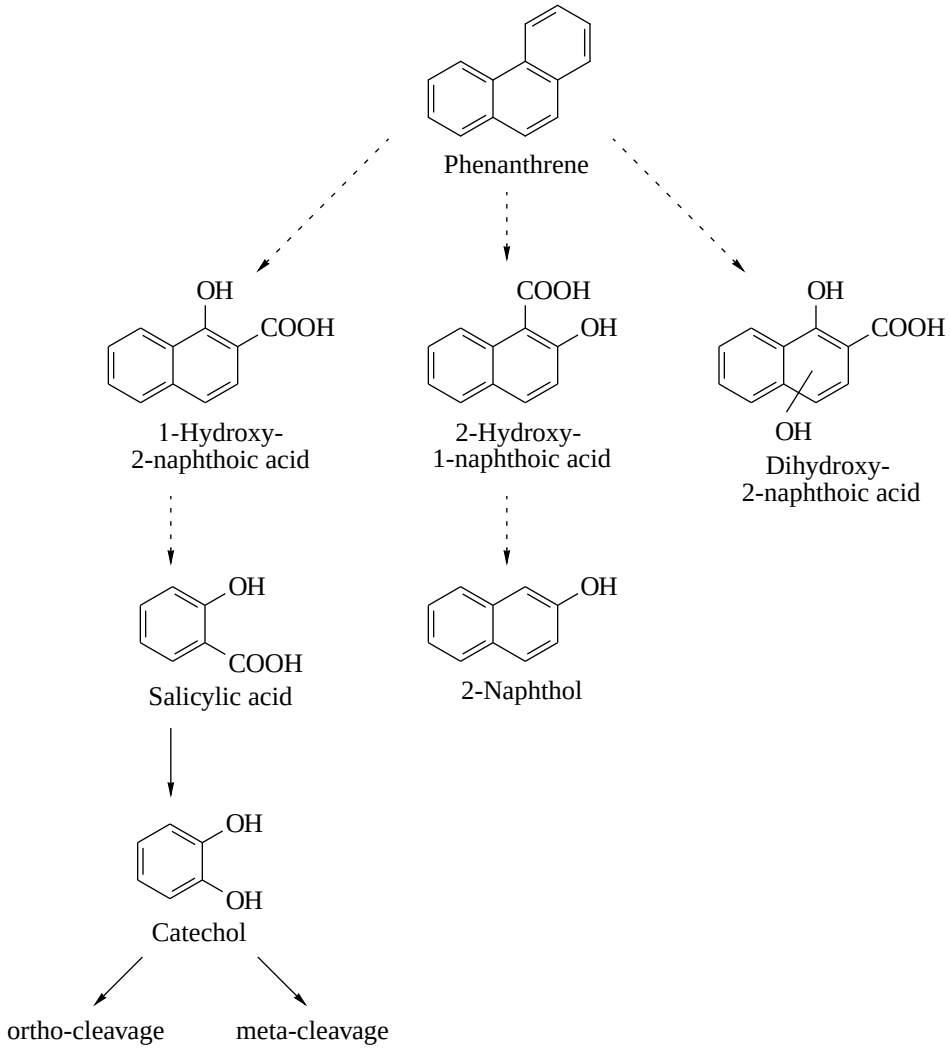

Fig. 2. Putative scheme of phenanthrene utilization by *P. putida* strains.

the parent and mutant strains. However, these enzymes were induced by salicylate in the parent strains and not in the phenanthrene-utilizing mutant strains.

Genetic Control over Phenanthrene Degradation

All of the strains studied, both parent and mutant, turned out to contain plasmids ranging in size from 50 to 120 kbp. Experiments on the elimination and conjugative transfer of these plasmids to the plasmid-free recipient strain BS394 showed that plasmids pBS216-1 (83 kbp), pBS101-1 (50 kbp), and pBS1181-1 (120 kbp), which are present in the strains BS590-P, BS575-P, and BS3750 with the phenotype $Nah^+Sal^+Phn^+Hna^+$, contain all of the genes that are necessary for the expression of this phenotype (Table 1). The transconjugants obtained were able to grow on naphthalene, grew poorly on phenanthrene, and failed to grow on salicylate or 1-hydroxy-2-naphthoate. The also transconjugants accumulated considerable amounts of 1H2N, which was not further metabolized.

Thus, the degradation of phenanthrene to 1H2N and of naphthalene to salicylate in the strain BS3701-P is controlled by the plasmid pBS1141-1. The further oxidation of 1-hydroxy-2-naphthoate is controlled by both plasmid and chromosomal genes. Chromosomal genes are also responsible for the ability of strain BS3701-P to grow on salicylate, which is utilized by the *ortho*-pathway of catechol oxidation.

In strains BS202-P1 and BS202-P2, plasmids pBS1180 and pBS1180-1, like the plasmid pBS1141-1 of strain BS3701-P, control the first steps of degradation of naphthalene and phenanthrene. The salicylate hydroxylase and 1-hydroxy-2-naphthoate hydroxylase genes are located on the chromosome.

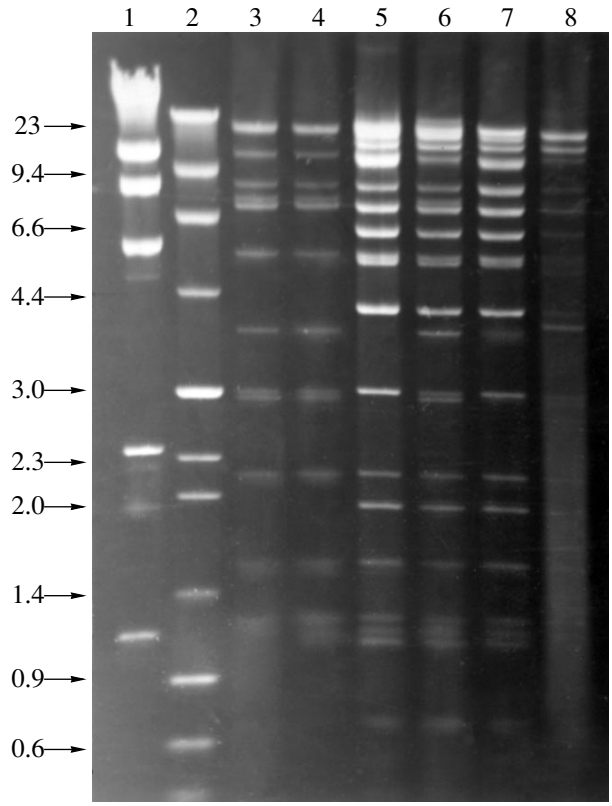

Fig. 3. Electrophoresis of plasmid DNA digested with the restriction endonuclease *Hin*dIII. Lanes: 1, NAH7–*Xho*I; 2, marker DNA; 3 and 4, NPL-1; 5, pBS1180; 6, pBS1180-1; 7, pBS1141-1 and pBS1142; 8, pBS1141 and pBS1142.

The *Eco*R1 and *Hin*dIII restriction fragment maps of plasmids isolated from parent strains with the phenotype $Nah^+Sal^+Phn^-Hna^-$ differed from those of plasmids isolated from mutant strains with the phenotype $Nah^+Sal^+Phn^+Hna^+$. It should be noted that the restriction fragment maps of plasmids isolated from strains BS202-P1 and BS202-P2 differed from each other (Fig. 3), although these plasmids have the same parent plasmid, NPL-1, and control the same mutant characteristic (the constitutive synthesis of naphthalene dioxygenase). Thus, the phenotype Phn^+Hna^+ of mutant *P. putida* strains is related to structural changes in the plasmids governing naphthalene catabolism. These changes make it possible for mutant strains to grow on phenanthrene, and the ability to grow on naphthalene is preserved.

DISCUSSION

Typically, naphthalene-degrading microorganisms are able to transform phenanthrene, although the ability to utilize phenanthrene as the sole source of carbon and energy is very uncommon to these microorganisms. The data presented in this paper demonstrate the possibility of obtaining phenanthrene-utilizing mutants from naphthalene-degrading strains. The fact that all of the phenanthrene-utilizing strains obtained in this study also grew on 1H2N suggests that 1-hydroxy-2-naphthoate hydroxylase, which converts 1H2N to 1,2-dihydroxynaphthalene, is the key enzyme of phenanthrene degradation. In our previous genetic and biochemical studies of salicylate hydroxylase and its gene *nahG*, we showed that it is this enzyme that is responsible for the 1-hydroxy-2-naphthoate hydroxylase activity of strain BS202-P [18, 19]. The 1-hydroxy-2-naphthoate hydroxylase activities of strains capable of growth on phenanthrene and 1H2N do not exceed the respective activities of strains incapable of growth on these substrates (Table 2). Therefore, the presence of 1-hydroxy-2-naphthoate hydroxylase activity in a microorganism is insufficient for its ability to grow on 1H2N.

Studies of the naphthalene and phenanthrene degradation pathways and the genetic control over them showed that both PAHs are utilized with the involvement of the same enzymes with a broad substrate specificity. Analysis of the expression of the cloned gene *nahA* that encodes naphthalene dioxygenase showed that this enzyme is also active with phenanthrene (unpublished data). It is known that the naphthalene degradation genes of many pseudomonads are organized into two operons, one of which (*nah* operon) controls the upper pathway of naphthalene conversion into salicylate, and the other (*sal* operon) of which controls the lower pathway of salicylate cleavage to the Krebs

cycle products. The enzymes encoded by the respective genes of these operons are induced by salicylate (the product of naphthalene oxidation) in the presence of the regulatory *nahR* gene product [2]. The inducible character of expression of the genes of both operons is typical of all the strains with the phenotype $Nah^+Sal^+Phn^-Hna^-$ (Table 2). At the same time, strains with the phenotype $Nah^+Sal^+Phn^+Hna^+$ are characterized by the constitutive synthesis of phenanthrene and naphthalene dioxygenases (the initiating enzymes of degradation of these substrates). In this case, salicylate, which induces the expression of genes of the upper and lower pathways of naphthalene oxidation, is produced by naphthalene dioxygenase from naphthalene. In microorganisms with the phenotype Nah^+Phn^-, the basal level of activity of naphthalene dioxygenase is obviously insufficient to form the necessary amounts of salicylate and thus to induce the relevant enzymes.

The similarity of the restriction fragment maps of plasmids NPL-1 and pBS1141, which control the inducible synthesis of naphthalene dioxygenase, suggests that both plasmids may have a similar structure. These plasmids are not expressed in the pseudomonads whose chromosomes do not contain the salicylate hydroxylase gene linked to a regulatory gene. As shown earlier, the inversion of a DNA segment in plasmid NPL-1 gives rise to the mutant plasmid NPL-41, which governs the constitutive synthesis of the upper pathway of naphthalene oxidation and thus makes pseudomonads capable of growth on naphthalene with the formation of salicylate [20]. Like NPL-41, the mutant plasmids pBS1180-1, pBS1180-2, and pBS1141-1, when conjugatively transferred to recipient strains, impart the phenotype Nah^+Sal^-.

Thus, the acquired ability of naphthalene-degrading strains to grow on phenanthrene is explained by the fact that the inducible character of expression of genes controlling the upper pathway of naphthalene oxidation becomes constitutive. Mutants with the constitutive synthesis of degradative enzymes play an important role in the evolution of microorganisms, since they become able to utilize a wider range of chemical compounds. Constitutively expressed genes and operons can be maintained in bacterial populations by means of horizontal transfer.

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