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EXPERIMENTAL ARTICLES

The Microbial Transformation of Phenanthrene and Anthracene

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Abstract—The transformation of phenanthrene and anthracene by *Rhodococcus rhodnii* 135, *Pseudomonas fluorescens* 26K, and *Arthrobacter* sp. K3 is studied. Twenty-one intermediates of phenanthrene and anthracene transformation are identified by HPLC, mass spectrometry, and NMR spectroscopy. *P. fluorescens* 26K and *Arthrobacter* sp. K3 are found to produce a wide range of intermediates, whereas *R. rhodnii* 135 oxidizes phenanthrene, resulting in the formation of a sole product, 3-hydroxyphenanthrene. Putative transformation pathways of phenanthrene and anthracene are proposed for the three bacterial strains studied. These strains can be used to obtain valuable compounds (such as hydroxylated polycyclic aromatic hydrocarbons) that are difficult to produce by chemical synthesis.

Key words: phenanthrene, anthracene, biotransformation, *Rhodococcus, Pseudomonas*, *Arthrobacter.*

Phenanthrene and anthracene are polycyclic aromatic hydrocarbons (PAHs). Their bacterial degradation is being extensively studied with the aim of developing approaches to the bioremediation of PAH-contaminated soils and water [1]. The productive metabolism of phenanthrene (i.e., that associated with bacterial growth) begins with its oxidation by dioxygenase at positions C3 and C4. The resultant dihydrodiol sequentially transforms into 1-hydroxy-2-naphthoic acid, which can be metabolized through salicylate [2], phthalate [3], or, in some cases, 1-naphthol [4]. The productive metabolism of anthracene also occurs through the formation of 1-hydroxy-2-naphthoic acid, which is further transformed through salicylate [2] or, according to recent findings, through *o*-phthalate [5]. Some reactions occurring during the bacterial oxidation of phenanthrene and anthracene (such as oxidation at C9 and C10 atoms) are similar to the respective nonenzymatic reactions [6], but, usually, bacteria do not oxidize the most reactive atoms (for instance, C1 and C2) [7]. In fact, the reactions occurring during phenanthrene and anthracene oxidation are very diverse and far from being well understood.

The enzyme systems involved in PAH oxidation are not specific. For instance, the naphthalene dioxygenase of *Pseudomonas* sp. NCIB 9816 oxidizes to a greater extent than 50 PAHs, including phenanthrene and anthracene. This enzyme's broad substrate specificity, together with its strong regio- and stereospecificity toward particular substrates [7], is responsible for the relatively small degree of PAH conversion by whole bacterial cells, which often do not have the enzymes necessary for further utilization of the primary oxidation products of PAHs.

The microbial conversion of PAHs is considered to be a promising method for the production of compounds that are difficult to obtain by chemical synthesis [8]. According to Davies, the hydroxylation of inactive centers in hydrocarbons is potentially the most useful biotransformation reaction [9]. Some dihydroxylated and dehydrated derivatives of phenanthrene are pharmacologically important due to their antiallergic and anticancer properties.

It should be noted that most of the research in this area deals with the study of bacterial strains that produce the maximum degradation of PAHs, i.e., up to their complete mineralization, whereas bacterial strains that implement incomplete degradation of PAHs have remained beyond the interest of researchers. In order to fill this gap, the aim of the present study was the isolation of phenanthrene- and anthracene-transforming strains from hydrocarbon-polluted natural sources and the screening of laboratory collections for such strains. Our other objectives were the investigation of PAH transformation, the identification of PAH metabolites, and the study of their dynamics during transformation.

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The products of anthracene (1–6) and phenanthrene (7–21) biotransformation by the bacterial strains *P. fluorescens* 26K and *Arthrobacter* sp. K3

No.	R_f, TLC	Empirical formula	Major characteristic peaks in the mass spectrum, *m/z* (%)	Compound
1	0.35	$C_{14}H_8O_2$	M^+ 208 (96), 180 (100), 152 (83), 151 (43)	Anthraquinone
2	0.31	$C_{10}H_8O_2$	M^+ 160 (100), 144 (40), 131 (60), 128 (20), 120 (20), 115 (40), 91 (40)	2,3-Dihydroxynaphthalene
3	0.40	$C_{11}H_8O_3$	M^+ 188 (35), 189 (3), 171 (12), 170 (100), 143, 142, 115, 114, 86	2-Hydroxy-3-naphthoic acid
4	0.31	$C_{13}H_{10}O_2$	M^+ 198 (100),170 (30), 156 (80), 128 (40), 115 (100)	Reduced 6,7-benzocoumarin
5	0.68	$C_{14}H_{14}O_3$	M^+ 230 (52), 231 (8), 199 (24), 198 (100), 171 (15), 170 (62), 157 (27), 156 (25), 142 (16), 141 (28), 128 (52), 115 (25)	1-Hydroxy-2-naphthylpropionic acid, methyl ester
6	0.08	$C_{15}H_{16}O_3$	M^+ 244 (15), 172 (12), 171 (100), 170 (12), 143 (45), 142 (20), 141 (13), 115 (22)	1-Hydroxy-2-naphthylbutanoic acid, methyl ester
7	0.67	$C_{10}H_{10}O_4$	M^+ 194 (14), 163 (100), 164 (13), 135 (22), 120 (6), 104 (6), 103 (4), 92 (8), 76 (12), 59 (9)	*o*-Phthalic acid, dimethyl ester
8	0.40	$C_{14}H_{12}O_4$	M^+ 244 (64), 245 (9), 199 (22), 198 (73), 170 (47), 157 (56), 156 (100), 141 (25), 129 (31), 128 (70), 115 (27)	1-Carboxy-2-naphthylpropionic acid
9	0.7	$C_{16}H_{16}O_4$	M^+ 272 (100), 273 (17), 213 (20), 195 (25), 181 (35), 170 (28), 157 (93), 156 (62), 141 (32), 129 (31), 128 (64), 115 (28)	1-Carboxy-2-naphthylpropionic acid, dimethyl ester
10	0.55	$C_{13}H_{12}O_3$	M^+ 216 (43), 199 (13), 198 (76), 170 (98), 169 (24), 156 (100), 141 (30), 129 (24), 128 (68), 127 (59), 115 (24)	1-Hydroxy-2-naphthylpropionic acid
11	0.55	$C_{14}H_{14}O_3$	M^+ 230 (30), 212 (40), 184 (100)	1-Hydroxy-2-naphthylbutanoic acid
12	0.37	$C_{11}H_8O_3$	M^+ 188 (35), 189 (3), 171 (13), 170 (100), 142 (15), 116, 115 (61), 114 (11)	1-Hydroxy-2-naphthoic acid
13	0.31	$C_{13}H_{10}O_2$	M^+ 198 (100), 170 (30), 156 (80), 128 (40), 115 (100)	Reduced 7,8-benzocoumarin
14	0.40	$C_{10}H_8O$	M^+ 144 (100), 145 (11), 136 (3), 137 (4), 116 (10), 115 (40), 89 (7), 63 (5)	2-Hydroxynaphthalene
15	0.31	$C_{10}H_8O_2$	M^+ 160 (100), 144 (40), 131 (60), 128 (20), 120 (20), 115 (40), 91 (40)	Dihydroxynaphthalene
16	0.70	$C_{13}H_8O_2$	M^+ 196 (84), 168 (100), 139 (50), 140 (18), 113 (5)	7,8-Benzocoumarin
17	0.65	$C_{14}H_{10}O$	M^+ 194 (100), 165 (38), 139 (4)	3-Hydroxyphenanthrene
18	0.27	$C_{14}H_{12}O_2$	M^+ 212 (45), 194, 166 (72), 165 (100), 140 (26)	3,4-Dihydroxy-3,4-dihydrophenanthrene
19	0.30	$C_{10}H_8O_2$	M^+ 160 (100), 131 (13), 114 (42)	2,3-Naphthalenediol
20	0.70	$C_{13}H_8O_2$	M^+ 196 (100), 168 (35), 139 (38)	6,7-Benzocoumarin
21	0.52	$C_{13}H_{14}O_4$	M^+ 234 (20), 203 (100), 174 (30), 121 (10)	Carboxyphenylbutenic acid, dimethyl ester

MATERIALS AND METHODS

Reagents. The main reagents used in this work were 99% HPLC-pure phenanthrene and anthracene, purchased from Fluka; high-purity acetone and chemical-grade methanol, from Reakhim (Russia); and chemical-grade ethylacetate, from Ekos-1 (Russia). The methionine and salts (all of chemical grade) used for the preparation of media were purchased from Reakhim.

Cultivation medium. The mineral medium for the cultivation of bacterial strains contained (g/l) NH_4NO_3, 1.0; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; and $CaCl_2$, 0.02 (pH 7.5). The medium was supplemented with $FeCl_3$ (two drops of saturated solution per liter). The medium was filtered, dispensed in 100-ml portions into 750-ml flasks, and sterilized. Phenanthrene and anthracene were added to the medium in the form of acetone solutions. The medium was inoculated with bacterial cells after the flasks had been incubated on a shaker for 1 day to allow the acetone to evaporate.

The bacterial strains used in this study were mainly taken from the collection of xenobiotic-degrading microorganisms held by the Laboratory of Enzymatic Degradation of Organic Compounds at the Skryabin Institute of Biochemistry and Physiology of Microorganisms. One strain, however, was isolated from the wastewater of a tractor plant using an enrichment culture, with phenanthrene as the sole source of carbon and energy. The latter strain was identified by using the identification criteria of *Bergey's Manual* [10].

Cultivation conditions. The strains were cultivated at 29°C on a shaker (120 rpm). For experiments involving the collection strains and *Arthrobacter* sp. K3 grown without methionine, the inoculum consisted of 2-day-old cells washed off nutrient agar slants (the cells from one slant were used to inoculate one cultivation flask). The initial concentrations of phenanthrene and anthracene in the medium were 50 and 12 mg/ml, respectively. For experiments involving *Arthrobacter* sp. K3 grown in the presence of L-methionine

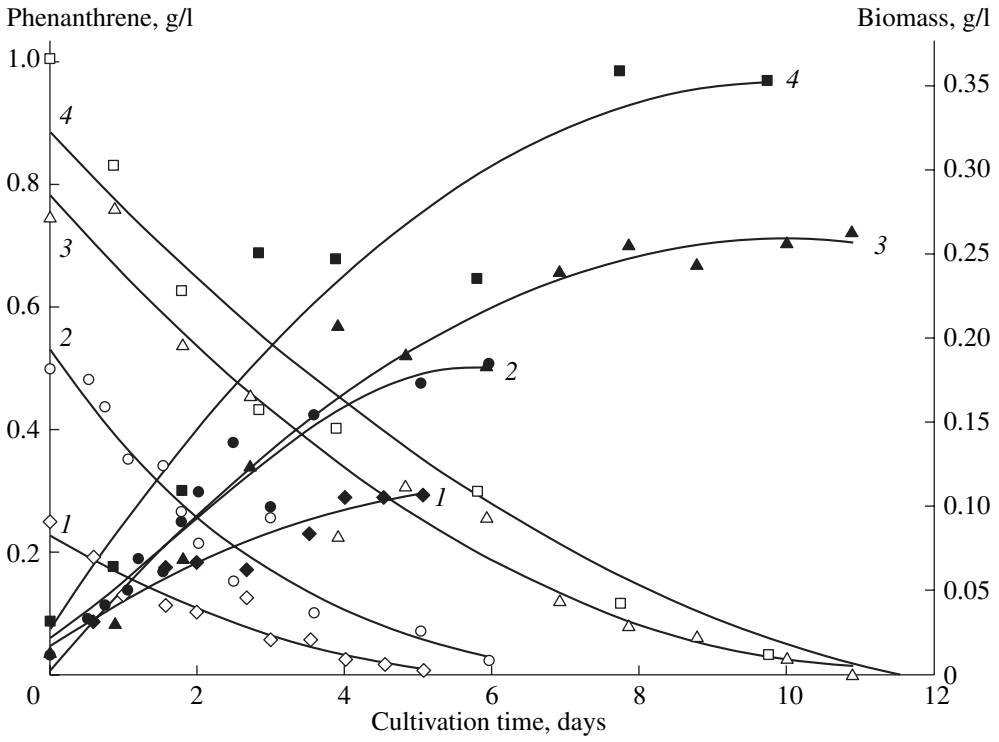

Fig. 1. Growth of *Arthrobacter* sp. K3 (filled symbols) and phenanthrene consumption (open symbols) in the mineral medium after supplementation with L-methionine (20 mg/l) and phenanthrene at concentrations of (*1*) 0.25, (*2*) 0.5, (*3*) 0.75, and (*4*) 1 g/l.

(20 mg/ml) and phenanthrene (0.1–1 g/l), the mineral medium was inoculated with 1 ml of a culture grown to the stationary phase in the presence of 0.2 g/l phenanthrene. In this case, the initial culture density was 0.68 mg/l.

Bacterial growth. The biomass of *Arthrobacter* sp. K3 was evaluated from the difference in the optical densities of the whole culture and the culture liquid from which bacterial cells were removed by centrifugation. The optical densities were measured at 540 nm using a KFK colorimeter. The biomass was calculated by using a calibration curve, which was constructed by measuring the dry weight of cells in suspensions with known optical densities [11].

Analytical methods. Phenanthrene and anthracene metabolites were extracted with ethylacetate from the culture liquid after it had been acidified with 1 N HCl to pH 2. The extract was evaporated at 37°C using a vacuum rotary evaporator. The dry residue was dissolved in a small volume of acetone. The acetone solution was qualitatively analyzed by thin-layer chromatography (TLC) on silica gel plates (Merck, Germany), which were developed using a benzene–dioxane–acetic acid (90 : 10 : 1) solvent mixture. The resulting metabolite spots were visualized under UV light. Hydroxylated PAH derivatives were identified after treatment with diazotized benzidine [12]. The phenanthrene and anthracene metabolites were isolated in preparative amounts by TLC.

The phenanthrene and anthracene contained in the extracts were quantitatively analyzed using a Pye-Unicam gas–liquid chromatograph equipped with a flame ionization detector and a (1.5 m × 2 mm) column

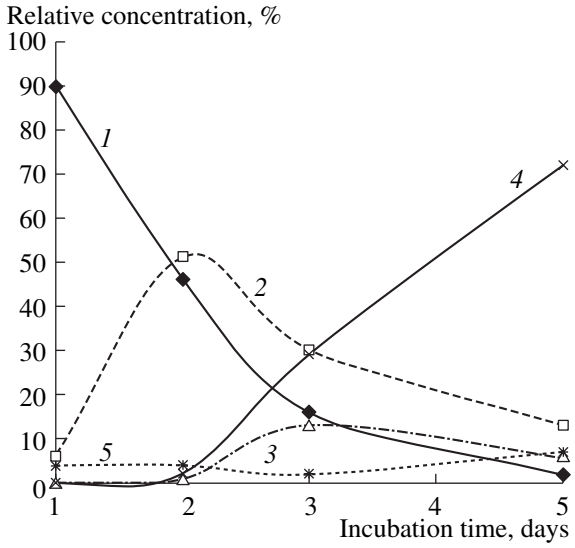

Fig. 2. The consumption of phenanthrene (*1*) and the dynamics of its bioconversion products during the incubation of *Arthrobacter* sp. K3 cells in the mineral medium: (*2*) 1-hydroxy-2-naphthoic acid, (*3*) 7,8-benzocoumarin, (*4*) *o*-phthalic acid, and (*5*) minor products.

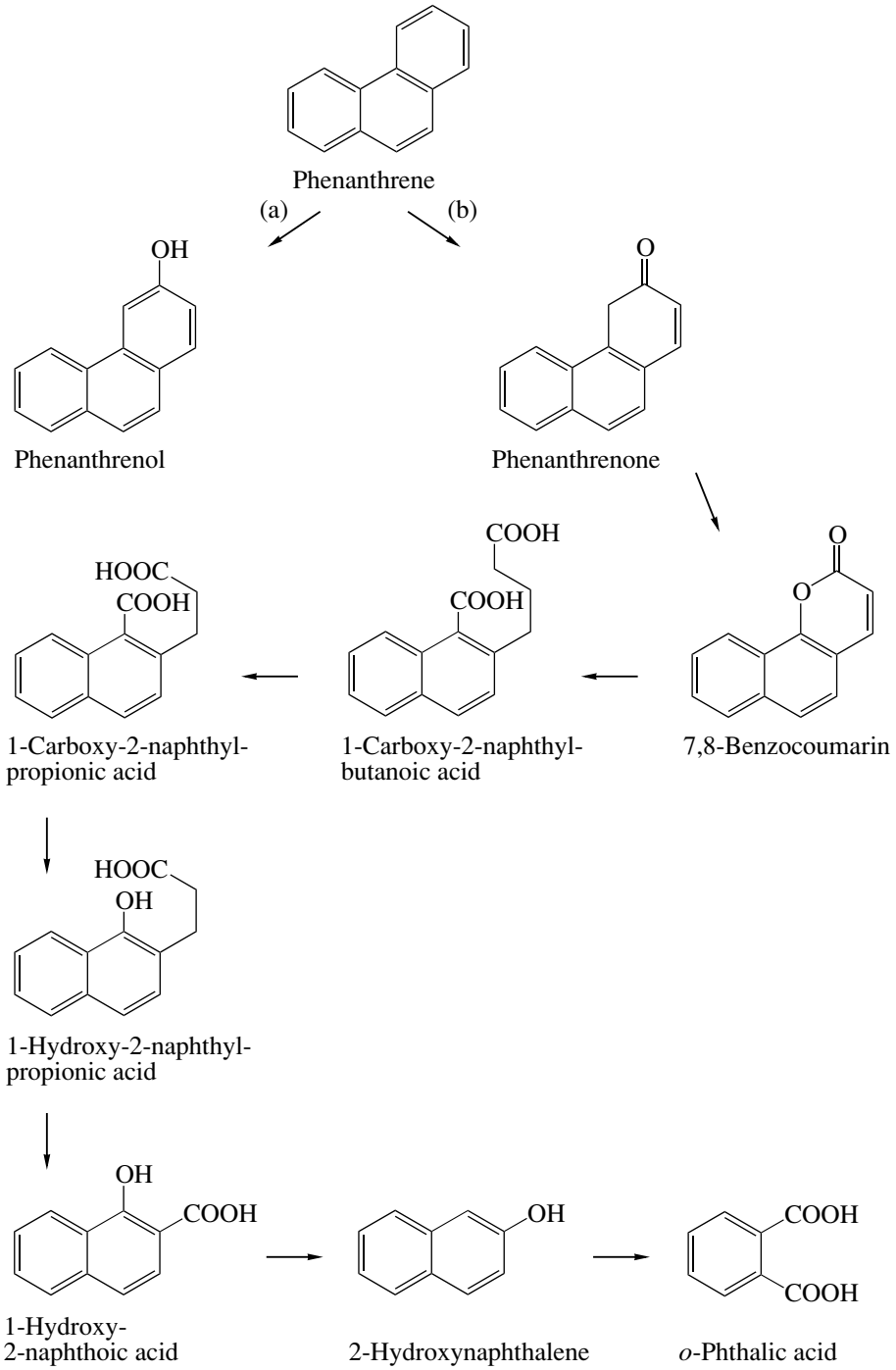

Fig. 3. Putative schemes of phenanthrene transformation by (a) *R. rhodnii* 135, (b) *P. fluorescens* 26K, and (c) *Arthrobacter* sp. K3.

packed with Chromosorb G-AW-DMSC containing 3% SE-30. Helium was used as a carrier gas at a flow rate of 40 ml/min. The injector, column, and detector were kept at 150, 185, and 290°C, respectively.

The phenanthrene and anthracene metabolites were analyzed using a Waters 996 HPLC chromatograph equipped with an Alltima C_{18} 5U (150 × 4.6 mm) column and a UV detector (254 nm). The column was eluted for 30 min with a gradient of methanol (40–85%) containing 0.1% trichloroacetic acid. The metabolites were identified using a Finnigan MAT 8430 mass spectrometer with ionization by electron impact at 70 eV and the direct injection of samples into the ionization zone. The structure of the analyzed compounds was determined based on the data of mass spectrometry, chromatography, and NMR spectrometry using authen-

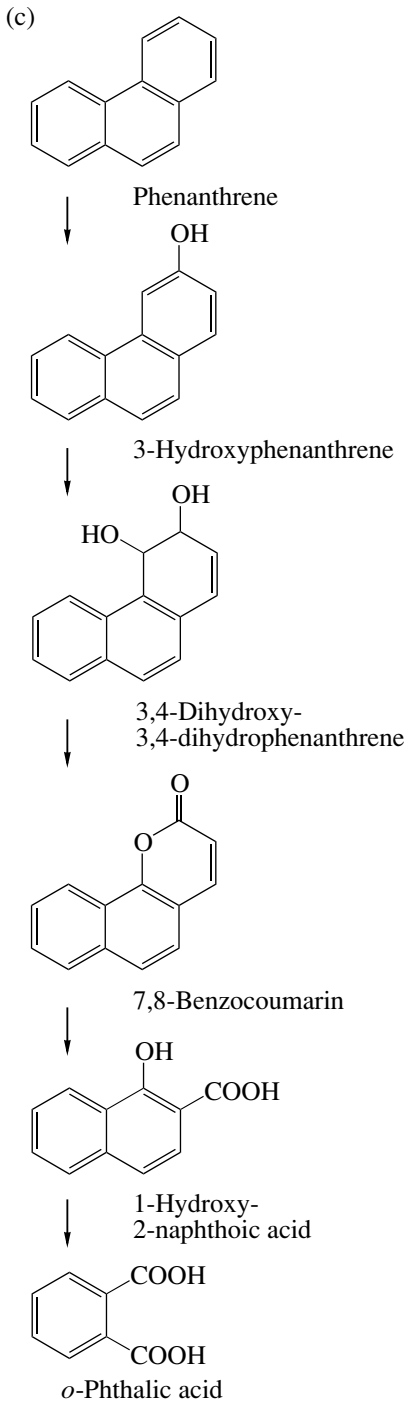

Fig. 3. (Contd.)

tic samples of these compounds (if they were available), and their spectra were taken from the data of other publications.

In preparation for NMR spectrometry, the samples were dissolved in 0.5 ml of deuterated acetonitrile. The NMR spectra were recorded at 400.131962 MHz using a Bruker DPX-400 spectrometer.

RESULTS AND DISCUSSION

The screening of the 46 bacterial strains available in the laboratory collection allowed us to reveal that two strains, *Rhodococcus rhodnii* 135 and *Pseudomonas fluorescens* 26K, were able to transform phenanthrene and anthracene.

Strain K3, which was isolated from the wastewater of a tractor plant, was identified as *Arthrobacter* sp. K3

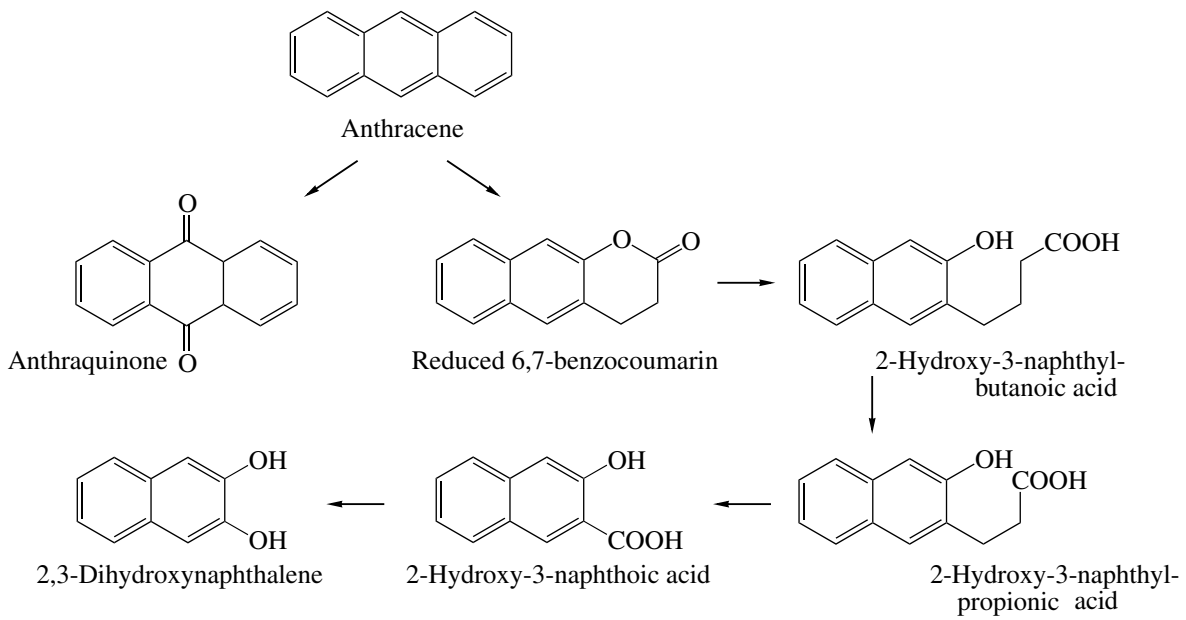

Fig. 4. Putative scheme of anthracene transformation by *P. fluorescens* 26K and *Arthrobacter* sp. K3.

based on its chemotaxonomic characteristics and specific growth cycle (rods–cocci) [10]. This strain was found to be auxotrophic for L-methionine.

The incubation of *R. rhodnii* 135 and *P. fluorescens* 26K in the medium containing phenanthrene led to the consumption this RAH, so that, at a concentration of 50 mg/l, it was completely consumed by *P. fluorescens* 26K within 3 days. Under these conditions, the degree of phenanthrene transformation by *R. rhodnii* 135 was 56%. In the sterile control, the concentration of phenanthrene did not decrease.

In the presence of L-methionine, *Arthrobacter* sp. K3 grew on phenanthrene (Fig. 1) with a growth yield $Y_{\text{biomass/phenanthrene}}$ equal to 0.34 ± 0.05. This strain was able to transform phenanthrene at concentrations of up to 1 g/l. If the *Arthrobacter* sp. K3 cells used for inoculation were grown on phenanthrene, the lag phase was virtually absent.

The transformation of phenanthrene and anthracene was accompanied by the formation of a great number of intermediates, some of which accumulated in the medium (this was typical of *R. rhodnii* 135) whereas others were further converted with the formation of new products. Overall, we isolated and identified 21 phenanthrene and anthracene metabolites (table).

The greatest number of metabolites were identified when phenathrene was transformed by the *P. fluorescens* 26K cells. In this case, the metabolites included phenanthrenone, 7,8-benzocoumarin, and cleavage products of one of the aromatic rings (1-carboxy-2-naphthylbutanoic, 1-carboxy-2-naphthylpropionic, and 1-carboxy-2-naphthoic acids). The transformation of phenanthrene to 1-hydroxy-2-naphthoic acid was rather rapid, whereas the further conversion of the latter metabolite into 2-hydroxynaphthalene and *o*-phthalate occurred after a lag phase.

The incubation of *R. rhodnii* 135 with phenanthrene gave rise to only one product, 3-hydroxyphenanthrene (phenanthrenol), suggesting that this bacterium is only able to hydroxylate phenanthrene. This reaction is of biotechnological interest.

Under nongrowth conditions (in the absence of methionine in the medium), *Arthrobacter* sp. K3 transformed phenanthrene via mono- and dihydroxylation, giving rise to 3,4-dihydroxydihydrophenanthrene, which was further metabolized (through 7,8-benzocoumarin) into 1-hydroxy-2-naphthoic acid and then to *o*-phthalic acid. An HPLC study of the dynamics of the phenanthrene metabolites in the medium showed that phenanthrene was mainly consumed during the first three days of incubation, giving rise to 1-hydroxy-2-naphthoic acid. The concentration of this acid in the medium reached a maximum on the second day and then decreased. This decrease was accompanied by the formation of 7,8-benzocoumarin and *o*-phthalic acid, whose concentration reached a maximum on the fifth day of incubation. The minor products of the phenanthrene transformation were detected in the medium throughout the incubation period (Fig. 2).

Based on the analysis of the transformation products and their dynamics in the medium, we proposed putative pathways for the transformation of phenanthrene by *R. rhodnii* 135, *P. fluorescens* 26K, and *Arthrobacter* sp. K3 (Figs. 3a–3c).

P. fluorescens 26K transformed anthracene much more slowly than phenanthrene, leaving 54% of anthracene in the medium untransformed after 14 days of incubation. The major product of anthracene trans-

formation was found to be 2-hydroxy-3-naphthoic acid, with 2-hydroxy-2-naphthylbutanoic acid, 2-hydroxy-3-naphthylpropionic acid, benzocoumarin, 2,3-dihydronaphthalene, and anthraquinone (a nonmetabolizable compound) being minor transformation products.

The *Arthrobacter* sp. K3 transformation of anthracene resulted in the same products as above. The putative pathway of anthracene transformation by *Arthrobacter* sp. K3 and *P. fluorescens* 26K is shown in Fig. 4. It should be noted that the bacterial oxidation of phenanthrene at the C3–C4 bond and anthracene at the C1–C2 bond is well known [1–6], whereas, to the best of our knowledge, the microbial transformation of phenanthrene into 3-hydroxyphenanthrene has not yet been described any publications.

Thus, we obtained strains that are capable of transforming phenanthrene and anthracene, isolated and identified the transformation products of these PAHs, and described their dynamics in the culture liquid. Two of the strains studied are of particular interest: *R. rhodnii* 135, which transforms phenanthrene into 3-hydroxyphenanthrene in one step, and *Arthrobacter* sp. K3, which transforms phenanthrene with a high yield of conversion products. The data obtained has the potential to form the basis for a purposeful biosynthesis of compounds (such as hydroxylated PAHs, benzocoumarins, and hydroxy-naphthylalkanoic acids) that are important chemicals and antioxidants in biomedicine and the cosmetics industry. The initial concentrations of phenanthrene and anthracene susceptible to bacterial transformation can be augmented using amphiphilic compounds, either of artificial or natural (microbial) origin. The regulation of the microbial transformation of phenanthrene and anthracene may improve the yield of their transformation products. The strains described in this paper can be used to degrade phenanthrene and anthracene in soils, water, and sewage contaminated with these PAHs. This type of utilization is to be the subject of our future investigations.

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