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EXPERIMENTAL ARTICLES

Fluorene Transformation by Bacteria of the Genus *Rhodococcus*

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Abstract—Of the four investigated *Rhodococcus* strains (*R. rhodochrous* 172, *R. opacus* 4a and 557, and *R. rhodnii* 135), the first three strains were found to be able to completely transform fluorene when it was present in the medium as the sole source of carbon at a concentration of 12–25 mg/l. At a fluorene concentration of 50–100 mg/l in the medium, the rhodococci transformed 50% of the substrate in 14 days. The addition of casamino acids and sucrose (1–5 g/l) stimulated fluorene transformation, so that *R. rhodochrous* 172 could completely transform it in 2–5 days. Nine intermediates of fluorene transformation were isolated, purified, and structurally characterized. It was found that *R. rhodnii* 135 and *R. opacus* strains 4a and 557 hydroxylated fluorene with the formation of 2-hydroxyfluorene and 2,7-dihydroxyfluorene. *R. rhodochrous* 172 transformed fluorene via two independent pathways to a greater degree than the other rhodococci studied.

Key words: fluorene, degradation, rhodococci, *Rhodococcus.*

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants. The major sources of their discharge into the environment are the oil and coal industries and the combustion of organic materials. PAHs are detected in the atmosphere, surface waters, marine sediments, and soils. Being very persistent, toxic, and carcinogenic, PAHs are of great environmental concern [1–4].

Fluorene is one of the 16 most hazardous PAHs specified by the US Environmental Protection Agency [1, 5]. The transformation of fluorene by bacteria of the genera *Pseudomonas*, *Arthrobacter*, *Staphylococcus*, and *Mycobacterium* was studied using microbial associations and pure cultures [1, 2, 5–11].

Rhodococci are widespread in nature and play an important role in the detoxification of persistent pollutants. This work was undertaken to search for novel fluorene-transforming rhodococci, to select optimal conditions of fluorene transformation, and to study the fluorene transformation pathways.

MATERIAL AND METHODS

Bacterial strains and cultivation conditions. Fluorene-transforming strains were selected from the laboratory collection of xenobiotic-degrading strains and strains isolated from sewage of the coking industry.

The ability of bacterial strains to grow on fluorene as the sole carbon source was studied using a mineral agar medium containing (g/l) NH_4NO_3, 1.0; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; $FeCl_3$, 0.002; and agar, 20. The same medium but without agar was used for cultivation in flasks with 100–200 ml of the medium on a shaker (200 rpm) at 28°C. Fluorene was added to the medium at concentrations of 25, 50, 100, and 200 mg/l in the form of an acetone solution (50 mg/l). The medium was inoculated with bacterial cells after the flasks had been incubated on the shaker for 20–30 min to remove acetone (after the removal of acetone, fluorene appeared in the medium as fine crystals). The addition of 3% casamino acids to the medium (1 ml per 100 ml) or 4% yeast extract (0.5 ml per 100 ml) improved growth but was not a necessary condition of cultivation. Sucrose as a cosubstrate was added to the medium at concentrations between 1 and 5 g/l.

Bacterial growth was monitored by measuring the optical density of cultures at 540 nm using a KFK-2ML colorimeter and 0.5-cm cuvettes. The control cuvette contained the uninoculated mineral medium with fluorene.

Analytical methods. To analyze the content of fluorene and its transformation products in the culture

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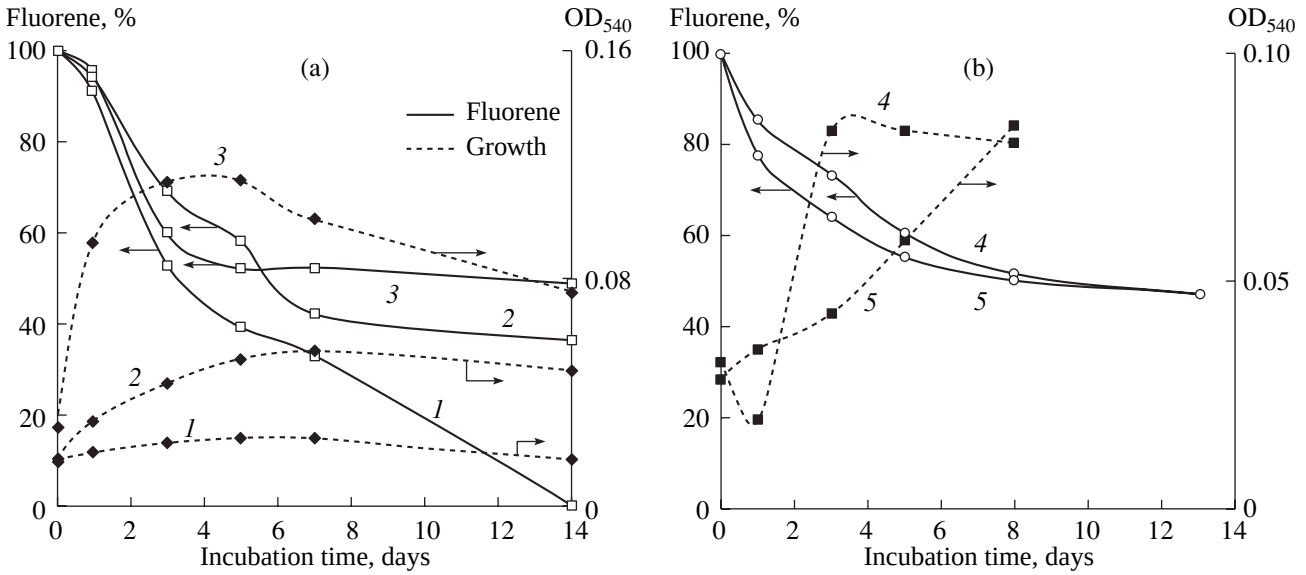

Fig. 1. The transformation of (*1*) 25, (*2*) 50, and (*3*) 100 mg/l fluorene by (a) *R. rhodochrous* 172 and 100 mg/l fluorene by (b) the *R. opacus* strains 4a (*4*) and 557 (*5*).

liquid, the latter was extracted twice with ethylacetate, acidified with 1 N HCl to pH 2, and again extracted twice with ethylacetate. The extracts were pooled and evaporated at 37°C using a vacuum rotary evaporator. The dry residue was dissolved in 0.1–0.4 ml acetone.

The acetone solution was analyzed qualitatively by thin-layer chromatography (TLC) on DC-plasticfolien silica gel plates (Merck, Germany). The plates were developed using a benzene–dioxane–acetic acid (90 : 10 : 1) solvent mixture. Spots were visualized under UV light and treatment with diazotized benzidine [12]. Fluorene intermediates were isolated in the same way.

Fluorene in the culture liquid extracts was analyzed quantitatively using a GCD gas–liquid chromatograph equipped with a flame ionization detector and a (1.5 m × 2 mm) column packed with Chromosorb G-AW-DMSC containing 3% SE-30. The carrier gas was helium at a flow rate of 40 ml/min. The injector, column, and detector were kept at 150, 175, and 290°C, respectively.

Fluorene intermediates were analyzed using a Waters 996 HPLC chromatograph equipped with an

The chromatographic and mass spectrometric characteristics of the products of fluorene transformation by rhodococci

Compound	R_f (TLC)	Empirical formula	Major peaks in mass spectra, *m/z* (%)	Identified compound
1	0.62	$C_{13}H_{10}O$	M^+ 182(90), 181(100), 165(18), 153(32), 152(66), 151(18), 127(3), 126(5), 76(11)	9-Hydroxyfluorene
2	0.63	$C_{13}H_{10}O$	M^+ 182(100), 181(68), 165(14), 153(23), 152(43), 151(13), 127(2), 126(3), 76(14)	2-Hydroxyfluorene
3	0.75	$C_{13}H_8O$	M^+ 180(100), 152(41), 151(18), 150(12), 126(3), 76(8)	9-Fluorenone
4	0.66	$C_{13}H_8O_2$	M^+ 196(100), 168(23), 140(7), 139(51)	Hydroxyfluorenone
5	0.35	$C_{13}H_{10}O_2$	M^+ 198(100), 197(35), 181(18), 169(14), 168(9), 152(7), 141(10), 139(16), 115(10)	Dihydroxyfluorene
6	0.35	$C_{11}H_8O_4$	M^+ 204(40), 186(40), 145(80), 131(100), 115(55), 103(27), 91(34), 77(24)	β-Indanone-β-hydroxyacetic acid
7	0.40	$C_{10}H_6O_2$	M^+ 158(95), 130(34), 129(84), 128(25), 115(100), 89(10)	Formylindanone
8	0.40	C_9H_8O	M^+ 132(100), 131(33), 104(83), 103(41), 102(9), 78(31), 77(30), 51(22)	1-Indanone
9	0.32	$C_9H_8O_2$	M^+ 148(100), 147(42), 119(36), 105(54), 91(48), 77(37)	3,4-Dihydroxycumarine

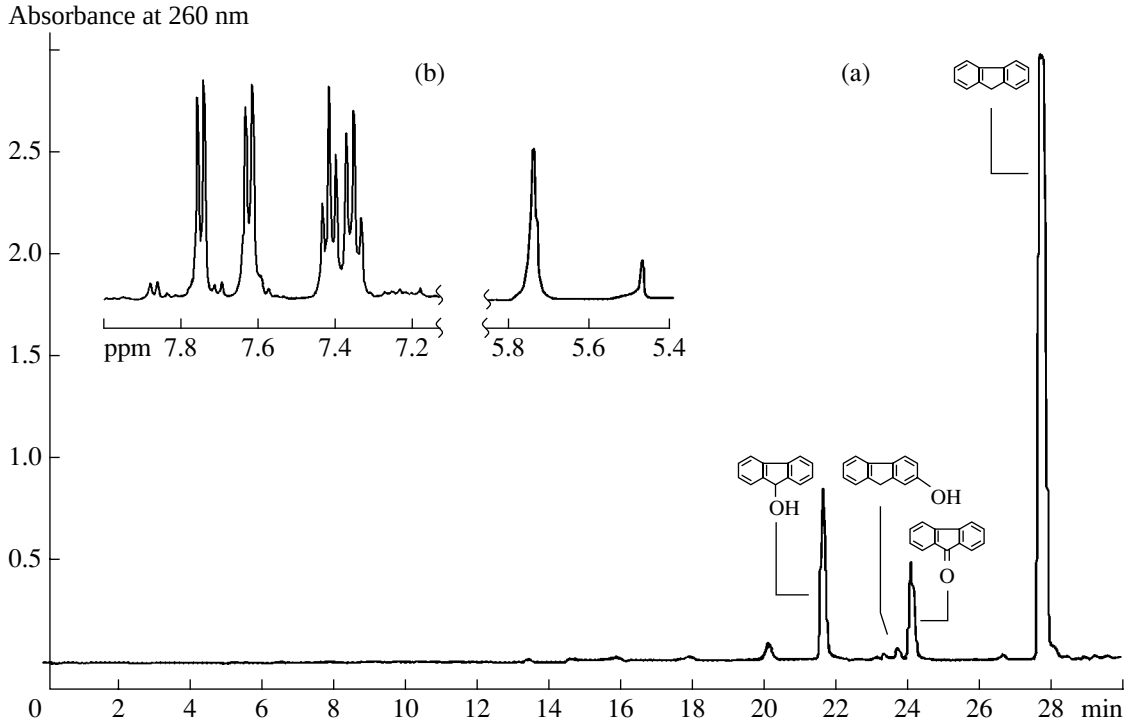

Fig. 2. (a) The HPLC profile of the extract of the *R. rhodochrous* 172 culture incubated with fluorene for 3 days, and (b) the ^{1}H NMR spectra of 9-hydroxyfluorene.

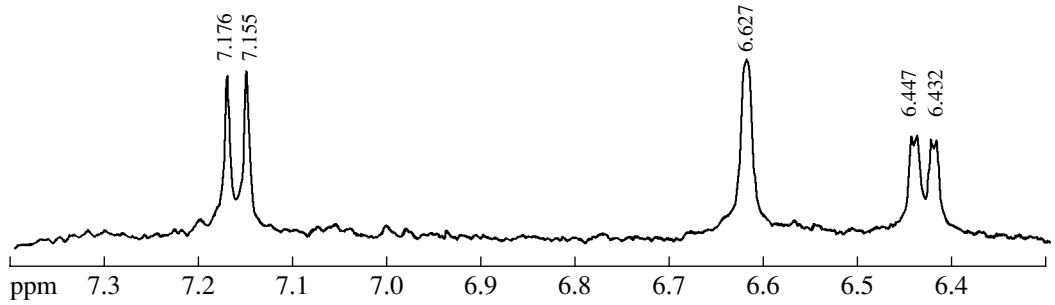

Fig. 3. The ^{1}H NMR spectrum of 2,7-dihydroxyfluorene.

Alltima C_{18} (150 × 4.6 mm) column and a UV detector (260 nm). The column was eluted with a 20–100% gradient (30 min) of methanol containing 0.1% trichloroacetic acid at a flow rate of 1 ml/min. The sample size was 10 μl. The products of fluorene transformation 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 analyzed compounds was determined based on the data of mass spectrometry, chromatography, and NMR spectrometry using the authentic samples of these compounds (if they were available) and their spectra known from the literature.

Samples for NMR spectrometry were dissolved in 0.5 ml of deuterated acetonitrile. NMR spectra were recorded at 400.1319762 MHz using a Bruker DPX-400 spectrometer.

Reagents. The major reagents used in this work were 99% pure (by HPLC) fluorene and 9-fluorenone from Fluka, acetone (high-purity) and methanol (chemical grade) from Reakhim (Russia), and ethylacetate (chemical grade) from Ekos-1 (Russia).

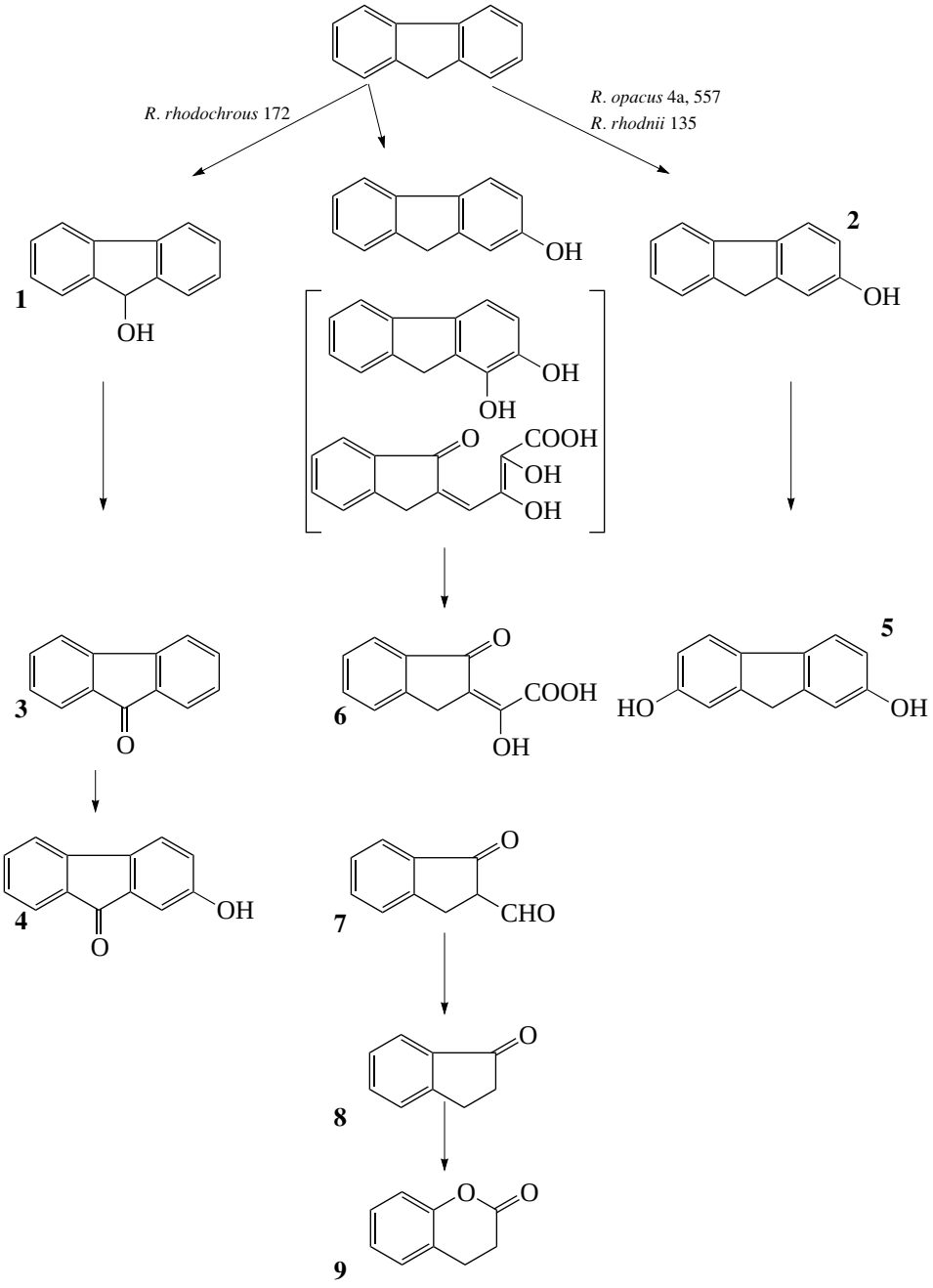

Fig. 4. Putative pathways of fluorene transformation by rhodococci.

RESULTS AND DISCUSSION

Of the 22 bacterial degraders available in the laboratory collection, four strains of the genus *Rhodococcus* (*R. rhodochrous* 172, *R. opacus* 4a and 557, and *R. rhodnii* 135) and two strains of the genus *Pseudomonas* (*P. fluorescens* 26k and 17k) were found to be able to transform fluorene. Further studies were carried out with only rhodococci.

The strains *R. rhodochrous* 172 and *R. opacus* 4a and 557 were found to be able to completely transform fluorene in 10–14 days when it was present in the medium as the sole source of carbon at concentrations between 12 and 25 mg/l. At fluorene concentrations between 50 and 100 mg/l, these rhodococci transformed 50% of the substrate in 14 days (Figs. 1a, 1b). In the presence of casamino acids or yeast extract in the medium, *R. rhodochrous* 172 transformed 100 mg/l flu-

orene by 67% in 10 days, compared to about 50% in the absence of these energy sources. In the presence of sucrose (1–5 g/l) as the cosubstrate, *R. rhodochrous* 172 completely transformed fluorene in 2–5 days [13].

Chromatographic and mass spectrometric analyses allowed us to identify nine products of fluorene transformation in the culture liquid extract (table). Compounds **1** and **2** had the same empirical formula $C_{13}H_{10}O$ and were probably hydroxyfluorenes. The different mobilities of these compounds during TLC and the different intensities of their molecular ions suggested that the positions of hydroxyl groups in their molecules are different. The ^{1}H NMR spectrum of compound **1** had two doublets and two triplets, indicating that the two aromatic rings of this compound are not modified. The signal at 5.5 ppm indicated the presence of one aliphatic proton at C9 (Fig. 2b). All this showed that compound **1** is 9-hydroxyfluorene.

The intensity of the molecular ion of compound **2** and its fragments in the mass spectrum indicated that the hydroxyl group of this compound occurs in position 2, which was confirmed by ^{1}H NMR spectroscopy. Compounds **3** through **9** were identified based on their mass and UV spectra available in the literature [1, 2, 9, 14]. The hydroxyl group of compound **4** (hydroxyfluorenone) was most probably in position 2. The positions of hydroxyl groups in compound **5** (dihydroxyfluorene) differed from those described by Gasellas *et al.* [9]. The low intensities of its fragment ions and the absence of an *ortho*-effect in the mass spectrum of compound **5** indicated that its molecule is stable and that the hydroxyl groups are not in the *ortho*-position. The ^{1}H NMR spectrum of compound **5** had only three signals of six protons (Fig. 3), indicating that the positions of hydroxyl groups in the different benzene rings of this compound are symmetric. A comparison of the ^{1}H NMR spectrum of compound **5** with the theoretical spectra of compounds with a hydroxyl group in position 2 or position 3 allowed the position of one hydroxyl group in compound **5** to be identified at the C2 atom and the other hydroxyl group to be identified at the symmetric C7 atom. It should be noted that the formation of 2,7-dihydroxyfluorene during the microbial transformation of fluorene has so far not been reported.

As is evident from the data obtained in this study, the rhodococci transform fluorene via different pathways (Fig. 4). *R. rhodochrous* 172 transformed fluorene with the production of compounds **1**, **3**, and **4**, whereas compounds **2** and **6**–**9** were detected in trace amounts, which can be explained by their further conversion. Analysis of the HPLC profile of the extract of the *R. rhodochrous* 172 culture incubated in the medium with 100 mg/l fluorene for 3 days showed that the relative peak areas of fluorene, 9-fluorenone, 9-hydroxyfluorene, and 2-hydroxyfluorene were 70, 8, 16, and 1%, respectively (Fig. 2a). Such a pathway of fluorene transformation was described earlier for *Arthrobacter* sp. 101 [9]. The fluorene intermediates 1-indanone and 3,4-dihydroxycumarine can be utilized via the central metabolism.

Three strains, *R. opacus* 4a and 557 and *R. rhodnii* 135, transform fluorene to 2-hydroxyfluorene and 2,7-dihydroxyfluorene. This pathway of fluorene transformation has so far not been described in the literature.

Thus, four strains of rhodococci transform fluorene via different species-specific pathways. The data obtained in this study show that rhodococci play an important role in the bioremediation of fluorene-contaminated soil sites. The fluorene-transforming strains can also be used for the production of particular fluorene intermediates that are difficult to obtain chemically. There are grounds to believe that the use of inverse micelles may increase the tolerable initial concentration of fluorene in the medium and augment the yield of fluorene transformation products by 60–70 times.

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