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EXPERIMENTAL ARTICLES

Horizontal Transfer of Catabolic Plasmids in the Process of Naphthalene Biodegradation in Model Soil Systems

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Abstract—The process of naphthalene degradation by indigenous, introduced, and transconjugant strains was studied in laboratory soil microcosms. Conjugation transfer of catabolic plasmids was demonstrated in naphthalene-contaminated soil. Both indigenous microorganisms and an introduced laboratory strain BS394 (pNF142::Tn*Mod*-OTc) served as donors of these plasmids. The indigenous bacterial degraders of naphthalene isolated from soil were identified as *Pseudomonas putida* and *Pseudomonas fluorescens.* The frequency of plasmid transfer in soil was 10^{-5}–10^{-4} per donor cell. The activity of the key enzymes of naphthalene biodegradation in indigenous and transconjugant strains was studied. Transconjugant strains harboring indigenous catabolic plasmids possessed high salicylate hydroxylase and low catechol-2,3-dioxygenase activities, in contrast to indigenous degraders, which had a high level of catechol-2,3-dioxygenase activity and a low level of salicylate hydroxylase. Naphthalene degradation in batch culture in liquid mineral medium was shown to accelerate due to cooperation of the indigenous naphthalene degrader *P. fluorescens* AP1 and the transconjugant strain *P. putida* KT2442 harboring the indigenous catabolic plasmid pAP35. The role of conjugative transfer of naphthalene biodegradation plasmids in acceleration of naphthalene degradation was demonstrated in laboratory soil microcosms.

Key words: catabolic plasmids, horizontal transfer, naphthalene biodegradation, *Pseudomonas.*

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Polycyclic aromatic hydrocarbons (PAH) are a group of ubiquitous recalcitrant pollutants; they are present in sewage and gas emissions of coke, gas, and oil industry. Naphthalene, phenanthrene, anthracene, chrysene are components of heavy oil fractions and are released into the environment as a result of emergency spills of oil products and fuel combustion under oxygen deficiency. PAH have a tendency to be accumulated in soil because of low bioavailability caused by their low solubility in water. Therefore, the ability of *Pseudomonas* and related bacterial genera to utilize PAH is a subject of particular scientific interest, especially in respect to the application of degrading microorganisms for environmental bioremediation.

The genes of PAH catabolism in pseudomonads are often located on plasmids. The plasmids of PAH biodegradation are conjugative and can be transferred horizontally between and within bacterial populations, intensifying the degradation potential of soil microorganisms and promoting their adaptation to the changing environmental conditions [1].

The goal of this work was to study horizontal transfer of catabolic plasmids and population dynamics of indigenous and introduced degrader strains in the process of naphthalene biodegradation in sterile and nonsterile model soil.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. The plasmid-free strain *Pseudomonas putida* KT2442 was kindly granted by Prof. Kornelia Smalla (Braunschweig, Germany); the auxotrophic strain *P. putida* BS394 and strain *P. putida* KT2442 with the labeled naphthalene degradation plasmid pNF142::Tn*Mod*-OTc were constructed earlier [2].

Cultivation conditions and media. Bacteria were grown at 24°C on rich Luria–Bertani (LB) medium [3], King B medium [4], Pseudomonas Isolation Agar P (PIA) (Difco, USA), and mineral Evans medium [5]

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Table 1. Strains and plasmid used in the work

Strain	Plasmid	Strain characteristics	Source of isolation
		Initial strains	
Pseudomonas sp. 142NF	pNF142	Nah^+ Rif^r Sm^r	Collection of Laboratory of Plasmid Biology
P. putida KT2442	pNF142::Tn*Mod*-OTc	Nah^+ Gfp Km^r Tc^r	Collection of Laboratory of Plasmid Biology
P. putida KT2442	–	Gfp Km^r	K. Smalla, Germany
P. putida BS394	pNF142::Tn*Mod*-OTc	Cys^- Nah^+ Tc^r Sm^r Nal^r	Collection of Laboratory of Plasmid Biology
		Indigenous naphthalene degraders	
P. fluorescens AP1-1	–	Nah^+ Km^r Nb^r	Present work
P. fluorescens AP1-2	–	Nah^+ Km^r Nb^r	Present work
P. fluorescens AP1-3	–	Nah^+ Km^r Nb^r	Present work
P. putida AP4-1	pAP4	Nah^+ Rif^r Nb^r	Present work
P. putida AP4-2	pAP5	Nah^+ Rif^r Nb^r	Present work
		Transconjugants	
P. putida KT2442 clone 35	pAP35	Nah^+ Gfp Km^r	Present work
P. putida KT2442 clone 36	pAP36	Nah^+ Gfp Km^r	Present work
P. putida KT2442 clone 1	pNF142::Tn*Mod*-OTc	Nah^+ Gfp Km^r Tc^r	Present work
P. putida KT2442 clone 21	pNF142::Tn*Mod*-OTc	Nah^+ Gfp Km^r Tc^r	Present work

Note: Nah^+, ability for growth on naphthalene; Gfp^+, presence of green fluorescent protein; Cys^-, cysteine auxotroph; resistance to antibiotics: Km^r, kanamycin, Nal^r, nalidixic acid, Nb^r, novobiocin, Rif^r, rifampicin, Sm^r, streptomycin, Tc^r, tetracycline.

supplemented with a carbon source. Agarized media were obtained by addition of 2% (wt/vol) Difco agar.

Naphthalene, glucose, succinate, and salicylate were used as carbon and energy sources in a final concentration of 0.1–0.2%. When growing the bacteria on agarized media, naphthalene was applied to the inner side of petri dish cover. When microorganisms were cultivated in a liquid medium, 1 g of naphthalene per liter was added. Cysteine (0.05 g/l) was added into the mineral medium for auxotrophic strain BS394.

Antibiotics of domestic manufacture were used in the work. The final concentration of antibiotics in selective media was (μg/ml): 10, 50, and 100 for Tc (tetracycline), and 30 and 100 for Km (kanamycin).

Conjugative plasmid transfer. Plasmid transfer by conjugation was carried out according to [6].

Plasmid DNA was isolated by alkaline lysis [7].

Measurement of the activities of biodegradation enzymes. Cell-free extracts for measuring enzyme activities were obtained by disruption of frozen biomass in an IBPM press (Russia). Cell debris was precipitated in a Beckman J2-21 centrifuge (Beckman Instruments, USA) (32000 *g*, 0°C, 60 min). Specific enzyme activities were measured on a UV-160A spectrophotometer (Shimadzu, Japan) as described previously [8]. Protein concentration was measured by spectrophotometry [9]. Specific activities of the enzymes were expressed in micromoles of consumed substrate (cofactor) per minute per 1 mg of total bacterial protein.

Model soil systems. Model soil systems were made on the basis of gray forest soil collected in the vicinity of Pushchino (Moscow oblast), which of the following composition: 91.00% ash (SiO_2, 72.50%; C, 2.89%; H, 1.05%; N, 0.25%; P, 0.06%; Ca, 0.48%; Mg, 0.14%; Fe, 1.20%; K, 2.47%); the pH of the aqueous extract was 7.05. The soil (40 g) was placed into glass petri dishes (100 mm in diameter). For preparing sterile microcosms, the soil was autoclaved three times with daily intervals for 1 h at 103.1 kPa (121°C). Naphthalene powder was added into the soil of all microcosms in a concentration of 2 mg of naphthalene per 1 g of soil.

Strain BS394(pNF142::Tn*Mod*-OTc) grown in liquid Evans medium with naphthalene as a sole carbon and energy source and plasmid-free strain KT2442 grown in LB broth were used as inoculum. The cells were cultivated to the middle of the exponential growth phase (to a concentration of $1.0–5.0 \times 10^8$ colony forming units (CFU)/per ml), washed twice in phosphate buffer, resuspended in sterile tap water, and introduced into microcosms in the amount of $7.0 \times 10^4–8.0 \times 10^5$ CFU/g of dry soil. The amount of water was calculated on the basis of 40% (wt/wt) final soil humidity in the microcosms.

Soil sampling and determination of naphthalene content in the samples were performed as described in [10].

Monitoring of the tagged plasmid-bearing degrader strains in the course of naphthalene degradation in soil. To monitor the plasmid-bearing strains, the markers of antibiotic resistance and UV fluorescence on agarized LB medium (strain KT2442) and by auxotrophy (strain BS394) were used. After appropriate dilutions in phosphate buffer, soil samples were plated onto agarized LB medium or mineral Evans medium with naphthalene and with (or without) cysteine.

For the isolation of KT2442 transconjugants, 500 clones from each variant of the model system were re-picked from solid Evans medium with naphthalene onto selective media containing kanamycin (30 μg/ml) and/or tetracycline (10 μg/ml).

Salicylate quantification in culture broth samples. The suspension containing bacterial cells grown in Evans medium with naphthalene and metabolic products were precipitated from the culture liquid by centrifugation in an Eppendorf miniSpin table-top centrifuge (Eppendorf AG, Germany) at 10000 *g* for 4 min. The supernatant (200 μl) was analyzed using an LKB-2150 HPLC chromatograph; Pharmacia LKB Biotechnology column; Spherisorb ODS2 sorbent, 5 μm, 4 mm × 250 mm; flow rate, 1 ml/min; detection wavelength, 310 nm; and eluent, 75% methanol. Salicylate concentration was calculated by the peak area as compared with the peak area of the control sample.

Isolation of total DNA. The cells grown in LB liquid medium for 12 h (1 ml) were precipitated in an Eppendorf miniSpin centrifuge at 10000 *g* for 2 min. The cells were washed with 1 ml of 1M NaCl and resuspended in 1 ml of solution containing Tris–HCl (20 mM), NaCl (1 M), Na-SDS (1%), EDTA (50 mM), and proteinase K (2 mg/ml). The test tubes were heated in a thermostat at 37°C for 30 min. Then 80 μl of cetyltrimethylammonium bromide was added and the mixture was incubated in a thermostat at 65°C for 30 min. Phenol (100 μl) and chloroform (200 μl) were added to the lysate, which was then centrifuged for 8 min. Supernatant (1 ml) was collected into another test tube and the procedure was repeated. DNA was precipitated from the supernatant by adding 600 μl of isopropyl alcohol and centrifuging for 5 min. The pellet was washed with 200 μl of 70% ethanol, resuspended in 100–200 μl of Tris–EDTA buffer, and kept in a thermostat for 15 min at 65°C.

Polymerase chain reaction. Polymerase chain reaction (PCR) was carried out in a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer, United States) under standard conditions. The PCR mixture (25 μl) contained buffer for the enzyme (10 mM Tris–HCl (pH 8.4), 50 μmol KCl, and 0.1 μg/μl gelatin), 5–10 ng of target DNA, 18 pM of each primer, deoxyribonucleotide triphosphates in the final concentration of 200 μM, 1.5 mM or 2.0 mM of $MgCl_2$ (to amplify the *nahAc* gene), and 1.5–2.5 units of *Taq* polymerase (Amersham Life Science, United Kingdom). Primers 63f and 1387r were used to amplify the 16S rRNA gene [11]. DNA was denatured at 94°C for 5 min, then 35 cycles were performed of 94°C (30 s), 55°C (30 s), and 72°C (2 min), and PCR was completed by an extension step at 72°C for 14 min. Primers Acl49f and Ac1014r were used to amplify the gene of the large subunit of naphthalene dioxygenase (*nahAc*) [12]. DNA was denatured at 95°C for 5 min and 35 cycles of 94°C (30 s), 43°C (20 s), and 72°C (1 min) were performed, with a final extension step at 72°C for 4 min.

DNA hydrolysis by restriction endonucleases. Buffer solutions and restriction endonucleases from Fermentas (Lithuania) were used in the work. The total volume of reaction mixture was 20 μl. For hydrolysis of 100–300 ng of plasmid DNA, 20–30 U of an appropriate enzyme were added to the reaction mixture. The reaction was performed according to the manufacturer's protocol at 37°C for 1 h. The reaction was stopped by heating at 65°C for 15 min.

Amplified ribosomal DNA restriction analysis (ARDRA). For identification of indigenous naphthalene-degrading strains, the 16S rRNA gene amplification product was hydrolyzed with restriction endonucleases *Msp*II and *Rsa*I. Restriction digests obtained by electrophoresis were compared with those obtained earlier for different *Pseudomonas* species [13].

Electrophoresis was carried out in horizontal agarose gel according to the standard procedure [14].

Results were processed using embedded Excel statistical software (MS Office 2000).

RESULTS

Scheme of the experiment. The processes of horizontal transfer of catabolic plasmids, population kinetics of degrader strains, and naphthalene biodegradation in soil were studied in laboratory microcosms according to the following scheme (Fig. 1). Evaporation and abiotic degradation of naphthalene were assessed in microcosm 1 with sterile soil. The contribution of indigenous microorganisms to its degradation was determined from naphthalene decrease in microcosm 2 containing nonsterile soil. The population kinetics of microorganisms and horizontal transfer of catabolic plasmids were analyzed in microcosms 3, 4, and 5. In the beginning of the experiment, the quantity of indigenous and introduced naphthalene-degrading bacteria was $2.0–4.0 \times 10^4$ and 1.3×10^4 CFU g^{-1} of dry soil, respectively (about 3.2–6.2% and 2.1% of the total number of soil microorganisms) (Fig. 2). The fate of the plasmid-free strain KT2442 introduced into soil was followed due to the presence of chromosomal reporter genes *gfp*, kanamycin and rifampicin resistance genes; the fate of the plasmid-bearing strain BS394(pNF142::Tn*Mod*-OTc) was followed due to its

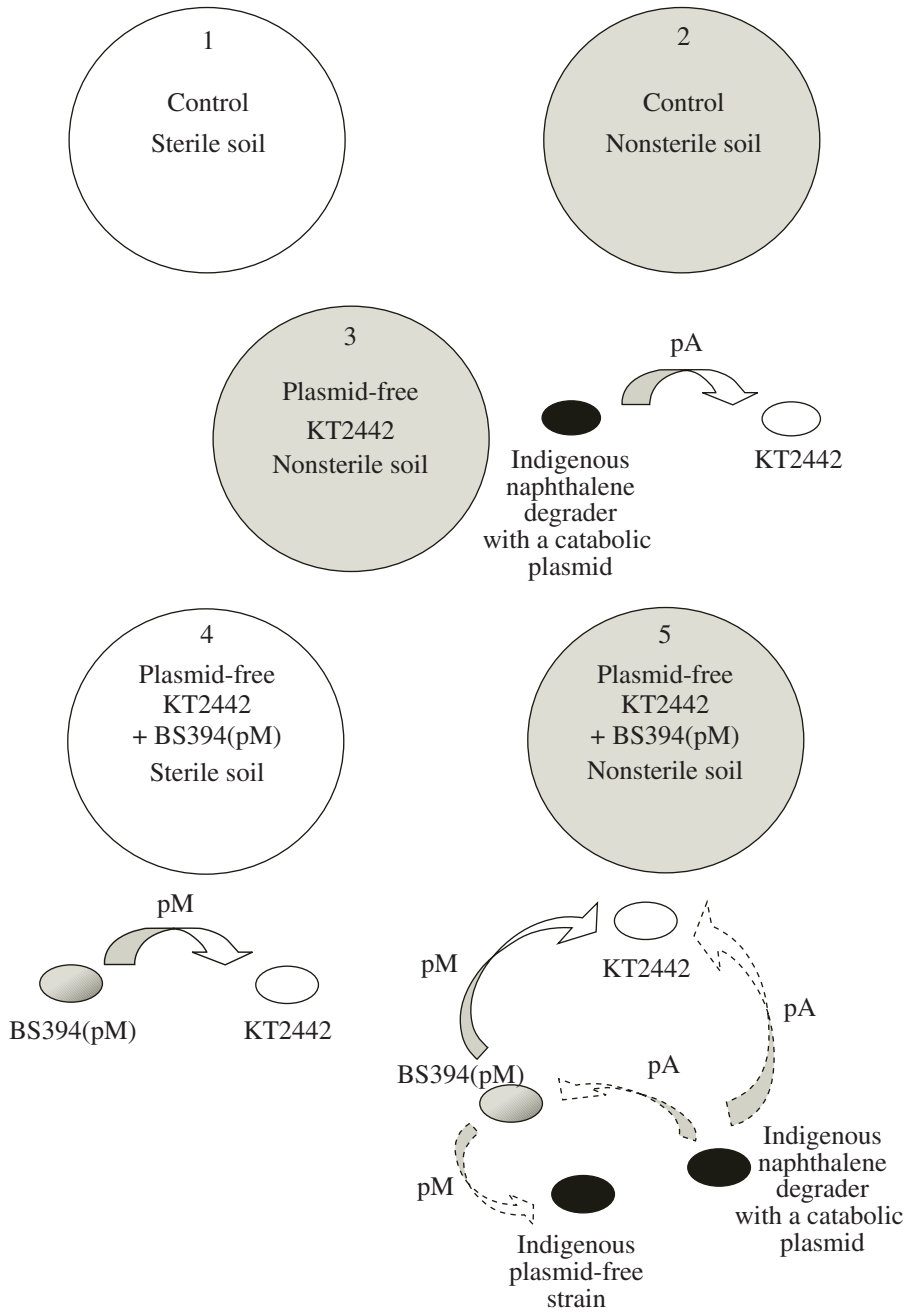

Fig. 1. The scheme of laboratory experiment (in all variants, naphthalene was added to soil in the concentration of 2 g/kg soil) and possible directions of transfer of naphthalene biodegradation plasmids: solid-line arrows, transfer demonstrated in the experiment; broken-line arrows, transfer not demonstrated; pA, indigenous plasmid of naphthalene biodegradation; pM, labeled plasmid pNF142::Tn*Mod*-OTc.

auxotrophy. The genes for naphthalene biodegradation and tetracycline resistance as components of the labeled plasmid made it possible to observe its transfer in the model soil. Monitoring of the transfer of the labeled plasmid pNF142::Tn*Mod*-OTc from strain BS394 and indigenous plasmids from indigenous naphthalene degraders into the plasmid-free strain KT2442 was performed during the work.

Characterization of indigenous naphthalene degraders. Two indigenous naphthalene-degrading strains, designated as AP1 and AP4, were isolated from non-sterile soil used in the experiment. The strains differed from each other in morphological characteristics (color, shape and size of the colonies), antibiotic resistance, and the spectrum of utilized substrates. The microorganisms were gram-negative and fluorescent on King B medium; they grew on PIA (*Pseudomonas*

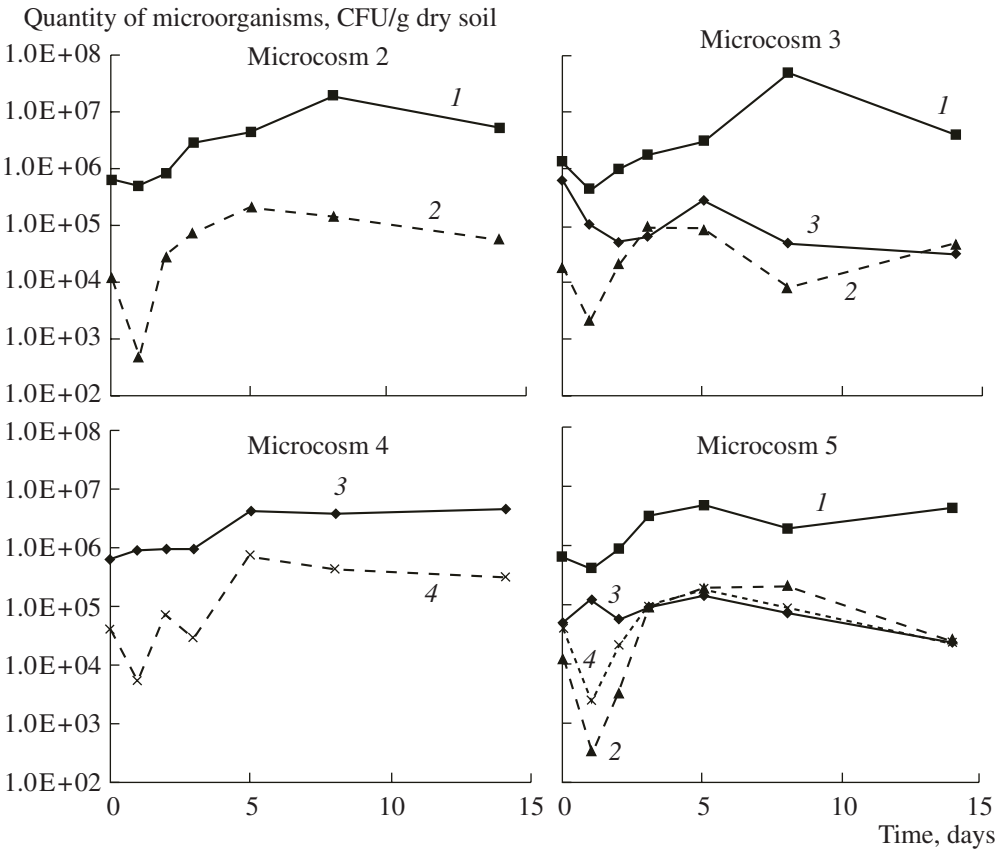

Fig. 2. The kinetics of microbial numbers in soil microcosms: microcosm 2, control with nonsterile soil; microcosm 3, nonsterile soil with plasmid-free strain KT2442; microcosm 4, sterile soil with strains BS394 (pNF142::Tn*Mod*-OTc) and KT2442; microcosm 5, non-sterile soil with strains BS394 (pNF142::Tn*Mod*-OTc) and KT2442. Total quantity of soil microorganisms (*1*); quantity of indigenous naphthalene degraders (*2*); quantity of plasmid-free strain *P. putida* KT2442 (*3*); quantity of strain BS394 (pNF142::Tn*Mod*-OTc) (*4*).

Isolation Agar). The results of restriction analysis of amplified 16S rDNA using the *Msp*II and *Rsa*I endonucleases (comparison of restriction digests with the previously obtained digests for some species of fluorescent pseudomonades, i.e., strains *P. putida* mt-2, *P. fluorescens* 2-79, etc. [13]) confirmed the identification of strains AP1 and AP4 as *Pseudomonas fluorescens* and *Pseudomonas putida*, respectively.

Plasmid localization of the naphthalene biodegradation genes and the conjugativity of the harboring plasmids were confirmed by a series of conjugations, with indigenous naphthalene degraders AP1 and AP4 as donors and the plasmid-free strain KT2442 as a recipient. Naphthalene was used for selection; rifampicin for strain AP1 and kanamycin for strain AP4, for counterselection. Transconjugants were obtained only on plates with strain AP4. The frequency of transfer was 1.0×10^{-7}–5.0×10^{-8} per donor cell.

Total DNA was isolated from soil naphthalene degraders AP1 and AP4. The presence of the *nahAc* gene in these strains was demonstrated by PCR (data are not presented). Strain AP4 was shown to contain a naphthalene biodegradation plasmid of 80–90 kb. Restriction digests of the plasmid DNA preparations (Fig. 3), isolated from the indigenous strain (clones AP4-1 and AP4-2) and hydrolyzed with the *Eco*RI and *Pst*I restriction endonucleases, were similar to each other and to the native plasmid pNF142 [15].

Microcosms 4 and 5 also showed horizontal transfer of the labeled catabolic plasmid pNF142::Tn*Mod*-OTc from donor strain BS394 introduced in to soil to plasmid-free strains KT2442 with a frequency of 10^{-5}per donor cell, which was registered on day 8 in microcosm 4 and on day 2 in microcosm 5. Moreover, the transfer of indigenous plasmids to strain KT2442 in microcosm 3 was registered. PCR with primers Acl49f and Ac1014r [12] showed the presence of the *nahAc* gene in the transconjugants of strain KT2442 isolated from the soil of microcosm 3 (clones 35 and 36), and of strain KT2442(pNF142::Tn*Mod*-OTc) isolated from the soil of microcosms 4 and 5 (clones 1 and 21). Restriction analysis of the indigenous naphthalene biodegradation plasmids isolated from transconjugant strains KT2442, clones 35 and 36, revealed their similarity with the indigenous plasmids isolated from native strain AP4 (Fig. 3), which is evidence of transfer of indigenous

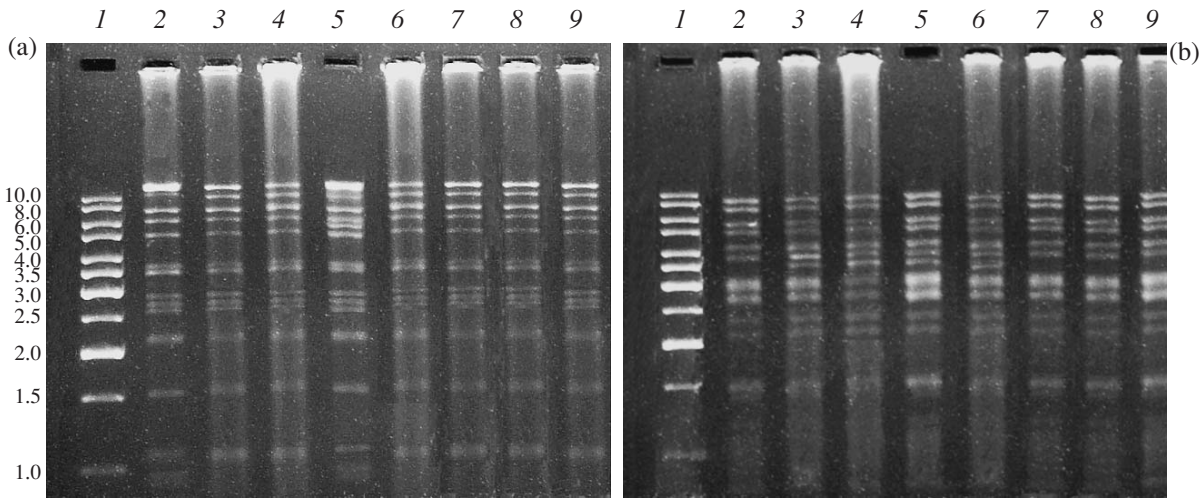

Fig. 3. Restriction patterns of plasmid DNA hydrolyzed with restriction endonucleases *Eco*RI (a) and *Pst*I (b): 1-kb Ladder Gene Ruler (*1*); pNF142 (*2*); pAP35 (*3*); pAP36 (*4*); pAP4-1 (*5*); pAP4-2 (*6*); pNF142::Tn*Mod*-OTc (KT2442 clone 1) (*7*); pNF142::Tn*Mod*-OTc (KT2442 clone 21); initial pNF142::Tn*Mod*-OTc (*9*).

catabolic plasmids. Moreover, the restriction patterns of the labeled plasmid pNF142::Tn*Mod*-OTc and of the indigenous plasmid from strain A4 were also shown to be similar to the digest of the previously described naphthalene biodegradation plasmid pDTG1 isolated from soil near Bangor, Wales, United Kingdom [16].

Estimation of activity of the key enzymes of naphthalene catabolism in degrading bacteria. Activities of the key enzymes of naphthalene catabolism were assessed in indigenous microorganisms AP1 and AP4 (Table 2). The activity of catechol-2,3-dioxygenase (C23O) was shown to be high (0.3–0.9 μmol/min per mg of protein), while the activity of salicylate hydroxylase was comparatively low (0.039–0.004 μmol/min per mg of protein). In all strains, the activity of catechol-1,2-dioxygenase (C12O) was an order of magnitude lower than the activity of C23O.

The activity of the key enzymes of naphthalene biodegradation was also examined in strains 142NF (pNF142), BS394(pNF142::Tn*Mod*-OTc), KT2442 (pNF142::Tn*Mod*-OTc), and transconjugant strains KT2442 with indigenous and labeled plasmids obtained as a result of horizontal transfer in the model soil (Table 2). In the initial strain 142NF(pNF142), the C23O activity prevails over the C12O activity; i.e., catechol oxidation proceeds mainly through the *meta*-pathway. Tranconjugants of strain KT2442 with indigenous catabolic plasmids (clones 35 and 36) showed different activities of catechol oxidation enzymes. The C12O activity in clone 36 was the highest among the strains under study, while the C23O activity was not revealed. It should be noted that the transfer of the labeled plasmid pNF142::Tn*Mod*-OTc from strain BS394 to strain KT2442 in soil results in appearance of transconjugants (clones 1 and 21) in which, similarly to the initial strain KT2442(pNF142::Tn*Mod*-OTc), the enzyme activity of catechol oxidation via the *ortho*-pathway (C12O) predominates (Table 2); i.e., the plasmid gene of catechol degradation is not expressed. At a reverse transfer of plasmid pNF142::Tn*Mod*-OTc to strain BS394, the activity of the enzyme of catechol oxidation via the *meta*-pathway (C23O) was restored. Significant differences in the naphthalene oxygenase activities were not observed. It should be noted that the transconjugants of strain KT2442 (clones 35 and 36) showed a high activity of salicylate hydroxylase (0.05–0.11 μmol/min per g of protein), which was an order of magnitude higher than the activity in both indigenous naphthalene-degrading strains (Table 2).

The study of the phenomenon of cooperation of indigenous naphthalene degraders and transconjugants formed in the course of transfer of indigenous plasmids. The quicker loss of naphthalene in microcosm 3 where transconjugants with indigenous plasmids were detected as compared with microcosm 2 with non-sterile soil (Fig. 4b) suggests possible cooperation of indigenous naphthalene degraders and transconjugants formed during biodegradation. This assumption was confirmed by the experiment with the indigenous naphthalene-degrading strain AP1 (clone AP1-1), which has low SH activity and high C23O activity, and the transconjugant strain KT2442 (pAP35), clone 35, bearing an indigenous plasmid, under batch cultivation in a liquid mineral medium with naphthalene. The numbers of microorganisms and accumulation of salicylate, an intermediate product of naphthalene degradation, were assessed in the course of this experiment. As is seen from Figure 5a, strain AP1 and mixed culture of the strains show comparable population kinetics of bacteria. It should be noted that the quantity of KT2442(pAP35), clone 35, in mixed culture in the first 20 h was an order of magnitude lower than the quantity of strain AP1 (about 10% of the quantity of clone AP1-1).

Table 2. Activities of the key enzymes of naphthalene biodegradation in strains under study

Strain	Enzyme activity, μmol/min mg protein			
	NO	SH	C23O	C12O
Initial strains				
KT2442	ND	ND	0	0.0730
142NF(pNF142)	0.0392	0.0486	0.2849	0.0917
394(pNF142::Tn*Mod*-OTc)	0.0235	0.0302	0.3520	0.0284
KT2442(pNF142::Tn*Mod*-OTc)	0.0322	0.0801	0.0057	0.1853
Indigenous naphthalene degraders				
P. fluorescens AP1-1	0.0383	0.0042	0.7298	0.0738
P. fluorescens AP1-2	0.0171	0.0391	0.3739	0.0797
P. fluorescens AP1-3	0.0601	0.0050	0.9506	0.0849
P. putida AP4-1(pAP4-1)	0.0158	0.0053	0.3603	0.0680
P. putida AP4-2(pAP4-2)	0.0211	0.0136	0.5242	0.1359
Transconjugants				
P. putida KT2442(pAP35) clone 35	0.0230	0.1074	0.0169	0.0086
P. putida KT2442(pAP36) clone 36	0.0131	0.0485	0.0000	0.2480
P. putida KT2442(pNF142::Tn*Mod*-OTc) clone1	0.0376	0.0620	0.0033	0.2017
P. putida KT2442(pNF142::Tn*Mod*-OTc) clone 21	0.0305	0.0934	0.0053	0.1696

Note: NO, naphthalene dioxygenase; SH, salicylate hydroxylase; C23O, catechol-2,3-dioxygenase; C12O, catechol-1,2-dioxygenase; ND, not determined.

Salicylic acid accumulation was observed in both cases, with salicylate concentration growing twice as fast in mixed culture (Fig. 5b). The maximal accumulation of salicylate was detected after 12 h of cultivation; salicylate concentration was 89.64 mg/ml in the AP1-1 culture on naphthalene and 168.33 mg/ml in the mixed culture. After 12 h of cultivation, salicylate consumption was observed in both cases, but in the latter case it was quicker (as the previous salicylate accumulation). Thus, the kinetics of salicylate concentration was indirect evidence of a more active metabolism of the mixed culture, because the accumulation and consumption of salicylate and therefore of naphthalene was more active.

DISCUSSION

Research on the effect on biodegradation processes of inoculation of degrader strains into soil or activated sludge and on the effect of dissemination of catabolic plasmids among indigenous microorganisms commenced more than ten years ago [17–22]. However, there are no works in which PAH biodegradation and the associated transfer of degradation plasmids were studied simultaneously. The donors used in the previous studies of the processes of xenobiotic destruction and horizontal transfer of catabolic plasmids under laboratory conditions were active degraders of pollutants, mainly gram-negative microorganisms of the genera *Pseudomonas* [18, 19] and *Alcaligenes* [17, 20]. Donor strains harbored plasmids of biodegradation of 2,2-dichloropropionate (pFL140) [17]; 3-chlorbenzoate, 4-methylbenzoate, and 4-ethylbenzoate (pFRC20 and pWW0-EB62) [18]; 2,5-dichlorbenzoate (pPB111) [19]; and 2,4-dichlorophenoxyacetate (pJP4 [20–22] and pEMT1k [21, 22]). Recipients were the introduced strains of the genus *Pseudomonas* [18], indigenous soil microorganisms [19–22], and both simultaneously [17].

Researchers [21, 22] mainly studying the influence of transconjugants on the 2,4-dichlorophenoxyacetate destruction process purposefully used for introduction in soil a donor with low degradation activity, which most likely resulted from elimination of the pEMT1k plasmid. The formed transconjugants had a selective advantage and their quantity in the population increased.

In our work, the active naphthalene degrader *P. putida* BS394(pNF142::Tn*Mod*-OTc) was used as a donor strain with a stably maintained plasmid [2]. The plasmid-free strain *P. putida* KT2442 incapable of naphthalene degradation was used as a recipient. The presence of indigenous naphthalene degraders in test

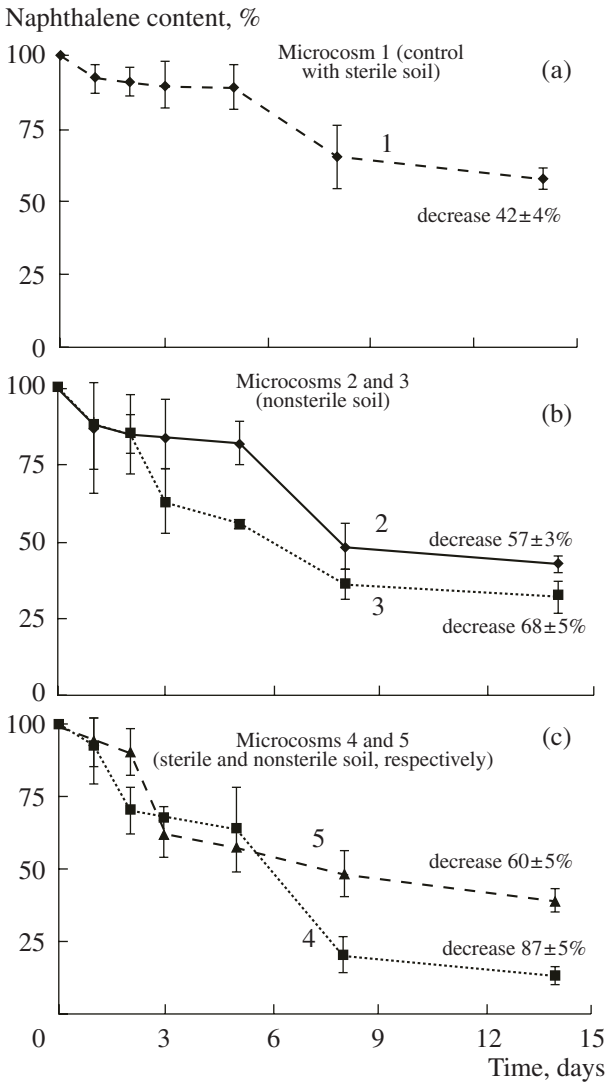

Fig. 4. Comparative kinetics of naphthalene decrease in soil microcosms.

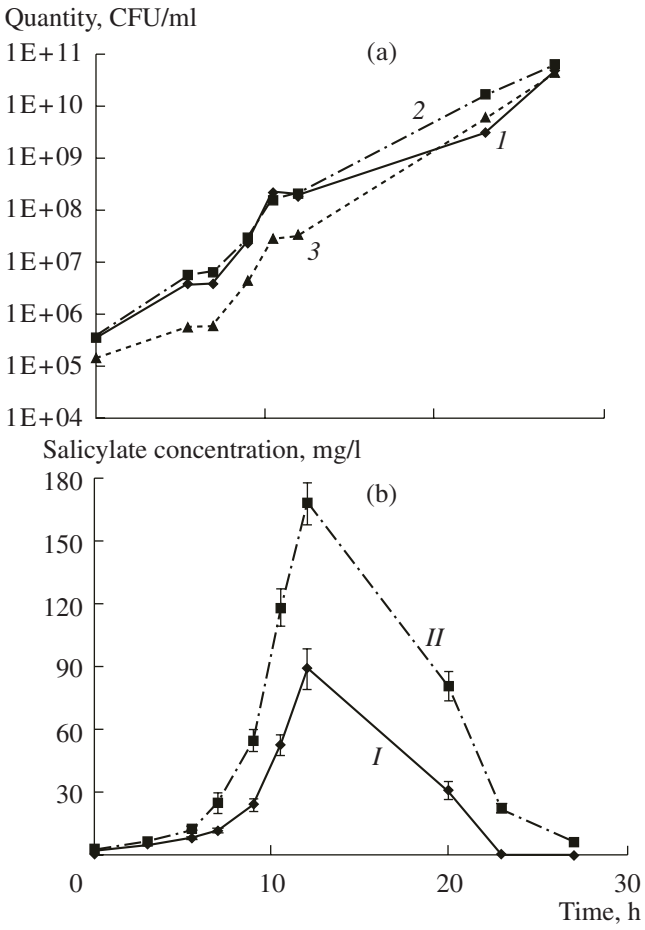

Fig. 5. The dynamics of bacterial numbers (a) and salicylate accumulation (b) of indigenous degrader AP1-1 and transconjugant KT2442(pAP35), clone 35 in batch culture in a liquid mineral medium with naphthalene: quantity of clone AP1-1 in pure culture (*1*); quantity of clone AP1-1 in mixed culture (*2*); quantity of KT2442(pAP35), clone 35, in mixed culture (*3*); the kinetics of salicylate in pure culture (*I*); the kinetics of salicylate in mixed culture (II).

soil was shown; they could act as potential recipients and donors of catabolic plasmids. The scheme of possible directions of the transfer of the degradation plasmids is shown in Figure 1. Three of the four possible directions of plasmid transfer have been confirmed in our work.

Transconjugant clones of the introduced plasmid-free strain KT2442 that had accepted indigenous plasmids were revealed in microcosm 3. Previously, the exogenous isolation of plasmid has been performed by conjugation of a recipient strain and a suspension of indigenous bacteria isolated from manure on a rich solid medium [23]. In our work, the endogenous isolation of indigenous catabolic plasmids has been carried out. Since the number of indigenous naphthalene degraders was at the level of 10^4–10^5 CFU g/dry soil, the plasmid transfer frequency could not be lower than 10^{-5}–10^{-4} per donor cell. Isolation of indigenous transconjugants in microcosm 5 was not achieved, probably because of the low frequency of transfer of the labeled plasmid pNF142::Tn*Mod*-OTc from the introduced degrader strain BS394 BS394(pNF142::Tn*Mod*-OTc) into indigenous microorganisms (less than 10^{-5} per donor cell). Moreover, these transconjugants were most likely unable to reach the level of detection quickly, because the decrease of selective pressure due to the loss of naphthalene was rather rapid: on day 14, the content of naphthalene was about 13% of the initial concentration. The transfer of the labeled catabolic plasmid pNF142::Tn*Mod*-OTc into plasmid-free recipient strain KT2442 was observed in microcosms 4 and 5. The frequency of plasmid pAP4 transfer in conjugations on plates with LB agar was 10^{-8}–10^{-7} per donor cell.

The presence of microbial degraders in microcosms 2–5 accelerated the process of naphthalene biodegradation

as compared with the control microcosm 1 with sterile soil, where the loss of the pollutant was 42 ± 4% on day 14.

It is interesting to note that in our experiment in non-sterile soil with the introduced strain KT2442 (microcosm 3), the process of naphthalene degradation was faster in the second half of the experiment than in the similar soil without this strain (microcosm 2). The activity of the key enzymes of naphthalene catabolism was measured in the analyzed strains in order to find out the cause of accelerated naphthalene decrease. The activity of salicylate hydroxylase in transconjugant clones 35 and 36 (isolated from the soil of microcosm 3), which had received indigenous catabolic plasmids, was an order of magnitude higher than in the four indigenous naphthalene-degrading strains (Fig. 4). However, the activity of the catechol degradation enzyme C23O in indigenous microorganisms was an order of magnitude higher than in clone 35 and three orders of magnitude higher than in clone 36. Apparently, the phenomenon of cooperation occurred in microcosm 3, when clones 35 and 36 cleave naphthalene to salicylate and then to catechol much faster than indigenous degraders. The latter, which have high C23O activity, are capable of rapid catechol cleaving followed by inclusion of the intermediates into the TCA cycle. This fact supports our previous assumption [10] of the two-stage character of naphthalene utilization by strain *P. putida* G7(NAH7) in soil.

The catabolic plasmids isolated from indigenous naphthalene-degrading microorganisms were ca. 90 kb in size. The PCR analysis with the primers specific for the *nahAc* gene of the large subunit of naphthalene dioxygenase showed the presence of the *nahAc* genes in the total DNA of both the indigenous strains and transconjugants. The analysis of plasmid DNA treated with restriction endonucleases revealed the similarity of restriction digests between the indigenous plasmid pAP4 and the labeled naphthalene biodegradation plasmid pNF142::Tn*Mod*-OTc (Fig. 3), as well as with the known plasmid pDTG1 [16]. Plasmids similar to pDTG1 have previously been isolated from strains found in the soil of a coal-tar contaminated site in Glen Falls, United States [24].

The experiment in liquid minimal medium demonstrated that strain KT2442(pAP35), clone 35, in a ten times lower concentration, as compared with the indigenous naphthalene-degrading strain present in the same system, contributes to quicker (twofold) accumulation in the medium of salicylate, a naphthalene degradation intermediate, and to its further rapid consumption (Fig. 5). Thus, the presence of KT2442 transconjugants with indigenous biodegradation plasmids in microcosm 3 apparently provided a slight but reliable acceleration of naphthalene degradation in the end of experiment. Previously, it has been demonstrated that the transfer of 2,4-dichlorophenoxyacetate biodegradation plasmids into appropriate adapted indigenous bacteria in soil accelerates pollutant degradation [21]. Our results demonstrate another intriguing effect: the introduction of a plasmid-free laboratory strain followed by the transfer of indigenous catabolic plasmids into it can create conditions for acceleration of naphthalene destruction in soil.

It should be noted that the quantity of the donor strain BS394(pNF142::Tn*Mod*-OTc) in sterile soil (microcosm 4) was an order of magnitude higher in comparison with its quantity in non-sterile soil (microcosm 5). This can be explained by the fact that high-molecular organic matter and microbial cells are decomposed in the course of soil sterilization, thus increasing the quantity of easily available organic matter [25]. Thus, as a consequence of the high abundance of the donor strain BS394(pNF142::TnM*od*-OTc) in microcosm 4, naphthalene degradation accelerated as compared with microcosm 5. Naphthalene degradation in microcosm 5 was carried out by strain BS394(pNF142::Tn*Mod*-OTc) and indigenous bacterial degraders. The KT2442(pNF142::Tn*Mod*-OTc) transconjugants formed in microcosms 4 and 5 seemed to have no effect on naphthalene degradation process due to their low quantity in soil.

Thus, it has been demonstrated under laboratory conditions that the transfer of naphthalene biodegradation plasmids takes place in soil and the transconjugants formed in soil can contribute to the acceleration of this process.

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