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EXPERIMENTAL ARTICLES

Horizontal Transfer of Catabolic Plasmids and Naphthalene Biodegradation in Open Soil

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Abstract—The horizontal transfer of naphthalene biodegradation plasmids and the parallel process of its microbial degradation were studied for the first time. The tagged naphthalene-degrading strains bearing labeled biodegradation plasmids were used for the monitoring of horizontal plasmid transfer in open soil. The population kinetics of microorganisms, the survival rate and competitiveness of introduced strains, and the transfer of biodegradation plasmids to indigenous strains were investigated. The transfer of the labeled plasmid pNF142::Tn*Mod*-OTc to the introduced plasmid-free recipient *P. putida* KT2442 and to indigenous soil microorganisms of the genus *Pseudomonas* was shown both under selection pressure (in the presence of naphthalene) and in its absence. The 16S rRNA gene sequencing showed that the soil strains that had acquired plasmids were close to the species *P. lini, P. frederiksbergensis, P. jessenii, P. graminis, P. putida*, and *P. alcaligenes*. Thus, direct evidence of dissemination of the naphthalene biodegradation plasmids in microbial populations in open soil under selective and nonselective conditions has been obtained.

Key words: horizontal transfer, plasmids, naphthalene, soil, *Pseudomonas*.

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The cleanup of contaminated soils by introducing active microbial destructors is attracting the ever-increasing attention of researchers. This approach is especially attractive when pollutants are stable and recalcitrant and when their low concentration in soil makes microbiological remediation the sole alternative.

Catabolic genes are known to be often localized on plasmids [1]. The possibility of increasing the biodegradative potential of soil microorganisms and accelerating the biodegradation process by transfer of the catabolic genes is discussed in many papers [2, 3].

The transfer of resistance plasmids between microorganisms is rather well-studied [4, 5]. Recently, interest in the transfer of catabolic plasmids has increased [3, 6–8]. The works are dedicated, mainly, to the study of transfer of catabolic plasmids in model soil systems [9]. Plasmid transfer in seawater [10], activated sludge [11], and epilithone [12], as well as on the surface of leaves [13] and plant sprouts [6], has been studied as well. Due to the methodical problems of monitoring strains under field conditions, few works consider the transfer of biodegradation plasmids in situ which is associated with. For example, the transfer of catabolic plasmids into indigenous soil bacteria was investigated [9, 10], but in relation to the process of pollutant destruction. At the same time, there are no works dedicated to the horizontal transfer of catabolic plasmids and its role in the biodegradation of polycyclic aromatic hydrocarbons in open environment.

The objective of this work was to study the horizontal transfer of catabolic plasmids in open soil both in the presence and absence of the selection pressure factor (naphthalene) and to assess the effect of this process on naphthalene biodegradation in soil.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmid-free strain *Pseudomonas putida* KT2442 Gfp^+ Km^r Rif^r, kindly provided by Prof. Kornelia Smalla (Braunschweig, Germany), and strains *P. putida* BS394 Cys^- Sm^r Nal^r and *P. putida* KT2442 Gfp^+ Km^r Rif^r with the labeled naphthalene biodegradation plasmid pNF142::Tn*Mod*-OTc [8] were used in this work.

Cultivation conditions and media. The bacteria were grown at 24°C on the following rich media: Luria–Bertani (LB) [14], King B [15], and *Pseudomonas* Isolation Agar (Difco, United States), as well as the Evans mineral medium [16], with naphthalene. Agar medium was obtained by adding 2% (wt/vol) Difco agar. For cultivation on agar medium, naphthalene was added onto the inside of a petri dish cover. For the

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auxotrophic strain BS394, the mineral medium was supplemented with cysteine (0.05 g/l). Antibiotics by Sigma-Aldrich and ICN were used. The final antibiotic concentrations in selective media were (μg/ml): 10, 50, and 100 for tetracycline (Tc) and 30 and 100 for kanamycin (Km).

Monitoring of tagged plasmid-bearing degrader strains during naphthalene degradation in soil. Plasmid-bearing strains were monitored by the markers of antibiotic resistance and fluorescence under UV light on LB agar (strain KT2442) and the marker of auxotrophicity (strain BS394). Serial dilutions of soil samples in phosphate buffer were plated on selective agar media. Transconjugants were isolated on the Evans agar medium with naphthalene and tetracycline (20 μg/ml).

Naphthalene concentration in soil samples was determined as described earlier [17].

Plasmid DNA was isolated by the method of alkaline lysis [18].

DNA hydrolysis by restriction endonucleases was carried out in accordance with the manufacturer's protocol (Fermentas, Lithuania) at 37°C for 1 h.

Total DNA was isolated according to [19].

Polymerase chain reaction (PCR) was carried out in a GeneAmp PCR System 2400 amplifier (Perkin-Elmer, United States) under standard conditions. The PCR mixture (25 μl) contained enzyme buffer (Fermentas); target DNA, 5–10 ng; 18 pM of each primer; deoxyribonucleotide triphosphates (final concentration 200 μM); $MgCl_2$, 1.5 mM; and *Taq*-polymerase, 1.5–2.5 units (Amersham Life Science, United Kingdom). Primers 63f and 1387r were used for the 16S rRNA gene amplification [20].

Sequencing of the 16S rRNA gene of a number of tested strains was performed in a CEQ™ 2000XL DNA Analysis System with the DTCS reagent kit (Beckman Instruments, United States) in accordance with the manufacturer's protocol.

Electrophoresis was performed in horizontal agarose gel by the standard procedure [21].

Preparation of the experimental site. The experiment was carried out in the period from September to November on specially allocated sites on the territory of the water treatment facilities (Pushchino, Moscow oblast). The soil of the experimental site was defined as gray forest soil and contained the following: ash, 91.00% (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%; and K, 2.47%); water infusion pH was 7.05.

The site consisted of six soil plots (1 m^2) separated from each other (Fig. 1). Naphthalene as a powder (2.5 g/kg soil) was added to plots 2, 4, and 6. The soils of plots 3 and 4 were inoculated with the strain *P. putida* KT2442(pNF142::Tn*Mod*-OTc); the soils of plots 5 and 6 were inoculated with the following strains: plasmid-free recipient KT2442 and auxotrophic donor BS394(pNF142::Tn*Mod*-OTc).

Plot 1 Control	Plot 2 Control with naphthalene
Plot 3 *P. putida* KT2442 (pNF142::Tn*Mod*-OTc)	Plot 4 P. putida KT2442 (pNF142::TnMod-OTc) naphthalene
Plot 5 *P. putida* KT2442 and BS394(pNF142::Tn*Mod*-OTc)	Plot 6 P. putida KT2442 and BS394(pNF142::TnMod-OTc) naphthalene

Fig. 1. The scheme of the field experiment on the horizontal transfer of catabolic plasmids.

Bacteria grown in the liquid Evans medium with naphthalene as the sole carbon and energy source were used as inocula. The cells were grown to the midexponential growth phase ($1.0–5.0 \times 10^8$ of colony-forming units (CFU) per 1 ml), precipitated by centrifugation, washed twice in phosphate buffer ($NaH_2PO_4 \cdot 2H_2O$, 6 g/l; $Na_2HPO_4 \cdot 12H_2O$, 22 g/l), and resuspended in sterile tap water.

Soil sampling. The population kinetics of indigenous and introduced degraders and naphthalene concentration were assessed in soil samples (50 g) taken every 24 h after the onset of the experiment and then every 3–5 days. Averaged soil samples (5 g) were suspended in 45 ml of phosphate buffer and stirred in a Paramix shaker (Germany) for 1 min at room temperature. Standard serial dilutions of the suspension were plated on selective media. The plates were incubated at 24°C for 1–3 days. CFU were counted and recalculated per 1 g of dry soil.

RESULTS

Indigenous naphthalene-degrading soil microorganisms. Soil samples from experimental plots were tested for the presence of indigenous naphthalene destructors. Their quantity was low: $3 \times 10^2–1 \times 10^3$ CFU/g soil, i.e., less than 1% of the total quantity of microorganisms. Indigenous naphthalene destructors were isolated and designated as AP1 and AP4 [22]. These strains differed by morphological traits (color, size and shape of the colonies), antibiotic resistance, and the range of utilized substrates. Strain AP1 was preliminarily identified as *Pseudomonas fluorescens* and strain AP4 was identified as *P. putida* by comparing the restriction profiles of amplified 16S rDNA with the previously obtained profiles for different species of fluorescent pseudomonads (strains *P. putida* mt-2, *P. fluorescens* 2-79, etc.).

It was shown that both strains possessed the gene of the large subunit of naphthalene dioxygenase, *nahAc*,

and that strain AP4 contained a plasmid of about 80–90 kb [22].

Survival and competitiveness of introduced tagged plasmid-free and plasmid-bearing strains in soil. For the study of horizontal transfer of catabolic plasmids, the tagged naphthalene destructors *P. putida* KT2442(pNF142::Tn*Mod*-OTc), BS394(pNF142::Tn*Mod*-OTc), and the plasmid-free strain KT2442 were introduced into soil in accordance with the scheme (Fig. 1). The introduced strains were monitored by chromosome markers Gfp, Km^r, Rif^r, and Cys^-; the labeled plasmids were monitored by the markers Nah^+ and Tc^r.

In control plot no. 1 (without introduced bacteria and without naphthalene), the number of indigenous naphthalene destructors in the first 12 days increased from 2.0×10^2 to 5.0×10^4 CFU g^{-1} of dry soil (Fig. 2) concurrently with a change in the total number of microorganisms, which probably resulted from the higher humidity of soil due to intensive autumn rains and a short-term rise of the ambient temperature (Fig. 3). The number of indigenous naphthalene destructors in plot no. 2 (without introduced bacteria but with naphthalene) increased by 3–4 orders of magnitude (from 6.0×10^2 to 3.2×10^6 CFU g^{-1} of dry soil) during the same period (Fig. 2).

The strain KT2442(pNF142::Tn*Mod*-OTc) was introduced into the soil of plots 3 and 4. In the absence of selection pressure (plot 3 without naphthalene), this strain was noncompetitive compared to indigenous naphthalene destructors. As a result, its number decreased by three orders of magnitude by day 12 of the experiment (Fig. 2). In the presence of naphthalene (plot 4, Fig. 2), this strain predominated over indigenous destructors and its number increased during the first 12 days by more than an order of magnitude (from 2.0×10^5 to 4.0×10^6 CFU g^{-1} dry soil). However, it number decreased sharply after 20 days, probably due to the decrease of naphthalene concentration in the soil (Fig. 2).

Two strains, plasmid-bearing auxotrophic BS394 (pNF142::Tn*Mod*-OTc) and plasmid-free KT2442, were introduced into the soil of plots 5 and 6 as a potential plasmid donor and recipient, respectively. In plot 5 without naphthalene (Fig. 2), the strain BS394(pNF142::Tn*Mod*-OTc) was noncompetitive and was eliminated from the soil. Under selection pressure (plot 6 with naphthalene), its number remained at the high initial level and decreased after day 20. The number of plasmid-free strain KT2442 on day 12 expectedly decreased by two orders of magnitude in both plots, as it was not a destructor but a potential recipient of catabolic plasmids.

Horizontal transfer of biodegradation plasmids in soil microbial populations and its effect on the efficiency of naphthalene biodegradation in open soil. In the naphthalene-contaminated soil of plots 4 and 6, the plasmid pNF142::Tn*Mod*-OTc was transferred to indigenous soil bacteria at a frequency of 4×10^{-6}/donor cell (mainly to fluorescent pseudomonads), as well as to the plasmid-free strain KT2442 (transfer frequency of 2×10^{-7}/donor cell). Nearly all transconjugants isolated from the soil of plot 6 were indigenous bacteria that had acquired the labeled plasmid pNF142::Tn*Mod*-OTc. Their taxonomic affiliation was determined by the 16S rRNA gene sequencing. These microorganisms were identified as *P. lini*, *P. frederiksbergensis*, *P. jessenii*, *P. graminis*, *P. putida*, and *P. alcaligenes*. Restriction profiles of the plasmid DNA isolated from transconjugants were similar to the profile of plasmid pNF142::Tn*Mod*-OTc (Fig. 4). Restriction patterns of the preparations of plasmid DNA isolated from the strains *P. frederiksbergensis* OSP3 and *Pseudomonas* sp. OSP10 show the presence of additional fragments (Fig. 4, lanes *2* and *3*), which seems to result from intraplasmid rearrangements.

The effect of horizontal transfer of catabolic plasmids on the efficiency of naphthalene degradation in the soil of plots 2, 4, and 6 was assessed by measuring the content of this pollutant. In all three plots, the rate of naphthalene consumption was different. The slowest decrease of naphthalene concentration was observed in the control plot no. 2 (about 0.2 g/kg soil on day 30). In plot no. 4, naphthalene concentration decreased by that time to 0.06 g/kg soil; in plot no. 6, only residual naphthalene concentrations were found already on day 20 (0.01 g/kg soil).

DISCUSSION

Investigation of the problems of gene transfer in nature requires the monitoring of both microorganisms and plasmids carrying the catabolic genes. Direct observation of strains and plasmids under field conditions is extremely difficult due to uncontrolled aspects of field experiments. The process of horizontal transfer of biodegradation plasmids in nonsterile soil and in open environment is therefore poorly studied. In the tagged strains used in this work, the chromosome contained reporter genes *gfp, Km*, and *Rif*, while the naphthalene biodegradation plasmid was labeled by the gene of tetracycline resistance. The chromosomal fluorescence gene *gfp*, together with the plasmid marker of tetracycline resistance, made it possible to monitor the introduced strain KT2442 (used as a plasmid donor and recipient in different plots) and the transfer of the labeled biodegradation plasmid pNF142::Tn*Mod*-OTc with direct selection of transconjugants.

Our work demonstrated that the tagged plasmid-bearing strains *P. putida* KT2442(pNF142::Tn*Mod*-OTc) and BS394(pNF142::Tn*Mod*-OTc) could dominate over indigenous naphthalene destructors under selection pressure (soil contamination) and were quickly eliminated in the absence of the pollutant.

The expected directions of transfer of naphthalene biodegradation plasmids under selective conditions were as follows (Fig. 5): in plot 4, the labeled plasmid could be transferred from *P. putida* KT2442(pNF142::Tn*Mod*-

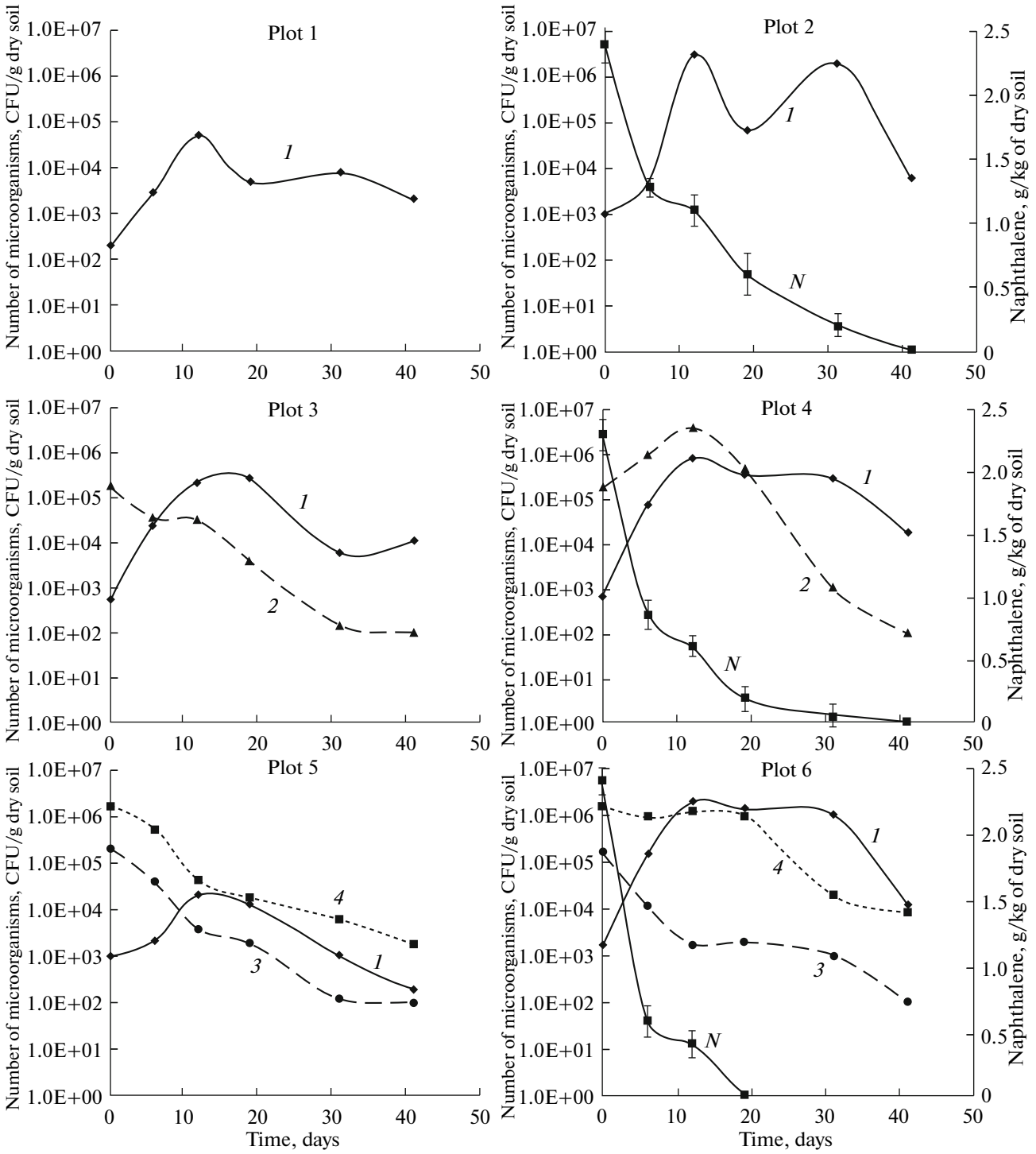

Fig. 2. Dynamics of microbial number and naphthalene concentration in soil: indigenous naphthalene destructors (*1*); plasmid-bearing strain *P. putida* KT2442(pNF142::Tn*Mod*-OTc) (*2*); plasmid-free strain *P. putida* KT2442 (*3*); strain *P. putida* BS394(pNF142::Tn*Mod*-OTc) (*4*); and the curve of naphthalene decrease (*N*).

OTc) to indigenous microorganisms and, besides, indigenous plasmids could be transferred to the introduced strain; in plot 6, the labeled plasmid could be transferred from strain BS394(pNF142::Tn*Mod*-OTc) to the plasmid-free KT2442, from BS394(pNF142::Tn*Mod*-OTc) to indigenous microorganisms, and from indigenous plasmid-bearing microorganisms to plasmid-free KT2442. The transfer of indigenous plasmids to strain KT2442 was not registered, probably because of its low abundance in soil.

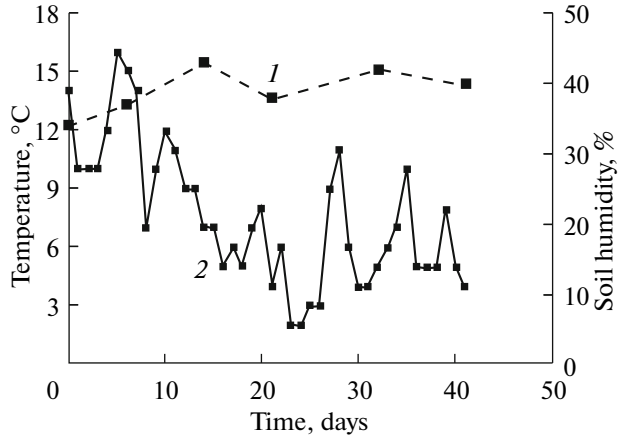

Fig. 3. Dynamics of soil humidity (*1*) and air temperature (*2*) during the experiment.

It is known that selection pressure in soil can substantially influence on the distribution of genes [2, 3, 11]. The frequency of plasmid transfer was 10^{-7}–10^{-6} per donor cell. The frequency of plasmid transfer was higher in plot no. 6, with naphthalene as a selective factor.

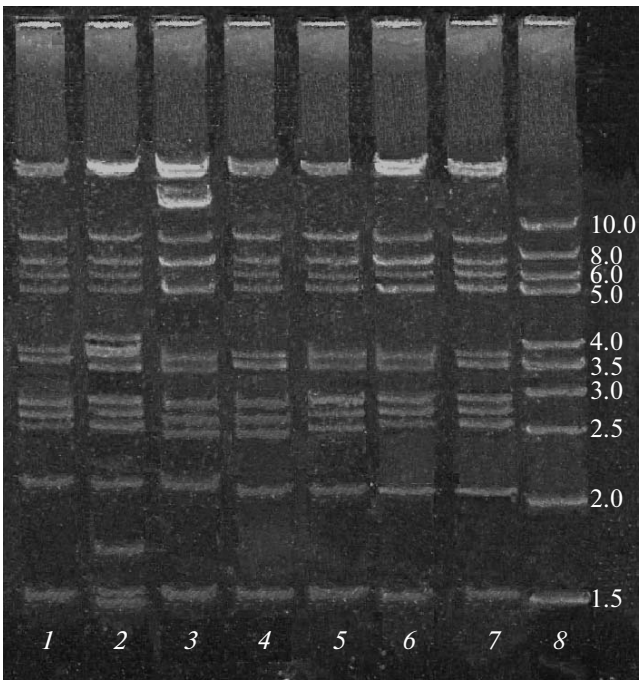

Fig. 4. Restriction patterns of plasmid pNF142::Tn*Mod*-OTc isolated from different strains and treated with restriction endonuclease *Eco*RI: initial strain KT2442 (*1*); *2–5*, indigenous transconjugants: *P. frederiksbergensis* OSP3 (*2*); *Pseudomonas* sp. OSP10 (*3*); *P. putida* OSP16 (*4*); *P. putida* OSP19 (*5*), *6–7*, transconjugants of strain KT2442: clone 25 (*6*); clone 22 (*7*); and 1-kb Ladder Gene Ruler^{TM} Fermentas (Lithuania) (*8*).

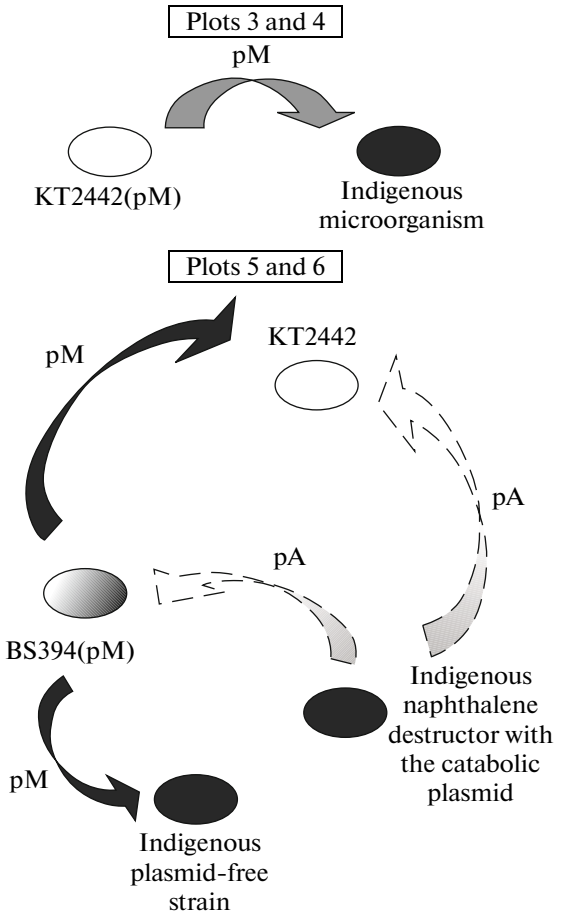

Fig. 5. The scheme of possible directions of the transfer of naphthalene biodegradation plasmids in open soil (arrows with a solid boundary indicate the transfer demonstrated in the experiment; arrows with a dash boundary indicate unregistered transfer).

It is interesting to note that the labeled plasmids were transferred from the strain BS394(pNF142::Tn*Mod*-OTc) to indigenous (fluorescent and non-fluorescent) bacteria in plot no. 6 and, in the absence of selection pressure in plot no. 5, from the donor BS394(pNF142::Tn*Mod*-OTc) to plasmid-free recipient KT2442. The isolated transconjugant strains belonged to several species of the genus *Pseudomonas*.

The total activity of indigenous destructors and introduced strains KT2442(pNF142::Tn*Mod*-OTc) and BS394(pNF142::Tn*Mod*-OTc) ensured accelerated naphthalene degradation in plots 4 and 6, respectively. The transconjugants formed in plot 6 may have made a certain contribution to the process of naphthalene degradation.

During the transfer, plasmid DNA was rearranged in some of the transconjugants (*P. frederiksbergensis* OSP3 and *Pseudomonas* sp. OSP10), which could be evidence of the presence of transposons in the plasmid. The presence of transposons as components of

catabolic plasmids has been shown previously: for the toluene biodegradation plasmid pWW0 [23] and naphthalene biodegradation plasmids NLP-1 [24] and pDTG1 [25] (plasmid pNF142 is known to be pDTG-like [22]).

Thus, direct evidence of dissemination of naphthalene biodegradation plasmids in microbial populations in open soil under selective and nonselective conditions was obtained. The transfer of naphthalene biodegradation plasmids in soil microbial populations seems to enhance the efficiency of hydrocarbon biodegradation under field conditions due to the increase of microbial degradative potential.

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