Microbiology, Vol. 72, No. 5, 2003, pp. 597–604. Translated from Mikrobiologiya, Vol. 72, No. 5, 2003, pp. 672–680.

EXPERIMENTAL ARTICLES

Identification of the Key Genes of Naphthalene Catabolism in Soil DNA

D. V. Mavrodi*, N. P. Kovalenko, S. L. Sokolov*, V. G. Parfenyuk***, I. A. Kosheleva*[1], and A. M. Boronin***

**Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

***Kuban State University, Krasnodar, 350040 Russia*

****Pushchino State University, pr. Nauki 3, Pushchino, Moscow oblast, 142290 Russia*

Received November 26, 2002; in final form, March 11, 2003

Abstract—The key genes *nahAc* and *xylE* of the naphthalene catabolism of fluorescent *Pseudomonas* spp. in total soil DNA samples were detected by the polymerase chain reaction (PCR) technique. The collection of fluorescent *Pseudomonas* spp. was screened for the occurrence of these genes. The results obtained show the possibility of using this approach in the goal-directed search for plasmid-containing naphthalene-degrading fluorescent pseudomonads in soil. The distribution of the naphthalene catabolism genes in soils contaminated with creosote and petroleum products was also studied.

Key words: fluorescent *Pseudomonas*, naphthalene catabolism genes, total soil DNA, polymerase chain reaction.

Soil microflora plays a key role in the natural decontamination of the environment polluted with aromatic hydrocarbons [1]. There is a large number of publications devoted to the isolation of microbial strains capable of utilizing particular aromatic hydrocarbons and to their study in pure cultures. At the same time, little is known about the distribution of the key catabolic genes (usually of plasmid origin) among microbial populations living in contaminated soils and, hence, about the role of particular microbial species in natural bioremediation. The problem was aggravated by the fact that most relevant microorganisms cannot be cultivated under laboratory conditions [2]. In recent years, however, new analytical methods have been developed that allow ecological studies to be carried out by analyzing the total DNA extracted from soil [1, 3].

Naphthalene and other polycyclic aromatic hydrocarbons (PAHs) can be degraded by some of both gram-negative and gram-positive microorganisms [4]. It is known that the genetic systems of naphthalene catabolism control the catabolism of more complex PAHs as well [5]. Consequently, naphthalene can be used as a model substrate for the study of the general degradation principles of the whole class of PAHs. As a rule, the naphthalene catabolism genes of *Pseudomonas* spp. are carried by large conjugative plasmids and are organized into two *nah* operons, which control the oxidation of naphthalene to salicylate, followed by its conversion into catechol and then to the Krebs cycle intermediates [6]. In spite of the fact that the genes that control the initial steps of naphthalene oxidation were cloned from a number of *Pseudomonas* spp., the diversity, evolution, and the hosts of the naphthalene catabolism plasmids have until now been poorly studied. It is known that these plasmids differ in size, conjugative properties, and the disposition of the *nah* operons, although the arrangement of genes in the operons is typically the same [7].

The aim of this work was to detect naphthalene-degrading microorganisms in soil by means of the PCR analysis of the total soil DNA for the presence of the key genes of naphthalene catabolism, as well to isolate and to study these microorganisms.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Nutrient media. The bacterial strains were grown in an Evans medium containing 8.87 g/l KH_2PO_4, 5 mM NH_4Cl, 0.1 mM Na_2SO_4, 62 μM $MgCl_2$, 1 μM $CaCl_2$, and 5 μM $(NH_4)_6Mo_7O_2 \cdot 4H_2O$. The medium was supplemented with 1 ml of a trace element solution containing (g/l) ZnO, 0.41; $FeCl_2 \cdot 6H_2O$, 5.4; $MnCl_2 \cdot 4H_2O$, 2; $CuCl_2 \cdot 4H_2O$, 0.17; $CoCl_2 \cdot 6H_2O$, 0.48; and H_3BO_3, 0.06 (pH 7.0).

The medium was solidified by adding 20 g/l Difco agar (United States). Naphthalene was placed on the59

[1] Corresponding author. E-mail: kosheleva@ibpm.serpukhov.su

0026-2617/03/7205-0597$25.00 © 2003 MAIK "Nauka / Interperiodica"

Table 1. The bacterial strains and plasmids used in this work

Strain (plasmid)	Relevant phenotype*	Source
Pseudomonas putida BS202 (NPL-1)	Nah^+ Sal^+ Gen^+	Soil nearby a coal mine, Makeevka, the Ukraine
P. putida BS3701 (pBS1141, pBS1142)	Nah^+ Sal^+ Gen^+ Phn^+	Soil nearby a coking plant, Vidnoe, Moscow region
P. putida BS3710 (pBS216)	Nah^+ Sal^+ Gen^- Phn^+ Cys^-	Soil nearby the Magnitogorsk iron- and steelworks
P. putida BS3750 (pBS1181)	Nah^+ Sal^+ Phn^+	Oil-polluted soil, West Siberia
P. putida 8C, 15C, 16C, 24C, 25C	Nah^+ Sal^+ Phn^+	Ditto
Pseudomonas sp. 16L	Nah^+ Sal^+	Ditto
Pseudomonas sp. SN11 (pSN11)	Nah^+ Sal^+	Soil nearby a salt pile, Berezniki
Pseudomonas sp. HK21, HK22, HK32, HK33, HK43, HK72	Nah^+ Sal^+	Oil sludge, Nizhnekamsk
P. putida BS238 (pBS2)	Nah^+ Sal^+ $xylE^-$	Soil nearby the Nizhnetagil'skii iron- and steelworks
P. putida BS575 (pBS101)	Nah^+ Sal^+ $xylE^+$	Derived from pBS2
P. putida BS627 (pBS213)	Nah^+ Sal^+ Cys^-	Soil nearby the Nizhnetagil'skii iron- and steelworks
P. putida BS3790 (pBS1191, pBS1192)	Nah^+ Sal^+ Gen^+ Phn^+ Ant^+	Oil-polluted soil, West Siberia
P. putida BS394 (pBS1192)	Nah^- Sal^+ Cys^- Sm^R	Derived from BS3790
P. putida BS3790-E5 (pBS1192)	Nah^- Sal^+	Derived from BS3790
Burkholderia sp. BS3702 (pBS1143)	Nah^+ Sal^+ Phn^+	Soil contaminated with fuel oil, Pushchino, Moscow region
Burkholderia sp. BS3770 (pBS1170)	Nah^+ Sal^+ Phn^+	Oil-polluted soil, West Siberia
P. putida BS394 (pBS265), *P. putida* BS394 (pBS267)	Cap^+	Waste treatment plant of a chemical plant, Severo-Donetsk, the Ukraine
P. putida BS394 (pBS268)	Cap^+	Waste treatment plant of a chemical plant, Kemerovo
P. putida BS247 (NAH7)	Nah^+ Sal^+	Laboratory collection
P. putida KT2442	$Nah^-Sal^-GfpKm^R$	K. Smalla (Germany)

* Nah^+, Sal^+, Phn^+, Gen^+, Ant^+, and Cap^+ are the ability to grow on, respectively, naphthalene, salicylate, phenanthrene, gentisate, anthracene, and caprolactam as the sole source of carbon and energy. Cys^- is cysteine auxotrophy. $xylE^+$ is the possession of catechol 2,3-oxygenase activity. Sm^R and Km^R are streptomycin and kanamycin resistance, respectively. Gfp is green fluorescent protein.

inner side of the petri dish lid. Amino acids were added to a final concentration of 40 μg/ml.

The isolation of naphthalene-degrading strains. Microorganisms capable of utilizing naphthalene as a sole source of carbon and energy were isolated through enrichment cultures. Soil samples (2 g) were placed in flasks containing 50 ml of Evans medium. The flasks were incubated at 28°C on a shaker. The second subculture was plated onto agar medium with naphthalene as the sole source of carbon and energy. Microorganisms grown on this medium were tested for the ability to utilize salicylate (a naphthalene intermediate) and various PAHs. The fluorescence of pseudomonads was tested using King B medium [8]. Fluorescent pseudomonads were preliminarily identified by amplified ribosomal DNA restriction analysis (ARDRA) with *Rsa*I restriction endonuclease and *Pseudomonas putida, P. fluorescens, P. aureofaciens, P. chlororaphis*, and *P. aeruginosa* as the reference species. Plasmid DNA was isolated by the rapid alkaline extraction procedure of Birnboim and Doly [9] with minor modifications. Plasmids were conjugatively transferred on the agar medium as described by Rheinwald *et al.* [10].

The isolation of DNA from soil. Soil DNA was isolated using a Fast DNA Kit (Bio 101, United States) according to the manufacturer's instruction. The lysis of soil microorganisms was enhanced by using glass beads. The isolated DNA was purified with the aid of a Wizard DNA Cleanup System (Promega, United States), eluting DNA with 50 μl of deionized water heated to 65°C.

Quantification of DNA in soil samples. The amount of soil DNA was determined in a TKO-100 fluorimeter (Hoefer Scientific Instruments, United States) using the dye Hoechst 33258 (Bio-Rad, United States) dissolved in a TNE buffer (10 mM Tris–HCl (pH 7.4)

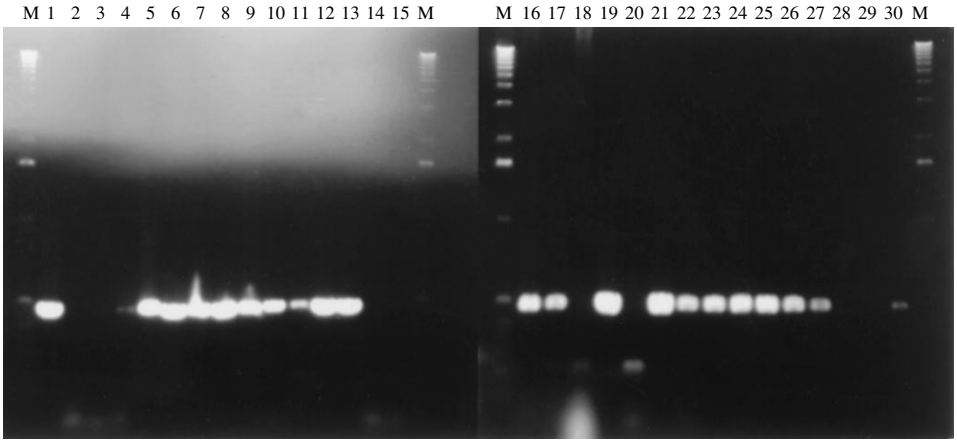

Fig. 1. Amplification of the *nahAc* gene of strains (1) 16L, (2) HK21, (3) HK22, (4) HK32, (5) HK33, (6) HK43, (7) HK72, (8) 8C, (9) BS3790(pBS1191 + pBS1192), (10) BS3790-E5, (11) BS575(pBS101), (12) BS3710(pBS216), (13) BS3750(pBS1181), (14) BS3702, (15) BS3770, (16) BS394(pBS265), (17) BS394(pBS267), (18) BS394(pBS268), (19) SN11(pSN11), (20) BS627(pBS213), (21) BS238(pBS2), (22) 15C, (23) 16C, (24) 24C, (25) 25C, (26) BS202(NPL1), (27) BS3701(pBS1141 + pBS1142), (28) BS247(NAH7), (29) BS3790-E5(pBS1192), and (30) BS394(pBS1192). M is the marker.

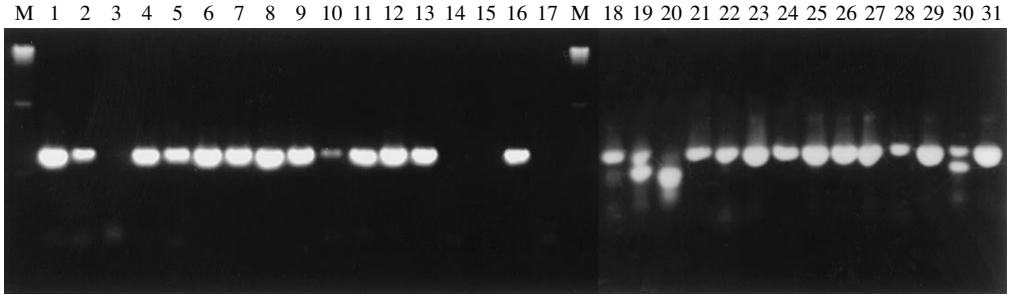

Fig. 2. Amplification of the *xylE* gene of strains (1) 16L, (2) HK21, (3) HK22, (4) HK32, (5) HK33, (6) HK43, (7) HK72, (8) 8C, (9) BS3790(pBS1191 + pBS1192), (10) BS3790-E5, (11) BS575(pBS101), (12) BS3710(pBS216), (13) BS3750(pBS1181), (14) BS3702, (15) BS3770, (16) BS202(NPL1), (17) H_2O (negative control), (18) BS394(pBS265), (19) BS394(pBS267), (20) BS394(pBS268), (21) SN11(pSN11), (22) BS627(pBS213), (23) 15C, (24) 16C, (25) 24C, (26) 25C, (27) BS3701(pBS1141 + pBS1142), (28) BS238(pBS2), (29) BS247(NAH7), (30) BS394(pBS1192), and (31) BS202(NPL1). M is the marker.

containing 1 mM EDTA and 0.2 M NaCl) according to the manufacturer's instructions.

Polymerase chain reaction (PCR). PCR amplifications were performed in a GeneAmp 2400 thermal cycler (Perkin-Elmer, United States) and in a Hybaid gradient thermal cycler (United Kingdom). The reaction mixture contained PCR buffer (10 mM tris-HCl, pH 8.4, 50 μM KCl, 0.1 μg/ml gelatin), 5–10 ng of target DNA, 18 nM of each primer, 200 μM of each deoxyribonucleotide triphosphate, 1.5 mM $MgCl_2$, and 1.5–2.5 U of *Taq* DNA polymerase (Amersham, United Kingdom). In some experiments, 3% DMSO (Sigma, United States) was used as the denaturing agent.

Electrophoresis in agarose gel. Electrophoresis was carried out in horizontal 1% agarose slabs [11] using a 1-kb DNA ladder (Invitrogen, United States) as the standard.

DNA–DNA hybridization. DNA samples were analyzed by electrophoresis in agarose gel and transferred onto Hybond N^+ nylon membranes (Amersham) in 0.4 M NaOH for 3–4 h. The membranes were rinsed in 2× SSC (0.03 M sodium citrate buffer (pH 7.0) with 0.3 M NaCl) for 5 min. Prehybridization was carried out at 65°C for 2 h in 50 ml of the reaction mixture containing 5× Denhardt solution, 1% SDS, and 300 mg/ml denatured calf thymus DNA in 2× SSC. Hybridization was carried out at 65°C for 12–16 h in 1 ml of the prehybridization mixture supplemented with [^{32}P]dATP (1–2 million cpm) as the probe. The probe was labeled with the DECAprime II™ system (Ambion, United States) according to the manufacturer's instruction. After hybridization, the filter was washed as described in the handbook [12], dried, and exposed to Hyperfilm ECL (Amersham) for 6–16 h.

RESULTS

The amplification of the marker genes *nahAc* and *xylE* of soil fluorescent pseudomonads. PCR

Table 2. Soil samples used in this work

Soil sample no.	Soil sampling location*
11	Soil from the tar spill site, the *Metoxyl* plant, Vetluzhskii settlement, Nizhegorod region
12	Soil at a 1-m distance from the tar spill site, the *Metoxyl* plant
13	Soil at a 1-m distance from the outlet valve of a tank with creosote, the *Metoxyl* plant
14	Soil below the outlet valve of the tank with creosote, the *Metoxyl* plant
15	Soil from the creosote spill site, the *Metoxyl* plant
16	Soil nearby the gasoline station of Vetluzhskii settlement
17	Soil from a garden, Voskresenskoe village, Nizhegorod region
18	Soil nearby the Shurgovashka River, Nizhegorod region
19	Soil nearby the gasoline station no. 215, Voskresenskoe village
1004	Soil from the Oka Terrace State Nature Reserve, Moscow region
1010	Soil from the Oka Terrace State Nature Reserve, Moscow region
1011	Soil from the Oka Terrace State Nature Reserve, Moscow region

* Soil samples were collected in May–June 2001 at 15–20°C.

amplifications were carried out with the total DNA of fluorescent pseudomonad strains from the collection of the Laboratory of Plasmid Biology of the Institute of Biochemistry and Physiology of Microorganisms (Table 1) and two primers, Ac114F (5'-CTGGCWWTTYCTCACYCAT-3') and Ac596R (5'-CRGGTGYCTTCCAGTTG-3'), which amplify a 482-bp fragment of the naphthalene dioxygenase gene [13]. PCR was performed with the initial DNA denaturation step at 94°C for 1 min, followed by 29 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 57°C for 1 min (the annealing temperature was lowered at a rate of 0.5°C per cycle), and primer extension at 72°C for 1.5 min, and 15 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 42°C for 1 min, and primer extension at 72°C for 1.5 min, with the final extension step at 72°C for 5 min.

The data presented in Fig. 1 demonstrate that most of the 29 plasmid DNA samples of fluorescent pseudomonads exhibited an intense specific amplification of the *nahAc* gene. The amplification of this gene in the plasmid DNA samples of *P. putida* BS3701, BS575, BS394(pBS1192), BS394(pBS267), and *Pseudomonas* sp. HK32 was negligible, and it was completely absent in the case of plasmid DNA from *P. putida* BS627, BS3790-E5, and BS394(pBS268), *Pseudomonas* sp. HK21 and HK22, and *Burkholderia* sp. BS3702 and BS3770.

The *xylE* gene in the same DNA samples was amplified with primers 23OF (5'-ATGGATDTDATGGGDTTCAAGGT-3') and 23OR (5'-ACDGTCADGAADCGDTCGTTGAG-3'), which amplify a 721-bp fragment of the catechol 2,3-oxygenase gene [14]. Optimal reaction conditions were determined with the aid of the Hybaid gradient thermal cycler by varying the primer annealing temperature from 45 to 60°C. The optimal annealing temperature of primers 23OF and 23OR was found to be 52°C (Fig. 2). The PCR amplification of the *xylE* gene was carried out with the initial DNA denaturation step at 94°C for 1 min, followed by 35 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 52°C for 30 s, and primer extension at 72°C for 45 s, with the final extension step at 72°C for 4 min.

As can be seen from the data presented in Fig. 2, most of the 29 plasmid DNA samples of fluorescent pseudomonads exhibited an intense specific amplification of the *xylE* gene. The specific amplification of this gene in the plasmid DNA samples of *P. putida* BS238, BS3790-E5, and *Pseudomonas* sp. HK32 was negligible, while completely absent in the plasmid DNA of *Burkholderia* sp. BS3702 and BS3770. The amplification of the *xylE* gene in the plasmid DNA of *P. putida* BS394(pBS1192) and BS394(pBS267) was nonspecific.

The isolation and purification of DNA from soil. Soil samples were collected at different sites in the Moscow and Nizhegorod regions (Table 2). We succeeded in isolating the total DNA from all the soil samples taken for analysis, except for soil samples nos. 11 and 15, which were heavily contaminated with tar and creosote and contained only trace amounts of DNA. These samples were excluded from further analysis. The isolated DNA was purified as described in the Materials and Methods section and quantified fluorimetrically. The amount of DNA in soil samples varied from 0.6 ng/μl in sample no. 14 to 49 ng/μl in sample no. 19.

The optimization of the marker gene amplification conditions. The optimal conditions of PCR with the DNA extracted from soil samples were determined with the modified primers 27fm (5'-AGA GTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGHTACCTTGTTACGACTT-3'), which amplify a 1.5-kb fragment of the 16S rDNA gene [15]. PCR amplifications were performed with the initial DNA denaturation step at 94° for 5 min, followed by 35 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and primer extension at 72°C for 2 min, with the final extension step at 72°C for 7 min. Gene amplification was most intense when the reaction mixture contained 5–10 ng of soil DNA and was supplemented with DMSO (under these conditions, the amplification was observed in all of the DNA samples taken for analysis) (Fig. 4).

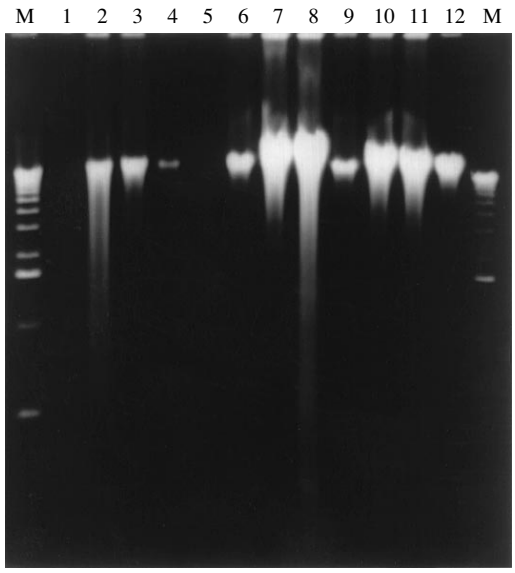

Fig. 3. Electrophoresis of the total DNA extracted from soil samples no. (1) 11, (2) 12, (3) 13, (4) 14, (5) 15, (6) 16, (7) 17, (8) 18, (9) 19, (10) 1004, (11) 1010, and (12) 1011. M is the marker.

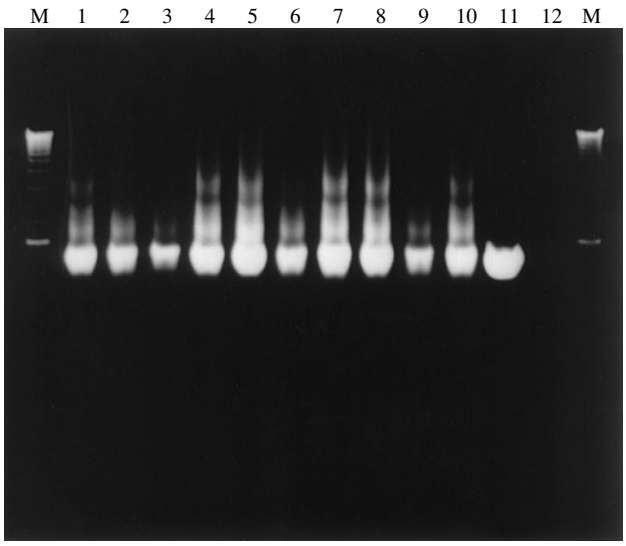

Fig. 4. Amplification of the 16S rRNA gene of the total DNA extracted from soil samples no. (1) 12, (2) 13, (3) 14, (4) 16, (5) 19, (6) 1004, (7) 17, (8) 18, (9) 1010, (10) 1011, and (11) from *Escherichia coli* (positive control). Lanes (12) and (M) are H_2O (negative control) and the marker.

The amplification of the marker genes *nahAc* and *xylE* in soil DNA and the analysis of PCR specificity by DNA–DNA hybridization. The marker gene *nahAc* in the soil DNA samples was amplified with primers Ac114F and Ac596R under the same conditions as in the case of plasmid DNA. The specificity of amplification was tested by DNA–DNA hybridization with the naphthalene 1,2-dioxygenase gene of plasmid NPL-1 as the probe. The hybridization showed that the amplification of the *nahAc* gene in the total DNA extracted from soil samples nos. 12, 14, 16, and 19 was specific (Fig. 5).

The marker gene *xylE* in the soil DNA samples was amplified with primers 23OF and 23OR under the same conditions as in the case of plasmid DNA. The DNA–DNA hybridization showed that the *xylE* gene was efficiently amplified in the total DNA extracted from soil sample no. 1004 and less efficiently in the total DNA of soil sample no. 19 (data not presented).

Table 3. The naphthalene-degrading microorganisms isolated from soil samples

Soil sample no.	Detection of *nahAc* in soil DNA	Detection of *xylE* in soil DNA	Naphthalene-degrading isolates
11	ND	ND	*P. putida* NF11(pNF11)
12	+	–	*P. putida* NF12(pNF12), *P. fluorescens* NF121(pNF121)
13	–	–	13N
14	+	ND	14N, *Pseudomonas* sp. 141NF, *Pseudomonas* sp. 142NF(pNF142), *Pseudomonas* sp. 143NF(pNF143)
15	ND	ND	–
16	+	–	*P. fluorescens* 16NF (pNF16), 161N, *Pseudomonas* sp. 162NF, *P. putida* 163NF, *P. putida* 164NF
17	–	–	17N
18	–	–	18N, 181N
19	+	+	19N, 191N(pN191), 192N(pN192)

Note: ND stands for "not determined". The letter N in the names of isolates indicates that they were not identified. The letter F in the names of isolates implies that they are fluorescent on a King B medium. Parenthesized are naphthalene degradation plasmids.

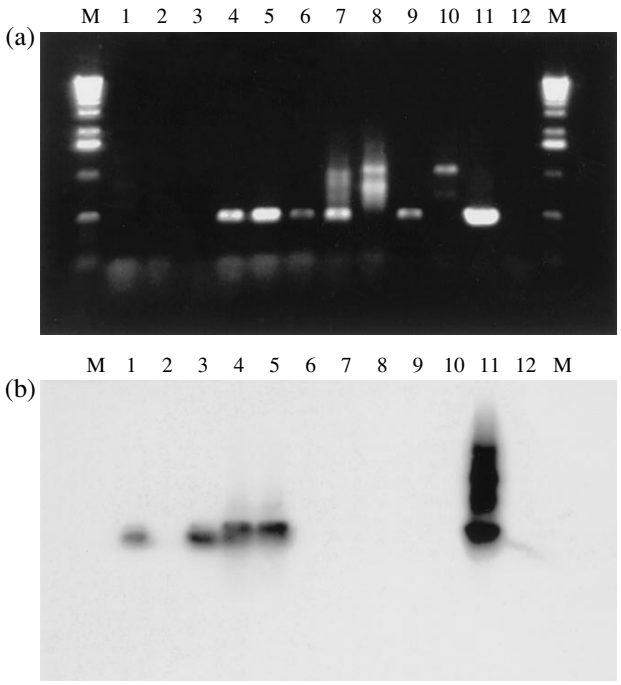

Fig. 5. (a) The amplification of the *nahAc* gene of the total soil DNA and (b) the hybridization of the PCR products with a probe containing the *nahA* gene sequence of plasmid NPL1. The total DNA was extracted from soil samples no. (1) 12, (2) 13, (3) 14, (4) 16, (5) 19, (6) 1004, (7) 17, (8) 18, (9) 1010, (10) 1011, and (11) from *Escherichia coli* (positive control). Lanes (12) and (M) are H_2O (negative control) and the marker.

The isolation of naphthalene-degrading microorganisms. As can be seen from Table 3, naphthalene-degrading strains were isolated from all of the soil samples listed in Table 2, except for soil samples nos. 1004, 1010, and 1011, which were collected in the Oka Terrace State Nature Reserve. Preliminary identification by ARDRA was carried out only for fluorescent pseudomonads, which were isolated from soil samples nos. 11, 12, 14, and 16. Strains 11NF, 12NF, 163NF, and 164NF were assigned to the species *P. putida*, and two strains (121NF and 16NF) were assigned to the species *P. fluorescens.*

Analysis of the isolated naphthalene-degrading strains for the presence of biodegradation plasmids. All naphthalene-degrading fluorescent pseudomonads, as well as the unidentified strains 19N, 191N, and 192N isolated from soil sample no. 19 (in which the *nahAc* and *xylE* genes were detected by PCR), were tested for the presence of plasmid DNA. All of the tested microorganisms turned out to contain plasmids, whose size varied from 83 to 100 kb. Experiments on the conjugal transfer of plasmids showed that strains 11NF, 12NF, 121NF, 141NF, 143NF, 16NF, and 164NF contained conjugative plasmids, which could be transferred to *P. putida* KT2442 cells at a frequency of 10^{-6} to 10^{-4} and which control the whole pathway of naphthalene catabolism through salicylate and catechol (i.e., the *P. putida* KT2442 transconjugants have the phenotype Nah^+Sal^+).

The amplification of the marker genes *nahAc* and *xylE* showed that they are able to amplify with the plasmid DNA of all of the naphthalene-degrading strains under study, including the nonfluorescent strains 19N and 191N.

DISCUSSION

The major goal of this work was to prove the possibility of using the screening of soil DNA samples for the presence of the key genes of naphthalene catabolism as a rapid method of searching for new naphthalene-degrading soil microorganisms. As marker genes, we chose the *nahAc* and *xylE* genes, which code for, respectively, the large subunit of naphthalene 1,2-dioxygenase [16] and catechol 2,3-oxygenase (an enzyme of the *meta*-cleavage pathway) [17]. Both of these enzymes are the key enzymes of naphthalene catabolism in fluorescent pseudomonads [18]. The marker genes were detected by PCR, which is one of the most rapid, specific, and sensitive modern analytical methods.

Our earlier studies showed that the *P. putida* strains BS202, BS3701, BS3710, BS3750, BS575, BS238, BS3790, and BS247 and the *P. fluorescens* strain SN11 contain naphthalene degradation plasmids bearing the *nahAc* and *xylE* genes [5, 19]. The screening of the laboratory collection of naphthalene-degrading strains (Table 1) for the presence of these marker genes showed their occurrence in the *P. putida* strains 8C, 15C, 16C, 24C, 25C, and the *Pseudomonas* sp. strains 16L, HK32, HK33, HK43, and HK72 (Figs. 1, 2). This finding suggests that these strains contain the naphthalene degradation plasmids as well, since the simultaneous presence of the *nahAc* and *xylE* genes is a characteristic feature of the fluorescent pseudomonads that contain the naphthalene degradation plasmids [6]. The strains *P. putida* BS3790-E5 and *P. putida* BS394(pBS268), which are not able to grow on naphthalene, did not show the presence of the *nahAc* and *xylE* genes.

It should be noted that the strain *P. putida* BS394(pBS1192), which contains the salicylate degradation plasmid, and strains *P. putida* BS394(pBS267) and BS394(pBS265), which contain the caprolactam degradation plasmid, exhibited a weak amplification of the marker genes, although all of these strains are unable to grow on naphthalene. Conversely, the strains *P. putida* HK21, HK22, and BS627, which are able to grow on naphthalene, did not exhibit the amplification of the *nahAc* and *xylE* marker genes. The absence of correlation between the presence of the marker genes in the strains mentioned in this paragraph and their ability to grow on naphthalene requires further studies.

The *nahAc* and *xylE* marker genes failed to amplify in the DNA of the *Burkholderia* sp. strains BS3702 and BS3770 either (Figs. 1, 2), although these strains are able to grow on naphthalene. This can be accounted for

by the fact that the degradation of naphthalene in *Burkholderia* spp. is controlled by genes whose structure differs from that of the naphthalene degradation genes of fluorescent pseudomonads [20].

The amplification of the marker genes with the DNA isolated directly from soil samples (Table 2) is a convenient approach to the rapid detection of soil microorganisms capable of degrading naphthalene and other polycyclic aromatic hydrocarbons. This approach allows researchers to avoid tedious cultivation procedures. Moreover, it potentially allows the detection of nonculturable soil naphthalene-degrading microorganisms, which cannot be detected by conventional culture techniques.

The optimization of conditions for the amplification of soil DNA with the 16S rRNA gene and the 27fm and 1492r primers showed that the reaction mixture must contain DMSO and that the optimal concentration of DNA in the reaction mixture is 5–10 ng (Fig. 4). Higher concentrations of soil DNA inhibited PCR, which can be explained by the fact that humic acids likely present in the samples of soil DNA may inhibit PCR [14]. Furthermore, some soil samples used in this study were contaminated with various anthropogenic pollutants, which could inhibit PCR as well.

The results of the amplification of the marker genes *nahAc* and *xylE* indicated the presence of naphthalene-degrading microorganisms in soil samples nos. 16 and 19 (Fig. 5). Control studies with the aid of DNA–DNA hybridization revealed the amplification products of *nahAc* not only in these samples but also in samples nos. 12 and 14, in which the amplification products could not be revealed merely by electrophoresis in agarose gel. The specificity of PCR was confirmed by the direct plating of soil suspensions onto the agar medium with naphthalene. This conventional culture technique indicated the occurrence of naphthalene-degrading fluorescent pseudomonads in soil sample nos. 12, 14, and 16. The DNA of the naphthalene-degrading microorganisms isolated from soil sample no. 19 was amplified with the *nahAc* and *xylE* marker genes. As can be seen from Table 3, there is a good correlation between the presence of the marker gene *nahAc* in a soil sample and the presence of naphthalene-degrading microorganisms in this sample. At the same time, the other marker gene *xylE* did not always show such a correlation.

Thus, the PCR technique with the *nahAc* marker gene can conveniently be used in the goal-directed search for naphthalene-degrading microorganisms in soil.

ACKNOWLEDGMENTS

This work was supported by the INTAS, grant no. 99-01487; grant E00-6.0-288 from the Ministry of Education of the Russian Federation; and by a grant within the scope of the Russian federal scientific and technical program “High-Priority Research and Development in Science and Engineering,” the “Basic and Applied Research and Development” subprogram, the “Biotechnology for Environmental Protection” project.

REFERENCES

1. *Manual of Environmental Microbiology*, Hurst, C.J. *et al.*, Eds., Washington: ASM, 2002.
2. Felske, A., Wolterink, A., van Lis, R., de Vos, W.M., and Akkermans, A.D.L., Searching for Predominant Soil Bacteria: 16S rDNA Cloning versus Strain Cultivation, *FEMS Microbiol. Ecol.*, 1999, vol. 30, pp. 137–145.
3. Zhou, J., Bruns, M.A., and Tiedje, J.M., DNA Recovery from Soils of Diverse Composition, *Appl. Environ. Microbiol.*, 1996, vol. 62, pp. 316–322.
4. Cerniglia, C.E., Biodegradation of Polycyclic Aromatic Hydrocarbons, *Biodegradation*, 1992, no. 3, pp. 351–368.
5. Kosheleva, I.A., Balashova, N.V., Izmalkova, T.Yu., Filonov, A.E., Sokolov, S.L., Slepen’kin, A.V., and Boronin, A.M., Degradation of Phenanthrene by Mutant Naphthalene-degrading *Pseudomonas putida* Strains, *Mikrobiologiya*, 2000, vol. 69, no. 6, pp. 783–789.
6. Yen, K.-M. and Serdar, C.M., Genetics of Naphthalene Catabolism in Pseudomonads, *Crit. Rev. Microbiol.*, 1988, vol. 5, pp. 247–268.
7. Zylstra, G.J., Kim, E., and Goyal, A.K., Comparative Molecular Analysis of Genes for Polycyclic Aromatic Hydrocarbon Degradation, *Genetic Engineering*, Setlow, J.K., Ed., New York: Plenum, 1997, vol. 19, pp. 257–369.
8. King, O.E., Ward, W., and Raney, D.E., Two Simple Media for the Demonstration of Pyocyanin and Fluorescein, *J. Lab. Clin. Methods*, 1954, vol. 44, no. 2, pp. 301–307.
9. Birnboim, H.C. and Doly, J.A., A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA, *Nucleic Acids Res.*, 1979, vol. 7, pp. 1513–1519.
10. Rheinwald, J., Chakrabarty, A.M., and Gunsalus, I.C., A Transmissible Plasmid Controlling Camphor Oxidation in *Pseudomonas putida*, *Proc. Natl. Acad. Sci. USA*, 1973, vol. 70, pp. 885–889.
11. Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York: Cold Spring Harbor Lab., 1989.
12. *Short Protocols in Molecular Biology*, Ausubel, F.M., Ed., New York: John Wiley & Sons, 1995.
13. Wilson, M.S., Bakermans, C., and Madsen, E.L., In Situ, Real-Time Catabolic Gene Expression: Extraction and Characterization of Naphthalene Dioxygenase mRNA Transcripts from Groundwater, *Appl. Environ. Microbiol.*, 1999, vol. 65, pp. 80–87.
14. Wilkstrom, P., Wilklund, A., Anderson, A.C., and Forsman, M., DNA Recovery and the PCR Quantification of Catechol 2.3-Dioxygenase Genes from Different Soil Types, *J. Biotechnol.*, 1996, vol. 52, pp. 107–120.

15. Weisburg, W.G., Barnes, S.M., Pelletier, D.A., and Lane, D.J., 16S Ribosomal DNA Amplification for Phylogenetic Study, *J. Bacteriol.*, 1991, vol. 173, pp. 697–703.

16. Simon, M.J., Osslund, T.D., Saunders, R., Ensley, B.D., Suggs, S., Harcourt, A., Suen, W.C., Cruden, D.L., Gibson, D.T., and Zylstra, G.J., Sequences of Genes Encoding Naphthalene Dioxygenase in *Pseudomonas putida* G7 and NCIB 9816-4, *Gene*, 1993, vol. 127, pp. 31–37.

17. Nakai, C., Kagamiyama, H., Nozaki, M., Nakazawa, T., Inouye, S., Ebina, Y., and Nakazawa, A., Complete Nucleotide Sequence of the Metapyrocatechase Gene on the TOL Plasmid of *Pseudomonas putida* mt-2, *J. Biol. Chem.*, 1983, vol. 258, pp. 2923–2928.

18. Eltis, L.D. and Bolin, J.T., Evolutionary Relationships among Estradiol Dioxygenases, *J. Bacteriol.*, 1996, vol. 178, pp. 5930–5937.

19. Balashova, N.V., Kosheleva, I.A., Golovchenko, N.P., and Boronin, A.M., Phenanthrene Metabolism by *Pseudomonas* and *Burkholderia* Strains, *Process Biochem.*, 1999, vol. 35, no. 3-4, pp. 291–296.

20. Laurie, A.D. and Lloyd-Jones, G., The *phn* Genes of *Burkholderia* sp. Strain RP007 Constitute a Divergent Gene Cluster for Polycyclic Aromatic Hydrocarbon Catabolism, *J. Bacteriol.*, 1999, vol. 181, pp. 531–540.