ELSEVIER

Process Biochemistry 41 (2006) 2417–2423

Process Biochemistry

www.elsevier.com/locate/procbio

Plant growth-promoting *Pseudomonas* bearing catabolic plasmids: Naphthalene degradation and effect on plants

Tatyana O. Anokhina [a,*], Olga V. Volkova [a,b], Irina F. Puntus [a], Andrei E. Filonov [a,b], Vladimir V. Kochetkov [a,b], Alexander M. Boronin [a,b]

[a] *G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Prosp. Nauki 5, Pushchino, Moscow Region 142290, Russia*

[b] *Pushchino State University, Prosp. Nauki 3, Pushchino, Moscow Region 142290, Russia*

Received 30 March 2006; received in revised form 24 June 2006; accepted 27 June 2006

Abstract

Two IncP-9 naphthalene degradative plasmids pOV17 and pBS216 were transferred into plant growth-promoting *Pseudomonas* which were represented by species *P. aureofaciens*, *P. chlororaphis, P. fluorescens*, and *P. putida*. The strains with the same plasmid differed significantly by their growth parameters, stability of the plasmid and plant protective effect from naphthalene action. Strains *P. putida* 53a(pOV17) and *P. chlororaphis* PCL1391(pOV17) demonstrated higher population number in the rhizosphere. Moreover, they protected the mustard plants from naphthalene toxic influence more effectively than the wild type strain *P. aureofaciens* OV17(pOV17). The activity of catechol-2,3-dioxygenase in the strains with the plasmid pOV17 was higher than that in strains with the plasmid pBS216. The strain *P. putida* 53a(pOV17) with high catechol-2,3-dioxygenase activity has been demonstrated to have the best protective effect. The strain *P. putida* 53a(pBS216) without catechol dioxygenases activities did not have protective effect but suppressed the plant germination.
© 2006 Elsevier Ltd. All rights reserved.

Keywords: Plant growth-promoting rhizosphere bacteria *Pseudomonas*; Naphthalene; Plasmids; Plant–bacteria interactions

1. Introduction

Fluorescent *Pseudomonas* strains are typical inhabitants of rhizosphere and rhizoplane. Some strains of *Pseudomonas* rhizosphere bacteria may stimulate plant growth by the synthesis of phytohormones, improve mineral nutrition of plants and protect plants from phytopathogenic fungi due to the synthesis of antibiotics and siderophores [1–4]. These strains are referred to plant growth-promoting *Pseudomonas* (PGPP).

Furthermore, some *Pseudomonas* strains are able to degrade various xenobiotics, including polycyclic aromatic hydrocarbons (PAHs) [5,6]. PAHs catabolic genes are usually located in plasmids. A number of conjugative degradative plasmids has been described [7]. The genetic control and biochemical pathways of naphthalene and phenanthrene degradation by *Pseudomonas* have been studied [8,9]. As a rule, the naphthalene catabolic genes are organized in three operons. *Nah*1-operon encoding for the upper-pathway enzymes is involved in the conversion of naphthalene to salicylate, *nah*2-operon encoding for the lower-pathway enzymes is involved in the oxidation of salicylate via the plasmid-encoded catechol *meta*-cleavage pathway to acetaldehyde and pyruvate. The third operon encodes for a regulatory protein. It has been shown that some degradative plasmids carry non-functioning genes of *meta*-ring cleavage. The strains bearing such plasmids are able to grow on naphthalene using the chromosome-encoded *ortho*-pathway of catechol cleavage [10,11]. The ‘switch-on’ of the silent genes of *meta*-pathway was possible in certain cases (cultivation of strains on a mineral medium containing methylated naphthalene and salicylate derivatives) [12,13].

It is known that the phytotoxicity of contaminated soil is determined by both the direct action of organic pollutants (PAHs, oil hydrocarbons, pesticides, herbicides) and the influence of various microbial toxins. The main group of microorganisms producing toxic metabolites is presented by non-symbiotic micromycetes belonging to the genera *Mucor*, *Aspergillus*, *Penicilum*, *Fusarium* [14]. Thus, the inoculation of plants with biocontrol bacteria can protect plant health,

* Corresponding author. Tel.: +7 4967 73 26 30; fax: +7 495 956 33 70.
E-mail address: to_anohina@rambler.ru (T.O. Anokhina).

1359-5113/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2006.06.026

increase plant productivity on contaminated soils and intensify phytoremediation therefore the construction of PGPP strains with degradative potential is an actual task.

The aim of this study was to investigate the effect of catabolic plasmid-bearing PGPP strains on plant growth in microcosms with naphthalene and estimate the stability and expression of plasmid genes in rhizosphere bacteria.

2. Materials and methods

2.1. Bacterial strains, nutrient media and cultivation conditions

Bacterial strains and plasmids used are given in Table 1. The plasmids pBS216 and pOV17 were isolated from wild type naphthalene-degrading strains *P. putida* M313 and *P. aureofaciens* OV17 correspondingly. Bacterial strain *P. aureofaciens* OV17 (pOV17) was isolated in 2001 year from the rhizosphere of randomly selected cereals growing on oil-contaminated soil in West Siberia, Russia. *P. putida* M313 was isolated in 1980s from the soil of metallurgical plant, Ural region, Russia. The plasmids are wild type mobile genetic elements bearing naphthalene catabolic genes.

Later the plasmid pBS216 was transferred in a cysteine auxotroph strain *P. putida* BS394. It was shown that the strain *P. putida* BS394 is a good recipient of the pBS216 plasmid. In our study *P. putida* BS394(pBS216) was used as the donor of this plasmid in conjugal matings.

Since neither *P. putida* M313 nor *P. putida* BS394 possess any plant growth promoting properties we did not include them in the experiments with plants and did not construct *P. putida* BS394 bearing the plasmid pOV17.

All these plasmid-bearing strains are stored in the collection of Laboratory of Plasmid Biology, G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms Russian Academy of Sciences.

Bacteria were cultivated on Luria-Bertani (LB) [18] and the mineral Evans medium supplemented with naphthalene as a sole carbon and energy source [19]. Evans mineral medium (pH 7.0) had the final composition—K_2HPO_4: 8.71 g l^{-1}; NH_4Cl: 0.25 g l^{-1}; Na_2SO_4: 14 mg l^{-1}; $MgCl_2$: 6.0 mg l^{-1}; $CaCl_2$: 0.1 mg l^{-1}; $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$: 6.5 mg l^{-1}; 1 ml microelement solution. Microelement solution was prepared and sterilized separately and contained (g l^{-1})—ZnO: 0.41; $FeCl_3\cdot 6H_2O$: 5.4; $MnCl_2\cdot 4H_2O$: 2.0; $CuCl\cdot 2H_2O$: 0.17; $CoCl_2\cdot 6H_2O$: 0.48; H_3BO_3: 0.06.

To cultivate bacteria on the agarized Evans medium in Petry dishes naphthalene was added as crystals inside the dish lid (bacteria grew in vapors of naphthalene). To cultivate bacteria in the liquid Evans medium this compound was added as powder without dissolution in any organic solvent.

Growth parameters were studied in batch culture with naphthalene (2 g l^{-1}) as a sole carbon and energy source at 24 °C on orbital shaker at 200 rpm according to Pirt [20]. The initial density of bacteria was $(4–7) \times 10^5$ colony-forming units (CFU) ml^{-1}.

To determine the enzyme activity, the plasmid-harbouring strains were grown in 500 ml flasks containing 200 ml mineral Evans medium and naphthalene (2 g l^{-1}) as a sole carbon and energy source. The initial density of bacteria was $(2–4) \times 10^7$ CFU ml^{-1}. The cultivation proceeded for 18–20 h at 24 °C.

The plasmid-free bacteria used in the experiment with the induction of catechol-1,2-dioxygenase were grown on the mineral Evans medium containing 1 g l^{-1} sodium glutamate. The enzymatic activity was induced by adding catechol (0.1 g l^{-1}) in the beginning of the exponential growth phase, and then bacteria were cultivated for 2 h.

Conjugational transfer was carried out as described by Dunn and Gunsalus [21].

Plasmid stability was studied estimating phenotypic properties of naphthalene utilization in strains. For this purpose, the strains were grown under non-selective conditions (enriched LB medium) for 10 days. Within this period every day 50 μl bacterial suspension was added to 5 ml fresh LB broth and cultivated for 24 h and then transferred to another 5 ml fresh LB broth. 1, 3, 6

Table 1
Bacterial strains and plasmids

Strain	Phenotype description	Source
P. fluorescens		
38a	Producer of pyoluteorin, $Phn^-Nah^-Sal^-$	IBPM RAS[a]
38a(pBS216)[b]	Producer of pyoluteorin, $Phn^+Nah^+Sal^+$	This work
P. chlororaphis		
PCL1391	Producer of phenazine-1-carboxamide, $Phn^-Nah^-Sal^-$	[15]
PCL1391(pBS216)[b]	Producer of phenazine-1-carboxamide $Phn^+Nah^+Sal^+$	This work
PCL1391(pOV17)[c]	Producer of phenazine-1-carboxamide $Phn^+Nah^+Sal^+$	This work
P. putida		
BS394	Cys^-, $Phn^-Nah^-Sal^-$	IBPM RAS
BS394(pBS216), donor of the plasmid pBS216	Cys^-, $Phn^+Nah^+Sal^+$	IBPM RAS
53a	Producer of indole-3-acetic acid, $Phn^-Nah^-Sal^-$	IBPM RAS
53a(pBS216)[c,d]	Producer of indole-3-acetic acid, $Phn^+Nah^+Sal^+$	This work
53a(pOV17)[c]	Producer of indole-3-acetic acid, $Phn^+Nah^+Sal^+$	This work
P. aureofaciens		
BS1393	Producer of phenazine antibiotics, $Phn^-Nah^-Sal^-$	IBPM RAS [16,17]
BS1393(pBS216)[b]	Producer of phenazine antibiotics, $Phn^+Nah^+Sal^+$	[16]
BS1393(pOV17)[c]	Producer of phenazine antibiotics, $Phn^+Nah^+Sal^+$	This work
OV17(pOV17), wild type	Producer of phenazine antibiotics, $Phn^+Nah^+Sal^+$	IBPM RAS
OV17, derivative of the wild type strain OV17(pOV17), cured from the plasmid pOV17	Producer of phenazine antibiotics, $Phn^-Nah^-Sal^-$	This work
OV17(pBS216)[c]	Producer of phenazine antibiotics, $Phn^+Nah^+Sal^+$	This work
Plasmids		
pBS216	$Phn^+Nah^+Sal^+$ Inc P9 Tra^+ 83 kb	IBPM RAS
pOV17	$Phn^+Nah^+Sal^+$ Inc P9 Tra^+ 83 kb	IBPM RAS

Phn^+: ability to grow on phenanthrene; Nah^+: ability to grow on naphthalene; Sal^+: ability to grow on salicylate; Cys^-: cysteine auxotroph.

[a] Strains from the collection of Laboratory of Plasmid Biology, G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences.

[b] Plasmid-bearing variants obtained by conjugal transfer of pBS216 plasmid from donor strain *P. putida* BS394(pBS216).

[c] Plasmid-bearing variants obtained by transformation.

[d] *P. putida* 53a(pBS216) forms small dark-brown colonies (<1 mm in size).

and 10 day-bacterial cultures were plated on LB agar after a series of dilutions. One hundred to 200 single colonies were plated by replica on the mineral Evans medium supplemented with naphthalene. Plasmid stability was estimated as a ratio of clones able to grow on the mineral Evans medium with naphthalene (Nah^+ phenotype) to general number of clones studied.

2.2. DNA techniques

Isolation of plasmid DNA and DNA electrophoresis in agarose gel were performed according to the recommendations [18]. Plasmid DNA was visualized by Eckhardt method [22].

Transformation of bacterial cells by plasmid DNA was performed using a MicroPulser Electroporator (Bio-Rad, CA, USA). Transformation conditions were as follows: voltage: 1.2 kV, capacity: 25 μF, impulse time: 4.6–5.2 ms. Cells for electro-transformation were obtained as described previously [18]. Transformed cells were incubated in LB broth for 2 h at 28 °C. Cells were then washed in 0.85% NaCl solution and were plated onto the mineral Evans medium supplemented with naphthalene. Transformation frequency was estimated as a number of plasmid-bearing clones per μg of plasmid DNA used for transformation.

2.3. Enzyme activity assay

Cell-free extracts for determining enzymatic activities were produced by destroying the frozen cell biomass in IBFM-press (Russia). Cell debris was sedimented in a Beckman J2-21 centrifuge (United States) at 26,000 × *g* and 0 °C for 60 min. Cell-free extract was used immediately for determination of the enzyme activity. The reaction was performed at 30 °C and was started by the addition of 100 μl cell-free extract to the 2.9 ml reaction mixture in a standard quartz cuvette. Specific activities of enzymes were measured using spectrophotometer UV-160A (Shimadzu, Japan).

Specific activity of naphthalene dioxygenase was determined by measuring the decrease of NADH extinction in reaction mixture ($\lambda = 340$ nm, $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) containing 100 μM NADH and cell-free extract in 50 mM Na–K phosphate buffer (pH 7.5) saturated with naphthalene, considering the endogenous NADH consumption by cell-free extract [23].

Specific activity of salycilate hydroxylase was determined by measuring the decrease of NADH extinction in reaction mix ($\lambda = 340$ nm, $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) containing 100 μM NADH, 100 μM sodium salycilate and cell-free extract in 50 mM Na–K phosphate buffer (pH 7.5), considering the endogenous NADH consumption by cell-free extract [24].

Specific activity of catechol-1,2-dioxygenase was assayed by measuring the rate of *cis,cis*-muconate formation ($\lambda = 260$ nm, $\varepsilon = 16.9\ mM^{-1}\ cm^{-1}$). The reaction mixture contained 50 mM Na–K phosphate buffer (pH 7.0), 5 mM EDTA, 1 mM catechol and 100 μl cell-free extract [25].

Specific activity of catechol-2,3-dioxygenase was assayed by measuring the rate of 2-hydroxymuconic semialdehyde formation ($\lambda = 375$ nm, $\varepsilon = 33.4\ mM^{-1}\ cm^{-1}$). The reaction mixture contained 50 mM Tris–HCl buffer (pH 7.5), 0.5 mM catechol and 100 μl cell-free extract [26].

Specific activities of enzymes were expressed in micromoles of the cofactor consumed or product formed in the minute per 1 mg total bacterial protein.

Protein concentration was determined by the spectrophotometric method [27].

2.4. Model system for plant growth

Microcosms were used to study the effect of plasmid-bearing PGPP strains on plant growth [28]. The microcosms were gnotobiotic systems consisting of sterile sand (150 g), naphthalene, plants (Indian mustard, *Brassica juncea* L.) and bacterial cultures placed into closed plastic vessels (77 mm × 77 mm × 97 mm) mm) (Sigma Chemical Co., St. Louis, Mo). Naphthalene (200 $\mu g\ g^{-1}$ sand) was added in sterile conditions as powder to sand and thoroughly mixed. Murashige and skoog basal salt medium (Sigma Chemical Co., St. Louis, Mo) was used to provide plants with mineral nutrition. The amount of the medium added was calculated on a basis of 10% moisture. Bacterial culture-infected seedlings were planted to the model system. Bacterial cultures were grown up to the exponential growth phase in LB broth. A model system with naphthalene and plants but without bacterial cultures was a negative control. A model system with plants but without naphthalene and bacterial cultures was a positive control.

Indian mustard seeds were sterilized in 10% sodium hypochlorite for 30 min, washed four times with sterile tap water for 2 h. The seeds were then placed on LB agar and incubated for 18–20 h at 24 °C to control seed sterility.

Sterile seedlings were put in a bacterial suspension for 15 min. The suspension density was 10^8 CFU ml^{-1}. Twenty mustard seedlings were placed in one plastic vessel and cultivated under the following conditions: 12 h of daylight and 12 h in the dark at 20 °C. After 7 days of cultivation the plants were taken from the systems. Plant roots with the adhering sand particles were then cut from the shoots, placed into the tube with 5 ml phosphate buffer and vigorously shaken. After appropriate dilutions samples were plated onto LB agar. Petri dishes were incubated for 1-day at 24 °C and the colonies grown were then counted. In parallel, shoot length was measured.

2.5. Thin-layer chromatography (TLC)

TLC of the culture liquid samples was performed on silica gel plates (Sigma Chemical Co., St. Louis, Mo) using a system of solvents: chloroform/acetic acid (5:1). Culture medium (4 ml, pH 2) was extracted with the same volume of chloroform at intensive shaking. The chlorophorm phase was evaporated to 15 μl and used for chromatography. The plates were sprayed with 3% $FeCl_3$ that resulted in specific staining of salicylate and catechol typical for these compounds.

2.6. Statistical analysis

All the experiments were triplicated. The results were processed using the statistic package of ‘‘Stadia’’ programs [29].

3. Results

3.1. Construction of PGPP strains bearing PAH degradative plasmids

Two naphthalene degradative plasmids pOV17 and pBS216 were transferred to rhizosphere strains *P. putida* 53a, *P. fluorescens* 38a, *P. aureofaciens* BS1393, and *P. chororaphis* PCL1391 by conjugation or transformation (Table 1). *P. aureofaciens* OV17(pOV17) was a natural plasmid-bearing strain isolated from the rhizosphere of cereals grown in oil-contaminated soil. The plasmid pBS216 was also transferred into the plasmid-free derivative *P. aureofaciens* OV17 (Table 1). Both plasmids were about 80 kb. These plasmids belong to IncP-9 group and contain a full set of naphthalene degradative genes. Because of similar antibiotic resistance of donor and recipient strains and very low frequency of plasmid transfer to some strains only three plasmid-bearing variants [*P. aureofaciens* BS1393 (pBS216), *P. chlororaphis* PCL1391(pBS216) and *P. fluorescens* 38a(pBS216)] were constructed by conjugation and the other five strains [*P. putida* 53a(pBS216), *P. putida* 53a(pOV17), *P. aureofaciens* BS1393(pOV17), *P. aureofaciens* OV17(pBS216), *P. chlororaphis* PCL1391(pOV17)] were obtained by transformation.

During our experiments all possible combinations of strains and plasmids with the exception of *P. fluorescens* 38a(pOV17) were obtained. *P. fluorescens* 38a(pOV17) was not obtained neither by transformation nor conjugation. The presence of plasmid DNA in all nine strains was confirmed by electrophoresis methods [22].

Eight plasmid-strains combinations were able to use naphthalene as a sole carbon and energy source, which indicated normal expression of the degradative plasmid genes in the obtained strains. The exception was *P. putida* 53a(pBS216) characterized by very weak growth on the agarized Evans medium with naphthalene. The strain formed small colonies of dark-brown color. Similarly, the culture broth became dark-brown in the process of bacterial cultivation in the liquid Evans medium. Numerous experiments with transformation of this strain with the plasmid pBS216 failed to generate clones with the normal $Nah^{+}Sal^{+}$ phenotype.

3.2. Growth parameters and plasmid stability in PGPP strains

The growth parameters of all constructed strains were studied. Data of the growth rate and plasmid stability that were observed in plasmid-bacterial host combinations are given in Table 2.

The growth kinetics of plasmid pOV17-bearing strains showed that the natural strain *P. aureofaciens* OV17(pOV17) had the maximum growth rate ($\mu_{max} = 0.41\ h^{-1}$), though its maximum CFU number was less by a factor of 1.4–2 than for *P. chlororaphis* PCL1391(pOV17) and *P. putida* 53a(pOV17). Although plasmid pBS216 was initially isolated from *P. putida*, the value of μ_{max} of *P. putida* 53a(pBS216) was lower by a factor of 4 than for *P. chlororaphis* PCL1391(pBS216). The CFU_{max} of *P. fluorescens* 38a(pBS216) was higher by a factor of 3 in comparison with *P. putida* 53a with the same plasmid.

The plasmid-bacterial strain combinations were unstable (Table 2). Plasmids were eliminated from the strains with high frequency when cultivated on rich nutrient medium. The exception was *P. putida* 53a(pOV17) and *P. chlororaphis* PCL1391(pBS216).

TLC analysis of culture broth after the cultivation of plasmid-bearing strains in the liquid Evans medium with naphthalene showed that neither salicylate ($R_f = 0.86$) nor catechol ($R_f = 0.54$) were found after 24, 48 and 72 h cultivation, excepting *P. putida* 53a(pBS216). Catechol and unidentified compounds with $R_f = 0.08$, 0.28, 0.35 and 0.44 were detected in the culture broth of this strain after 72 h cultivation.

3.3. Activity of the key enzymes of naphthalene catabolism in obtained PGPP strains

Analysis of the activities of enzymes involved in naphthalene catabolism in plasmid-bearing PGPP strains showed that they all had similar level of naphthalene dioxygenase (2–9 nmol/min/1 mg protein) and salicylate hydroxylase (4–11 nmol/min/1 mg protein) activities (Table 3).

The activity of catechol-1,2-dioxygenase was detected in obtained plasmid-bearing (Table 3) and original plasmid-free (Table 4) strains. These groups of strains had different phenotype—Nah^{+} and Nah^{-} correspondingly. Thus, their cultivation conditions were various (see section of culture condition). Catechol-1,2-dioxygenase in plasmid-free strains was induced by adding catechol ($0.1\ g\ l^{-1}$) in the beginning of the exponential growth phase. Therefore its values were higher (Table 4) than those ones of non-induced plasmid-bearing strains (Table 3).

Catechol-1,2-dioxygenase and catechol-2,3-dioxygenase activities were found in all the studied plasmid-bearing strains, excepting *P. putida* 53a(pBS216). Both catechol dioxygenase activities were absent in *P. putida* 53a(pBS216). Plasmid-free strain *P. putida* 53a did not have the catechol-1,2-dioxygenase activity in contrast to the other plasmid-free variants (Table 4).

The activity of catechol-2,3-dioxygenase in pOV17-bearing strains was higher by a factor of 3-5 than in pBS216-harboring combinations (200–300 and 40–60 nmol/min/1 mg protein, respectively).

3.4. Effect of plasmids-bearing PGPP strains on plant growth in microcosms with naphthalene

All plasmid-bearing PGPP strains were studied for the ability to protect mustard plants from toxic effect of naphthalene. The measurement of shoot length and the total

Table 2
Growth characteristics of plasmid-harboring PGPP strains in liquid Evans medium with naphthalene in batch culture

Strain	Lag-phase (h)	μ_{CFU} max (h^{-1})	CFU max (units ml^{-1})	Plasmid stability (%)
Strains with the plasmid pBS216				
BS394(pBS216)	6	0.30	$1.0 \times 10^9 \pm 2.2 \times 10^7$	0
PCL1391(pBS216)	9	0.50	$6.1 \times 10^8 \pm 6.7 \times 10^7$	100
OV17(pBS216)	14	0.27	$1.9 \times 10^8 \pm 1.3 \times 10^7$	0
BS1393(pBS216)	6	0.36	$2.1 \times 10^8 \pm 2.0 \times 10^7$	0
53a(pBS216)	10	0.13	$2.9 \times 10^6 \pm 2.3 \times 10^5$	50
38a(pBS216)	8	0.39	$1.3 \times 10^9 \pm 5.1 \times 10^7$	0
Strains with the plasmid pOV17				
PCL1391(pOV17)	15	0.30	$1.0 \times 10^9 \pm 6.0 \times 10^5$	69
OV17(pOV17)	19	0.41	$7.4 \times 10^8 \pm 2.2 \times 10^7$	14
BS1393(pOV17)	20	0.33	$7.5 \times 10^8 \pm 5.8 \times 10^6$	40
53a(pOV17)	43	0.31	$1.5 \times 10^9 \pm 1.8 \times 10^7$	100

μ_{CFU} max: maximal specific growth rate (h^{-1}); calculated using CFU, CFU: number of colony-forming units (units ml^{-1}); plasmid stability: the percent of clones with retained ability for growth on Evans mineral medium supplemented with naphthalene after 10 days of cultivation under nonselective conditions.

Table 3
Activities of key enzymes of naphthalene degradation in the initial and modified strains grown in mineral Evans medium with naphthalene

Strain	Specific enzyme activity (nmol/min/1 mg protein)			
	NO	SH	C12O	C23O
BS394(pBS216)	1.99 ± 0.01	3.98 ± 0.01	14.64 ± 1.83	41.31 ± 3.22
PCL1391(pBS216)	2.76 ± 0.01	4.41 ± 0.02	34.31 ± 3.34	63.49 ± 3.62
53a(pBS216)	3.79 ± 0.01	4.20 ± 0.01	0	0
OV17(pOV17)	8.83 ± 0.03	10.79 ± 0.08	57.77 ± 3.50	305.28 ± 11.33
PCL1391(pOV17)	3.55 ± 0.01	4.46 ± 0.01	30.97 ± 2.18	200.51 ± 8.45
53a(pOV17)	5.21 ± 0.01	5.20 ± 0.01	0	224 ± 6.45

Average values and standard deviations are provided according to results of three repeats of each experiment. NO: naphthalene dioxygenase; SH: salycilate hydroxylase; C12O: catechol-1,2-dioxygenase; C23O: catechol-2,3-dioxygenase.

Table 4
Activities of the catechol dioxygenases in the plasmid-free strains

Strain	Specific enzyme activity (nmol/min/1 mg protein)	
	C12O	C23O
P. fluorescens 38a	42.69 ± 1.15	0
P. chlororaphis PCL1391	101.73 ± 4.22	0
P. aureofaciens BS1393	154.94 ± 5.62	0
P. aureofaciens OV17	163.50 ± 5.18	0
P. putida BS394	18.93 ± 1.12	0
P. putida 53a	0	0

Average values and standard deviations are provided according to results of three repeats of each experiment. C12O: catechol-1,2-dioxygenase; C23O: catechol-1,3-dioxygenase.

dry plant biomass after week cultivation of mustard seedlings in the presence of naphthalene (200 μg g^{-1}) demonstrated that naphthalene had a strong phytotoxic effect (Fig. 1). The shoot length in negative control (a model system with naphthalene and plants but without bacterial cultures) was in average by 80% shorter than in positive control (plants without naphthalene and bacterial cultures).

Treatment of seedlings with plasmid-bearing rhizobacteria led to a pronounced protective effect from naphthalene. The exception was the seedlings treated with *P. putida* 53a(pBS216) (Fig. 1). In this case no seed germination was observed. *P. chlororaphis* PCL1391(pOV17) and *P. putida* 53a(pOV17) had the best protective effect on the plants. Shoot length of mustard seedlings treated with the strains mentioned was comparable with the control plants grown without naphthalene.

After 7 days of inoculation, bacterial numbers in the plant rhizosphere varied, depending on bacterial species and plasmids studied (Fig. 2). The number of all strains bearing plasmid pOV17 was greater than those with plasmid pBS216. *P. chlororaphis* PCL1391(pOV17) had the highest number in the rhizosphere of plants – 1.7 × 10^8 CFU g^{-1}. *P. chlororaphis* PCL1391(pBS216) was a good colonizer too (1.5 × 10^8 CFU g^{-1}), however, with a poor protective effect. *P. putida* 53a(pBS216) suppressed growth of plants, cell concentration of this strain in rhizosphere was low—2.7 × 10^7 CFU g^{-1}.

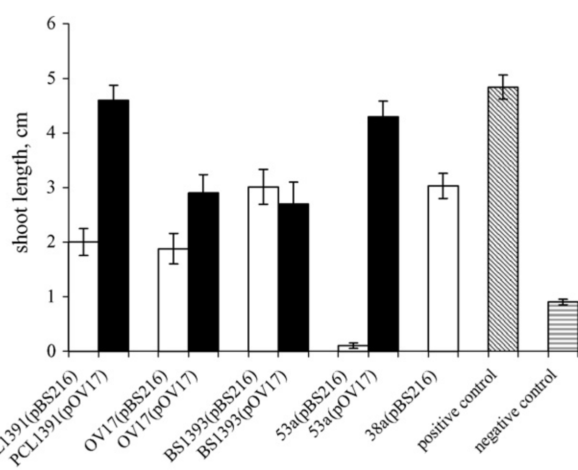

Fig. 1. Effect of plasmid-harboring strains on shoot length of mustard plants in microcosms (sterile sand supplemented with naphthalene). Indian mustard seedlings were inoculated with plasmid-bearing strains and grown in the microcosms. After 7 days the shoots length was measured. Values were calculated from 20 plants. The experiment was in triplicate. ▨: as a positive control, plants without naphthalene and bacterial culture were used. ▤: as a negative control, plants with naphthalene but without bacterial culture were used. □ : strains bearing the plasmid pBS216, ■ : strains bearing the plasmid pOV17.

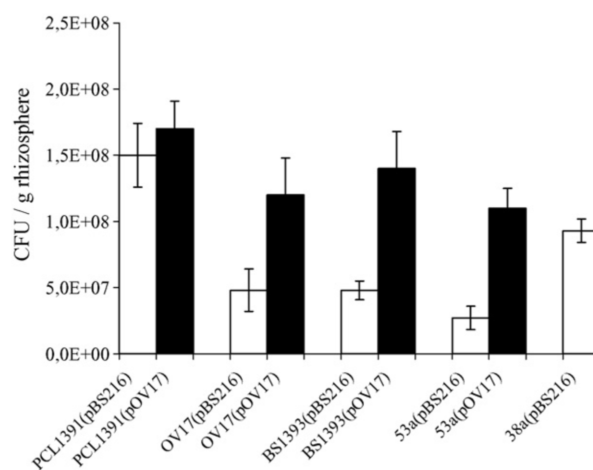

Fig. 2. Number of plasmid-harboring cells in the rhizosphere of mustard plants in microcosms (sterile sand supplemented with naphthalene). Indian mustard seedlings were inoculated with plasmid-bearing strains and grown in the microcosms for 7 days. Bacteria were isolated from the roots, diluted and plated on LB-medium. Values were calculated from 10 plants. □ : pBS216 plasmid-bearing strains, ■ : pOV17 plasmid-bearing strains.

4. Discussion

Eight naphthalene degrading PGPP strains were obtained in our study. Three strains were constructed by conjugation and five strains were obtained by transformation. The frequency of conjugation transfer was 10^{-7} to 10^{-8} per a donor cell. The frequency of transformation was 10^2 to 10^3 per μg of DNA. Most of the studied plasmid-bacterial strain combinations were unstable. When we isolated the wild type strain *P. aureofaciens* OV17(pOV17) in 2001 the plasmid stability was about 100% after 10–14 days of cultivation under nonselective conditions. Now stability of the plasmid became low. It may be because of *P. aureofaciens* species is not typical host for naphthalene catabolic plasmids. According to our data it is first mention about *P. aureofaciens* strain harbouring naturally occurring naphthalene catabolic plasmid. Possibly, during the storaging the strain *P. aureofaciens* OV17(pOV17) some genetic events occurred and the plasmid became low-stable.

All obtained plasmid-bacteria combinations utilized naphthalene as a sole carbon and energy source and had the activity of naphthalene degradative enzymes being similar with its plasmid donor strains (Tables 2 and 3). The sole exception was *P. putida* 53a(pBS216). Specific growth rate (μ_{max} = 0.13 h^{-1}) and maximum concentration of bacteria (CFU_{max} = 2.9×10^6) were the lowest and the Evans medium with naphthalene became dark-brown color, when *P. putida* 53a(pBS216) was cultivated. It was shown that both catechol dioxygenases activities were absent in the strain *P. putida* 53a(pBS216) (Table 3). The restriction analysis of plasmid DNA isolated from this strain revealed the presence of structural rearrangements [30], which could turn off catechol-2,3-dioxygenase gene. Thus, the cells were unable to utilize catechol. Earlier it was shown that some toxic intermediates formed in the oxidation of aromatic compounds led to the death of growing cell [31,32]. In this connection, the growth inhibition of *P. putida* 53a(pBS216) and the strong phytotoxic effect could be explained by the accumulation of catechol and its oxidation products.

Naphthalene degradative plasmids were shown to be capable of transferring to *Pseudomonas* rhizobacteria, and genes of degradative plasmids were found to express in *P. putida, P. fluorescens* and *P. aureofaciens* [6,16,33]. However, growth parameters and the efficiency of PAH degradation by plasmid-bearing PGPP were not studied. Kupier et al. reported that a rhizosphere strain *P. putida* PCL144 with degradative capabilities and the ability of efficient colonization of rye grass roots *(Lolium multiflorum*) was isolated [34], but the presence of catabolic plasmids in this strain was not investigated. Natural and obtained in laboratory conditions naphthalene degradative plasmid-PGPP combinations were studied in our experiments. These strains combined plant growth-promoting and degradative properties.

To assess the effect of plasmid-bacteria combinations on plant growth in microcosms with naphthalene, the experiments, where mustard seedlings were treated by plasmid-bearing PGPP strains, were carried out. *P. chlororaphis* PCL1391(pOV17) and *P. putida* 53a(pOV17) demonstrated the best protective effect on growing plants (Fig. 1). The CFU number in mustard rhizosphere of all strains bearing plasmid pOV17 was also greater than those with pBS216 (Fig. 2). This correlates with the fact of catechol-2,3-dioxygenase activity in pOV17-harboring strains being higher than that in pBS216-bearing strains. It is known the strains able to degrade catechol via the *meta*-pathway demonstrate high specific growth rates [35–37]. *P. chlororaphis* PCL1391(pBS216) had the maximum growth rate and colonized plant rhizosphere well (nearly 1.5×10^8 CFU g^{-1} rhizosphere). Despite its best growth parameters the strain showed no obvious protective effect on plants. This was probably because of low catechol-2,3-dioxygenase activity (*meta*-pathway) in the pBS216-bearing strain.

Thus, the plant protective effect from naphthalene phytotoxicity of the obtained strains is probably provided by the different level of the catechol-2,3-dioxygenase activity. The strain *P. putida* 53a(pOV17) with high catechol-2,3-dioxygenase activity demonstrated the best protective effect on plants. The strain *P. putida* 53a(pBS216) without catechol dioxygenases activities did not have protective effect but suppressed the plant germination.

Thus, before using PAH-degrading PGPP strains for phytoremediation, the level of colonization of the rhizosphere by bacteria, the efficiency of pollutant destruction, interaction between plasmid, bacterial host strain and plant, competitiveness of the ''plasmid-bacterial host'' combination in the polluted site should be studied.

Acknowledgments

This work was accomplished with support of the basic research program of the Presidium of the Russian Academy of Sciences ''Environmental and climatic changes: nature disasters'' (# 13), the project ''Change in microbial association of rhizosphere microorganisms under the impact of environmental anthropogenic pollution and introduction of plant growth-promotion microorganisms to the rhizosphere'', the CRDF (grant # RB2-2377-PU-02), ISTC (grant 2366), the Russian Federal scientific and technical program, Federal contract ''Environmental biotechnology'' # 43.073.1.1.2502, the Russian Foundation For Basic Research (grant # 03-04-49145) and the DOE-IPP-CRDF RBO-10118. We are grateful to Prof. Dr. B. J. J. Lugtenberg (Institute of Biology Leiden University, Leiden, The Netherlands) for providing bacterial strain *P. chlororaphis* PCL1391.

References

[1] Compant S, Duffy B, Nowak J, Clement C, Barka EA. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol 2005;71(9):4951–9.

[2] Haas D, Defago G. Biological control of soil-born pathogens by fluorescent pseudomonads. Nat Rev Microbiol 2005;3(4):307–19.

[3] Bloemberg GV, Lugtenberg BJ. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr Opin Plant Biol 2001;4(4): 343–50.

[4] Shoda M. Bacterial control of plant disease. J Biosci Bioeng 2000;89 (6):515–21.

[5] Cerniglia CE. Biodegradation of polycyclic aromatic hydrocarbons. Curr Opin Biotechnol 1993;4:331–8.

[6] Kochetkov VV, Boronin AM. Comparative study of plasmids controlling naphthalene degradation by *Pseudomonas*. Microbiology (translated from Mikrobiologiya) 1984;53(4):639–44.

[7] Yen KM, Serdar CM. Genetics of naphthalene catabolism in Pseudomonads. CRC Crit Rev Microbiol 1988;15(3):247–68.

[8] Habe H, Omori T. Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. Biosci Biotechnol Biochem 2003;67 (2):225–43.

[9] Kiyohara H, Nagao K. The catabolism of phenanthrene and naphthalene by bacteria. J Gen Microbiol 1978;105:69–75.

[10] Kosheleva IA, Tsoi TV, Kulakova AN, Boronin AM. Comparative analysis of the organization of the NPL-1 plasmid controlling naphthalene oxidation in *Pseudomonas putida* and its derivatives. Genetika (in Russian) 1986;22(10):2389–97.

[11] Boronin AM, Starovoitov II, Borisoglebskaya AN, Skryabin GK. Plasmid of *Pseudomonas putida* which controls initial stages of naphthalene oxidation. Dokl Acad Nauk SSSR (in Russian) 1976;228(4):962–5.

[12] Boronin AM, Kulakova AN, Tsoi TV, Kosheleva IA, Kochetkov VV. 'Silent' genes of meta-pathway of catechol oxidation in the composition of plasmids of naphthalene biodegradation. Dokl Acad Nauk SSSR (in Russian) 1988;299(1):237–40.

[13] Kulakova AN, Boronin AM. Mutants of plasmid of naphthalene biodegradation determining the oxidation of catechol along the meta-pathway. Microbiology (translated from Mikrobiologiya) 1989;58(2):298–304.

[14] Nazarov AV, Ilarionov SA. Potential of the use of microbial-plant interaction for bioremediation. Biotechnology (in Russian) 2005;5:54–62.

[15] Chin-A-Woeng TF, Bloemberg GV, Mulders IH, Dekkers LC, Lugtenberg BJ. Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. Mol Plant Microbe Interact 2000;13(12):1340–5.

[16] Kochetkov VV, Balakshina VV, Mordukhova EA, Boronin AM. Plasmids encoding naphthalene biodegradation in rhizosphere bacteria of the genus *Pseudomonas*. Mikrobiologiya (in Russian) 1997;66(2):211–6.

[17] Delaney SM, Mavrodi DV, Bonsall RF. Thomashow LS. *phz*O, a gene for biosynthesis of 2-hydroxylated phenazine compounds in *Pseudomonas aureofaciens* 30-84. J Bacteriol 2001;183(1):318–27.

[18] Sambrook J, Russel DW. Molecular Cloning: A laboratory manual, 3rd ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2001.

[19] Evans CGT, Herbert D, Tempest DB. The continuous cultivation of microorganisms. Construction of a Chemostat. Meth Microbiol 1970;2: 277–327.

[20] Pirt SJ. Principles of microbe and cell cultivation. Moscow: Mir, 1978.

[21] Dunn NW, Gunsalus IC. Transmissible plasmids coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. J Bacteriol 1973;114(3): 974–9.

[22] Eckhardt T. A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. Plasmid 1978;1(4):584–8.

[23] Dua RD, Meera S. Purification and characterization of naphthalene oxygenase from *Corynebacterium renale*. Eur J Biochem 1981;120(3):461–5.

[24] Shamsuzzaman KM, Barnsley EA. The regulation of naphthalene metabolism in pseudomonads. Biochem Biophys Res Commun 1974;60(2):582–9.

[25] Hegeman GD. Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. I. Synthesis of enzymes by the wild type. J Bacteriol 1966;91(3):1140–54.

[26] Feist CF, Hegeman GD. Phenol and benzoate metabolism by *Pseudomonas putida*: regulation of tangential pathways. J Bacteriol 1969;100(2): 869–77.

[27] Kalb VF, Bernlohr RW. A new spectrophotometric assay for protein in cell extract. Anal Biochem 1977;82(2):362–71.

[28] Simons M, van der Bij AJ, Brand I, de Weger LA, Wijffelman CA, Lugtenberg BJ. Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. Mol Plant Microbe Interact 1996;9(7):600–7.

[29] Kulaichev AP. Methods and software for data processing in the operational environment Windows. Stadia 6.0. In: Informatics and computers. Moscow, 1996.

[30] Volkova OV, Anokhina TO, Puntus IF, Filonov AE, Kochetkov VV, Boronin AM. Effects of naphthalene degradative plasmids on the physiological characteristics of rhizosphere bacteria of the genus *Pseudomonas*. Appl Biochem Microbiol (in Russian) 2005;41(5):460–4.

[31] Cane PA, Williams PA. The plasmid-coded metabolism of naphthalene and 2-methylnaphthalene in *Pseudomonas putida* strains: phenotypic changes correlated with structural modification of the plasmid pWW60-1. J Gen Microbiol 1982;128:2281–90.

[32] Reineke W, Jeenes DJ, Williams PA, Knackmuss HJ. TOL plasmid pWWO in constructed halobenzoate degrading *Pseudomonas* strains: prevention of *meta* pathway. J Bacteriol 1982;150(1):195–201.

[33] Mordukhova EA, Kochetkov VV, Polikarpova FY, Boronin AM. Synthesis of indole-3-acetic acid by rhizosphere pseudomonads: effect of biodegradative plasmids. Appl Biochem Microbiol (in Russian) 1998;34: 287–92.

[34] Kuiper I, Bloemberg GV, Lugtenberg BJ. Selection of a plant-bacterium pair as a novel tool for rhizostimulation of polycyclic aromatic hydrocarbon-degrading bacteria. Mol Plant Microbe Interact 2001;14(10): 1197–205.

[35] Boronin AM, Filonov AE, Gayazov RR, Kulakova AN, Mshensky YN. Growth and plasmid-encoded naphthalene catabolism of *Pseudomonas putida* in batch culture. FEMS Microbiol Lett 1993;113(3):303–7.

[36] Filonov AE, Duetz WA, Karpov AV, Gayazov RR, Kosheleva IA, Breure AM, Filonova IF, van Andel JG, Boronin AM. Competition of plasmid-bearing *Pseudomonas putida* strains catabolizing naphtalene via various pathways in chemostat culture. Appl Microbiol Biotechnol 1997;48(4): 493–8.

[37] Filonov AE, Karpov AV, Kosheleva IA, Puntus IF, Balashova NV, Boronin AM. The efficiency of salicylate utilization by *Pseudomonas putida* strains catabolizing naphthalene via different biochemical pathways. Process Biochem 2000;35:983–7.