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EXPERIMENTAL ARTICLES

Effect of Catabolic Plasmids on Physiological Parameters and Efficiency of Oil Destruction by *Pseudomonas* Bacteria

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Abstract—The ability of microbial degraders of polycyclic aromatic hydrocarbons to grow at 24°C in liquid mineral medium supplemented with oil as the sole source of carbon and energy was studied. Growth characteristics (CFU) and the level of oil destruction by plasmid-bearing and plasmid-free strains were determined after seven days of cultivation. The presence of catabolic plasmids in the degrader strains, including rhizosphere pseudomonads, was shown to increase cell growth and enhance the level of oil degradation. Strain *Pseudomonas chlororaphis* BS1391 bearing plasmid pBS216 was found to be the most effective oil degrader.

Key words: biodegradation, *Pseudomonas*, plasmids, oil.

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The contamination of the environment with oil and oil products is detrimental to the ecological situation in the world. The well-known capability of microorganisms to transform or degrade oil hydrocarbons allows their application for bioremediation of contaminated areas [1].

To date, more than 70 species of microorganisms capable of degrading various oil components have been isolated and described. Some representatives of the genus *Pseudomonas* are able to degrade various oil hydrocarbons, including aliphatic, mono- and polyaromatic, heterocyclic, halogenated, and methylated compounds [2], of which polyaromatic hydrocarbons are the most environmentally harmful [3].

Earlier, it was shown that the key enzymes involved in the catabolism of aromatic hydrocarbons pseudomonads are characterized by wide substrate specificity. For instance, naphthalene dioxygenase catalyzes 76 reactions belonging to five main groups (dioxygenation, monooxygenation, dehydration, O- and N-dealkylation, and sulfooxidation), whereas toluene dioxygenase catalyzes 109 reactions, which can be combined into five main groups with respect to the substrates catalyzed [4]. Bacterial genes responsible for the catabolism of polycyclic aromatic hydrocarbons (PAH) are often located on large biodegradation plasmids together with the regulatory genes and, sometimes, with the genes responsible for the cell chemotaxis to PAH [5].

Biodegradation plasmids are often conjugative and capable of horizontal transfer inside and between bacterial populations, thus extending the biodegradative potential of microorganisms and promoting their adaptation to changing environmental conditions [6]. It may be assumed that bacterial strains harboring the PAH biodegradation plasmids are characterized by increased level of oil destruction compared to plasmid-free strains. Since pseudomonads are good recipients of biodegradation plasmids [7], it seems promising to use certain *Pseudomonas* strains capable of biodegradation of various hydrocarbons for the bioremediation of contaminated soils.

The aim of this work was to elucidate the role of the PAH degradation plasmids in microbial oil destruction, i.e., to compare the efficiency of oil degradation by plasmid-bearing and plasmid-free strains and to study the effect of plasmids on the physiological characteristics of oil-grown strains.

MATERIALS AND METHODS

Bacterial strains. The study was carried out with ten bacterial strains obtained from the collection at the Laboratory of Plasmid Biology, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino), including two plasmid-free and eight plasmid-bearing PAH-degrading strains

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Table 1. Characteristics of bacteria and plasmids

Strain	Characteristics	CFU^*_{max}
Pseudomonas chlororaphis:		
PCL1391	Producer of phenazine-1-carboxyamide $Phn^- Nah^- Sal^- Npht^+ Dsf^+ Hde^+$	$96 \times 10^6 \pm 3 \times 10^6$
PCL1391(pBS216)	$Phn^+ Nah^+ Sal^+ Npht^+ Dsf^+ Hde^+$	$155 \times 10^6 \pm 9 \times 10^6$
PCL1391(pOV17)	$Phn^+ Nah^+ Sal^+ Npht^+ Dsf^+ Hde^+$	$212 \times 10^6 \pm 12 \times 10^6$
PCL1391(pNF142::Tn*Mod*-OTc)	$Phn^+ Nah^+ Sal^+ Tc^r Npht^+ Dsf^+ Hde^+$	$122 \times 10^6 \pm 20 \times 10^6$
Pseudomonas putida:		
BS394	$Cys^- Phn^- Nah^- Sal^- Npht^+ Dsf^+ Hde^+$	$7 \times 10^6 \pm 0.7 \times 10^6$
BS394(pBS216)	$Cys^- Phn^+ Nah^+ Sal^+ Npht^+ Dsf^+ Hde^+$	$12 \times 10^6 \pm 4 \times 10^6$
BS394(pNF142::Tn*Mod*-OTc)	$Cys^- Phn^+ Nah^+ Tc^r Sal^+ Npht^+ Dsf^+ Hde^+$	$32 \times 10^6 \pm 4 \times 10^6$
Pseudomonas aureofaciens:		
BS1393	Producer of phenazine antibiotics $Phn^- Nah^- Sal^- Npht^+ Dsf^+ Hde^+$	$6 \times 10^6 \pm 1 \times 10^6$
BS1393(pBS216)	$Phn^+ Nah^+ Sal^+ Npht^+ Dsf^+ Hde^+$	$140 \times 10^6 \pm 34 \times 10^6$
BS1393(pOV17)	$Phn^+ Nah^+ Sal^+ Npht^+ Dsf^+ Hde^+$	$109 \times 10^6 \pm 9 \times 10^6$
BS1393(pNF142::Tn*Mod*-OTc)	$Phn^+ Nah^+ Tc^r Sal^+ Npht^+ Dsf^+ Hde^+$	$121 \times 10^6 \pm 37 \times 10^6$
Plasmid		
pBS216	$Phn^+ Nah^+ Sal^+$ Inc P–9 Tra^+ 83kb	
pOV17	$Phn^+ Nah^+ Sal^+$ Inc P–9 Tra^+ 83kb	
pNF142::Tn*Mod*-OTc	$Nah^+ Tc^r$ Inc P–9 Tra^+ 83kb	

Note: * CFU_{max} was determined for strains grown in mineral Evans medium supplemented with oil as the sole source of carbon and energy; (Phn^+), (Nah^+), (Sal^+), (Dsf^+), ($Npht^+$), (Hde^+) stand for the cell growth on phenanthrene, naphthalene, salicylate, diesel fuel, crude oil, and hexadecane, respectively; (Cys^-) means cysteine auxotrophy; (Tc^r) denotes tetracycline resistance; (Tra^+) stands for plasmid transfer system; (Inc P-9) signifies plasmid incompatibility group.

(Table 1). Plasmid-free strain *Pseudomonas chlororaphis* PCL1391 was kindly provided by Professor Luchtenberg (The Netherlands). Strain PCL1391 (pNF142::Tn*Mod*-OTc) was obtained by conjugal transfer of the plasmid pNF142::Tn*Mod*-OTc from strain *P. putida* BS394 (pNF142::Tn*Mod*-OTc) to the plasmid-free strain *P. chlororaphis* PCL1391.

Growth media. Bacteria were grown on minimal Evans medium [8] and rich media, such as Luria–Bertani agar (LB) [9], King B agar (KB) [10], tryptose-soybean agar (TSA, Difco, United States), and agar for isolation of pseudomonads (Difco, United States).

Crude oil, diesel oil, toluene, naphthalene, phenanthrene, hexadecane, succinate, salicylate, *o*-phthalate, biphenyl, or benzoate were used as the sole source of carbon and energy. When bacteria were grown on minimal agar medium, naphthalene, phenanthrene, and biphenyl were placed on the inner side of the lids of inverted Petri dishes; salicylate, succinate, *o*-phthalate, and benzoate were added into the medium to the final concentration of 1 g/l; diesel oil, toluene, and hexadecane were introduced into a ring-shaped silicone hose fitted to the lid of a petri dish.

LB agar was supplemented with the following antibiotics (Russia) (μg/ml): ampicillin (500 and 1000); tetracycline (20 and 50); nalidixic acid, novobiocin, streptomycin, kanamycin, claforan, rifampicin, and chloramphenicol (50 and 100).

To prepare the medium supplemented with dispersed oil, a 300-ml flask containing 150 ml of minimal Evans medium, agar (3 g), and oil (1.5 g) was sterilized; the hot medium was sonicated using an MSE-150B ultrasonic disintegrator (United Kingdom) at the maximal amplitude for 3 min. If required, the dispersion procedure was repeated several times. The prepared agarized medium with dispersed substrate was immediately poured into petri dishes; inoculation was performed in the ordinary way.

Cultivation conditions. The growth of bacteria on different substrates and media (LB, KB, and TSA) at 24°C and their resistance to antibiotics were studied by using the replica plating method [11].

To study the strain capability for oil utilization, bacteria were primarily cultivated on agarized Evans medium supplemented with naphthalene as the source of carbon and energy, then plated onto petri dishes with diesel-oil-containing medium, and finally cultivated in Erlenmeyer flasks with 100 ml of minimal Evans medium supplemented with oil to the final concentration of 1.5% (by weight). The oil-containing medium was inoculated with the cell suspension (1 ml/100 ml of medium at the concentration of 1–5 $\times$ 10^6 cells/ml). Flasks were incubated on a orbital shaker (120 rpm) at 24°C for seven days.

To characterize cell growth, a 7-day culture was sampled (0.5 ml); serial tenfold dilutions of the samples were plated in triplicate onto petri dishes with the rich Luria–Bertani medium, and colony-forming units (CFU) were enumerated.

The conjugal transfer of bacterial plasmids was studied by the modified method of Dunn and Gunsalus [12]. Donor and recipient cells, taken in a proportion of 1 : 2, were plated onto LB agar and incubated for 12 h. Then the cells were washed off the plates with 0.85% NaCl. The serial tenfold dilutions of the washings were plated onto selective agar media. The transconjugants were plated successively three times onto selective media to obtain individual colonies.

Determination of emulsifying activity. Emulsifying activity was estimated both visually on a four-point scale by the method of Francy et al. [13] and by measuring the optical density of the mixture of the supernatant and hexadecane according to our modification of the Cirigliano and Carman method [14]. An aliquot (1 ml) of the culture grown in the hexadecane-containing Evans medium was centrifuged on a Beckman microcentrifuge (United States) at 10000 *g* for 3 min; the supernatant (1 ml) was mixed with 1 ml of 1 M phosphate buffer (pH 7.0) and 0.5 ml of hexadecane and stirred on a Paramix shaker (Germany) for 2 min. The mixture containing 0.5 ml of hexadecane and 2 ml of 1 M phosphate buffer (pH 7.0) was used as control. Surface activity was determined from the optical density of the samples measured with a FEK-56M-U42 spectrophotometer (Russia) at 540 nm.

Determination of oil destruction. The level of oil degradation was determined gravimetrically from a decrease in the total oil content of the medium after cell growth [15].

Determination of the fraction composition of crude oil The fraction composition of residual oil was analyzed by high-pressure liquid chromatography [15].

RESULTS AND DISCUSSION

Bacteria of the genus *Pseudomonas* were chosen as hosts of degradation plasmids, since they are known as (1) natural hosts of many catabolic plasmids, viz., NAH, TOL, CAM, OCT, SAL, and others [16]; (2) good recipients of catabolic plasmids; (3) degraders of a wide range of substrates; and (4) they are widely applied for the bioremediation of oil contaminations.

Catabolic properties of degrader strains. The replica plating method was used to study the ability of plasmid-bearing and plasmid-free strains of rhizosphere pseudomonads *P. putida*, *P. aureofaciens*, and *P. chlororaphis* (from the collection at the Laboratory of Plasmid Biology, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences) to grow at 24°C on media supplemented with various substrates as the sole sources of carbon and energy.

All the *Pseudomonas* strains studied were able to grow on crude oil, diesel oil, hexadecane, and succinate. No growth of either plasmid-bearing or plasmid-free strains was observed on minimal agarized Evans medium supplemented with toluene, *o*-phthalate, biphenyl, or benzoate. Only plasmid-bearing strains were able to utilize such substrates as salicylate, naphthalene, and phenanthrene owing to the expression of plasmid genes in the cells of the host bacteria. All the strains were resistant to nalidixic acid, novobiocin, and chloramphenicol. The growth of strains bearing plasmid pNF142::Tn*Mod*-OTc on LB agar with tetracycline was due to the presence of tetracycline resistance genes in this plasmid. All the strains studied were sensitive to streptomycin, kanamycin, claforan, and rifampicin.

The high environmental persistence of oil products is the result of their poor solubility in water. It is known that hydrocarbon-grown microorganisms are able to produce bioemulsifiers. Microbial biosurfactants comprise a structurally heterogeneous group of surface-active substances, which decrease the interphase and surface tension in both aqueous solutions and hydrocarbon mixtures and thus promote hydrocarbon biodegradation. Bioemulsifiers are often the intermediates of microbial destruction of pollutants [17]. Biosurfactant-producing microorganisms are known to exhibit high surface activity when grown on hexadecane [12, 13, 18]. This is why in this work, bacteria were cultivated on hexadecane-containing media to reveal their capacity for biosurfactant production. Pseudomonads are known to produce low-molecular-weight biosurfactants exhibiting extremely high activity. For instance, a *P. aeruginosa* strain produced cell-wall-associated rhamnolipids [19]. As seen from Table 2, the values of surface activity of all the strains were high when determined by the visual method and low when assayed by measuring the optical density of the culture liquid. Based on the results obtained, the studied bacteria were assigned to the endo-type of degraders, which produced cell-wall-associated emulsifiers. Thus, when grown on oil, the strains studied were capable of producing surface-active substances that decreased the surface tension of the medium and promoted oil biodegradation.

Three different naphthalene biodegradation plasmids were used in the study: pNF142::Tn*Mod*-OTc, pBS216, and pOV17 (Table 1). These plasmids belong

to the P-9 incompatibility group and are structurally similar to the known plasmid pDTG1 [20]. Plasmids similar to pDTG1 were obtained from bacterial strains inhabiting the soils of the Glen Fall site (NY, United States) contaminated by coal-tar pitch [21]. pDTG1-similar plasmids can be isolated from different geographic areas. Earlier, we have analyzed plasmid DNA digested with restriction endonucleases and revealed the similarity of the restriction profiles of the labeled naphthalene degradation plasmid pNF142::Tn*Mod*-OTc to the known plasmid pDTG1 [22]. It was also shown that plasmids pBS216 and pOV17 determined the activity level of catechol-2,3-dioxygenases [23]. Plasmid pNF142::Tn*Mod*-OTc contained the gene of tetracycline resistance; it was obtained by the introduction of a Tn*Mod*-OTc minitransposon into the naphthalene degradation plasmid pNF142. Plasmid pNF142::Tn*Mod*-OTc has earlier been used for the study of the role of horizontal transfer of catabolic plasmids between microorganisms in PAH biodegradation [22].

In this study, the conjugal transfer of plasmid pNF142::Tn*Mod*-OTc from the auxotrophic strain BS394(pNF142::Tn*Mod*-OTc) to the plasmid-free rhizosphere strain PCL1391 was performed at a frequency of 10^{-8} per donor cell. Selection of transconjugants was based on their ability to utilize naphthalene as the sole source of carbon and energy.

Physiological characteristics of bacteria. As seen from Fig. 1, in all groups of oil-grown host strains, plasmid-containing bacteria were characterized by a higher CFU value than plasmid-free strains. It should be noted that the transfer of biodegradation plasmids to strain *P. putida* BS394 did not increase the number of oil-growing bacteria, whereas the presence of plasmids in strains *P. aureofaciens* BS1393 and *P. chlororaphis* PCL1391 promoted strain growth on oil-containing media. The presence of plasmid genes of biodegradation appeared to increase the spectrum of substrates utilized by the host bacterium. Strains BS1393(pBS216) and PCL1391(pOV17) were characterized by the highest biomass yield, which may be due to more complete utilization of oil hydrocarbons. The transfer of plasmids (pNF142::Tn*Mod*-OTc, pBS216, and pOV17) to strain *P. aureofaciens* BS1393 resulted in reliable increase (by two orders of magnitude) in the maximum CFU number as compared with the plasmid-free strain (Table 1 and Fig. 1). The results obtained demonstrate the effect of the host surroundings on the expression of plasmid genes. Similar data were obtained by Anokhina et al. [23] when gnotobiotic plant systems were used in the study of the effect of plasmids of naphthalene biodegradation on the physiological characteristics of rhizosphere bacteria of the genus *Pseudomonas*.

Crude oil destruction by the studied bacteria. Oil degradation by the strains was estimated gravimetrically from the decrease in the total oil content of the medium after cell growth.

Table 2. Surface activity of degrader strains grown on hexadecane

Strain	Surface activity	
	Visual method [13]	Optical density method [14]
P. putida BS394	3	0.14
P.putida BS394 (pNF142::Tn*Mod*-OTc)	2.5	0.16
P. putida BS394 (pBS216)	3	0.08
P. aureofaciens BS1393 (pNF142::Tn*Mod*-OTc)	3	0.10
P. aureofaciens BS1393	3	0.09
P. aureofaciens BS1393 (pBS216)	3	0.14
P. aureofaciens BS1393 (pOV17)	3	0.11
P. chlororaphis PCL1391	3	0.05
P. chlororaphis PCL1391 (pBS216)	3	0.07
P. chlororaphis PCL1391 (pOV17)	3	0.11
P. chlororaphis PCL1391 (pNF142::Tn*Mod*-OTc)	2.5	0.10

As seen from Fig. 2, the presence of plasmid pBS216 in strain *P. chlororaphis* PCL1391 resulted in a tenfold increase in the level of oil degradation as compared with the plasmid-free strain, whereas the presence of plasmids pOV17 and pNF142::Tn*Mod*-OTc in the same strain showed no marked effect on oil destruc-

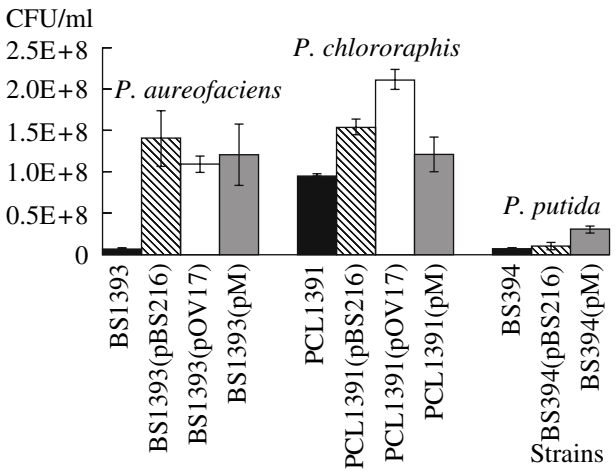

Fig. 1. The number of plasmid-bearing and plasmid-free strains after 7-day cultivation in oil-containing medium at 24°C (pM) stands for plasmid pNF142::Tn*Mo*d-OTc.

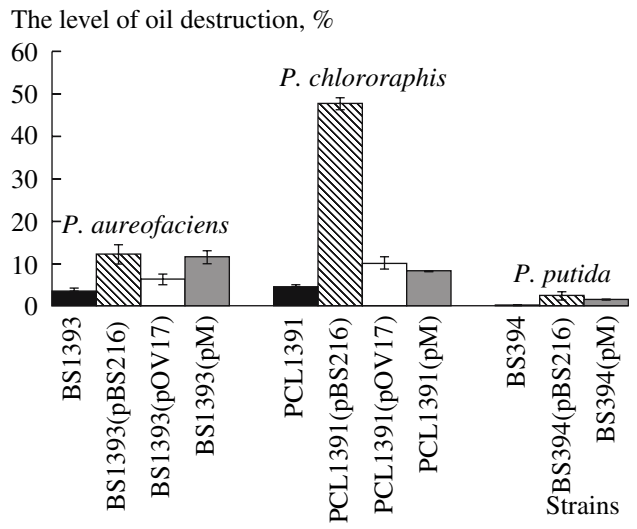

Fig. 2. The levels of oil destruction by pseudomonad strains grown at 24°C for seven days. (pM) stands for plasmid pNF142::Tn*Mod*-OTc.

tion. When plasmid pBS216 was introduced to strains *P. aureofaciens* BS1393 and *P. putida* BS394, the increase in the level of oil destruction was not so significant as in the case of strain *P. chlororaphis* PCL1391. It is known that plasmids pBS216 and pOV17 are structurally similar [23], but differ considerably in the activity of catechol-2,3-dioxygenase, which may affect the level of oil degradation by the same host strain harboring different plasmids. Strain *P. chlororaphis* PCL1391 bearing plasmid pBS216 was the most effective oil degrader among the strains tested. It should be noted that this strain was characterized by a rather low value of CFU_{max} (1.4×10^7 CFU/ml) (Fig. 1).

Changes in the fraction composition of crude oil after its biodegradation. Crude oil is a complex system composed of over 500 individual components. The fraction composition of oil was analyzed by liquid-adsorption chromatography, which allowed oil separation into three arbitrary fractions soluble, respectively, in hexane, benzene, or benzene–ethanol mixture. It is known that low-boiling (hexane-soluble) oil fractions can easily be removed from a contaminated surface by evaporation (up to 15% in summer), whereas heavy benzene–ethanol fractions containing asphaltenes and tars were practically not eroded and slowly seeped into the soil [24]. Variations in the percentages of individual oil fractions indicate a change in their levels in residual oil compared to control, rather than in their absolute amounts. Table 3 demonstrates the changes in the fraction composition of oil after its degradation by various bacterial strains. Oil degradation by the plasmid-free strain *P. chlororaphis* PCL1391 resulted in the largest decrease in the content of the benzene–ethanol fraction (to 9% of control), which was accompanied by changes in the relative amounts of hexane and benzene fractions. These results indicate that the plasmid-free strain possesses enzymes with wide substrate specificity. Strain *P. chlororaphis* PCL1391, bearing the plasmid pOV17, degraded mainly hydrocarbons of benzene–ethanol and benzene fractions of oil (18 and 15%, respectively) (Table 3). Strain *P. chlororaphis* PCL1391 (pNF142::Tn*Mod*-OTc) was able to degrade hexane and benzene–ethanol fractions (13 and 9%, respectively). The introduction of the PAH degradation plasmids to

Table 3. Changes in the fraction composition of crude oil after microbial degradation

Strain	Oil destruction, %	Fraction content of oil after destruction			Fraction content of degraded oil, % of control		
		hexane fraction, %	benzene fraction, %	benzene–ethanol fraction, %	hexane fraction, %	benzene fraction, %	benzene–ethanol fraction, %
P. chlororaphis	5	56	19	10	7	5	9
P. chlororaphis (pBS216)	48	26	9	5	38	31	26
P. chlororaphis (pNF142::Tn*Mod*-OTc)	8	52	19	10	13	5	9
P. chlororaphis (pOV17)	10	56	17	9	7	15	18
Control (oil without bacteria)	0	60	20	11	0	0	0

the host strain increased the level of degradation of benzene–ethanol fractions as compared to the plasmid-free strain; these fractions contain the most recalcitrant oil components. Strain *P. chlororaphis* PCL1391 (pBS216) was the most efficient degrader of paraffin naphthenes, mono- and polyaromatic hydrocarbons, asphalthenes, and tars; the content of hexane, benzene, and benzene–ethanol fractions decreased by 38, 31, and 26%, respectively, as compared with the control. This strain demonstrated the highest level of oil destruction obtained in this experiment (47.7%). The bacterial strains studied were characterized by degradation selectivity with respect to individual oil fractions.

Thus, the results obtained indicate that the presence of catabolic plasmids in the rhizosphere hydrocarbon-degrading strains influenced the physiological characteristics of plasmid-bearing strains by enhancing cell growth, and increased the level of oil degradation. It should be noted that the catabolic potential of bacteria depended considerably on the chosen "host strain–plasmid" combination. Strain *P. chlororaphis*, bearing the plasmid pBS216, was proved to be the most effective degrader of oil hydrocarbons.

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