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Possible Pathways for Destruction of Polyaromatic Hydrocarbons by Some Oil-Degrading Bacteria Isolated from Plant Endosphere and Rhizosphere

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Abstract—Six strains of oil-degrading bacteria isolated from the endosphere and rhizosphere of plants growing on oil polluted soils of the Irkutsk region were studied to determine the pathways for biodestruction of polyaromatic oil hydrocarbons. All strains were able to efficiently degrade polyaromatic hydrocarbons with the formation of pyrocatechin as a final product; strains 90, 108, and 112 additionally formed protocathechuic acid. The culture broth of the studied strains contained ferulic, *n*-coumaric, *n*-oxybenzoic, vanillic, and lilac acids, which probably represent metabolites of cinnamic alcohol, cinnamic aldehyde, and benzoic acid presenting in oil and metabolized by bacteria.

Keywords: oil-degrading bacteria, aromatic compounds, oil

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The annual world production of crude oil reaches 4220 million tons. Such production volumes inevitably cause losses connected mainly with emergencies occurring at oil wells and pipelines. Soil is significantly contaminated with oil. Due to a high oil adsorption by a soil surface, the level of oil accumulation in soil is very high that affects its physical, agrochemical, and microbiological properties. The need to eliminate oil spillages results in the development of various remediation methods. Among them, the use of hydrocarbon-oxidizing microorganisms is considered to be the most promising method [1, 2]. To use such microorganisms more efficiently, one should know the ways of oil decomposition under natural conditions.

Among all oil components, the alkane fraction is destructed by microbes in the most efficient way. The degradation of this fraction occurs via the β-oxidation pathway [3], since the majority of microorganisms have corresponding enzymes. Asphaltenes, high-molecular heteroatomic compounds, are the most hardly decomposed oil components, which are almost inaccessible for microorganisms. During oil biotransformation in soil, asphaltenes are included in humus as separate blocks or bind the soil space; alternatively, they remain unchanged [4]. The most toxic oil fraction contains 5–50% of aromatic compounds of naphthalene and phenanthrene classes. Microorganisms able to decompose these compounds should have a certain set of oxygenases. Therefore, bacteria able to decompose the aromatic oil fraction will probably able to degrade hardly-decomposing polyaromatic hydrocarbons (**PAH**s).

Endophytic and rhizosphere bacteria are considered to be promising destructors for the bioremediation of oil-contaminated soil [5]. In the case of endophytic microorganisms, the content of plasmids responsible for oil degradation is higher than in free-living soil microorganisms [6]. In the rhizosphere, the number of microorganisms able to destruct the pollutant is higher than in the distant soil layers [7, 8].

The purpose of this study was the analysis of culture broths of oil-degrading endophytic and rhizosphere bacteria to determine a composition of low-molecular phenolic compounds and characterize the pathways of biodegradation of oil PAHs.

MATERIALS AND METHODS

Strains and culture conditions. Bacterial cultures were isolated from the endosphere and rhizosphere of plants and from oil-contaminated soil samples collected near the Tyret village (Irkutsk region, Russia). Strain identification was carried out at the Institute of

Table 1. Oil degradation in liquid mineral medium by various bacteria

Oil loss, wt %	Strain no.	Species
40–45	102	*Pseudomonas* sp.
	109	*Pseudomonas oryzihabitats*
45–54	108	*Rhodococcus erytropolis*
	112	*Acinetobacter guillouiae*
	114	*Acinetobacter guillouiae*
	90	*Pseudomonas* sp.

Microbiology of the National Academy of Science of Belarus (Minsk, Belarus) by 16S rRNA sequencing.

To isolate endophytic bacteria, the surface of plants was sterilized by a 10-min treatment with 0.7% sodium hypochlorite [9]. To isolate rhizospheric bacteria, 10 g of roots with soil were put into 100 mL of sterile tap water and shaken for 15 min. The resulted suspensions were inoculated into accumulative medium of the following composition (g/L): KNO_3, 4.0; KH_2PO_4, 0.6; $Na_2HPO_4 \cdot 12H_2O$, 1.4; $MgSO_4 \cdot 7H_2O$, 0.8. The medium was supplemented with hexane, motor oil, waste motor oil, and gasoline as the sole carbon sources. Strains were grown on a shaker for 30 days at 26°C. To obtain pure culture of hydrocarbon-oxidizing bacteria, a solid agar medium was used [10].

Screening of the oil-degrading activity of strains. Two-day bacterial cultures (10^7 CFU/mL) were transferred into liquid mineral medium of the following composition (g/L): NH_4NO_3, 1.0; $MgCl_2$, 0.1; KH_2PO_4, 3.0; K_2HPO_4, 7.0, $CaCO_3$, 1.0 (pH 7.0). The medim was supplemented with 2% (vol/vol) of crude oil, which C : H : S ratio was equal to 85 : 11.76 : 0.92, as the sole carbon source. Cultures were grown in the dark at 26°C.

The initial oil degradation in flasks was assessed by the emulsification of the surface oil film, medium turbidity, and gas production [11, 12]. After two months of cultivation, the level of oil degradation was determined by a gravimetric analysis [13]. A sterile mineral medium containing the same volume of oil (2%) was used as a control.

Determination of the composition of low-molecular phenolic compounds. The composition of phenolic compounds (**PC**s) was determined using ethyl acetate extracts from culture broths. Bacteria (10^7 CFU/mL) were grown for eight weeks on 8E mineral medium supplemented with crude oil (10% vol/vol) [14]. Each week 50 mL of culture broth were sampled, acidulated with 2 N HCl to pH 3.0, and then the equal volume of ethyl acetate was added to extract PCs. The solvent was removed from the extract by a cold air flow, and the residue was dissolved in 0.2 mL of methanol. The PC composition was determined by HPLC. A sterile mineral medium without bacteria was used as a control.

HPLC analysis. Oil degradation products were determined by reversed-phase HPLC using a microcolumn Milichrom A-02 chromatographic system (Nauchpribor, Russia) with a ProntoSil 120-5-C18 column (5 μm, 2 × 75 mm; EkoNova, Russia). The separation was carried out in a linear AB gradient concentration at a flow-rate of 0.1 mL/min and a temperature of 40°C, where eluent A was 0.2N lithium perchlorate in 0.1% trifluoroacetic acid and eluent B was 100% acetonitrile (class "0"). To determine the nature of major components, the separation was carried out in the acetonitril (B) gradient concentration (5–100%) increasing from 5 to 28% within first nine minutes and from 46 to 100% within next nine minutes. The sample volume was 4 μL. The absorbance was measured at 250, 280, 290, 300, and 310 nm. The PCs were identified by the retention times of standard reference markers with the further determination of the UV spectra of the components. A preliminary PC identification was carried out using a BD-2003-500 spectrum library for the Milichrom A-02 chromatographic system.

The used reference markers included protocathechuic, vanillic, and cinnamic acids ("Serva", USA), benzoic acid ("Reakhim", Ukraine), *n*-coumaric, *n*-oxybenzoic, *o*-pyrocathechuic, salicylic, syringic, ferulic, caffeic, pyrocathechuic, and hydrocinnamic acids ("Reakhim", Russia), gentisic acid ("Sigma-Aldrich", USA), *o*-vanillin ("Merck", Germany), *o*-cresol ("Acros", Germany), cinnamic alcohol ("WOSMRI", China), and cinnamic aldehyde ("ZJP&Chemical CO, Ltd", China).

RESULTS AND DISCUSSION

During this study, 60 bacterial strains were isolated from the endosphere and rhizosphere of plants growing on the oil-contaminated territory of the Irkutsk region. Among them, six strains isolated from sedge and couch-grass demonstrated the highest oil-degrading activity (50% oil loss, Table 1) [15]. Strains 90, 102, 108, and 109 emulsified oil that probably evidenced the synthesis of surfactants by these bacteria [16, 17], whereas strains 112 and 114 caused the discoloring and thinning of the oil film with the accompanying gas formation. These results allowed us to suppose that selected strains used different pathways to destruct oil components.

To provide the most rational use of microorganisms, one should have a clear idea of the possible ways of degradation of separate oil fractions. Among these fractions, the aromatic one is of special interest. This fraction is most toxic for all living things, but contains compounds, which can be included into bacterial metabolism. Microbial mono- and dioxygenases catabolize naphthalene and phenanthrene to single-ring phenols and then to water-soluble acyclic hydrocarbons mainly via two pathways [18, 19]. Each of these pathways is characterized by specific com-

Table 2. Composition of aromatic compounds revealed in culture broths of the studied bacterial strains

Compound	Bacterial strain						Control
	108	112	114	90	102	109	
Benzoic acid	+	+	+	+	+	+	+
n-Oxybenzoic acid	+	+	+	+	–	–	–
Protocathechuic acid	+	+	–	+	–	–	–
Pyrocathechuic acid	–	+	–	–	–	–	–
Vanillic acid	–	Tr.*	+	+	–	–	–
Syringic acid	+	+	–	–	–	–	–
Salicylic acid	+	+	+	+	+	+	–
Gentisic acid	–	Tr.	+	–	+	–	–
Cinnamic acid	+	+	+	+	+	+	–
n-Coumaric acid	Tr.	+	+	+	+	+	–
Ferulic acid	Tr.	–	–	–	–	–	–
Cinnamic alcohol	+	–	–	+	–	+	+
Cinnamic aldehyde	+	+	–	+	–	+	+
o-Vanillin	+	+	+	+	+	+	–
Pyrocatechin	+	–	+	–	–	–	–
Maximum number of components**	30 (8)	15 (8)	18 (6)	12 (8)	15 (6)	13 (8)	11(8)

* Trace amount.
** The maximum number of components with the retention time within 7–17 min and adsorption within 250–300 nm observed during the corresponding growth period (shown in parentheses).

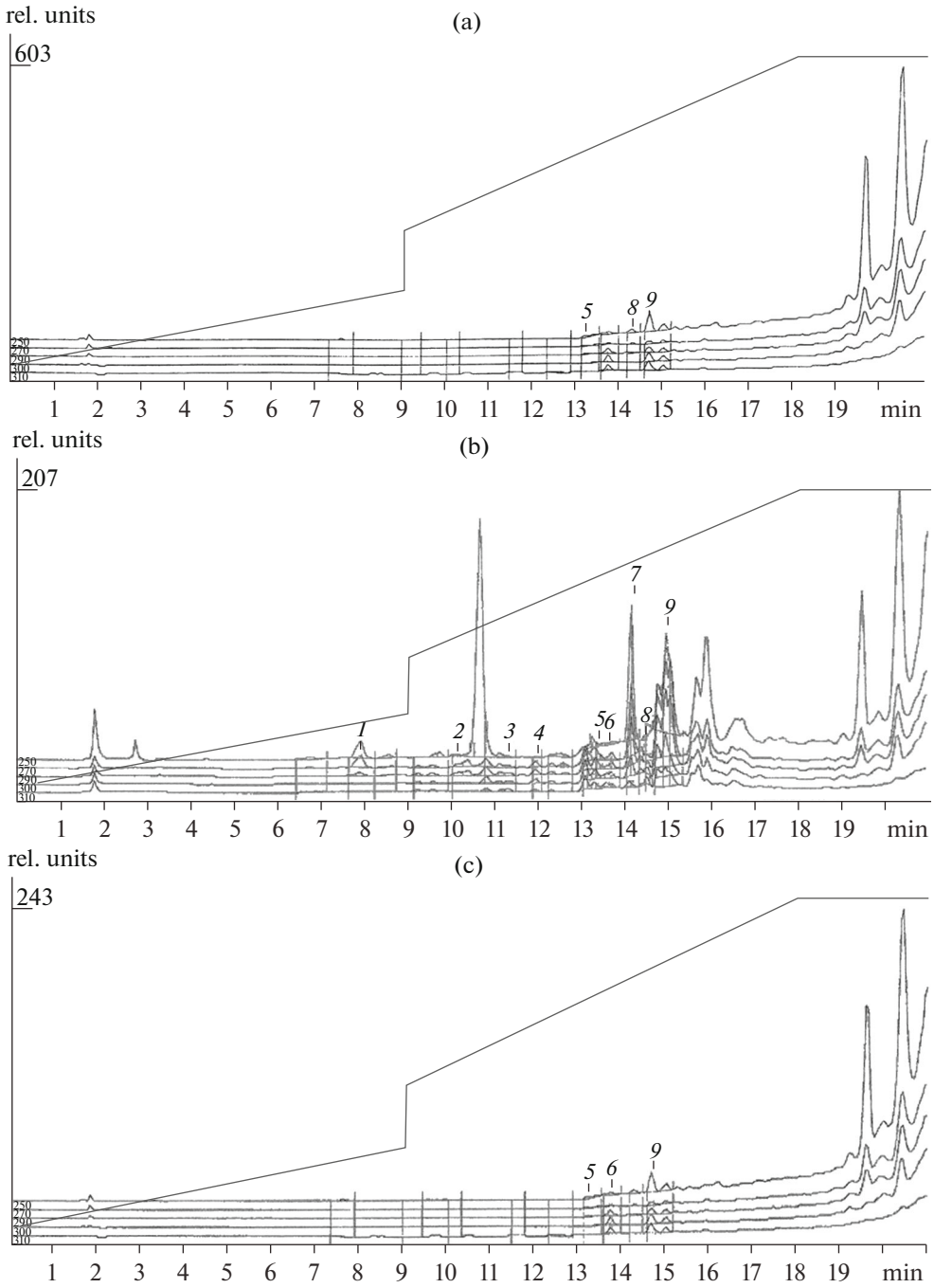
pounds, which detection in culture broth of the studied microorganisms would confirm the corresponding PAH degradation pathway. For example, the presence of pyrocatechin and protocathechuic acid in culture broth would indicate a simultaneous realization of two pathways of the naphthalene and phenanthrene degradation [18, 20]. The most frequent way of the phenanthrene degradation in bacteria includes the formation of naphthalene compounds and single-ring aromatic compounds, especially pyrocatechin and one of its precursors, salycilic acid [18, 20]. This PAH degradation pathway can be confirmed by the detection of gentisic acid, one of the products of the salycilic acid oxidation, in culture broth of bacteria [18]. Bacterial degradation of some flavonoids is also accompanied with a pyrocatechin formation [21]. In the case of this degradation pathway, aromatic compounds are transformed into such acyclic products as cys-cys-muconic acid and 2-hydroxymuconic semialdehyde, which are then catabolized to the organic acids of the Krebs cycle [18, 19].

The presence of such metabolites as protochatechuic and *o*-phthalic acid in culture broth can indicate another PAH degradation pathway [18], which, depending on either meta-, or ortho- opening of aromatic ring, results in the formation of either carbocy-cys-cys-muconic, or 3-oxoadipic acid, respectively [19]. It was shown that some bacteria do not have protocatechoate-3,4-dioxygenase catalyzing the transformation of *o*-phthalic acid into protocatechuic acid [20]. In this case, accumulation of *o*-phthalic acid results in a cell death.

The composition of aromatic compounds determined by a HPLC analysis of the extracts obtained from culture broths of the six studied bacterial strains is shown in Table 2. The presence of salicylic acid was observed for all strains indicating the bacteria catabolized polycyclic compounds via the pyrocatechin formation pathway. However, pyrocatechin was detected only for two strains, 108 and 114, at the second and fifth weeks of growth, respectively. Pyrocatechin is catabolized by bacteria to cys-cys-muconic acid and 2-hydroxymuconic semialdehyde [19]. The absence of this compound in the culture broths of the strains 90, 102, 109, and 112 and during certain growth stages of the strains 108 and 114 can be explained by a high level of its transformation to acyclic compounds.

Like pyrocatechin, protocatechuic acid was detected at different cultivation periods of strains 108, 112, and 90 indicating these strains have a second pathway for PAH degradation, which results in the formation of *o*-phthalic acid as an intermediate metabolite [18].

Culture broths of the studied strains also contained *n*-oxybenzoic, vanillic, and syringic acids (Table 2) that is explained by the presence of bacterial oxidases involved into the hydroxylation of the aromatic ring of benzoic acid [18, 19]. Metabolites of all strains included *n*-coumaric acid; culture broth of the strain 108 additionally contained ferulic acid (Table 2). These acids could be obtained from cinnamic acid due to a bacterial oxidation of such oil components as cinnamic aldehyde and cinnamic alcohol. Culture broths of strains 112, 104, and 90 contained gentisic acid (Table 2) that was probably caused by the oxidation of



HPLC analysis of the extracts from the control medium (a) and culture broths of the strains 108 (b) and 102 (c) after eight weeks of growth. *1*, protocatechuic acid; *2*, syringic acid; *3*, *n*-coumaric acid; *4*, ferulic acid; *5*, benzoic acid; *6*, salicylic acid; *7*, cinnamic alcohol; *8*, cinnamic acid; *9*, cinnamic aldehyde.

salicylic acid [18]. Culture broths of all strains also contained *o*-vanillin, but the pathways of its generation remained unclear. The composition of the identified compounds and their content were not constant during eight weeks of strain cultivation. The analysis of revealed changes allowed us to reveal the periods of the maximum and minimum degrading activity of bacteria. For strains 114 and 102, the maximum activity was reached to the sixth week of growth, whereas strains 108, 112, 90, and 109 were the most active at the eighth week.

Degradation products, which had the maximum adsorption at 250–310 nm and retention times within 7–17 min, were not identified. The composition of such compounds varied in all strains depending on the growth period (see figure). The analysis of chromatograms using a "BD-2003-500" spectrum library for

the Milichrom A-02 chromatographic system showed that some of these components belong to naphthalenes; the structure of other compounds remained unknown.

A comparison of the degrading activity of strains can be made by aromatic compounds generated during their life activity. According to the results of such comparison, strains 108, 112, and 114 were oil destructors, whereas strains 102, 90, and 109, which belonged to the genus *Pseudomonas*, were rather co-destructors (see figure).

Thus, we determined the main metabolites generated during a PAH degradation by the studied strains and shown that each strain had one main pathway for degradation of aromatic compounds characterized by a formation of salicylic acid and pyrocatechin. In the case of strains 112 and 114 belonging to the species *Acinetobacter guillouiae*, culture broth contained compounds, which presence indicated different PAH degradation pathways in these strains. The culture broth of the strain 112 contained both salicylic and protocatechuic acids, whereas the PAH degradation by the strain 114 occurred via only one pathway with the formation of salicylic acid and its derivative, pyrocatechin. In all strains, the oxidation of such oil components as cinnamic aldehyde and cinnamic alcohol resulted in the formation of cinnamic, *n*-coumaric, and, in some cases, ferulic acids. In addition, we identified benzoic acid and its derivatives, *n*-oxybenzoic, vanillic, syringic, pyrocatechuic, and gentisic acids, that evidenced the oxygenases of the studied bacteria were also able to catabolize more simple aromatic oil components.

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