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SUITMA 9: URBANIZATION — CHALLENGES AND OPPORTUNITIES FOR SOIL FUNCTIONS AND ECOSYSTEM SERVICES

Contribution of soil bacteria isolated from different regions into crude oil and oil product degradation

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Abstract

Purpose Crude oil and oil products are the most widespread environmental pollutants. The most efficient bioremediation is performed by using specific oil-degrading strains. Our objectives were to assess the role of soil bacteria, belonging to the following genera *Arthrobacter*, *Microbacterium*, *Rhodococcus*, *Gordonia*, and *Acinetobacter* in reduction of toxicity of environmental pollutants. Bacteria with different versatility were chosen: isolates from aromatic compounds or crude oil-contaminated soils and common representatives of the soil microflora.

Materials and methods In this work, crude oil from the field Aschisay (Kazakhstan) of the following composition: alkanes 78%, naphthenes 6.7%, arenes 3.7%, and other compounds 11.6% was used as carbon source. To investigate the metabolic activity of microorganisms, they were cultured in flasks for 10 days under different conditions (variations in pH range, temperature, salinity, carbon source). Infrared spectrophotometry method was employed to determine the residual oil content after cultivation of bacteria. The ability of bacteria to produce biosurfactants was assessed by measuring surface tension and emulsifying activity (the Francey et al. method); localization of biosurfactants was detected.

Results and discussion Forty-six strains from oil-spilled soils were isolated, with seven of these isolates showing the high degradation ability. Analysis of 16S-RNA gene sequences assigns these cultures to the genus *Rhodococcus*. Their degradation activity was then compared with the one of two rhodococci isolated from soil contaminated with chloroaromatics. The strains under study degraded crude oil, diesel fuel, and phenol; some of them destroyed benzene and naphthalene. The most active strains utilized up to 55–59% of crude oil hydrocarbons. The behavior of strains in the presence of petroleum components (benzene, toluene, nonane, decane, hexadecane) revealed bacterial persistence under severe conditions. Bacteria proved to be more sensitive to aromatic solvents than to aliphatic hydrocarbons. Most of the strains produced biosurfactants when grown on hydrophobic substrates.

Conclusions The obtained results show that bacteria highly adapted to oil contaminations play an important role in the biodegradation of recalcitrant pollutants. Such strains may serve as the basis of bioaugmentation approach for soil remediation in sites with high contamination degree. Furthermore, this study highlights a significant role of common representatives of soil microflora in reducing pollution level in the soil owing to various, however, not necessary high destructive activities of soil strains.

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1 Introduction

Crude oil and oil products are the most common environmental pollutants. According to the Toxic Release Inventory (EPA) report, oil refining industry is in the list of ten major sources releasing/emitting toxic chemicals into the environment (Varjani and Upasani 2016c). In terms of adverse effects on any ecosystem, crude oil and petroleum products rank second behind radioactive contamination. Oil consists of hundreds of different organic substances, such as hydrocarbons, including alkanes (paraffins), naphthenes (cycloparaffins), and arenes (aromatic hydrocarbons), volatile asphaltene components, and sulfur and porphyrins. The ratio of compounds varies depending on the origin of petroleum sources (Fuentes et al. 2014).

Oil pollution causes negative changes in soil biological communities, resulting in sharply reduced productivity and economic value of land. In many cases, the process of oil degradation can be activated by the application of mineral and/or organic fertilizers (biostimulation), as well as the introduction of specific strains of microorganisms capable of degrading a variety of oil hydrocarbons (bioaugmentation) (Fuentes et al. 2014; Varjani and Upasani 2017). The natural remediation process of contaminated soils may take 10 to 50 years. With the use of new biological products and technologies, the time required for the natural remediation process can be shortened to 1 to 3 years. Microbial associations consisting of two or more strains are used recently for bioremediation since the introduction of single microbial strains into hydrocarbon-contaminated environment can not completely solve the problem of soil clean-up.

Microorganisms represented by different taxonomic groups are able to utilize petroleum hydrocarbons (Das and Chandran 2011; Joshi et al. 2014). They are various species of micromycetes, yeasts, and bacteria. More than 20 genera of bacteria and over 25 genera of fungi capable of degrading different petroleum hydrocarbons have been described (Das and Chandran 2011; Fuentes et al. 2016; de Goes et al. 2017). To date, there is a considerable amount of research about the ability of microorganisms to recycle a wide range of petroleum hydrocarbons. It is recognized that an important role in reducing pollution belongs to bacterial genera *Rhodococcus*, *Arthrobacter*, *Acinetobacter*, and *Pseudomonas* (Tanase et al. 2013; Oberoi et al. 2015; Chaudhary 2016; Liu et al. 2016b).

The purpose of this work was to evaluate the contribution of bacteria—representatives of soil microflora with various biodegradative potential, to restoration of soils contaminated with crude oil and its components.

2 Materials and methods

2.1 Objects

The following groups of bacteria were used in the work: (1) bacteria with high destruction activity isolated from soils contaminated with crude oil, (2) degraders of a wide variety of aromatic compounds, (3) a strain isolated from the soil contaminated with chlorinated aromatic compounds, not exhibiting significant destructive activity, (4) typical representatives of the soil microflora (Table 1).

The strain *Rhodococcus opacus* 1CP can completely degrade benzoate, phenol, and chlorophenols (Gorlatov et al. 1989). The strain *R. wratislaviensis* G10 is able to grow on chlorobiphenyls (Plotnikova et al. 2006). *Gordonia polyisoprenivorans* 135 decomposes a number of aromatic compounds (Solyanikova et al. 2015). The strains *Microbacterium foliorum* BN52 and *Arthrobacter agilis* Lush 13 have not shown any significant destructive activity against biphenyl, phenol, or chlorophenols; however, they are able to maintain their long-term viability under the conditions of prolonged exposure by adverse factors, such as starvation or the presence of toxic compounds (Solyanikova et al. 2015, 2017b).

2.2 Nutrient media and cultivation conditions

Microorganisms were cultivated on an Evans mineral salt medium (E-MSM) (Evans et al. 1970) (per liter): K_2HPO_4, 8.71 g; 5-M solution of NH_4Cl, 1 mL; 0.1-M solution of Na_2SO_4, 1 mL; 62-mM solution of $MgCl_2$, 1 mL; 1-mM solution of $CaCl_2$, 1 mL; 0.005-mM solution of $(NH_4)_6Mo_7O_{24}$, 1 mL; trace element solution, 1 mL. The value of pH was adjusted to 7.5 with concentrated HCl. The composition of the trace element solution in 1% water solution of HCl (per liter) are as follows: ZnO, 0.41 g; $FeCl_2$, 2.9 g; $MnCl_2$, 1.28 g; $CuCl_2$, 0.13 g; $CoCl_2$, 0.26 g; H_3BO_3, 0.06 g. A sole source of carbon and energy (hexadecane, diesel fuel, crude oil) was added in the amount of 2% after sterilization at 0.5 atm.

The Luria–Bertani broth medium (LB broth) was also used (Carhart and Hegeman 1975) (per liter): bactotryptone (Difco, USA), 10 g; yeast extract (Difco, USA), 5 g; NaCl, 10 g.

Agar media were obtained by adding 2% bacteriological agar (by weight).

Table 1 Soil microorganisms used in the present work

Source		Group characteristics	Representatives	Region	Ref.
Contaminated soils	1	Destructors of crude oil	*Rhodococcus* sp. F2-1	"Festivalnoe" oil field in Western Siberia	This work
			Rhodococcus sp. F2-2	-//-	
			Rhodococcus sp. T3	"Tarasovskoye" oil field in Western Siberia	
			Rhodococcus sp. T3-4	-//-	
			Rhodococcus sp. B-1	"Barsukovskoye" oil field in Western Siberia	
			Rhodococcus sp. K1	"Kumkol" oil field, Republic of Kazakhstan	
			Rhodococcus sp. K2	-//-	
	2	Destructors of aromatic compounds	*Rhodococcus opacus* 1CP		(Gorlatov et al. 1989)
			Gordonia polyisoprenivorans 135		(Solyanikova et al. 1999)
			Rhodococcus wratislaviensis G10		(Plotnikova et al. 2006)
	3	Bacterium with weak destructive activity	*Microbacterium foliorum* BN52		(Solyanikova et al. 2017a)
Uncontaminated soils		Bacteria with weak destructive activity	*Arthrobacter agilis* Lush13		(Solyanikova et al. 2015)
			Acinetobacter sp. 894		This work

2.3 Cultivation of bacteria

To check the ability of microorganisms to grow under non-optimal conditions, microorganisms were cultivated as follows: (1) in 100 mL of E-MSM with 10% ($V\,V^{-1}$) LB at 4, 30, and 37 °C, optical density at 545 nm (OD_{545}) was assessed after 3 days of cultivation; (2) in 100 mL of E-MSM with 10% ($V\,V^{-1}$) LB with pH 4, 8, and 9, OD_{545} was determined after 3 days of cultivation at 30 °C; (3) in 100 mL of E-MSM with 10% ($V\,V^{-1}$) LB with NaCl 5, 7, and 9%, OD_{545} was measured after 4 days of cultivation at 30 °C. To check the ability to degrade crude oil or its components (nonane, decane, hexadecane), microorganisms were cultivated in flasks for 10 days at 30 °C in a liquid E-MSM supplemented with 2% of crude oil or alkanes as the sole carbon source.

The used crude oil from the field Aschisay (Republic of Kazakhstan) had the following composition: alkanes 78%, naphthenes 6.7%, arenes 3.7%, other compounds 11.6%, specific density 0.84 g $(sm^3)^{-1}$. E-MSM with added carbon sources was sterilized at 0.5 atm for 30 min before inoculation by bacteria.

2.4 Crude oil analysis

Infrared spectrophotometry method was employed to determine the residual oil content after cultivation of bacteria. Using of infrared spectrophotometry method allows for the determination of the hydrocarbon content by a number of C–C and C–H bonds. Oil hydrocarbons were extracted by CCl_4 from the test sample (1:1, $V\,V^{-1}$) and the measurement of the concentration (g L^{-1}) was performed using an instrument AN-2 (JSC Neftekhimavtomatika, Russia). Oil degradation degree was calculated in percentage related to the control (sterilized E-MSM with 2% crude oil without microorganisms):

$$D, \% = \frac{P_k - P_i}{P_k}.100\%,$$

where D is the degree of destruction %; P_k is the concentration of hydrocarbons in the control (liquid E-MSM + 2% oil); P_i is the concentration of oil hydrocarbons in the samples (liquid E-MSM + 2% crude oil + microorganisms).

2.5 Measurement of surface-active characteristics

The strains grown in liquid E-MSM containing diesel fuel were studied for their ability to produce biosurfactants. Bacteria were batch cultivated for 7 days in a 750-mL Erlenmeyer flask containing a 250-mL Evans liquid mineral salt medium (pH 7.0) (Evans et al. 1970) with diesel fuel (2%, $V\,V^{-1}$) as carbon and energy source. The flasks were shaken at the temperature of 24 °C, 200 rev min^{-1}. The emulsifying activity was visually assessed. Surface tension was analyzed by a du Noüy ring method (Petrikov et al. 2013) using the tensiometer K6 (Kruss, Germany) at a temperature of 25 °C. The surface tension of the reference solution (liquid E-MSM) was 77 mN m^{-1}.

2.6 Localization of biosurfactants

Localization of biosurfactants was detected by separating cells from the culture broth and determining the optical density of the cell-free culture broth after the addition of hexadecane. Surface activity was determined visually and by change in optical density (OD) of the culture broth with hexadecane according to Cirigliano and Carman (Cirigliano and Carman 1984; Francy et al. 1991). If the strains exhibited high surface activity in a course of visual evaluation and OD of the supernatant in the presence of hexadecane was low, surface-active compounds were referred to as the endotype (biosurfactants bound with cell surface). If the strains possessed both high surface activity by visual estimation and high OD of the supernatant in the presence of hexadecane, surface-active compounds were referred to as the exotype (biosurfactants excreted into culture medium).

2.7 Light microscopy

Cell cultures were examined using a Nikon Eclipse Ci microscope (Nikon, Japan) equipped with a camera ProgRes Speed XT core5 (Jenoptik, Germany).

2.8 DNA manipulations

Bacterial total DNA was isolated according to Ausbel et al. (Ausbel et al. 1995).

Amplification of 16S rRNA genes was carried out using standard primers f27 and r1492 under conditions described by Tiirola et al. (Tiirola et al. 2002). PCRs were performed on a GeneAmp PCR System 2400 (Perkin-Elmer, USA) thermal cycler using Taq-DNA polymerase. The reaction products were separated by electrophoresis in 1% agarose gel at 10 V cm^{-1}, stained with ethidium bromide solution (5 mg mL^{-1}), and examined on a Gel DocTM XR system (Bio-Rad Laboratories, USA).

Sequencing DNA was sequenced on a Genetic Analyzer Applied Biosystems 3130XL (Applied Biosystems, USA) using the BigDye sequencing kit v.3.1 according to manufacturer's recommendations in the Laboratory of Molecular Genetics at D.I. Ivanovskii Institute of Virology (Ministry of Health of the Russian Federation).

Preliminary analyses were done using BLAST software (www.ncbi.nlm.nih.gov). Each strain was identified based on 16S rRNA gene sequence. Search for homologous sequences was performed using EzTaxon Database (http://www.ezbiocloud.net). The 16S rRNA gene similarity percentage with the homologous gene of the type strain was calculated using online resources of the EzTaxon server.

3 Results and discussion

3.1 Metabolic versatility of the soil bacterial strains under study

To explore the ability to degrade petroleum hydrocarbons in microorganisms from different ecological niches we used the following: (1) bacteria with high petroleum-destructing activity isolated from oil-spilled soils; (2) aromatic degrader bacteria from soils polluted by chlorinated aromatic compounds; (3) microorganisms from native soil.

Initially, using enrichment culture, 46 bacterial strains with high petroleum-utilizing activity have been isolated from soils sampled from oil deposits in Western Siberia (Russia) (36 strains) and Republic of Kazakhstan (Kumkol) (ten strains). Of the 46 cultures grown on a minimal Evans medium with petroleum (15%) as a sole energy and carbon source, seven strains, which were efficient in oil degradation, have been chosen. According to the results of the analysis of the nucleotide sequences of 16S rRNA gene fragments, these strains are assigned to the genus *Rhodococcus*. The nucleotide sequences for the mentioned genes of the investigated bacteria were deposited in GenBank under accession numbers MF359738 (*Rhodococcus* sp. F2-2), MF359739 (*Rhodococcus* sp. T3-4), MF359740 (*Rhodococcus* sp. K1), MF359741 (*Rhodococcus* sp. T3), MF359742 (*Rhodococcus* sp. K2), MF359743 (*Rhodococcus* sp. B1), and MF359744 (*Rhodococcus* sp. F2-1). For these rhodococci, establishing the species affiliation using only 16S rRNA gene sequencing data appears to be not possible, since the cut-off areas show 99% similarity to those of several species (Table S1, Electronic Supplementary Material).

The belonging of many oil-degrading strains to the genus *Rhodococcus* is a common fact. These bacteria are known to be widely distributed in nature and are common representatives of the water (ground, surface, waste) and soil habitats, associated in particular with crude oil and gas fields (Ivshina et al. 1987; Zhukov et al. 2006; Martínková et al. 2009). Rhodococci play an important role in reducing the level of soil contamination by many types of recalcitrant pollutants (Bell et al. 1998; Larkin et al. 2005; Martínková et al. 2009). Bacteria of the genus *Rhodococcus* are able to interact with hydrophobic substrates and oxidize a wide range of substrates. These biodestructive properties are due to the presence of lipophilic cell wall in rhodococci. The cell wall, on the one hand, has a high affinity to hydrocarbons and provides interaction with them and, on the other hand, performs a barrier function for larger molecules, including antibiotics. Coronelli and Kalyuzhnaya (1983) showed the suppression of the synthesis of mycolic acids by bacterium result in the loss of their ability to oxidize hydrocarbons. Due to mycolic acids and the ability to change the fatty acid composition of membrane lipids, rhodococci are able to change the fluidity

and permeability of cell walls. This, in turn, leads to the acquisition of rhodococcal resistance to the action of chemical substances and facilitates degradation of the last ones (Coronelli and Kalyuzhnaya 1983; Sikkema et al. 1995; de Carvalho 2010).

The second group has included microorganisms previously isolated from soils polluted with chlorinated aromatic compounds (chlorophenols, chlorobenzoate, and chlorobiphenyls). They are or effective aromatics-degrading bacteria (genera *Rhodococcus* and *Gordonia*) or ones with low destructive activity (*Microbacterium*). The third group has united microorganisms isolated from control soil near the city of Pushchino. These bacteria are related to *Arthrobacter* and *Acinetobacter*.

Together with rhodococci, bacteria of the genera *Acinetobacter*, *Microbacterium*, and *Arthrobacter* (originally defined as micrococci) are known to be typical representatives of the soil microflora. They are heterotrophs and take an active part in increasing soil fertility (Bello-Akinosho et al. 2016; Liu et al. 2016a).The metabolic activity shown by representatives of these genera is important for the dissemination of chemical elements along food chains.

First of all, the ability of these bacteria to grow under non-optimal conditions was tested. The strains could grow well in the medium in a pH range of 4 to 8 and only four of the rhodococci (F2-2, F2-1, T3, T3-4) grew at pH 9. All the examined strains are able to grow at salt concentrations in the medium up to 7% in the temperature range of 4 to 37 °C (Table 2). These strains are able to grow at low pH; this could be explained by the fact that they have been isolated from acidic soils and seem to possess protection systems. Bacteria of the genus *Rhodococcus* are the most common hydrocarbon-oxidizing microorganisms, since they easily adapt to a number of extreme environmental conditions including high and low temperatures, salinity variations, low humidity.

All the microorganisms under study have been tested for the growth ability in a E-MSM with petroleum hydrocarbons. It is shown that the strains F2-1, F2-2, T3, B1, T3-4, K1, and K2 are capable of degrading crude oil, diesel fuel, and phenol; F2-1, T3, and K1 degrade benzene, and K2 degrades naphthalene (Table 3). The strain *R. wratislaviensis* G10 is catabolically active toward phenol and naphthalene, exhibiting a weak activity against crude oil and diesel fuel. *G. polyisoprenivorans* 135 isolated as a 3-chlorobenzoate destructor grows well on crude oil and diesel fuel but does not grow on benzene and naphthalene.

Actinobacteria under study are found to express some activity against aliphatic hydrocarbons, such as nonane (C9), decane (C10), and hexadecane (C16). Strains *Rhodococcus* spp. F2-1, K1, and B1 grow on nonane. The strain F2-1 gives the highest biomass yield. The strains F2-1, F2-2, T3-4, K1, and B1 grow on decane, when these substrates are added as a sole source of carbon. All rhodococci strains grow on a medium with hexadecane (Table 3). This growth varies depending on the individual strains. Thus, the cell growth pattern on hexadecane is not similar: for example, for cultures B1 and K1, there are individual multicellular granules assembled on the surface of broth. For K2 and T3-4 cultures, cells are evenly distributed throughout the mineral medium, with T3 cells forming small sinking conglomerates. F2-2 culture cells, as well as K2 and T3-4, are evenly distributed within the medium volume with the tendency to form some surface layer. F2-1 culture cells are assembled into one whitish clot floating in the subsurface layer, while the medium without adhered cells remains transparent. The translocation and growth in alkane phase were shown for cells of *R. erythropolis* PR4 (Takihara et al. 2014). Authors noted that this ability of rhodococci was beneficial for bioremediation.

Phase-contrast microscopy of cells of newly isolated rhodococci (B1, K2, T3-4, and F2–1) grown on a rich medium has shown their morphological similarity. These cells are represented by small short rods being in the process of division (Figs. 1, 2, 3, and 4). Figures 1, 2, 3, and 4 show images of newly isolated rhodococci when cultivated on alkanes, benzene, or toluene. A significant change in the morphology of the cells is shown for the culture of *Rhodococcus* sp. B1 (Fig. 1). The most active growth on all oil components was distinctive for the strain *Rhodococcus* sp. F2-1 (Fig. 4). Quasi-"negative" view of cells in phase-contrast microscopy appears to be common for cells growing in the lipophilic environment (Takihara et al. 2014).

Figure S1(a) of the Electronic Supplementary Material shows cells of *A. agilis* Lush13 cultivated with C9, C10, and C16 aliphatic hydrocarbons. Comparison with vegetative cells grown in the liquid LB broth has not revealed the difference in cell morphology. It has been shown that strains are not resistant to solvents such as benzene and toluene. For cells of strain *Rhodococcus* sp. F2-1, the tendency is the formation of shortened, shiny cells when cultured in a mineral medium with toluene (Fig. 4c). Figures S2 and S3 of the Electronic Supplementary Material demonstrate the cultivation of *G. polyisoprenivorans* 135 and *R. wratislaviensis* G10 with benzene. As seen from the figures, cells of both strains are sensitive to the solvent. Cultivation with benzene has led to cell morphology changing and appearing of shortened and more refractory cells as compared with the control ones (grown in rich LB broth). Thus, cultivation of cells in liquid E-MSM with toluene and benzene results in transforming of the majority of cells into the resting-like form.

As for a strain *Acinetobacter* sp. 894, the active growth on benzene or short-chain alkanes is not typical. Nevertheless, analysis of cell samples with electronic microscope revealed that the strain can survive, at least, in E-MSM with added benzene and alkanes as a sole carbon and energy source (Fig. S4 of the Electronic Supplementary Material). In the

Table 2 Growth of the bacteria under study in the rich medium with different pH, salinity, and temperatures

Strain	4 °C	37 °C	pH 4	pH 8	pH 9	NaCl 5%	NaCl 7%	NaCl 9%
Rhodococcus sp. B1	+	+	+	+	−	+	+	−
Rhodococcus sp. T3-4	+	+	+	+	+	+	+	−
Rhodococcus sp. F2-1	+	+	+	+	+	+	+	−
Rhodococcus sp. F2-2	+	+	+	+	+	+	+	−
Rhodococcus sp. K1	+	+	+	+	−	+	+	−
Rhodococcus sp. K2	+	+	+	+	−	+	+	−
Rhodococcus sp. T3	+	+	+	+	+	+	+	−
R. opacus 1CP	+	+	−	+	+	+	+	+
R. wratislaviensis G10	−	+	−	+	+	+	+	+
G. polyisoprenivorans 135	−	+	+	+	+	+	+	−
A. agilis Lush13	−	+	−	+	+	+	+	−
M. foliorum BN52	+	+	−	+	+	+	+	−
Acinetobacter sp. 894	−	+	−	+	+	+	−	−

+The ability to grow in a mineral medium with an appropriate source of C

−There is no growth

presence of different carbon sources, cell morphology remains unaltered.

3.2 Degradation of crude oil by microorganisms

The study of petroleum degradation process (15%) has shown that the *Rhodococcus* strains isolated from soils contaminated with crude oil are able to reduce the oil content at normal and low temperatures (Table 4), and the following three strains *Rhodococcus* sp. T3-4, *Rhodococcus* sp. F2-1, and *Rhodococcus* sp. F2-2 have the highest activity. For these strains, a degradation degree has achieved 55–59% at a temperature of 24 °C in 7 days and 25–36% at 4 °C in 10 days.

With regard to strains utilizing chloroaromatic compounds, they show different activity to crude oil (Table 4). The strain *G. polyisoprenivorans* 135 has demonstrated the highest degradation activity. Although the strain *R. wratislaviensis* G10 grows on diesel fuel, it has weak catabolic activity against crude oil. Bacteria of the genus

Table 3 Ability of the strains under investigation to grow with crude oil components

Strain	Crude oil	diesel fuel	Alkane			Phenol	Benzene	Naphthalene
			C16	C10	C9			
Rhodococcus sp. B1	+	+	+	+	+	+	−	−
Rhodococcus sp. T3-4	+	+	+	+	−	+	−	−
Rhodococcus sp. F2-1	+	+	+	+	+	+	+	−
Rhodococcus sp. F2-2	+	+	+	+	−	+	−	−
Rhodococcus sp. K1	+	+	+	+	+	+	+	−
Rhodococcus sp. K2	+	+	+	−	−	+	−	+
Rhodococcus sp. T3	+	+	+	−	−	+	+	−
R. opacus 1CP	+	+	+	+	+	+	−	−
R. wratislaviensis G10	+	±	+	+	±	+	−	+
G. polyisoprenivorans 135	+	+	+	+	+	+	−	−
A. agilis Lush13	+	+	±	±	±	−	−	−
M. foliorum BN52	±	−	−	−	−	−	−	−
Acinetobacter sp. 894	+	+	+	±	±	−	±	−

+The ability to grow in the liquid mineral medium E-MSM

±The ability to survive for a long time when cultivated in the liquid E-MSM

−There is no growth

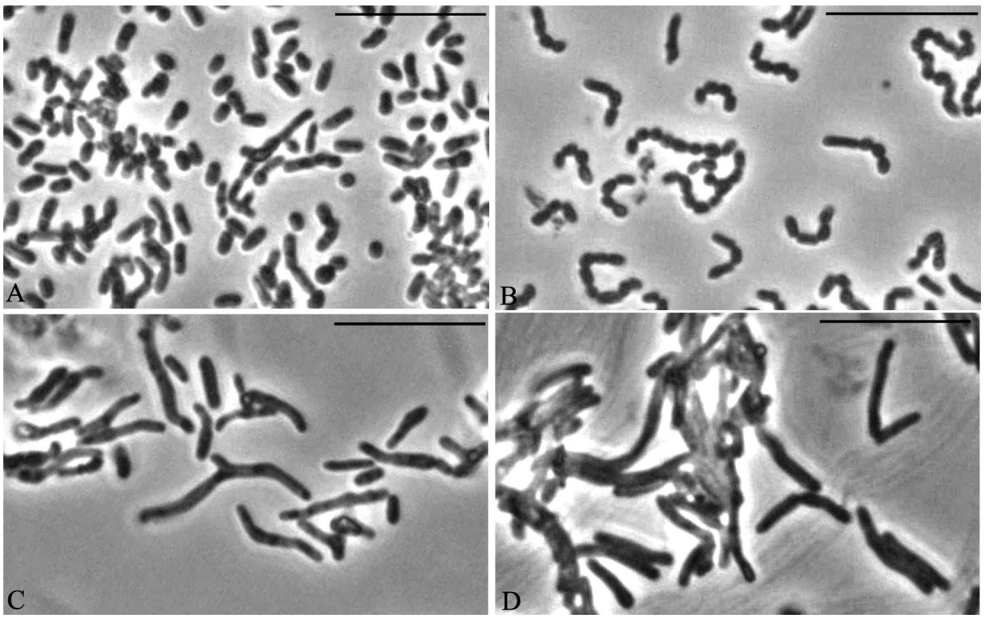

Fig. 1 *Rhodococcus* sp. B1 cells grown in LB broth (**a**) or after the incubation in liquid E-MSM with benzene (**b**) and C9 (**c**) and C10 (**d**) *n*-alkanes. Phase-contrast microscopy. Scale bar, 10 μm

Gordonia are known to be widespread soil inhabitants, including some representatives contributing significantly to the decomposition of persistent pollutants (Drzyzga 2012). The representatives of this genus degrade oil hydrocarbons (Romanowska et al. 2010), diesel oil (Hong et al. 2011), solid *n*-alkanes (Lo Piccolo et al. 2011), etc.

M. foliorum BN52 does not show a destructive ability toward petroleum hydrocarbons. *M. foliorum* BN52 cultivation in E-MSM with aliphatic hydrocarbons (C9, C10, C16) does not lead to the decomposition of these compounds, indicating a lack of oil-decomposing activity in vegetative cells as described earlier (Solyanikova et al. 2017a). However, cells that germinate after resting stage with formation of rough colonies possess some destructive activity (24% oil degradation) that is rather expected. This can be explained by gene activation that can occur after cell germination; their induction in vegetative cells of the strain might be difficult.

A destructive activity exhibited by representatives of soil microflora such as strains of *Acinetobacter* sp. 894 and *A. agilis* Lush13 seems to be significant. Vegetative cells of *A. agilis* Lush13 have also shown some oil-destructing activity. These data are in good agreement with the results previously obtained concerning Lush13 ability to survive under adverse environmental conditions (Solyanikova et al. 2017b). The strain *Acinetobacter* sp. 894 isolated from uncontaminated soil grows with diesel fuel, petroleum, and some alkanes (Tables 3 and 4).

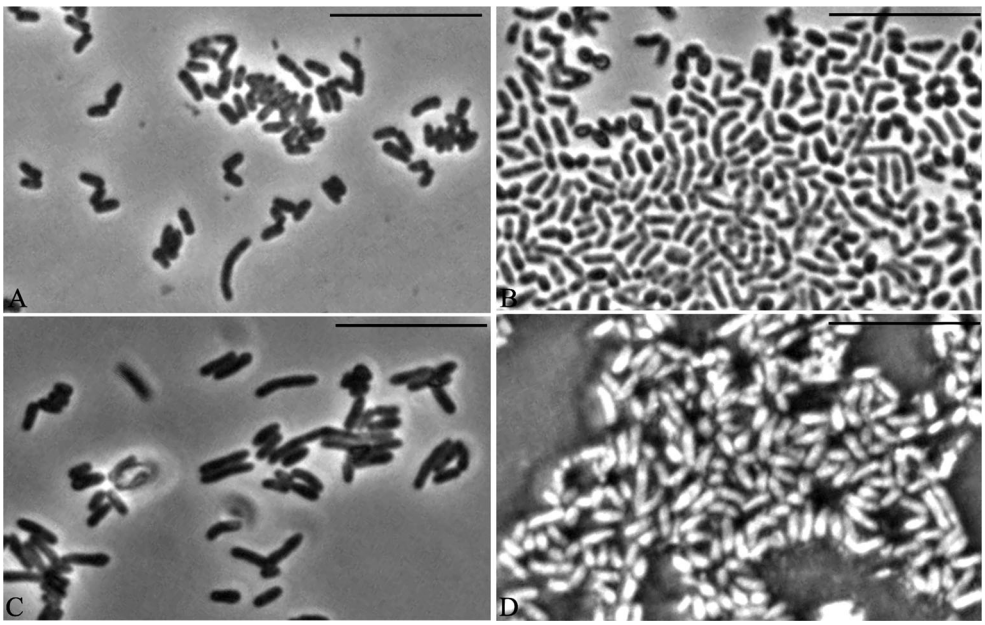

Fig. 2 *Rhodococcus* sp. K2 cells after the incubation in liquid E-MSM with benzene (**a**), toluene (**b**), and C10 (**c**) and C16 (**d**) *n*-alkanes. Phase-contrast microscopy. Scale bar, 10 μm

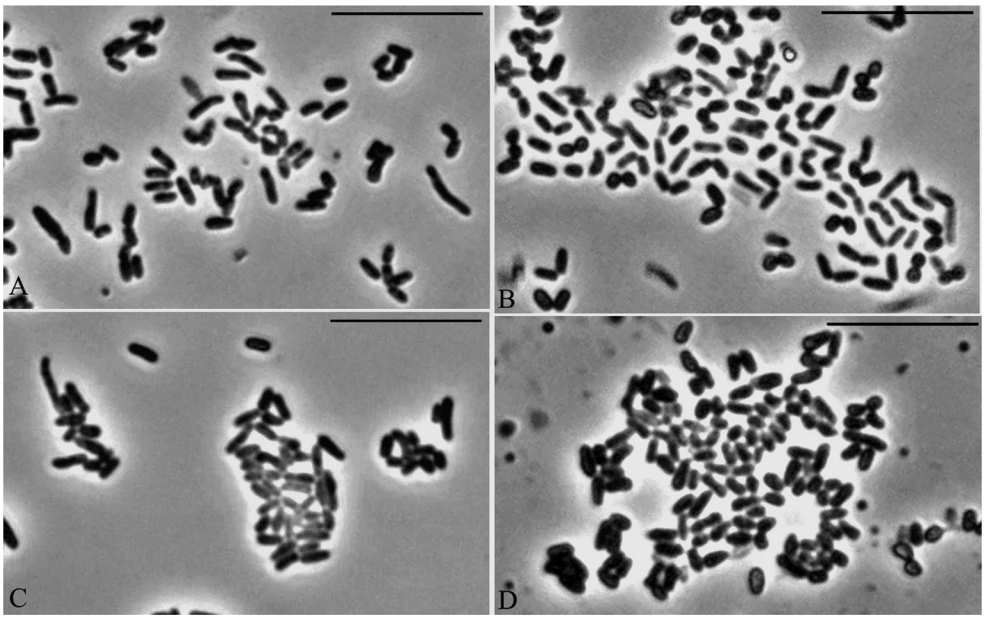

Fig. 3 *Rhodococcus* sp. T3-4 cells after the incubation in liquid E-MSM with benzene (**a**), toluene (**b**), and C10 (**c**) and C16 (**d**) *n*-alkanes. Phase-contrast microscopy. Scale bar, 10 μm

Bacteria of the genus *Acinetobacter*, as common representatives of soil microflora, are also capable of decomposing oil and its components, making a significant contribution to this process (Ishige et al. 2000; Throne-Holst et al. 2006; Yamahira et al. 2008; Kang et al. 2011). *Acinetobacter halotolerans* sp. nov. was capable of destroying C18, C20, and C22 hydrocarbons and oil (kerosene, diesel, and gasoline) (Dahal et al. 2017).

The data obtained highlight a significant role of soil microflora in removing contaminants from the soil. The bacteria of the genera *Acinetobacter*, *Microbacterium*, and

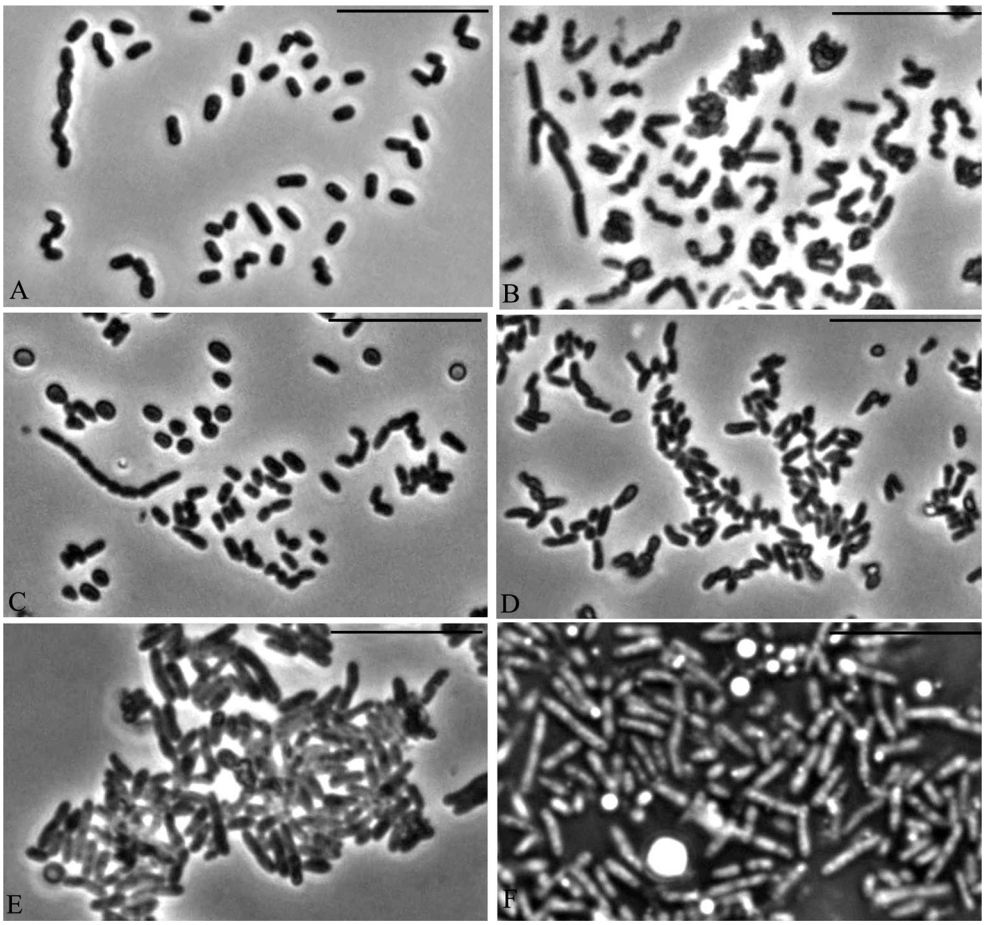

Fig. 4 *Rhodococcus* sp. F2-1 cells grown in LB broth (**a**) or after the incubation in liquid E-MSM with benzene (**b**), toluene (**c**), and C9 (**d**), C10 (**e**), and C16 (**f**) *n*-alkanes. Phase-contrast microscopy. Scale bar, 10 μm

Table 4 Characterization of the microorganisms under study with respect to crude oil destruction ability and surfactant production

Strain	Crude oil destruction (%)		Surface tension (mN m^{-1})
	24 °C	4 °C	
Rhodococcus sp. B1	16.8	6.2	55 ± 1
Rhodococcus sp. T3-4	58.4	35.6	49 ± 1
Rhodococcus sp. F2-1	54.8	29.5	40 ± 1
Rhodococcus sp. F2-2	54.7	24.5	36 ± 5
Rhodococcus sp. K1	11.8	10.2	55 ± 1
Rhodococcus sp. K2	19.1	12.4	59 ± 1
Rhodococcus sp. T3	17.6	23.1	53 ± 1
R. opacus 1CP	*	n.d.	37 ± 1
G.polyisoprenivorans 135	43.7	n.d.	51 ± 1
R. wratislaviensis G10	10.9	n.d.	66 ± 2
M. foliorum BN52	0.5	n.d.	66 ± 1
A. agilis Lush13	20.3	n.d.	66 ± 1
Acinetobacter sp. 894	8.6	n.d.	57 ± 1

n.d. not determined

*Non-significant data

Arthrobacter are known to be typical representatives of the soil microflora. They are heterotrophs and take an active part in increasing soil fertility (Bello-Akinosho et al. 2016; Liu et al. 2016a).The metabolic activity shown by representatives of these genera is important for the dissemination of chemical elements along food chains.

According to literature data, the most active strains against crude oil and its components are being isolated from various oil-contaminated sites. Halotolerant *Pseudomonas aeruginosa* NCIM 5514 was isolated from crude oil-polluted site of Gujarat (Varjani et al. 2015; Varjani and Upasani 2016a, b, c). *Pseudomonas stutzeri* was also obtained from petroleum-contaminated soil (Kaczorek et al. 2012). The use of the potential of such active strains makes the improvement of soil biorestoration processes possible applying bioaugmentation, which plays a significant role in enhancement of biodegradative capacities of polluted sites. For example, *Gordonia alkanivorans* and *Rhodococcus erythropolis* formed the augmented consortium during successful bioremediation of soils contaminated with diesel fuel (C10–C28) and fuel oil (C10–C40) (Lin et al. 2010).

3.3 The ability of degrader strains under study to produce biosurfactants

Low bioavailability of many organic pollutants such as crude oil, fuel oil, creosote, and polycyclic aromatic hydrocarbons for microbial destruction is mainly due to their extremely low solubility in water, which, in turn, is one of the reasons for the high recalcitrance of these pollutants in the environment. The decision of the problem of biodegradation for such pollutants can be in their suspension or emulsification in an aqueous medium by using synthetic surface-active compounds. But the latter themselves are stable environmental pollutants. Moreover, synthetic additives can suppress the growth of microorganisms. Another alternative is the use of microorganisms capable of producing biosurfactants (van Hamme et al. 2003; Satpute et al. 2010; Pacwa-Plociniczak 2011). The use of microbial biosurfactants was shown to make a significant increase onto the hydrocarbon substrates biodegradation (Cameotra and Singh 2008; Cui et al. 2008; Varjani et al. 2014; Sajna et al. 2015).

The strains under our study have been tested for the ability to produce biosurfactants during growth in E-MSM containing diesel fuel. The surface tension of E-MSM at the beginning of cultivation is 77 mN m^{-1}. As seen from the obtained data (Table 4), the surface tension of the culture broth has been reduced to 30–60 mN m^{-1}, which indicates a high emulsifying activity of the strains. Strains of the genus *Rhodococcus* proved to be the best producers of biosurfactants. Experiments on detection of biosurfactant localization have shown that biosurfactants in F2-2 and F2-1 strains are not associated with the cell wall of microorganisms. The biosurfactants have been produced into the culture broth during strain growth on hydrophobic substrates (crude oil and diesel fuel). For the remaining rhodococci strains, the produced biosurfactants are bound to the cell wall. Cultivation of the strain *R. wratislaviensis* G10 on diesel fuel is not accompanied by a decrease in the surface tension of the mineral medium, although cell-forming units increases by four orders of magnitude. The fact that in this case the cells of the strain have been in the diesel fuel layer is intriguing and requires further investigation.

The surface tension decreases because of the formation of glycolipids by pseudomonads or rhodococci with its values usually varying within the limits of 26–36 mN m^{-1} (Franzetti et al. 2010; Petrikov et al. 2013). It was shown (White et al. 2013) that cultivation of *Rhodococcus* sp. PLM026 at 28 °C is accompanied by excretion of tregalolipids into the culture broth (up to 300 mg L^{-1}) with surface tension being reduced to 29 mN m^{-1}. The authors of another study (Rapp and Gabriel-Jurgens 2003) also observed the decrease of surface tension to 29 mN m^{-1} during the growth of *Rhodococcus* sp. MS11 in a medium with *n*-alkanes (in a series from *n*-decane to *n*-heptadecane). Moreover, succynoiltregalolipids concentrated from a culture broth of bacteria *R. wratislaviensis* BN38 and *Rhodococcus* sp. SD74 reduced surface tension to 24 and 19 mN m^{-1}, respectively (Tuleva et al. 2008; Tokumoto et al. 2009). Thus, it can be considered as established that in a temperature range 20–30 °C, rhodococci when grown on hydrophobic substrates produce tregalolipid biosurfactants which effectively reduce the broth surface tension. Addition of rhamnolipids to a *Pseudomonas stutzeri* strain significantly increased diesel oil degradation giving 88% loss after 14 days as compared to 54% loss without the surfactant (Kaczorek et al. 2012).

Thus, the studied oil-degrading strains which produce biosurfactants seem to be promising and may be used to create microbial associations for environmental clean-up technologies and waste purification systems.

3.4 Conclusions

Crude oil and oil-derived products are the most common environmental pollutants. The experts state that about 47% of crude oil releases into the marine environment from natural seeps, and 53% are the result of leaks and spills during the extraction process, transportation, refining, storage, and use of oil. This amount of natural crude oil seepage is estimated to be approximately 600,000 metric tons per year (with a possible increase of 200,000 metric tons) (Kvenvolden and Cooper 2003). Being released into the environment, oil and its components lead to a significant reduction in the number of viable bacteria. Main in situ strategies for remediation of hydrocarbon polluted soils are biostimulation, bioaugmentation, and bioventing (Fuentes et al. 2014). Application of highly effective strains seems to be a reasonable and optimal approach. Meanwhile, sites exposed to long-term contamination are a source of such highly beneficial bacteria. At these locations, as a rule, one can sample the most effective strains are capable of catabolizing crude oil and its components, which can be used as a basis for biopreparations. The introduction of such strains into contaminated soils leads to their most rapid rehabilitation. The overwhelming majority of studies are devoted specifically to the isolation and determination of microbial destructive properties. However, in addition to places with a high pollutant load, there are numerous local areas, where the degree of contamination may not be so high, or contain residual amounts of contaminants after the remediation procedures applied. The use of targeted biopreparations in such places may not always be economically viable. In such cases, an important condition to clean up the site exposed to toxic compounds is the presence of indigenous microorganisms, and their metabolic activity will determine how rapidly the rehabilitation of contaminated sites occurs. So, in these cases, an important role in reducing the level of pollution belongs to soil bacteria, which can reduce the level of oil component contamination in the soil even in the absence of special techniques.

In this study, the possible contribution of different groups of microorganisms to degradation of the crude oil reducing environmental contaminants has been estimated.

(1) A number of new rodococci strains have been identified which can not only decompose oil and its components, but also survive under adverse conditions, such as low pH, high salinity, and low temperatures. It is shown that these strains, due to their peculiarities, can be successfully applied to clean up contaminated sites. The most promising strains of the studied ones are *Rhodococcus* spp. F2-1, F2-2, and F3-4, which not only utilize crude oil by more than 50%, but also synthesize a large amount of surfactants. In the future, these strains will be tested for the ability to degrade crude oil and its components under conditions not optimal for growth.
(2) It is shown that non-target strains destructing chloroaromatics, such as *G. polyisoprenivorans* 135, can be also used in the processes of contaminated sites bioremediation.
(3) Our study demonstrates a major role of soil microorganisms in eliminating pollutants from the local soil environment. Still, this aspect is poorly described in the literature.

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