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AQUATIC MICROBIOLOGY

Hydrocarbon-Oxidizing Bacteria from Urban Lake Beloye (Moscow): Identification and Phylogenetic Analysis

T. R. Kravzova[a, *], V. V. Ilinsky[a], I. V. Lazebnaya[b], O. E. Lazebny[c], A. U. Akulova[a], I. V. Mosharova[a, d], and O. A. Koksharova[e]

[a] *Biological Faculty, Moscow State University, Moscow, Russia*
[b] *Institute of General Genetics, Russian Academy of Sciences, Moscow, Russia*
[c] *Institute of Developmental Biology, Russian Academy of Sciences, Moscow, Russia*
[d] *Shirshov Institute of Oceanology, Russian Academy of Sciences, Moscow, Russia*
[e] *Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia*
**e-mail: skypoy-92@mail.ru*

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Abstract—Cultured hydrocarbon-oxidizing bacteria have been isolated for the first time from bacterioplankton in urban Lake Beloye (Moscow). The taxonomic positions of two bacterial strains (2012B and 2012C) isolated from this lake have been determined. Lipids of the strain 2012B comprise $C_{14:0}$–$C_{19:0}$ fatty acids, the most abundant of them being $C_{15:0}$ (54%), $C_{16:0}$ (17%), $C_{17:0}$ (10%), and 10-methyl $C_{18:0}$ (3.5%). Lipids of the strain 2012C comprise $C_{14:0}$–$C_{19:0}$ fatty acids, the most abundant of them being $C1_{5:0}$ (45%), $C_{16:0}$ (32%), and $C_{17:0}$ (9%). A phylogenetic analysis of strain 2012B is performed using the nucleotide sequences of the 16S rRNA (KP779654.1) and *alkB* (KR422620.1) genes and the strain is identified as a typical member of the genus *Rhodococcus* spp. (Actinobacteria, Nocardiaceae). The combination of molecular identification and analysis of biochemical and physiological properties makes it possible to identify strain 2012B as *Rhodococcus qingshengii* 2012B. A phylogenetic analysis of strain 2012C is performed using the nucleotide sequences of 16S rRNA (MG966152) and shows the highest identity (99.57%) of strain 2012C with *Pseudomonas psychrotolerans* and *Pseudomonas oryzihabitans*

Keywords: hydrocarbon-oxidizing bacteria, phylogenetic analysis, 16S rRNA, alkB, Lake Beloye
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INTRODUCTION

Urban lakes are the "ecological barometers of the health of a city" (Ravikumar et al., 2013) and bacterioplankton communities are their indispensable components. Pollutants, including polycyclic aromatic hydrocarbons from vehicle exhaust emissions, and hydrophobic organochlorine compounds such as chlordane, dieldrin, and polychlorinated biphenyls (Long et al., 2003) easily enter urban lakes through water drains and accumulate as bottom sediments.

The cheapest bioremediation technique is the application of HOB actively participating in the natural biodegradation of oil and other hydrocarbon pollutants (Brooijmans et al., 2009). In an aquatic environment, HOB utilize 0.003–100% of hydrocarbons (Das, Chandran, 2011), which leads to the more rapid cleanup of aquatic systems and provides biotope balance. It has been shown that a microbial community consisting of two isolates, *Pseudomonas aeruginosa* (Schroeter, 1872) Migula, 1900) and *Rodococcus* sp. (EU259892), can degrade up to 90% hydrocarbons in a liquid culture within 6 weeks under laboratory conditions (Cameotra, Singh, 2008).

The taxonomic composition of HOB inhabiting the urban lakes of Russia has not actually been described in literature, and the species taxonomic composition of cultured HOB isolated from Moscow lakes has never been studied. More than half a century ago it was only reported that the water of Lake Beloye contained bacteria capable of oxidizing methane, hexane, and naphthalene (Kuznetsov, 1952).

It is known that cultured HOB is <1% of the total number of bacteria in the natural bacteriocoenosis. In view of the above, it would be of particular interest to identify new cultured natural HOB strains undoubtedly influencing the state of the entire biota in urban Lake Beloye.

The goals of the present work were to characterize the cultured HOB strains isolated for the first time from urban Lake Beloye, carry out a phylogenetic analysis, and determine their taxonomic positions.

Abbreviations: PCR, polymerase chain reaction; HOB, hydrocarbon-oxidizing bacteria

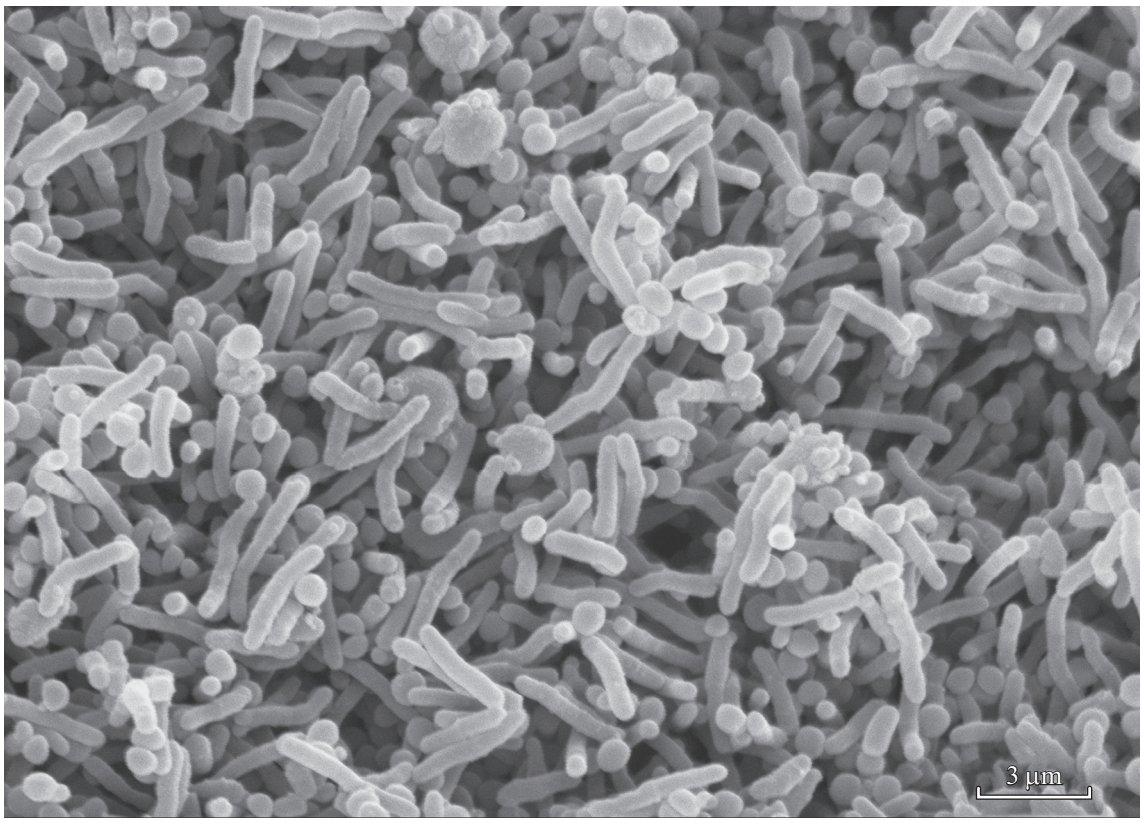

Fig. 1. Cell morphology of the 2012B strain (SEM).

MATERIALS AND METHODS

Characteristics of the water of Lake Beloye. This lake of natural origin is the deepest of three lakes in a natural historical park. Its depth and area are 4.2–13.5 m and 0.2 km^2, respectively (Rossolimo, 1925). It had a eutrophic status as early as in the 1930s; the processes of anaerobic destruction of organic matter were observed in its bottom sediments (Kuznetsov, 1970). In 1986, it became part of the city of Moscow.

In the period of observations, the temperature of the surface water layer in the lake varied from 0.1–0.5 to 27–30°C. The pH values varied from neutral 7.22 to weakly alkaline 7.99; the peaks of water alkalization were from 9.11 to 8.62. Concentrations varied within a range of 0.05–1.73 mg/L for ammonium ions, 0.22–4.73 mg/L for nitrates, 0.003–0.12 mg/L for nitrites, and 0.02–0.33 mg/L for phosphates. The content of easily degradable organic matter estimated by the parameters of permanganate oxidation reached 4.41–7.13 mg O_2/L.

Thus, the degree of saprobity of Lake Beloye with respect to the content of biogenic elements varied in different seasons from xenosaprobic to polysaprobic. The continuous presence of biogenic elements in water excludes them from the factors limiting the development of heterotrophic bacteria (Akulova et al., 2014).

Sampling. Water samples from Lake Beloye for microbiological analysis were taken from a depth of ~0.5 m at a distance of 5 m from the shore, where the depth was ≥1.5 m.

Isolation of pure HOB cultures. Pure cultures were grown on a 2% agarized synthetic MMS medium (Mills et al., 1978) with summer diesel fuel containing 20–30% naphthenic, 20–30% aromatic, and up to ≤~40% paraffin hydrocarbons. Bacterial cultures were stored on the MMS medium in a refrigerator at 4–6°C.

Morphological characteristics. The cell morphology of bacterial strains was studied with a Nikon Eclipse E 200F 200 light microscope and a Camscan S-2 Cambridge scanning electron microscope (Great Britain). Microscopy samples were taken after 1, 3, 7, 16, and 30 days of culture growth on MMS and a Muller medium (Hansen, Møller, 1975).

Enzymatic activity was assayed according to Sambrook (Sambrook et al., 1989). The biodegradation of amino acids and sugars was assessed by strain growth on the M9 medium with hydrocarbons (Sambrook et al., 1989). Fatty acids were assayed by MIDI (Microbial Identification System) (Sasser, 2001).

HOB strains were identified using Bergey's Manual (Bergey et al., 1989) and molecular techniques.

Extraction of genomic DNA. Genomic DNA was extracted from bacteria according to Koksharova et al.

Table 1. Comparative phenotypic characterization of *Rhodococcus* sp. 2012B with respect to the reference strains of the genus *Rhodococcus*

Parameter	*R.* sp. 2012B	*R. qingshengii* sp. djl-6^T (Xu et al., 2007)	*R. jialingiae* sp. djl-6-2^T (Wang et al., 2010)	*R. erythropolis* sp. DSM 43066^T (Xu et al., 2007)
Sampling source	Lake Beloye (Moscow)	Vegetable field soil polluted with carbendazim (Jiangsu Province, China)	Wastewater sediments from water treatment facilities of carbendazim production (Jiangsu, China)	nd
Colony color	Pink	Orange	Pale pink	Pale pink
Max NaCl, %	5	5	7	5
pH	6–9.2	5–8	4–9	5–8
Carbon and nitrogen as the sole nutrient source				
L-asparagine	+	–	–	+
Benomyl	–	+	+	–
Carbendazim	–	+	+	–
L-inosine	+	nd	nd	Nd
L-arabinose	+	nd	nd	nd
D-fructose	+	–	+	+
L-lactic acid	–	–	–	+
Maltose	+	–	–	+
D-mannitol	+	–	+	+
D-sorbitol	+	–	–	+
Sucrose	+	+	–	+
D-xylose	–	–	–	+

+ is a positive response and – is a negative response; nd indicates no data.

(Koksharova et al., 1998); the 16S rRNA gene sequence was amplified by PCR. The following primers were used: RodF (5'-CAGCA GCTCAACTGCTGGCT -3') and RodR (5'-CATGCTCCGCCGCTTGTG-3') (Fredriksson et al., 2013) for the strain 2012B; Ps-F (5'-GGTCTGAGAGGATGATCAGT-3') and Ps-R (5'-TTAGCTCCACCTCGCGGC|-3') for the strain 2012C (Widmer et al., 1998). The temperature–time regime for PCR was 94°C, 10 min; 25 cycles (94°C, 45 s; 54°C, 45 s; 68°C, 2 min) and storage at 4°C. PCR products were visualized in 1.5% agarose gel. The *alkB* gene sequence was amplified with primers alk-FI (5'-CATAATAAAGGGCATCACCG-3') and alk-RI (5'-GATTTCATTCTCGAAACTCC-3') (Kohno et al., 2002).

Cloning and sequencing of PCR products. DNA fragments were cloned using CloneJet PCR™ Cloning Kit #K1231 (Fermentas, EC). PCR products were sequenced with an Applied Biosystems 3730 DNA Analyzer using reagents ABI PRISM ® BigDye™ Terminator v. 3.1 at the Genome Center for Collective Use of Equipment (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences).

Nucleotide sequence analysis. Phylogenetic reconstruction of the sequences was performed by the Neighbor-Joining method (Saitou, Nei, 1987) using Blast Tree View (Fast minimum evolution tree method). GenBank (NCBI) and BLAST (www.blast.ncbi.nlm.nih.gov) were used for the primary comparative analysis of the sequences (Altschul et al., 1997). The sequences were tested for the presence of chimeras with Bellerophon (Huber et al., 2004). Bootstrap values (1000 iterations) are indicated next to the branches (Felsenstein, 1985). Branch lengths are equivalent to the evolutionary distances used for phylogenetic tree construction. Phylogenetic trees were constructed with MEGA X (Kumar et al., 2018).

RESULTS AND DISCUSSION

Two HOB strains capable of utilizing liquid hydrocarbons as the sole carbon and energy source were selected among the isolated cultured strains to determine their taxonomic position.

Characteristics of the strain 2012B. Cell colonies are pink, convex, and opaque with even edges, dry. Cells are Gram-positive, aerobic, immotile, non-spore-forming (Fig. 1).

Cell size is 1–2 μm on the MMS medium with diesel fuel and 3–5 μm on the Mueller medium. The cells are catalase positive and acid resistant. The fatty acid profile of the strain is represented by fatty acids with carbon chain lengths of $C_{14:0}$–$C_{19:0}$. The most abundant fatty acids are $C_{15:0}$ (54%), $C_{16:0}$ (17%), $C_{17:0}$ (10%), and 10-methyl $C_{18:0}$ (3.5%).

The 2012B strain utilizes sugars and some amino acids (L-inosine, L-arabinose, L-asparagine, D-fructose, maltose, D-manitol, and D-sorbitol) but does

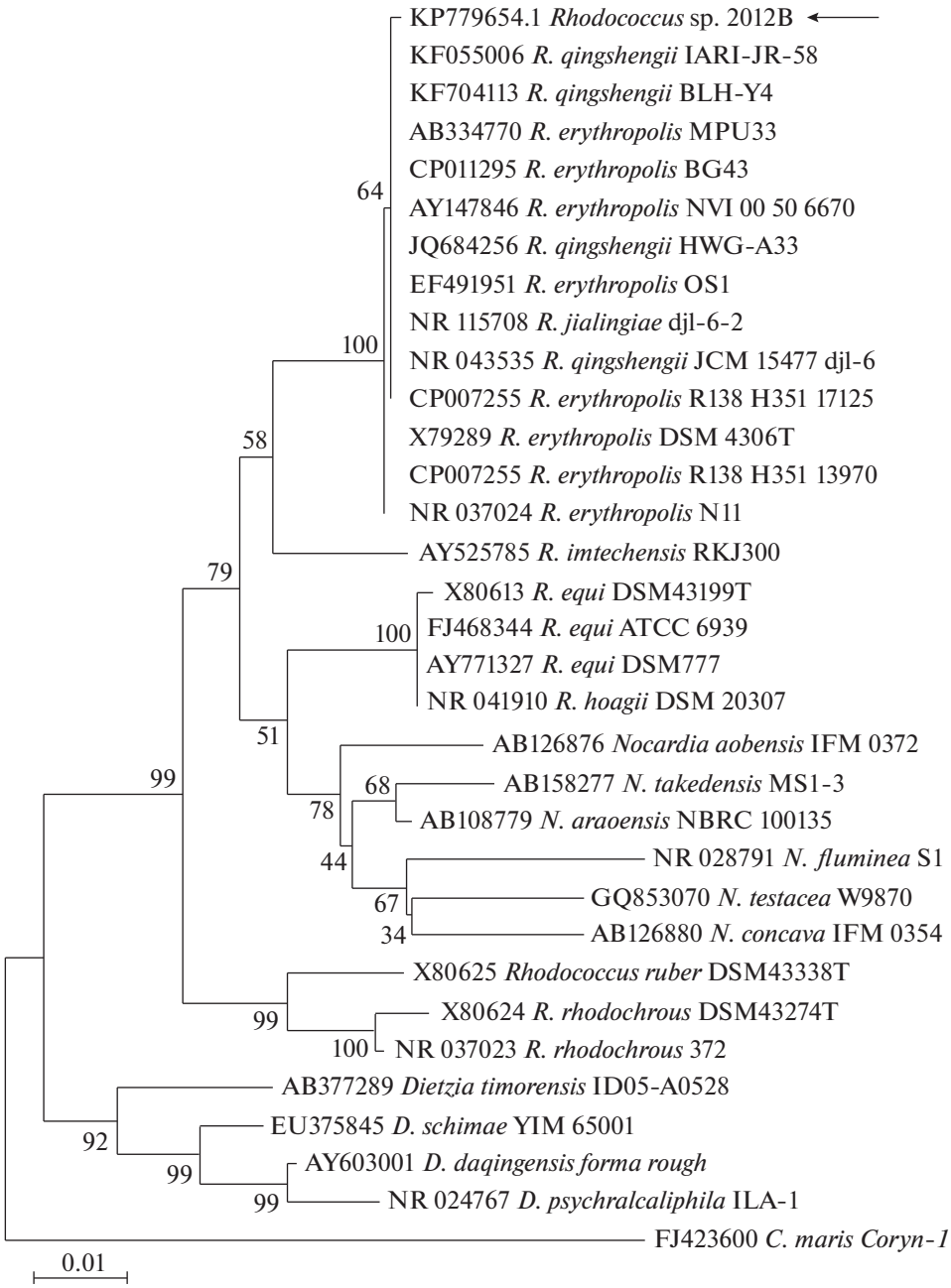

Fig. 2. Evolutionary relationships between strain Rhodococcus sp. 2012B (KP779654) and members of the genus Rhodococcus with respect to the 16S rRNA gene fragment (1286 bp). Evolutionary distances were calculated by the method of (Wang et al., 2010) and expressed in units representing the number of base substitutions per site. The changes in the rate of base substitution in the studied sequence were modeled using the gamma distribution (shape parameter = 0.44). The species Corynebacterium maris was used as an outgroup.

not utilize lactose and D-xylose from the fungicides benomyl and carbendazim (Table 1).

Recently, the 2012B strain was shown to have the unique ability to biodegrade nanodiamonds (Safronova, Koksharova, 2018). Environmental emissions of nanomaterials (to soil and water) entail negative consequences. Hence, the above strain can be considered a potential destructor of these carbon-containing materials.

PCR and phylogenetic analysis of the 2012B strain. PCR produced a DNA fragment containing the nucleotide sequence (1479 bp) of the 16S rRNA gene of a small ribosomal subunit of bacteria.

Phylogenetic analysis has shown that the 16S rRNA gene sequence falls within an independent cluster formed by *Rhodococcus* spp. and is maintained by three reference strains: *R. qingshengii* dj1-6^T (NR_043535.1), *R. jialingiae* dj1-6-2^T

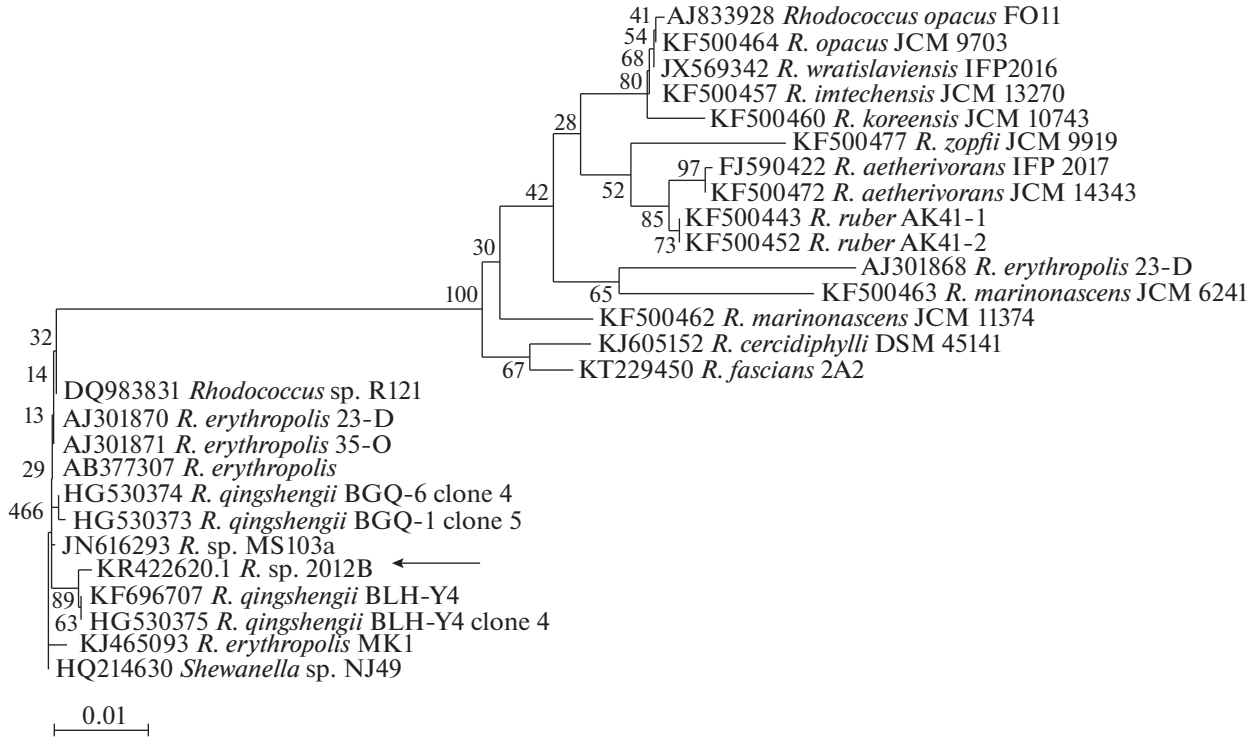

Fig. 3. Evolutionary relationships between the strain Rhodococcus sp. 2012B (KR422620) and members of the genus Rhodococcus with respect to the alkB gene fragment (272 bp). Evolutionary distances were calculated by the three-parameter method (van Beilen, Funhoff, 2007) and expressed in units representing the number of base substitutions per site. The changes in the rate of base substitution in the studied sequence were modeled using the gamma distribution (shape parameter = 0.41). The strain NJ49 of the genus Shewanella was used as an outgroup.

(NR_115708.1), and *R. erythropolis* DSM 43066^T (X79289) with >99.9% homology and the strain *R. qingshengii* BLH-Y4 (KF704113) with degree of similarity (99.93%) (Fig. 2). All 16S rRNA gene sequences falling in the cluster with the *Rhodococcus* sp. 2012B sequences are representatives of the same monophyletic group.

Species identification was also performed by comparing morphological and physiological-biochemical characteristics with those of the reference strains: *R. qingshengii* dj1-6^T, *R. jialingiae* dj1-6-2^T, and *R. erythropolis* DSM 43066^T (Wang et al., 2010; Xu et al., 2007) (Table 1).

The results of ribosomal gene-based phylogenetic analysis were corrected using the protein-encoding sequence of the *alkB* gene as an additional marker gene. The product of this gene is alkane monooxygenase providing the oxidation of *n*-alkanes with carbon chain lengths of C_6–C_{17} and catalyzing the reaction of hydrocarbon degradation (Chernyavskaya et al., 2012; van Beilen, Funhoff, 2007).

The nucleotide sequence of 333 bp corresponding to the *alkB* gene fragment, which was produced by PCR for the 2012B strain, made it possible to construct a phylogenetic tree (Fig. 3). One can see that the closest relative of strain *Rhodococcus* sp. 2012B is *R. qingshengii* BLH-Y4 (KF696707) (97.3% identity) isolated from the soil of the Qinghai–Tibet Plateau (Xu et al., 2007). Thus, the level of similarity between the 16S rRNA and *alkB* gene sequences has shown that, with respect to molecular taxonomic criteria (Stackebrandt, 2011), the 2012B strain can be attributed to the species *Rhodococcus qingshengii* sp. nov. (Xu et al., 2007).

Characteristics of the strain 2012C. The cells are Gram-negative, aerobic, single rods (Fig. 4). On the MMS medium with diesel fuel they form convex glistening colonies, beige, with even edges, with a cell size of 2–3 μm. The cells on the Mueller medium are larger (3–5 μm). The cells fluoresce in transmitted light. The bacteria were catalase positive and had no acid resistance.

The fatty-acid composition shows the predominance of long-chained saturated and polyunsaturated fatty acids with carbon chain lengths of $C_{14:0}$–$C_{19:0}$ with the maximum presence of $C_{15:0}$ (45%), $C_{16:0}$ (32%), and $C_{17:0}$ (9%).

The strain 2012C utilizes sugars and amino acids (L-asparagine, L-inosine, D-maltose, D-sorbitol, L-arabinose, and D-arabitol) but does not utilize lactose or the fungicides benomyl and carbendazim.

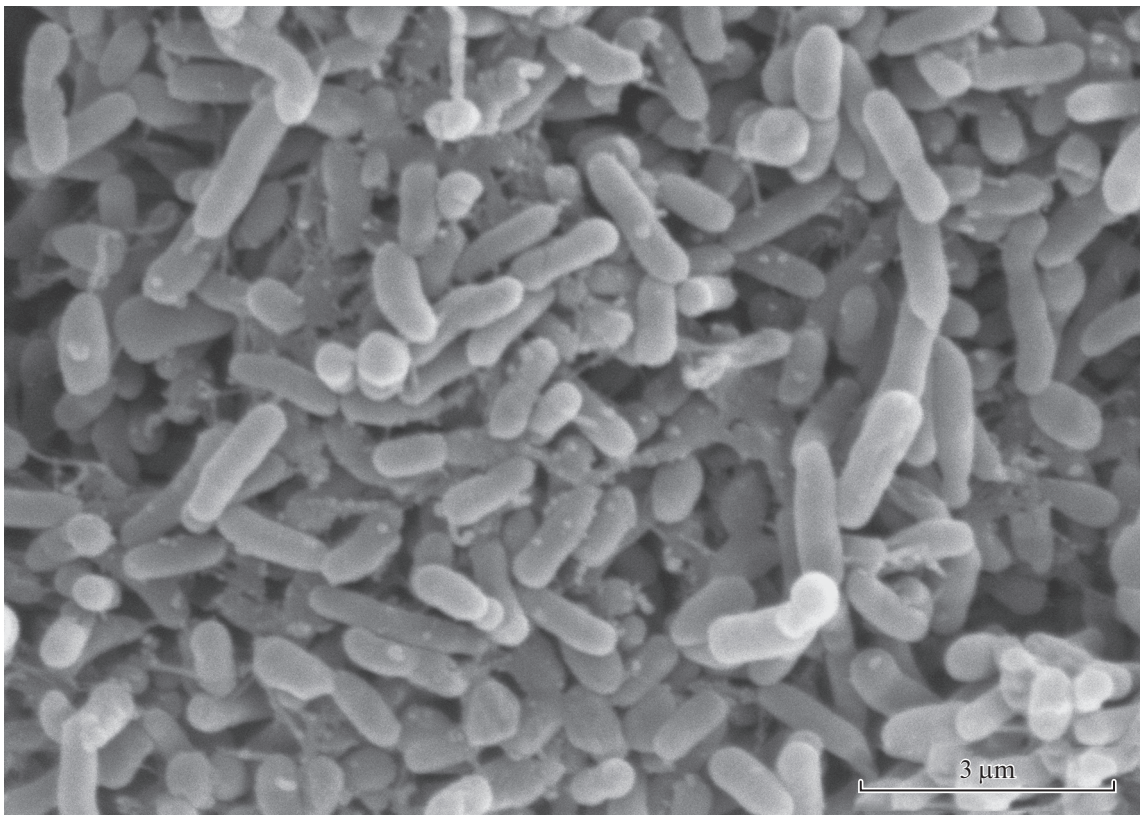

Fig. 4. Cell morphology of the strain 2012C (SEM).

PCR and phylogenetic analysis of the strain 2012C. PCR produced a DNA fragment containing the 16S rRNA gene sequence (936 bp) of the small ribosomal subunit of bacteria.

It has been shown that the nucleotide sequence of the 16S rRNA gene is highly similar to those of different representatives of the family Pseudomonadaceae and makes it possible to attribute the strain 2012C to the genus *Pseudomonas* (Proteobacteria, Pseudomonadaceae) (Fig. 5). Phylogenetic analysis showed that 2012C formed a taxonomically homogenous cluster representing strains of the genus *Pseudomonas* with a 99.57% homology; at the same time, the cluster combines bacteria from different ecosystems and different geographical zones. For example, *P. mendocina* (KF318795) was isolated from rhizospheric soil in India; *P. psychrotolerans* (MG778875) was isolated from Brazilian passionflower (*Passiflora*). *P. oryzihabitans* (MG571765.1) from polluted soil in Saudi Arabia can degrade hydrocarbons.

The 2012C strain was cultivated on a solid synthetic MMS medium with summer diesel fuel as the sole carbon source, which indicates the ability of the strain 2012C to degrade aliphatic hydrocarbons. PCR with specific primers (Kohno et al., 2002) was used to obtain the nucleotide sequence of ~220 bp corresponding to the *alkB* gene fragment. The findings suggest that the genome of *Pseudomonas* sp. 2012C contains genes encoding the enzymes responsible for the oxidation of alkanes with "short" carbon chains. Thus, phylogenetic analysis made it possible to identify strain *Pseudomonas* sp. 2012C as the closest one to the species *P. psychrotolerans* and *P. oryzihabitans*.

CONCLUSIONS

The cultured hydrocarbon-oxidizing strains have been isolated from a freshwater reservoir situated within the city limits of Moscow and characterized. The species taxonomic position of strain *Rhodococcus* 2012B has been defined as *Rh. qingshengii*; strain *Pseudomonas* sp. 2012C is closest to the species *P. psychrotolerans* and *P. oryzihabitans*. Novel strains can become a basis for the development of biopreparations stimulating the natural processes of environmental cleanup from oil hydrocarbons, including the most stable polycyclic aromatic compounds.

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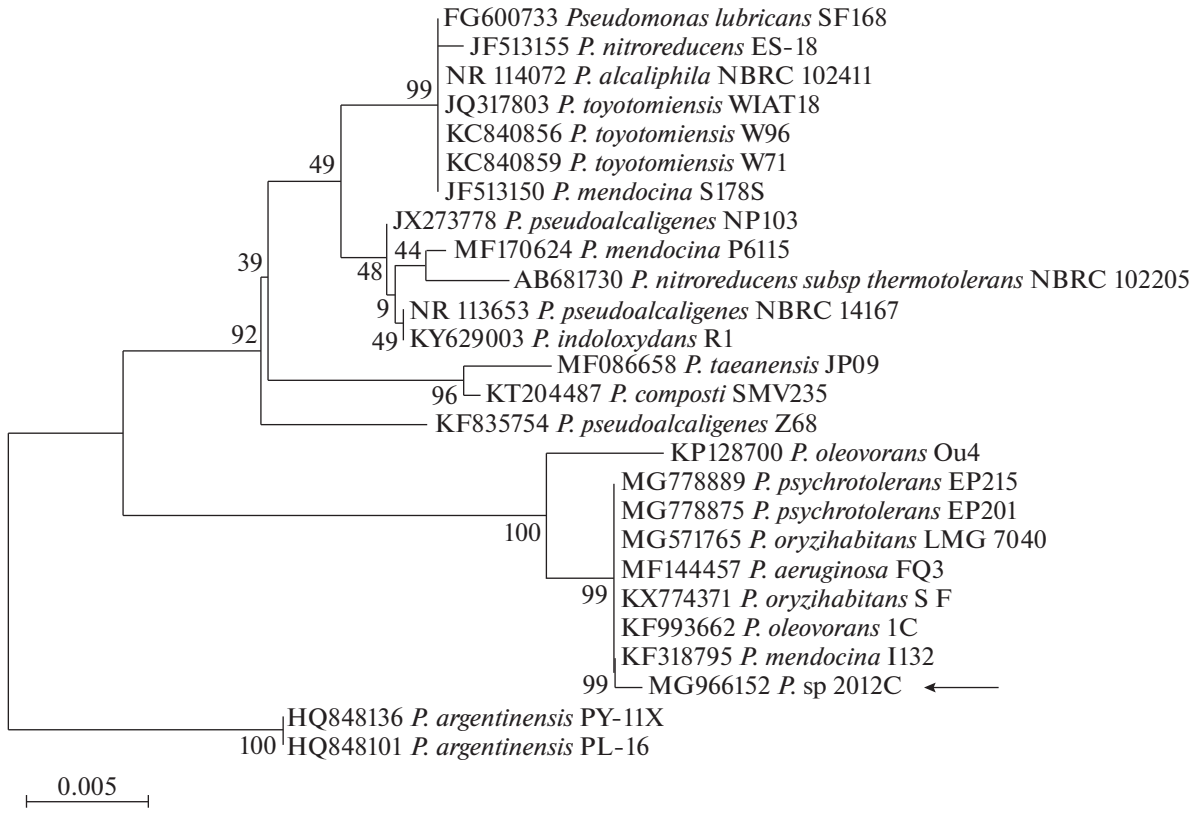

Fig. 5. Evolutionary relationships between the strain Pseudomonas sp. 2012C (MG966152) and members of the genus Pseudomonas with respect to the 16S rRNA gene fragment (917 bp). Evolutionary distances were calculated by Kimura's 2-parameter method (Long et al., 2003) and expressed in units representing the number of base substitutions per site. The changes in the rate of base substitution in the studied sequence were modeled using the gamma distribution (shape parameter = 0.44).

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Translated by E. V. Makeeva

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