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Genetic Diversity of Bacteria Adapted to Cyanide-Bearing Compounds in the Technogenic Ecosystems as Detected by 16S rDNA Sequences

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Abstract—The genetic diversity of microbial communities that developed naturally within the system of ore heap–solution of heap leaching process has been studied. The difference in the microbial community structure is identified. It is found that phylotypes *Serratia* and *Achromobacter* dominated within the ore heap and *Hydrogenophaga* and *Acinetobacter* dominated in the solution. Phylogenetic analyses revealed that there are microorganisms among the closest homologues that are able to destruct toxic compounds and/or exhibit their enzyme activity at low temperature. It is shown that aerobic organoheterotrophs are the most promising for the isolation from autochthonous microbial communities of technogenic complexes in East Siberia, as well for studying their destructive potential and use in bioremediation.

Keywords: cyanide, heap leaching, process solution, ore heap, 16S rDNA, *Serratia*, *Achromobacter*, *Exiguobacterium*, *Acinetobacter*, *Hydrogenophaga*

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INTRODUCTION

The methods for biological decontamination are of particular interest in solving environmental problems of gold-mining waste detoxification. In the world practice for developing new cost-effective and environmentally efficient technologies for biodegradation of cyanide compounds, active strains of microorganisms are isolated for further use in bioremediation activities. The biotechnology potential of bacteria is currently being studied. These bacteria belong to different taxonomic groups: Proteobacteria: *Achromobacter* sp., *Acinetobacter* sp. (Baxter, Cummings, 2006), *Methylobacterium thiocyanatum* (Wood et al., 1998), *Pseudomonas* spp. (Harris and Knowles, 1983; Grigorieva et al., 2008), *Serratia marcescens* (Kumar et al., 2013); Firmicutes: *Bacillus* spp. (Perumal et al., 2013; Mirizadeh et al., 2014); Actinobacteria: *Rhodococcus* spp. (Maniyam et al., 2011), etc. Most of the isolated strains have been successfully used in cyanide technology waste processing in regions with warm climates (Harris and Knowles, 1983; Kumar et al., 2013; Mirizadeh et al., 2014). However, their use for gold-mine waste processing in Russia faces the challenge of maintaining their active status with rapid daily and seasonal temperature fluctuations (Belykh et al., 2014).

Under the conditions of severely continental climate in the autochthonous microbial communities of natural technogenic complexes of heap leaching (HL) waste microorganisms develop. These microorganisms are adapted not only to high concentrations of certain substances, but also to environmental conditions. A study of their diversity make it possible to adapt nutrient media and culture conditions for isolation of strains involved in the destruction of toxic compounds, taking into account regional specificity and characteristics of the chemical composition of rocks. Currently there are almost no researches on the diversity, composition, and structure of microbial communities from gold-mine HL wastes in Eastern Siberia. Therefore, there is a necessity for identifying microorganisms which developed in situ in two basic types of HL wastes: process solution and ore heap. They are negative factors of technogenic impact on air, soil, open- and groundwater, and may for a long time determine the state of the environment in the area of HL plants. Carrying out this kind of research will allow one to offer optimal conditions for the cultivation of physiologically important groups of bacteria involved in the detoxification of cyanide and its derivatives. The aim of this study was to investigate the genetic diversity of natural microbial communities developed in the system ore heap–HL process solu-

tion, compare the composition of microorganisms, and determine their phylogenetic relationships with their closest homologues.

MATERIALS AND METHODS

Sampling for the study was carried out in 2012 on one of the fields of Krasnoyarsk krai. Samples of the process solution and ore were collected from the functional gold HL ore heap in sterile containers, transported, and stored at 4°C before the experiments.

Chemical analysis of the process solution was carried out using the following methods: ionic composition of calcium, magnesium and chloride was measured by titration method (Environmental Regulation at the Federal level (ER F) 14.1: 2.95-97, ER F 14.1: 2.98-97, ER F 14.1: 2.96-97, respectively); ionic composition of sulfates was measured by turbidimetric method (ER F 14.1: 2.159-2000), that of cyanide and thiocyanate by the photometric method with pyridine and barbituric acid (ER F 14.1: 2.56-96, ER F 14.1: 2: 4.156-99, respectively), and the total salt content was measured by the gravimetric method (ER F 14.1: 2: 4.114-97). The elemental composition of metals was determined by atomic emission spectrometry with inductively coupled plasma (ER F 14.1: 2: 4.135-98). To determine the chemical composition of the ore solid phase, X-ray fluorescence quantitative analysis was used (on the MA RAC-53-2004 (FR.1.31.2014.18483)).

The diversity of microbial communities of the process solution and the ore heap was investigated by the molecular genetic analysis of 16S rRNA gene fragments (Belkova and Andreeva, 2009). DNA extraction was performed with a AxyPrep Bacterial Genomic DNA commercial kit (Axygen Biosciences, United States). Amplification was carried on bacterial conserved primers (500L–1350R); amplicons were analyzed on agarose gel, eluted by freezing and thawing, followed by cloning and sequencing (Belkova and Andreeva, 2009). The nucleotide sequences were determined on an ABI310A ABI PRISM 310 Genetic Analyzer automatic sequencer (United States) in the Genomic Core Facility, Siberian Branch, Russian Academy of Sciences (Novosibirsk, Russia). A comparative analysis of the sequences was performed using the FASTA (http:/ www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic relationships with their closest homologues were evaluated by Mega software package v. 6.06. The nucleotide sequences were deposited in the international databases (accession numbers: LK392396–LK392456).

RESULTS AND DISCUSSION

Chemical composition and content of cyanide compounds in the system process solution–ore heap. Considering that samples were collected from an functional ore heap, the chemical composition of the process solution corresponds to the composition of the interporous ore moisture. The characteristic features of these solutions are high concentrations of cyanide (2640 TLV) and thiocyanate (190 TLV), as well as a significant concentration of heavy metals: aluminum (53 TLV), copper (50 TLV), nickel (18 TLV), and zinc (14 TLV) (Table 1).

The main components of the ore solid phase are silica (the mass fraction of 55.6%) and alumina (21.1%) due to the presence of quartz and mica. The proportion of total iron is less than 4.19%; most of it is in an oxidized form as goethite and only 0.314% is in the form of sulphide in the pyrite. The content of alkali and alkaline earth metal is no more than 5.5% (K_2O 3.8%, Na_2O 1.0%, CaO 0.042%, MgO 0.7%) (Table 2).

Heavy metals enter the interporous ore moisture due to increased oxidation processes in gold HL during their transition to mobile form in the composition of soluble cyanide complexes (Nekrasov, 1973). The presence of cyanide in the interporous ore moisture in the concentration higher than threshold limit value (TLV) (Table 1) is associated with irrigation of the ore heap with the process solution of their high content.

Thus, taking into account the chemical composition of the liquid and solid phases of gold HL wastes, the development of specific microbial communities that are adapted to the high content of cyanide-containing compounds and heavy metals can be assumed in these conditions.

Comparative characteristics of ore heap and process solution microbial communities structure. Molecular cloning of 16S rDNA amplicons revealed a low diversity of microbial communities in the ore heap and process solution. Comparative analysis showed a difference in the representation of phylotypes: microorganisms belonging to the Actinobacteria and Proteobacteria were identified in the ore, and in the solution Fimicutes were detected additionally. It should be noted that there are almost no data on the total microbial diversity in the gold-mining HL waste obtained by molecular cloning or metagenomic analysis. The investigations with these methods are actively carried out for different types of soil microbial communities (Petrova et al., 2010).

A comparative analysis of the sequences with the closest homologues from international databases (Genbank and EMBL) revealed a similarity level from 95.1 to 100%, which made it possible to determine their tribal affiliation (Pinevich, 2006). We identified the genera: *Achromobacter* (homology of 98.0 to 100%), *Acinetobacter* (95.8–96.3%), *Alcaligenes* (95.1%), *Exiguobacterium* (95.8–96.2%), *Hydrogenophaga* (95.3–97.5%), *Pseudomonas* (99.5%), *Propionibacterium* (99.3%), *Rhodococcus* (98.7–99.2%), and *Serratia* (99.5–100%) (Fig. 1).

Table 1. The chemical composition of the HL process solution (ore interporous moisture)

Defined components	Concentration, mg/L	TLV (water bodies of fishery), mg/L
pH	12.1	
	Total salt content	
Calcium	375.0	180.0
Magnesium	Not found	40.0
Sulphates	Not found	100.0
Chlorides	11.0	300.0
Cyanides	132.0	0.05
Thiocyanates	19.0	0.1
Aluminium	2.12	0.04
Arcenic	Not found	0.05
Bismuth	Not found	0.1
Cadmium	Not found	0.005
Cobalt	0.01	0.01
Copper	0.05	0.001
Iron	Not found	0.1
Manganes	Not found	0.01
Nickel	0.018	0.001
Lead	Not found	0.1
Antimony	Not found	0.05
Zink	0.14	0.01

Table 2. Chemical composition of the ore heap solid phase

Components	Mass content, %	Components	Mass content, %
SiO_2	55.6	MnO	0.014
Al_2O_3	21.1	P_2O_5	0.033
TiO_2	1.20	Sb	<0.001
MgO	0.7	Hg	<0.001
K_2O	3.8	Ni	0.005
Na_2O	1.0	WO_3	0.004
CaO	0.042	Mo	<0.001
Fe_{total}	4.19	Cu	0.0024
$Fe_{oxidized}$	3.99	Zn	0.004
$Fe_{sulphide}$	0.314	Pb	0.0099
S_{total}	0.035	C_{total}	0.14
$S_{oxidized}$	Not found	$C_{organic}$	<0.050
As_{total}	0.105		
$As_{sulphide}$	0.063		
$As_{oxidized}$	0.039		

It should be noted that, in previous studies from ores, contaminated with cyanide compounds and waste water of gold mining, the following strains were isolated and studied: *Achromobacter* sp., *Alcaligenes* spp. (Baxter and Cummings, 2006), *Pseudomonas* spp. (Harris and Knowles, 1983; Grigor'eva et al., 2008, and others), *Rhodococcus* spp. (Maniyam et al., 2011), and *Serratia* sp. (Kumar et al., 2013). The representatives of

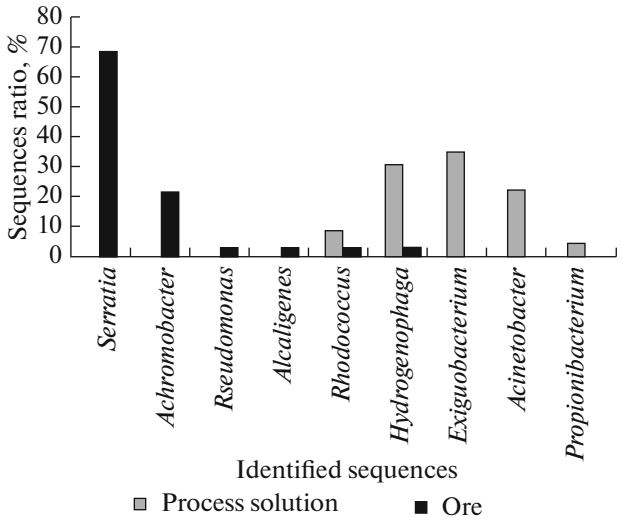

Fig. 1. Species diversity of microbial communities developed in the system ore heap–process solution.

genera *Exiguobacterium* and *Propionibacterium* were not detected in gold HL waste, but they were isolated from other polluted habitats (Collins et al., 1983). In addition, using the same molecular methods in similar technogenic ecosystems (tailing damps, waste water (Zhang et al., 2010), activated sludge, and soil polluted by heavy metals (Brodie et al., 2006)), representatives of microorganisms belonged to all identified genera have been determined.

Also, differences in phylotype quantitative ratio of the same genus in different types of waste were found. The phylotypes of Proteobacteria—*Serratia* (68.4%) and *Achromobacter* (21.1%)—dominate in the ore heap (Fig. 1). Their closest homologues were isolated from contaminated soils; they are resistant to heavy metals and are capable of destroying polycyclic aromatic hydrocarbons (Ji et al., 2012). The proportion of minor forms of this microbial community is 10.5%. They are represented by different phylotypes of Proteobacteria and Actinobacteria: *Alcaligenes*, *Hydrogenophaga*, *Pseudomonas*, and *Rhodococcus*. An important feature of their closed relatives is the ability to degrade toxic compounds, including cyanides and thiocyanates (Grigor'eva et al., 2008; Maniyam et al., 2011; Belykh et al., 2014).

In the microbiocenosis of the process solution, besides the representatives of Proteobacteria, *Hydrogenophaga* (30.4%), and *Acinetobacter* (21.7%), Firmicutes were identified: *Exiguobacterium* (34.8%). Their homologues were mainly, isolated from soils contaminated with heavy metals from activated sludge of treatment plants and industrial waste-water industry (Yoon et al., 2008). Minor forms (13.5%) were presented by only two types of Actinobacteria: *Propionibacterium* and *Rhodococcus*; their nearest relatives were isolated from the technogenic ecosystem and had the ability to degrade polycyclic aromatic hydrocarbons (Thomassin-Lacroix et al., 2001).

Thus, the representatives of Proteobacteria, Actinobacteria, and Firmicutes were identified in the microbial community of the system ore heap–process solution. Identified microorganisms were adapted to high concentrations of cyanide compounds and heavy metals; according to the food type, they belonged to organoheterotrophs and are aerobic. It was shown that in the process of HL under ore heap irrigation with cyanide solution, changes in both dominant and minor phylotypes of microbial communities occur.

Comparative characteristics of the phylogenetic relationships of ore heap dominant phylotypes with the closest homologues. Taking into account the different levels of similarity (95.1–100%) of the obtained sequences with the closest homologues from international databases (Genbank and EMBL), a phylogenetic analysis of the type of strains and the closest homologues isolated or identified in the similar ecosystems was conducted for the comparative characteristics and identification.

The representatives of the genus *Serratia* are dominant in the microbial community of the ore heap. The sequences are clustered together with species *S. marcescens* subsp. *sakuensis* KRED^T and *S. nematodiphila* DZ0503SBS1^T (Fig. 2). The variability among the sequences vary from complete homology to the five nucleotide substitutions.

Genus *Serratia* belongs to the family Enterobacteriaceae, Gammaproteobacteria. Currently, genus contains 20 species predominantly isolated from natural sources: soil, water, and plants. The type species is *S. marcescens* DSM 30121^T. It is an opportunistic pathogen for humans. The sequences derived from the ore heap form a separate cluster from the type species of the genus *Serratia* on the phylogenetic tree. They grouped together with a strain of *S. marcescens* subsp. *sakuensis* KRED^T that was isolated from activated sludge (Ajithkumar et al., 2003). Taking into account that *S. nematodiphila* DZ0503SBS1^T, which differs from *S. marcescens* subsp. *sakuensis* KRED^T by only three nucleotide substitutions, fall in the same cluster, it is correct to identify obtained sequences only at a genus level. It should be noted that the sequences of the representatives of the *Serratia* genus were obtained by molecular methods from other ecosystems similar in ecology: soils contaminated with heavy metals (HM461145, KF596616, KF511906), a polluted river (AB698033), and waste water (KJ136643) also belong to this cluster (Fig. 2).

The sequences related to the genus *Achromobacter* form a single cluster with sequences of the type strains *A. insuavis* LMG 26845^T, *A. pulmonis* R-16442^T, and *A. xylosoxidans* DSM 10346^T (Fig. 3). Variability among sequences obtained is 1 to 7 nucleotide substitutions.

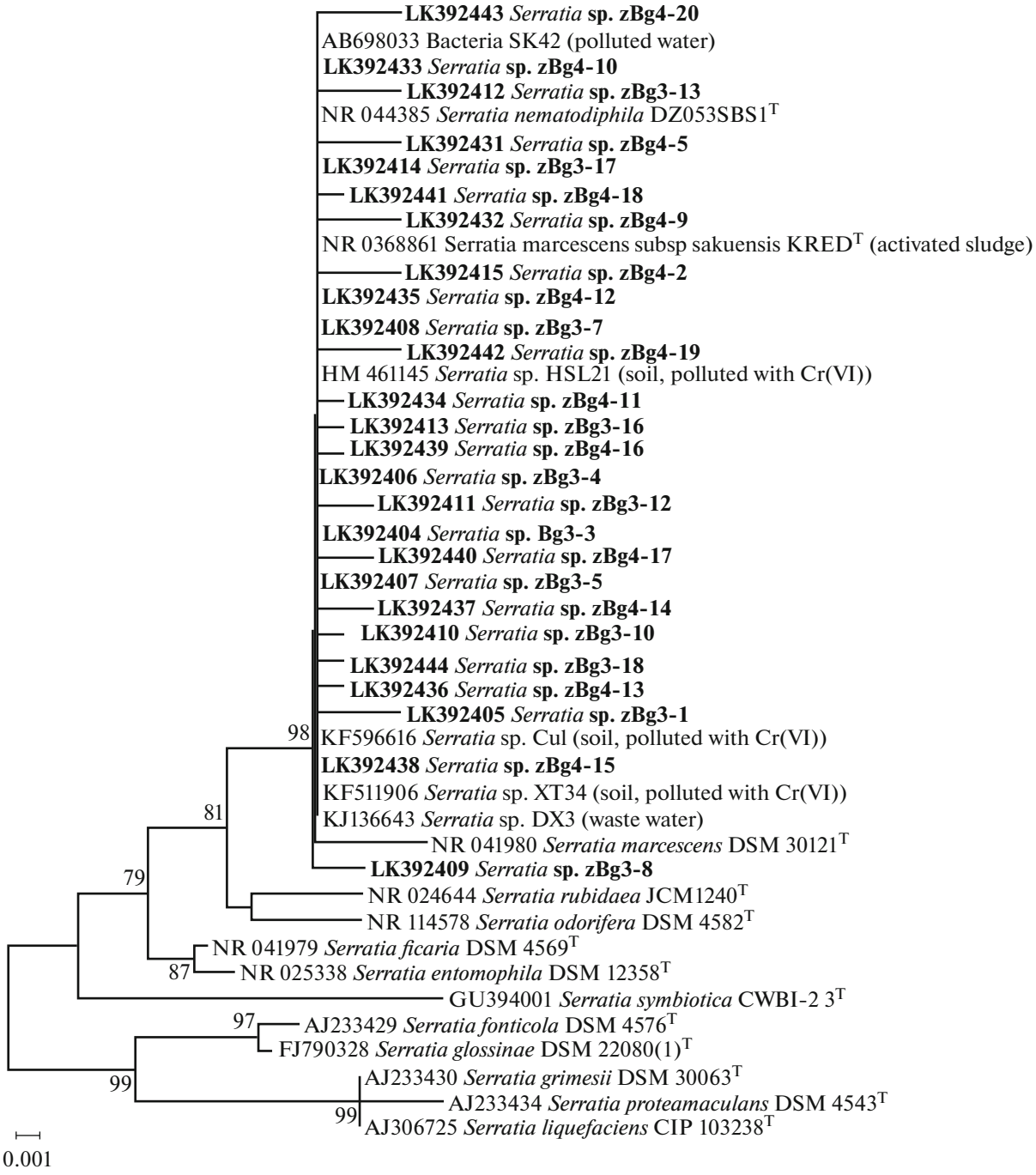

Fig. 2. Phylogenetic tree constructed on the fragments of the 16S rRNA gene of the representatives of *Serratia*. The sequences obtained in this study are shown in bold. The scale corresponds to 1 nucleotide substitution per 1000 bp. The numbers showed a statistical significance of the order branch determined by a bootstrap analysis of 1000 alternative trees; values below 75% are not indicated.

Genus *Achromobacter* is polyphyletic, belonges to the family Alcaligenaceae, class Betaproteobacteria. There is now a thorough revision of its individual members, especially close to the genera *Alcaligenes* and *Bordetella* (*Bergey's Manual...*, 2005). The closest homologues of the obtained sequences included genus *Bordetella*; however, the phylogenetic relationship analysis showed that they are grouped into different clusters (Fig. 3). Genus *Achromobacter* contains 18 species isolated from a variety of sources: water (*A. piechaudii*, *A. ruhlandi*) and soil (*A. marplatensis*). The type species of this genus is *A. xylosoxidans*

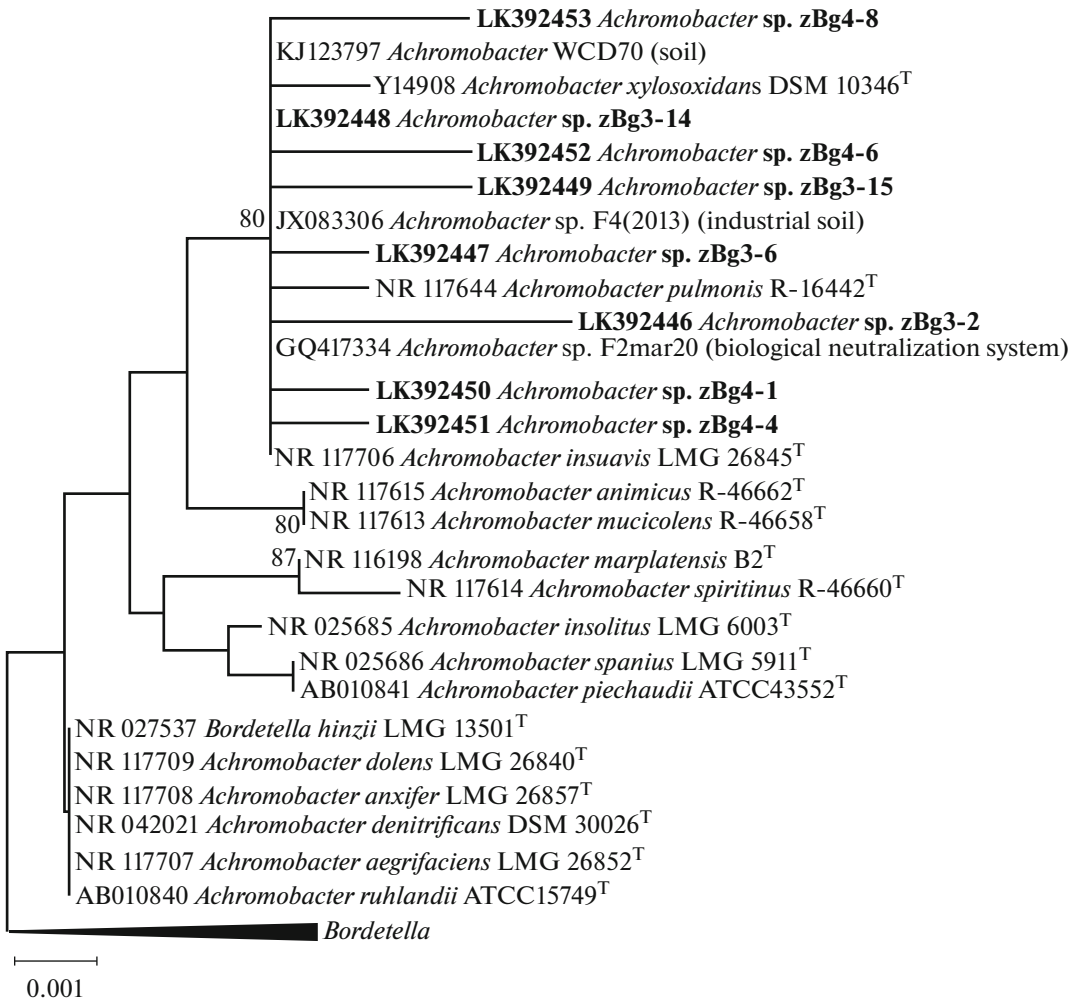

Fig. 3. Phylogenetic tree constructed on the fragments of the 16S rRNA gene of the representatives of genera *Achromobacter* and *Bordetella*. The sequences obtained in this study are shown in bold. The scale corresponds to 1 nucleotide substitution per 1000 bp. The numbers showed a statistical significance of the order branchdetermined by a bootstrap analysis of 1000 alternative trees; values below 75% are not indicated.

DSM 10346^T, an opportunistic pathogen for humans, but it is widely distributed in oligotrophic aquatic ecosystems (*Bergey's Manual...*, 2005). Despite the fact that recently the strains isolated from clinical specimens are being actively studied (Vandammea et al., 2013), many strains (NR116198, KJ123797, JX083306, GQ417334 and others) have been isolated from natural and technogenic ecosystems, but not all of them are identified to the species level.

An analysis of phylogenetic relationships has shown that sequences derived from the ore heap grouped into a same cluster with several type species of the genus *Achromobacter* and can be identified at genus level as the *Achromobacter* spp. It should be noted that, in addition to the type sequences, the sequences of *Achromobacter* spp., obtained from biological neutralization systems (GQ417334) and industrial soils (JX083306) are also in this cluster.

Thus, the results of phylogenetic analysis confirmed not only the tribal affiliation of the obtained sequences, but also their ecological apartness.

Comparative characteristics of the phylogenetic relationships between dominant phylotypes of the process solution and the closest homologues. In the microbiocenosis of HL process solution, the sequences of the genera *Exiguobacterium* and *Acinetobacter* were identified. Additionally, *Hydrogenophaga* was detected in the HL solution and in the ore heap.

Members of the genus *Exiguobacterium* are the dominants in the microbial community of HL process solution. The sequences are included in the cluster with *E. indicum* HHS 31^T and *E. acetylicum* DSM 20416^T (Fig. 4). The variability among the sequences is from 22 to 48 nucleotide substitutions.

Exiguobacterium belongs to a class of bacilli and is currently defined in the independent family Family XII.

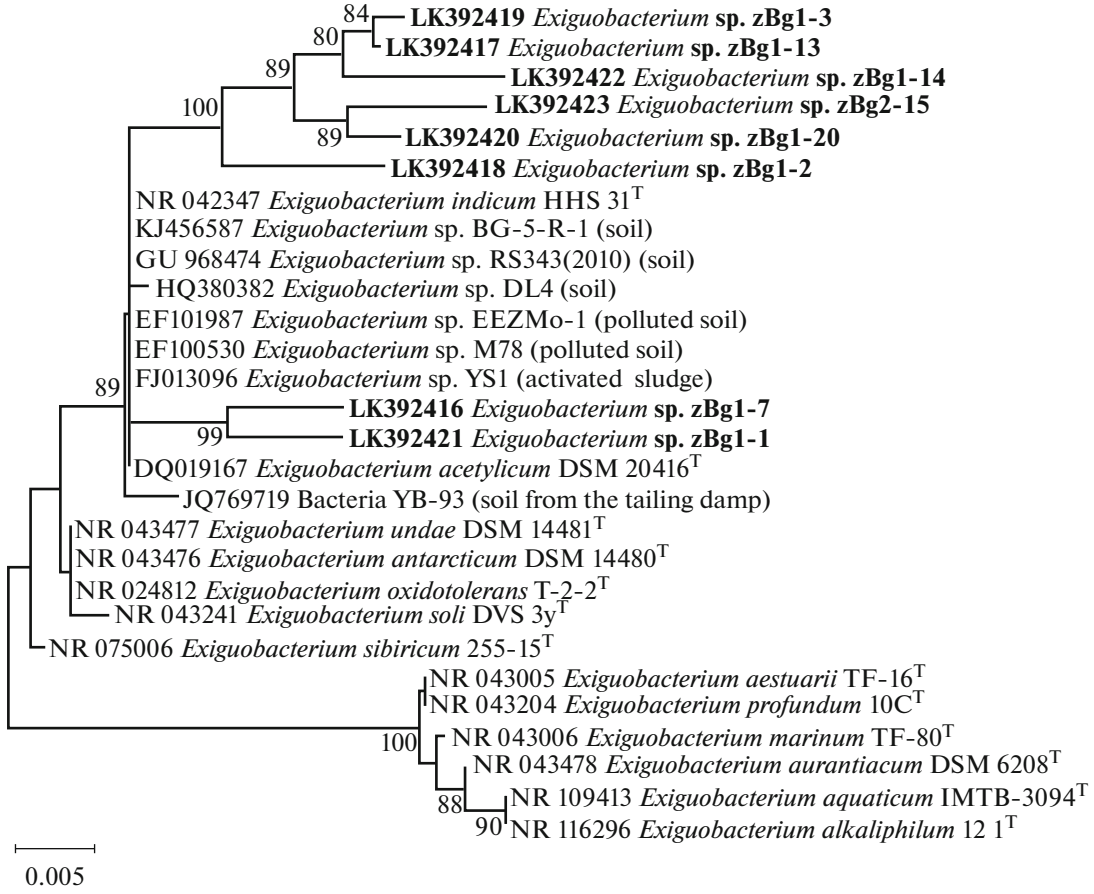

Fig. 4. Phylogenetic tree constructed on the fragments of the 16S rRNA gene for the representatives of genera *Exiguobacterium*. The sequences obtained in this study are shown in bold. The scale corresponds to 5 nucleotide substitutions per 1000 bp. The numbers showed a statistical significance of the order branch determined by a bootstrap analysis of 1000 alternative trees; values below 75% are not indicated.

Incertae Sedis (*Bergey's Manual...*, 2009). This genus contains 15 species that are mainly isolated from natural sources: water (*E. undae*, *E. aquaticum*), hydrothermal springs (*E. profundum*), or contaminated habitats: waste water (*E. aurantiacum*, *E. oxidotolerans*, and *E. alkaliphilum*) (Fig. 4). Type species is *E. aurantiacum* DSM 6208^T. It was isolated from the waste water and showed the ability to reduce nitrates (Collins et al, 1983). Some members of this genus are thermotolerant and can develop at low temperatures: *E. antarcticum* DSM 14480^T was isolated from a microbial mat of Lake Fryxell (Antarctica); *E. sibiricum* 255-15^T was isolated from permafrost (Siberia) (*Bergey's Manual...*, 2009).

An analysis of phylogenetic relationships showed that the sequences obtained from the process solution form two independent branches in a cluster separate from the type species of *Exiguobacterium* together with species *E. indicum* HHS 31^T and *E. acetylicum* DSM 20416^T (Fig. 4). It should be mentioned that the strain *E. indicum* HHS 31^T (NR042347) belongs to psychrotrophic bacteria and was isolated from the glacier of the Himalayan mountain range (India); *E. acetylicum* DSM 20 416^T was isolated from waste waters (*Bergey's Manual...*, 2009). Other than typical, the cluster includes sequences obtained by molecular methods from similar ecosystems: waste water (FJ013096) and natural and contaminated soils (KJ456587, HQ380382, EF101987, JQ769719, and others). Considering the independence of the branches (Fig. 4), it is correct to identify the sequences at a genus level as *Exiguobacterium* spp.

Figure 5 shows a phylogenetic tree constructed on the fragments of the 16S rRNA gene for the representatives of the genus *Acinetobacter*. Sequences identified in the process solution are formed the cluster with the representatives of *Acinetobacter*, including those obtained by molecular methods (Fig. 5). The variability among the identified sequences is from 24 to 38 nucleotide substitutions.

The genus *Acinetobacter* belongs to the family Moraxellaceae, class Gammaproteobacteria, and contains 35 species. Despite the fact that the type species of the genus, *Acinetobacter calcoaceticus*

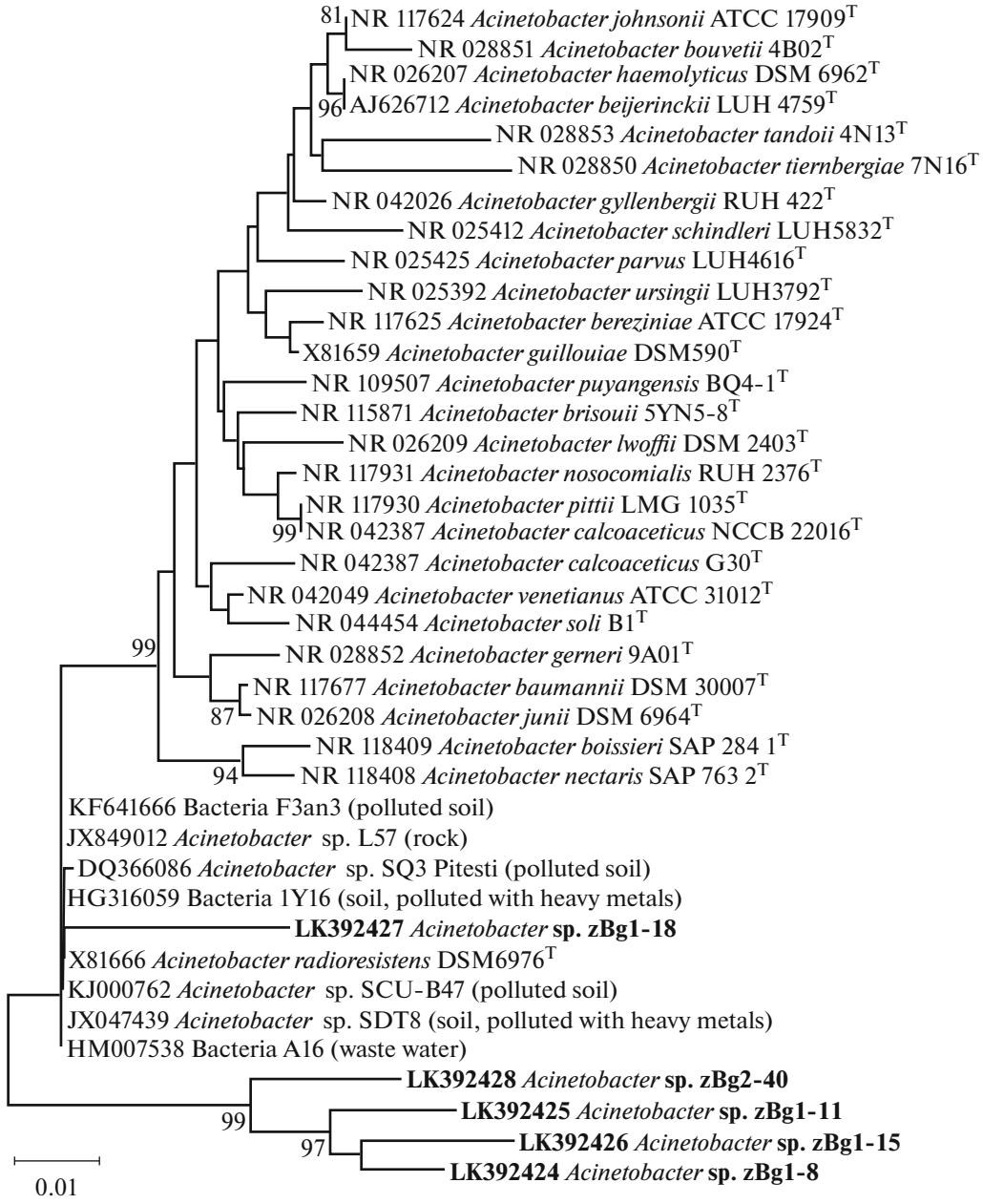

Fig. 5. Phylogenetic tree constructed on the fragments of the 16S rRNA gene for the representatives of genera *Acinetobacter*. The sequences obtained in this study are shown in bold. The scale corresponds to 1 nucleotide substitution per 1000 bp. The numbers showed a statistical significance of the order branch determined by bootstrap analysis of 1000 alternative trees; values below 75% are not indicated.

NCCB 22016^T, was isolated from clinical specimens, many species of the genus *Acinetobacter* were isolated from natural sources: soil (*A. soli*) and plants (*A. puyangensis*, *A. boissieri*, and *A. nectaris*), as well as from men-made places: activated sludge (*A. tjernbergiae*, *A. towneri*, *A. tandoii*, *A. junii* and *A. gerneri*) and waste water (*A. rudis*).

An analysis of phylogenetic relationships showed that four sequences (LK392424–LK392426, LK392428) identified in the HL process solution form a separate cluster phylogenetically located far from the type strains of *Acinetobacter*. The sequence LK392427 forms a cluster with the type strain *A. radioresistens* DSM6976^T, which was isolated from soil (Nishimura et al., 1988). The same cluster includes sequences obtained by molecular methods from ecosystems with a similar ecology: rock (JX849012), waste water (HM007538), and polluted soils (KF641666,

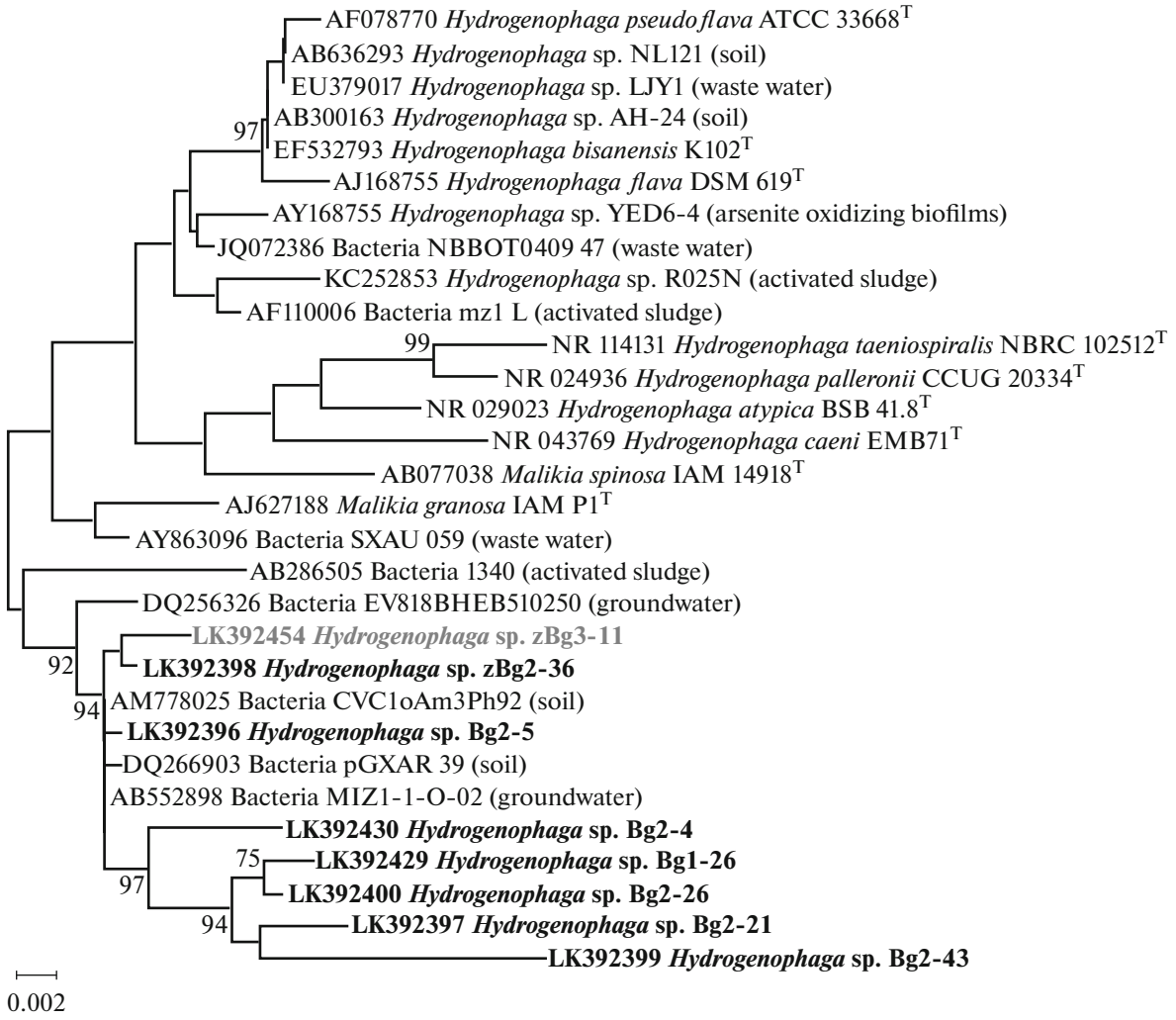

Fig. 6. Phylogenetic tree constructed on the fragments of the 16S rRNA gene for the representatives of genera *Hydrogenophaga*. The sequences obtained in this study are shown in bold: in black are sequences obtained from the process solution; sequences obtained from ore are gray. The scale corresponds to 2 nucleotide substitutions per 1000 bp. The numbers showed a statistical significance of the order branch determined by a bootstrap analysis of 1000 alternative trees; values below 75% are not indicated.

DQ366086, HG316059, JX047439). Based on the phylogenetic position, the sequences are correctly identified at only the genus level as *Acinetobacter* spp.

Members of the genus *Hydrogenophaga* were identified both in a process solution (LK392396–LK392400, LK392429, LK392430), and in an ore heap (LK392454). The variability among the obtained sequences is from 6 to 34 nucleotide substitutions. Among the closest homologues with a low percentage of homology (from 96.3 to 97.0%), the sequence of two type strains of the genera of the phylum Proteobacteria, *Hydrogenophaga* and *Malikia,* were detected. An analysis of phylogenetic relationships confirmed the independence of the obtained sequences (Fig. 6). It should also be noted that this cluster only includes sequences of microorganisms whose taxonomic position is not defined, but they are isolated from soils (AM778025, DQ266903), ground (DQ256326, AB552898) and waste waters (AB28650).

Hydrogenophaga and *Malikia* belong to the family Comamonadaceae, class Betaproteobacteria. Currently, the genus *Hydrogenophaga* includes nine species, which are mainly isolated from natural sources (soil, mud, and water) and from places under man-made influence (*H. atypica*, *H. defluvii*, *H. bisanensis*, and *H. caeni*). Four species, which belong to the genus *Hydrogenophaga* (*H. flava*, *H. pseudoflava*, H. *taeniospiralis*, and *H. palleronii*), have been reclassified from the genus *Pseudomonas* (Willems et al., 1989). The genus *Malikia* contains only two species (*M. granosa* and *M. spinosa*), which have also been reclassified from the genus *Pseudomonas* (Spring et al., 2005). Given the complexity of the taxonomic characteristics of both genera, derived sequences can be identified

only at the level of family as representatives of the family Comamonadaceae.

CONCLUSIONS

Thus, on the basis of the results, we can say that specific microbial communities adapted to the high content of cyanide compounds and heavy metals developed in the conditions of the system of the ore heap–process solution. Their diversity varies in representation and quantitative ratio of phylotypes. Phylotypes *Serratia* spp. and *Achromobacter* spp. dominate in the ore heap. Representatives of *Exiguobacterium* dominated in the process solution microbiocenosis in addition to the representatives of *Hydrogenophaga* spp., *Acinetobacter* spp.

Phylogenetic analysis determined not only the tribal affiliation of the identified sequences, but also their ecological apartness. The characteristics of the biochemical features of closest homologues indicate the possible presence of similar enzymes that catalyze degradation of toxic compounds in microorganisms from HL waste and/or the ability to exhibit their enzymatic activity at low temperatures. Characterized microorganisms are adapted to high concentrations of cyanide compounds and heavy metals. They are aerobic organotrophs; this should be taken into account while the preparation of culture media. Isolation and investigation of the destructive abilities of microorganisms of autochthonous microbial communities of natural technogenic complexes of HL gold mining is a promising direction in biotechnology HL waste treatment.

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