БИОЛОГИЧЕСКИЕ НАУКИ

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ISOLATION AND CHARACTERIZATION *ACIDITHIOBACILLUS FERROOXIDANS* AND *ACIDITHIOBACILLUS FERRIVORANS* FROM THE ARSENOPYRITE FLOTATION CONCENTRATE IN KAZAKHSTAN

Abstract. Seven arsenic resistant ferrous oxidizers were isolated from Aksu and Bestobe mine samples. The isolates are a rod-shaped, motile Gram-negative bacterium. Characterization of these isolates was done using standart microbiological, biochemical and phylogenetic comparative methods. The morphological, biochemical, physiological analysis based on 16S rRNA gene sequence showed that the strains 385A, 377B, 536B, 538J, 537C are most closely related to *Acidithiobacillus ferrivorans*, the strains 383A and 393B identified as *Acidithiobacillus ferrooxidans* and *Acidithiobacillus ferriphilus*, respectively. The results showed, in the flask experiments all the seven isolates at 8mM of arsenic were able to oxidize ferrous iron to ferric iron. Isolate 383A showed high resistance to As concentration of 64mM. This is very promising results since, the native isolated strains especially strain 383A can be used in bioleaching and bioremoval of As processes.

Аннотация. Из шахтной воды и руды рудных месторождений Аксу и Бестобе было выделено семь устойчивых к мышьяку железных окислителей. Изоляты представляют собой палочковидную, подвижную грамотрицательную бактерию. Характеристика этих изолятов проводилась с использованием стандартных микробиологических, биохимических и филогенетических сравнительных методов. Морфологический, биохимический, физиологический анализ, основанный на последовательности гена 16S рРНК, показал, что штаммы 385A, 377B, 536B, 538J, 537C принадлежат к виду *Acidithiobacillus ferrivorans*, штаммы 383A и 393B, идентифицированные как *Acidithiobacillus ferrooxidans* и *Acidithiobacillus ferriphilus*, соответственно. Результаты показали, что в экспериментах с колбами все семь изолятов при 8 мМ мышьяка были способны окислять двухвалентное железо в трехвалентное железо. Изолят 383A показал высокую устойчивость к концентрации As 64 мМ. Это очень многообещающие результаты, поскольку природно выделенные штаммы, в особенности штамм 383A, могут быть использованы в процессах биологического выщелачивания и биовосстановления As.

Keywords: Bioleaching, Acidithiobacillus ferrooxidans, Acidithiobacillus ferrivorans, biooxidation of ferrous iron.

Ключевые слова: биовыщелачивание, Acidithiobacillus ferrooxidans, Acidithiobacillus ferrivorans, биоокисление двухвалентного железа.

1. Introduction

Arsenic is a toxic metalloid which widely distributed in many environments and highly toxic to all life forms. It occurs primarily in inorganic form, as arsenate (AsV) and arsenite (AsIII)(1). It is found in the environment in a wide range of more than 200 mineral forms in soils in several forms of inorganic compounds, of which about 60% are arsenates, 20% sulfides and sulfosalts, the remaining 20% are arsenites, arsenides, silicates, oxides and elemental arsenic. Most of them are ore minerals or their alteration products. The greatest concentrations of these minerals can be found in mineralised areas which in close associated with the Cd, Pb, Ag, Au, Sb, P, W and Mo. The most abundant arsenic-containing ore minerals are arsenopyrite or mispickel (FeAsS), which contains 46% arsenic by mass, realgar (AsS), and orpiment (As2S3) (2) which are As(III) compounds that were formed under reducing, subsurface conditions. Arsenic has been presented in the Earth's crust in an average amount of about 2–5 mg/kg(3). The optimization of industrial heap bioleaching, mainly aimed recovery of copper and uranium, and stirred reactor processes for the recovery of precious metals such as gold and silver requires knowledge of the main types of microorganisms involved in bioleaching processes and associated with them. Furthermore, physiological and taxonomic characterization of microbial species involved in oxidative dissolution of sulfide mineral is also exceedingly important for comprehension disturbances in natural environments resulting from acid mine drainage(4,5). Since arsenic has been associated with gold ores, gold mining may contribute to arsenic pollution. Practically, gold mining activities are played as the key source of arsenic contamination in many regions(6).

Bioleaching is extraction of valuable metals from their ores using bacteria or their metabolites. The most familiar representatives of acidophilic bacteria used for bioleaching are mainly ferrous iron and/or sulfur oxidizing chemoautotrophs, such as *Acidithiobacillus, Leptospirillum, Acidimicrobium, Sulfobacillus and Sulfolobus* spp.(7), that derive energy by oxidization of ferrous iron and/or sulfur-containing minerals for

growth(8). The genus *Acidithiobacillus* is a Gram-negative, chemoautotrophic microorganism that derive energy from the oxidation of reduced sulfur compounds. The mesophilic bioleaching bacteria *A. ferrooxidans* is considered the most widely studied and the most important microorganisms in the bioleaching of sulfide ores(9,10), nevertheless other sulfur or ferrous iron oxidizing bacteria might have significant roles in these processes(11). Although it is found in many types of low pH natural environments in various geoclimatic zones, it is more commonly referred to in anthropogenic environments. The main reason of using *A. ferrooxidans* is its ability to utilize both ferrous iron (Fe^{2+}) and sulfur moieties in sulfide ores for growth. There are two mechanisms of solubilization metal ions from sulfide minerals: metal ions solubilized directly by *A. ferrooxidans* cells (the direct) and metal sulfides are chemically attacked by ferric iron produced by *A. ferrooxidans* cells (the indirect)(12,13). On the other hand, *A. ferrivorans* is the psychrotolerant bacteria that has the ability to oxidize ferrous iron with more efficiency than *A. ferrooxidans* at low temperature(14–16). These a polyphyletic group of Gram-negative, rod-shaped bacteria have been isolated from sulfur-containing mineral deposits(17)·(18). Although these microorganisms have many common physiological features, *A. ferrooxidans* and *A. ferrivorans* vary in growth in low pH and temperatures, which suggests that the two microorganisms would tend to dominate a diverse environment. In addition to the function of metal oxidation and the production of sulfuric acid in the process of bioleaching, this microorganism also makes an important contribution to the biogeochemical cycle of metals and sulfur in the environment(19). The oxidation of arsenopyrite (FeAsS), depending on the oxidation conditions, leads to a number of products with different final sulfur and arsenic oxidation rates. Biological oxidation of arsenopyrite also results in the dissolution of the arsenic in the form of As(III) and generate As(V) in the medium(20).

In the present study, we described the isolation and characterization of the mesophilic and acidophilic iron oxidizing microorganisms of novel strains of *A. ferrooxidans, A. ferriphilus* and *A. ferrivorans* which were isolated from mine tailings from gold ores in Kazakhstan and studied its ability for oxidizing ferrous iron at high concentration of arsenic.

2. Materials and methods

2.1 Sample

The liquid sample was collected from a gold deposits Bestobe (52°36'18" N. 73°13'48" E.) and Aksu (52° 25' 08" N. 72° 00' 00" E) in Northern Kazakhstan. The ore minerals in this deposits are mainly pyrite, galena, chalcopyrite, etc. which are known to contain a high As content. The arsenopyrite containing ores and concentrate was provided by mining and metallurgical complex Kazakhaltyn JSC.

2.2 Culture media

9K-Fe modified solid medium and liquid medium(21) were used for enrichment and isolation of the sulfur-oxidizing microorganisms at a temperature of 28°C. The medium used for the isolation of iron-oxidizing bacteria of the genus *Acidithiobacillus* consisted of the following basal salts (in grams per liter): KH_2PO_4 (3,0), $MgSO_4 \cdot 7H_2O$ (0,5), $(NH_4)_2SO_4$ (3,0), KCl (0,1), $Ca(NO_3)_2$ (0,01), adjusted to pH 2.0 at room temperature using 10 N H_2SO_4 and autoclaved at 121 °C for 20 min; the medium also supplemented with filter-sterilized $FeSO_4 \cdot 7H_2O$ (44,2) were used for flasks enrichment and liquid culture.

2.3 Isolation and phenotypic characteristics

The cultures were isolated and purified on agar solid 9K medium. The agar plates were incubated at 28 °C for 12-14 days until microbial growth. Then single milky colonies were carefully selected and streak cultures on solid medium were repeated at least five times until a pure culture was obtained. The purity of the selected cultures was confirmed by a phase-contrast microscope. Isolated samples in an amount of 5 g were introduced into flasks with 100 ml of 9K medium to obtain accumulative cultures of mesophilic iron-oxidizing microorganisms. Growth experiments was carried out on a shaker-incubator at a temperature of 28 ° C at 200 rpm. Samples were removed for pH measurements and iron analysis. The presence and growth of microorganisms was recorded by indirect signs — by the transition of ferrous iron (Fe^{2+}) to its oxidized ferric iron form (Fe^{3+}) and a decrease in the pH of the solution. Morphological properties of the isolates were investigated by under a phase-contrast microscopy (Standard 25 Carl Zeiss, Germany). The physiological and biochemical characterizations, tinctorial properties of the isolated pure cultures (the shape and size of cells, size, color, edge of the colonies, pH, temperature, growth characteristics on solid and liquid media) were studied in accordance with described in Bergey's Manual of Determinative Bacteria.

2.4 Screening and selection of microorganism strains and arsenic resistance

Arsenic resistance of the isolated bacteria was conducted using modified 9K-Fe where different concentration of As (III) (8-64 mM) was added. Cultivation was carried out in 750 mL Erlenmeyer flasks containing 180 mL of 9K at 28 ° C on rotary shaker at 200 rpm. Control experiments were also carried out with the arsenic-free media. Growth of the microorganisms were measured in terms of ferrous oxidation. Growth of the isolates was measured in terms of ferrous iron oxidation.

2.5 Chemical analysis

The pH and redox potential were measured using saturated calomel electrode (Mettler Toledo Seven Multi S47-K) while redox potential (Eh) was measured in terms of mV by combined platinum ring indicator and S7 screw head. The concentration of Fe^{3+} and Fe^{2+} ions in the liquid phase was determined spectrophotometrically using a Biomate 3 spectrophotometer (Thermo Ficher Scientific). The elemental composition of the ore and concentrate was determined by atomic emission spectrometry on an iCAP 7200 ICP-OES Analyzer spectrometer from ThermoScientific.

2.6 Phylogenetic analysis of 16S rRNA genes

Genomic DNA of all the four iron oxidizers was isolated using pQIAamp DNA Mini Kit according to the manufacturer's protocol. The concentration of extracted DNA was measured on a NanoDrop 1000 (Thermo Scientific, USA). The 16S rRNA gene of the isolated strains were amplified by PCR using bacterial primers 8F (5' AGAGTTTGATCCTGGCTCAG 3') and 806 R (5' GGACTACCAGGGTATCTAAT 3') DNA Engine Tetrad 2 Cycler (Bio-Rad). The composition of the reaction mixture: 10 × dNTPs (2 mmol / l each) 2.5 μl, 10 × PCR buffer 2.5 μl, target DNA (0.04 μg / μl) 5 μl, Taq DNA polymerase 1U, 10 pmol primers 0, 5 μl. The total volume of the mixture was adjusted to 25 μl with nuclease-free water. Thermo Cycling procedure was as follow: pre-denaturation at 95 ° C for 5 min, followed denaturation at 95 ° C for 30 sec, annealing at 55 ° C for 40 sec and elongation at 72 ° C for 50 sec. Total PCR consisted of 30 cycles. The last elongation was carried out at 72 ° C for 7 min. The PCR products were analyzed by 2% agarose gel electrophoresis at 120V in1×TAE (0.04 M tris acetate and 0.001 M EDTA and 57.1 mL of glacial acetic acid, pH 8.0) buffer and the bands were visualized by ethidium bromide stained gel. The resulting PCR product was purified then screened and sequenced using BigDye terminator v3.1 sequencing kit (Applied Biosystems) on a 3730 DNA analyzer (Applied Biosystems, USA) according to the manufacturers' protocols. Sequences were analyzed and aligned using the Vector NTI Advance 10 software. Gene fragments were separated using an ABI 3730xl automatic sequencer (Applied Biosystems, USA).

The obtained 16S rRNA nucleotide sequences of the strains were compared with sequences of other microorganisms through the GenBank NCBI database and the phylogenetic trees were constructed by MEGA X software.

3. Results and discussion

3.1 Isolation of the strains

The arsenopyrite ores and wastes of a gold deposits Bestobe and Aksu showed extreme environmental conditions in terms of pH and temperature. Four mine water samples and 13 ore samples were taken from ore deposits to isolate acidophilic thionic bacteria (Table 1). Isolates of samples of natural materials (ore, mine water) that were not processed to the technological process were used to isolate microorganisms and the pH of the samples was in the range of 4.0-6.5. The temperature of the samples was from 8 to 21 ° C. The presence of high content of sulfur and iron in the samples (Bestobe 1.09-1.48%, Aksu 0.42-0.93%) conducive to the development of chemolithotrophic microflora. The high arsenic content (Bestobe 0.16-0.32%; Aksu 0.10-0.23%) conducive the presence of microflora resistant to arsenic (Table 2-3).

Table 1

Physico-chemical characters of water samples and ore samples from ore deposits

Gold deposits	(No.) samples	No. of samples	pH	Temperatures °C
Aksu	(1)Mine water	383	4.5	13.5
	(2)Mine drains No. 39 (1)	759	4.5	13.5
	(3)Mine drains No. 39 (2)	385	6.5	14.0
	(4)Ores: Mine rock dumps No.39	373	5.0	16.0
	(5)Mine	537	4.4	10.0
	(6)Mine	539	4.0	13.5
	(7)quarry	777	4.6	18.0
Bestobe	(1)Mine drains(1)	393	4,5	15,5
	(2)Mine drains(2)	439	6,0	20,0
	Ores:			
	(3)mine “Zapadnaya”	374	5,0	16,0
	(4)quarry	536	4,2	18,0
	(5)mine No.2	377	5,5	18,5
	(6)flotation concentrate	381	5,7	17,0
	(7)mine	437	5,5	21,0
	(8)mine tailings	538	4,5	20,0
	(9)heap	435	5,6	21,0
	(10)quarry No.38	44	6,8	8,0

In total, seven pure cultures were isolated from different samples of the 9K medium by sowing on silica gel plates saturated with 9K culture medium and numerous serial dilutions in the nutrient medium. Beige-colored colonies of accumulative crops 383, 385,537 (Aksu), 377, 393, 536, 538 (Bestobe), developed on the 9K–Fe-agarose plate after 5 days’ incubation at 28 °C. These microorganisms were considered as efficient iron-oxidizing bacteria named as 385A, 383A, 377B, 393B, 536B, 538J, 537C1.

Table 2.

Results of a general chemical analysis of Bestobe deposits

Elements	Contents, mg/L									
	Sample No.1	Sample No.2	Sample No.3	Sample No.4	Sample No.5	Sample No.6	Sample No.7	Sample No.8	Sample No.9	Sample No.10
Fe	25687	23941	27455	26462	27346	27930	28514	29098	29682	30266
As	2858	3045	3232	2419	1606	2793	2980	2167	2354	2541
Pb	94	150	210	116	174	187	199	212	224	237
Sb	15	12	9	16	23	14	8	17	96	11
Cu	324	304	71	27	115	75	84	197	213	151
S	12301	12487	14878	13451	14623	13230	12791	11352	10913	13474

Table 3.

Results of a general chemical analysis of Aksu deposits

Elements	Contents, mg/L						
	Sample No.1	Sample No.2	Sample No.3	Sample No.4	Sample No.5	Sample No.6	Sample No.7
Fe	14968	16862	16588	12896	17954	21320	18630
As	1713	1914	2271	1614	1652	1885	1991
Pb	109	162	219	132	197	201	207
Sb	61	58	55	62	69	60	54
Cu	219	196	40	77	18	31	28
S	6214	6601	9349	7265	7475	7316	6982

3.2 Phenotypic characteristics

The isolated strains were described by the same morphological and physiological characteristics (Table 4). Colonies of the isolates on solid 9K–Fe-agarose medium appeared flat, pasty, reddish-brown after 5 days' incubation (Fig.1. a). Microscopic observations of the isolated microorganisms showed that all the isolates are small, rod shaped, single or double cells and motile (Fig. 1. b).

Table 4.

Primary identification of isolates

Isolate	393B	383A	385A	377B	537C1	538J	536B
Cell shape and size (μm)	Rod 0,4-0,6 0,7-1,0	Rod 0,8-1,0 1,1-1,5	Rod 0,5-0,8 0,9-1,1	Rod 0,2-0,5 0,8-0,9	Rod 0,2-0,5 0,8-0,9	Rod 1,0-1,3 1,4-1,6	Rod 1,1-1,2 0,9-1,4
Gram stain	-	-	-	-	-	-	-
Growth with $S_2O_3^{2-}$	-	+	-	+	-	+	-
Growth with Fe^{2+}	+	+	+	+	+	+	+
Optimum pH	1,7-3,0	1,8-4,0	1,9-2,9	1,7-2,8	1,6-3,2	1,9-2,8	1,9-2,8
Growth temperature, °C	28-30	28-30	8-18	28-30	8-18	12-28	8-18

3.3 Genotypic characteristics

The isolated strains were further identified by the partial nucleotide sequence of 16S rRNA. The determined partial nucleotides sequences of 16S rRNA were used to find the most similarity with the bacterial strains in the GenBank database. The results of molecular genetic analysis using sequencing analysis of 16S rRNA genes confirm the data obtained by studying the phenotypic characteristics of isolates and according to the nucleotide sequences of 16S rRNA strains 383A, 393B, 377B and 538J were most closely related to *A. ferrooxidans* ATCC 23270 (NR_074193) with 99.36%, *A. ferriphilus* strain M20 (NR_147744) with 100%, *A. ferrivorans* NO-37 (NR_114620) with 99% and *A. ferrivorans NO-37 (NR_114620)* with 100% similarity, respectively. The strains 385A, 536B and 537C1 closest relatives were *A. ferrivorans* strain NO-37 (NR_114620) with 99.31%, *A. ferrivorans strain NO-37* (NR_114620) with 99.83% and A. *ferrivorans* strain NO-37 (NR_114620) with 98.57% sequence similarity, respectively. Phylogenetic relationships based on 16S rRNA gene sequences are shown in Fig.2.

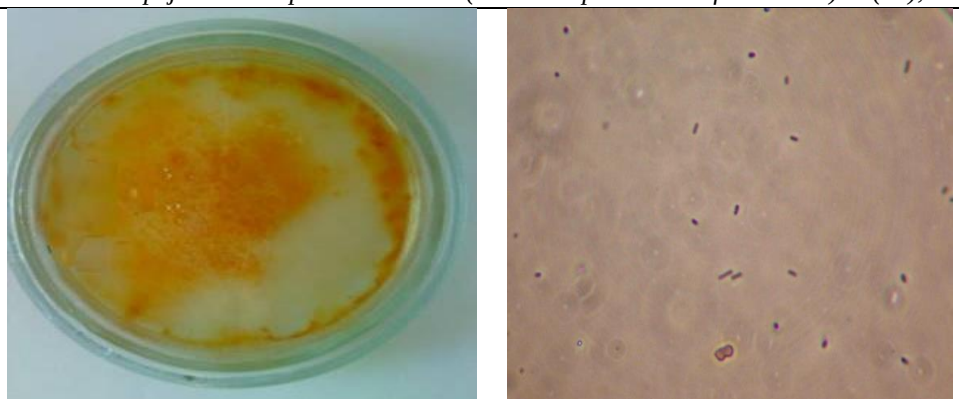

Figure. 1. Colony morphology of the strains. a: colonies on 9K–Fe solid medium; b: phase-contrast microscopy

Table 5.

The effect of different arsenic concentration on the oxidizing ability of the isolated strains

Isolates	Control	Concentration of As^{3+},mM				
		8	16	32	48	64
Acidithiobacillus ferrivorans 385A	+++	+++	++	+	+	---
Acidithiobacillus ferrooxidans 383A	+++	+++	+++	++	++	+
Acidithiobacillus ferrivorans 377B	+++	+++	---	---	---	---
Acidithiobacillus ferriphilus 393B	+++	+++	---	----	---	---
Acidithiobacillus ferrivorans 536B	+++	+++	+++	++	---	---
Acidithiobacillus ferrivorans 538J	+++	+++	+	++	+	---
Acidithiobacillus ferrivorans 537C	+++	+++	+++	++	---	

(+++) - abundant growth; (++) - average growth; (+) - growth is very weak; (----) - no growth.

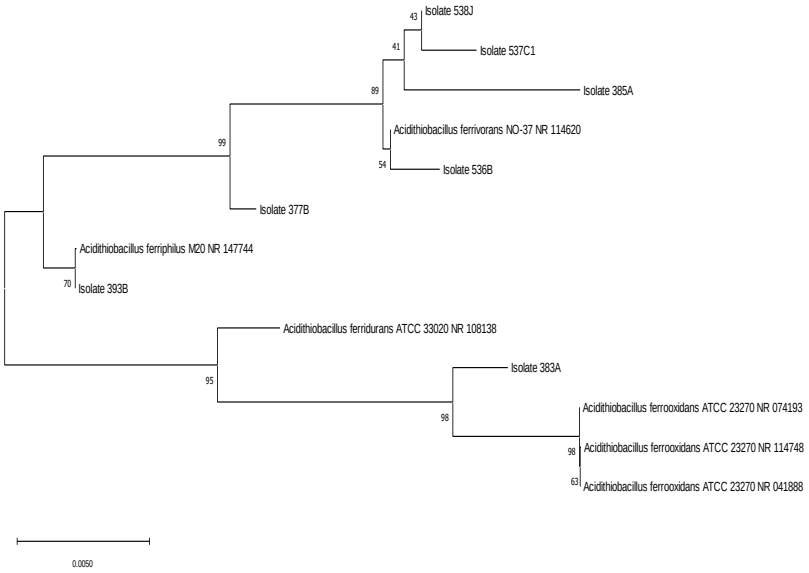

Fig. 2. Neighbour-joining phylogenetic tree of isolated strains based on 16S rRNA.

3.4 Arsenic resistance experiments

Iron oxidation ability of the selected seven isolates in the presence of different concentration and absence of arsenite [As(III)] is shown in Table 5. According to the data, it can be seen that at all the isolates were able to oxidize ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) in the presence of 8 mM of arsenic. At arsenic concentration above 16 mM, the strains of 377B and 393B growth was completely inhibited as no oxidation of iron has happened. Meanwhile, in the medium with other strains, no adverse effects on oxidation were observed. The ferrous iron oxidation at a concentration of 48mM of As, the isolated strains named as *A. ferrivorans* 385A, *A. ferrooxidans* 383A and *A. ferrivorans* 538J showed a slow oxidation rate. Isolate 383A was found to be the most efficient oxidizing iron at high concentration of As (64mM). Although isolate 385A and 537C were isolated from almost same ecosystems (Aksu deposit), these isolates showed in terms of their resistance to 64 mM of arsenite. These results suggest a physiological diversity among these isolates.

Conclusions

Due to some limitations and ecological harm of physical and chemical methods in removal processes for the treatment arsenic-contaminated water resources; the application of acidophilic ferrous iron-oxidizing bacterium will be the best choice. In the bioremoval experiments, native microorganisms that isolated from their region might be considered as the best object due to their reconcilability with the environment and resistance to the toxic minerals. In this work we have isolated and characterized an native acidophilic iron oxidizing microorganisms from Bestobe and Aksu deposits in Kazakhstan. According to the cell morphology, physiological and phylogenetic analyses, the isolates have identified as a new strains of *A. ferrooxidans, A. ferriphilus* and *A. ferrivorans*. All the seven isolates were able to oxidize iron at concentration of arsenic (8mM). Whereas, the isolated strain *A. ferrooxidans* 383A was able to oxidized ferrous iron at 64mM of As.

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