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EXPERIMENTAL ARTICLES

Strain Polymorphism of the Plasmid Profiles in *Acidithiobacillus ferrooxidans*

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Abstract—Plasmid profiles were studied in 27 *Acidithiobacillus ferrooxidans* strains isolated from different geographic zones and substrates differing in composition of the main sulfide minerals, and also in experimentally obtained strains with acquired enhanced resistance to the ions of heavy metals (Fe, Ni, Cu, Zn, As). In 16 out of 20 strains isolated from different substrates, one to four 2- to 20-kb and larger plasmids were revealed. Plasmids were found in all five strains isolated from gold-containing pyrite–arsenopyrite ores and concentrates, in nine of 11 strains isolated from the ores and concentrates containing nonferrous metals, and in two of four strains isolated from the oxidation substrates of simple composition (mine waters, pyritized coals, active sludge). Changes in the plasmid profiles in some *A. ferrooxidans* strains (TFZ, TFI-Fe, TFV-1-Cu) with experimentally enhanced resistance to Zn^{2+}, Fe^{3+}, and Cu^{2+}, respectively, were noted as compared with the initial strains. After 30 passages on a S^0-containing medium, strain TFBk showed changes in the copy number of plasmids. The role of plasmids in the processes of oxidation of energy substrates and in the acquired enhanced resistance to heavy metal ions is discussed.

Key words: Acidithiobacillus ferrooxidans strains, plasmid profiles, mineralogical composition of the oxidation substrates, resistance to heavy metal ions.

The acidophilic chemolithoautotrophic gram-negative motile bacterium *Acidithiobacillus ferrooxidans* derives energy required for growth from the oxidation of inorganic substrates, such as ferrous iron, elemental sulfur, its reduced compounds, and sulfide minerals. *A. ferrooxidans* is a typical representative of the mesophilic consortium of microorganisms developing under specific extreme conditions at low pH values, from 2 and lower, and high concentrations of heavy metals, up to 70 g/l. The habitats of *A. ferrooxidans* differ in their geographical location, composition of sulfide minerals, content of heavy metals in the liquid phase, and pH and temperature values. Researchers from different countries on all continents isolated a great number of *A. ferrooxidans* strains. The strains described in the literature differ in genome size, DNA G+C content, and level of DNA–DNA homology of total genomes [1]. Each *A. ferrooxidans* strain possesses a unique chromosomal DNA structure analyzed by the method of pulsed-field gel electrophoresis of native DNA digested by the same restriction endonuclease [2]. One or more plasmids ranging in size from 2 to 75 kb were present in 75% of *A. ferrooxidans* strains [3–12]. Two of them, pTF-FC2 of 12.2 kb [6, 9] and pTFO of 20 kb [7], had a broad host range. The latter plasmid is carried by many *A. ferrooxidans* strains. For example, seven out of 12 strains isolated from different habitats contained plasmid pTFO [7]. Plasmids belonging to the same family pTFI91 were revealed in some *A. ferrooxidans* strains isolated on different continents [7, 10, 13, 14]; one such plasmid was isolated independently by researchers in three different laboratories. The restriction maps of these plasmids were identical [10]

The aim of the present work was to analyze the plasmid profiles in *A. ferrooxidans* strains isolated from natural sources and processing pulps, as well as from strains with experimentally increased resistance to heavy metal ions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. In this work, we used 27 *A. ferrooxidans* strains isolated from different habitats and strains experimentally adapted to high concentrations of metal ions. The strains, the places of their isolation, and the composition of the main sulfide minerals in the substrates are presented in the table.

The strains were grown in 250-ml Erlenmeyer flasks containing 100 ml of Silverman–Lundgren medium with 9 g/l of Fe^{2+} or 10 g/l of S^0 [15] on a rotary shaker (150 rpm) at 28 ± 2°C. The inoculum was introduced at a rate of 10% by volume. When growing strains B-458-Cu, TFV-1-Cu, TFBk-Cu, TFZ, TFI-Fe, TFAs2, and TFNi, 15 g/l Cu^{2+}, 17.5 g/l Cu^{2+}, 20 g/l Cu^{2+}, 50 g/l Zn^{2+}, 50 g/l Fe^{2+}, 3 g/l As^{3+}, and 30 g/l Ni^{2+}, respectively, were additionally introduced into the medium. Bacterial cells for isolation of plasmid DNA were grown in 5-l bottles containing 3 l of medium under

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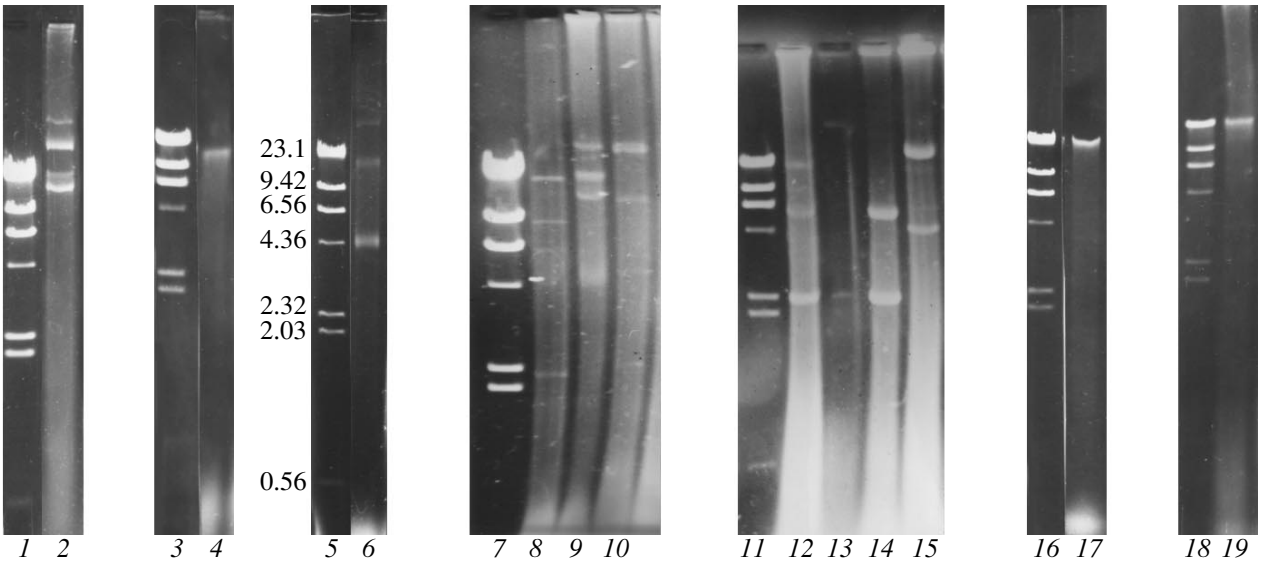

Fig. 1. Plasmid profiles of *A. ferrooxidans* strains grown on medium with ferrous oxide: *2*, TFBk; *4*, TFO; *6*, TFN-d; *8*, TFD; *9*, TFN; *10*, TFT; *12*, TFI; *13*, TFZ; *14*, TFI-Fe; *15*, TFV-1; *17*, TFL-2; *19*, TFUch. *1, 3, 5, 7, 11, 16, 18*: phage lambda DNA cleaved by restriction endonuclease *Hin*dIII. At the side of lane 5, the sizes of the DNA fragments in kb are given.

forced aeration (3 l/min) and harvested in the late exponential growth phase. The biomass was washed according to the standard procedure [2].

Isolation of plasmid DNA. A thick cell suspension (25 to 50 μl) was introduced into 200 μl of solution 1 (0.9% glucose, 0.75% EDTA in 0.025 M Tris–HCl, pH 8.0) containing 2 mg/ml of lysozyme, mixed thoroughly, and kept for 15 min at 4°C. The resulting suspension was added to 400 μl of solution 2 (1% SDS in 0.8% NaOH solution), mixed thoroughly, and incubated for 5 min at room temperature until the solution became clear. Sodium acetate (300 μl 3 M), pH 4.8 to 5.0, was then added to the solution and mixed using a Mikrowstrzasarka typ ML-1 microshaker until a caseous sediment was formed. The mixture was allowed to stand in a refrigerator at –20°C for 30 min and then centrifuged for 10 min at 5000 *g* using an ELMI centrifuge. The supernatant was transferred to another test tube. Isopropanol (0.8 of volume) was added, the contents were mixed thoroughly and maintained for 20 min at –20°C. The test tube was centrifuged for 3 min at 9000 *g*. The supernatant was decanted, the sediment was dried and dissolved in 200 μl of TE buffer, pH 8.0 (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). NaCl (5 M, 200 μl) was added to the solution, mixed, maintained for 30 min at 4°C, and then centrifuged 3 min at 9000 *g*. The supernatant was transferred to a new test tube, mixed with 0.8 volume of isopropanol and maintained in the refrigerator for 15 min at –20°C. The DNA was sedimented by centrifugation for 3 min at 9000 *g*, dried, and then dissolved in 200 μl of TE buffer. The resultant solution was mixed with 200 μl of 9 M lithium chloride solution, maintained in the refrigerator for 15 min at –20°C and centrifuged for 3 min at 9000 *g*. The supernatant containing DNA was transferred to another tube, 0.8 volume of isopropanol was added, the contents were mixed and maintained in the refrigerator for 15 min at –20°C. The mixture was centrifuged for 3 min at 9000 *g*. The DNA-containing sediment was dried and then dissolved in 50 μl of TE buffer.

The method used allowed the DNA preparations to be obtained in a supercoiled form.

Analysis of plasmid profiles. The plasmid profiles of the strains of *A. ferrooxidans* were analyzed using the standard technique of electrophoresis in 1% agarose gel [16] in TAE-buffer (40 mM Tris, 2 mM EDTA, 20 mM sodium acetate, pH 8.0) at a constant voltage of 90 V. The fragments of phage lambda DNA digested with the restriction endonuclease *Hin*dIII served as molecular weight standards.

RESULTS

Ecological diversity of the strains. The strains of *A. ferrooxidans* used in the study were isolated from different ecological niches and various types of substrates differing in composition of the main sulfide minerals (table). Most strains (14) were isolated from ores and concentrates in Russia; the other 6 strains were isolated in the Ukraine, Kazakhstan, Peru, India, and Yugoslavia. Seven strains with enhanced resistance to metal ions (B-458-Cu, TFAs2, TFNi-3, TFV-1-Cu, TFBk-Cu, TFZ, and TFI-Fe) were experimentally obtained from the strains isolated from natural sources. The isolation substrates are divided in the table into five groups: 1, gold-containing pyrite–arsenopyrite ores and concentrates; 2, ores and concentrates of a complex composition containing nonferrous metals in the miner-

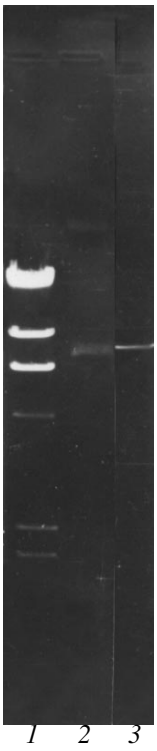

Fig. 2. Plasmid profiles of *A. ferrooxidans* strain TFY: *1*, phage lambda DNA cleaved by restriction endonuclease *Hin*dIII; *2*, TFY plasmid DNA; *3*, TFY plasmid DNA cleaved by restriction endonuclease *Eco*RI.

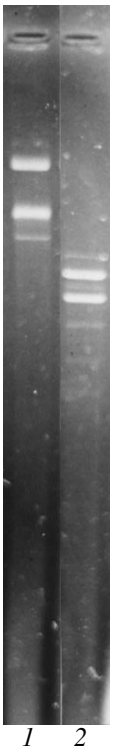

Fig. 3. Plasmid profiles of *A. ferrooxidans* strain TFBk grown on medium with elemental sulfur: *1*, TFBk plasmid DNA; *2*, TFBk plasmid DNA cleaved by restriction endonuclease *Hin*dIII.

als ZnS, CuS, $CuFeS_2$, etc; 3, mine waters of the copper–zinc deposit; 4, mine waters of pyritized coals; 5, active sludge of municipal sewage containing heavy metals.

The plasmid profiles of the strains isolated from natural substrates. All *A. ferrooxidans* strains isolated from gold-containing pyrite–arsenopyrite ores and concentrates (TFBk, TFO, TFN-d, TFD, and TFT) contained plasmid DNAs (Fig. 1, lanes *2*, *4*, *6*, *8*, *10*). One plasmid was present in strain TFO (Fig. 1, lane *4*); two plasmids, in strains TFBk and TFN-d (Fig. 1, lanes *2, 6*); four plasmids, in strains TFD and TFT (Fig. 1, lanes *8, 10*).

No plasmids were revealed in two (TFS and TFUd3) out of 11 *A. ferrooxidans* strains isolated from the ores and concentrates containing nonferrous metals. All the remaining nine strains carried plasmid DNA (Figs. 1, 2). For example, strains TFL-2 and TFY harbored one plasmid each (Fig. 1, lane *17*; Fig. 2, lane *3*); strain TFV-1, two plasmids (Fig. 1, lane *15*); strains TFN and TFI, no less than three plasmids (Fig. 1, lanes *9, 12*). The electrophoregram of the plasmid DNA of strains TFP, TFG, and TFUd2 shows two minor DNA bands; strain TFKm has four bands.

One plasmid was found in strain TFUch isolated from the mine waters of the Uchalinskoe copper–zinc ore deposit (Fig. 1, lane *19*). Of two *A. ferrooxidans* strains, VKM B-458 and TFR1, isolated from pyritized coals, a low-copied plasmid was revealed in the latter strain. Strain TFWc isolated from the active sludge of municipal sewage carried no plasmids at all.

In some samples of the plasmid DNA isolated from *A. ferrooxidans* strains TFBk, TFN-d, TFY, and TFZ, the chromosomal DNA traces were present that formed the band located above the 23.1 kb fragment of the phage lambda DNA (Figs. 1–3). This was proven by digestion of the plasmid DNA from strains TFBk and TFY with restriction endonucleases *Hin*dIII and *Eco*RI, respectively (Figs. 2, 3). In the *Hin*dIII restriction pattern of the plasmid DNA from strain TFBk the band with the lowest electrophoretic mobility disappeared from the electrophoregram (Fig. 3). The plasmid DNA was cleaved by this enzyme into seven fragments. Digestion of the plasmid DNA from strain TFY with restriction endonuclease *Eco*RI also resulted in the disappearance of the corresponding band (Fig. 2). The plasmid DNA of strain TFY had no restriction sites of this endonuclease. When the plasmid DNAs from strains TFN and TFT were digested with restriction endonuclease *Eco*RI, the DNA bands located on the lanes above the band of 23.1 kb fragments of phage lambda DNA (Fig. 1, lanes *9, 10*) did not disappear. This gives evidence of the fact that they did not contain chromosomal DNA and that the largest plasmids of

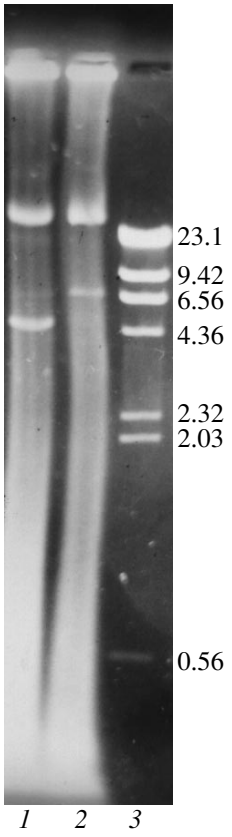

Fig. 4. Plasmid profiles of *A. ferrooxidans* strains: *1*, TFV-1; *2*, TFV-1-Cu; *3*, phage lambda DNA cleaved by restriction endonuclease *Hin*dIII. At the side of lane 3, the sizes of the DNA fragments in kb are given.

strains TFN and TFT were not digested with restriction endonuclease *Eco*RI.

The plasmid profiles of experimentally obtained strains with enhanced resistance to heavy metal ions. No plasmids were revealed in the following related strains: the parent strain VKM B-458, strain B-458-Cu resistant to 15 g/l Cu^{2+}, strain TFNi-3 resistant to 40 g/l Ni^{2+} and strain TFAs2 resistant to 4 g/l As^{3+}. Strain TFBk and strain TFBk-Cu adapted to 20 g/l Cu^{2+} harbored two plasmids of the same size each.

Changes in the plasmid profiles were observed in three pairs of related *A. ferrooxidans* strains (the 1st pair, TFI and TFI-Fe resistant to 50 g/l Fe^{3+}; the 2nd pair, TFY and TFZ resistant to 70 g/l Zn^{2+}; the 3rd pair, TFV-1 and TFV-1-Cu resistant to 17.5 g/l Cu^{2+}). The largest of the three plasmids disappeared in strain TFI-Fe (Fig. 1, lanes *12, 14*). In strain TFZ, a smaller plasmid (Fig. 1, lane *13*) appeared in the process of adaptation to a high zinc concentration, as compared with the plasmid size of the parent strain TFY (Fig. 2, lane *3*). In strain TFZ, the DNA band located closer to the start in the electrophoregram represents a set of chromosomal DNA fragments, as it was shown for strain TFY. Strains TFV-1 and TFV-1-Cu carried two plasmids each; the larger plasmids are similar, and smaller plasmids differ in size (Fig. 4).

Plasmid sizes and the copy number. The plasmids revealed in different *A. ferrooxidans* strains were of different size. The sizes of two plasmids were determined based on the results of restriction analysis in strain TFBk: the large plasmid was 30 kb in size, and the small plasmid was 13.5 kb (unpublished data). The relative sizes of other plasmids was determined by comparing their electrophoretic mobility with the mobility of the above two plasmids. The restriction fragments of phage λ DNA digested with endonuclease *Hin*dIII were used in parallel as the measuring standard. Phage lambda DNA sized 48.6 kb is cleaved by this enzyme into fragments of 23.1, 9.42, 6.56, 4.36, 2.32, 2.02, and 0.56 kb. It was shown that *A. ferrooxidans* strains carried small plasmids whose mobility was comparable with that of the small fragments of phage lambda DNA 2.32 kb and less in size (TFD, TFT, TFI, TFZ, TFKm, TFG, and TFR1) and also the plasmids whose mobility was lower than that in the 23.1 kb phage lambda DNA fragment (TFN, TFT, and TFV-1). Plasmids as big as 20 kb were present in many strains. These are strains TFI, TFL-2, TFUch, TFN, and TFUd2. As was noted earlier, the plasmid of the same size (20 kb) was revealed in many *A. ferrooxidans* strains [7].

The difference in the copy number of plasmids in the strains of *A. ferrooxidans* merits attention. The relative copy number was assessed by the luminescence intensity of the corresponding bands in the electrophoregrams. The plasmids were present in a small number of copies in strains TFZ, TFKm, TFG, TFUd2, and TFR1. In strains TFBk, TFO, TFI-Fe, TFL-1, and TFUch, the copy number of plasmid DNA was higher.

In *A. ferrooxidans* TFBk grown on medium with ferrous iron, a smaller plasmid was present in a larger number of copies than a larger one (Fig. 1, lane *2*). The adaptation to a new oxidation substrate (30 passages on medium with elemental sulfur) led to changes in the copy number of plasmids: a larger plasmid was present in a larger number of copies (Fig. 3, lane *1*). The influence of adaptation of *A. ferrooxidans* strains to new oxidation substrates on the plasmid profiles will be the subject of our further investigations.

DISCUSSION

Since the 1980s, many researchers studied the plasmid composition of *A. ferrooxidans* strains of different origin and the role of plasmids in their oxidation activity. This was due to the fact that *A. ferrooxidans* is dominant in mesophilic microbial communities involved in the bacterial–chemical processes of gold extraction or leaching nonferrous metals from sulfide ores and concentrates under extreme environmental conditions (at low pH values or high concentrations of heavy metal ions).

Isolation sites of *A. ferrooxidans* strains and composition of the main sulfide minerals in the substrate

Substrate type	Strains	Site isolation	Main sulfide minerals
1. Gold-containing pyrite–arsenopyrite ores and concentrates	TFBk	Kazakhstan, Bakyrchikskoe deposit	FeS_2, FeAsS, CuS, $CuFeS_2$
	TFBk-Cu	The strain derived from TFBk, resistant to 20 g/l Cu^{2+}	
	TFO	Russia, Olimpiadinskoe deposit	FeS_2, FeAsS, Sb_2S_3, FeS, ZnS, PbS, $CuFeS_2$
	TFN-d	Russia, Nezhdaninskoe deposit	FeS_2, FeAsS, ZnS, $CuFeS_2$, PbS
	TFD	Russia, Darasunskoe deposit	FeS_2, FeAsS, $CuFeS_2$, ZnS, PbS
	TFT	Russia, the concentrate pulp	FeS_2
2. Ores and concentrates containing nonferrous metals	TFI	India, zinc tailings	FeS_2, ZnS, FeS
	TFI-Fe	Mutant derived from strain TFI, resistant to 50 g/l Fe^{3+}	
	TFS	Russia, Ural, Saf'yanovsk gray ores	FeS_2, ZnS, $CuFeS_2$
	TFY	Yugoslavia, copper deposit	FeS, FeS_2, CuS, Cu_2S, Cu_5FeS_4, $CuFeS_2$
	TFZ	The strain derived from TFY, resistant to 70 g/l Zn^{2+}	
	TFL-2	Russia, Ural, copper–zinc industrial product	ZnS, FeS_2, FeAsS, $CuFeS_2$, FeS, gray ore
	TFV-1	Russia, Ural, Volkovskoe deposit	$CuFeS_2$, Cu_2S, Cu_5FeS_4, copper oxides
	TFV-1-Cu	The strain derived from TFV-1, resistant to 17.5 g/l Cu^{2+}	
	TFKm	Russia, Kamchatka, Shanuchskoe deposit	FeS, $(Ni, Fe)_9S_8$, $CuFeS_2$
	TFN	Kazakhstan, Nikolaevskoe deposit	FeS_2, $CuFeS_2$, ZnS, PbS, Cu_2S, CuS, oxides
	TFP	Peru, copper deposit	$CuFeS_2$, Cu_5FeS_4, Cu_2S, CuS, FeS_2
	TFG	Russia, Ural, Gaiskoe deposit	FeS_2, $CuFeS_2$, Cu_2S, Cu_5FeS_4, ZnS, oxides
	TFUd 2	Russia, Siberia, Udokansk ore	$CuFeS_2$
	TFUd 3	"	"
3. Mine waters	TFUch	Russia, Ural, Uchalinskoe deposit	ZnS
4. Pyritized coals	BKM B-458	Russia, mine runoff of coal field	FeS_2
	TFAs2	The strain derived from B-458, resistant to 4 g/l As^{3+}	
	TFNi-3	The strain derived from B-458, resistant to 40 g/l Ni^{2+}	
	B-458-Cu	The strain derived from B-458, resistant to 15 g/l Cu^{2+}	
	TFR1	Ukraine, Donbass, mine runoff	FeS_2
5. Sewage active sludge	TFWc	Russia, Moscow water canal	Fe^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}

Martin *et al.* [3] found plasmids ranging from 7.4 to 75 kb in 11 out of 15 strains analyzed. Valenti *et al.* [7] isolated plasmids in 7 of 12 strains isolated from different geographic areas. Shiratori *et al.* [8] isolated more than 100 *A. ferrooxidans* strains from six ore deposits of Japan, 73% of which contained one and more plasmids ranging from 2 to 30 kb. Chisholm *et al.* [17] found plasmids in 13 of 15 *A. ferrooxidans* isolates. The authors noted variability in the plasmid size, but most of them were larger than 4.3 kb. No relationship between the habitat of *A. ferrooxidans* strains and the presence of plasmids in them was revealed [7, 18].

As is seen from our results, of 27 *A. ferrooxidans* strains studied, only four strains isolated from natural sources (TFS, TFUd3, B-458, TFWc) did not harbor plasmids. Plasmids were also absent from three strains (TFAs2, B-458-Cu, TFNi) derived experimentally from strain B-458 containing no plasmids.

Earlier, we showed the role of the oxidation substrate in the change of nucleotide sequences in the chromosomal DNA of *A. ferrooxidans* strains [19]. Is there any correlation between the substrate from which the strains of *A. ferrooxidans* were isolated and the presence of plasmids in them? Plasmids were revealed in all five *A. ferrooxidans* strains isolated from gold-containing pyrite–arsenopyrite ores and concentrates and in nine of 11 natural *A. ferrooxidans* strains containing nonferrous metals. Of four *A. ferrooxidans* strains isolated from the oxidation substrates whose composition was relatively simple (TFUch, B-458, TFR1, TFWc), only two strains (TFUch and TFR1) were found to have one plasmid each. These results suggest a relation between the mineralogical composition of the oxidation substrate and the presence of plasmids in *A. ferrooxidans* strains. The simpler the substrates in composition, the greater the share of plasmid-free strains isolated from these substrates. Thus, plasmids were present in 50% of *A. ferrooxidans* strains isolated from mine waters, pyritized coals, and the active sludge of municipal sewage, whereas 82 to 100% of the strains isolated from the ores and concentrates were found to harbor plasmids. It is known from the literature that the presence or absence of plasmids does not affect the ability of *A. ferrooxidans* strains to oxidize Fe^{2+}, S^0, and sulfide minerals or their resistance to the ions of heavy metals or antibiotics [9]. The strains of *A. ferrooxidans* are considered to be cryptic. Their phenotype is not known. However, the results obtained in our work suggest a possible role of plasmids in the regulation of processes of substrate oxidation.

We showed earlier that five of the plasmid-containing *A. ferrooxidans* strains studied in this work (TFBk, TFN-d, TFO, TFL-2, and TFV-1) differ in growth rate, the activity of oxidation of Fe^{2+}, S^0, FeS_2, and sulfide minerals in the concentrates of pyrite–arsenopyrite ores, in the rate and effectiveness of adaptation to new oxidation substrates, and attain the adaptation threshold characteristic of every strain [20]. In three of them (TFBk, TFN-d, and TFO) we revealed changes in the chromosomal DNA structure analyzed by pulsed-field gel electrophoresis when their metabolism was switched to the oxidation of another substrate [19]. One of the possible mechanisms of changes in the chromosomal DNA nucleotide sequences can be plasmid–chromosomal recombinations.

As mentioned above, the plasmids of *A. ferrooxidans* do not contain determinants of resistance to metal ions. However, as was shown in this work, the adaptation of three strains (TFZ, TFI-Fe, and TFV-1-Cu) to 70 g/l Zn^{2+}, 50 g/l Fe^{3+}, or 17.5 g/l Cu^{2+}, respectively, induced changes in their plasmid profiles. Strain TFZ contains a plasmid of a smaller size than that of the parent strain TFY, and earlier we revealed amplification of one of the chromosomal DNA fragments in strain TFZ adapted to 70 g/l of Zn^{2+} [2]. In strain TFV-1-Cu, the size of a smaller plasmid has changed as compared with the plasmid size of the parent strain TFV-1. In the work of other researchers, the adaptation of *A. ferrooxidans* strain MAL4-1 to 20 and 30 g/l Cu^{2+} resulted in the disappearance of all plasmids, but they reappeared when the initial growth conditions were restored [21]. The pair of the related strains TFI and TFI-Fe is of special interest. Inheritable changes in the chromosomal DNA structure appeared in strain TFI-Fe in the process of adaptation to high Fe^{3+} concentrations in the medium. A new fragment was noted in the *Xba*I restriction pattern of its chromosomal DNA, as compared with the restriction pattern of the parent strain [19]. The loss of one of the three largest plasmids present in the parent strain TFI was also revealed in strain (TFI-Fe). It is possible that the increased resistance of the mutant strain to 50 g/l Fe^{2+}/Fe^{3+} is connected with chromosomal–plasmid recombinations.

Of obvious importance is further analysis of the plasmid profiles and the chromosomal DNA restriction profiles in *A. ferrooxidans* strains adapted to new oxidation substrates and increased concentrations of heavy metal ions, as well as the study of plasmid–chromosomal interactions as another means of gaining an insight into the role of plasmids in the activity of *A. ferrooxidans.*

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