Microbiology, Vol. 71, No. 4, 2002, pp. 438–443. Translated from Mikrobiologiya, Vol. 71, No. 4, 2002, pp. 514–521.

EXPERIMENTAL ARTICLES

Restriction Profiles of the Chromosomal DNA from *Acidithiobacillus ferrooxidans* Strains Adapted to Different Oxidation Substrates

T. F. Kondrat'eva, S. N. Ageeva, T. A. Pivovarova, and G. I. Karavaiko

Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia

Received July 9, 2001

Abstract—Restriction profiles of chromosomal DNA were studied in different *Acidithiobacillus ferrooxidans* strains grown on medium with Fe^{2+} and further adapted to another oxidation substrate (S^0, FeS_2, or sulfide ore concentrates). The restriction endonuclease *Xba*I digested the chromosomal DNA from different strains into different numbers of fragments of various sizes. Adaptation of two strains (TFBk and TFN-d) to new oxidation substrates resulted in structural changes in *Xba*I-restriction patterns of their chromosomal DNA. Such changes in the DNA restriction patterns occurred in strain TFBk after the adaptation to precyanidated gravitational pyrite-arsenopyrite concentrate (no. 1) from the Nezhdaninskoe deposit or to copper-containing ore from the Udokanskoe deposit and also in strain TFN-d adapted to untreated pyrite-arsenopyrite concentrate (no. 2) from the Nezhdaninskoe deposit. No changes in the number or size of the *Xba*I-restriction patterns of chromosomal DNA were revealed in either strain TFBk cultivated on media with pyrite from the Angren and Tulun deposits or in strains TFN-d and TFO grown on media with S^0 and pyrite. Neither were changes observed in the *Xba*I-restriction patterns of the DNA from strain TFV-1, isolated from the copper ore of the Volkovskoe deposit, when Fe^{2+} was substituted with alternative substrates—S^0, pyrite or concentrate no. 2 from the ore of the Nezhdaninskoe deposit. In strain TFO, no differences in the *Xba*I-restriction patterns of the chromosomal DNA were revealed between the culture grown on medium containing concentrate no. 2 or the concentrate of surface-lying ore from the Olimpiadinskoe deposit and the culture grown on medium with Fe^{2+}. When strain TFO was cultivated on the ore concentrate from deeper horizons of the Olimpiadinskoe deposit, which are characterized by lower oxidation degrees and high antimony content, mutant TFO-2 differing from the parent strain in the chromosomal DNA structure was isolated. The correlation between the lability of the chromosomal DNA structure in *A. ferrooxidans* strains and the physical and chemical peculiarities of the isolation substrate and habitat is discussed.

Key words: *Acidithiobacillus ferrooxidans* strains, oxidation substrates, pulsed-field gel electrophoresis, chromosomal DNA, restriction endonucleases, restriction patterns.

The acidophilic chemolithoautotrophic gram-negative bacterium *Acidithiobacillus ferrooxidans* derives energy required for growth from the oxidation of Fe^{2+}, S^0, reduced sulfur compounds, and sulfide minerals at low pH values in the medium and uses carbon dioxide as the source of cellular carbon. The main natural habitats of this bacterium are specific ecological niches, such as sulfide ores and mine waters. A great number of *A. ferrooxidans* strains were isolated in different geographic areas from acid environments differing in element composition. Strains of *A. ferrooxidans* most actively oxidizing sulfide minerals were isolated from the pulps produced during biohydrometallurgical processing of ore concentrates from different deposits. It is known that sulfide ores vary in quantitative and qualitative composition of minerals, and ore solutions differ in the heavy metal content. *A. ferrooxidans* strains developing in various ecological niches and adapted to specific oxidation substrates and environmental conditions are characterized by different growth rates and activities of ferrous iron oxidation. They also vary in their genetically determined ability to adapt to other oxidation substrates and in growth rate on the new substrates [1]. *A. ferrooxidans* strains described in the literature differ in the genome size, DNA G+C content, and the number and sizes of plasmids [2, 3]. Pulsed-field gel electrophoretic analysis of the DNA restriction patterns allowed us to reveal restriction fragment length polymorphism of the chromosomal DNA in various strains of this bacterium [4]. Each *A. ferrooxidans* strain possesses a unique endonuclease restriction pattern of the chromosomal DNA, and this provides a means for its identification among other strains and its monitoring in nature, in biotechnological processes, and under changed cultivation conditions. In some *A. ferrooxidans* strains, the substitution of the oxidation substrate caused nonheritable changes in the chromosomal DNA structure [5, 6]. Prolonged continuous cultivation of an *A. ferrooxidans* strain in medium with increasing concentrations of Fe^{3+} allowed us to obtain a mutant with a

0026-2617/02/7104-0438$27.00 © 2002 MAIK "Nauka / Interperiodica"

changed chromosomal DNA structure and resistant to 50 g Fe^{3+} per liter [7]. We suggested that the changes in nucleotide sequences in the chromosomal DNA may be one of the mechanisms responsible for strain polymorphism in *A. ferrooxidans*, while the energy substrate appears to be the main factor selecting for the most competitive genotype in a heterogeneous population. It may be assumed that *A. ferrooxidans* strains occurring in natural environments containing ores of a complex composition or growing in the presence of technologically processed concentrates or industrial products have developed more efficient systems regulating the metabolism switching to the oxidation of a new substrate than the strains isolated from more simple substrates and not adapted to their frequent changes in the process of evolution. The correlation between the adaptational capacities of *A. ferrooxidans* strains and their prehistory has been experimentally proved [1]. It is known that the changes in the location of nucleotide sequences occurring close to the structural operon promoters, e.g., due to the integration of IS-elements, transposons or plasmids, can influence the expression of chromosomal genes [8]. Therefore, the strains with a more efficient adaptational capacity to oxidize new substrates may possess a more labile genome than the strains with a lower adaptation threshold.

In this work, we studied the restriction profiles of chromosomal DNA in various *A. ferrooxidans* strains upon switching them to new oxidation substrates.

Sizes (kb) of *Xba*I-generated restriction fragments of the chromosomal DNA from *A. ferrooxidans* strains grown on medium with Fe^{2+}

Band no.	Strains			
	TFN-d	TFBk	TFO	TFV-1
1	205 ± 2	328 ± 6	218 ± 1	352 ± 8
2	177 ± 1	269 ± 4	185 ± 1	304 ± 10
3	160 ± 1	214 ± 4	155 ± 1	262 ± 10
4	145 ± 1	199 ± 3	143 ± 1	202 ± 6
5	120 ± 4	186 ± 2	128 ± 0.5	193 ± 1
6	110 ± 2	169 ± 3	118 ± 1	182 ± 1
7	105 ± 1	147 ± 2	100 ± 1	170 ± 4
8	95 ± 1	129 ± 2	94 ± 0.5	140 ± 3
9	90 ± 0.5	88 ± 1	74	129 ± 2
10	86 ± 0.5	77 ± 1	68	108 ± 1
11	77 ± 0.5	65 ± 1	54	87
12	72 ± 0.5	60 ± 1	47	76
13	53 ± 1	54 ± 0.5	42	71
14	48 ± 0.5	46 ± 0.5	–	61
15	40 ± 1	36 ± 0.5	–	56
16	38 ± 0.5	Less than 30	–	51
17	–	–	–	48

* Arithmetic mean error was calculated according to Peters using the Moldengauer factor or constant *k*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. In this work, we used the following four strains of *Acidithiobacillus ferrooxidans* from the culture collection of the Laboratory of chemolithotrophic microorganisms at the Institute of Microbiology, Russian Academy of Sciences: TFN-d, TFBk, TFO, and TFV-1. Strains TFN-d, TFBk, and TFO were isolated from dense pulps formed in the reactors during experimental biohydrometallurgical processing of gold concentrates obtained through the enrichment of pyrite–arsenopyrite ores from the Nezhdaninskoe, Bakyrchikskoe, and Olimpiadinskoe deposits, respectively. Strain TFV-1 was isolated from the solution formed during dump leaching of the waste ore from the Volkovskoe deposit. Characteristics of the employed substrates and growth conditions of *A. ferrooxidans* strains in technological processes were described earlier [1].

Batch cultivation of strains was performed at 28 ± 2°C on a shaker (150 rpm) in 250-ml Erlenmeyer flasks containing 100 ml of Silverman–Lundgren medium [9], in 500-ml flasks containing 200 ml of medium, or in 5-l bottles containing 3 l of medium under forced aeration (3 l/min). One of the following substrates was used as the energy source: ferrous iron; elemental sulfur; pyrites from the Akchatau and Tulun deposits; two types of gravitational pyrite–arsenopyrite concentrate from the Nezhdaninskoe deposit: no. 1, cyanidated and additionally ground to a particle size of 0.044 mm (95% grade) and no. 2, untreated concentrate with a particle size of 0.074 mm (95% grade); two types of pyrrhotite-containing pyrite–arsenopyrite ore concentrate from the Olimpiadinskoe deposit: surface-lying ore and ore from deeper horizons; copper-containing ore from the Udokanskoe deposit. Exponential-phase cultures were used as the inoculum (10 vol%). All strains were preadapted to a new oxidation substrate. Adaptation involved successive passages on a mineral base of Silverman–Lundgren medium containing a new substrate instead of Fe^{2+} at a pulp solid : liquid phase ratio of 1 : 50. The structure of the chromosomal DNA from *A. ferrooxidans* strains was studied after no less than 10 passages.

Preparation of intact chromosomal DNA. Harvesting and washing of the biomass and preparation of intact genomic DNA were described earlier [4].

Analysis of the chromosomal DNA restriction profiles. Chromosomal DNA structure of *A. ferrooxidans* strains grown on media with different oxidation substrates was analyzed by pulsed-field gel electrophoresis (PFGE) [4]. Intact genomic DNA was digested with three restriction endonucleases: *Xba*I (40 U/30 μl), restriction site T↓CTAGA; *Bcu*I (20 U/30 μl), restriction site A↓CTAGT; *Smi*I (40 U/30 μl), restriction site ATTT↓AAAT. Chromosomal DNA fragments were

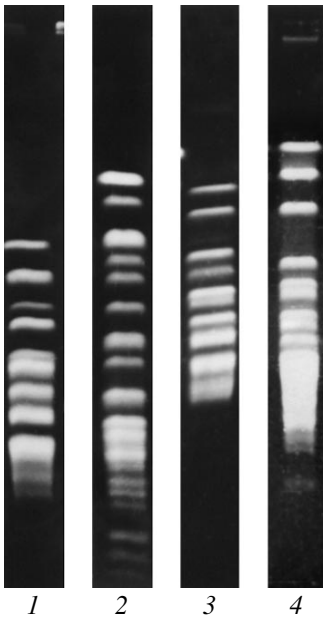

Fig. 1. *Xba*I-restriction patterns of chromosomal DNA from *A. ferrooxidans* strains grown on medium with Fe^{2+}. Pulsed-field gel electrophoresis was run at 120 V/cm and a 25-s pulse (40-s for strain TFV-1) for 44 h at 10–13°C: (*1*) TFN-d; (*2*) TFBk; (*3*) TFO; (*4*) TFV-1.

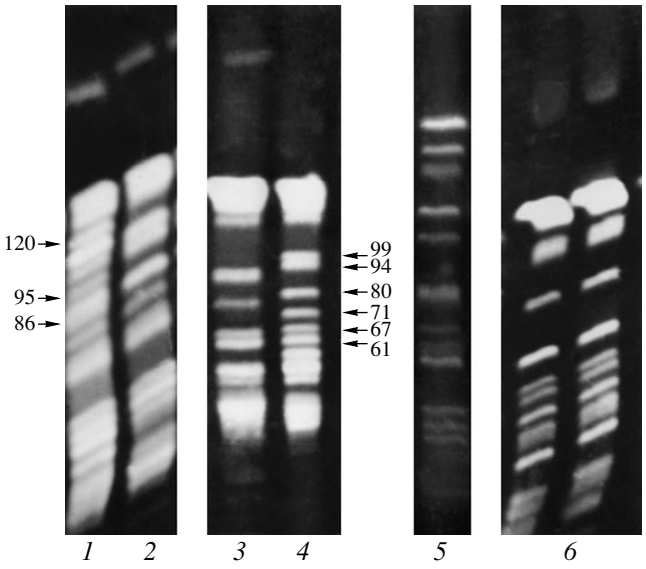

Fig. 2. *Xba*I-restriction patterns of chromosomal DNA from *A. ferrooxidans* strains. Pulsed-field gel electrophoresis was run at 130 V/cm and a 10-s pulse for 68 h at 14–16°C. (*1*, *2*) TFN-d; (*3*) TFBk; (*4*) VKM B-458; (*5*) TFO; (*6*) TFV-1. Energy sources: (*1*, *3–6*) Fe^{2+}; (*2*) untreated concentrate no. 2 of the ore from the Nezhdaninskoe deposit. Figures at the side of the panels are fragment sizes (kb).

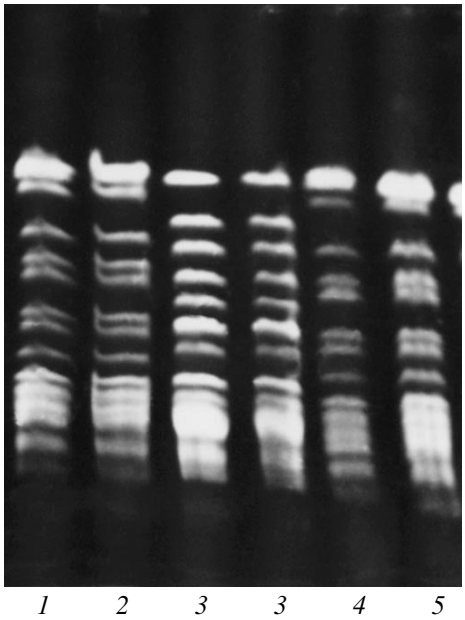

Fig. 3. *Xba*I-restriction patterns of chromosomal DNA from *A. ferrooxidans*, strain TFV-1, grown in media with (*1*) Fe^{2+}, (*2*) S_0, (*4*) FeS_2, (*5*) concentrate and (*3*) from strain VKM B-458 grown in medium with Fe_{2+}. Pulsed-field gel electrophoresis was run at 120 V/cm and a 25-s pulse for 44 h at 15–17°C.

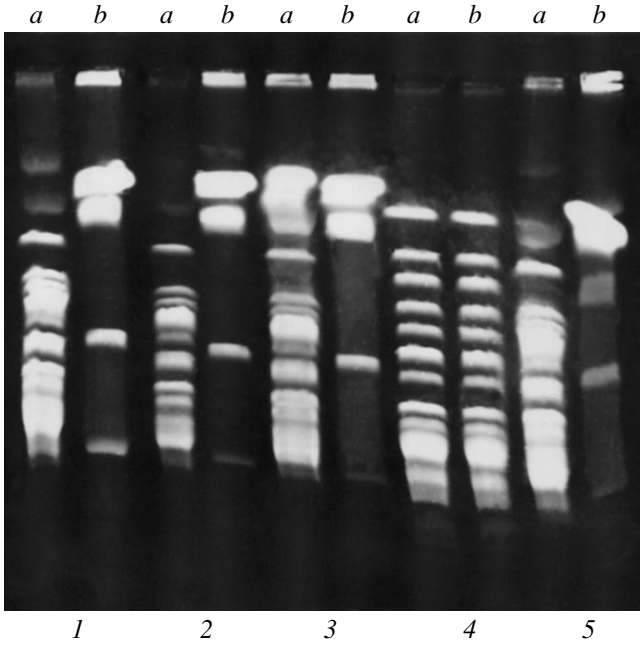

Fig. 4. Restriction patterns generated from chromosomal DNA of *A. ferrooxidans* TFV-1 with endonucleases (a) *Bcu*I and (b) *Smi*I and (*4*) *Xba*I-restriction pattern of strain VKM B-458 grown in medium with Fe^{2+}. Strain TFV-1 was grown in media with (*1*) Fe^{2+}, (*2*) S_0, (*3*) FeS_2, and (*5*) concentrate. The pulsed-field gel electrophoresis conditions were as in Fig. 3.

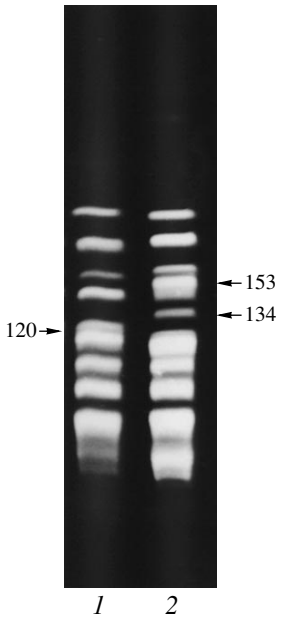

Fig. 5. *Xba*I-restriction patterns of chromosomal DNA from *A. ferrooxidans* TFN-d grown in media with (*1*) Fe^{2+} and with (*2*) gravitational ore concentrate no. 2 from the Nezhdaninskoe deposit. The pulsed-field gel electrophoresis conditions were as in Fig. 1.

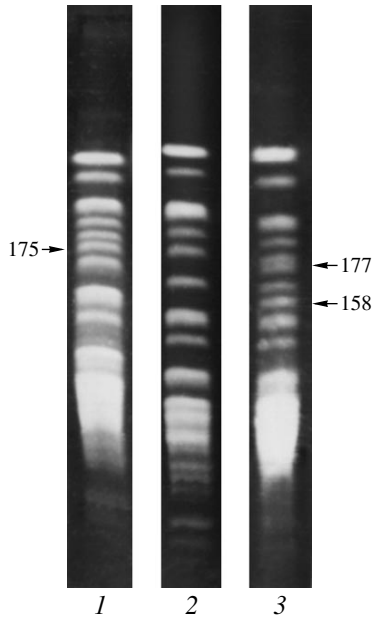

Fig. 6. *Xba*I-restriction patterns of chromosomal DNA from *A. ferrooxidans* TFBk grown in media (*1*) with ore from the Udokanskoe deposit, (*2*) with Fe^{2+}, and (*3*) with cyanidated gravitational concentrate no. 1 from the Nezhdaninskoe deposit. The pulsed-field gel electrophoresis conditions were as in Fig. 1.

separated in a "Diapul's-1" chamber designed for PFGE. Separation was performed in 0.5 × TBE buffer (1 × TBE is 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.0). The voltage, pulse time, and duration of electrophoresis were varied depending on the size of the fragments separated. The DNA fragments of the known size from *A. ferrooxidans* VKM B-458 served as molecular mass markers [10].

RESULTS AND DISCUSSION

Previously we showed that *A. ferrooxidans* strains TFBk and VKM B-458, isolated from the substrates of different mineral composition, differ in their response to a change of the energy source in the growth medium [5, 6]. In strain TFBk, isolated from pyrite–arsenopyrite concentrate characterized by a complex mineral composition, we revealed nonheritable changes in the chromosomal DNA structure caused by switching strain metabolism from ferrous iron oxidation to the oxidation of elemental sulfur, pyrite from the Akchatau deposit, or sulfide concentrate from the Olimpiadinskoe deposit. No changes in the chromosomal DNA structure were observed in strain VKM B-458 isolated from the mine waters of the Moscow region coal field (containing FeS_2). We suggested that the changes in nucleotide location in the chromosomal DNA of strain TFBk could influence the activity of oxidation of a new substrate.

Four *A. ferrooxidans* strains grown on medium with ferrous iron differed in the number and sizes of *Xba*I-generated chromosomal DNA fragments (table, Figs. 1, 2). The following results were obtained after the adaptation of these strains to other oxidation substrates.

No structural changes in the chromosomal DNA digested with restriction endonuclease *Xba*I were observed in either strain TFV-1, (isolated from a poor copper-bearing ore from the Volkovskoe deposit containing only $CuFeS_2$, Cu_2S, and Cu_5FeS_4) or in strain VKM B-458 [5, 6] during the adaptation of these strains to the oxidation of elemental sulfur, pyrites from various deposits, and pyrite–arsenopyrite concentrates (Fig. 3). Stability of the chromosomal DNA structure in strain TFV-1 was confirmed by using two other restriction endonucleases: *Bcu*I and *Smi*I. The restriction patterns of chromosomal DNA from strain TFV-1 grown in medium with ferrous iron did not differ from the *Bcu*I- and *Smi*I-restriction patterns recorded after growth of this strain on other oxidation substrates (Fig. 4).

The adaptation of strain TFN-d to growth on gravitational pyrite–arsenopyrite concentrate no. 2 caused structural changes in the chromosomal DNA (Fig. 2, lanes *1, 2*; Fig. 5, lanes *1, 2*): the electrophoregram of the *Xba*I-digest showed two new bands of 153 and 134 kb that were absent in the restriction digests of the DNA from parent cells grown in medium with ferrous iron, whereas the fragments sized 120, 95, and 86 kb disappeared from strain TFN-d. However, no structural changes in the chromosomal DNA were revealed in strain TFN-d after its adaptation to pyrite from the

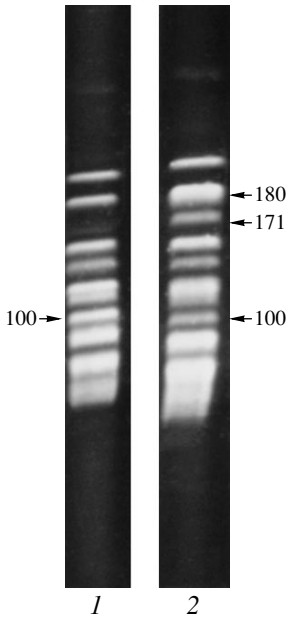

Fig. 7. *Xba*I-restriction patterns of chromosomal DNA from *A. ferrooxidans* strains (*1*) TFO and (*2*) TFO-2. The pulsed-field gel electrophoresis conditions were as in Fig. 1.

Akchatau and Tulun deposits or to elemental sulfur as the single sources of energy in the medium.

In strain TFBk, the change of the oxidation substrate from ferrous iron to gravitational pyrite–arsenopyrite concentrate no. 2 did not cause any changes in the chromosomal DNA structure. After prolonged cultivation of strain TFBk on the medium with concentrate no. 1, the electrophoregram of the *Xba*I-restriction pattern showed a new band formed by 177-kb fragments that was absent from the restriction pattern of the initial strain (Fig. 6, lanes *2*, *3*). In addition, a distinct band consisting of 158-kb fragments appeared in the restriction pattern of strain TFBk adapted to this new oxidation substrate, whereas, as shown earlier [5], it was either missing or showed occasionally in the chromosomal DNA of the initial strain. The changes in the nucleotide sequences in the genomic DNA, causing the changes in location of restriction sites and, respectively, in the number and sizes of the *Xba*I-generated DNA fragments, were also revealed after prolonged cultivation of strain TFBk on the medium with the copper-containing ore from the Udokanskoe deposit as the only source of energy. In the *Xba*I-restriction pattern of the chromosomal DNA, we observed a new band formed by 175-kb fragments that was absent from the restriction pattern of the initial strain TFBk (Fig. 6, lanes *1*, *2*). No structural changes in the chromosomal DNA were induced by switching strain TFBk from the oxidation of ferrous iron to the oxidation of pyrite from the Angren [5] and Tulun deposits.

In strain TFO, the adaptation to growth on pyrite from the Tulun deposit or untreated gravitational pyrite–arsenopyrite concentrate no. 2 as the only sources of energy in the medium instead of ferrous iron caused no changes in the chromosomal DNA structure.

Structural changes in the chromosomal DNA seem to essentially correlate with physical and chemical characteristics of the new oxidation substrates. In strain TFBk, we analyzed the variations in the genomic DNA structure upon switching the strain from the oxidation of ferrous iron to the oxidation of eleven other energy sources. No changes in the restriction profiles of chromosomal DNA were revealed in cultures switched to the oxidation of pyrite from the Angren and Tulun deposits, a concentrate containing only arsenopyrite, or pyrite–arsenopyrite ore concentrates from the Bakyrchikskoe, Nezhdaninskoe, and Sayakskoe deposits [5, 6, present paper]. Changes were found in strain TFBk adapted to elemental sulfur, mineralogically pure pyrite from the Akchatau deposit, ore concentrates from the Olimpiadinskoe and Udokanskoe deposits, or precyanidated ore concentrate from the Nezhdaninskoe deposit. It should be noted that a new band containing similar 177-kb fragments was revealed only in the *Xba*I-restriction patterns of chromosomal DNA from cultures adapted to growth on pyrite from the Akchatau deposit and on ore concentrate no. 1 (but not on concentrate no. 2) from the Nezhdaninskoe deposit. Conceivably, essential changes could have been caused in the composition of concentrate no. 1 by cyanidation of gold. As indicated above, the cyanidated concentrate was additionally ground, which lead to more complete and fast oxidation of FeAsS, FeS_2, and S_0 contained in it.

The influence of the mineral composition of pyrite–arsenopyrite concentrate on the structure lability of genomic DNA was demonstrated by the example of the ore concentrate from the Olimpiadinskoe deposit. Strain TFO-2 isolated from the sulfide ore concentrate obtained from deep horizons had essential structural distinctions in the chromosomal DNA as compared with strain TFO isolated from mixed surface-lying ores. The electrophoregram of *Xba*I-digested DNA from strain TFO-2 shows two additional bands consisting of 180- and 171-kb fragments (Fig. 7). In addition, the fluorescence intensity of the 100-kb fragment decreased. Strain TFO-2 appears to be a new strain and not the result of nonheritable modifications caused by the influence of new oxidation substrates, as in the case of strains TFBk and TFN-d: the peculiarities of the chromosomal DNA structure were retained after multiple passages of strain TFO-2 on medium with ferrous iron. This is a second case of adaptive evolution, when we observed the appearance of a new strain upon the change of growth conditions. Previously, prolonged continuous cultivation of *A. ferrooxidans* strain in medium with increasing concentrations of Fe^{3+} allowed us to obtain a new strain TFI-Fe with a changed chromosomal DNA structure and resistant to 50 g Fe^{3+} per liter [7].

Thus, in this work, we have obtained additional data on the role of the oxidation substrate in the divergence

of *A. ferrooxidans* strains in various ecological niches and on the changes in location of nucleotide sequences in the chromosomal DNA as one of the microevolution mechanisms.

Of great interest is the fact that the genome of one and the same strain showed different responses to the pyrite from different deposits (Angren, Akchatau, or Tulun). Conceivably, there exist more intricate relations between the microorganism and the substrate and a more significant role of physicochemical, crystallochemical, and electrophysical properties of the substrate in the initiation of genetic modifications. These issues will be the subject of our further investigations.

ACKNOWLEDGMENTS

We are grateful to L.N. Muntyan for providing *A. ferrooxidans* strains from the culture collection.

This work was supported by the State Scientific and Technical Program "Novel Bioengineering Methods," the Russian Foundation for Basic Research (RFBR) "Leading Scientific Schools," project no. 00-15-97765, and the RFBR Initiative Research Project, project no. 01-04-48270.

REFERENCES

1. Ageeva, S.N., Kondrat'eva, T.F., and Karavaiko, G.I., Phenotypic Characteristics of *Thiobacillus ferrooxidans* Strains, *Mikrobiologiya*, 2001, vol. 70, no. 2, pp. 226–234.
2. Harrison, A.P., Jr., Physiological Diversity amongst Strains of *Thiobacillus ferrooxidans*, *Arch. Microbiol.*, 1982, vol. 131, no. 1, pp. 68–76.
3. Rawlings, D.E. and Kusano, T., Molecular Genetics of *Thiobacillus ferrooxidans*, *Microbiol. Rev.*, 1994, vol. 58, no. 1, pp. 39–55.
4. Kondratyeva, T.F., Muntyan, L.N., and Karavaiko, G.I., Zinc- and Arsenic-Resistant Strains of *Thiobacillus ferrooxidans* Have Increased Copy Numbers of Chromosomal Resistance Genes, *Microbiology* (UK), 1995, vol. 141, no. 5, pp. 1157–1162.
5. Kondrat'eva, T.F., Pivovarova, T.A., Muntyan, L.N., and Karavaiko, G.I., Structural Changes in the Chromosomal DNA of *Thiobacillus ferrooxidans* Cultivated on Media with Various Oxidation Substrates, *Mikrobiologiya*, 1996, vol. 65, no. 1, pp. 67–73.
6. Kondrat'eva, T.F., Pivovarova, T.A., and Karavaiko, G.I., Peculiarities in the Structure of Chromosomal DNAs from *Thiobacillus ferrooxidans* Strains Adapted to Growth on Media with Pyrite or Elemental Sulfur, *Mikrobiologiya*, 1996, vol. 65, no. 5, pp. 675–681.
7. Kondratyeva, T.F., Pivovarova, T.A., Muntyan, L.N., and Karavaiko, G.I., Strain Diversity of *Thiobacillus ferrooxidans* and Its Significance in Biohydrometallurgy, *Biohydrometallurgy and Environment toward the Mining of the 21st Century*, Amils, R. and Ballester, A., Eds., Amsterdam: Elsevier, 1999, Part B, pp., 89–96.
8. Dybvig, K., DNA Rearrangements and Phenotypic Switching in Prokaryotes, *Mol. Microbiol.*, 1993, vol. 10, no. 3, pp. 465–471.
9. Silverman, M.P. and Lundgren, D.C., Study on the Chemoautotrophic Iron Bacterium *Ferrobacillus ferrooxidans*: I. An Improved Medium and Harvesting Procedure for Securing High Cell Yield, *J. Bacteriol.*, 1959, vol. 77, no. 5, pp. 642–647.
10. Kondrat'eva, T.F. and Karavaiko, G.I., Restriction Analysis of *Thiobacillus ferrooxidans* DNA with the Use of Pulsed-Field Electrophoresis, *Mol. Gen. Mikrobiol. Virusol.*, 1992, no. 3–4, pp. 9–12.