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EXPERIMENTAL ARTICLES

Plasmid Profiles of *Acidithiobacillus ferrooxidans* Strains Adapted to Different Oxidation Substrates

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Abstract—Plasmid profiles were studied in five *Acidithiobacillus ferrooxidans* strains of various origin cultivated on a medium with Fe^{2+}, as well as adapted to such oxidation substrates as S^0, FeS_2, and sulfide concentrate. The method used revealed plasmids in all *A. ferrooxidans* strains grown on a medium with Fe^{2+}. One plasmid was found in strain TFL-2; two plasmids, in strains TFO, TFBk, and TFV-1; and three plasmids were detected in strain TFN-d. The adaptation of strain TFN-d to sulfide concentrate and the adaptation of strain TFV-1 to S^0, FeS_2, or sulfide concentrate resulted in a change in the number of plasmids occurring in cells. In cells of strain TFN-d adapted to sulfide concentrate, the number of plasmids decreased from three to two. The number of plasmids in cells of strain TFV-1 adapted to different substrates varied from three to six depending on the energy source present in the medium: three plasmids were found after growth on FeS_2, four after growth on S^0, and six after growth on sulfide concentrate. The possible role of plasmids in the adaptation of *A. ferrooxidans* to new energy substrates and in the regulation of the intensity of their oxidation is discussed.

Key words: *Acidithiobacillus ferrooxidans* strains, plasmid profiles, oxidation substrates, adaptation, chromosomal DNA structure, plasmid–chromosome recombination.

Acidithiobacillus ferrooxidans is a gram-negative acidophilic chemolithoautotrophic mesophilic bacterium which gains energy at the expense of oxidation of inorganic substrates, such as ferrous iron, elemental sulfur, reduced sulfur compounds, and sulfide minerals. In natural ecosystems, its main habitats are deposits of sulfide ores. The species *A. ferrooxidans* is characterized by a wide range of interstrain genotypic variability with respect to the DNA G + C content, level of DNA–DNA homology of total genomes, and chromosomal DNA structure revealed by pulsed-field gel electrophoresis [1]. Cells of 75% of *A. ferrooxidans* strains were found to harbor from one to seven plasmids 2–70 kb in size [2]. Our study of 20 *A. ferrooxidans* strains isolated in various geographical regions from substrates differing in qualitative and quantitative composition of sulfide minerals showed that 16 strains contained one to four plasmids differing in size and copy numbers [3].

All *A. ferrooxidans* strains can oxidize the aforementioned substrates regardless of the presence of plasmids, suggesting chromosomal localization of the genes involved in these oxidation processes. Plasmids of *A. ferrooxidans* do not contain genes determining resistance to heavy metal ions or to antibiotics [2, 4]. The plasmid phenotype remains unknown, and, thus, these plasmids are to be termed cryptic [2]. However, they are maintained in cells of most strains in spite of the additional energy expenditures required. Thus, *A. ferrooxidans* cells harboring plasmids are to have selective advantages over cells devoid of plasmids. So, what is the function of these plasmids? This problem has puzzled researchers since the discovery of plasmids in *A. ferrooxidans* [5]. In an early work of Martin *et al.* [6], it was noted that a change of the oxidation substrate from ferrous iron to tetrathionate did not alter the plasmid profile of the *A. ferrooxidans* strain studied. Cells of the cultures grown on the two different substrates contained a single plasmid, pTF-350, sized 13×10^6 Da. Amils *et al.* [7] also revealed no dependence of the plasmid number on the oxidation substrate (ferrous iron, thiosulfate, elemental sulfur, hydrogen). Rawlings *et al.* [8], who studied protein synthesis in a cell-free *Escherichia coli* system in the presence of recombinant plasmids containing genetic material of *A. ferrooxidans*, hypothesized that the plasmids of the chemolithotrophic bacterium may contain certain regulatory signals recognized by the transcriptional and translational systems of the heterotrophic bacterium. Pramila *et al.* [9] reported a negative correlation between the presence of plasmids in *A. ferrooxidans* cells and their resistance to copper. Adaptation of *A. ferrooxidans* to elevated concentrations of copper ions in the medium (more than 10 g/l) led to the elimination of plasmids from the cells. Several passages on copper-free medium resulted in the appearance of plasmids. The authors suggested that the elevated concentration of copper ions decreased the rate of plasmid replication.

Earlier, we revealed by pulsed-field gel electrophoresis that the chromosomal DNAs of some *A. ferrooxidans* strains undergo changes during the adapta-

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tion to increased concentrations of metal ions or toxic elements (Zn^{2+}, Fe^{3+}, As^{3+}), as well as upon switch over of the metabolism from the oxidation of ferrous iron to other substrates (S^0, FeS_2, sulfide concentrates). These are either changes in the number and sizes of fragments yielded by *Xba*I digestion or changes in the nucleotide composition of equally sized fragments or amplification of certain fragments [10–14].

In some *A. ferrooxidans* strains exhibiting increased resistance to heavy metal ions (Fe^{3+}, Zn^{2+}, Cu^{2+}), acquired as a result of adaptation, changes in the plasmid profiles were revealed. Thus, cells of strain TFI-Fe, resistant to 50 g/l Fe^{3+}, lacked the largest of the three plasmids present in cells of the original strain TFI [3]. At the same time, strain TFI-Fe exhibited irreversible alternations in the structure of chromosomal DNA, whose *Xba*I restriction profile displayed a fragment absent from the profile of the original strain [10]. Strain TFV-1 and strain TFV-1-Cu, which is resistant to 17.5 g/l Cu^{2+}, harbor a common large plasmid. Each of these strains also harbors one more plasmid; the sizes of these plasmids in the two strains are different [3]. No distinctions were detected in the chromosomal DNAs in terms of the profile of fragments having a size greater than 50 kb. Strain TFZ, resistant to 70 g/l of Zn^{2+}, harbored a plasmid that was smaller than the plasmid of the original strain TFY [3]. The *Xba*I restriction profile of the chromosomal DNA of strain TFZ indicated amplification of fragments sized 98 kb [11]. These results suggested an assumption that the integration or excision of plasmids may be one of the reasons for the changes observed in the nucleotide sequences of chromosomal DNA. A hypothesis was put forward about the role of plasmid–chromosome recombination in the regulation of the mechanisms of heavy metal resistance and of the processes of oxidation of energy substrates.

The aim of this work was to investigate plasmid profiles of *A. ferrooxidans* strains adapted to different oxidation substrates.

MATERIALS AND METHODS

***A. ferrooxidans* strains and conditions of their cultivation.** This work used strains TFV-1, TFN-d, TFBk, TFO, and TFL-2, adapted to elemental sulfur, pyrite, or gravitational pyrite–arsenopyrite concentrate of the ore of the Nezhdaninskoe deposit. The sites of strain isolation, the composition of the main sulfide minerals in natural substrates, and the conditions of the growth of strains in the course of technological processes were described elsewhere [15]. Cultivation was performed in 250-ml Erlenmeyer flasks containing 100 ml of medium at 28 ± 2°C on a shaker (150 rpm). The inoculum was introduced in a dose of 10 vol %. Ferrous iron (Silverman and Lundgren medium with 9 g/l Fe^{2+} [16]), elemental sulfur (10 g/l), pyrite, or gravitational pyrite–arsenopyrite concentrate of the ore of the Nezhdaninskoe deposit (hereafter, concentrate) were used as the energy source; the solid phase–liquid phase ratio was 1 : 50. All strains were preadapted to each new substrate by at least 20 successive passages on a Silverman–Lundgren medium devoid of Fe^{2+} and supplemented with a corresponding substrate.

For DNA isolation, biomass was grown to the end of the exponential phase in 5-l bottles with 3 l of medium sparged with air (3 l/min). The biomass was washed according to a standard method described earlier [11].

Isolation of plasmid DNA was performed according to the method described in [3].

Analysis of plasmid profiles of *A. ferrooxidans* strains involved electrophoresis in 1% agarose gel [17] in TAE buffer (40 mM Tris, 2 mM EDTA, 20 mM sodium acetate, pH 8.0) at a constant voltage of 90 V.

Fragments of phage lambda DNA digested with *Hin*dIII restriction endonuclease served as molecular mass markers. Phage lambda DNA, sized 48.6 kb, was digested with *Hin*dIII into fragments with sizes of 21.1, 9.42, 6.56, 4.36, 2.32, 2.02, and 0.56 kb. Before electrophoresis, linear forms of DNA and circular forms with single-strand nicks were removed from the preparations of plasmid DNA via treatment with *Exo*III restriction exonuclease (25U/10 μl) and by heating for 2 min at 95°C [6].

To make the illustrations more convenient, some of the figures (Figs. 3, 5) were composed of lanes taken from different gels. *Hin*dIII-digested phage lambda DNA was always applied onto each gel as a molecular weight marker. Some of the figures were composed of fragments of gels in which the mobility of the standard was the same.

RESULTS AND DISCUSSION

Cells of all of the *A. ferrooxidans* strains studied were found to contain plasmids ([3] and the present work). One plasmid was found in strain TFL-2 (Fig. 1, lane *2*), two plasmids were revealed in strains TFO, TFBk, and TFV-1 (Figs. 2, 3, 5, lanes *2*, *3*, *2*, respectively), and three plasmids were detected in strain TFN-d (Fig. 4, lane *2*). Four strains of five contained in their cells plasmids of different sizes. Thus, cells of strains *A. ferrooxidans* TFL-2, TFO, TFN-d, and TFV-1 harbored plasmid with an electrophoretic mobility comparable to that of the 23.1-kb linear fragment of phage lambda DNA. In addition to this plasmid, cells of strains TFO, TFN-d, and TFV-1 harbored larger plasmids, and cells of strain TFN-d and TFV-1 also contained smaller plasmids. One of the plasmids harbored by strain TFN-d and TFV-1 cells exhibited higher electrophoretic mobility than that of the 23.1-kb linear fragment of phage lambda DNA; the other plasmid of strain TFBk displayed a lower mobility. The plasmids that we found in *A. ferrooxidans* stains differed in their copy numbers, as indicated by the lower luminescence intensity of the bands of larger plasmids in the plasmid profiles of strains TFO and TFN-d. Some samples of plasmid DNA contained its various conformations; therefore,

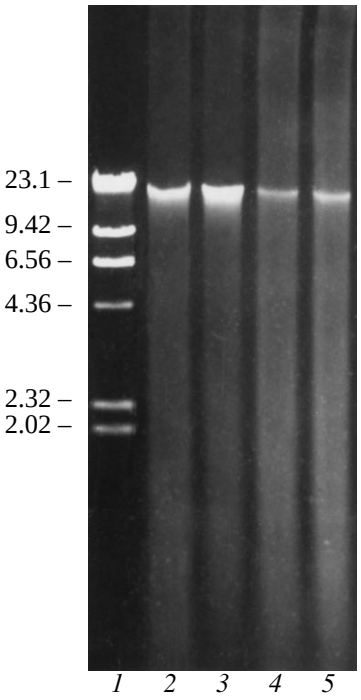

Fig. 1. Plasmid profiles of *A. ferrooxidans* TFL-2 cells adapted to different oxidation substrates—(*2*) Fe^{2+}, (*3*) S^0, (*4*) FeS_2, and (*5*) gravitational concentrate—studied by electrophoresis in parallel with (*1*) phage lambda DNA digested with restriction endonuclease *Hin*dIII (fragment sizes are given in kb).

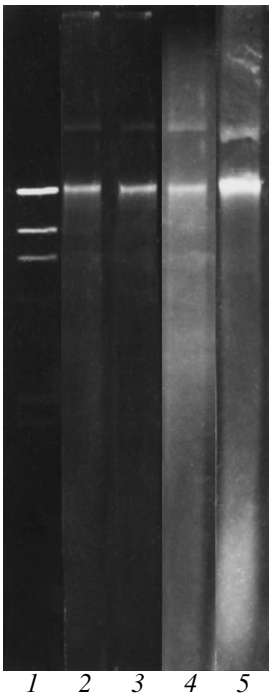

Fig. 2. Plasmid profiles of *A. ferrooxidans* TFO cells adapted to different oxidation substrates. Designations are as in Fig. 1.

the actual plasmid number was judged from the results of treatment of such preparations with restriction exonuclease *Exo*III or thermal treatment. For example, the electrophoregram of plasmid DNA of strain TFBk exhibited four bands differing in both luminescence intensity and mobility in agarose gel (Figure 3, lane *2*). After digestion of the DNA of this strain with restriction nuclease *Exo*III, two lower-intensity bands disappeared (Fig. 3, lane *3*).

Earlier, we showed that strains TFV-1, TFN-d, TFBk, TFO, and TFL-2 differ in the growth rate and the activity of oxidation of ferrous iron and other substrates (elemental sulfur, pyrite, arsenopyrite concentrate), as well as in the time needed for the adaptation to these substrates and the efficiency of adaptation [15]. In three of these strains (TFN-d, TFBk, and TFO), the adaptation to new substrates was accompanied by changes in the structure of chromosomal DNA, revealed by pulsed-field gel electrophoresis [10, 14]. A question arose as to the mechanism of the structural rearrangements in chromosomal DNA. Chromosome–plasmid recombination can be one of the possible mechanisms. Integration of plasmid DNA into chromosomal DNA or its excision may lead to an increase or a decrease in the size of certain fragments of chromosomal DNA and to the appearance or disappearance in chromosomal DNA restriction profiles of bands corresponding to DNA fragments comparable in size with the plasmids known for *A. ferrooxidans*. Thus, the *Xba*I restriction profile of strain TFBk adapted to elemental sulfur exhibited a new fragment 164 kb in size, which is 35 kb greater than the size of a 129-kb fragment revealed in the *Xba*I restriction profile of the original strain. On the other hand, it can be hypothesized that plasmid–chromosome recombination may influence readiness of the culture to switch to the oxidation of a new energy source; i.e., plasmids may be involved in the regulation of the structural genes of chromosomal DNA responsible for substrate oxidation. It can be assumed that strains possessing such a regulation mechanism may exhibit faster adaptation to new substrates and unfavorable environmental factors as compared to strains lacking such a mechanism. This possible role of plasmids in the adaptation of *A. ferrooxidans* to new substrates has not been studied earlier; therefore, it was of interest to investigate the plasmid composition in *A. ferrooxidans* strains adapted to different oxidation substrates and differing in the efficiency of adaptation to new substrates.

Our study of the effect of the adaptation of *A. ferrooxidans* strains to different oxidation substrates on their plasmid profiles yielded the following results. In three *A. ferrooxidans* strains (TFL-2, TFO, and TFBk), the adaptation to elemental sulfur, pyrite, or gravitational pyrite-arsenopyrite concentrate did not lead to changes in the plasmid profiles. Earlier, it was shown that the adaptation of strain TFO to elemental sulfur and of strains TFL-2 and TFBk to pyrite and the con-

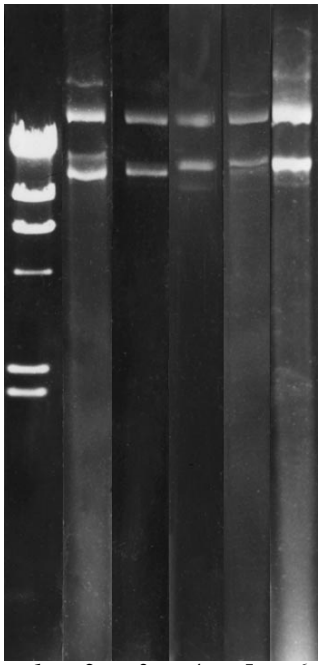

Fig. 3. Plasmid profiles of *A. ferrooxidans* TFBk cells adapted to different oxidation substrates (*2*) Fe^{2+}, (*4*) S^0, (*5*) FeS_2, and (*6*) gravitational concentrate studied by electrophoresis in parallel with (*1*) phage lambda DNA digested with restriction endonuclease *Hin*dIII. Lane *3* is *Exo*III-digested plasmid DNA of Fe^+ adapted cells.

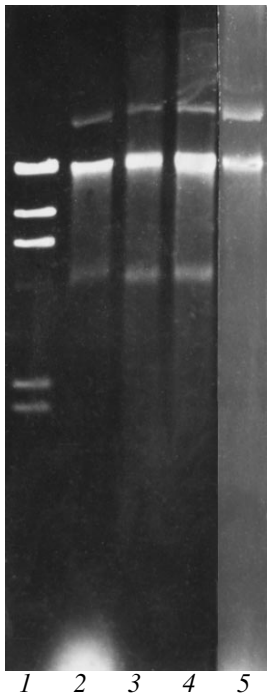

Fig. 4. Plasmid profiles of *A. ferrooxidans* TFN-d cells adapted to different oxidation substrates. Designations are as in Fig. 1.

centrate was not accompanied by changes in the *Xba*I restriction profiles of chromosomal DNA [14].

Structural changes in the chromosomal DNA were observed after the switch of the *A. ferrooxidans* strain TFBk from the oxidation of ferrous iron to the oxidation of elemental sulfur [10]. The present work shows that the change of the oxidation substrate from ferrous iron to elemental sulfur did not result in changes in the size and number of plasmids harbored by this strain. It may be concluded that plasmids are not involved in the exchange of genetic material between plasmid and chromosomal DNA as a possible mechanism of the structural rearrangement of chromosomal DNA observed in this strain upon the switch of its metabolism to another substrate.

In strain TFN-d, the adaptation to elemental sulfur or pyrite did not cause changes in the plasmid profile. Like the cells of the original strain, the cells of adapted cultures harbored three plasmids: a multicopy plasmid and two plasmids with low copy numbers (Fig. 4, lanes *2–4*). Neither is the adaptation of strain TFN-d to these substrates accompanied by changes in the structure of chromosomal DNA [14]. Changes in the nucleotide sequences of chromosomal DNA resulting in changes in the location of restriction sites and, hence, in the number and sizes of *Xba*I restriction fragments were noted in strain TFN-d only after its adaptation to the ore concentrate from which it had been isolated: the gravitational pyrite–arsenopyrite concentrate of the ore from the Nezhdaninskoe deposit [10, 14]. The analysis of the plasmid composition in the culture adapted to the concentrate showed that the adaptation of strain TFN-d to this substrate also resulted in changes in the plasmid profile: only two plasmids could be revealed (Fig. 4, lane *5*) out of the three found in the cells of the original strain. Earlier, we showed that strain TFN-d exhibits higher efficiency of adaptation to the concentrate than the *A. ferrooxidans* strains TFBk, TFO, TFL-2, and TFV-1: after the shift from the oxidation of ferrous iron, it showed a higher growth rate and higher activity of concentrate oxidation [15]. The present work shows that the above fact can be explained in terms of the involvement of plasmids in the regulation of concentrate oxidation by strain TFN-d.

In strain TFV-1, the adaptation to any new substrate tested in this work was accompanied by changes in the plasmid profile but, as shown earlier, did not lead to changes in the structure of chromosomal DNA [10, 14]. After growth on a medium with ferrous iron, cells of strain TFV-1 contained two plasmids. Their electrophoretic mobilities were similar to those of fragments of phage lambda DNA sized 23.1 and 4.36 kb (Fig. 5, lanes *1*, *2*). Adaptation of the strain to new oxidation substrates was accompanied by changes in the number of plasmids detectable in the cells. Thus, the electrophoregram of plasmid DNA of strain TFV-1 adapted to elemental sulfur demonstrates the absence of a small plasmid that occurs in cells grown on a medium with

ferrous iron, as well as the presence of two additional larger plasmids that cannot be detected after growth with ferrous iron (Fig. 5, lane *3*). Interestingly, the analysis of the DNA of this strain adapted to elemental sulfur and maintained for a long time on a medium with this substrate showed the presence of the small plasmid harbored by the original strain but undetectable in cells that experienced a few passages on medium with elemental sulfur (Fig. 5, lane *4*). In cells of strain TFV-1 adapted to pyrite, three plasmids were detected (Fig. 5, lane *5*). Two of them were also present in cells of the original strain grown on medium with ferrous iron, and the third, larger one, also occurred in cells adapted to elemental sulfur. In strain TFV-1, the greatest changes in the plasmid profile occurred upon the shift of its metabolism from ferrous iron oxidation to gravitational pyrite–arsenopyrite concentrate. The electrophoregram of the plasmid DNA of this strain grown on the latter substrate exhibited six bands corresponding to plasmid DNA (Fig. 5, lane *6*), whereas in the original strain, only two plasmids were detectable. Treatment of the plasmid DNA of strain TFV-1 grown on a medium with the concentrate with restriction exonuclease *Exo*III did not change the number and intensity of bands visible in the electrophoregram. The cells of this strain evidently harbor at least six plasmids, which manifest themselves in different combinations depending on the energy source present in the growth medium.

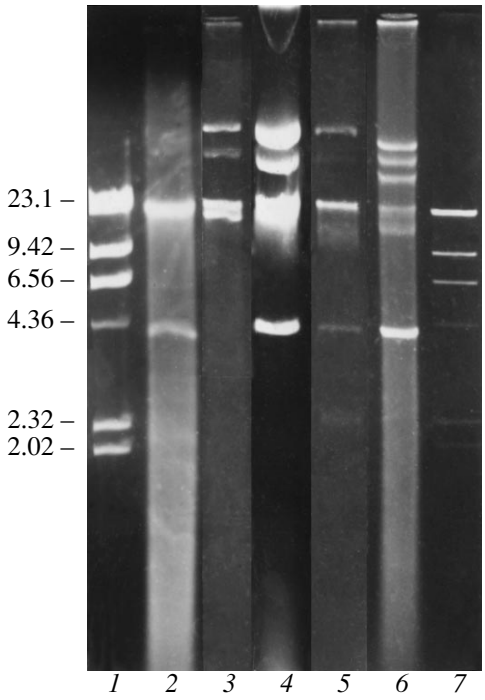

Fig. 5. Plasmid profiles of *A. ferrooxidans* TFV-1 cells adapted to different oxidation substrates—(*2*) Fe^{2+}, (*3, 4*) S^0, (*5*) FeS_2, and (*6*) gravitational concentrate—studied by electrophoresis in parallel with (*1, 7*) phage lambda DNA digested with restriction endonuclease *Hin*dIII. On the left, fragment sizes (kb) are shown.

Earlier we showed that upon adaptation to new oxidation substrates, strain TFV-1 showed lower growth rate and lower oxidative activity than other *A. ferrooxidans* strains (TFN-d, TFBk, TFO, and TFL-2). The adaptation of this strain to elemental sulfur or pyrite required five culture passages, and its adaptation to the concentrate required seven passages [15]. Our present work showed that the plasmid composition of strain TFV-1 changed with any new substrate; the greatest changes in the plasmid profile were caused by its adaptation to the concentrate. Earlier, changes in the plasmid profile of strain TFV-1 were reported to occur during its adaptation to copper ions (17.5 g/l) [3]. Thus, although *A. ferrooxidans* plasmids are considered cryptic, the data obtained for strain TFV-1 suggest their involvement in the adaptation of *A. ferrooxidans* to changes in environmental factors.

The reaction of *A. ferrooxidans* strains to the change of the energy source in the growth medium depends on the strain's prehistory, i.e., on the nature of the substrate to which the strain was adapted in its natural habitat or in the laboratory [15]. In some *A. ferrooxidans* strains that have evolved on complex multicomponent ores as substrates (strains TFN-d, TFBk, TFO), the mechanisms of regulation of the switch of metabolism to a new substrate may include changes in the nucleotide sequences of chromosomal DNA. This regulation mechanism, as distinct from the induction–repression by a substrate, characteristic of all *A. ferrooxidans* strains, is apparently an additional mechanism that appeared as a result of evolution under extreme and frequently changing environmental conditions. Strain TFV-1, isolated from a more simple substrate, has not evolved the ability to adapt to its frequent change; the structure of the chromosomal DNA of this strain is stable, and its ability to adapt to new substrates is lower than that of other *A. ferrooxidans* strains [14, 15]. In this connection, of interest is our finding of the change in the number of plasmids occurring in this strain in response to the change of the energy source in the medium.

Our study of the plasmid profiles of *A. ferrooxidans* strains TFBk and TFN-d characterized by the lability exhibited by the structure of their chromosomal DNA upon the switch of their metabolism from oxidation of ferrous iron to other substrates failed to reveal common regularities in the reactions of their genomes to new substrates. Strain TFBk, which exhibits changes in the structure of its chromosomal DNA during the adaptation to elemental sulfur, did not show changes in the plasmid profile. In strain TFN-d, adaptation to the concentrate was accompanied by changes both in the structure of chromosomal DNA and in the plasmid profile. These results do not contradict the assumption of the possible involvement of plasmids in the exchange of genetic material with chromosomal DNA, which may be one of the mechanisms of the changes in the nucleotide sequences of chromosomal DNAs of some *A. ferrooxidans* strains during their adaptation to new oxida-

tion substrates. It is possible that the integration of plasmids into chromosomal DNA does not lead to their disappearance; a part of plasmid DNA integrates into the chromosome, causing a change in the location of restriction sites and, hence, the number and size of the restriction fragments, while another part of plasmid DNA remains in the free state. It is evident that plasmid–chromosome recombinations in *A. ferrooxidans* strains adapted to different oxidation substrates should be studied in more detail and with the use of molecular biological methods.

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