ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 4, pp. 466–473. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © T.F. Kondrat'eva, V.N. Danilevich, G.I. Karavaiko, 2008, published in Mikrobiologiya, 2008, Vol. 77, No. 4, pp. 524–532.

EXPERIMENTAL ARTICLES

The Primary Structure and Characteristics of ISAfe600, an Insertion Sequence from *Acidithiobacillus ferrooxidans* Strains

T. F. Kondrat'eva[a,1], V. N. Danilevich[b], and G. I. Karavaiko[a†]

[a] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7/2, Moscow, 117312 Russia*

[b] *Shemyakin–Ovchinnicov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, Moscow, 117871 Russia*

Received May 18, 2007

Abstract—A new IS-like element (604 bp) was revealed in the genome of several *Acidithiobacillus ferrooxidans* strains isolated from diverse biotopes. It includes 26-bp imperfectly matched terminal inverted repeats (TIRs), similar in structure to the TIRs of the ISAfel insertion element. The 60-bp DNA fragment adjacent to the right TIR (TIR_R) exhibits pronounced homology with the similarly located DNA fragments in ISAfel and IST445, as well as with the internal fragment of ISAfel encoding the transposase gene (nucleotides from 254 to 311 bp). The central section of ISAfe600 is unique and exhibits no homology with any prokaryotic DNA. A duplication of 8 bp of the target DNA was found in the ISAfe600 insertion site. One to four copies of ISAfe600 were revealed by Southern hybridization in the genome of *A. ferrooxidans* strains studied. The number of ISAfe600 copies varies depending on the growth conditions (energy substrate). Since open reading frames big enough to encode transposase are not presert in the structure of ISAfe600, it may be a deficient IS element; its translocation is possibly achieved under control of the ISAfel transposase.

Key words: Acidithiobacillus ferrooxidans, insertion element, DNA sequencing, inverse PCR, Southern blot.

DOI: 10.1134/S0026261708040139

Acidithiobacillus ferrooxidans is a gram-negative, mesophilic, acidophilic, chemolithoautotrophic bacterium. *A. ferrooxidans* uses inorganic substrates (ferrous iron, elemental sulfur, reduced sulfur compounds, and a number of sulfide minerals) as a source of energy and CO_2 as a source of carbon. *A. ferrooxidans* strains isolated from different biotopes exhibit diverse genotypic and phenotypic traits [1, 2]. Each strain of *A. ferrooxidans* has a unique pattern of chromosomal DNA *Xba*I restriction, which has been analyzed by pulsed-field gel electrophoresis [3]. The structural polymorphism of the chromosomal DNA is a consequence of the rearrangements of large genomic segments. Homologous recombinations between dispersed copies of transposable elements can rearrange these segments. Repetitive transposable DNA sequences are present in the genome of many *A. ferrooxidans* strains. The genome of *A. ferrooxidans* contains several families of insertion sequences, e.g., ISAfel, IST2, IST3091, and IST445 [4–8]. We have previously applied PCR (polymerase chain reaction) to the analysis of IS elements in genomes of five *A. ferrooxidans* strains isolated from diverse environments [9]. The oligonucleotide primers complementary to the terminal inverted repeats (TIRs) of ISAfel and IST2 elements were used in this work. When using genomic DNA from *A. ferrooxidans* strains as templates, two types of DNA fragments were obtained by PCR, some similar in size and structure to ISAfel (1300 bp) and some shorter DNA fragments of approximately 600 bp. In the case of two strains (TFL-2 and TFBk), only ISAfel elements were produced, while in the other three strains (TFO, TFN-d, and TFV-1), only the 600 bp DNA fragment was amplified. At the same time, only the 1300 bp fragment corresponding to the ISAfel element was amplified in the case of strains ATCC 19859 and ATCC 23270^T, which were used as reference standards. Since the nucleotide sequences of the PCR primers corresponded precisely to the TIRs of the ISAfel element (20 of 26 nucleotides), it was logical to expect the 600 bp DNA fragment to be an insertion element as well. It was, however, unclear whether this IS element was a deletion derivative of ISAfel or a new IS of unique structure. In order to elucidate this problem, the 600 bp DNA fragments from strains TFO and TFN-d were cloned into the pBluescriptII(SK+) vector plasmid and sequenced. The preliminary sequencing results revealed that the DNA being analyzed had unique structure; the 600 bp DNA fragment is therefore

[1] Corresponding author; e-mail: kondr@inmi.host.ru
[†] Deceased.

Oligonucleotide primers used in the experiments

Oligonucleotide primers	Sequence (5'–3')	Application
El.If	TCG TCG GAT TGA GTG GGT AG	PCR amplification of ISAfel DNA
E1.2r	TCG TCA TTT CAA GTG GGT AG	
IPlf	CCT TGC CTT GCT TGG TTT CC	Inverse PCR
IPlr	AGA CCA CGG AAG CAT CCT GA	
IP2f	TGC AGG AGG CAA GGT GC	
P2r	GTA TCG TGA ACG CAA GC	
IP3f	TGA TCT CTC TGT GCT GC	
IP3r	CAT GGA GCA ACA AGG TC	
IPdir	CCA ACC TCC ATC AAT GG	Probe design for DNA-DNA hybridization
IPrev	GAC CTT GTT GCT CCA TG	

a new insertion element, tentatively designated ISAfe600 [9].

The aim of the present work was to determine the complete nucleotide sequence of ISAfe600 and of the DNA adjacent to its two ends, to find out the number of ISAfe600 copies in the genomes of the five *A. ferrooxidans* strains under study (by means of Southern hybridization), and to elucidate its possible involvement in genomic rearrangements occurring in the course of adaptation of *A. ferrooxidans* cells to new energy substrates.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Experiments were carried out with five *A. ferrooxidans* strains isolated from diverse environments: TFV-1, TFL-2, TFBk, TFN-d, and TFO [9]. Two collection strains, *A. ferrooxidans* ATCC 19859 and ATCC 23270^T were used as reference strains. The strains were purified by repeated serial dilutions in a Silverman and Lundgren medium (9K) [10], followed by repeated (three times) single colony isolation on plates of the same medium with 0.5% agarose (Difco, USA) as solidifying agent. The culture purity was confirmed both by microscopy and by the physiological tests based on the ability of *A. ferrooxidans* to oxidize ferrous iron, elemental sulfur, and sulfide minerals under autotrophic conditions and on its inability to grow on organic substrates [10]. The strains were grown in a 9K medium at 28–30°C on a shaker (150 rpm) in 250-ml Erlenmeyer flasks containing 100 ml of the medium or in 5-l bottles containing 3 l of the medium, with aeration (3 1 min^{-1}). The inoculum ratio was 10% v/v. The strains were adapted to elemental sulfur by means of sequential transfers onto solutions of mineral salts [10] containing 10 g l^{-1} of elemental sulfur instead of ferrous iron (more then 20 passages). The cultures adapted to sulfur were maintained on the same medium as sulfur and transferred to a fresh medium at least once a month.

Subcloning of *A. ferrooxidans* strains. Subcloning was performed by plating of *A. ferrooxidans* cells on solid nutrient medium. The medium consisted of three solutions. Solution 1 contained the following (g/l): $(NH_4)_2SO_4$, 5.0; KCl, 0.17; $K_2HPO_4 \cdot 3H_2O$, 0.83; $MgSO_4 \cdot 7H_2O$, 0.83. Solution 2 contained the following (per 1 l): $FeSO_4 \cdot 7H_2O$, 100 g; and 10 N H_2SO_4, 8 ml. Solution 3 contained (g/l) agarose for electrophoresis (La Chema, Czechoslovakia), 25.0. Solution 1 was autoclaved at 1 atm; solutions 2 and 3, at 0.5 atm. Agarose solution was liquefied and cooled to 41°C; the other two solutions were heated to 41°C. Three parts of solution 1, one part of solution 2, and one part of solution 3 were combined and distributed (30 ml per plate). The isolated colonies were collected after 12 days of incubation, transferred to test tubes with 10 ml of medium [10] and incubated in batch mode for six days at 28°C. The genomic DNA was obtained from the harvested biomass according to the microscale procedure (which is a modification of the large scale method) developed in our laboratory.

Large scale procedure for preparation of genomic DNA. The large scale procedure for preparation of genomic DNA and other DNA manipulations was performed as described previously [9, 11].

PCR procedure. PCR amplification of ISAfe600 elements from the genomic DNA of *A. ferrooxidans* strains was carried out as described previously [9]. In all the PCR experiments, a mixture of *Taq*- and *Pfu*-DNA polymerases was used (at a 50 : 1 ratio of the activities). Ten picomol of each of the two primers (El.lf + E1.2r) (table, Fig. 1) and 10–20 ng of genomic DNA (template) were added to 50 µl of the reaction mixture. The DNA fragments produced by PCR were purified electrophoretically followed by elution from agarose (Sigma, USA) gel, phosphorylated by phage T4 polynucleotide kinase (Amersham, England), and cloned using phage T4 DNA ligase (Promega, USA) into the pBluescriptII(SK+) vector plasmid (Stratagene, USA), linearized by *Eco*RV restriction endonuclease (Fermentas, Lithuania), and dephosphorylated by alkaline phosphatase from calf intestine (CIAP) (USB, USA). Recombinant DNA was introduced into the cells of the recipient *Escherichia coli* XL-1 blue

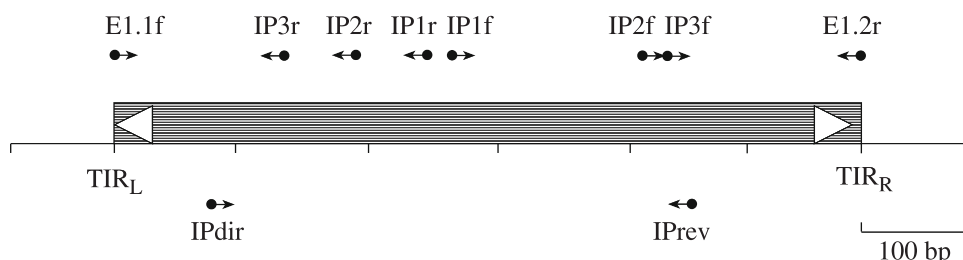

Fig. 1. Schematic representation of ISAfe600 and localization of primers annealing sites. The arrows (5' ⟶ 3') indicate oligonucleotide primer orientation. TIR$_L$ and TIR$_R$ are the left and the right terminal inverted repeats.

strain (Stratagene, USA) by the technique of electrotransformation.

Inverse PCR. The genomic DNAs isolated from three individual clones of *A. ferrooxidans* TFO were used for inverse PCR. The procedure was performed according to [12]. For this the samples of genomic DNA (100 ng) were digested with pairs of blunt cutting restriction endonucleases: *Ksp*AI + *Hind*II; *Ksp*AI + *Ssp*I; *Hind*II + *Ssp*I; *Sca*I + *Pvu*II; *Sca*I + *Eco*RV; and *Pvu*II + *Eco*RV (Fermentas, Lithuania). All these restriction endonucleases generate blunt ends and cut outside the insertion sequence. The treatment with restriction endonucleases was performed overnight (12 h) at 37°C in 20 μl of the buffers supplied by the manufacturer of the enzymes. At the next stage, circular DNAs were produced from the linear DNA fragments (restricts) by phage T4 DNA ligase (Promega, USA); 3 activity units of DNA ligase were added to 10 ng of restricted DNA. Ligation was performed in 30 μl of the reaction mixture for 14 h at 16°C. After ligation, the DNA samples were frozen and stored at –20°C. In the inverse PCR experiments, Encyclo polymerase mix and Encyclo buffer (Evrogen, Moscow) were used. This DNA polymerase mixture exhibits high 5' > 3' DNA polymerase activity, contains proof reading 3' > 5' exonuclease activity, and ensures automatic hot start. The PCR reaction mixture (50 μl) contained 3 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each of the two primers, and 1 μl of the solution containing circular DNAs. The PCR products (individual DNA fragments) were obtained by two-round PCR with nested primers. Specific primers IP1f + IP1r (Table) were used in the first round. The PCR conditions were as follows: 30 s denaturing at 93°C; 30 s annealing at 65°C; and 3 min synthesis at 72°C. The number of cycles was 30–33. The PCR mixtures were then diluted 50–100-fold with distilled water; and 1 μl of these dilutions were used as PCR templates with another pair of nested primers, IP2f + IP2r or IP3f + IP3r (Table, Fig. 1). The PCR conditions for the second round differed only in the annealing (30 s at 60°C) and synthesis (1.5 min at 72°C) stages; the number of cycles was 19–21. The individual DNA fragments obtained by nested PCR were fractioned by electrophoresis in 0.8% low-melting agarose (Agarose ultra-pure, Gibco BRL, USA). The DNA-containing gel zones were excised and transferred to clean 0.7-ml centrifuge test tubes. The DNA samples in agarose gel after melting at 60°C were used for re-amplification by PCR with the appropriate primers and the mixture of *Taq* and *Pfu* DNA polymerases (at a 50 : 1 ratio of the activities). The re-amplified DNA was purified by preparative electrophoresis in 0.8% agarose gel according to the standard procedure [13] and cloned into the EcoRV site of the pBluescriptII(SK+) vector as described above.

DNA sequencing. DNA fragments incorporated into a vector plasmid were sequenced by the dideoxy chain termination method with a model 3 100 Avant genetic analyzer (USA) using the M13/pUC direct and M13/pUC reverse primer [14].

Southern analysis of genomic fragments. The conditions for digestion of the genomic DNA by *Eco*RI restriction endonuclease and electrophoresis of DNA fragments in agarose gels, as well as the transfer of DNA fragments to nylon membranes (blotting) were performed as described previously [9]. Genomic DNA samples (about 300 ng each) were digested by *Eco*RI endonuclease and resulting *Eco*RI-restricts were separated by electrophoresis in a 1% (w/v) agarose gel prepared with Tris-acetate buffer [13] at 3.5 V/cm for 6 h. DNA fragments were transferred from an agarose gel to hybridization membranes in vacuum using Vacugene 2016 model (LKB Bromma, Sweden). GeneScreenplus (DuPont, USA) nylon membranes were used for the blotting procedure. Once the transfer was complete, the membranes with immobilized DNA were dried and then used for hybridization with probes containing a radioactive label.

Preparation of probes with radioactive label. Specific probe for Southern hybridization was obtained by PCR amplification of the unique internal DNA fragment of ISAfe600, which was limited by the primers IPdir and IPrev (Table, Fig. 1). The derivatives of pBluescriptII(SK+) containing corresponding DNA fragment were used as template in this PCR. Uniformly labeled DNA probe was synthesized using Klenow's fragment of *E. coli* polymerase I (Promega, USA) and the appropriate DNA template with a random 9-oligonucleotide primer annealed on it [13]. [α-^{32}P] dATP was used as a label. The reaction between DNA (PCR

```
                                                                   60
aaagggttca cgagaaagct ataccattGG CTCTTCGTCG GATCGAGTGG GTAGGGGGGT

                                                                  120
GGTAGAGGGC CGGGCCGAAA GACATCACCT GGTCGGCGGC GGGCGCGGCT GCCAACCTCC

                                                                  180
ATCAATGGGG TGGCTTCGCC TTCACCTTCC AAGCGGGCAG CACAGAGAGA TCAATGATCC

                                                                  240
CACTGTACAT CTCGGGCGCT GCCCGCCTCG CTCGCTTGCG TTCACGATAC GCCGCCGACG

                                                                  300
CCGCCTTGCT TGCCGCCTTC GCGTCAGGAT GCTTCCGTGG TCTGCCGCCG ACCCCCTTGC

                                                                  360
CTTGCTTGGT TTCCATAGCC TCGCCCTCTT GCCGTTGGCA CCTTTATGTA TATGACGGTG

                                                                  420
ACATAAACAT AAACGAATTT ATATAACGGT GACATAAATT AGTCAGCCAA AATCACCCCA

                                                                  480
ATCAATCCCG GGCCCTGCTG GCTGCAAGAG GCAAGGTGCG CATGGAGCAA CAAGGTCGGC

                                                                  540
GGCACAGGTC TTCAGTGTCC GCCGACCCCA TCACCTGGTT GGCGGACGCT CCCGTCGGCG

                                                                  600
GCTGAGCACC CGCAAAAACT TCATCAACAT GGCCTACCTG ATCCTTTGGG TAAGCTGGAT

                                                                  660
CTCGGGCTAC CCACTGGAAA TGACGAGGAA CCataccatt ccgttctgcc agatttggtg
```

Fig. 2. Nucleotide sequence of ISAfe600 and adjacent regions. Lowercase indicates unique genomic sequences outside of ISAfe600. Target-site duplications are indicated by letters enclosed in boxes. The nucleotide sequence of ISAfe600 is shown in uppercase letters. The terminal inverted repeats are marked with arrows. The 60 bp DNA fragment of the internal part of ISAfe600 which exhibits pronounced homology with the similarly located DNA fragments in ISAfel and IST445 is underlined and is directly adjacent to the right TIR.

product, 200 ng), a random primer (75 ng) and 2.5 MBq [α-^{32}P] dATP was conducted as described [13].

Prehybridization and hybridization were performed as described [9]. The nucleotide sequence data of the ISAfe600 element and the DNA fragment adjacent to ISAfe600 reported in this paper were deposited in GenBank under accession number EF459502.

RESULTS AND DISCUSSION

The nucleotide sequence of the internal part of ISAfe600 The 600 bp DNA fragments, PCR products obtained with El.If + El.2r primers from genomic DNA of two *A. ferrooxidans* strains, TFO and TFNd, were cloned into the pBluescriptII(SK+), as described in the Materials and Methods section. The inserted 600 bp DNA fragments in recombinant plasmids were sequenced from both termini with the universal primers M13/pUCdir and M13/pUCrev. The DNAStar software package was used to analyze the sequences obtained. The analyzed DNA sequences have all the characteristics of insertion elements: they contain internal part and are flanked with 20 bp terminal inverted repeats, completely corresponding in structure to those of ISAfel. The ISAfe600 sequences from two *A. ferrooxidans* strains, TFO and TFNd, were found to be identical; their precise length was 596 bp (Fig. 2). The BlastN search revealed that the 60 bp fragment of ISAfe600, adjacent to the right TIR (nucleotides position from 522 to 581 of ISAfe600), is homologous to the similarly located DNA fragment of ISAfel and IST445, as well as to the internal part of ISAfel, encoding the transposase

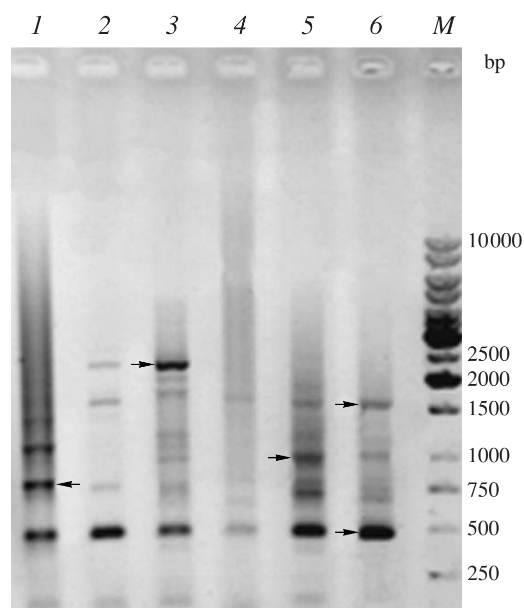

Fig. 3. Electrophoretic analysis of inverse PCR products in 0.8% agarose gel. DNA fragments containing the left and right segments of ISAfe600 were obtained by nested PCR with IP2f + IP2r primers. DNA samples (diluted 100-fold) from the first PCR round with IP1f + IPlr were used as templates. As original templates, circular DNA fragments (restricts) were obtained by restriction of *A. ferrooxidans* TFO genomic DNA by the following pairs of restriction endonucleases: 1, (*1*) *Ksp*AI + *Hind*II; (*2*) *Ksp*AI + *Ssp*I; (*3*) *H ind*II + *Ssp*I;(*4*) *Sca*I + *Pvu*II; (*5*) *Sca*I + *Eco*RV; and (6) P*v*ull + *Eco*RV. *M*. is markers of the DNA fragment length DNA (1 kb DNA ladder). The DNA fragments used for cloning and sequencing are indicated by arrows.

gene (nucleotides from 254 to 311 of ISAfel) [7, 8]. At the same time, the nucleotide sequence of the central part of ISAfe600 (region from 27 to 521 bp) was unique; i.e., it had no significant homology to any known prokaryotic and eukaryotic DNA (the exception is the short 19–21 bp DNA segments, which were found to be homologoes to other DNA, data not shown). The nucleotide sequence of ISAfe600 was translated in all the six frames using the MapDraw program (included in the DNAStar software package). Visual analysis of translation products revealed no open reading frames (ORF) in the structure of ISAfe600 large enough to encode transposase (TPase). It is therefore clear that ISAfe600 does not encodes TPase; i.e., it is a functionally inactive IS element incapable of independent transposition. We suggest that it is a defective IS element derived from ISAfel or another IS-element of the ISL3 family, which has lost the transposase gene but retained TIRs similar in structure to those of ISAfel (the complete primary structure of TIRs in ISAfe600; see below). We believe that ISAfel TPase is possibly responsible for the transposition of this element. Indeed, several copies of ISAfel are usually present in ISAfe600-containing *A. ferrooxidans* cells [9].

The structure of TIRs of ISAfe600 and of the duplicated target DNA. The complete nucleotide sequences of TIRs in ISAfe600 and the structure of the target DNA were determined by inverse PCR [12]. Strain TFO was maintained in our laboratory for 9 years. Previously we observed a spontaneous loss of IS elements in *A. ferooxidans* cultures after multiple passages in the laboratory conditions (our unpublished observations). To test if ISAfe600 is still present in the majority of the cells in our TFO culture, fifty separate clones were isolated and transferred to liquid media. The samples of genomic DNA purified by microscale procedure from the biomass of these clones were used as templates in PCR with the primers El.lf+ E1.2r (Table, Fig. 1). The single PCR product, a 600 bp DNA fragment, was obtained for all template DNAs. It should be noted that in no case were 1300 bp fragments (ISAfel) observed in this set of PCR experiments. From this result we conclude that ISAfe600 is present in the overwhelming majority of the cells in bacterial population of the original *A. ferrooxidans* TFO strain.

Three TFO subclones of the 50 investigated were chosen for the subsequent work. These subclones were cultivated in 100 ml of liquid medium [10] with ferrous iron and the biomass obtained was used for large-scale preparation of genomic DNA. The samples of genomic DNA from the three subclones were treated with six pairs of restriction endonucleases followed by treatment with phage T4 DNA ligase. The samples of circular DNAs obtained in this way were used in inverse PCR (see Materials and Methods).

For every pair of restriction endonucleases, inverse PCR products were obtained. No differences in DNA fragments distribution were revealed between the three *A. ferrooxidans* TFO clones. It should be noted that smears are usually formed in the course of the first round PCR; we did not succeed in obtaining individual DNA fragments when only one pair of primers was used (e.g., IP1f + IPlr or IP2f + IP2r). Sufficiently pure and homogeneous DNA fragments were obtained only in the second PCR round with the nested primers (Fig. 3).

The size of the DNA fragments obtained (inverse PCR products) varied from 0.5 to 2.5 kb. As a rule, two or three PCR fragments of different size were generated for each pair of restriction endonucleases. The bands corresponding to the DNA fragments of ca. 0.5 and 0.75 kb can be seen in almost every lane presented on Fig. 3. Sequencing of these DNA fragments revealed deletions in the 5' part of the IS element and in adjoining segments of the DNA (see below). Only larger DNA fragments (over 0.75 kb) were found to contain full-sized left and right segments of the ISAfe600-containing products of double cleavage.

Five DNA fragments the products of inverse PCR were cloned into pBluescriptII(SK+) and sequenced (partially, from both ends): 0.75 kb fragment obtained from (*Ksp*AI + *Hind*II) digestion; 2.5 kb fragment from

(*Hind*II + *Ssp*I) digestion; 1 kb fragment – from (*Sca*I + *Eco*RV); and 1.6 kb and 0.5 kb fragments – from (*Pvu*II + *Eco*RV) library. Sequencing of the 0.5-kb fragment obtained from the (*Pvu*II + *Eco*RV) library revealed a deletion including 112 nucleotides at 5' terminus of ISAfe600 and a large adjacent fragment of the DNA. The mechanism of formation of deletion derivatives is not clear. On the one hand, these deletions may be induced by ISAfe600 (it is well known that IS elements induce deletions [15]). On other hand, deletions could have possibly occurred in the course of formation of circular DNA (at the ligation stage); their formation in the course of two-round PCR is less probable.

The sequencing results for three big DNA fragments cloned into pBluescriptII(SK+) indicated that the nucleotide sequences flanking ISAfe600 element were identical in all cases (data not shown). In another words, inverse PCR of strain TFO revealed only one site for the insertion of ISAfe600 into the genomic DNA. On the other hand, DNA/DNA hybridization revealed three copies of ISAfe600 in TFO cells grown on the medium with ferrous iron. As can be seen from Fig. 4a, these copies are located in various portions of the bacterial genome (in different *Eco*RI restricts). One of the possible explanations for this discrepancy is that ISAfe600 is a component of a larger system, probably of a transposon, and moves together with it. One can suggest several copies of this transposon may be simultaneously present in the bacterial genome. The results of Southern hybridization do not exclude the possibility of the plasmid localization of some of ISAfe600 copies. Indeed, early in the cells of TFO strain a cryptic plasmid was observed by us [16]. Its size (approximately 14 kb) corresponds well to the most intensive hybridization band obtained for TFO strain (see the upper band on Fig. 4a, line *2*).

The visual analysis of the sequences thus obtained revealed duplication of the target DNA at the boundary between ISAfe600 and the chromosomal DNA; similar to ISAfel [8], it consisted of 8 AT-rich nucleotides: ATACCATT (Fig. 2). The complete sequences of TIR_L and TIR_R (26 nucleotides) were also determined. Figure 2 presents the complete nucleotide sequence of ISAfe600 along with the adjacent short pieces (30 bp) of DNA.

A comparison of TIR nucleotide sequences of ISAfe600 and four other members of the ISL3 family is shown in Fig. 5. The TIRs of the ISL3 family representatives vary in size from 15 to 39 bp [15]. The 26-bp-long TIRs of ISAfe600 fit well into this range. The sequences of left and right TIRs are not identical in ISAfe600 containing the nucleotide substitutions in both their internal parts and terminal regions.

The experimental data suggest the two-domain composition of TIR_S. One of these functional domains is located inside TIR and is involved in transposase binding. The second one, including two or three terminal nucleotides, is involved in splitting and in the transfer of the DNA chain resulting in the IS element transposition [15]. Unlike ISAfel, the three terminal nucleotides in the TIRs of ISAfe600 (GGC and GGT) are not identical (Fig. 5). Similar cases have been reported for other IS elements of the ISL3 family [15], for instance, three terminal nucleotides (GCC and GGC) in the TIRs of the ISAel.

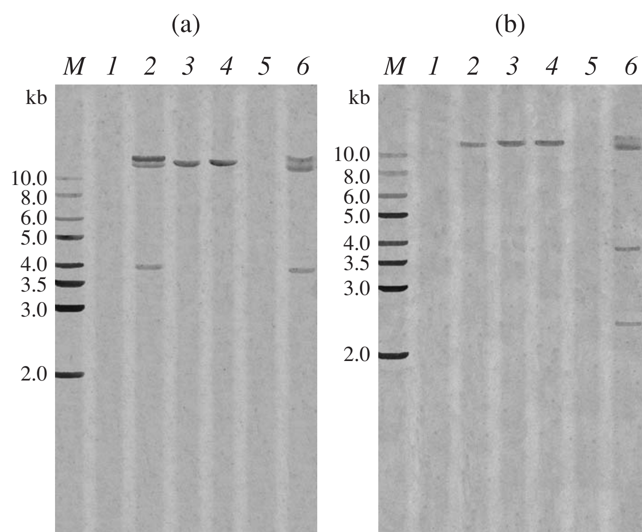

Fig. 4. Southern blot analysis several strains of *A. ferrooxidans* grown in a medium with ferrous iron (a), or adapted to elemental sulfur (b). Genomic DNA was cut with *Eco*RI and hybridized with ^{32}P-labeled probe, derived from an internal fragment of ISAfe600, and generated in PCR with IPdir + IPrev primers. Lines: (*1*) ATCC 23270^T; (*2*) TFO; (*3*) TFBk; (*4*) TFL-2; (*5*) TFV-1; (*6*) TFN-d; M is 1 kb DNA ladder (Fermentas, Lithuania). At the side: DNA fragments length in kb. The DNA of ATCC 23270^T strain in a and b, grown in the medium with ferrous iron, served as a reference.

The differences in the internal structure of TIR_L and TIR_R in ISAfe600 and ISAfel revealed by sequencing are also presented in Fig. 5 (they are shown in bold). We believe that these small differences can not affect the ability of ISAfe600 to form an active complex with the transposase of ISAfel element.

The structure of DNA fragment flanking ISAfe600 copy. We have read ca. 430 bp to the left and over 340 bp to the right of the ISAfe600 element. A total of ca. 770 bp of the *A. ferrooxidans* genome DNA fragment was sequenced. The IS element was inserted into this fragment. Apart from the 19–21 bp DNA segments, which were homologous to other DNAs, including eukaryotic ones, BlastN analysis of the ISAfe600 sequence and of the DNA sequences flanking this IS element (total length, 1385 bp) revealed no significant homology to known prokaryotic sequences. BlastN detects only relatively high homologies and requires the presence of long identical fragments (11 bp by default) for reliable operation. The tBlastx search, detecting homologies at the level of amino acid sequences encoded by a given nucleotide sequences,

Insertion seguence	Left and Right terminal IR sequence
ISAfe600	5′ GGC TCT TCG TCG GAT CGA GTG GGT AG TIR_L
	5′ GG**T** TC**C** TCG TC**A** **TT**T C**C**A GTG GGT AG TIR_R
ISAfe1	5′ GGC TCT TCG TCG GAT TGA GTG GGT AG TIR_L
	5′ GGC TCT TCG TC**A** **TT**T **CA**A GTG GGT AG TIR_R
ISAe1	5′ GCC TCT TTT GAA TTT CAA GTG GGT GG TIR_L
	5′ G**G**C TCT TTT GAA **AAA** **TG**A GTG GGT **TA** TIR_R
IS13869	5′ GGC TCT TCC GTT TTT AGA GTG CAT TG TIR_L
	5′ GGC TCT TCC GTT TTT AGA GTG CAT TG TIR_R
ISL3	5′ GGC TCT ATA ATT TTT TTT ACT GAT GG TIR_L
	5′ GGC TCT **T**T**G** **TCA** **AA**T **C**TT ACT GAT G**C** TIR_R

Fig. 5. Alignment of TIR sequences of ISAfe600 and some other members of the ISL3 family. The sequences of ISAel, IS13869 and ISL3 TIRs are adopted from [15], where additional TIRs of the ISL3 family can be found. TIRr sequences are presented as a complementary strand to facilitate the search for similarity. The differences between right and left TIRs in each IS element are highlighted by bold.

was therefore used. Analysis of the 1385 bp sequence revealed high homology (over 50%) with bacterial transcription regulators at the 5' region and with DNA topoisomerases of various plasmids at the 3' terminus (homology over 60%). The 3'-terminal homologous fragment is located between 1385 and 1155 bp; the 5'-terminal one, between 260 and 68 bp. In this coordinate system, ISAfe600 occupies the 439–1024 bp region. Thus, the genome DNA sequences flanking ISAfe600 in strain TFO probably also encode important cellular proteins. This issue can be elucidated by sequencing of longer fragments to the left and to the right of ISAfe600.

The number of ISAfe600 copies in the genome of five *A. ferrooxidans* strains. *A. ferrooxidans* strains were either grown on media with ferrous iron or have been cultivated with elemental sulfur for extensive periods of time. Genomic DNA was isolated from the harvested biomass. DNA was treated with *Eco*RI restriction endonuclease (*Eco*RI does not cut the inside of ISAfe600); the fragments thus obtained were separated by electrophoresis in 0.8% agarose gel and were then transferred to nylon filters. The ^{32}P-labeled internal fragment of ISAfe600 DNA was obtained with E1.3f + E1.4r primers and used as the specific probe for Southern hybridization (Table, Fig. 1). This DNA fragment was unique and contained no homologies with c ISAfel. Fig. 4 demonstrates the results of Southern hybridization. One can see that no hybridization signals with the probe were registered for the *Eco*RI fragments of the DNA of the control strain ATCC 23270^T. No hybridization signals occurred also with the DNA fragments of another control strain, ATCC 19859 (data not shown). However, clear hybridization signals were obtained for the DNA of strains TFO, TFN-d, TFBk and TFL-2, grown either with ferrous iron (Fig. 4a) or with elemental sulfur (Fig. 4b). Three hybridization bands were observed for the DNA from strains TFO and TFN-d grown with ferrous iron. One hybridization band was recorded for strains TFBk and TFL-2. The DNA fragments from strain TFV-1 did not hybridize with the probe. It should be mentioned that in the course of our experiments clear and relatively intense hybridization signals were obtained with most of the 10 kb DNA ladder fragments; these fragments were used as molecular mass standards. Small DNA fragments of less than 2.0 kb, however, produced no hybridization signal. The probe nucleotide sequences probably contained homologies with some fragments of the 10 kb DNA ladder. Under the experimental conditions of hybridization and washing (with annealing temperature of 65°C), 20 bp DNA-DNA homology is known to be sufficient to obtain a hybridization signal. Thus, our data indicated the presence of one to three copies of ISAfe600 element in *A. ferrooxidans* genome. As for the strain TFV-1, for which no hybridization signal was recorded, we suggest that this IS element was present only in the genome of a small fraction

of the bacterial population, as indicated by the PCR detection of ISAfe600 in one of TFV-1 clones [9].

Fig. 4b illustrates the distribution of hybridization bands in the case of growth of the cells on elemental sulfur as an energy substrate. One can see from this figure only one hybridization signal was revealed in the DNA of strain TFO grown under these conditions, i.e., the >10 kb hybridization band was preserved. In the case of strain TFN-d, an additional 2.4 kb hybridization band emerged. In two other strains, TFBk and TFL-2, the band distribution did not change; only one hybri-dization band persisted. These results support ambiguous interpretation. Selection of cells (clones) with specific phenotypes (genotypes) possibly occurs when the substrate is changed. The presence of ISAfe600 in specific loci of the bacterial DNA can influence the ability to utilize specific energy substrates. Alternatively, the adaptation to a new energy substrate (elemental sulfur instead of ferrous iron) can be accompanied by ISAfe600 transposition into a new locus of the chromosomal DNA (as in strain TFN-d) or by its excision from the chromosome (as in strain TFO). These phenomena can be related to the regulation of activity of certain specific structural loci. Transpositions of IS elements in the chromosomal DNA of *A. ferrooxidans* caused by changed cultivation conditions were reported earlier [17–21].

REFERENCES

1. Ageeva, S.N., Kondrat'eva, T.F., and Karavaiko, G.I., Phenotypic Characteristics of *Thiobacillus ferrooxidans* Strains, *Microbiology (Engl. Transl. Mikrobiologiya)*, 2001, vol. 70, no. 2, pp. 226–234.
2. Karavaiko, G.I., Turova, T.P., Kondrat'eva, T.F., Lysenko, A.M., Kolganova, T.V., Ageeva, S.N., Muntyan, L.N., and Pivovarova, T.A., Phylogenetic Heterogeneity of the Species *Acidithiobacillus ferrooxidans, Int. J. Syst. Evol. Microbiol*, 2003, vol. 53, no. 1, pp. 113–119.
3. 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*, 1995, vol. 41, no. 5, pp. 1157–1162.
4. Yates, J.R. and Holmes, D.S., Two Families of Repeated DNA Sequences in *Thiobacillus ferrooxidans, J. Bacteriol.*, 1987, vol. 169, no. 5, pp. 1861–1870.
5. Yates, J.R., Cunningham, R.P., and Holmes, D.S., IST2: An Insertion Sequence from *Thiobacillus ferrooxidans, Proc. Natl. Acad. Sci. USA*, 1988, vol. 85, no. 9, pp. 7284–7287.
6. Chakravarty, L., Kittle, J.D., and Tuovinen, O.H., Insertion Sequence IST3091 of *Thiobacillus ferrooxidans, Can. J. Biol*, 1997, vol. 43, no. 6, pp. 503–508.
7. Chakraborty, R., Deb, C., Lohia, A., and Roy, P., Cloning and Characterization of a High Copy Number Novel Insertion Sequence from Chemolithotrophic *Thiobacillus ferrooxidans, Plasmid*, 1997, vol. 38, no. 2, pp. 129–134.
8. Holmes, D.S., Zhao, H.L., Levican, G., Ratouchniak, J., Bonnefoy, V., Varela, P., and Jedlicki, E., ISAfel and ISL3 Family Insertion Sequence from *Acidithiobacillus ferrooxidans* ATCC 19859, *J. Bacteriol.*, 2001, vol. 183, no. 14, pp. 4323–4329.
9. Kondrat'eva, T.F., Danilevich, V.N., Ageeva, S.N., and Karavaiko, G.I., Identification of IS Elements in *Acidithiobacillus ferrooxidans* Strains Grown in a Medium with Ferrous Iron or Adapted to Elemental Sulphur, *Arch. Microbiol.*, 2005, vol. 183, no. 6, pp. 401–410.
10. Silverman, M.P. and Lundgren, D.G., Studies 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.
11. Chirgwin, J.M. and Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. Isolation of Biologically Active Ribonucleic Acid from Sources Enriched in Ribonuclease, *Biochemistry*, 1979, vol. 18, no. 24, pp. 5294–5299.
12. Triglia, T., Peterson, M.G., and Kemp, D.J., A Procedure for *in vitro* Amplification of DNA Segments That Lie Outside the Boundaries of Known Sequences, *Nucleic Acid Res.*, 1988, vol. 16, no. 16, pp. 81–86.
13. Maniatis, T., Fritsch, E.F., and Sambrook, J., *Molecular cloning: A Laboratory Manual*, Cold Spring Harbor: Cold Spring Harbor Lab, 1982.
14. Yanisch-Perron, C., Viera, J., and Messing, J., Improved Ml3 Phage Cloning Vector and Host Strains: Nucleotide Sequences of the M13mpl8 and PUC19 Vectors, *Gene*, 1985, vol. 33, no. 9, pp. 103–119.
15. Mahillon, J. and Chandler, M., Insertion Sequences, *Microbiol. Mol. Biol. Rev*, 1998, vol. 62, no. 3, pp. 725–774.
16. Kondrat'eva, T.F., Ageeva, S.N., Muntyan, L.N., Pivovarova, T.A., and Karavaiko, G.I., Strain Polymorphism of the Plasmid Profiles in *Acidithiobacillus ferrooxidans, Microbiology,* 2002, vol. 71, no. 3, pp. 319–325.
17. Schrader, J.A. and Holmes, D.S., Phenotypic switching of *Thiobacillus ferrooxidans*, *J. Bacteriol,* 1988, vol. 170, no. 9, pp. 3915–3923.
18. Holmes, D.S. and Haq, R.U., Adaptation of *Thiobacillus ferrooxidans* for Industrial Application, in *Biohydrometallurgy*, Salley, J. and McCready, R.G.L., Eds., Ottawa: CANMET, 1989, pp. 115–123.
19. Zhao, H. and Holmes, D.S., Insertion sequence IST1 and Associated Phenotypic Switching in *Thiobacillus ferrooxidans*, in *Biohydrometallurgical Technologies*, Torma, A.E., Apel, M.L., and Brierley, C.L., Eds., Wyoming: Minerals, Metals, Materials, 1992, pp. 667–671.
20. Cadiz, R., Gaete, L., Jedlicki, E., Yates, J., Holmes, D.S., and Orellana, O., Transposition of IST2 in *Thiobacillus ferrooxidans*, *Mol. Microbiol.*, 1994, vol. 12, no. 1, pp. 165–170.
21. Holmes, D.S., Jedlicki, E., Cabrejos, M.E., Bueno, S., Guacucano, M., Inostroza, C., Levican, M., Varela, P., and Garcia, E., The Use of Insertion Sequence to Analyze Gene Function in *Thiobacillus ferrooxidans*: a Case Study Involving Cytochrome *c*-Type Biogenesis Proteins in Iron Oxidation, in *Biohydrometallurgy and the Environment: Toward the Mining of the 21st century*, Amils, R. and Ballester, A., Eds., Amsterdam: Elsevier, 1999, pp. 139–147.