Phylogenetic heterogeneity of the species *Acidithiobacillus ferrooxidans*

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Polyphasic genotypic analysis of 25 *Acidithiobacillus ferrooxidans* strains isolated from ores and ore concentrates collected in different regions of the world showed considerable strain heterogeneity. Restriction patterns of the chromosomal DNA of these strains obtained by PFGE were specific for each strain. According to the degree of DNA relatedness, 17 of the 23 strains studied were divided into four genomovars. Six independent, considerably divergent strains could not be assigned to any of the genomovars. A comparison of nearly complete nucleotide sequences of the 16S rDNA of five representatives of the genomovars (including the type strain of *A. ferrooxidans*, ATCC 23270^T) with those of species of the genus *Acidithiobacillus* available from GenBank showed that most of the *A. ferrooxidans* strains, together with the type strain and some other strains of the species *Acidithiobacillus thiooxidans*, comprised a monophyletic cluster. Within this major cluster, *A. ferrooxidans* strains fell into four phylogenetic groups that were equidistant from the phylogenetic group of *A. thiooxidans* strains. In general, the distribution of strains among the phylogenetic groups correlated with their distribution among the genomovars, except that the representatives of two different genomovars fell into one phylogenetic group. Thus, at least two levels of phylogenetic heterogeneity for *A. ferrooxidans* have been found. The phylogenetic heterogeneity of *A. ferrooxidans* strains, which are phenotypically indistinguishable, suggests the occurrence of microevolutionary processes in different ecotopes. This should be taken into account in the biohydrometallurgical applications of *A. ferrooxidans* strains.

INTRODUCTION

The development of biohydrometallurgy calls for a comprehensive investigation of mesophilic chemolithotrophic microbial communities in extreme ecotopes characterized by low pH and high concentrations of metal ions. These communities are able to oxidize Fe²⁺ ions, sulfur and sulfide minerals and comprise a few phylogenetically unrelated groups of micro-organisms, i.e. some archaea, sulfobacilli, *Leptospirillum ferrooxidans* and bacteria of the genus *Thiobacillus*, the taxonomy of which has recently been revised (Kelly & Wood, 2000). On the basis of the results of 16S rDNA sequence analysis, *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* and *Thiobacillus caldus* were combined into a new genus, *Acidithiobacillus*, within the γ -subclass of the *Proteobacteria*. Arbitrarily, the species *Thiobacillus albertensis* was also assigned to this

genus, though the 16S rRNA genes of this species have yet to be sequenced.

The distinguishing feature of the species *Acidithiobacillus ferrooxidans* is its ability to derive energy from the oxidation of ferrous ions. Various strains of this species have been isolated from natural (rocks, ores and mine waters) and technological (ore concentrates and pulps of the gold and non-ferrous industries) sources. The habitats of *A. ferrooxidans* strains are geographically extremely diverse and vary in their physico-chemical conditions (presence of particular sulfide minerals and their ratio, pH, temperature and the content of toxic compounds in the liquid phase). This might explain the polymorphism of *A. ferrooxidans* strains, in terms of both their physiological properties (Tuovinen *et al.*, 1971; Harrison, 1982; Valenti *et al.*, 1989; Leduc & Ferroni, 1994; Chisholm *et al.*, 1998; Frattini *et al.*, 2000; Ageeva *et al.*, 2001) and genotypic characteristics (Martin *et al.*, 1981; Harrison, 1982; Shiratori *et al.*, 1991; Kondratyeva *et al.*, 1993, 1995; Rawlings & Kusano, 1994; Rawlings, 1999). The study of the natural

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of *Acidithiobacillus ferrooxidans* strains ATCC 23270^T, TFN-d, TFI, TFD and TFY are AF465604–AF465608.

genotypic and phenotypic variability of bacterial strains is of great importance in discerning the microevolutionary mechanisms of species formation and for their monitoring in biohydrometallurgical processes and in nature.

This study involved a polyphasic genotypic analysis of a large group of *A. ferrooxidans* strains isolated from different habitats and the evaluation of the phylogenetic heterogeneity of these strains.

METHODS

Strains. Experiments were conducted with 25 strains isolated from natural sulfide ores, their concentrates and dense pulps taken from pilot bacteriochemical reactors used for gold recovery and leaching of non-ferrous metals from sulfide ores and their concentrates (Table 1). All of the novel isolates were Gram-negative, motile, acidophilic, mesophilic, chemolithoautotrophic bacteria that obtained energy by the oxidation of Fe^{2+} , S^0 or sulfide minerals (Karavaiko *et al.*, 1997; Kondratyeva *et al.*, 1999; Ageeva *et al.*, 2001). This allowed us to assign all of the novel isolates to the species *A. ferrooxidans*. This was supported by the genotypic analysis performed in this work. Nineteen strains, two of which (strains VKM B-458 and VKM B-1160) have been deposited in the All-Russia Collection of Microorganisms, were isolated in the Laboratory of Chemolithotrophic Micro-organisms, the Institute of Microbiology and the Russian Academy of Sciences. Two strains (ATCC 23270^T and ATCC 19859) were obtained from the American Type Culture Collection. Four identified strains (TFY, TF-16, TFP and TFR-2) were kindly donated by M. Vrvic, A. Torma, J. Hurtado and E. S. Kalyaeva.

Cultivation. *A. ferrooxidans* strains were cultivated at 28–30 °C in batch mode in 250 ml Erlenmeyer flasks containing 100 ml of a medium described by Silverman & Lundgren (1959). The flasks were

shaken at 160 or 240 r.p.m. The initial pH of the medium was 1.8–2.0. The inoculum size comprised 10 % (v/v) of the growth medium.

DNA isolation. *A. ferrooxidans* cells for the isolation of DNA were grown in unshaken 5 l bottles containing 3 l growth medium aerated at a rate of 3 l air min⁻¹. Native DNA was isolated from cells as described by Kondratyeva *et al.* (1995). In DNA–DNA hybridization experiments, DNA was isolated by the method of Marmur (1961).

DNA analysis. Chromosomal DNA was analysed by PFGE (Kondratyeva *et al.*, 1995). The G+C content of the DNA was determined by the thermal denaturation method. The genome size and the level of DNA–DNA hybridization were evaluated from the DNA renaturation rates (De Ley *et al.*, 1970).

16S rRNA gene amplification and sequencing. 16S rRNA genes were amplified and sequenced using universal prokaryotic primers (Edwards *et al.*, 1989). PCR amplifications were carried out using thermostable *BioTaq* polymerase (Dialat), according to the manufacturer's instructions. The volume of the reaction mixture was 20 µl. The PCR program consisted of one cycle of DNA denaturation at 94 °C for 3 min, primer annealing at 50 °C for 3 min and DNA synthesis at 72 °C for 3 min, five cycles of 94 °C for 30 s, 50 °C for 2 min and 72 °C for 30 s, 30 cycles of 94 °C for 30 s, 40 °C for 30 s and 72 °C for 30 s and a final incubation at 72 °C for 7 min. PCR products were analysed by electrophoresis in 1 % agarose gel. Bands were visualized by illuminating ethidium bromide-stained gels on a BioKom UV transilluminator. 16S rDNA fragments were extracted from agarose gels using a Wizard PCR Preps kit (Promega) and sequenced by the method of Sanger *et al.* (1977) using a Silver Sequencing kit from the same manufacturer. These procedures were performed according to the manufacturer's instructions, with minor modifications. Electrophoresis was conducted using a MacroPhor device (Pharmacia) and an SQ3 Sequencer (Hoefer) at a gel thickness of 0.19 mm.

Table 1. *A. ferrooxidans* strains and their isolation sites

Strains are grouped according to the type of ore/main sulfide minerals from the isolation site, as described in the text.

Strain	Isolation site
Gold–arsenic ores and concentrates/FeS_2, FeAsS, FeS	
TFD, TFO, TF-1292, TFM, TFN-d, TF 97	Siberia, Russia
TFBk, TFT-92	Kazakhstan
TFT	Kyrgyzstan
Copper–zinc and copper ores, concentrates/FeS_2, CuFeS_2, Cu_2S, CuS, Cu_5FeS_4, ZnS	
TFI	India
TFV-1, TFL-2, TFL-3, TFS	Central Urals, Russia
VKM B-1160, TFG	Southern Urals, Russia
TFN	Altai mountains, Russia
TFY	Yugoslavia
TF-16	Canada
TFP-15	Peru
ATCC 23270 ^T , ATCC 19859	USA
Other sources/FeS_2, Fe^{2+}	
TFR-2	Ferri-ferrous stream on a volcano slope (i.-d. Kunashir), Russia
VKM B-458	Ferri-ferrous stream in Submoscow coalfield, Russia
TFWc	Activated sludge from cleaning building, Moscow, Russia

Phylogenetic analysis. The *Xba*I restriction profiles of native DNA of the strains under study were formalized according to the presence or absence of DNA fragments of a certain length and analysed using the maximum-parsimony algorithm realized in the PHYLIP software package (Felsenstein, 1989) and the neighbour-joining algorithm of the FREETREE program (Pavlicek *et al.*, 1999). A dendrogram based on DNA–DNA hybridization data was constructed using an approximate similarity matrix, whose unknown terms were calculated based on mean values for the cluster, with the aid of the UPGMA algorithm of the PHYLIP software. The 16S rRNA gene sequences of analysed strains were aligned with those of the closest micro-organisms using CLUSTAL W (Thompson *et al.*, 1994). An unrooted phylogenetic tree was generated with the aid of the neighbour-joining algorithm realized in the TREECON package (Van de Peer & De Wachter, 1994).

RESULTS

Ecological diversity of strains

The sources of *A. ferrooxidans* strains (sulfide minerals from different geographical zones) can be divided into three groups (Table 1): (i) gold–arsenic ores and their concentrates; (ii) copper and copper–zinc ores and their concentrates; and (iii) other sources. The major sulfide minerals of gold–arsenic ores are pyrite (FeS₂), arsenopyrite (FeAsS) and, in ores from the Olimpiadinskoe and Maiskoe deposits, pyrrhotite (FeS). Copper and copper–zinc ores from different deposits were mainly polymetallic, except that the copper ore from the Volkovskoe deposit (from which strain TFV-1 was isolated) contained primarily copper sulfide minerals. The major source of *A. ferrooxidans* strains isolated from coal deposits is pyrite. The pH of ore pulps depends on the composition of sulfide minerals and the activity of bacteriochemical processes in bioreactors. Correspondingly, the amounts of metal ions leached from ores into the liquid phase of ore pulps are different.

Analysis of chromosomal DNA relatedness by PFGE

PFGE of *Xba*I-digested DNA of various *A. ferrooxidans* strains showed that their genomes differ somewhat (Kondratyeva *et al.*, 1993, 1995). Analysis of the DNA restriction profiles of 20 *A. ferrooxidans* strains by the maximum-parsimony algorithm showed that they fell into three clusters (Fig. 1). However, the confidence levels of most branching points of the dendrogram evaluated by bootstrap analysis were less than 70 %. This dendrogram correlated with that generated by the neighbour-joining algorithm (data not presented) with respect to only three branching points, whose bootstrap values exceeded 70 %.

The G + C content of DNA and the genome size

The G + C content of *A. ferrooxidans* strains varied from 56.1 to 58.1 mol%, and the genome size was 2.2–2.8 × 10⁶ kDa (Table 2). The most frequent genome size was 2.3–2.4 × 10⁶ kDa and the most frequent G + C content was about 57 mol%.

Fig. 1. Phylogenetic tree of *A. ferrooxidans* strains derived from analysis of the lengths of nucleotide fragments obtained by the digestion of native chromosomal DNA with the restriction endonuclease *Xba*I.

Similarity analysis of the total genomes by DNA–DNA hybridization

DNA–DNA hybridization analysis of 23 *A. ferrooxidans* strains with respect to 17 reference strains showed their considerable genetic heterogeneity (Fig. 2). The strains

Table 2. DNA G + C content and genome size of *A. ferrooxidans* strains

Strain	G + C content (mol%)	Genome size (× 10 ⁶ kDa)
TFD	58.1	2.2
TFV-1	57.6	2.3
VKM B-458	57.6	2.3
TF-16	56.8	2.3
TFS	57.6	2.3
TFG	57.3	2.3
TFP-15	56.4	2.3
TFN	57.8	2.3
TFWc	57.4	2.3
TFI	57.5	2.4
TFT-92	57.1	2.4
TFN-d	56.9	2.4
TFT	57.7	2.4
VKM B-1160	56.5	2.4
TFR-2	56.3	2.4
TFM	56.9	2.4
TFBk	56.7	2.4
TFL-2	57.2	2.4
TFO	57.3	2.4
ATCC 23270 ^T	56.1	2.7
ATCC 19859	57.3	2.7
TF-1292	57.2	2.7
TFY	57.5	2.8

Fig. 2. Approximate relatedness dendrogram of *A. ferrooxidans* strains derived from DNA–DNA hybridization data. The scale bar corresponds to the level of genome similarity.

were divided into four groups characterized by a high degree of genome similarity. Such genome groups are presently called genomovars (Ursing *et al.*, 1995; Rossello-Mora & Amann, 2001).

Genomovars are commonly distinguished at a genome similarity level of 70 %, which is the typical level of genome similarity within a species (Wayne *et al.*, 1987). However, some authors believe that this value for genome variability within a species should be raised to 50 % (Ursing *et al.*, 1995; Vandamme *et al.*, 1996). According to this criterion, the *A. ferrooxidans* strains under study were divided into four genomovars: (1) strains ATCC 23270^T, ATCC 19859, TF-1292 and TFBk, with genome similarity levels of 59–96 %; (2) strains VKM B-1160, TFM, TFR-2, TFO, TFN-d and TFT, with genome similarity levels of 86–97 %; (3) strains TFY and TFL-2, with a genome similarity level of 92 %; and (4) strains TF-16, TFN, TFD, TFI and TFT-92, with genome similarity levels of 50–99 %. The remaining six *A. ferrooxidans* strains could not be assigned to any of the genomovars, since the similarity of their genomes to one another and to those of the strains comprising the genomovars was only 13–43 %.

Phylogenetic analysis of 16S rRNA genes

We determined nearly complete nucleotide sequences (about 1450 nt) of the 16S rRNA genes of five *A. ferrooxidans* strains: the type strain, ATCC 23270^T (genomovar 1), and strains TFN-d (genomovar 2), TFY (genomovar 3), TFI and TFD (genomovar 4). A comparative phylogenetic analysis of the 16S rRNA genes was performed with respect to a large group of *A. ferrooxidans* strains, two validly described species of the genus *Acidithiobacillus* (*Acidithiobacillus thiooxidans* and *Acidithiobacillus caldus*), a species of this genus that has not yet been validly described (*Acidithiobacillus cuprithermicus*) and several

strains of this genus whose 16S rDNA nucleotide sequences are available from the GenBank database.

According to the results of this analysis, most of the *A. ferrooxidans* strains, including the type strain, ATCC 23270^T, constituted a monophyletic cluster with a 100 % bootstrap value (Fig. 3). Within this cluster, *A. ferrooxidans* strains fell into three phylogenetic groups. The only exception was *A. ferrooxidans* strain IFO 14245, which showed sequence similarity of 97.8–98.7 % to the 16S rRNA genes of the other members of this cluster and fell into none of the groups. The strain *Acidithiobacillus* sp. SSP, which was not identified to the species level, was assigned to phylogenetic group III, while phylogenetic group IV included the strains *Acidithiobacillus* spp. NO-8, NO-25, NO-37 and KSC-1, which were also not identified to the species level. The major phylogenetic cluster included not only *A. ferrooxidans* strains but also several strains of *A. thiooxidans* (including the type strain of this species) and the unidentified strain *Acidithiobacillus* sp. THA, all of which constituted phylogenetic group V. The high bootstrap values of the branching points of the phylogenetic tree (not less than 87 %) confirm the statistical significance of the above phylogenetic grouping. DNA

Fig. 3. Unrooted phylogenetic tree of various representatives of the genus *Acidithiobacillus* showing the position of the *A. ferrooxidans* strains ATCC 23270^T, TFN-d, TFI, TFD and TFY. Bar, 5 nucleotide substitutions per 100 nucleotides (evolutionary distance). Numerals near internal branches refer to bootstrap replications (out of 100 resampling) confirming the grouping of the species to the right of the branch. Bootstrap values of less than 85 % are not shown. Square brackets indicate that the current identification of a given strain is not confirmed by 16S rRNA gene sequence data.

similarity levels within and between the phylogenetic groups were respectively 97.8–100 and 96.7–99.2 %. The highest level of intergroup divergence of 16S rRNA gene sequences (1.8–3.3 %) was observed for phylogenetic group V of *A. thiooxidans* strains.

The strains of *A. caldus*, including the type strain of this species, constituted another monophyletic cluster with a bootstrap value of 97 %. This cluster also included '*A. cuprithermicus*' (91.3–94.5 % similarity) and *Acidithiobacillus* spp. strains TC-1 and GO-1. The similarity of the 16S rRNA gene sequences of *A. caldus* strains within this cluster was 98.3–99.7 %, whereas the similarity of this cluster with respect to *A. ferrooxidans* and *A. thiooxidans* strains was considerably lower (88.1–96.7 %).

Some *A. ferrooxidans* and *A. thiooxidans* strains could not be affiliated with the major phylogenetic cluster. Strains [*A. thiooxidans*] DSM 612 and [*A. thiooxidans*] KCTC 8929P took an intermediate position between *A. ferrooxidans*, *A. thiooxidans* and *A. caldus*, showing 88.7–96.9 % 16S rRNA gene sequence similarity (square brackets indicate that the current identification of a given strain is not confirmed by 16S rRNA gene sequence data). Strain [*A. ferrooxidans*] LM2 was found to be close to the *A. caldus* type strain, DSM 8584^T (99.2 % similarity). On the basis of 16S rRNA gene sequence similarity, three *Acidithiobacillus* strains, [*A. ferrooxidans*] DSM 2392 (79.9–88.4 % similarity), [*A. thiooxidans*] KCTC 8928P (76.1–82.2 %) and [*A. thiooxidans*] KCTC 8930P (68.1–76.6 %), could not be assigned to this genus.

All newly studied *A. ferrooxidans* strains belonged to the major phylogenetic cluster, which also included *A. thiooxidans* strains (Fig. 3). The type strain of *A. ferrooxidans* fell within phylogenetic group I (99.3–100 % 16S rRNA gene sequence similarity), strain TFN-d fell within group II (99.7–99.9 %) and strains TFI, TFD and TFY fell within phylogenetic group III (99.0–100 %).

DISCUSSION

Comparative analysis of the 16S rRNA gene sequences of presently known *A. ferrooxidans* strains showed their close phylogenetic relatedness and therefore, their common evolutionary history. According to the results of this analysis, the previously unidentified *Acidithiobacillus* strains NO-8, NO-25, NO-37, KSC-1 and SSP should be assigned to the species *A. ferrooxidans*. The common origin of most *A. ferrooxidans* strains was confirmed by the low level of variability of the nucleotide composition of the DNA and the genome size of the strains studied. At the same time, strains of this species isolated from different habitats exhibited considerable genotypic diversity, which manifested itself as a noticeable divergence of their 16S rRNA gene sequences, a low level of similarity for total DNA and significant differences in the structure of chromosomal DNA revealed by PFGE.

The genotypic heterogeneity of the species *A. ferrooxidans* is evident even from the analysis of 16S rRNA gene sequences, which are generally much more conservative than the total bacterial genome sequence. This heterogeneity shows up in the grouping of strains belonging to this species into four phylogenetic groups. The data on DNA–DNA hybridization also indicate high genotypic divergence of *A. ferrooxidans* strains, which, with the exception of six independent considerably divergent strains, constitute four groups characterized by a high level of genome similarity (genomovars). These data agree well with the earlier findings of Harrison (1982), who also revealed five groups of *A. ferrooxidans* strains corresponding to genomovars and several strains that could not be affiliated with any of these groups. The high level of DNA–DNA relatedness (96 %) of the genomes of strain ATCC 19859 and the type strain ATCC 23270^T suggests that all the allied strains studied by Harrison and us constitute one genomovar and therefore, that the members of this genomovar must be widespread in nature. It can also be suggested that analysis of a larger group of *A. ferrooxidans* strains than those studied by Harrison and us may disclose several additional genomovars among these strains.

Analysis of 16S rRNA gene sequences of the strains studied by DNA–DNA hybridization by Harrison (1982) allowed us, in addition, to compare the results yielded by the two methods, and showed that *A. ferrooxidans* strains ATCC 19859 and F221 from phylogenetic group I belong to one genomovar, while strains IFO 14262, from phylogenetic group II, and IFO 14245, from a separate phylogenetic branch, represent two other genomovars.

Therefore, in general, the results of the DNA–DNA hybridization analysis of *A. ferrooxidans* strains do not contradict the results of analysis of 16S rRNA gene sequences, except that the latter analysis combines the representatives of two different genomovars (strain TFY from genomovar 3 and strains TFI and TFD from genomovar 4) into one phylogenetic group. The data presented provide evidence that there are at least two levels of genotypic divergence for *A. ferrooxidans*. Phylogenetic grouping based on analysis of 16S rRNA gene sequences represents a higher level of the genotypic divergence of *A. ferrooxidans* strains, whereas the division of these strains into genomovars in accordance with the species criterion of Wayne *et al.* (1987) reflects the lower level of their divergence.

The DNA–DNA hybridization analysis of *A. ferrooxidans* and *A. thiooxidans* strains carried out by Harrison (1982) showed low genome similarity (not more than 23 %). Moreover, most of the *A. thiooxidans* strains studied, including the type strain but excluding strain DSM 612, were found to belong to one genomovar. Analysis of the 16S rRNA gene sequences of *A. thiooxidans* strains, including the type strain, showed that they belong to the same phylogenetic cluster as the majority of *A. ferrooxidans* strains, constituting a separate phylogenetic

group equidistant from the other phylogenetic groups of this cluster. These data indicate a close relatedness of the species *A. ferrooxidans* and *A. thiooxidans* and allow the suggestion to be made that the latter species has diverged from the former species because of a gradual accumulation of phenotypic differences. According to the findings of Harrison (1982), *A. thiooxidans* strain DSM 612 does not belong to the same genomovar as other *A. thiooxidans* strains. Analysis of 16S rRNA gene sequences confirmed the incorrect taxonomic identification of this strain, as well as of some other strains such as KCTC 8929P and LM2 (the latter strain probably belongs to the species *A. caldus*). As for strains DSM 2392, KCTC 8928P and KCTC 8930P, they do not belong to the genus *Acidithiobacillus* at all.

Analysis of 16S rRNA gene sequences showed that the degree of relatedness of the species *A. caldus* to *A. ferrooxidans* and *A. thiooxidans* is lower than the degree of relatedness between the latter two species. The species '*A. cuprithermicus*' was affiliated with the phylogenetic cluster of the species *A. caldus* (with a bootstrap value of 98 %), which casts doubt on the existence of '*A. cuprithermicus*' as a separate species.

The genotypic heterogeneity of *A. ferrooxidans* and other species brings up the question of whether they appear as a result of microevolutionary processes within one species or arise through the convergence of several species. The data presented here, in particular those indicating the monophylicity of strains, suggest that their genotypic differences are due to microevolutionary processes. As is evident from the results of analysis of chromosomal DNA by PFGE, these processes may change the location of some genes, while their expression will be retained.

Another problem is the degree of intraspecies variability and the validity of using various physiological, biochemical and molecular genetic methods in the taxonomy of *A. ferrooxidans*. If we insist that the isolates studied in this work do belong to *A. ferrooxidans*, then this heterogeneous species is a combination of several groups of strains that have similar phenotypes but differ in their 16S rRNA gene sequences and in the composition of their total genomes. This difficulty is a specific example of the general taxonomic problem concerning agreement between the results of phenotypic and genotypic analyses. The taxonomic division of a phylogenetically heterogeneous species into genomovars and then into separate species occurs as the number of distinguishing phenotypic characteristics of these genomovars increases. It can be suggested that some genomovars of *A. ferrooxidans* may turn out to be separate species, as was the case with the species *A. thiooxidans*.

The DNA restriction profiles, obtained by PFGE, for *A. ferrooxidans* strains cannot adequately characterize the degree of their genome similarity, as is evident from the differences in the dendrograms (of these strains) that were generated using different algorithms. Even the most statistically significant clusters of these dendrograms do

not coincide with genomovars determined by DNA–DNA hybridization. This can be accounted for by the fact that PFGE is able to detect point mutations at restriction sites, changes in the locations of IST elements (insertion sequences; Yates & Holmes, 1987) and plasmid–chromosome recombinations, i.e. events that do not alter significantly the nucleotide sequence of the total genome. PFGE is the most sensitive method of DNA analysis applied to the evaluation of intraspecies genome divergence. Unlike chromosomal DNA restriction patterns, the less-sensitive 16S rDNA fingerprinting technique fails to differentiate *A. ferrooxidans* strains from each other, but can differentiate them from *A. caldus* strains (Kamimura *et al.*, 2001). In other words, study of the structure of the chromosomal DNA of *A. ferrooxidans* strains by PFGE cannot evaluate the degree of similarity of the genomes of these strains (and, therefore, the degree of their relatedness) but can detect genetic variability. This method is also of particular value in the individual characterization ('passportization') of microbial strains, especially those used in biohydrometallurgy, and for monitoring natural microbial communities.

It is interesting to ask whether there is a geographical correlation between the diversity of *A. ferrooxidans* strains and their origin. Like Harrison (1982), we failed to reveal such a correlation, although Harrison interpreted his data as an indication of selection of particular genotypes in microniches of particular geographical zones. Our data may be interpreted as indicating the existence of a certain degree of correlation between the similarity of the genomes of *A. ferrooxidans* strains and the mineralogical characteristics of their habitats. For instance, most strains of genomovar 2 (TFO, TFM, TFN-d and TFT) were isolated from gold–arsenic pyrite–arsenopyrite ores and concentrates, although some other strains of this genomovar, such as VKM B-1160 and TFR-2, were isolated from other natural substrates. It is conceivable that large-scale investigations of the genotypic features of indigenous bacterial strains in relation to the properties of their habitats might provide an insight into the role of natural substrates in the microevolution of *A. ferrooxidans*. This is illustrated by our data on the degree of adaptation of particular strains to oxidation substrates (Fe^{2+} , S^0 and sulfide minerals) (Ageeva *et al.*, 2001): each strain attained its own adaptation level determined by the strain's history. At the same time, each indigenous *A. ferrooxidans* strain isolated from an econiche with a particular mineralogical composition of oxidation substrates was characterized by its own chromosomal DNA restriction pattern. In biohydrometallurgical technologies, indigenous strains are always dominant in the pulps of gold and non-ferrous metal ores and concentrates, gradually displacing other introduced strains. Intense oxidative processes in these pulps activate microevolutionary processes and thus promote the formation of new *A. ferrooxidans* strains. These strains differ in terms of the structure of chromosomal DNA, plasmid composition, optimal pH and temperature

values for growth, the growth and substrate oxidation rates, resistance to heavy-metal ions, adaptability and adaptation thresholds. This may explain the failure of attempts to introduce, into natural environments, artificially constructed microbial strains that are highly productive under laboratory conditions.

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