engineering proceedings

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Approaches to Improve the Bioleaching of Arsenopyrite Flotation Concentrate with *Acidithiobacillus ferrooxidans*: A Comparison of Two Strains of Different Origin †

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† Presented at the 3rd International Electronic Conference on Processes—Green and Sustainable Process Engineering and Process Systems Engineering (ECP 2024), 29–31 May 2024; Available online: https://sciforum.net/event/ECP2024.

Abstract: Bacterial leaching is a well-known green technology proposed for the extraction of valuable metals into solution. However, this biotechnology has some "bottle neck" problems too. Arsenopyrite, a gold-bearing ore, is a refractory mineral material that is hardly soluble and contains toxic arsenic compounds which decrease any bioleaching production. The most common biotechnology used for this process is provided with the species *Acidithiobacillus ferrooxidans*: autotrophic and acidophilic bacterial strains including ones resistant to inorganic arsenic compounds. Common attempts to dissolve arsenopyrite with increasing volumes of sulfuric acid provoke acidification of the environment and its pollution with toxic compounds. In our research, we compared two *A. ferrooxidans* strains of different origin: TFBk isolated from arsenopyrite ore (pre-adopted to arsenic), the Republic of Kazakhstan, and ShA-GNK isolated from silicate nickel-ferrous ore (laterite, without arsenic), the Russian Federation. The studied genomes of both strains showed the presence of the same genes providing defense against arsenic compounds, but the resistance to toxic compounds was higher in the strain that had never been exposed to any high As concentration under the natural conditions. Both strains showed a weak oxidation of the arsenopyrite flotation concentrate (AFC). In accordance with the published data, supplementation of the medium with formate stimulated bacterial growth in the culturing medium. However, this supplementation to the leaching solution decreased the arsenopyrite oxidation during the first stage of the AFC leaching because formate was used as an alternative energy substrate, but subsequently gave a higher iron yield later.

Keywords: green technology; bioleaching; arsenopyrite; *Acidithiobacillus ferrooxidans*; strain origin; genes of resistance; supplements

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Citation: Abashina, T.; Yachkula, A.; Shaikin, A.; Vainshtein, M. Approaches to Improve the Bioleaching of Arsenopyrite Flotation Concentrate with *Acidithiobacillus ferrooxidans*: A Comparison of Two Strains of Different Origin. *Eng. Proc.* **2024**, *67*, 60. https://doi.org/10.3390/engproc2024067060

Academic Editor: Young-Cheol Chang

Published: 30 September 2024

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1. Introduction

Bioleaching of valuable metals is a well-known green technology. Nevertheless, its application showed a row of problems related with the presence of toxic elements in various mineral raw materials (low-grade ore, flotation concentrates, etc.). Arsenopyrite, a gold-bearing ore, is a refractory mineral material which is hardly soluble and contains toxic arsenic compounds; the latter property strictly worsens bioleaching results [1]. The most common bioleaching agents for arsenopyrite leaching are the bacteria *Acidithiobacillus ferrooxidans* which are autotrophic, acidophilic, and resistant to inorganic arsenic compounds [2]. Attempts to dissolve arsenopyrite with increasing volumes of sulfuric acid and supplements of iron, which can be used by iron-oxidizing leaching bacteria as an additional energy source, provoke the following acidification of the environment and its

pollution with excess of iron [3]. Comparison of various *A. ferrooxidans* strains isolated from different sulfide ores, including arsenopyrite, showed their resistance for the mineral arsenic compounds is widely expanded [4].

Researchers made numerous attempts to intensify leaching, including use of various strains [5,6], technical improvements in the leaching process (for example, the solid–liquid ratios in the pulp, changes in the process temperature or aeration and redox conditions [7–11], etc.), and use of stimulant supplements including formate, which was used as energy substrate for the autotrophic bacteria [12–17]. At the same time, a number of researchers rely on the general proposal that organic compounds, including some of low molecular weight, inhibit the growth of autotrophic bacteria. In particular, it was shown that the addition of formate reduced iron oxidation by *A. ferroxidans* bacteria, which was interpreted by the researchers as bacterial suppression [18–20].

This paper presents comparison of two strains of *A. ferrooxidans* which are isolated from two principally different ores, namely the strain TFBk from arsenopyrite deposit (sulfide ore with As) and the strain ShA-GNK from silicate nickel-ferrous ore (laterite ore without As). The research goals were to compare (i) bacterial genomes for their resistance to arsenic, (ii) bacterial leaching arsenopyrite flotation concentrate, and (iii) bacterial response on the formate supplementation as a possible additional energetic substrate for the autotrophic bacteria *A. ferrooxidans*.

2. Materials and Methods

2.1. Materials

All experiments on bioleaching were carried out with arsenopyrite flotation concentrate (AFC) as a model of the As-bearing refractory mineral material. The AFC samples were received from the Bioprom Technologies Ltd., (Stepnogorsk, the Republic of Kazakhstan. The company also presented data on the content of the chemical elements in the AFC which are important for the industrial recovery of gold (Table 1). First of all, these elements include arsenic because it is toxic for humans, microorganisms, and the environment as a whole. The content of iron in comparison with arsenic permits us to suggest and calculate some share of arsenopyrite mineral by the formula FeAsS. According to this formula, the AFC also could contain other arsenic-bearing minerals in addition to arsenopyrite.

Table 1. Element content in arsenopyrite concentrate.

Element	Content, mg/kg
Arsenic	77,279
Iron	24,740
Copper	953
Nickel	150
Gold	100

2.2. Microorganisms

As it has already been mentioned above, two strains of *A. ferrooxidans* were used in the presented research investigations, namely the (i) strain TFBk that had been isolated from the Bakyrchik arsenopyrite deposit, the Republic of Kazakhstan, received from Dr. A. Bulaev and deposed in the All-Russian collection of Microorganisms (VKM) [4]; and (ii) the new strain ShA-GNK isolated from the iron-nickel silicate ore/laterite nickel ore (Orenburg region, the Russian Federation).

2.3. Identification of the Strain ShA-GNK by Molecular Methods

Then, molecular identification was conducted using sequencing of the 16S rDNA gene analysis. Genomic DNA was isolated as described by [21] and the extracted DNA was quantified by NanoDrop 2000c (Thermoscientific, Waltham, MA, USA). The uni-

versal primers 16S rRNA prokaryotes: 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) [21] were used for amplification of the 16S rDNA genes.

The amplified DNA was purified as described by [21]. Sequencing of obtaining fragments was performed on an Applied Biosystems Genetic Analyzer automatic sequencer.

Primary phylogenetic screening of the obtained sequences was performed using the EzBioCloud database (www.ezbiocloud.net, accessed on 1 September 2023) and the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 1 September 2023). For phylogenetic analysis the nucleotide sequences of the 16S rRNA gene obtained for the ShA-GNK strain were manually aligned with the sequences of reference strains of the related microorganisms. The 16S rRNA gene of the ShA-GNK strain has been deposited in the NCBI GenBank database under the number OR946237.

Sequencing for the whole genomic DNA of the strain ShA-GNK was performed on Illumina HiSeq500 equipment at the BioSpark biotechnological laboratory (Troitsk, Moscow, Russia).

Average nucleotide identity (ANI) value was calculated using the EzBioCloud web service [22]. The value of digital DNA-DNA hybridization (DDH) was calculated using the Genome-to-Genome Distance Calculator 2.1 as described by [23]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) [24] was used for functional annotation.

2.4. Analyses of Genomes and Genes

Analyses of genomes and genes were performed with support of the BV-BRC institution https://www.bv-brc.org/, accessed on 1 September 2023.

2.5. Bench Scale Experiments on the Bioleaching Arsenopyrite Flotation Concentrate (AFC)

The bioleaching experiments were carried out in the stationary flasks at a bench scale. The pulp density, presented as its relation of solid and liquid phases (S:L), was 1:10 (5 g of concentrate per 50 mL of liquid medium) for the strain ShA-GNK. The duration of the incubation was 18 days at 24 °C. To carry out the experiments, we used a liquid nutrient medium that was adapted after classical Silverman-Lundgren 9K and contained the following components, g/L: $(NH_4)_2SO_4$—3.0, KCl—0.1, K_2HPO_4—0.5, $MgSO_4 \cdot 7H_2O$—0.5, $Ca(NO_3)_2$—0.01, $FeSO_4 \cdot 7H_2O$—0.0, 10 N H_2SO_4—4.5 mL/L. The used reagents were produced by Sigma-Aldrich/Merck (Darmstadt, Germany) or Reakhim (Moscow, Russia). In contrast to the initial classical medium composition, ferrous iron was replaced with the arsenopyrite concentrate. In the special experiments with formate, formic acid was added. The initial pH was adjusted by adding 5 mL of concentrated sulfuric acid per 1 L to pH 2.0. The starting cultures used as an inoculum has been pre-grown in the same medium. Total number of bacterial cells was calculated using direct cell counting with the light phase-contrast microscopy using LM-2 microscope (LOMO, Saint-Petersburg, Russia) in preparation with the cover glass, magnification ×100 ×1.5 ×15.

2.6. Experiments on the Resistance the Strains to Heavy Metals

The strains were cultivated in the mentioned adapted medium supplemented with various concentrations of heavy metal salts, namely $NiCl_2 \cdot 6H_2O$, $CuSO_4$, Na_3AsO_4 (0; 0.1; 0.5; 1.0; 1.5%). The strains were cultivated at 30 °C.

2.7. Chemical Analyses

Degradation of the flotation concentrate was evaluated by the increase in the sulfate concentration in the leaching medium. The sulfate content in the solution was estimated with the high-pressure ion chromatography using a Metrohm 761 Compact IC (Metrohm, Herisau, Switzerland), equipped with a conductometric detector and a chemical anion suppression module. A separation column Metrohm Metrostep A Supp 5 100/4.0 mm (Switzerland) was used. The eluent was a carbonate buffer with concentrations of 3.2 mM

Na_2CO_3 and 1.0 mM $NaHCO_3$. Analysis time was 30 min. The MultiChrom 3.4 Pro software package (Ampersend, Moscow, Russia) was installed to control the process.

2.8. Statistical Analyses

Experimental analyses were performed in triplicate. Data are presented as arithmetic means where the confidence intervals did not exceed ±5%. Statistical analysis was performed using the standard methods provided by Excel (https://support.microsoft.com/en-us/office/use-the-analysis-toolpak-to-perform-complex-data-analysis-6c67ccf0-f4a9-487c-8dec-bdb5a2cefab6, accessed on 14 May 2024). ANOVA was used to estimate the significance of the data. All presented data are reliable ($p > 99\%$).

3. Results and Discussion

3.1. Identification and Relationships of the Strain ShA-GNK

The new strain ShA-GNK was identified by molecular genetic methods as *A. ferrooxidans* (Figure 1). As it is clearly seen from Figure 1, it is close to the type strain *A. ferrooxidans* ATCC 23270 and very close to the second strain presented in the current research—*A. ferrooxidans* TFBk.

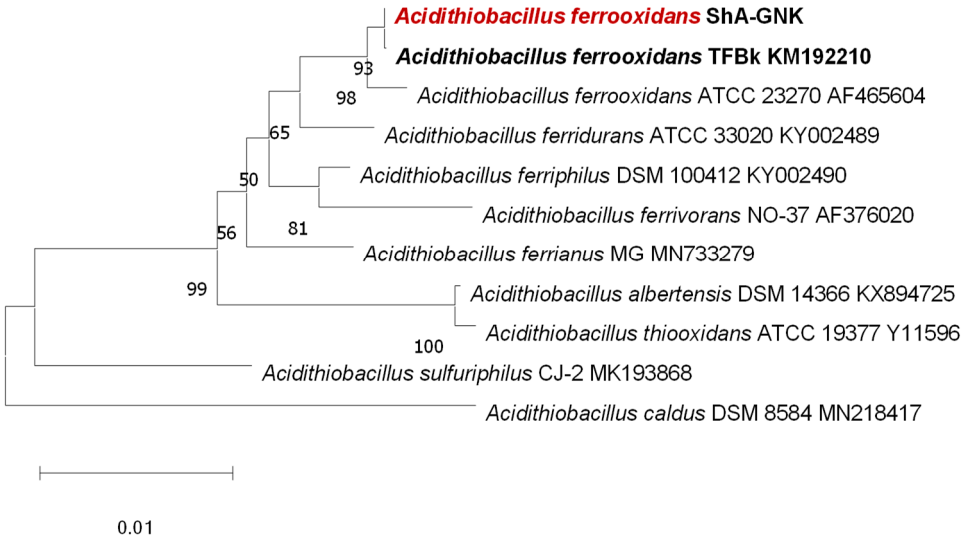


Figure 1. Neighbor-joining phylogenetic tree based on the 16S rRNA gene sequences and presents both the position of the strain Sha-GNK among the members of the genus *Acidithiobacillus*, and the relationships of the strain. The scale bar is equal to 0.01 substitutions per nucleotide position.

It has to be mentioned that all these strains have a different origin by the sources of their isolation, and thus by their pre-adaptation. The type strain *A. ferrooxidans* ATCC 23270 was isolated from acid bituminous coal mine effluent from Pennsylvania, the USA (https://bacdive.dsmz.de/strain/118, access on 14 May 2024); the strain TFBk was isolated from an arsenopyrite deposit from Bakyrchik, the Republic of Kazakhstan [4]; the strain ShA-GNK was isolated from silicate iron-nickel ore from Orenburg region, the Russian Federation. According to so different habitats and different natural sources of energy, it was also possible to discover some different pre-adaptations and varying abilities of the strains. Thus, the strain TFBk has already been adapted both to S-compounds and Fe as energy sources and has survived in the presence of high As concentration, while the strain ShA-GNK lived in the absence of sulfides and arsenic and was pre-adapted to nickel.

3.2. Bioleaching Arsenopyrite Flotation Concentrate (AFC)

The AFC degradation was evaluated by the accumulation of dissolved sulfate in the leaching medium. The experiments showed that the application of *A. ferrooxidans* ShA-GNK

resulted in 4-fold higher production of dissolved sulfate in comparison with the chemical leaching (Table 2).

Table 2. Leaching of the arsenopyrite flotation concentrate (AFC) by *A. ferrooxidans* ShA-GNK calculated per 1 kg AFC/L.

Sulfate, Concentration in the Leaching Solution	Initial	Final		
		Chemical Oxidation (Sterile Blank)	Chemical and Biological Oxidation without Formate	Chemical and Biological Oxidation with Formate
µS/cm·sec (peak area)	152.01	183.61	729.93	1058.42
mg/L per 1 kg AFC	36,600	44,400	176,200	255,495

During sterilization of the flotation concentrate samples by autoclaving, 36.6 g sulfates/L (calculated from the specimen to 1 kg of AFC) was passed into the solution. Subsequent chemical oxidation over 18 days calculated for 1 kg AFC amounted to an additional 7.8 g sulfates/L. Biological oxidation with the over 18 days, excluding chemical oxidation, amounted to 131.8 g sulfates/kg-L. The experiments showed that the application of *A. ferrooxidans* ShA-GNK, with the arsenopyrite flotation concentrate as a single source of energy, resulted in 4-fold higher production of dissolved sulfate.

It was shown in our former publication [4] (Table 4) that the bio-oxidation by the *A. ferrooxidans* strain TFBk amounted to 1170 mg of sulfate/L from 40 g AFC in 15 days, which in calculation from the specimen to 1 kg of AFC, was equal to 29.25 g sulfates/L. Thus, the sulfate output from the AFC was 13 w.% with the ShA-GNK strain and only 2.9 w.% with the TFBk strain. These results were obtained for the specimens taken from the same AFC sample but not simultaneously. In general, they can be considered as approximate. Nevertheless, we can conclude that the TFBk strain isolated from the Kazakhstan arsenopyrite ore had no advantages in comparison with the strain ShA-GNK isolated from the laterite ore.

3.3. Bioleaching Arsenopyrite Flotation Concentrate (AFC) with Formate Supplementation

In the last century, it was shown that the bacteria *A. ferrooxidans* are capable of oxidizing formate and using it as an energy source [15]. However, the bacteria do not use this compound as a direct source of carbon but oxidize it to carbonate forms. Considering the low solubility of atmospheric carbon dioxide in the acidic medium for *A. ferrooxidans* (pH 1.5–2.5), it can be assumed that the secondary carbonate formed during formate oxidation also stimulates bacterial growth. In our work with *A. ferrooxidans* [4] and, as well, with other autotrophic thiobacilli *Guyparkeria halophila* [12], we confirmed that these microorganisms can oxidize formate and that this substrate can stimulate growth.

As was mentioned above, these results seem to contradict the data of other researchers who showed that the addition of formate to an iron-containing medium increased iron oxidation by *A. ferroxidans* [18–20]. The authors interpreted this decrease as bacterial suppression. However, the contradiction here is apparent, since the decrease in iron oxidation just means the transition of bacteria to an alternative energy source, namely from the Fe-oxidation to formate oxidation.

In our bioleaching experiments, an additional source of energy for bacteria, formate, was added to the leaching solution. Bacterial consumption of formate in 18 days measured with ion chromatography was 15.52%. At the same time, the biological oxidation of arsenopyrite over 18 days (excluding some chemical oxidation) amounted to 449 mg sulfates/L, which was 31.87% less than with formate. Thus, the presence of formate decreased the use of the mineral substrates at the first stage only when formate is not yet exhausted. Similar results were achieved in our former experiments with the strain TFBk [4]. It seems that the bacteria switched to a noticeable extent to the additionally introduced alternative energy source, namely, in our case, formate.

Thus, we showed that the possible use of formate to stimulate the bioleaching process is limited at the first stage—as long as formate is not exhausted. These data are in good agreement with the patent claimed by Pronk and co-authors [16], where the use of formate was proposed for preliminary growth of the culture, but not for leaching. However, we can state, also in good agreement with the same patent [16], that during the formate consumption, the activity and number of *A. ferroxidans* cells rapidly increased, which ensured the better and later oxidation of iron. As a consequence, the later result after formate usage was higher than without formate (Table 2).

3.4. Resistance to Arsenic Compounds and Its Genetic Basis

The main advantage of one strain over others for use in the bioleaching process is the mechanism of resistance of the strain to toxic compounds, heavy metals, or, in the case of AFC, to arsenic. The "simplest" and most common—or, at least, the most expanded upon in bacteria—mechanism of As-resistance is provided with the Ars (arsenic-resistance system). In accordance with its name, this system is encoded with the *ars* operon. Its role in bacterial As-resistance has already been investigated by numerous researchers. The most remarkable feature of this system is the existence of different mechanisms for its implementation in various strains [22].

We discovered the Ars gene group in both studied strains, despite their very different origins. As far as the strain TFBk was isolated from arsenopyrite, the Ars gene presence was logical. However, the natural substrate for the ShA-GNK strain was ferrous iron while arsenic compounds were never found in the laterite ore. Anyway, the ShA-GNK genome showed presence of the same genes. The *ars* gene group of the strains included *arsA*, *arsB*, *arsC* (*arsC1*, *arsC2*), *arsD*, *arsH*, *arsM*, *arsR*. Despite the different functions of these genes, they work together as a united single complex providing the As-resistance. Some of these genes (*arsC*) provide production and activity of the arsenate reductase, i.e., they reduce As^{5+} to As^{3+}. Two different two arsenate reductases encoded with the *arsC1* and *arsC2* were discovered in both studied ShA-GNK and TFBk genomes, but in the genome strain ShA-GNK we discovered *arsC3*. Other mentioned ars genes mainly provide a pump which carries the reduced As-form out of the bacterial cell, as well as a balance between the carriers and antiporters of the As-compounds.

According to the presented data, we have to conclude that both studied strains, ShA-GNK and TFBk, isolated from the drastically different habitats (arsenopyrite and laterite ores), have the same genetic Ars defense against arsenic. It is interesting also that both strains showed the presence of the *arsM* genes which provide production of the arsenic methyltransferases, the biological role of which is not clear yet.

In addition, when analyzing the genomes of the strains, genes for resistance to heavy metals, such as chromium (*chr*AC), copper (*cop*DZ), Cobalt/zinc/cadmium resistance protein (*czc*D) and nickel, were discovered.

In experiments on the resistance of the strains to heavy metals and arsenic, data were obtained that showed that the strain Sha-GNK was more resistant than the strain TFBk (Figure 2).

Thus, despite the same set of resistance genes, the resistance threshold differs in different strains.

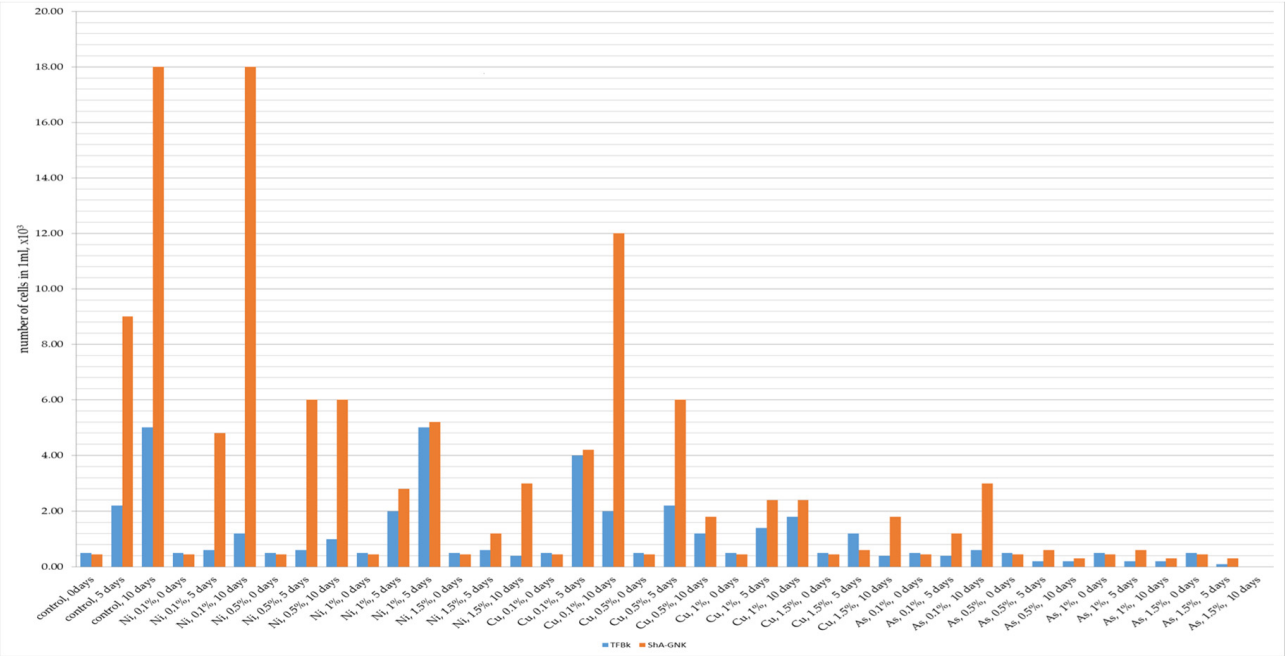


Figure 2. Resistance of the strains ShA-GNK (orange columns) and TFBk (blue columns) to heavy metals and arsenic. The added toxic chemical elements, their concentrations, and their incubation time (days) are shown in the figure.

4. Conclusions

The genus *Acidithiobacillus* includes a group of obligate acidophilic chemolithotrophic Gram-negative bacteria that carry out their vital functions by oxidizing reduced inorganic sulfur compounds such as thiosulfate, sulfide, and elemental sulfur. This property is used for industrial purposes in the bioleaching of valuable metals from sulfide ores. The most well-known bioleaching agent is the species *A. ferrooxidans* which is preferable due to its abilities to use ferrous iron (Fe^{2+}) as another energy substrate and to be active at extremely low pH.

It is obvious that the *Acidithiobacillus* species used for the ore bioleaching must have certain highly effective and advanced metal-resistance mechanisms and the selection of industrial strains have to take this feature into account. Working in this area of investigations, the researchers [25] have already shown that representatives of the genus *Acidithiobacillus* showed a wide range of genes which provide resistance to various metals. Most likely, the presence of these genes is caused by an evolutionary adaptation to difficult conditions and is realized both via horizontal gene transfer and gene duplication.

After analyzing genomes of the Sha-GNK and TFBk strains, we suggested that the Sha-GNK strain has a higher degree of resistance to arsenic and toxic metals due to additional copies of resistance genes in the strain chromosome. On the whole, it is shown that bacterial resistance to arsenic and heavy metals can be provided with a complex of the same genes that are distributed in the *A. ferrooxidans* strains of principally different origin. Moreover, these genes are also distributed in other representatives of the order *Acidithiobacillales*, including other species of the genus and representative of the relative family [25]. Despite the fact that genes with resistance to arsenic are widespread in the genus *Aciditniobacillus*, the task of the industrial researcher working in area of bioleaching of arsenopyrite has to include a selection of the strains by their resistance to high concentrations of arsenic and concomitant leaching by heavy metals.

It seems that the natural habitancy and pre-adaptation of an isolated strain is not a guarantee of its abilities. We discovered that the *A. ferrooxidans* strain isolated from the laterite/iron-nickel ore showed no less ability to leach AFC than the strain isolated from

arsenopyrite ore. The search and selection of new effective leaching strains continues to present an important objective for mining microbiologists.

The ability of autotrophic leaching agents to oxidize formate as an energy substrate is an interesting approach to stimulate *A. ferrooxidans*. The oxidation of formate by autotrophic bacteria serves not only as a source of energy but also directly provides the cells with carbon dioxide directly. However, the possible new biotechnologies must take into account the appearance of some additional stages related to switching from an inorganic substrate to formate, and back.

Author Contributions: Conceptualization, T.A. and M.V; methodology, T.A., A.Y. and A.S.; validation, T.A.; investigation, T.A., A.Y. and A.S.; resources, T.A.; data curation, T.A. and M.V.; writing and editing, T.A. and M.V; supervision, T.A.; project administration, T.A.; funding acquisition, T.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Russian Science Foundation, No. 23-24-00380 (https://rscf.ru/project/23-24-00380/, accessed on 10 May 2024).

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

Conflicts of Interest: The authors declare no conflicts of interest.

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