






Article

Attempts to Stimulate Leaching Activity of *Acidithiobacillus ferrooxidans* Strain TFBk

Alyona Yachkula ¹, Olga Rozova ¹, Tatiana Abashina ¹, Mikhail Vainshtein ¹, Denis Grouzdev ²
and Aleksandr Bulaev ^{3,*}

¹ Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS, Federal Research Center “Pushchino Scientific Center for Biological Research of the Russian Academy of Sciences”, Prospect Nauki 5, 142290 Pushchino, Russia

² SciBear OU, 10115 Tallinn, Estonia

³ Research Center of Biotechnology, Russian Academy of Sciences, 119071 Moscow, Russia

* Correspondence: bulaev.inmi@yandex.ru; Tel.: +7-499-135-04-21

Abstract: Autotrophic acidophilic bacteria *Acidithiobacillus ferrooxidans* is a model species for studying metal bioleaching from low-grade sulfide ores and concentrates. Arsenopyrite gold-bearing concentrates are refractory and often processed using biohydrometallurgical approaches; therefore, it is important to develop methods to improve arsenopyrite bioleaching. In the present work, we have studied the possibility of improving arsenopyrite concentrate bioleaching by the strain of *A. ferrooxidans*. For this purpose, we have analyzed the genome of the strain *A. ferrooxidans* TFBk to reveal the genes potentially important in the bioleaching process. Genes determining resistance to arsenic, as well genes involved in the utilization of C₁-compounds and resistance to oxidative stress, were revealed. Therefore, the possibility of increasing the rate of arsenopyrite concentrate bioleaching using C₁-compounds (methanol and formate) was studied. Formate was able to increase both the biomass yield of the strain *A. ferrooxidans* TFBk as well as the bioleaching rate. In addition, the effect of redox potential increase by means of the addition of sodium persulfate in the medium on arsenopyrite concentrate bioleaching was studied. It was shown that the addition of 0.1% sodium persulfate stimulated strain growth, while a higher concentration inhibited it. Despite this, the rate of concentrate bioleaching increased in the presence of 0.5–1.0% of persulfate, which may be explained by the interactions of added oxidizer with concentrate components.

Keywords: *Acidithiobacillus ferrooxidans*; genome analysis; arsenic resistance; bioleaching; growth stimulation; formate; oxidizer; persulfate



Citation: Yachkula, A.; Rozova, O.; Abashina, T.; Vainshtein, M.; Grouzdev, D.; Bulaev, A. Attempts to Stimulate Leaching Activity of *Acidithiobacillus ferrooxidans* Strain TFBk. *Minerals* **2022**, *12*, 1051. <https://doi.org/10.3390/min12081051>

Academic Editor: Naoko Okibe

Received: 28 July 2022

Accepted: 17 August 2022

Published: 20 August 2022

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Metal extraction from low-grade ores, refractory sulfide concentrates, and different mining wastes using pyrometallurgical approaches is often not profitable, while hydrometallurgical methods in this case are more suitable both from economic and technological points of view. In the last decades, bioleaching methods have been widely used. Bioleaching processes are based on microbially mediated destruction/oxidation of mineral components (mainly sulfide minerals) performed by acidophilic bacteria and archaea. Successful application of acidophilic microorganisms for mineral raw materials processing is possible since (1) their growth optima is in the low pH level range that also corresponds to the optima of active metal leaching; (2) most of the acidophilic microorganisms used for bioleaching are chemolithoautotrophs, i.e., they do not require organic nutrients for growth and, on the contrary, use components of sulfide minerals to gain energy for metabolism that in turn simplify its participation in technological processes [1,2].

Since the 1950s, iron- and sulfur-oxidizing microorganisms have been used on an industrial scale for bioleaching processes [1]. Bacteria *Acidithiobacillus ferrooxidans* (formerly *Thiobacillus ferrooxidans*) are first described as acidophilic bacteria oxidizing both ferrous

iron and reduced sulfur compounds. These bacteria are model organisms, which are successfully used for bioleaching studies up to now due to their properties (pH optima < 2, capability of ferrous iron and sulfur oxidation, resistance to various metal and metalloid ions) [2].

In general, optimization of metal extraction is a complex task, which may be solved by different methods depending on the features of mineral raw materials and extracted metals. One of the most complex problems is the processing of arsenic-containing ores and concentrates. To date, a lot of different arsenic minerals have been described; arsenopyrite (FeAsS), enargite (Cu₃AsS₄), tennantite (Cu₁₂As₄S₁₃), realgar (As₄S₄), and auripigment (As₂S₃) are the most widespread ones [3]. Arsenopyrite is the most widespread arsenic mineral, which contains up to 45% arsenic [4–6] and gold in the form of fine particles in a crystal lattice [7]. Acid mine drainage (AMD) formed due to arsenopyrite biooxidation is a serious environmental issue as it is highly toxic due to the arsenic presence [6,8]. In this regard, the study of the resistance of leaching strains to arsenic and their application is one of the important areas of hydrometallurgy and microbial ecology.

In general, the main approaches for the intensification of metal bioleaching involve: (1) the selection of strains and study of their metabolic and genetic potential [9,10]; (2) the study of the use of stimulating additives for the activation of microorganisms and microbially mediated processes [11–18]; (3) the optimization of physicochemical conditions of the process [19].

In modern hydrometallurgy, the search for new industrial strains as well as attempts to intensify bioleaching by stimulating bacteria with introduced chemical compounds has continued for decades [15–17]. *A. ferrooxidans* is one of the major microorganisms in consortia used for the industrial leaching of various valuable metals, which is an obligate autotroph. Three decades ago, J. Pronk et al. [20] already showed that the growth of *A. ferrooxidans* (formerly *Thiobacillus ferrooxidans*) can be stimulated with formic acid. According to the literature data [20], formic acid as an organic compound was not used as a substrate for growth but provided some energy requirements. Moreover, the mentioned authors also proposed pre-cultivation of *A. ferrooxidans* with formate for subsequent leaching of metals from ores and applied for a patent regarding this approach [21]. Developing these works, we obtained similar results for neutrophilic leaching bacteria [15]. Moreover, it was shown that genomes of some representatives of the genus *Acidithiobacillus* contain the genes involved in the utilization of C₁-compounds [10]. Thus, C₁-compounds, including formate and methanol, may be considered stimulating additives that increase the activity of the bacteria of the genus *Acidithiobacillus*, involved in the bioleaching process.

Another approach, which may be used to improve the bioleaching of different ores and concentrates, is the application of additional oxidizing agents, which are able to increase redox potential and enhance the bioleaching rate [18,22]. Persulfate as a peroxide compound is a more powerful oxidizing agent than dissolved atmospheric oxygen. It was shown that persulfate may be used as a disinfectant, inhibiting bacterial growth and activity [23–27] and for the destruction of a wide range of pollutants (including processes of bioremediation in which persulfate is applied in combination with microorganisms) [28–31]. It has also been shown that persulfate interacts with different sulfide minerals as a strong oxidant [25,32–34]. Therefore, persulfate may be used for oxidative leaching sulfide ores and concentrates in different hydrometallurgical processes [32–38], including bioleaching of metals from metallurgical industry sludge [22]. Thus, the application of persulfate may be considered as an approach to increase the rate of bioleaching of sulfide ore and concentrates. At the same time, it can act as an agent with an antimicrobial effect. Therefore, its effect on the growth of different microorganisms possessing various systems involved in oxidative stress resistance should be studied.

The goal of the present work was to study acidophilic autotrophic strain *Acidithiobacillus ferrooxidans* TFBk and the possibility to increase its bioleaching activity. For this purpose, the genes determining arsenic and oxidative stress resistance, as well as utilization of C₁-compounds, were analyzed, while the effect of C₁-organic compounds of formate and

methanol, as well as the effect of the additional oxidizing agent persulfate on the growth of *A. ferrooxidans* TFBk and bioleaching of metals from arsenopyrite concentrate, were studied.

2. Materials and Methods

2.1. Concentrate

The floatation arsenopyrite concentrate as well as information on the content of various metals and arsenic (Table 1) were provided by Bioprom Technologies Ltd. (Stepnogorsk, Republic of Kazakhstan). The amounts of metals, which are important from an industrial point of view, as well as arsenic, which is important as the main toxicant that interferes with bioleaching, were determined. The weight ratio of iron and arsenic in the concentrate showed a higher content of arsenic than its calculated presence according to the arsenopyrite formula FeAsS. Thus, the concentrate could also contain other arsenic minerals, for example: lollingite (FeAs₂), orpiment (As₂S₃), realgar (AsS), etc.

Table 1. Element content in arsenopyrite concentrate.

Element	Content, mg/kg	Method of the Analysis (GOST)
Arsenic	77,279	GOST R ISO 27085-2013
Aluminum	25,456	GOST R ISO 27085-2012
Iron	24,740	GOST R ISO 27085-2012
Calcium	16,672	GOST R ISO 27085-2012
Magnesium	11,893	GOST R ISO 27085-2012
Potassium	6801	GOST R ISO 27085-2012
Sodium	995	GOST R ISO 27085-2012
Copper	953	GOST R ISO 27085-2012
Silicon	793	GOST R ISO 27085-2019
Manganese	732	GOST R ISO 27085-2012
Zinc	514	GOST R ISO 27085-2012
Phosphorus	405	GOST R ISO 27085-2012
Cobalt	278	GOST R ISO 27085-2015
Nickel	150	GOST R ISO 27085-2012
Strontium	139	GOST R ISO 27085-2012
Barium	124	GOST R ISO 27085-2012
Gold	100	GOST R ISO 27085-2020
Lead	88	GOST R ISO 27085-2018
Chromium	32.3	GOST R ISO 27085-2012
Cadmium	19.0	GOST R ISO 27085-2014
Silver	12.3	GOST R ISO 27085-2012
Molybdenum	7.1	GOST R ISO 27085-2016

2.2. Microorganisms

We used the acidophilic chemolithoautotrophic strain *A. ferrooxidans* TFBk, isolated from the gold-bearing pyrite-arsenopyrite concentrate obtained from the ore of the Bakyrchik deposit (Republic of Kazakhstan), and deposited in the Laboratory of Chemolithotrophic Microorganisms of Winogradsky, Institute of Microbiology, Research Centre of Biotechnology RAS (Moscow, Russia) [39,40]. The strain was cultivated in the Silverman-Lundgren 9K medium [41] containing the following components (g/L): (NH₄)₂SO₄—3, KCl—0.1, K₂HPO₄—0.5, MgSO₄·7H₂O—0.5, Ca(NO₃)₂—0.01, FeSO₄·7H₂O—44.22, 5M H₂SO₄—4.5 mL/L. We used reagents produced by Sigma-Aldrich/Merck (Darmstadt, Germany) and Reakhim (Moscow, Russia).

Recently, the strain has also been deposited in the All-Russian Collection of Microorganisms (VKM) as *A. ferrooxidans* VKM B-3655 (IBPM RAS, Pushchino, Moscow region, Russia).

2.3. Molecular Genetic Analysis of Arsenic Resistance Genes

Genomic DNA was isolated from cells using the Fungal/Bacterial DNA Kit (ZymoResearch, Irvine, CA, USA) according to the manufacturer's recommendation. Arsenic

resistance genes were amplified by PCR using specific primers developed in the present work.

Based on the genomes of different strains of *A. ferrooxidans* available in the database NCBI, degenerate primers for this species were compiled:

- | | | |
|-----|---|-------------------------|
| (1) | <i>arsC</i> -F | ATGAAAACCCYGRAMATCCT |
| | <i>arsC</i> -R | GTGCCGATGCGCKCCAGTTC |
| | (PCR fragment length of <i>arsC</i> gene was about 500 bp) | |
| (2) | <i>arsB</i> -F | ATGCTGGCSGTMGYCATATT |
| | <i>arsB</i> -R | TCAAGCCAGCGGCARCCACCA |
| | (PCR fragment length of <i>arsB</i> gene was about 1300 bp) | |
| (3) | <i>arsM</i> -F | ATGAGCMAACAGAAAYGCCTGCT |
| | <i>arsM</i> -R | AGCCACCAGGYTTGAKCGCCTC |
| | (PCR fragment length of <i>arsM</i> gene was about 860 bp). | |

2.4. Genome Analysis

Genomic DNA for the sequencing was extracted using the QIAamp DNA minikit (Qiagen, Hilden, Germany). The DNA libraries were constructed with the MGIEasy universal DNA library prep set, according to the protocol for the kit. Sequencing of genomic DNA was carried out using the DNBSEQ-G400 platform (MGI Tech, Shenzhen, China), with 150-bp paired-end reads. A total of 4,013,413 paired-end reads were obtained from strain TFBk. These raw sequence reads quality was checked with FastQC v.0.11.9 (Illumina, San Diego, CA, USA), and low-quality reads were trimmed with Trimmomatic v.0.39 [42], using the default settings for paired-end reads. These quality-filtered reads were then de novo assembled with SPAdes v.3.15.0 (CAB SPbU, St. Petersburg, Russia) using the default settings [43]. The resulting assembly was quality assessed with QUAST v5.0 (CAB SPbU, St. Petersburg, Russia) [44]. The genome coverages were as estimated by QualiMap 2 v.2.2.2 [45] and Bowtie 2 v.2.3.5.1 [46]. The estimated completeness was evaluated with CheckM v.1.1.3 [47]. The taxonomic position of the assembled genome was determined using GTDB-Tk v.1.5.0 [48], and the assembly's average nucleotide identity (ANI) with closely related genomes was calculated with FastANI 2.0 [49]. Genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP; v.4.7) (NCBI, Rockville, MD, USA) [50]. The presence of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) was evaluated with CRISPRCasFinder [51]. The reCOGNizer was used to identify the Clusters of Ortholog Groups (COGs) [52] and Pfam domains [53] in the predicted protein sequences. Transmembrane helices and signal peptides were predicted by the online bioinformatic tools TMHMM v.2.0 (Department of Health Technology, Copenhagen, Denmark) [54] and SignalP v.5.0 (Department of Health Technology, Copenhagen, Denmark) [55], respectively.

2.5. Growth of Bacteria and Bioleaching Experiments

The effect of sodium persulfate, as well as C_1 compounds (formate and methanol), was studied using 9K medium supplemented with different concentrations of studied compounds. Experiments with these compounds included a comparison of growth in the 9K medium or leaching with and without (control) formate and methanol. Formate and methanol, as well as persulfate, were added at various concentrations. The inoculum was 10 mL of the starting culture, 10^6 – 10^7 cells/mL. Growth of the culture was determined by optical density (OD) measured using a Spekol 221 spectrophotometer (Carl Zeiss Industrielle Messtechnik GmbH, Jena, Germany) at a wavelength of 600 nm. Data show the average of three replicated experiments; variations were within 5%.

Bioleaching experiments were performed using 9K medium without ferrous sulfate supplemented with studied concentrate. A sample of a fine fraction (<1 mm) of a solid mineral raw material (solid phase, S) and a leaching solution (liquid phase, L) were placed in a 750 mL shake flask. Thus, the flask contained 200 mL of the 9K medium as the leach solution and 40 g of the concentrate, i.e., the solid and liquid phases were in ratio S:L = 1:5.

All experiments were carried out in triplicate under aerobic conditions at 28 °C using the IBPM shaker (IBPM RAS, Pushchino, Russia) at 180 rpm. Thus, the experiments modeled stirred tank leaching at a constant temperature.

2.6. Chemical Analyzes

The content of leached iron in the solution was determined by spectrophotometry according to GOST 13195-73. The method is based on the formation of a complex compound during the interaction of iron ions with potassium ferricyanide in an acidic medium. The optical density (OD) was measured at a wavelength of 540 nm using Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Iron (Fe^{3+}) concentration in the solution was determined by the constructed calibration curve.

The content of sulfate, formed during the oxidation of sulfides of the arsenopyrite concentrate, was determined by ion chromatography. The analyzes were performed using a liquid ion chromatograph for anion detection (Metrohm, Herisau, Switzerland) controlled by 761 Compact IC PC equipped with injection valve for manual injection or an autosampler with a Metrohm 838 Advanced Sample Processor; 6.1010.300 Metrosep Anion (3×150 mm) chromatographic column, as well as with conductometric detector. The 844 UV/VIS Compact IC chromatograph was controlled using the IC Net software and PC. 15 mmol/L NaHCO_3 /2.0 mmol/L Na_2CO_3 in deionized water (Milli-Q) was used as an eluent (flow rate 1.0 mL/min).

The pH and redox potential (ORP) values were determined using an Ecotest-112 pH meter/potentiometer (Econix, Moscow, Russia).

2.7. Statistical Processing

Statistical data validation was performed using ANOVA method and MS 15.0.459.1506 Excel 2013 software (Microsoft, Redmond, WA, USA) software. The analyzes were carried out in triplicate. The results presented in the article showed a high level of reliability ($p > 0.99$).

3. Results

3.1. Molecular Genetic Analysis of the Strain

According to literature data and analyzes of known genomes of *A. ferrooxidans* strains, differences were found in terms of arsenic resistance and the ability to leach arsenopyrite, therefore, in recent years, research has shifted towards the selection of novel arsenic-resistant species and strains.

Microorganisms have several strategies for arsenic resistance and transformation: (1) the cytoplasmic (*ars* genes)/periplasmic (*arr* genes) reduction of As(V) and isolation of As(III); (2) the As(III) oxidation (*aio* genes) and As(V) release via the phosphate transport system; (3) the methylation of As(III) to the gaseous compound $\text{As}(\text{CH}_3)_3$ (via *ArsM*), also called biomethylation. An analysis of complete bacterial genomes shows that a large number of phylogenetically diverse prokaryotes are able to transform As(V) and As(III) in various terrestrial and aquatic habitats, as well as in a wide range of environmental conditions [56–58]. Arsenotrophy, defined as the ability to grow by means of As(III) oxidation or As(V) reduction, requires membrane-associated proteins that transfer electrons from or to arsenic (*AioBA* and *ArrAB*, respectively). More common in many different bacteria is arsenic resistance based on the presence of *Ars* detoxification systems. In this process, As(V) is reduced intracellularly to As(III) by *ArsC*, a small 13–16 kDa protein. As(III) is then expelled from the cell by an efflux pump, *ArsB* or *ACR3*.

The “simplest” and most common system for protecting bacteria from arsenic is the *Ars* system. The resistance mechanisms encoded by the *ars* operon have been extensively studied. The operon configuration is different in different strains [59]. The simplest configuration (*arsRBC*) includes *ArsR* regulatory protein, which has an As(III)-specific binding site, an As(V) *ArsC* reductase, and an As(III) *ArsB* efflux pump. *ArsC* mediates As(V) reduction by glutaredoxin, glutathione, or thioredoxin. This detoxification system

requires energy in the form of ATP [60]. ArsC is localized in the cytoplasm and can only reduce As(V) that enters cells; thus, it is unable to reduce As(V) outside the bacterial cells [61]. Two families of transmembrane efflux pumps, ArsB and ACR3, are known. The ACR3 type is more widely distributed in nature and occurs in bacteria, animals, and plants, while ArsB is present only in bacteria [62]. Another operon configuration (*arsRDABC*) contains the additional ArsA ATPase, which provides energy for ArsB, which is the chaperone for arsenic efflux through ArsAB. In the third operon configuration, the *ars* genes are located in two operons (*arsRC* and *arsBH*) transcribed in opposite directions. The function of ArsH is not completely clear: it is present in almost all Gram-negative bacteria carrying the operon and absent in Gram-positive bacteria.

By performing PCR with the designed primers, we obtained data that confirmed the presence of arsenic resistance genes in the strain *A. ferrooxidans* TFBk.

The results of the analysis for the presence of arsenic resistance genes are shown in Figure 1. The data obtained for arsenic resistance genes for *A. ferrooxidans* TFBk with degenerate primers were confirmed by the data obtained from the analysis of the complete genome of *A. ferrooxidans* TFBk obtained in the present study.

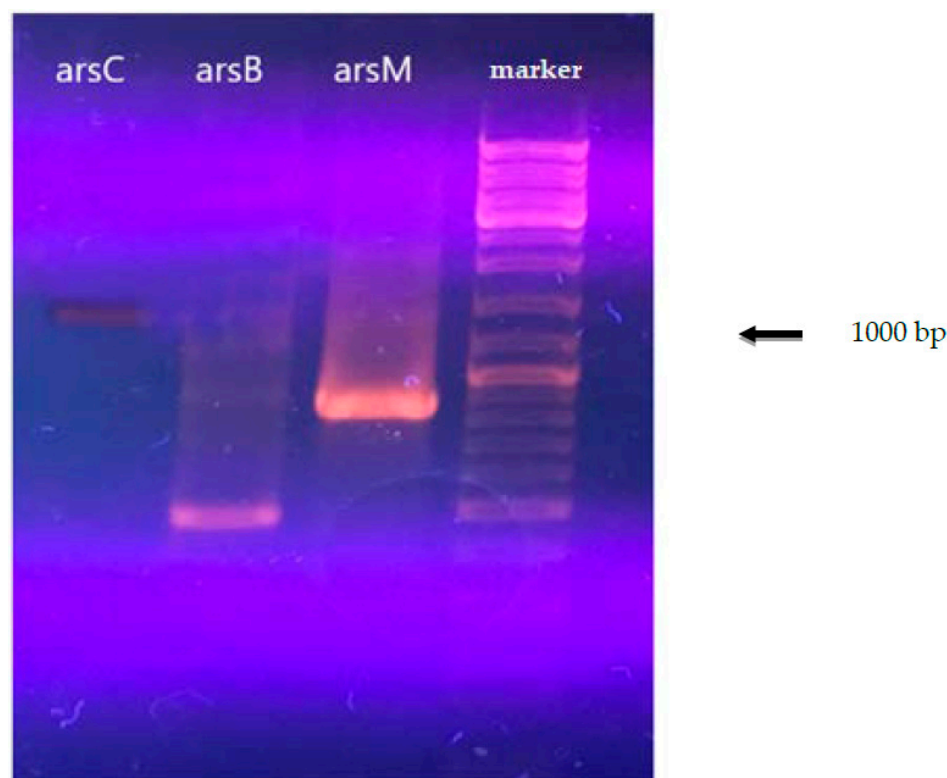


Figure 1. Electrophoresis of the PCR product of *A. ferrooxidans* total DNA with primers for the *arsC*, *arsM*, and *arsB* genes.

The final assembled genome was 3,620,318 bp long and includes 159 scaffolds with an N50 value of 86,581 bp; the G+C content was 57.91%, and coverage was 280.61x. The completeness, estimated using CheckM v.1.1.3 software [47], was 99.34%, and the estimated contamination was 0.0%. The taxonomic position of the assembled genome determined using GTDB-Tk v.1.5.0 software [48] and average assembly nucleotide identity (ANI) with closely related genomes calculated using FastANI 2.0 software [49] confirmed that the TFBk strain belongs to the species *A. ferrooxidans*. Genome annotation performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP; v.4.7) (NCBI, Rockville, MD, USA) [50] identified 3781 genes, 3558 protein coding sequences, 171 pseudogenes, and 52 RNA genes. The genome sequence parameters of the strain *A. ferrooxidans* TFBk are summarized in Table 2.

Table 2. Results of the strain TFBk genome annotation.

Parameter	Value
CDSs (total)	3729
Genes (coding)	3558
CDSs (with protein)	3558
Genes (RNA)	52
rRNAs	1, 1, 1 (5S, 16S, 23S)
Complete rRNAs	1, 1, 1 (5S, 16S, 23S)
Partial rRNAs	
tRNAs	45
ncRNAs	4
Pseudo Genes (total)	171
CDSs (without protein)	171
Pseudo Genes (ambiguous residues)	1 of 171
Pseudo Genes (frameshifted)	64 of 171
Pseudo Genes (incomplete)	118 of 171
Pseudo Genes (internal stop)	25 of 171
Pseudo Genes (multiple problems)	34 of 171

Analysis of the *A. ferrooxidans* TFBk genome revealed the presence of the *ars* (arsenic resistance system) gene group (Figure 2). The *arsC* gene encodes arsenate reductase and is involved in the transformation of As(V) into As(III), which is then excreted by the ArsB arsenite pump. This mechanism provides arsenic resistance for bacteria, although it increases environmental toxicity. In prokaryotes, there are three families of ArsC arsenate reductases, distinguished on the basis of the protein structures, reduction mechanisms, and the location of catalytic cysteine residues: (i) glutathione-dependent (GSH)/glutaredoxin, (ii) thioredoxin-dependent (Trx)/thioredoxin-reductase, and (iii) mycothiol-dependent (MSH)/mycoredoxin, recently discovered in actinobacteria. *A. ferrooxidans* TFBk possesses dependent arsene reductases with 69% identity of the translated amino acid sequences. The *arsC1* gene is surrounded by the genes *ArsA*, the arsenite/antimonite pump-driving ATPase, the arsenic metal chaperone *ArsD*, which carries trivalent metalloids to the *ArsAB* pump, the *ArsR* repressor, and the arsenite/antimonite: H⁺ antiporter *ArsB*. The second *arsC2* gene is also in close proximity to the *ars* genes, namely the arsenic resistance protein *arsH*, the arsenite/antimonite: H⁺ antiporter *arsB* and repressor *arsR*.

Thus, unlike other strains, *A. ferrooxidans* TFBk has all the genetic determinants for the operation of the arsenic resistance system in two variants. The genes responsible for other arsenic resistance strategies encoded by the *arr* and *aio* operons were not found in *A. ferrooxidans* TFBk.

In addition, *A. ferrooxidans* TFBk also has the *arsM* genes encoding putative arsenite methyltransferases, showing 95% identity. However, the *arsI* gene encoding the demethylase that breaks the As-C bond is absent. As shown by metagenomic studies, the *arsM* and *arsI* genes are often found in representatives of different species of the same community, forming an arsenate biogeochemical cycle in nature [63].

To better understand the possible mechanisms of formate and persulfate use, the presence of formate dehydrogenase genes and oxidative stress enzymes in the genome was studied. It can be assumed that formate dehydrogenase works according to the mechanism described for sulfurtransferase in *E. coli* [64]. Known strains of *A. ferrooxidans* have *fdhF* and *fdhD* genes in several variants (Table 3). They also have genes for the glutathione-dependent detoxification system, which detoxifies formaldehyde (the last one can be a by-product or produced during non-specific oxidation of methanol by alcohol dehydrogenase).

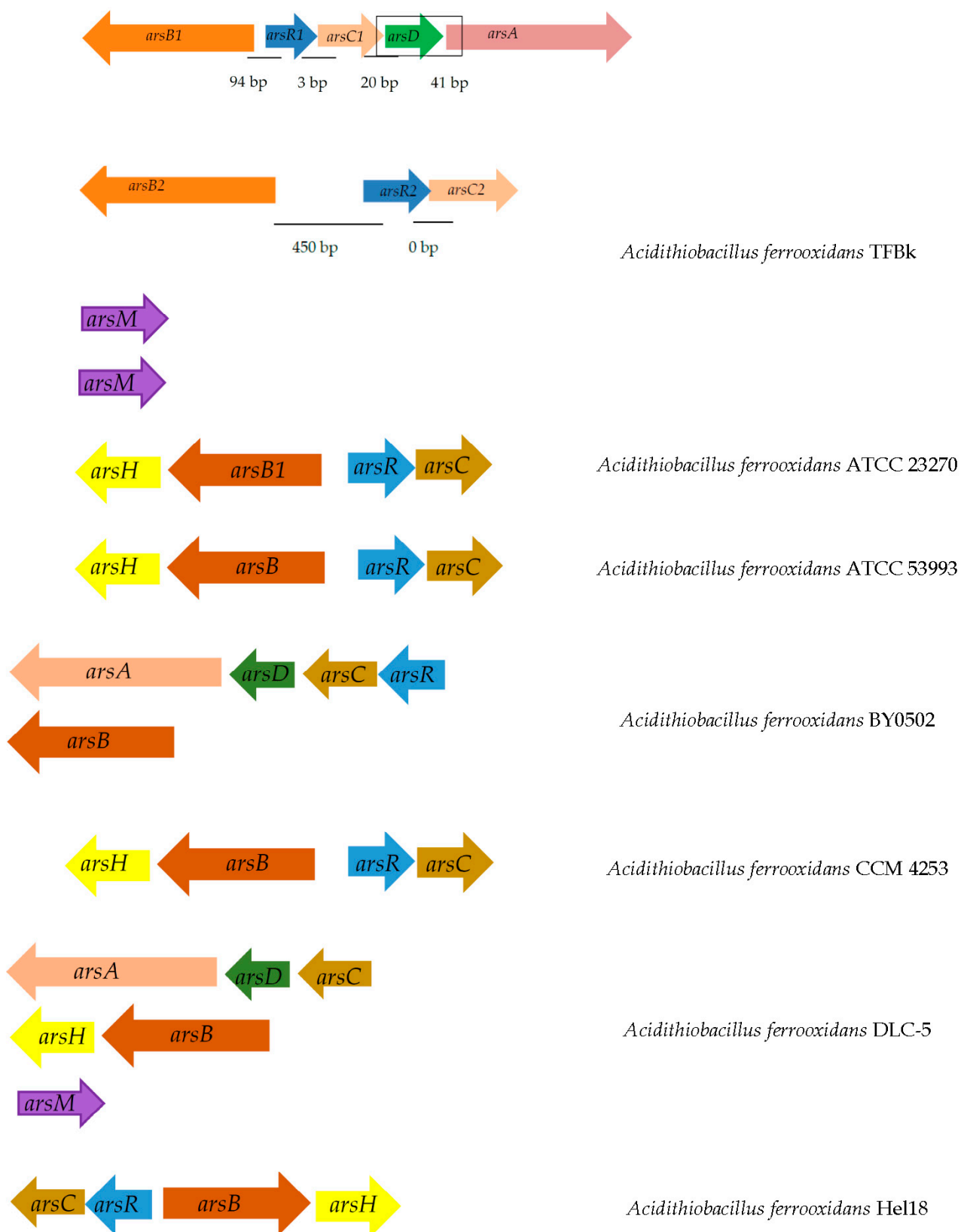


Figure 2. Cont.

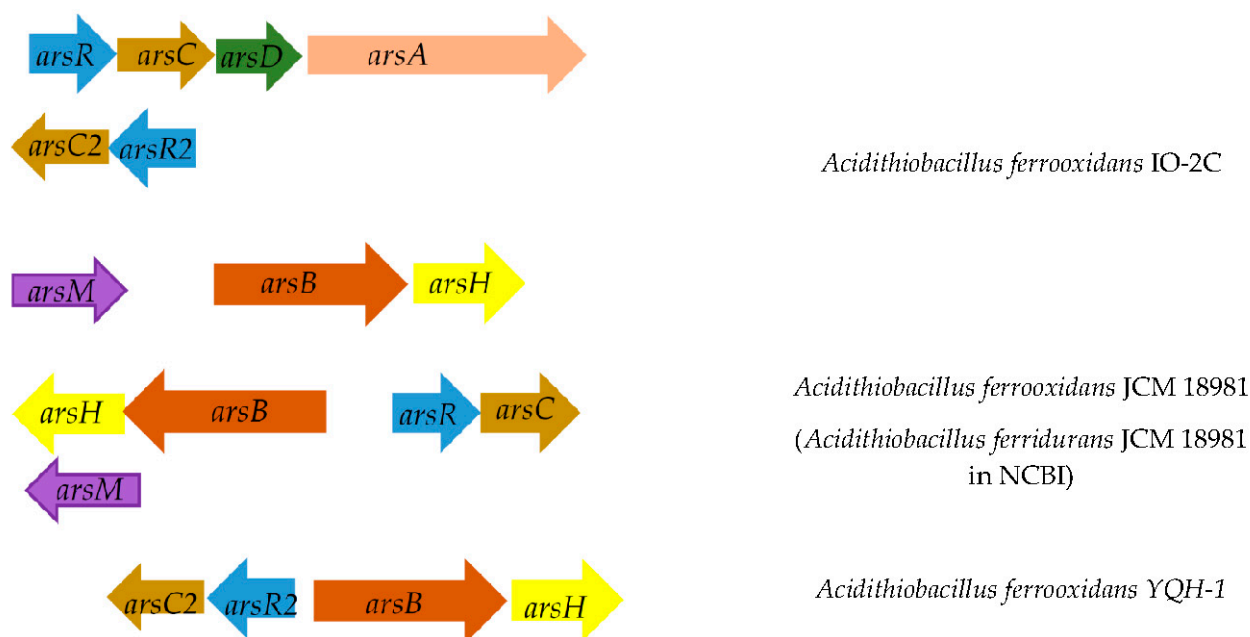


Figure 2. Location of arsenic resistance genes on the chromosome in different strains of *A. ferrooxidans*. Below is the distance between genes. Genes encoding: ArsB, arsenite/antimonite: H⁺ antiporter; ArsR, transcriptional repressor of the arsenic resistance operon; ArsC, arsenate reductase; ArsD, transcriptional repressor and arsenic metal chaperone.

Table 3. Distribution of formate dehydrogenase and oxidative stress enzymes in strains of *A. ferrooxidans*.

<i>A. ferrooxidans</i> Strains	FdhF/FdhD	Peroxioredoxin	Superoxide Dismutase	Glutathione Peroxidase	Catalase
TFBk	++	++	+	+	—
ATCC 23270	++	++	+	+	—
ATCC 53993	++	++	+	+	—
BY0502	+	+	+	+	—
CCM 4253	++	+	+	+	—
DLC-5	+	++	+	+	—
Hel18	++	+	+	+	—
YQH-1	++	+	+	+	—
IO-2C	++	+	+	+	—
JCM 18981	+++	+	+	+	—

The presence of genes is marked as “+”, “++”, and “+++” depending on their quantity, and the absence of genes is marked as “—”.

Bacteria can use superoxide dismutase and/or catalase to use or protect against peroxide compounds. As the study showed, all studied *A. ferrooxidans* strains have superoxide dismutase genes and no catalase genes. It can be assumed that superoxide dismutase plays the main role in the antioxidant defense of *A. ferrooxidans*.

3.2. Bioleaching Stimulation with Organic C₁ Compounds

Possible stimulation of acidophilic strain *A. ferrooxidans* TFBk was studied according to the scheme: growth was checked in a 9K medium with formate or methanol in comparison with the control. 5 mL of a 4-day culture of *A. ferrooxidans* TFBk was added as an inoculum.

The results on the effect of C₁ compounds in the 9K medium without concentrate are shown in Figures 3 and 4. The addition of methanol did not affect the growth of the bacteria (Figure 3). Formate concentration of 0.3% accelerated the growth of *A. ferrooxidans* TFBk only in the first three days compared to control, but did not provide an increase in final growth yield (Figure 4).

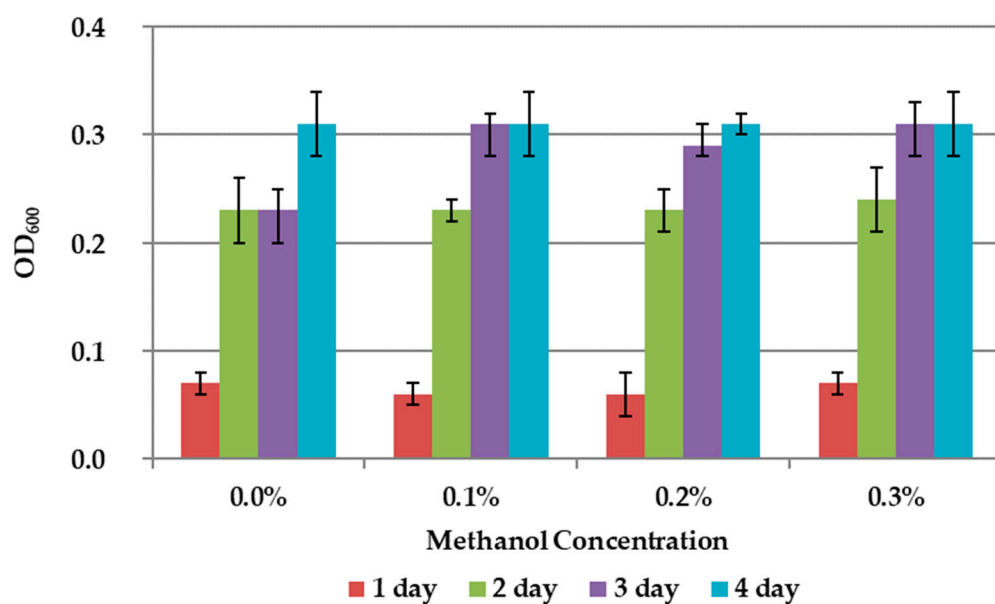


Figure 3. Growth dynamic of *A. ferrooxidans* TFBk in 9K medium with different concentrations of methanol. The y-axis shows the optical density (OD₆₀₀) of the medium with bacteria.

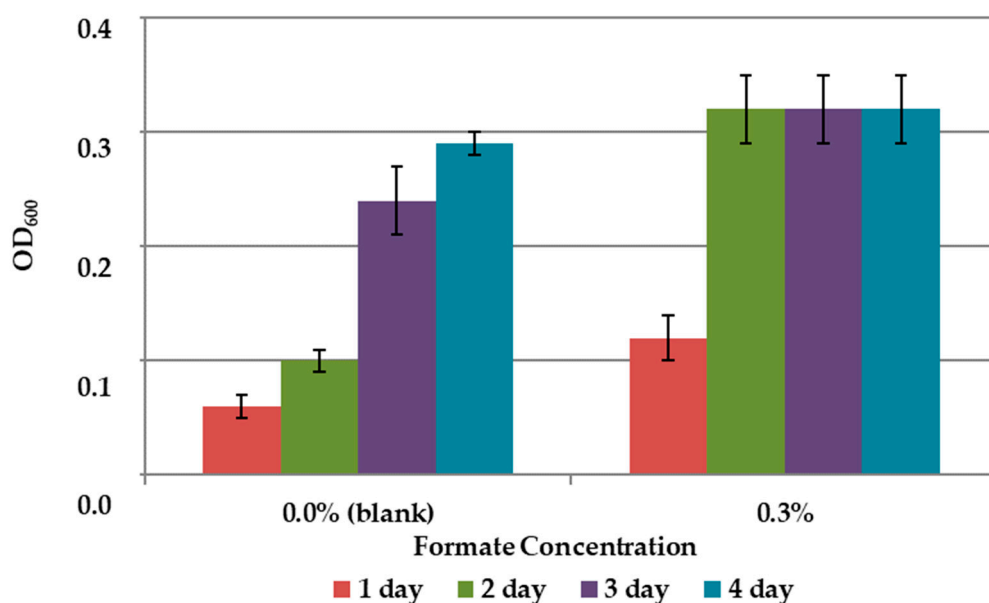


Figure 4. Growth dynamics of *A. ferrooxidans* TFBk in 9K medium with various formate concentrations. The y-axis shows the optical density of the medium with bacteria.

Addition of formate and methanol during the arsenopyrite concentrate leaching by the bacteria *A. ferrooxidans* TFBk showed that the addition of methanol not only did not stimulate the process, but even inhibited it. The addition of formate accelerated the appearance of iron and sulfate in solution (Table 4). However, after two weeks of incubation, the content of iron and sulfates in the variant with formate and the control were almost equal. The decrease in the concentration of iron in the solution during the leaching of the arsenopyrite concentrate after 2 weeks suggests secondary precipitation reactions.

Table 4. The effect of formate and methanol additions on the leaching of iron and sulfates from a sample of arsenopyrite concentrate by *A. ferrooxidans* TFBk bacteria. Control is the leaching in the medium without addition of formate and methanol.

Time, Days	Methanol, 0.3%			Formate, 0.3%			Control		
	pH	Fe ³⁺ , mg/L	SO ₄ ^{2−} , mg/L	pH	Fe ³⁺ , mg/L	SO ₄ ^{2−} , mg/L	pH	Fe ³⁺ , mg/L	SO ₄ ^{2−} , mg/L
0	3.4	800	500	3.4	800	500	3.4	800	500
1	3.6	1000	560	3.3	1995	1214	3.4	807	952
3	3.5	1715	944	3.3	1855	1352	3.4	1855	1044
6	3.2	1785	1060	3.3	1900	1670	3.2	1876	1346
9	3.2	1680	1080	3.3	1700	1670	3.1	1855	1668
15	3.2	1680	1100	3.3	1714	1670	3.1	1790	1670

3.3. Bioleaching Stimulation with Additional Oxidizing Agent, Persulfate

In patents devoted to biooxidation of gold-bearing mineral raw materials, it was noted that the intensification of the process can be achieved by increasing the redox potential (ORP) of the leaching medium [65,66]. At the same time, it is well known that the introduction of hydrogen peroxide into the medium can be accompanied by a sterilizing effect and inhibition of bacterial growth. We tested the possibility of stimulating bioleaching of the concentrate by the strain *A. ferrooxidans* TFBk by means of introducing a peroxide oxidant into the medium 9K that differs from hydrogen peroxide and is a more convenient form for transportation and storage: sodium persulfate.

The results showed slight stimulation of biomass growth in 9K medium without mineral concentrate at 0.1% persulfate concentration and suppression of growth at higher concentrations (Figure 5).

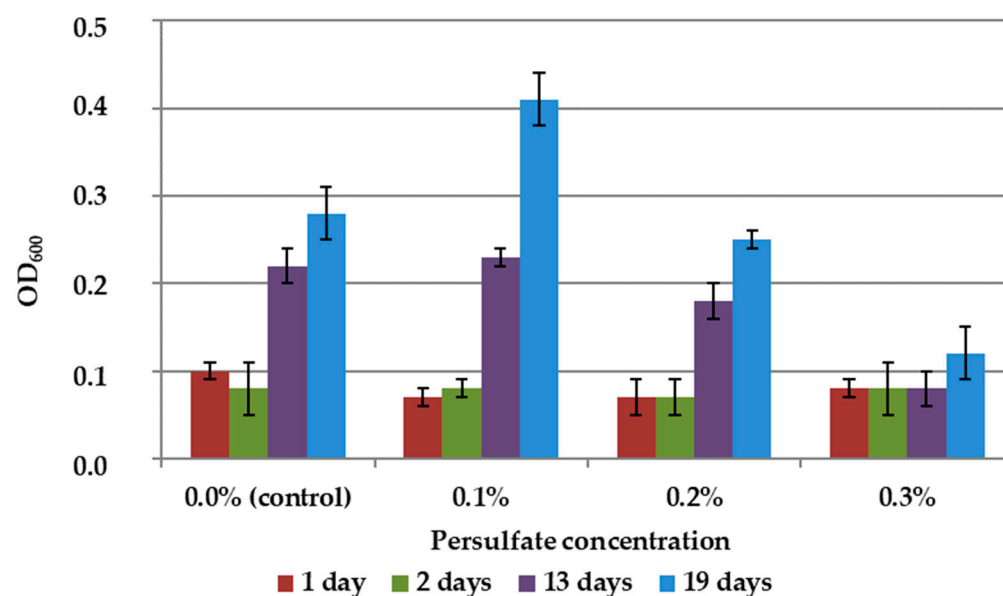


Figure 5. Growth dynamics of *A. ferrooxidans* TFBk culture in 9K medium with various persulfate concentrations. The y-axis shows the optical density of the medium with bacteria.

At the same time, the effect of persulfate on bacteria in a liquid culture medium was not identical to its effect in the presence of leached concentrate, since the interaction of the oxidizing agent with the concentrate reduced its toxic effect and increased the availability of minerals for bacteria. We have previously shown that the addition of persulfate or persulfate in combination with organic acids can intensify the leaching of minerals [13]. The results of the experiments on the effect of persulfates on the bioleaching of arsenopyrite raw materials in a 9K medium when inoculating *A. ferrooxidans* TFBk are shown in Table 5.

Table 5. Effect of sodium persulfate on the bioleaching of iron and sulfates from arsenopyrite flotation concentrate by *A. ferrooxidans* TFBk.

Time, Day	Sodium Persulfate, 0.0%			Sodium Persulfate, 0.3%			Sodium Persulfate, 0.5%			Sodium Persulfate, 1.0%		
	pH	Fe ³⁺ , mg/L	SO ₄ ^{2−} , mg/L	pH	Fe ³⁺ , mg/L	SO ₄ ^{2−} , mg/L	pH	Fe ³⁺ , mg/L	SO ₄ ^{2−} , mg/L	pH	Fe ³⁺ , mg/L	SO ₄ ^{2−} , mg/L
0	3.4	800	500	3.3	800	750	3.3	800	900	3.1	800	1300
5	2.8	1300	520	3.3	1716	1280	3.3	2094	1500	2.9	2694	2118
10	2.7	1016	700	3.2	1855	1300	3.3	2284	1712	3.0	3315	2400
15	3.1	778	930	3.1	1890	1600	3.2	2300	1980	3.0	3350	2360

Thus, the rate of leaching of arsenopyrite raw materials was higher with the addition of persulfate than in the control, i.e., in the absence of an additional oxidizing agent (Table 4). At the same time, in full accordance with the inhibition data presented in Figure 5, microscopy revealed an altered cell morphology and a decrease in the number of unsorbed cells in suspension. The results obtained may mean that the chemical and biological leaching of mineral raw materials is intensified by persulfate due to the interaction of the oxidizing agent with minerals.

4. Discussion

There are different mechanisms of bacterial resistance to arsenic [67]. These strategies of defense are based primarily on the following genes/processes: (1) cytoplasmic (*ars* genes)/periplasmic (*arr* genes) As(V) reduction and As(III) excretion; (2) As(III) oxidation (*ain* genes) and As(V) release via the phosphate transport system; (3) methylation of As(III) to the gaseous compound As(CH₃)₃ (via *arsM*), also called biomethylation. Our genetic analysis permitted us to determine which resistance mechanism is present in the strain *A. ferrooxidans* TFBk.

We carried out a genetic analysis of the *A. ferrooxidans* TFBk strain, originally isolated from the arsenopyrite deposit, and showed that it has a set of arsenic resistance genes. An analysis of the *A. ferrooxidans* TFBk genome revealed the presence of the *ars* gene group (arsenic resistance system). The *arsC* gene encodes arsene reductase and is involved in the transformation of As(V) into As(III), which is then excreted by the *ArsB* arsenite pump. *A. ferrooxidans* TFBk also possesses two thyredoxin-dependent arsene reductases. Thus, *A. ferrooxidans* TFBk has genetic determinants for resistance to arsenic in two variants. In addition, *A. ferrooxidans* TFBk also has the *arsM* genes encoding putative arsenite methyltransferases.

The applied genome-wide research methods provide more information than PCR amplification with degenerate primers, since they not only allow the detection of arsenic resistance genes, but also suggest possible bioleaching pathways. This approach is especially important when the leached mineral raw material is multicomponent (in our case, it contains not only arsenopyrite but also other mineral arsenic compounds). However, these primers can be useful when analyzing samples from different arsenic-contaminated environments to search for new strains with specific features. Thus, our work is one of the first attempts to use this approach for *A. ferrooxidans* for bioleaching intensification. At the same time, we expect that the development of this approach will make it possible to predict the phenotype and its applied use and, thus, will be included in the bioleaching methodology.

To better understand the possible mechanisms of formate and persulfate use, the presence of formate dehydrogenase genes and oxidative stress enzymes in the genome was studied. It can be assumed that formate dehydrogenase works according to the mechanism described for sulfurtransferase in *E. coli* [64]. All known strains of *A. ferrooxidans* have *fdhF* and *fdhD* genes in several variants (Table 5). *Acidithiobacillus* are acidophilic chemolithotrophs and assimilate carbon from carbon dioxide using the Calvin–Benson–Bassham cycle. Thus, an increase in the growth rate at the first stages with the addition of

formate can be associated with formate dehydrogenase, oxidizing formate to CO₂, which is assimilated by ribulose 1,5-bisphosphate carboxylase/oxygenase, i.e., formate can serve not only as a complementary source of energy, but also as a source of carbon. They also have genes for the glutathione-dependent detoxification system, which detoxifies formaldehyde (the last one can be a by-product or produced during non-specific oxidation of methanol by alcohol dehydrogenase). Bacteria can use superoxide dismutase and/or catalase to use or protect against peroxide compounds. As the study showed, all studied *A. ferrooxidans* strains have superoxide dismutase genes and no catalase genes. It can be assumed that superoxide dismutase plays the main role in the antioxidant defense of *A. ferrooxidans*.

Despite the resistance of *A. ferrooxidans* TFBk to arsenic, the rate of the leaching of refractory arsenopyrite concentrates was comparatively low. In this regard, since the strain possessed by the genes involved in C₁-compounds utilization and oxidative stress resistance, we investigated the possibility of intensifying bioleaching in two ways: (1) by means of stimulation with organic C₁ compounds as energy substrates and (2) the introduction of an additional oxidizing agent, sodium persulfate.

The possibility of stimulating the growth of biomass and bioleaching of *A. ferrooxidans* TFBk metals by adding the organic C₁ compound formate to the medium was confirmed. Previously, this ability was shown for another strain of this species [20]. It was shown that the organic C₁ compound methanol did not stimulate *A. ferrooxidans* TFBk and could even inhibit growth.

The possibility of using additions of a peroxide-type oxidant, sodium persulfate, to intensify bioleaching by *A. ferrooxidans* TFBk bacteria was shown. The addition of persulfate to the 9K medium without arsenopyrite stimulated the growth of biomass only at low concentrations (0.1%). However, high concentrations (0.5–1.0%) markedly intensified chemical-biological leaching of arsenopyrite, possibly due to the oxidation/deliberation of the ore minerals by means of both direct oxidation of the minerals with persulfate and increase of Fe³⁺/Fe²⁺ ratio, which, in turn, lead to the increase in mineral oxidation rate.

5. Conclusions

The genome of the leaching strain *A. ferrooxidans* TFBk was studied. It has been shown that it has genes responsible for resistance to arsenic. It was also found that its set of genes was fundamentally different from known other representatives of the species *A. ferrooxidans*. In addition, an analysis of the strain was carried out to assess its potential resistance to adverse factors (oxidative stress in the presence of persulfate), as well as the ability to utilize C₁ compounds.

In experiments with the strain *A. ferrooxidans* TFBk, the possibility of stimulating its growth and leaching activity with formate additives was shown. The possibility of stimulating autotrophic bacteria with organic C₁-compounds is an interesting direction for general and applied microbiology.

In bioleaching experiments, regulation of the redox potential was carried out by the addition of sodium persulfate. It was shown that supplementation with this additional oxidizing agent at low concentrations leads to an intensification of bioleaching. The study showed that the strain *A. ferrooxidans* TFBk is a promising object for its use in the bioleaching of arsenic-containing ores and concentrates. In general, the study showed the possibility of intensifying the process of bioleaching, justified by the methods of genetic analysis.

Author Contributions: Conceptualization, T.A., M.V. and A.B.; validation, A.Y., T.A. and D.G.; investigation, A.Y. and O.R.; data curation, T.A.; writing—original draft preparation, review and editing, T.A., M.V. and A.B. All authors have read and agreed to the published version of the manuscript.

Funding: The study was partly supported by the Ministry of Science and Higher Education of the Russian Federation (Grant agreement no. 075-15-2021-1051).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Sequence data associated with the annotated genome have been deposited in the NCBI BioProject accession number PRJNA866167. This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number JANJYU000000000. The version described in this paper is version JANJYU000000000.1.

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

References

1. Zimmerley, S.R.; Wilson, D.G.; Prater, J.D. Cyclic Leaching Process Employing Iron Oxidizing Bacteria. U.S. Patent 2,829,964, 8 April 1958.
2. Natarajan, K.A. Bioleaching Mechanisms. In *Biotechnology of Metals*; Natarajan, K.A., Ed.; Elsevier: Amsterdam, The Netherlands, 2018; pp. 49–80.
3. Diaz, A.J.; Serrano, J.; Leiva, E. Bioleaching of arsenic-bearing copper ores. *Minerals* **2018**, *8*, 215. [\[CrossRef\]](#)
4. Mikhlin, Y.L.; Romanchenko, A.S.; Asanov, I.P. Oxidation of arsenopyrite and deposition of gold on the oxidized surfaces: A scanning probe microscopy, tunneling spectroscopy and XPS study. *Geochim. Cosmochim. Acta* **2006**, *70*, 4874–4888. [\[CrossRef\]](#)
5. Murciego, A.; Alvarez-Ayuso, E.; Pellitero, E.; Rodriguez, M.A.; Garcia-Sanchez, A.; Tamayo, A.; Rubio, J.; Rubio, F.; Rubin, J. Study of arsenopyrite weathering products in mine wastes from abandoned tungsten and tin exploitations. *J. Hazard. Mater.* **2011**, *186*, 590–601. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Paikaray, S. Arsenic geochemistry of acid mine drainage. *Mine Water Environ.* **2015**, *34*, 181–196. [\[CrossRef\]](#)
7. Merkulova, M.; Mathon, O.; Glatzel, P.; Rovezzi, M.; Batanova, V.; Marion, P.; Boiron, M.-C.; Manceau, A. Revealing the chemical form of “invisible” gold in natural arsenian pyrite and arsenopyrite with high energy-resolution X-ray absorption spectroscopy. *ACS Earth Space Chem.* **2019**, *3*, 1905–1914. [\[CrossRef\]](#)
8. Meng, X.; Zhao, H.; Sun, M.; Zhang, Y.; Zhang, Y.; Lv, X.; Kim, H.; Vainshtein, M.; Wang, S.; Qiu, G. The role of cupric ions in the oxidative dissolution process of marmatite: A dependence on Cu²⁺ concentration. *Sci. Total Environ.* **2019**, *675*, 213–223. [\[CrossRef\]](#)
9. Li, S.-P.; Guo, N.; Wu, H.-Y.; Qiu, G.-Z.; Liu, X.-X. High efficient mixed culture screening and selected microbial community shift for bioleaching process. *Trans. Nonferrous Met. Soc. China* **2011**, *21*, 1383–1387. [\[CrossRef\]](#)
10. Yin, Z.; Feng, S.; Tong, Y.; Yang, H. Adaptive mechanism of *Acidithiobacillus thiooxidans* CCTCC M 2012104 under stress during bioleaching of low-grade chalcopyrite based on physiological and comparative transcriptomic analysis. *J. Ind. Microbiol. Biotechnol.* **2019**, *46*, 1643–1656. [\[CrossRef\]](#)
11. Yuehua, H.; Guanzhou, Q.; Jun, W.; Dianzuo, W. The effect of silver-bearing catalysts on bioleaching of chalcopyrite. *Hydrometallurgy* **2002**, *64*, 81–88. [\[CrossRef\]](#)
12. Hernández, P.; Dorador, A.; Martínez, M.; Toro, N.; Castillo, J.; Ghorbani, Y. Use of seawater/brine and caliche’s salts as clean and environmentally friendly sources of chloride and nitrate ions for chalcopyrite concentrate leaching. *Minerals* **2020**, *10*, 477. [\[CrossRef\]](#)
13. Ren, Z.; Krishnamoorthy, P.; Sanchez, P.Z.; Asselin, E.; Dixon, D.C.; Mora, N. Catalytic effect of ethylene thiourea on the leaching of chalcopyrite. *Hydrometallurgy* **2020**, *196*, 105410. [\[CrossRef\]](#)
14. Abashina, T.N.; Yachkula, A.A.; Vainshtein, M.B. Prevention of sulfuric acid pollution: Intensification of metal leaching with organic acids. *IOP Conf. Ser. Earth Environ. Sci.* **2022**, *981*, 032029. [\[CrossRef\]](#)
15. Abashina, T.; Yachkula, A.; Kaparullina, E.; Vainshtein, M. Intensification of nickel bioleaching with neutrophilic bacteria *Guyparkeria halophila* as an approach to limitation of sulfuric acid pollution. *Microorganisms* **2021**, *9*, 2461. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Aston, J.E.; Apel, W.A.; Lee, B.D.; Peyton, B.M. Growth effects and assimilation of organic acids in chemostat and batch cultures of *Acidithiobacillus caldus*. *World J. Microbiol. Biotechnol.* **2011**, *27*, 153–161. [\[CrossRef\]](#)
17. Bulaev, A.; Nechaeva, A.; Elkina, Y.; Melamud, V. Effect of carbon sources on pyrite-arsenopyrite concentrate bio-oxidation and growth of microbial population in stirred tank reactors. *Microorganisms* **2021**, *9*, 2350. [\[CrossRef\]](#)
18. Zhang, X.; Feng, Y.-L.; Li, H.-R. Enhancement of bio-oxidation of refractory arsenopyritic gold ore by adding pyrolusite in bioleaching system. *Trans. Nonferrous Met. Soc. China* **2016**, *26*, 2479–2484. [\[CrossRef\]](#)
19. Mahmoud, A.; Cezac, P.; Hoadley, A.F.A.; Contaminea, F.; D’Hugues, P. A review of sulfide minerals microbially assisted leaching in stirred tank reactors. *Int. Biodeterior. Biodegrad.* **2017**, *119*, 118–146. [\[CrossRef\]](#)
20. Pronk, T.; Meijer, W.M.; Hazen, W.; van Dijken, J.P.; Bos, P.; Kuenen, J.G. Growth of *Thiobacillus ferrooxidans* on formic acid. *Appl. Environ. Microbiol.* **1991**, *57*, 2057–2062. [\[CrossRef\]](#)
21. Pronk, J.T.; van Dijken, J.P.; Bos, P.; Kuenen, J.G. High Yield Method of Growing *Thiobacillus ferrooxidans* on Formate. Patent ZA 923117B, 6 May 1991.
22. Chen, C.; Li, H.; Cui, F.; Wang, Z.; Liu, X.; Jiang, G.; Cheng, T.; Bai, R.; Song, L. Novel combination of bioleaching and persulfate for the removal of heavy metals from metallurgical industry sludge. *Environ. Sci. Pollut. Res. Int.* **2022**, *29*, 33751–33763. [\[CrossRef\]](#)
23. Tsitonaki, A.; Smets, B.F.; Bjerg, P.L. Effects of heat-activated persulfate oxidation on soil microorganisms. *Water Res.* **2008**, *42*, 1013–1022. [\[CrossRef\]](#)

24. Afonyushkin, V.N.; Tabanyukhov, K.A.; Cherepushkina, V.S.; Khomenko, Y.S.; Tatarchuk, O.P. Effect of disinfectants based on potassium persulfate, hydrogen peroxide, glutaraldehyde and quaternary ammonium compounds on the genetic material of the pathogen bacteria specific to meat processing industry. *Theory Pract. Meat Process.* **2016**, *1*, 54–61. [\[CrossRef\]](#)
25. Wu, B.; Gu, G.; Deng, S.; Liu, D.; Xiong, X. Efficient natural pyrrhotite activating persulfate for the degradation of O-isopropyl-N-ethyl thionocarbamate: Iron recycle mechanism and degradation pathway. *Chemosphere* **2019**, *224*, 120–127. [\[CrossRef\]](#)
26. Qi, H.; Huang, Q.; Hung, Y.-C. Efficacy of activated persulfate in inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *Int. J. Food Microbiol.* **2018**, *284*, 40–47. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Qi, H.; Huang, Q.; Hung, Y.-C. Efficacy of activated persulfate in pathogen inactivation: A further exploration. *Food Res. Int.* **2019**, *120*, 425–431. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Venâncio, J.P.F.; Rodrigues, C.S.D.; Nunes, O.C.; Madeira, L.M. Application of iron-activated persulfate for municipal wastewater disinfection. *J. Hazard. Mater.* **2022**, *426*, 127989. [\[PubMed\]](#)
29. Lee, J.; von Gunten, U.; Kim, J.-H. Persulfate-based advanced oxidation: Critical assessment of opportunities and roadblocks. *Environ. Sci. Technol.* **2020**, *54*, 3064–3081. [\[CrossRef\]](#)
30. Bajagain, R.; Lee, S.; Jeong, S.-W. Application of persulfate-oxidation foam spraying as a bioremediation pretreatment for diesel oil-contaminated soil. *Chemosphere* **2018**, *207*, 565–572. [\[CrossRef\]](#)
31. Balaberda, A.; Ulrich, A.C. Persulfate oxidation coupled with biodegradation by *Pseudomonas fluorescens* enhances naphthenic acid remediation and toxicity reduction. *Microorganisms* **2021**, *9*, 1502. [\[CrossRef\]](#)
32. Ma, J.; Tang, Y.; Yang, D.Q.; Pei, P. Kinetics of advanced oxidative leaching of pyrite in a potassium peroxydisulphate solution. *J. S. Afr. Inst. Min. Metall.* **2020**, *120*, 165–172. [\[CrossRef\]](#)
33. Tang, Y.; Li, G.; Yang, Y.; Ma, J.; Zhi, Y.; Yao, Y.; Zheng, L.; Tuo, B. Oxidation of gold-bearing pyrite by ammonium persulfate. *J. Sustain. Metall.* **2021**, *7*, 1280–1292. [\[CrossRef\]](#)
34. Dakubo, F.; Baygents, J.C.; Farrell, J. Peroxodisulfate assisted leaching of chalcopryrite. *Hydrometallurgy* **2012**, *121*, 68–73. [\[CrossRef\]](#)
35. Turan, M.D.; Altundoğan, H.S. Leaching of a copper flotation concentrate with ammonium persulfate in an autoclave system. *Int. J. Min. Met. Mater.* **2014**, *21*, 862–870. [\[CrossRef\]](#)
36. Liu, Z.-X.; Yin, Z.-L.; Hu, H.-P.; Chen, Q.-Y. Leaching kinetics of low-grade copper ore containing calcium-magnesium carbonate in ammonia-ammonium sulfate solution with persulfate. *Trans. Nonferrous Met. Soc. China* **2012**, *22*, 2822–2830. [\[CrossRef\]](#)
37. Turan, M.D.; Arslanoğlu, H.; Altundoğan, H.S. Optimization of the leaching conditions of chalcopryrite concentrate using ammonium persulfate in an autoclave system. *J. Taiwan Inst. Chem. Eng.* **2015**, *50*, 49–55. [\[CrossRef\]](#)
38. Liu, Z.; Xiang, Y.; Yin, Z.; Wu, X.; Jiang, J.; Chen, Y.; Xiong, L. Oxidative leaching behavior of metalliferous black shale in acidic solution using persulfate as oxidant. *Trans. Nonferrous Met. Soc. China* **2016**, *26*, 565–574. [\[CrossRef\]](#)
39. Kondratyeva, T.F.; Muntyan, L.N.; Karavaiko, G.I. Zinc- and arsenic-resistant strains of *Thiobacillus ferrooxidans* have increased copy numbers of chromosomal resistance genes. *Microbiology* **1995**, *141*, 1157–1162. [\[CrossRef\]](#) [\[PubMed\]](#)
40. Kanaev, Z.K.; Bulaev, A.G.; Kanaev, A.T.; Kodrateva, T.F. Physiological properties of *Acidithiobacillus ferrooxidans* strains isolated from sulfide ore deposits in Kazakhstan. *Microbiology* **2015**, *84*, 370–376. [\[CrossRef\]](#)
41. Silverman, M.P.; Lundgren, D.C. Study on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. An improved medium and harvesting procedure for securing high cell yield. *J. Bacteriol.* **1959**, *77*, 642–647. [\[CrossRef\]](#)
42. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30*, 2114–2120. [\[CrossRef\]](#)
43. Bankevich, A.; Nurk, S.; Antipov, D.; Gurevich, A.A.; Dvorkin, M.; Kulikov, A.S.; Lesin, V.M.; Nikolenko, S.I.; Pham, S.; Pribelski, A.D.; et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* **2012**, *19*, 455–477. [\[CrossRef\]](#)
44. Gurevich, A.; Saveliev, V.; Vyahhi, N.; Tesler, G. QUAST: Quality assessment tool for genome assemblies. *Bioinformatics* **2013**, *29*, 1072–1075. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Okonechnikov, K.; Conesa, A.; García-Alcalde, F. Qualimap 2: Advanced multi-sample quality control for high-throughput sequencing data. *Bioinformatics* **2016**, *32*, 292–294. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Langmead, B.; Salzberg, S.L. Fast gapped-read alignment with Bowtie 2. *Nat. Meth.* **2012**, *9*, 357–359. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Parks, D.H.; Imelfort, M.; Skennerton, C.T.; Hugenholtz, P.; Tyson, G.W. CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res.* **2015**, *25*, 1043–1055. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Chaumeil, P.A.; Mussig, A.J.; Hugenholtz, P.; Parks, D.H. GTDB-Tk: A toolkit to classify genomes with the genome taxonomy database. *Bioinformatics* **2019**, *36*, 1925–1927. [\[CrossRef\]](#)
49. Jain, C.; Rodriguez, R.L.M.; Phillippy, A.M.; Konstantinidis, K.T.; Aluru, S. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat. Commun.* **2018**, *9*, 5114. [\[CrossRef\]](#)
50. Tatusova, T.; DiCuccio, M.; Badretdin, A.; Chetvernin, V.; Nawrocki, E.P.; Zaslavsky, L.; Lomsadze, A.; Pruitt, K.D.; Borodovsky, M.; Ostell, J. NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res.* **2016**, *44*, 6614–6624. [\[CrossRef\]](#)
51. Couvin, D.; Bernheim, A.; Toffano-Nioche, C.; Touchon, M.; Michalik, J.; Neron, B.; Rocha, E.P.C.; Vergnaud, G.; Gautheret, D.; Pourcel, C. CRISPRCasFinder, an update of CRISPRfinder, includes a portable version, enhanced performance and integrates search for Cas proteins. *Nucleic Acids Res.* **2018**, *46*, W246–W251. [\[CrossRef\]](#)
52. Galperin, M.Y.; Makarova, K.S.; Wolf, Y.I.; Koonin, E.V. Expanded microbial genome coverage and improved protein family annotation in the COG database. *Nucleic Acids Res.* **2014**, *43*, D261–D269. [\[CrossRef\]](#)

53. Finn, R.D.; Coghill, P.; Eberhardt, R.Y.; Eddy, S.R.; Mistry, J.; Mitchell, A.L.; Potter, S.C.; Punta, M.; Qureshi, M.; Sangrador-Vegas, A.; et al. The Pfam protein families database: Towards a more sustainable future. *Nucleic Acids Res.* **2016**, *44*, D279–D285. [[CrossRef](#)]
54. Krogh, A.; Larsson, B.; von Heijne, G.; Sonnhammer, E.L. Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *J. Mol. Biol.* **2001**, *305*, 567–580. [[CrossRef](#)] [[PubMed](#)]
55. Almagro Armenteros, J.J.; Tsirigos, K.D.; Sønderby, C.K.; Petersen, T.N.; Winther, O.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat. Biotechnol.* **2019**, *37*, 420–423. [[CrossRef](#)] [[PubMed](#)]
56. Rhine, E.D.; Phelps, C.D.; Young, L.Y. Anaerobic arsenite oxidation by novel denitrifying isolates. *Environ. Microbiol.* **2006**, *8*, 899–908. [[CrossRef](#)] [[PubMed](#)]
57. Handley, K.M.; Héry, M.; Lloyd, J.R. Redox cycling of arsenic by the hydrothermal marine bacterium *Marinobacter santoriniensis*. *Environ. Microbiol.* **2009**, *11*, 1601–1611. [[CrossRef](#)] [[PubMed](#)]
58. Osborne, T.H.; Jamieson, H.E.; Hudson-Edwards, K.A.; Nordstrom, D.K.; Walker, S.R.; Ward, S.A.; Santini, J.M. Microbial oxidation of arsenite in a subarctic environment: Diversity of arsenite oxidase genes and identification of a psychrotolerant arsenite oxidizer. *BMC Microbiol.* **2010**, *10*, 205. [[CrossRef](#)] [[PubMed](#)]
59. Páez-Espino, D.; Tamames, J.; de Lorenzo, V.; Cánovas, D. Microbial responses to environmental arsenic. *Biomaterials* **2009**, *22*, 117–130. [[CrossRef](#)] [[PubMed](#)]
60. Ordóñez, E.; Letek, M.; Valbuena, N.; Gil, J.A.; Mateos, L.M. Analysis of genes involved in arsenic resistance in *Corynebacterium glutamicum* ATCC 13032. *Appl. Environ. Microbiol.* **2005**, *71*, 6206–6215. [[CrossRef](#)]
61. Langner, H.W.; Inskeep, W.P. Microbial reduction of arsenate in the presence of ferrihydrite. *Environ. Sci. Technol.* **2000**, *34*, 3131–3136. [[CrossRef](#)]
62. Achour, A.R.; Bauda, P.; Billard, P. Diversity of arsenite transporter genes from arsenic resistant soil bacteria. *Res. Microbiol.* **2007**, *158*, 128–137. [[CrossRef](#)]
63. Ben Fekih, I.; Zhang, C.; Li, Y.P.; Zhao, Y.; Alwathnani, H.A.; Saquib, Q.; Rensing, C.; Cervantes, C. Distribution of arsenic resistance genes in prokaryotes. *Front Microbiol.* **2018**, *23*, 2473. [[CrossRef](#)]
64. Thomé, R.; Gust, A.; Toci, R.; Mendel, R.; Bittner, F.; Magalon, A.; Walburger, A. A sulfurtransferase is essential for activity of formate dehydrogenases in *Escherichia coli*. *J. Biol. Chem.* **2012**, *287*, 4671–4678. [[CrossRef](#)] [[PubMed](#)]
65. Sekisov, A.G.; Piskunov, S.A.; Filatov, B.L. Method for leaching of gold-bearing complex ores. Patent RU 2,044,875, 27 September 1995.
66. Bely, A.V.; Malashonok, A.P.; Leskiv, M.V.; Potylitsyn, N.V. Method for Bioleaching of Refractory Gold-Bearing Sulfide Flotation Concentrates. Patent RU 2,637,204, 30 November 2017.
67. Andres, J.; Bertin, P.N. The microbial genomics of arsenic. *FEMS Microbiol. Rev.* **2016**, *40*, 299–322. [[CrossRef](#)] [[PubMed](#)]