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# Isolation of an extremely acidophilic and highly efficient strain *Acidithiobacillus* sp. for chalcopyrite bioleaching

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**Abstract** An extremely acidophilic sulfur-oxidizing bacterium was isolated from an industrial-scale bioheap of the Zijinshan copper mine and was named ZJJN. A tuft of flagella and a layer of thick capsule outside the cell envelope were clearly observed under transmission electron microscopy (TEM), which might be closely related to the extremely acid-proof capacity of ZJJN cells in the bioleaching system; 16S ribosomal RNA (rRNA) phylogeny showed that the isolated strain was highly homologous to the genera of *Acidithiobacillus*. The optimum temperature of ZJJN was determined at 30 °C and pH at 1.0. It was capable of growth at even pH 0. Strain ZJJN can utilize reduced sulfur as an energy source but not with organics or ferrous ion. Strain ZJJN was sensitive to all antibiotics with different concentrations; when it showed a certain resistance to different concentrations of $Cu^{2+}$. In the mixed strains of ZJJN and *A. ferrooxidans* system (initial pH 1.0), the copper-leaching efficiency was up to 60.1 %, which was far higher than other systems. Scanning electron microscopy (SEM) analysis showed that less jarosite precipitation was produced in the most efficient system. The extremely acidophilic strain ZJJN would be of great potential in the application of chalcopyrite bioleaching.

**Keywords** *Acidithiobacillus* · Extremophile · Jarosite · Chalcopyrite · Highly efficient bioleaching

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## Introduction

With the decreased output of high-grade minerals, the demand for efficient use of low-grade minerals has been, and continues to be, more urgent due to overexploited mineral resources. Bioleaching is an interdisciplinary subject of biotechnology and metallurgy engineering. Owing to lower operating costs, lower investment in infrastructure, and higher recovery, bioleaching is considered a green metallurgical technology [4, 23, 24]. The Zijinshan copper mine is the first and largest commercial application of a bioleaching heap in China. It has an ore reserve >400 million tons with an average copper grade of 0.43 %. In December 2005, bioleaching was employed for dealing with its large amounts of low-grade ore capacity of 10,000 t copper per year [13]. From 2006 to 2009, total copper cathode production was 37,633.6 t, and the average operation cost for copper cathodes was US $1.10 lb, when total profit and tax exceeded US $149 million [8]. Under the extreme environmental conditions, the commonly used microbial species in the bioleaching processes might involve some extremophiles (particularly acidophilic) belonging to genera *Acidithiobacillus*, *Acidiphilium*, *Ferroplasma*, and *Leptospirillum* [3, 16]. For process optimization, research into microbial communities focused on leaching solution has become more important. *Acidithiobacillus* (66.78 %), *Leptospirillum* (28.29 %), *Sulfobacillus* (3.29 %), and *Ferroplasma* (1.64 %) were detected in a commercial nonaerated copper bioleaching heap in Zijinshan copper mine [13]. However, study regarding isolation and characterization of extremely acidophilic microbes is limited.

Due to the excessive acid and nonaerated control in Zijinshan copper mine, the operating pH in bioleaching decreased to 0.85 after several years. Only a small amount of species survived in the leaching solution due to

excessive acid and iron ions [18, 19]. After long-term selection and domestication, bacteria flourishing in the hostile environment of an industrial bioheap were able to tolerate extremely acid conditions and would provide a model for studying the mechanism of acid resistance. Additionally, in the process of chalcopyrite bioleaching, the high content of iron ions might easily form jarosite precipitation in certain conditions (pH > 2). It would attach to the mineral surface and decrease bioleaching efficiency [11]. It was reported that there was less jarosite precipitation formed in the systems with pH < 1.6 [12]. Compared with iron-oxidizing bacteria, sulfur-oxidizing bacteria could generally tolerate lower operating pH and play much more significant roles in chalcopyrite bioleaching [1, 14]. Therefore, isolation and characterization of extremely acidophile bacteria, especially sulfur oxidizers, is of great importance in the promotion of efficient chalcopyrite bioleaching.

In this study, a mesophilic, extremely acidophilic, and sulfur-oxidizing bacterium capable of growth at pH 0 was isolated from an industrial bioleaching heap in Zijinshan copper mine, China. To further understand the role *Acidithiobacillus* sp. strain ZJJN in bioleaching, its physiological and molecular traits and its role in chalcopyrite bioleaching were studied.

## Materials and methods

### Media and strain

Starkey medium was used in biochemical and physiological characterization studies. Starkey medium consisted of the following basal salts (g/l): $(NH_4)_2SO_4$ 3.0, $KH_2PO_4$ 3.0, $MgSO_4{\cdot}7H_20$ 0.5, $CaCl_2{\cdot}2H_20$ 0.25 [26]. Elemental sulfur 10.0 g/l was added as an energy source, and the medium was adjusted to pH 1.0 with 2 M HCl. The solid medium was prepared by addition of 20.0 g/l $Na_2S_2O_3{\cdot}5H_2O$ 10.0 g/l. *A. ferrooxidans* strain CUMT-1 was kindly provided by the Mining University of China, Jiangshu [6, 9]. This strain was isolated from a coal mine in Yanzhou, Shandong, China, and was cultured in 9 K medium consisting of the following basal salts (g/l): $(NH_4)_2SO_4$ 3.0, KCl 0.1, $K_2HPO_4$ 0.5, $MgSO_4{\cdot}7H_20$ 0.5, $Ca(NO_3)_2$ 0.01 [23]. Then, 44.7 g/l $FeSO_4{\cdot}7H_2O$ was added as an energy source, and the medium was adjusted to pH 2.0 with 2 M HCl. The basal medium was autoclaved using a high-pressure steam sterilizer (LDZX-40II, Shenan, China) at 121 °C for 20 min.

### Enrichment and isolation

Acid mine drainage samples were collected in October 2009 from the bioleaching heap consisting of secondary copper sulfides in Zijinshan copper mine (Fujian, China). The heap had been worked for 6 years, and the volume scale was about 2,000 $m^3$. The operating temperature and pH of the heap were about 10–40 °C and 0.5–1.0, respectively. The bacterium was enriched from the sample described above with Starkey basal media added by 10 g chalcopyrite per liter. When the enrichment sample liquid became dark yellow and turbid, serial dilution was adopted to isolate the strain on Starkey-$Na_2S_2O_3$ medium. After 10 days of incubation at 30 °C, single milky colonies began to develop, and the isolation process was repeated five times to ensure strain purity.

### Morphological and phylogenetic analysis

Morphology of the pure culture strain ZJJN was observed by light (DM-2500, Leica, China) and transmission electron (TEM) (H-7000, Hitachi, Japan) microscopy. The total genomic DNA of the culture was extracted using small bacteria genomic DNA Fast Extraction Kit (FastaGen, Shanghai, China). The 16S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR) using forward primer 27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 1492R 5′-GGTTACCTTGTTACG ACTT-3′ [5]. The PCR program was 94 °C for 4 min, 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 90 s, with a final extension step of 72 °C for 10 min after 30 cycles. The PCR product was purified using B-type small DNA fragment Gel Extraction Kit (BioDev-Tech, Beijing, China). After sequencing of 16S rRNA, the sequence was analyzed with those of similar types of strains in the National Center for Biotechnology Information using the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi/). A phylogenetic tree was constructed using CLUSTAL X version 1.81 and MEGA version 4.0 programs to determine the relationship of strain ZJJN with other acidophilic micro-organisms. Details of the relative strains used in this study are listed in Table 1.

### Determination of optimal culture condition

To determine the optimum temperature and pH for growth, strain ZJJN was grown at the range of 10 to 45 °C and initial pH range of 0.5 to 2.5. To determine the optimal energy source for strain ZJJN, Starkey basal medium with trace element was supplemented with one of the following compounds (g/l): yeast extract 1.0; peptone 1.0; glucose 1.0; fructose 1.0; sucrose 1.0; lactose 1.0; galactose 1.0; maltose 1.0; thiamine 1.0; asparagines 1.0; tyrosine 1.0; $Na_2S_2O_3{\cdot}5H_2O$ 10.0; $FeSO_4{\cdot}7H_2O$ 10.0; $FeCl_2$ 10.0; chalcopyrite 10.0; element sulfur 10.0; yeast extract 1.0, element sulfur 10.0; peptone 1.0, element sulfur 10.0; glucose 1.0, element sulfur 10.0. Inocula were washed twice with

**Table 1** Referenced strains in the analysis of molecular phylogeny

| Species | Strain | Sequence number | Similarity with ZJJN (%) | Description and source |
|---|---|---|---|---|
| *Acidithiobacillus* sp. | ZJJN | JQ259048 | 100 | Zijinshan copper mine, Fujian, China |
| *Acidithiobacillus* sp. | DBS-4 | EU084710 | 99 | Effluent from copper mine from Qujiang County, Guangdong, China |
| *Acidithiobacillus* sp. | YP-5 | EU084708 | 99 | Effluent from Yongping copper mine, Jiangxi, China |
| *Acidithiobacillus* sp. | D41 | FJ890903 | 98 | Zijinshan copper mine, Fujian, China |
| *A. thiooxidans* | ATCC19377 | AJ459803 | 97 | Braunschweig, Germany |
| *A. thiooxidans* | DFI | FJ998186 | 97 | Sulfate-reducing bioreactor, Qingdao, China |
| *A. thiooxidans* | NR | AF359940 | 97 | Acid mine drainge, Daehyundong, Korea |
| *A. caldus* | S2 | DQ256484 | 97 | Coal heap drainge, Changsha, China |
| *A. caldus* | N39-30-02 | EU499920 | 96 | Spent copper sulfide heap, Western Australia, Australia |
| *A. albertensis* | BY-05 | DQ423683 | 95 | Acid mine drainge of copper ore, Gansu, China |
| *A. albertensis* | DSM14366 | AJ459804 | 95 | Braunschweig, Germany |
| *A. ferrooxidans* | CHMS | EF059761 | 95 | Effluent from Chengmenshan copper mine, Jiangxi, China |
| *A. ferrooxidans* | CUMT-1 | JX266060 | 95 | Acid mine drainge of coal ore, Jiangsu, China |
| *A. ferrivorans* | NO-37 | AF376020 | 95 | Copper mine spoil drainage, Norway |

basal medium after harvesting via centrifugation. The initial cell density was $5.0 \times 10^7$ ml, pH was adjusted to 1.0, and all cultures were incubated at 30 °C for 5 days. The colony-forming unit (CFU) data was determined, and all experiments were carried out at least in duplicate.

### Antibiotics sensitivity and metal ion tolerance

The growth of strain ZJJN in the presence of metal ions was measured in batch cultures containing basal medium, trace elements, and elemental sulfur (as described above). Different concentrations of metal ions and antibiotics were added to the medium. After incubation at 30 °C for 5 days, CFU data was determined, and all experiments were carried out at least in duplicate.

### Bioleaching experiment

Chalcopyrite used for bioleaching was ground and sieved through a 200-mesh grid, and the diameter of particles was <75 μm, with the average copper content of the mineral at about 1.0 %. Bioleaching experiments were designed as follows: blank control; pure-strain *A. ferrooxidans*; pure-strain ZJJN; mixed strain *A. ferrooxidans* and ZJJN (initial pH 3.5), mixed strain *A. ferrooxidans* and ZJJN (initial pH 1.0). Cell densities (CFU) were controlled at $5.0 \times 10^7$ ml after being inoculated, and the cell density of each strain was $2.5 \times 10^7$ cells/ml in mixed system. Bioleaching experiments were carried out in 500-ml flasks containing 50 ml Starkey medium and 50 ml 9 K medium, respectively. The basal medium was adjusted to pH 1.5 (at 30 °C), and flasks were shaken at 170 r/min. Then, 2.0 ml basal medium was added to the bioleaching system each day to balance loss by evaporation. The same amplifying experiment was conducted in a 3-l glass cylindrical stirred reactor. Ferric and total iron were analyzed by *o*-phenanthrolene spectrophotometry [7]. The concentration of dissolved copper was monitored by atomic absorption spectrometry (Spectr AA-220/220Z, Varian, USA). The 16S rRNA-based sandwich hybridization assay was employed to detect cell densities of *A. ferrooxidans* and ZJJN in bioleaching [6]. Media were filtered after leaching, and residues were dried using a freeze drier. Morphology and surface of leached residues were analyzed by scanning electron microscopy (SEM) (Quanta-200, FEI, The Netherlands).

## Results and discussion

### Analysis of sample and morphology

The key index of the sample is listed as Table 2. The sample contained high contents of acid, iron, and heavy metal ion. In particular, the pH was determined at only 0.8 and iron at 51.7 g/l. Cell density was determined at only $10^6$ ml; it was widely accepted that most micro-organisms could not survive under the stress of high concentrations of acid and metal. As previously reported, bacteria active in this low acid system (pH < 1) were generally acidophilic micro-organisms [3]. Selection on media with $Na_2S_2O_3 \cdot 5H_2O$ as the sole energy source was a straightforward method for screening acidophilic sulfur-oxidizing microbes. After five isolations and incubations on plates, strain ZJJN was isolated from the leached solution. The colonies were milky and raised, with a regular edge. The diameter of

**Table 2** Analysis of collection sample

| Parameter | Value |
|---|---|
| pH | 0.8 |
| ORP/mv | 321.2 |
| $SO_4^{2-}$ (g/l) | 1.8 |
| $Fe^{2+}$ (g/l) | 9.1 |
| $Fe^{3+}$ (g/l) | 42.6 |
| $Cu^{2+}$ (g/l) | 5.1 |
| $Zn^{2+}$ (mg/l) | 32.8 |
| Cell number (lg cells/ml) | 6.4 |

The sample was collected from the leaching solution of the bioleaching heap in Zijinshan copper mine, Fujian, China

the colony was 1.0 mm at 30 °C after 10 days. Cells of strain ZJJN were 1.75 ± 0.15 μm in length and 0.62 ± 0.10 μm ($n = 50$) in width, which was a short rod, motile, and Gram negative. Tuft flagella and capsule were also observed around the cell by TEM (Fig. 1). Interestingly, flagella of strain ZJJN were significantly bushier than the other reported bacteria in the bioleaching process. Owing to the multiflagella, strain ZJJN might resist the extremely hostile environment when the capsule was clearly thick, which would enhance attachment to the mineral surface for acquiring a more limited energy source. These structures would improve the efficiency of the "direct mechanism" in the bioleaching process [18].

## Analysis of phylogeny

After reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* by Kelly and Wood in 2000 [10], genera of *Acidithiobacillus* consisting of five common species were divided into iron-oxidizing and sulfur-oxidizing groups. In the former group, the typical specie was *A. ferrooxidans*, which was the most broadly studied bacterium in the bioleaching process, especially with the release of the genome sequence [21]. Some novel species were recently reported, such as *A. ferrivorans*, which was isolated from copper mine drainage in Norway [7]. In the latter group, the model species were *A. thiooxidans* and *A. caldus*. Some relatively novel species, such as *A. albertensis*, were first isolated in acidic soil of a sulfur stockpile in Canada [25]. The 16S rRNA gene of strain ZJJN (1,411 bp) was sequenced and submitted to GenBank with the accession number JQ259048. Then, the 16S rRNA sequences of strain ZJJN were blasted in GenBank with 13 other related strains, and the phylogenetic tree was depicted based on their 16S rRNA gene sequences. Descriptions of referenced strains are listed in Table 1. The result indicated that the phylogenetic tree was divided into five groups, and strain ZJJN was located in the same clade with *Acidithiobacillus*. Strain ZJJN had a rather high sequence identity with strains belonging to the genera of *Acidithiobacillus* sp. (EU084708, 99 % of similarity), *A. thiooxidans* (FJ998186, 97 %), *A. caldus* (DQ256484, 97 %), *A. ferrooxidans* (EF059761, 95 %), and *A. albertensis* (AJ459804, 95 %). Strains of group 1, belonging to genera of *Acidithiobacillus* without species identification, showed the highest homology with ZJJN. Group 2 circumscribed those bacteria related to *A. thiooxidans*, whereas group 3, represented by *A. caldus*. *A. albertensis*, *A. ferrooxidans*, and *A. ferrivorans*, was included in groups 4 and 5, which showed just 95 % genetic similarity with ZJJN (Fig. 2).

## Effects of temperature, pH, and energy source on microbial growth

Based on phylogenetic and genotypic data, strain ZJJN should be a species of the genus *Acidithiobacillus*. Strain ZJJN exhibited a broad temperature range (20–35 °C), and the optimum temperature was 30 °C. Incubated at 30 °C, strain ZJJN could survive at even pH 0, and it revealed the fastest growth at pH 1.0 (Fig. 3). The growth rate slightly fluctuated at lower pH and dramatically decreased at higher pH. The optimum pH range was 0.2–2.0, and it was more acid tolerant than *A. ferrooxidans*, *A. thiooxidans*, or *A. caldus*. This trait provided potential in bioleaching under higher acid condition for jarosite inhibition. Some significant properties, such as flagella, might contribute to its successful survival in the extremely acid environment in Zijinshan copper mine. It was also reported that a novel lipoprotein, Licanantase, enhanced the contact mechanism of the mineral–bacteria interphase in the extremely acid environment [2]. However, incubated at the optimum environment (pH 1.0 and 30 °C), the culture doubling time of isolate SJJN was 14 h, which was significantly slower than those of other reported sulfur-oxidizing bacteria, such as *A. ferrooxidans* (about 6 h) [20]. Strain ZJJN was not able to grow in any sole organic medium reported in "Materials and methods," except for the presence of reduced sulfur. Additionally, $FeCl_2$ or $FeSO_4$ could not be used as energy sources. Interestingly, the growth of strain ZJJN was mildly promoted in the elemental sulfur system containing traces of yeast extract or peptone. Comparison of its taxonomic traits with the other *Acidithiobacillus* species is listed in Table 3.

## Sensitivity to antibiotics and tolerance to metal ions

Degrees of sensitivity or tolerance to antibiotics and metal ions of strain ZJJN are listed in Table 4. It was sensitive to all antibiotics listed with different concentrations. This might be because cell-wall formation was inhibited by

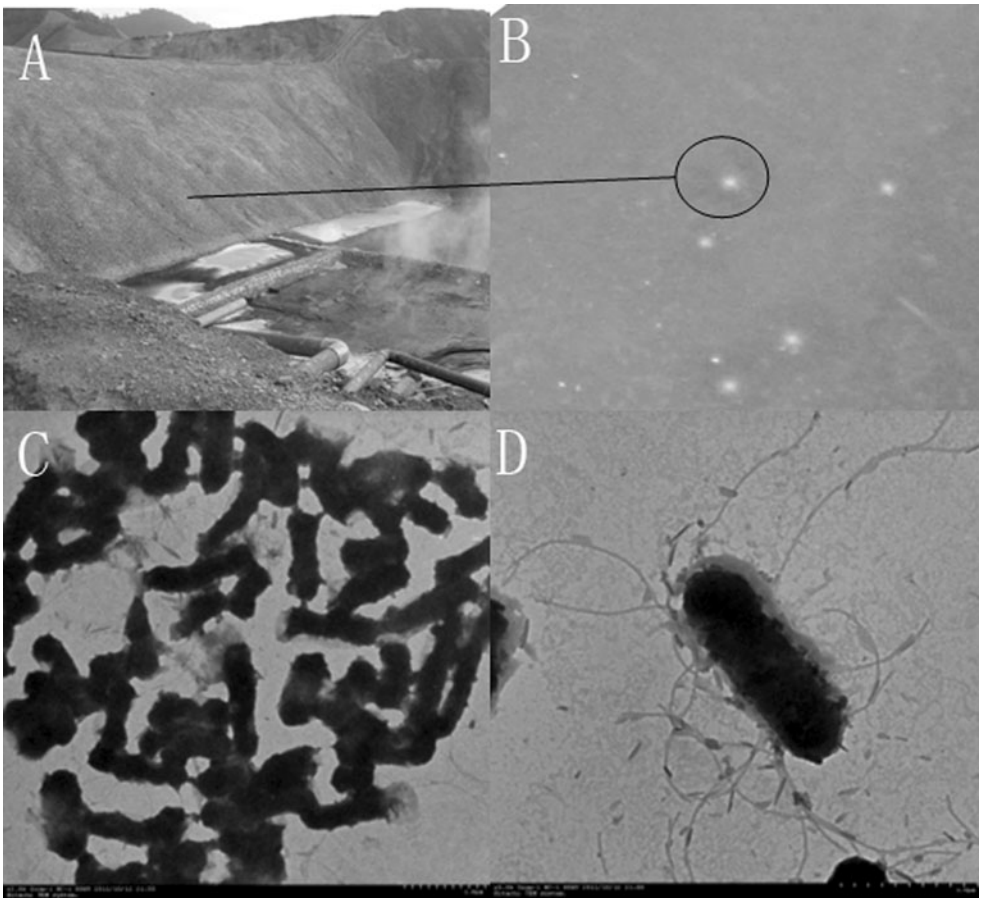


**Fig. 1** Collection site and morphological characterization of a ZJJN cell. **a** Bioheap at Zijinshan copper mine. **b** Colony of isolate ZJJN. **c** Transmission electron micrograph (TEM) (*bar*, 1 μm; 25.5 × k) of clustered ZJJN cells. **d** TEM micrograph (*bar*, 1 μm; 42.5 × k) of a single ZJJN cell. Images of ZJJN cells were viewed on a solid Starkey-$Na_2S_2O_3$ plate, and strain ZJJN was cultivated at 30 °C for 15 days

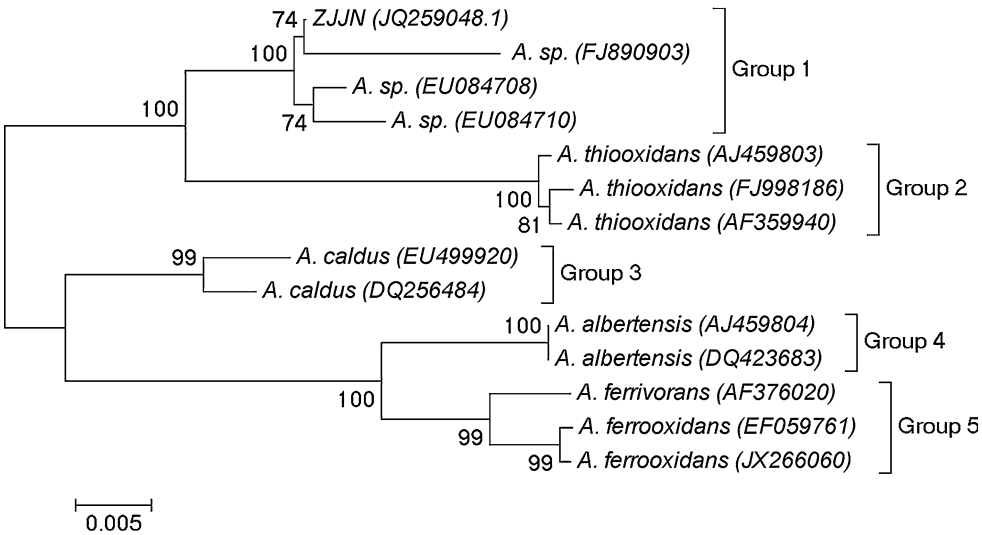


**Fig. 2** Neighbor-joining tree based on 16S ribosomal RNA (rRNA) gene sequences of strain ZJJN and related *Acidithiobacillus* isolates. *Numbers* in *parentheses* are GenBank accession numbers. *Scale bar* 0.005 indicates evolutionary distance

ampicillin, kanamycin, chloramphenicol, streptomycin, and erythromycin; and the synthesis of protein was greatly inhibited by tetracycline [28]. The order of tolerance to these metal ions was $Mg^{2+} > Mn^{2+} > Cu^{2+} > Ni^{2+} > Zn^{2+} > Pb^{2+} > Co^{2+}$. This is probably because strain ZJJN is accommodative with the high concentration

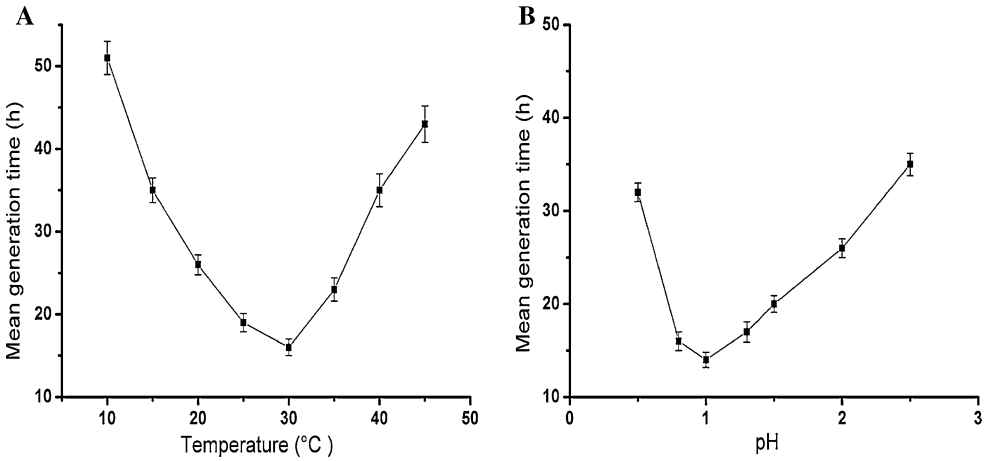


**Fig. 3** Effects of growth condition on strain ZJJN growth. **a** Temperature; **b** initial pH

**Table 3** Comparison of taxonomic traits of strain ZJJN with other *Acidithiobacillus* species

| Characteristic | Strain ZJJN | *A. ferrooxidans* CUMT-1 [9, 10] | *A. thiooxidans* ATCC19377 [15, 26] | *A. caldus* S2 [7, 17] | *A. albertensis* DSM14366 [7, 26] |
|---|---|---|---|---|---|
| Cell size (μm) | 1.0−2.0 × 0.6 | 1.0−2.0 × 0.5−0.7 | 1.0−2.0 × 0.5−0.7 | 1.0−2.0 × 0.7 | 1.0−2.0 × 0.5 |
| Motility | + | + | + | + | + |
| Flagella | Tuft polar | Single polar | Single polar | ND | Tuft polar |
| Optimum temperature (°C) | 28–30 | 30–35 | 28–33 | 45 | 25–30 |
| Optimum pH | 0–2.0 | 2.0–3.0 | 0.6–3.0 | 2.0–2.5 | 3.5–4.0 |
| Growth using electron donor | | | | | |
| Organics | − | − | − | − | − |
| Ferrous iron | − | + | − | − | − |
| Sulfur | + | + | + | + | + |
| Tetrathionate | + | + | + | + | + |
| Thiosulfate | + | + | + | + | + |
| Chalcopyrite | + | + | + | + | + |
| Anaerobic growth with ferric ion | − | + | − | − | ND |
| $N_2$ fixation | − | + | − | − | ND |
| Leaching rate in chalcopyrite (%) | 60.1[a] | 30.4[a] | ND | 30.6[b] | 46.97[c] |

+ positive, − negative, *ND* not determined

[a] This study

[b] Mixed-strains system with a strain of *A. ferrooxidans* after 20 days' bioleaching

[c] Mixed-strains system with a strain of *A. ferrooxidans* after 25 days' bioleaching

of metal ion in the Zijinshan copper mine, such as 5.1 g/l $Cu^{2+}$ after 6 years or longer. However, levels of tolerance to some of the metals were significantly influenced by increased concentrations of the heavy metal (for example, manganese inhibition). Additionally, different concentrations of copper ion indistinctively influence the growth of strain ZJJN, making it of great potential in industrial chalcopyrite bioleaching.

## Analysis of chemical indexes in bioleaching

The experiment of chalcopyrite bioleaching was carried out using strain ZJJN based on its superiority, as described above: 30.4 % of copper was released from chalcopyrite after 31 days in the leaching systems inoculated with pure-strain *A. ferrooxidans*, 29.4 % with pure-strain ZJJN, 53.4 % with *A. ferrooxidans*/strain ZJJN initial pH 3.5, and 60.1 % with *A. ferrooxidans*/strain ZJJN initial pH 1.0 (Fig. 4a). Compared with the pure-strain system, the consortium of sulfur-oxidizing bacteria strain ZJJN and iron-oxidizing bacteria *A. ferrooxidans* was more efficient; pure-strain *A. ferrooxidans* or ZJJN showed almost the same lower efficiency; leaching efficiency of mixed strains with initial pH 1.0 was the highest among all designed systems; efficiency of systems assisted with bacteria was much greater than the blank control system (4.8 %).

**Table 4** Sensitivity and tolerance of strain ZJJN to some antibiotics and metal ions

| Antibiotics | Concentration of antibiotics (mg/l) | Growth of strain ZJJN (lg CFU/ml)[a] | Metal | Concentration of metal (g/l) | Growth of strain ZJJN (lg CFU/ml)[a] |
|---|---|---|---|---|---|
| Blank control | 0 | 7.82 ± 0.10 | $MgSO_4{\cdot}7H_2O$ | 0.5 | 7.80 ± 0.09 c |
| | | | | 3 | 7.99 ± 0.10 z |
| Ampicillin | 10 | 7.15 ± 0.13 c | $MnSO_4{\cdot}H_2O$ | 0.5 | 7.60 ± 0.06 c |
| | 100 | 6.80 ± 0.11 z | | 3 | 7.08 ± 0.12 xy |
| Kanamycin | 10 | 7.02 ± 0.12 b | $CuSO_4{\cdot}5H_2O$ | 0.5 | 7.48 ± 0.20 bc |
| | 100 | 6.78 ± 0.10 z | | 3 | 7.25 ± 0.15 y |
| Chloramphenicol | 10 | 6.88 ± 0.09 b | $NiCl_2{\cdot}6H_2O$ | 0.5 | 7.22 ± 0.21 ab |
| | 100 | 6.75 ± 0.11 z | | 3 | 6.95 ± 0.13 x |
| Erythromycin | 10 | 6.81 ± 0.13 b | $ZnSO_4{\cdot}7H_2O$ | 0.5 | 7.23 ± 0.13 ab |
| | 100 | 6.33 ± 0.12 y | | 3 | 7.06 ± 0.12 xy |
| Streptomycin | 10 | 6.23 ± 0.17 a | $Pb(NO_3)_2$ | 0.5 | 7.12 ± 0.15 b |
| | 50 | 5.81 ± 0.10 x | | 1 | 6.90 ± 0.14 x |
| Tetracycline | 5 | 6.01 ± 0.08 a | $CoCl_2{\cdot}6H_2O$ | 0.1 | 6.96 ± 0.14 a |
| | 10 | 5.65 ± 0.10 x | | 0.5 | 6.56 ± 0.16 w |

[a] Growth was cultured in Starkey-$S^0$ medium at 30 °C and pH 1.0 for 5 days; experiments were carried out in triplicate. Criteria of sensitivity versus tolerance: compared with the blank control (100 %), cell density [colony-forming units (CFU)] of sample with antibiotics or metal ions decreased to 50 %; a–c and w–z represent statistically significant differences (c > b > a; z > y > x > w)

As an acid-soluble metal sulfide, chalcopyrite was oxidized to copper ion, ferrous ion, and elemental sulfur by ferric iron or proton (Eqs. 1 and 2). In the system consisting of *A. ferrooxidans* or ZJJN, ferrous ions produced in the reactions described above (Eqs. 1 and 2), was oxidized by *A. ferrooxidans*, whereas reduced sulfur was utilized by sulfur-oxidizing bacteria, such as strain ZJJN or *A. ferrooxidans* (Eqs. 3 and 4). This phenomenon would enhance the cycle of ferrous ion and improve the efficacy of bioleaching [27].

$$CuFeS_2 + 4Fe^{3+} = Cu^{2+} + 2S + 5Fe^{2+} \quad (1)$$

$$CuFeS_2 + 4H^+ + O_2 = Cu^{2+} + 2S + Fe^{2+} + 2H_2O \quad (2)$$

$$4Fe^{2+} + 4H^+ + O_2 = 4Fe^3 + 2H_2O \quad (3)$$

$$2S + 3O_2 + 2H_2O = SO_4^{2-} + 4H^+ \quad (4)$$

The final pH of pure-strain *A. ferrooxidans* was 1.9 and of pure-strain ZJJN and mixed-strains (initial pH 3.5) 1.2 (Fig. 4b). This should be due to the fact that sulfur was more efficiently oxidized by strain ZJJN and produced sulfuric acid than did *A. ferrooxidans*. Correspondingly, the concentration of sulfate was significantly higher in the strain ZJJN system (Fig. 4d). Compared with pure-strain ZJJN, ferrous iron concentration with pure-strain *A. ferrooxidans* was lower, indicating that *A. ferrooxidans* had a higher iron oxidation rate. In the first 14 days, the concentration of ferrous ion rose with the iron ions dissolving from chalcopyrite. Subsequently, it presented a decreasing trend after 15 days (Fig. 4c). This should be because bacteria entered a rapid growth phase after the lag period in the mineral system. Ferrous ions were utilized extensively as electron donors by *A. ferrooxidans* for obtaining energy during this phase. Moreover, the ferrous iron of concentration in the pure *A. ferrooxidans* system was significantly decreased, along with copper-leaching rates, after 18–30 days. This might be because the formation of jarosite precipitation caused passivation of the mineral surface. Jarosite precipitation was produced as shown in Eq. 5. Jarosite precipitation was frequently observed on the mineral surface, shielding the surface and greatly influencing the efficacy of bioleaching. The pH was the primary parameter affecting the formation of jarosite precipitation, which seldom formed in the system with pH <1.6 [11]. Therefore, in the mixed-strains systems, the system with initial pH 1.0 (60.1 %) was much more efficient than that with initial pH 3.5 (53.4 %).

$$3Fe^{3+} + 2SO_4^{2-} + 6H_2O + K^+ = KFe_3(SO_4)_2(OH)_6 + 6H^+ \quad (5)$$

The same experiment was conducted and verified in a 3-l-glass stirred reactor. Comparison of bioleaching efficiency is listed in Table 5. Bioleaching efficiency at the reactor level was closely consistent with that in the 500-ml flask system and demonstrated that the ZJJN has potential for use in industrial chalcopyrite bioleaching.

## Analysis of microbial community in mixed-strain bioleaching

A novel 16S rRNA-based sandwich hybridization assay with the aid of S1 nuclease treatment and fluorescent

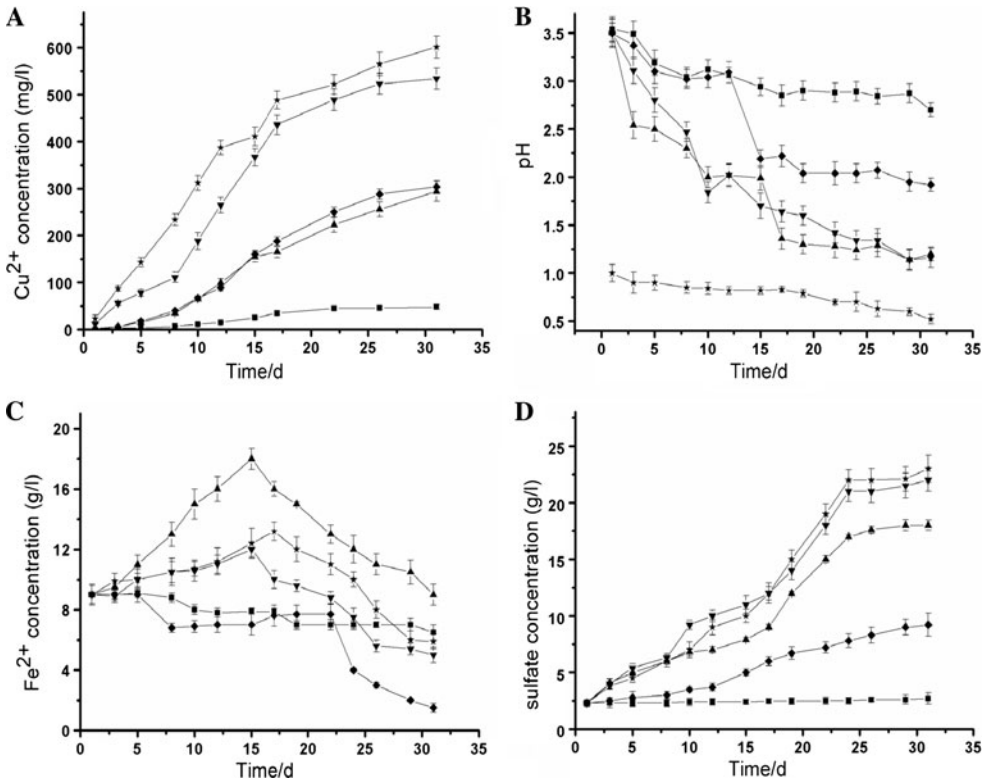


**Fig. 4** **a** Copper release from chalcopyrite; **b** pH changes during bioleaching of chalcopyrite; **c** ferrous oxidization during bioleaching of chalcopyrite; **d** sulfate oxidization during bioleaching of chalcopyrite. *Square* blank control; *diamond* pure-strain *Acidithiobacillus ferrooxidans*; *triangle* pure-strain ZJJN; *inverted triangle* mixed-strain *A. ferrooxidans* and ZJJN with initial pH 3.5; *star* mixed-strain *A. ferrooxidans* and ZJJN with initial pH 1.0

**Table 5** Comparison of bioleaching efficiency in flask and reactor systems

| Sample | Concentration of $Cu^{2+}$(mg/l) 10th | | Concentration of $Cu^{2+}$(mg/l) 20th | | Concentration of $Cu^{2+}$ (mg/l) 31st | |
|---|---|---|---|---|---|---|
| | Flask | Reactor | Flask | Reactor | Flask | Reactor |
| Blank | 11.2 | 12.5 | 37.2 | 41.0 | 47.6 | 52.1 |
| Pure-strain *A. ferrooxidans* | 67.3 | 72.3 | 211.5 | 204.7 | 304.3 | 301.6 |
| Pure-strain ZJJN | 64.6 | 67.3 | 207.6 | 215.2 | 294.1 | 305.6 |
| Mixed-strains pH 3.5 | 188.1 | 193.0 | 461.7 | 472.7 | 534.0 | 525.1 |
| Mixed-strains pH 1.0 | 312.0 | 335.3 | 498.1 | 485.2 | 601.3 | 595.2 |

labeling was employed to determine cell densities of *A. ferrooxidans* and strain ZJJN, respectively. As shown in Fig. 5a, there were at least five base pairs of the target 16S rRNA region between ZJJN and *A. ferrooxidans*. It was accurate enough to distinguish homologous species, with the exclusion of only two base pair differences [6]. Generally, *A. ferrooxidans* and strain ZJJN stepped into the rapid growth phase after a certain period of mineral domestication. In the mixed-strains system with initial pH 3.5, the cell density of *A. ferrooxidans* was higher than in strain ZJJN after 10 days. Sequentially, the cell density of strain ZJJN began to exceed *A. ferrooxidans* on the 20th day, and the phenomenon was more obvious on the 30th day. This is probably because the pH of the system with initial pH 3.0 was <1.5 on the 20th day and the extreme acid thus greatly inhibited the growth of *A. ferrooxidans*, which is optimum at pH 2.0–3.0. However, the pH was fairly optimal for the growth of strain ZJJN, which has a lower optimal range of 0.2–2.0. Therefore, strain ZJJN showed superiority compared with *A. ferrooxidans* from the beginning in the other mixed-strains systems with initial pH 1.0 (Fig. 5). Some sulfur-oxidizing bacteria could tolerate an extremely acid environment and were the dominant organism replacing *A. ferrooxidans* in some extreme acid mine drainages [22].

## Physical changes of chalcopyrite analyzed by SEM

SEM analysis of chalcopyrite residues revealed that the surface of the mineral particles was covered with etched pits and polyporous layers, especially in the presence of

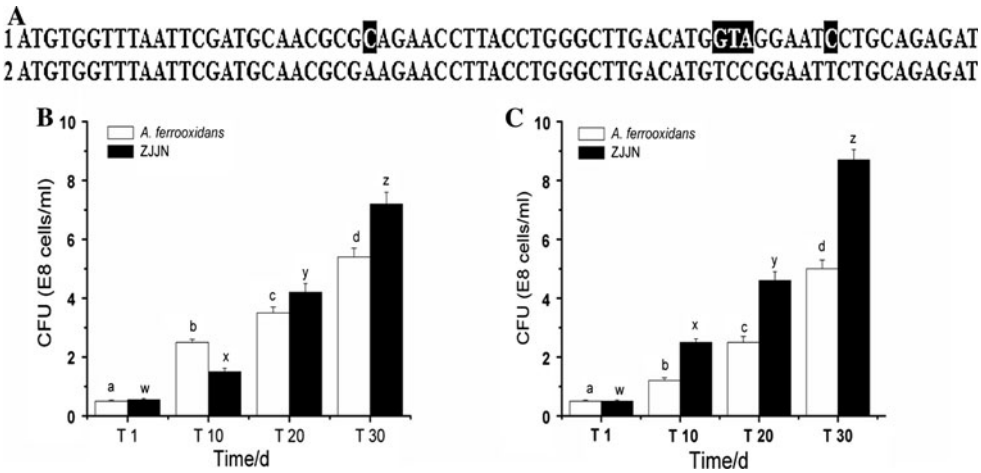


**Fig. 5** Community changes of bacteria in mixed-strains systems by 16S ribosomal RNA (rRNA)-based sandwich hybridization assay. **a** Differences in target 16S rRNA region between ZJJN and *Acidithiobacillus ferrooxidans* by 16S rRNA-based sandwich hybridization assay. *1* ZJJN, *2 A. ferrooxidans*; **b** initial pH 3.5; **c** initial pH 1.0. *a–d* and *w–z* represent statistically significant differences (d > c > b > a; z > y > x > w)

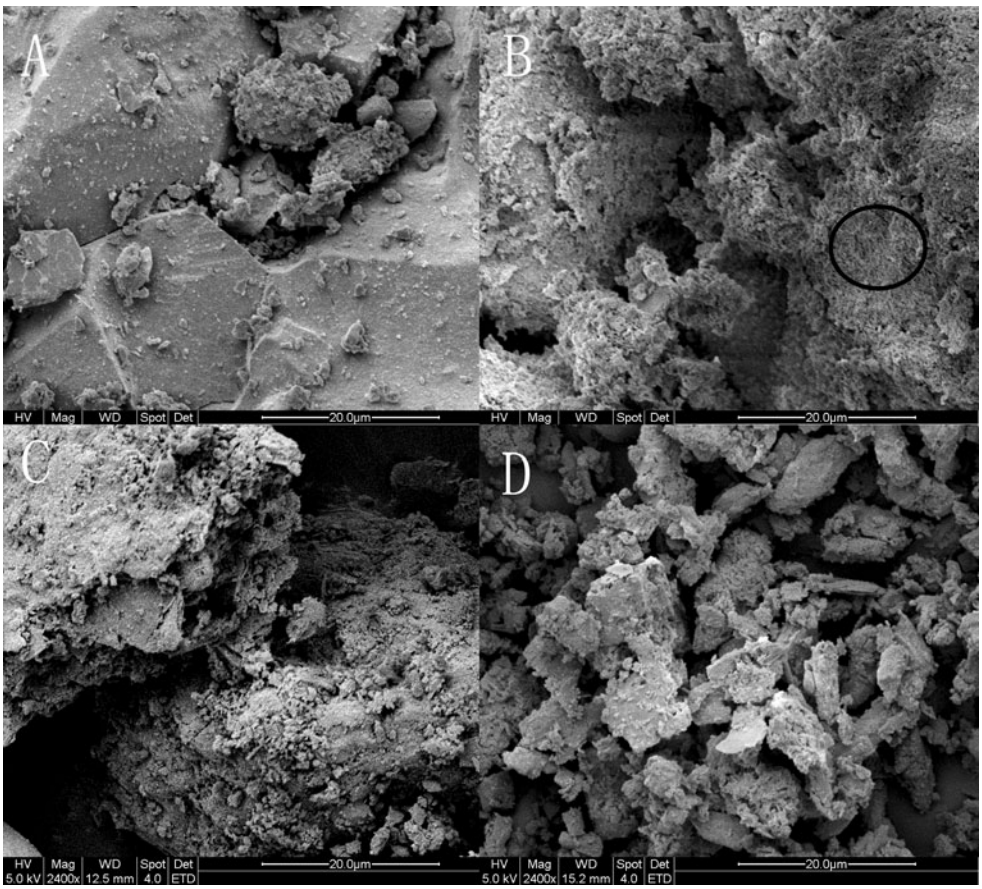


**Fig. 6** Scanning electron microscope (SEM) images (*bar*, 20 μm) of chalcopyrite residues after 31 days of bioleaching. **a** Blank control; **b** pure-strain *Acidithiobacillus ferrooxidans*; **c** mixed-strain system with initial pH 3.5; **d** mixed-strain system with initial pH 1.0

mixed strains of *A. ferrooxidans* and ZJJN (Fig. 6). Large quantities of short rod-shaped bacteria, such as *A. ferrooxidans* and ZJJN, were attached or embedded in these etched pits, as there was direct bioleaching besides indirect electrochemical leaching in the *A. ferrooxidans* and ZJJN system. Comparatively, the surface of mineral particles was smoother in the blank control system. Additionally, visible jarosite precipitations were observed in the pure-strains *A. ferrooxidans* system, whereas there were only tiny precipitations in the mixed-strain system (Fig. 6b), which was especially clear in those with initial pH 1.0. This might be because iron hydrolysis and precipitation was significantly reduced in the low-acidity system. Jarosite precipitation is mainly influenced by pH and the

concentration of ferric ions [12], and some other factors, such as phosphate and bacterial growth, would also affect its formation [12]. SEM of residues and bioleaching also demonstrated that the least amount of jarosite precipitation was produced in the low pH system, and metal extraction yield increased (Fig. 6).

In summary, a novel *Acidithiobacillus* sp. ZJJN, which was active in an extremely acid environment, was isolated from leaching solution of an industrial-scale bioheap in Zijinshan copper mine, China. Strain ZJJN showed the highest homology with *Acidithiobacillus* genus after morphological and phylogenetic identification. However, compared with the other *Acidithiobacillus* species, strain ZJJN could bear lower pH (0–1) and was capable of growth even at pH 0. This characteristic was employed in chalcopyrite bioleaching to decrease production of jarosite precipitation and improve efficacy. The recovery of copper in the mixed-strain system with initial pH 1.0 was up to 60.1 %, which was far higher than with other systems. Results of microbial community and SEM analyses showed that ZJJN flourished well in the extremely acid system, with less production of jarosite precipitation. As an extreme acidophile, strain ZJJN might be of potential use in chalcopyrite bioleaching and for studying the acid resistance of microorganisms. The strain has been deposited within the China Center for Type Culture Collection (CCTCC) (M 2012104).

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