



Transformation of iron in pure culture process of extremely acidophilic microorganisms

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Received 8 January 2016; accepted 21 June 2016

Abstract: Jarosite and extracellular polymer substance generated during pure culture and bioleaching process have been widely accepted the main transformation of decreasing iron in the medium. In the present work, acidophilus bioleaching organisms *Ferroplasma thermophilum*, *Leptospirillum ferriphilum* and *Acidithiobacillus ferrooxidans* were cultured. It was found that they can live in low pH environment, and more than 10 particles in each cell intracellular nano-particles are synthesized in the cells. By analyzing the morphology and chemical composition of nano-particles, they were found to contain iron, and the three microorganisms belonged to high-yielding strains. The results show that the transformation of the decreasing iron ions is not only generating jarosite, but also taken into cells and synthesizing ferruginous nano-particles.

Key words: jarosite; extracellular polymer substance; bioleaching; ferruginous nano-particles

1 Introduction

Acidithiobacillus ferrooxidans is a kind of acidophilus, autotrophic, obligate aerobic and gram-negative bacterium that thrives optimally at 30 °C and pH 2 [1–4]. *Leptospirillum ferriphilum* is gram-negative bacteria, which can grow in extremely acidic environment and grow optimally in inorganic media within pH range of 1.3–2.0 [5,6]. *Ferroplasma thermophilum* is a kind of acidophilus archaea that thrives optimally at 45 °C and pH 1.0 [7,8]. All those three kinds of microorganisms have the ability of oxidation of ferrous ions and can commonly grow in 9K medium [9].

Jarosite precipitation is a very widespread phenomenon observed in many bacterial cultures, especially in bioleaching process [10]. The jarosites are ammoniojarosites with the formula $\text{MFe}_3(\text{SO}_4)_2(\text{OH})_6$, where $\text{M}=\text{K}^+$, Na^+ , NH_4^+ , Ag^+ , or H_3O^+ . The formation of jarosite precipitation is a reaction in competition with the hydrolysis reaction giving products of basic ferric

hydroxysulphates. In the cultures process of *Ferroplasma thermophilum*, *Leptospirillum ferriphilum* and *Acidithiobacillus ferrooxidans*, the medium is 9K which contains a high concentration of NH_4^+ ions [9], and the jarosites produced are ammoniojarosites with formula $\text{NH}_4\text{Fe}_3(\text{SO}_4)_2(\text{OH})_6$. Abundant iron ions will reduce and form jarosites in the medium.

Extracellular polymer substance (EPS) is also a very widespread phenomenon observed in *Acidithiobacillus* and *Leptospirillum* cultures [11,12]. The extracellular polymeric substances of both species mainly consist of neutral sugars and lipids [13]. The functions of the exopolymers seem to mediate attachment to a (metal) sulfide surface, and to concentrate iron ions by complexation through uronic acids or other residues at the mineral surface, thus allowing an oxidative attack on the sulfide. In addition, it also contains iron ions [13,14].

Small-angle X-ray scattering [15,16] is well designed to study the nanoscale structures in a material, which is especially appropriate to measure nanoparticles because of their nanoscale sizes. In addition, nanoparticles are also the focus research object to seek after

Foundation item: Project (51374248) supported by the National Natural Science Foundation of China; Project (NCET-13-0595) supported by the Program for New Century Excellent Talents in University of China; Project (2016-SSRF-PT-006152) supported by the Shanghai Synchrotron Radiation Facility (SSRF), China; Project (2016-BEPC-PT-000855) supported by the Beijing Synchrotron Radiation Facility (BSRF), China

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DOI: 10.1016/S1003-6326(17)60134-1

nucleation mechanism and growth manner of materials. LI et al [15] studied the use of the small-angle X-ray scattering technique to probe the nucleation and growth regularity of nanomaterials. WANG et al [17] studied the size and shape evolution of gold nanoparticles in aqueous solution by using real-time small-angle X-ray scattering and ultraviolet-visible spectra at the 1W2A small-angle X-ray scattering station of Beijing synchrotron Radiation Facility, China.

Organisms have evolved complex strategies to maintain optimal intracellular iron pools without compromising cell viability, which include iron-dependent gene regulation using an array of transcriptional regulators from Fur, DtxR and RirA families, iron acquisition in either free form (e.g. Fe21/Mn21 NRAMP family transporters) or sequestered by small molecules (siderophores), intracellular storage proteins such as ferritins, and readily inducible defenses against free-radical formation [15,16].

In the present work, the transformation of decreasing iron in the medium was investigated. The intracellular nano-particles were observed by transmission electron microscope (TEM), the chemical composition of nano-particles was analyzed by energy spectrum analysis, and the grain size of nano-particles was analyzed by small angle X-ray scattering.

2 Materials and methods

A. ferrooxidans f1, *F. thermophilum* L1^T (EF062309) and *L. ferriphilum* YSK used in this work were provided by the Key Laboratory of Biohydrometallurgy, Ministry of Education, China. The strains were cultivated in basal salts of modified 9K medium, the pH of *A. ferrooxidans* f1 was adjusted to 2.0, by adding 50% H₂SO₄ (volume fraction), and the culture temperature was 30 °C; the pH of *F. thermophilum* L1^T was adjusted to 1.0, and the culture temperature was 45 °C; the pH of *L. ferriphilum* YSK was adjusted to 1.6, and the culture temperature was 45 °C. FeSO₄·7H₂O was added into the medium, after that these microorganisms were cultured at rotation

speed of 170 r/min. Additional 0.2% yeast extract was added to *F. thermophilum* L1^T.

Ultrathin sections were obtained with an ultramicrotome (LKB-5, LKB, Sweden) and then stained with uranyl acetate and lead citrate. The morphologies were observed by TEM (FEI Tecnai Spirit) at an accelerating voltage of 80 kV.

A drop of cell was deposited onto a copper grid (62 μm) covered by a carboncoated formvar film. The cells were allowed to settle on the grid for 10 min, and excess liquid was removed with a filter paper. All grids were rinsed at least twice with distilled water before TEM observation. In case of negative staining, wet grids were treated with a drop of negative staining solution (1.5% uranyl acetate) for 1 min and then observed by TEM (JEM2100F) at an accelerating voltage of 200 kV.

The concentrations of total iron in solution were measured by atomic absorption spectrometry, the ferrous iron concentration was determined by titration with potassium dichromate and the ferric iron concentration was equal to the difference between the concentrations of total iron and ferrous iron. The grain size of nano-particles was analyzed by small angle X-ray scattering at the small angle scattering experiment station (1W2A) at Beijing Synchrotron Radiation Facility, China.

3 Results and discussion

3.1 Morphology of nano-particles

TEM observation was used to analyze the morphology inside the microorganisms. As shown in Fig. 1, nano-particles are seen in *A. ferrooxidans* f1, *F. thermophilum* L1^T and *L. ferriphilum* YSK. The arrangements of the nano-particles inside the three strains are different, the arrangement of the nano-particles is random and disperse in the *A. ferrooxidans* f1 and *L. ferriphilum* YSK. However, in *F. thermophilum* L1^T, the nano-particles are gathered. The size of the nano-particles is inhomogenous, and there are more than 10 nano-particles in each cell.

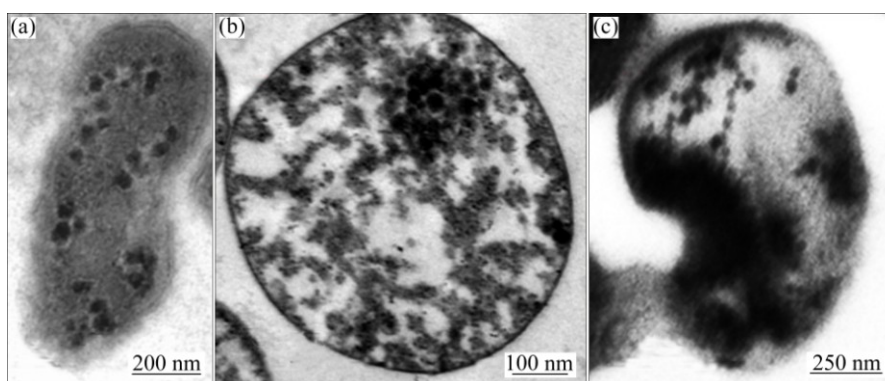


Fig. 1 TEM images of microorganisms: (a) *A. ferrooxidans*; (b) *L. ferriphilum*; (c) *F. thermophilum*

It has been reported that magnetotactic bacteria (MTB) [18–20], living in the anaerobic or microaerobic neutral or alkaline environment [21–23], biomineralize unique organelles called magnetosomes which are usually arranged as a chain within the cell, thereby maximizing the magnetic dipole moment of the cell and causing the cell to passively align along magnetic field lines as they swim [24]. Magnetosomes consist of magnetic mineral crystals, either magnetite (Fe_3O_4) or greigite (Fe_3S_4), enveloped by a bilayer membrane composed mostly of phospholipids, called the magnetosome membranes, which contain a number of proteins not present in the cytoplasmic and outer membranes (OMs) and are unique to MTB [19]. Obviously, there are differences between the nanocrystals and magnetosomes. TEM images can prove magnetosomes are membrane-enveloped. However, for the nanoparticles, there is no direct evidence demonstrating the nanocrystals are membrane-enveloped. Normally, magnetosomes are organized into one or multiple chain-like structures within the cell in order to optimize the cellular magnetic dipole moment. It is more like a compass needle to facilitate the navigation of MTB using the Earth's magnetic field, which causes MTB respond and orient along the lines of magnetic fields to find the anaerobic or microaerobic environment for living [24]. However, the arrangement of nanocrystals in

A. ferrooxidans f1, *F. thermophilum* L1^T and *L. ferriphilum* YSK is irregular, not in a line, and it may get together, as shown in Fig. 2(a). Take *F. thermophilum* for example, the nano-particles seem globular (Fig. 2(b)) and tooth-shaped (Fig. 2(c)), respectively. The size of magnetosomes is generally 20–30 nm, while the size of the nano-particles is inhomogeneous, the size of nano-particles of *A. ferrooxidans* f1 is 35–45 nm; for *F. thermophilum* L1^T, the size of nano-particles is approximately 20–30 nm; for *L. ferriphilum* YSK, the size of nano-particles approximately is 40–50 nm, as shown in Fig. 3. It has previously reported that *Acidithiobacillus ferrooxidans* can produce magnetite, and the number of those magnetite is 1–3 per cell [25,26]. In this work, more than 10 nano-particles are found in *A. ferrooxidans* f1, *F. thermophilum* L1^T, *L. ferriphilum* YSK in each cell. It is suggested that *A. ferrooxidans* f1, *F. thermophilum* L1^T and *L. ferriphilum* YSK not only can synthesize intracellular nanometre-sized particles, but also form a kind of high-yield nano-particle producing strains.

3.2 Chemical composition of nano-particles

There are plenty of nano-particles in the cell, however, the morphology of nano-particles is not the same. The energy dispersive X-ray (EDX) analysis results show that the nano-particles contain iron. Hence,

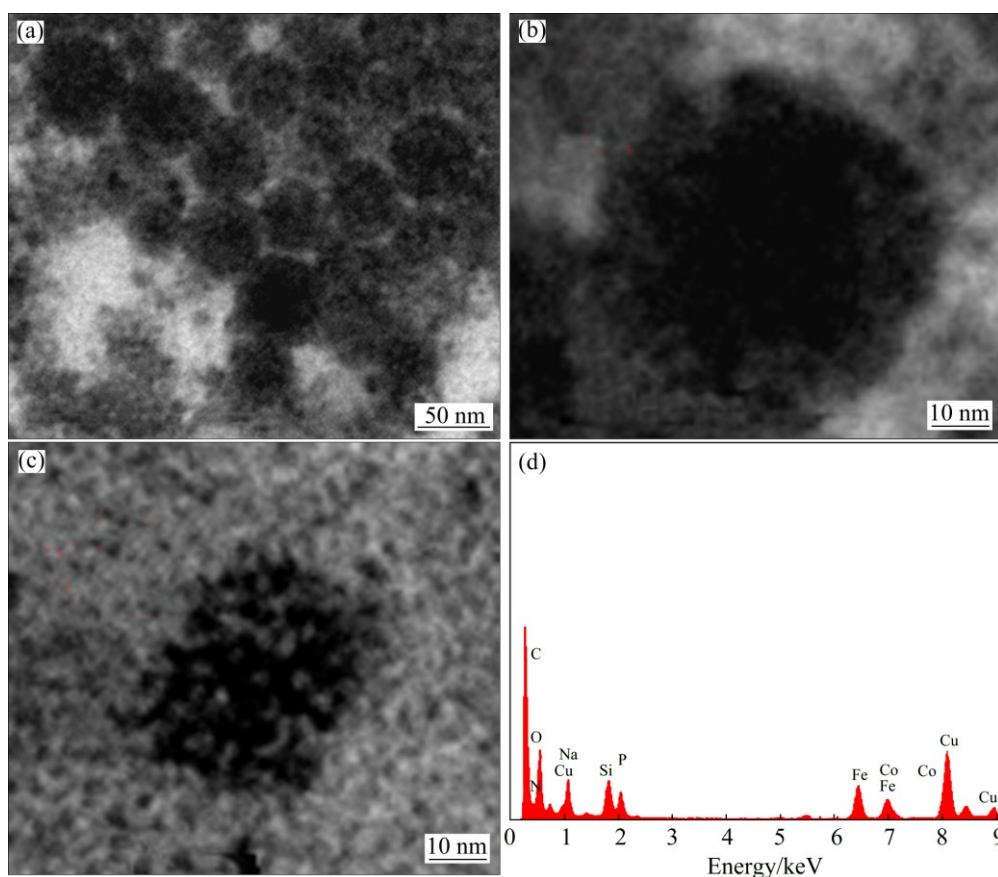


Fig. 2 TEM images (a, b, c) of microorganisms in *F. thermophilum* and corresponding EDX spectra (d)

it can be inferred that the transformation of the decreasing iron in the medium not only form jarosite and EPS, but also form intracellular ferruginous-nano-particles.

However, the chemical composition of the nano-particles is different from that of magnetosomes which contains iron minerals of magnetite (Fe_3O_4) and/or greigite (Fe_3S_4) and high amount of iron, oxygen and/or sulfur [19,20]. As shown in Fig. 2(d), the nano-particles contain iron, cobalt and oxygen, but no sulphur. It is found that the nano-particles of *A. ferrooxidans* f1, *F. thermophilum* L1^T and *L. ferriphilum* YSK contain cobalt as well in nature conditions. It is notable that no cobaltic ions are directly added into the culture medium, the source of cobalt is from the $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ that was added into the medium, which is not pure and mixed with cobalt ions, as indicated by ICP-OES analysis.

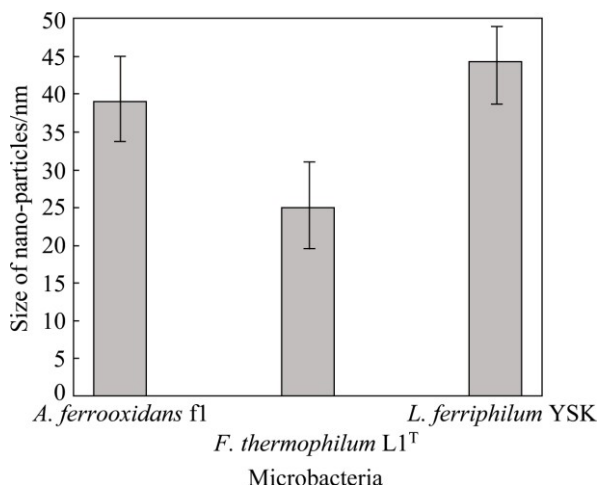


Fig. 3 Size of nano-particles of three microorganisms obtained by small angle X-ray scattering

3.3 Transformation of decreasing iron in medium

In the bioleaching process, the concentration of total iron decreases after an initial increase, as shown in Fig. 4. The reason of this phenomenon is that the iron is dissolved step by step from minerals containing iron, such as copper pyrites and bornite, hence, there is an initial increase. However, with the formation of jarosite and other ferruginous substance, the concentration of iron decreases. In the pure culture process, the concentration of total iron constantly decreases, as shown in Fig. 5. By the biological oxidation of microorganisms, the ferrous irons are constantly transformed to ferric irons, hence, the ferrous irons constantly decrease, and the ferric irons increase. Because the energy source is $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, it immediately dissolves once added into medium, so it does not have an initial increasing stage. Anyway, the total iron decreases both in bioleaching and pure culture

process. Previous research suggested that the transformation of the decreasing iron ions in the medium is forming ferruginous sediment and uptaken by cells [27,28]. As iron is an essential nutrient, the cell uptakes it into cytosolic, it is clear that microorganisms thrive in acidic ecosystems with high iron loads requiring a comprehensive investigation of the strategies to acquire iron and to coordinate this acquisition with utilization, storage and oxidation of iron through metal responsive regulation, however, the content of iron is very low, hence, the main transformation of the decreasing iron ions in the medium is forming jarosite and EPS. In this work, the decreasing iron ions in the medium are also uptaken by cell and form abundant ferruginous nano-particles, as shown in Fig. 6. By the interaction of biological oxidation, Fe^{2+} is transferred to Fe^{3+} . After a pure culture for a long time, part of them would transfer to jarosite, and the cell would uptake Fe^{2+} and Fe^{3+} to form EPS.

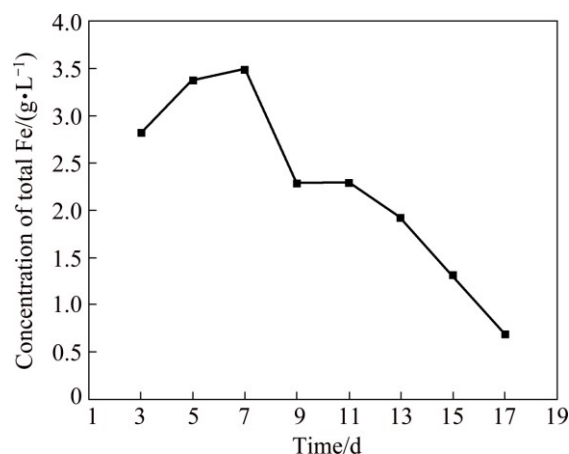


Fig. 4 Total iron concentration in bioleaching process

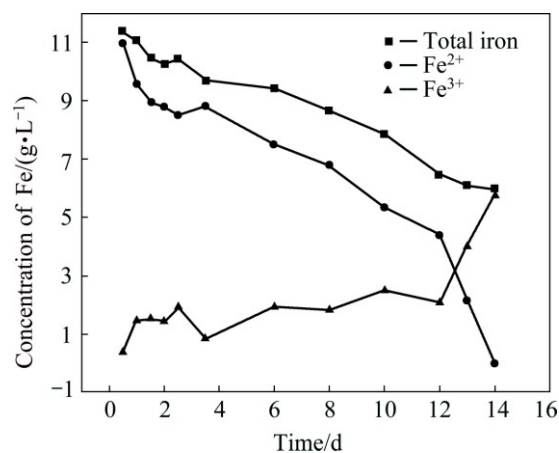


Fig. 5 Iron concentration in pure culture process

3.4 Cycle of iron in extreme acid environment

In the nature extreme acid environment, the main valence states of iron are Fe^{2+} and Fe^{3+} , which can interconvert, and the Fe^{2+} is oxidized to Fe^{3+} by

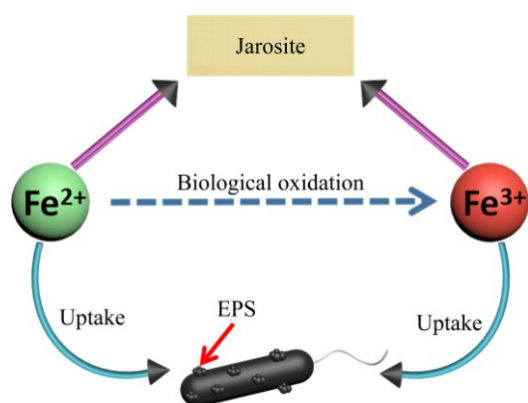


Fig. 6 Schematic diagram showing bioleaching process

biological or chemical oxidation. *L. ferriphilum* YSK, *A. ferrooxidans* f1 and *F. thermophilum* L1^T have enzymes of iron (II) ion oxidation, so they have the ability to oxidize Fe^{2+} to Fe^{3+} and also the Fe^{2+} can be reduced to Fe^{3+} by biological and chemical reduction [1,5,8]. Meanwhile, the cell will uptake iron ions both Fe^{2+} and Fe^{3+} into cytosolic by ion channel. *Acidithiobacilli* and the *Leptospirilla* have been predicted FeoB-like Fe (II) and Nramp-like Fe(II)-Mn(II) transporters exhibiting a plethora of predicted TonB-dependent Fe(III) transport systems [29]. It will accumulate within the cells during the formation of intracellular ferruginous nano-particles, however the biochemical/chemical pathways of which are still not completely understood. When cells die, parts of or entire magnetofossils could be dissolved thereby releasing Fe^{2+} and Fe^{3+} ions back to environmental iron cycling, as shown in Fig. 7. While other magnetofossils may be deposited into sediments eventually leading to mineral iron formation.

Up to now, it is just discovered that three kinds of extreme acid microorganisms can synthesize ferruginous nano-particles, the species involve bacteria and archaea, and the morphology involves rhabditiform, heliciform and globular, which may be a universal function of extreme acid microorganisms. More research involving the distribution and ecology of extreme acid microorganisms that can synthesize ferruginous nano-particles is necessary, which helps to better understand the conditions under which and to what extent extreme acid microorganisms affect the biogeochemical cycle of iron. As shown in Fig. 7, by the interaction of biological or chemical oxidation, Fe^{2+} is transferred to Fe^{3+} ; while by the interaction of biological or chemical reduction, Fe^{3+} is transferred to Fe^{2+} , so the bacteria uptakes both Fe^{2+} and Fe^{3+} . When the bacteria die, the bacteria transfer to magnetofossils. Then the magnetofossils dissolve into iron biominerals, and they dissolve into Fe^{2+} and Fe^{3+} , meanwhile the iron biominerals also dissolve into Fe^{2+} and Fe^{3+} .

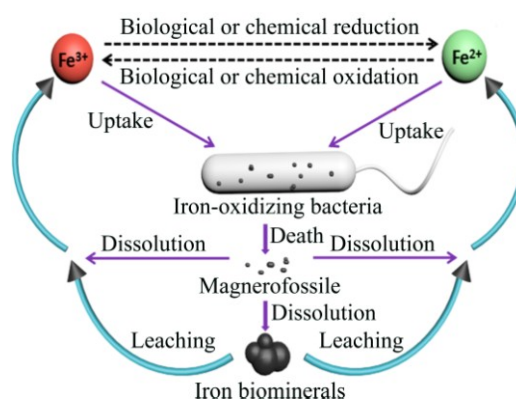


Fig. 7 Circle process of iron, bacteria and biominerals

4 Conclusions

1) The transformation of decreasing iron in media is investigated, in the pure culture process, the total iron constantly decreases. While in the bioleaching process, the total iron decreases after an initial increase.

2) *Acidithiobacillus ferrooxidans* f1, *Ferroplasma thermophilum* L1^T and *Leptospirillum ferriphilum* YSK have the ability to synthesize intracellular nano-particles containing iron, and the size of nano-particles is inhomogenous. There are more than 10 particles in each cell, so the three microorganisms belong to high-yielding strains.

3) The transformation of iron is not only generating jarosite and EPS, but is also taken into cell and synthesizing ferruginous nano-particles.

References

- [1] AMOURIC A, BROCHIER-ARMANET C, JOHNSON D B, BONNEFOY V, HALLBERG K B. Phylogenetic and genetic variation among Fe (II)-oxidizing *acidithiobacilli* supports the view that these comprise multiple species with different ferrous iron oxidation pathways [J]. Microbiology-SGM, 2011, 157: 111–122.
- [2] KELLY D P, WOOD A P. Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov and *Thermithiobacillus* gen. nov [J]. International Journal of Systematic and Evolutionary Microbiology, 2000, 50: 511–516.
- [3] LILJEQVIST M, RZHEPISHEVSKA O I, DOPSON M. Gene identification and substrate regulation provide insights into sulfur accumulation during bioleaching with the psychrotolerant acidophile *Acidithiobacillus ferrivorans* [J]. Applied and Environmental Microbiology, 2013, 79: 951–957.
- [4] LILJEQVIST M, VALDES J, HOLMES D S, DOPSON M. Draft genome of the psychrotolerant acidophile *Acidithiobacillus ferrivorans* SS3 [J]. Journal of Bacteriology, 2011, 193: 4304–4305.
- [5] CORAM N J, RAWLINGS D E. Molecular relationship between two groups of the genus *Leptospirillum* and the finding that *Leptospirillum ferriphilum* sp nov dominates South African commercial biooxidation tanks that operate at 40 °C [J]. Applied and Environmental Microbiology, 2002, 68: 838–845.
- [6] LIU J S, XIE X H, XIAO S M, WANG X M, ZHAO W J, TIAN Z L.

- Isolation of *Leptospirillum ferriphilum* by single-layered solid medium [J]. Journal of Central South University of Technology, 2007, 14: 467–473.
- [7] ZHANG L, ZHOU W, LI K, MAO F, WAN L, CHEN X, ZHOU H, QIU G. Synergetic effects of *Ferroplasma thermophilum* in enhancement of copper concentrate bioleaching by *Acidithiobacillus caldus* and *Leptospirillum ferriphilum* [J]. Biochemical Engineering Journal, 2015, 93: 142–150.
- [8] ZHOU H, ZHANG R, HU P, ZENG W, XIE Y, WU C, QIU G. Isolation and characterization of *Ferroplasma thermophilum* sp nov., a novel extremely acidophilic, moderately thermophilic archaeon and its role in bioleaching of chalcopyrite [J]. Journal of Applied Microbiology, 2008, 105: 591–601.
- [9] SILVERMAN M P, LUNDGREN D G. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. II. Manometric studies [J]. Journal of Bacteriology, 1959, 78: 326–331.
- [10] DAOUD J, KARAMANEV D. Formation of jarosite during Fe^{2+} oxidation by *Acidithiobacillus ferrooxidans* [J]. Minerals Engineering, 2006, 19: 960–967.
- [11] CRUNDWELL F K. How do bacteria interact with minerals? [J]. Hydrometallurgy, 2003, 71: 75–81.
- [12] VERA M, SCHIPPERS A, SAND W. Progress in bioleaching: Fundamentals and mechanisms of bacterial metal sulfide oxidation—Part A [J]. Applied Microbiology and Biotechnology, 2013, 97: 7529–7541.
- [13] SAND W, GEHRKE T. Extracellular polymeric substances mediate bioleaching/biocorrosion via interfacial processes involving iron (III) ions and acidophilic bacteria [J]. Research in Microbiology, 2006, 157: 49–56.
- [14] JENSEN A B W C. Ferrous sulphate oxidation using *Thiobacillus ferrooxidans*: A review [J]. Process Biochemistry (Oxford), 1995, 30 (3): 225–236.
- [15] LI Z Z, WU Z H, MO G, XING X Q, LIU P. A small-angle X-ray scattering station at Beijing Synchrotron Radiation Facility [J]. Instrumentation Science & Technology, 2014, 42(2): 128–141.
- [16] LI Z Z. A program for SAXS data processing and analysis [J]. Chinese Physics C, 2013, 37(10): 110–115.
- [17] WANG W, ZHANG K, CAI Q, MO G, XING X Q, CHENG W D, CHEN Z J, WU Z H. Real-time SAXS and ultraviolet-visible spectral studies on size and shape evolution of gold nanoparticles in aqueous solution [J]. European Physical Journal B, 2010, 76(2): 301–307.
- [18] KOMEILI A. Molecular mechanisms of compartmentalization and biomineralization in magnetotactic bacteria [J]. Fems Microbiology Reviews, 2012, 36: 232–255.
- [19] FAIVRE D, SCHUELER D. Magnetotactic bacteria and Magnetosomes [J]. Chemical Reviews, 2008, 108: 4875–4898.
- [20] UEBER R, SCHUELER D. Magnetosome biogenesis in magnetotactic bacteria [J]. Nature Reviews Microbiology, 2016, 14: 621–637.
- [21] LEFEVRE C T, BAZYLINSKI D A. Ecology, diversity, and evolution of magnetotactic bacteria [J]. Microbiology and Molecular Biology Reviews, 2013, 77: 497–526.
- [22] BAZYLINSKI D A, LEFEVRE C T. Magnetotactic bacteria from extreme environments [J]. Life, 2013, 3: 295–307.
- [23] LEFEVRE C T, FRANKEL R B, POSFAI M, PROZOROV T, BAZYLINSKI D A. Isolation of obligately alkaliphilic magnetotactic bacteria from extremely alkaline environments [J]. Environmental Microbiology, 2011, 13: 2342–2350.
- [24] LIN W, BAZYLINSKI D A, XIAO T, WU L, PAN Y. Life with compass: Diversity and biogeography of magnetotactic bacteria [J]. Environmental Microbiology, 2014, 16: 2646–2658.
- [25] YAN L, YUE X, ZHANG S, CHEN P, XU Z, LI Y, LI H. Biocompatibility evaluation of magnetosomes formed by *Acidithiobacillus ferrooxidans* [J]. Materials Science & Engineering C, 2012, 32: 1802–1807.
- [26] YAN L, ZHANG S, CHEN P, WANG W, WANG Y, LI H. Magnetic properties of *Acidithiobacillus ferrooxidans* [J]. Materials Science & Engineering C, 2013, 33: 4026–4031.
- [27] POTRYKUS J, JONNA V R, DOPSON M. Iron homeostasis and responses to iron limitation in extreme acidophiles from the *Ferroplasma* genus [J]. Proteomics, 2011, 11: 52–63.
- [28] WEAVER E A, WYCKOFF E E, MEY A R, MORRISON R, PAYNE S M. FeoA and FeoC are essential components of the vibrio cholerae ferrous iron uptake system, and FeoC interacts with FeoB [J]. Journal of Bacteriology, 2013, 195: 4826–4835.
- [29] OSORIO H, MARTINEZ V, NIETO P A, HOLMES D S, QUATRINI R. Microbial iron management mechanisms in extremely acidic environments: Comparative genomics evidence for diversity and versatility [J]. BMC Microbiology, 2008, 8(1): 1–18.

极端嗜酸微生物纯培养过程中铁元素的转化

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摘要: 在培养浸矿微生物过程中, 培养基中的铁含量不断减少, 一般认为主要是生成了黄钾铁矾沉淀与胞外多聚物。对三种嗜酸浸矿菌 *Ferroplasma thermophilum*, *Leptospirillum ferriphilum* 和 *Acidithiobacillus ferrooxidans* 进行培养, 发现他们能在嗜酸环境下生存, 而且平均每个细胞生成超过 10 个纳米颗粒。通过分析纳米颗粒的形态与成分, 发现颗粒中含有铁, 而且纳米颗粒的产量很高。结果表明, 在纯培养过程中减少的铁不仅参与生成黄钾铁矾, 同时也被吸收进入细胞, 合成含铁的纳米颗粒。

关键词: 黄钾铁矾; 胞外聚合物; 生物冶金; 含铁纳米颗粒

(Edited by Sai-qian YUAN)