

## Sulfur activation-related extracellular proteins of *Acidithiobacillus ferrooxidans*

ZHANG Cheng-gui(张成桂), ZHANG Rui-yong(张瑞永), XIA Jin-lan(夏金兰),  
ZHANG Qian(张倩), NIE Zhen-yuan(聂珍媛)

Key Laboratory of Biometallurgy of Ministry of Education, School of Minerals Processing and Bioengineering,  
Central South University, Changsha 410083, China

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**Abstract:** The fractions of the extracellular proteins of *Acidithiobacillus ferrooxidans* grown on two different energy substrates, elemental sulfur and ferrous sulfate, were selectively prepared with hot water treatment and distinctly shown by two-dimensional gel electrophoresis. Some protein spots with apparently higher abundance in sulfur energy substrate than in ferrous sulfate energy substrate were identified by using MALDI-TOF/TOF. Based on peptide mass fingerprints and bioinformatical analysis, the extracellular proteins were classified according to their functions as conjugal transfer protein, pilin, vacJ lipoprotein, polysaccharide deacetylase family protein, Ser/Thr protein phosphatase family protein and hypothetical proteins. Several extracellular proteins were found abundant in thiol groups and with CXXC functional motif, these proteins may be directly involved in the sulfur activation by use of their thiol group (Pr-SH) to bond the elemental sulfur.

**Key words:** sulfur oxidation; extracellular proteins; *Acidithiobacillus ferrooxidans*

### 1 Introduction

Elemental sulfur as the intermediate product of the bioleaching process may be accumulated and form a sulfur layer on the mineral surface, leading to the modification of the electrochemical properties of mineral surface and the inhibition of the bioleaching process [1–2]. It is apparently significant to elucidate and find the efficient way to eliminate this sulfur layer. Actually, the elemental sulfur in bioleaching is basically chemically inert, and it can exclusively be eliminated by bio-oxidation of acidophilic sulfur-oxidizing bacteria in typical acidic environments [1,3].

It is known that oxidation of elemental sulfur by acidophilic sulfur-oxidizing bacteria is a complex process that involves principally the attachment of cells to sulfur particles, the transportation of sulfur through the outer membrane and the oxidation of sulfur in the periplasmic space [4–5]. A prerequisite for initial attachment of bacterial cells to the sulfur particles is that the extracellular polymeric substances (EPS) excreted by

cells mediate this contact, by modifying the surfaces of both the sulfur particles and the cells themselves [6–7]. EPS bridge the contact between sulfur and cells to overcome the sulfur surface hydrophobicity, and assure cells to adsorb onto sulfur surface. The extracellular proteins and outer-membrane proteins were considered playing the key role in the sulfur mobilization and sulfur transportation, respectively [8–9]. *Acidithiobacillus thiooxidans* grown on elemental sulfur exposed extensive membranous blebbing of outer membrane which coated the surface of elemental sulfur particles to promote the dissolution of sulfur to colloidal sulfur [3]. A sulfur binding surface protein of 40 ku has been identified in *A. ferrooxidans* grown on elemental sulfur, which locates on the flagella, has thiol groups and adheres to elemental sulfur particles [10], and several outer-membrane proteins have been suggested to be involved in the adhesion of *A. ferrooxidans* to various surfaces and highly regulated by different energy substance [10–12]. However, up to now, few studies have been devoted to evaluating the extracellular proteins for adaptation of bacterial cells to the hydrophobic sulfur and activation/mobilization of

sulfur by *A. ferrooxidans* cells.

The present work was dedicated to analyzing the changes of *A. ferrooxidans* extracellular proteins when the bacteria cells are cultured in two different energy substrates, elemental sulfur and ferrous sulfate, in order to further understand the sulfur mobilization process.

## 2 Experimental

### 2.1 Bacterial strains and cultivation conditions

*A. ferrooxidans* ATCC23270 was obtained from the American Type Culture Collection. It was cultivated at 30 °C in 9K medium containing 5.0 g/L of S<sup>0</sup> or 22.5 g/L ferrous sulfate and the following components (g/L): (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5; KCl, 0.1; K<sub>2</sub>HPO<sub>4</sub>, 0.5 and Ca (NO<sub>3</sub>)<sub>2</sub>, 0.01. The initial pH of these media was adjusted to 2.0 with KOH.

### 2.2 Isolation and partial purification of extracellular proteins

Bacteria cells were harvested in the exponential growth phase by centrifugation from an initial 8 L sample of bacterial culture. The pellet was washed with distilled water (pH=2), and then resuspended in 10 mL of distilled water (pH=2) at 60 °C for 40 min in a shaking water bath to shed the extracellular proteins from cells. The supernatants obtained on centrifugation after all treatments were filtered through 0.22 μm cellulose acetate filters in a Pyrex filtering apparatus (Millipore) to ensure that samples were free of cells. The supernatants with extracellular proteins were extensively dialyzed against distilled water at 4 °C in sterilized dialysis bag (cutoff, 3 500 Da). The remaining solution of crude extracellular proteins was freeze-dried and stored at -80 °C.

### 2.3 Two-dimensional gel electrophoresis (2-DE)

2-DE was performed with the IPGphor system and Ettan DALT II system (GE). After electrophoresis, proteins were visualized by Coomassie brilliant blue R-250 staining.

### 2.4 Image acquisition and analysis

All experiments were duplicated and the corresponding gels were realized in triplicate. Two-dimensional gels were scanned using a Uniscan scanner (Tsinghua Unisplendour). The number of protein spots on the 2-DE gels was analyzed using Imagemaster software version 7.2 (GE).

### 2.5 In-gel tryptic digestion of 2-DE spots and MALDI-TOF/TOF

Protein spots were excised and subjected to in-gel digestion. Peptide mass fingerprints were obtained by

using an Autoflex MALDI-TOF/TOF tandem mass spectrometer (Bruker Daltonik Bremen), with delayed extraction and reflection[13-14].

### 2.6 Database searches for protein identification and protein sequence analysis

Peptide mass fingerprints were analyzed by using MASCOT search engine (www.matrixscience.com) based on SwissProt and AFE-3217 protein databases. The functional categories for the different identified proteins were obtained from the genomic sequence of *A. ferrooxidans* (www.tigr.org). The presence of export signals for each protein found in the periplasmic fraction was predicted by using SignalP (www.cbs.dtu.dk/services/signalP), TatP (www.cbs.dtu.dk/services/TatP-1.0), SecretomeP (www.cbs.dtu.dk/services/SecretomeP), and LipoP(www.cbs.dtu.dk/services/LipoP). The predicted subcellular locations of the proteins coded by the different ORFs analyzed were obtained using PENCE proteome analyst (<http://pasub.cs.ualberta.ca:8080/pa/Subcellular>) and CELLO (cello.life.nctu.edu.tw) programs. The possible presence of transmembrane domains in the ORFs analyzed was predicted by TMHMM (www.cbs.dtu.dk/services/TMHMM).

## 3 Results and discussion

### 3.1 Electrophoresis of extracellular proteins of *A. ferrooxidans* grown on sulfur and ferrous sulfate

The 1-D and 2-D electrophoregrams of the extracellular proteins of *A. ferrooxidans* grown on sulfur and ferrous sulfate are shown in Figs.1(a) and (b), respectively. It was found that the extracellular proteins of *A. ferrooxidans* grown on sulfur were much more complex than those of *A. ferrooxidans* grown on ferrous sulfate. Some extracellular polymeric substances were actively expressed when *A. ferrooxidans* cells were grown on sulfur, and the sulfur became bio-modified when the sulfur was treated by *A. ferrooxidans* cells[9]. So it is reasonable to infer that extracellular polymeric substances are responsible for overcoming the hydrophobic barrier during colonization of sulfur, and the extracellular proteins have the possible role to bond the sulfur with the direct biochemical reaction.

### 3.2 Identification and predication of extracellular proteins spots

As shown in Fig.1, there were some protein spots with relatively high abundance in sulfur energy substrate but not in ferrous sulfate energy substrate. Eighteen protein spots with relatively high abundance in sulfur substrate were excised in gel. Identification and predication of these eighteen extracellular proteins were made by MALDI-TOF/TOF followed by database

searches against SwissProt and AFE-3217 based on the peptide mass fingerprints, and the results are listed in Table 1.

### 3.3 Classification and analysis of extracellular proteins of *A. ferrooxidans* in elemental sulfur

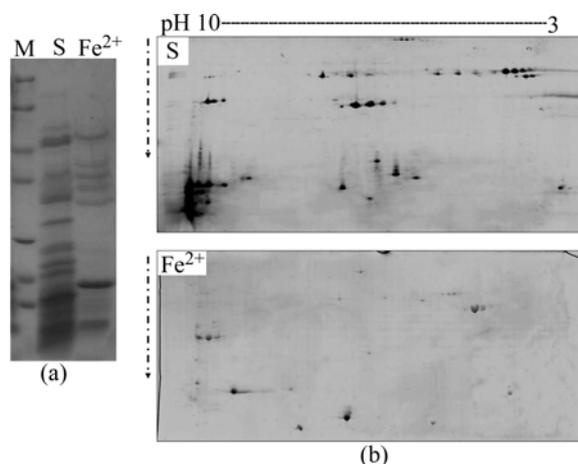
The identification and predication of extracellular protein spots were divided into different categories according to their potential biological functions in the cell, i.e., AFE\_1391: conjugal transfer protein; AFE\_2621: pilin; AFE\_1847: lipoprotein; AFE\_0982: vacJ lipoprotein; AFE\_0927: polysaccharide deacetylase

**Table 1** Identification and predication of extracellular proteins spots of *A. ferrooxidans* in sulfur energy substrate by MALDI-TOF/TOF followed by database searches against SwissProt and AFE-3217 based on peptide mass fingerprints

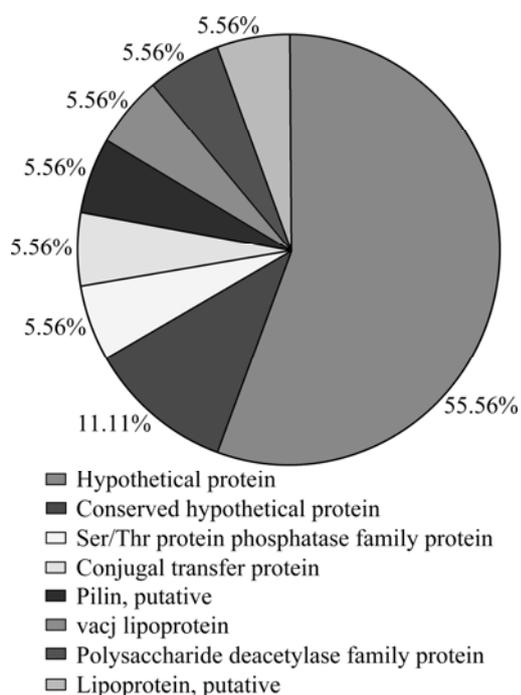
Spot No.	Locus	Common name	TIGR annotation	TheoreticalMr (KDa)/pI	Export signaland	No. of TM*	CELLO prediction localization	TMHMM prediction result
1	AFE0859	Hypothetical protein		36.71/4.63	Sec.,		Extracellular or outer membrane	Outside of membrane
2	AFE0206	Conserved hypothetical protein		28.36/5.04			Cytoplasmic or extracellular	Outside of membrane
3	AFE1932	Ser/Thr protein phosphatase family protein	Unknown function, enzymes of unknown specificity	32.31/5.06		1: 77-82 aa	Cytoplasmic or extracellular	Outside of membrane
4	AFE1511	Hypothetical protein		37.46/5.94			Cytoplasmic or extracellular	Outside of membrane
5	AFE1006	Hypothetical protein		27.91/9.51	Sec., Tat		Periplasmic or extracellular	Outside of membrane
6	AFE0982	vacJ lipoprotein	Cell envelope	30.04/6.97	Sec., LipoP		Periplasmic or extracellular	Outside of membrane
7	AFE0927	Polysaccharide deacetylase family protein	Energy metabolism, biosynthesis and degradation of polysaccharides	28.84/9.3		1: 10-24 aa	Inner membrane or extracellular	Outside of membrane
8	AFE2857	Hypothetical protein		30.95/10.41		1: 64-81 aa	Outer membrane or extracellular	Outside of membrane
9	AFE2770	Hypothetical protein		19.91/5.04	Sec., Tat		Extracellular or cytoplasmic	Outside of membrane
10	AFE2621	Pilin, putative	Cell envelope, surface structures	17.68/5.09			Extracellular or periplasmic	Outside of membrane
11	AFE1391	Conjugal transfer protein	Cellular processes, conjugation	24.35/7.14		1: 33-42 aa	Periplasmic or extracellular	Outside of membrane
12	AFE1857	Hypothetical protein		21.42/9.17			Extracellular or periplasmic	Outside of membrane
13	AFE1336	Hypothetical protein		20.85/10.2		1: 81-93 aa	Periplasmic or extracellular	Outside of membrane
14	AFE1847	Lipoprotein, putative	Cell envelope	10.15/7.72	Sec., LipoP		Extracellular or periplasmic	Outside of membrane
15	AFE2537	Conserved hypothetical protein		14.69/6.87			Periplasmic or extracellular	Outside of membrane
16	AFE1043	Hypothetical protein		7.31/9.93	Tat		Periplasmic or extracellular	Outside of membrane
17	AFE1774	Hypothetical protein		13.82/11.01			Periplasmic or extracellular	Outside of membrane
18	AFE1922	Hypothetical protein		11.96/7.76			Cytoplasmic or extracellular	Outside of membrane

\*: Number of Transmembrane

family protein; AFE\_1932: Ser/Thr protein phosphatase family protein and hypothetical proteins. The percents of each classified extracellular protein category of *A. ferrooxidans* grown on elemental sulfur are shown in Fig.2.



**Fig.1** Extracellular proteins of *A. ferrooxidans* grown on sulfur and ferrous sulfate separated by 1-D (a) and 2-D (b) electrophoresis



**Fig.2** Percents of each classified extracellular protein category of *A. ferrooxidans* grown on elemental sulfur

All the 18 identified extracellular proteins of *A. ferrooxidans* grown on elemental sulfur are predicted by their extracellular subcellular localization. Most of the identified proteins are hypothetical proteins (12 of the 18) which are not functionally classified in the TIGR database ([www.tigr.com](http://www.tigr.com)). Only six identified proteins have their own known functions according to the

classification in TIGR database. One conjugal transfer protein (conjugal transfer protein: AFE\_1391) distributes on the surface of the bacteria, and it performs an important role in gene transformation and recombination through contacting with bacteria, especially the transformation of resistance plasmid by the conjugal transfer protein, which can improve the ability of the bacteria grown in adverse circumstances.

The pilus protein (AFE\_2621: pilin, putative, cell envelope, surface structures), which is classified to the cell envelope proteins based on functional prediction, takes part in the composition of cell surface structure. Common pilus gives many kinds of Gram-negative bacteria the ability of adherence and ecesis on various cell surface, which is related to the pathogenicity of some bacteria[15]. The elemental sulfur has a hydrophobic solid surface. The presence of pilus protein in the present experiment may be explained by the bacteria adsorption to the sulfur solid particles, just like the adsorption of pathogenic bacteria to the cell.

There are two kinds of extracellular proteins of the typical signal peptide sequence of lipoproteins (AFE\_1847: lipoprotein, putative, cell envelope; AFE\_0982: vacJ lipoprotein, cell envelope). vacJ lipoprotein is an extracellularly anchored protein necessary for the *Shigella flexneri* transmission between bacteria[16]. The function of presence of vacJ lipoprotein during the process of *A. ferrooxidans* grown on sulfur remains to uncover.

The polysaccharide deacetylase family protein (AFE\_0927) and Ser/Thr protein phosphatase family protein (AFE\_1932) do not have short signal peptide fragments, but each peptide has a section of transmembrane peptide fragment. They may take part in the synthesis of polysaccharide and signal transduction of cell surface, respectively.

Interestingly, 12 of the 18 proteins are all unknown proteins. Some of these unknown proteins don't have the characteristics of signal peptide sequence, neither the transmembrane features. Such short peptide that localizes in the extracellular may be secreted to the extracellular space by the non-classical secretory pathway[17]. Additionally, six of these peptide sequences of the low molecular mass (AFE\_1336, AFE\_1847, AFE\_2537, AFE\_1043, AFE\_1774 and AFE\_1922) contain abundant of cysteine residues (Cys), and four of the six proteins have CXXC functional motif.

The thiol groups and the CXXC functional motif are the important active sites of proteins in biochemical reactions. The extracellular hydrophilic peptides abundant of cysteine residues may play a very important role in the chelation with heavy metals, protein modification, stress tolerance, signal transduction and other functions. Many redox proteins are involved in the

formation of disulfide bonds. Many oxidation-reduction reactions in organisms are mediated by the thiol oxidoreductase that contains CXXC functional motif as active sites. Protein structure can change also through the conversion between thiol group (—SH) and disulfide bond (—SS—), thus affecting the activity and function of protein molecules.

On the sulfur bio-oxidation, first of all, the acidophilic sulfur-oxidizing bacteria are required to contact and adsorb to sulfur. The effective absorption of bacteria determines an efficient oxidation on sulfur particles. The bacterial attachment to sulfur particles is attained by active expressing of some extracellular polymers substance, and the most important change is that the sulfur particle becomes bio-modified through the biochemical reactions between bacterial extracellular proteins and sulfur. The bio-sulfur is the available energy material for *A. ferrooxidans* cells. It is no doubt that these biochemical reactions are dominated by the extracellular proteins. It is reported that the sulfur must be used on the premise of its activation, but this step relies on the existence of low-molecular-mass mercaptan (such as glutathione: GSH) [8, 16, 18]. The proteins rich in thiol groups on *A. ferrooxidans* cell surface may be directly involved in the sulfur activation by using their thiol groups (Pr-SH) to bond sulfur and become Pr-Sn-SH. This assumption, however, needs to prove by further researches.

#### 4 Conclusions

1) The fractions of the extracellular proteins of *A. ferrooxidans* grown on elemental sulfur and ferrous sulfate were selectively prepared and separated by 2-D electrophoresis. The general pattern of the extracellular proteins for sulfur-grown *A. ferrooxidans* showed obvious differences from that observed for the ferrous iron-grown bacterium.

2) Some protein spots with apparent higher abundance in sulfur energy substrate were identified by using MALDI-TOF/TOF and the extracellular proteins were classified according to their functions as conjugal transfer protein, pilin, vacJ lipoprotein, polysaccharide deacetylase family protein, Ser/Thr protein phosphatase family protein and hypothetical proteins. Several extracellular proteins abundant of thiol groups and with CXXC functional motif may be directly involved in the sulfur activation by use of their thiol group (Pr-SH) to bond the elemental sulfur and form Pr-Sn-SH.

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