Proteomic Analysis of Differential Protein Expression in *Acidithiobacillus ferrooxidans* Grown on Ferrous Iron or Elemental Sulfur

Jianping Ouyang · Qian Liu · Bo Li ·
Jingqun Ao · Xinhua Chen

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Abstract In this study, a proteomic analysis of *Acidithiobacillus ferrooxidans* by two-dimensional electrophoresis identified 24 proteins that were differentially expressed when the cells were grown on ferrous iron (Fe^{2+}) or elemental sulfur (S^0). Sixteen of these proteins were upregulated by growth on S^0 or downregulated by growth on Fe^{2+} , including four proteins involved in disulfide bond reduction such as pyridine nucleotide-disulfide oxidoreductase, heterodisulfide reductase subunit B, thioredoxin-disulfide reductase, and cysteine desulfurase IscS, and three proteins involved in saccharide metabolism. A total of eight proteins were upregulated by growth on Fe^{2+} or downregulated by S^0 . Northern blots further confirmed the differences in transcription for these differentially expressed proteins. We functionally characterized cysteine desulfurase IscS, and found that its overexpression in *E. coli* promoted the growth of the cells in LB containing 2.5 % sodium thiosulfate. Our results provide new insights into the molecular basis for S^0 and Fe^{2+} oxidation by this extreme acidophile.

Keywords *Acidithiobacillus ferrooxidans* · Comparative proteomics · Cysteine desulfurase IscS · Ferrous iron oxidation · Sulfur oxidation

Introduction

The microbe *Acidithiobacillus ferrooxidans* (*A. ferrooxidans*), which is found in such acidic environments as mining dumps and acid mine drainages, is a chemolitho-autotrophic gram-negative γ -proteobacterium. *A. ferrooxidans* oxidizes ferrous iron, elemental sulfur and its catalysed compounds, or sulfide minerals to obtain energy [1, 2]. This ability makes it suitable for use in industrial mineral processing.

The proteins involved in ferrous iron oxidation and electron transfer have been studied extensively in *A. ferrooxidans*. The electron transfer during iron oxidation has been found to be related to two transcription units, the *petI* and *rus* operons [3]. The *petI* operon encoded three subunits of the *bc1* complex, a predicted short chain dehydrogenase, and a cytochrome c_4 . The *rus* operon encoded two c-type cytochromes, cytochromes c oxidase, and rusticyanin [3]. Reduced inorganic sulfur compounds (RISCs) were widespread in prokaryotes. Experiments confirmed that the *petII* operon, quinol oxidases (from the *bd* and *bo3* families), and sulfide/quinone oxidoreductase were involved in the oxidation of sulfide to sulfur in *A. ferrooxidans*, while the tetrathionate hydrolase encoded by *tetH* was found to be related to the oxidation of tetrathionate [3]. In addition, three sulfurtransferases, a heterodisulfide reductase complex, an ATP sulfurylase and a NADH complex accessory protein were considered to be involved in the electron transfer during RISCs oxidation [4].

Jianping Ouyang, Qian Liu and Bo Li contributed equally to this work.

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J. Ouyang · B. Li · J. Ao · X. Chen (✉)
Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography State Oceanic Administration, 184 Daxue Road, Xiamen 361005, People's Republic of China
e-mail: chenxinh@tom.com

Q. Liu
School of Resource Processing and Bioengineering, Central South University, Hunan, People's Republic of China

Two-dimensional electrophoresis (2-DE) has been used to study the expression of *A. ferrooxidans* proteins under different growth conditions, including heat shock [5], pH stress [6], phosphate limitation [7], different energy resources [8], and the presence of copper [9]. To further identify the components involved in the oxidation of sulfur and ferrous iron, we applied a comparative proteomic approach to analyze differentially expressed proteins when *A. ferrooxidans* was grown on ferrous iron or elemental sulfur. Twenty-four differentially expressed proteins were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Of the novel proteins involved in the oxidation of sulfur and ferrous iron that were found, we functionally characterized cysteine desulfurase IscS, which was upregulated by growth on S⁰.

Materials and Methods

Bacterial Strains and Growth Conditions

The bacterium *A. ferrooxidans* was isolated from samples from the Yufu mine of the Hunan province in China. The *A. ferrooxidans* was grown in 9K medium containing ferrous iron that was modified as described previously [10]. Growth on elemental sulfur was carried out by adding 1 % (w/v) sulfur pills to the 9K medium. The pH of the medium was initially adjusted to 2.0.

Protein Extraction

The cells were harvested by centrifugation when they reached the late exponential phase of growth. The cells were resuspended in the lysis buffer containing 2 % β -mercaptoethanol, and 2 mM PMSF and sonicated. The cell lysate was extracted with an equal volume of phenol that was saturated with water. The precipitated protein was washed with ice-cold methanol and then dried. Next, the protein was dissolved in a rehydration solution consisting of 50 mM DTT and 2 % carrier ampholyte for 2-DE analysis. Protein concentration was measured using a Coomassie protein assay kit with BSA as the standard protein (Pierce Biotechnology).

2-DE and Image Analysis

Two-DE was performed as previously described [11]. One microgram of the protein was analyzed for each sample. Isoelectric focusing (IEF) was carried out with an IPGphor unit (Amersham Biosciences) using precast 18-cm pH 4–7 nonlinear IPG gel strips (Amersham Biosciences). Proteins were separated according to mass in the second dimension

by SDS-PAGE with 12 % gels. The Coomassie Brilliant Blue G250-stained gels were scanned with a SHARP JX-330 laser densitometer. The spots were each quantitatively analyzed with ImageMaster v5.00 software (Amersham Biosciences). The protein spots with expression variation of more than 1.5-fold were considered significant.

In-Gel Tryptic Digestion

In-gel tryptic digestion was performed according to the procedure previously reported [11]. The extracts were evaporated in the SpeedVac and dissolved in 0.5 % TFA (Trifluoroacetic Acid) for mass spectrometric [12] analysis.

Protein Identification by Peptide Mass Fingerprinting

MS analysis was done as previously described [11]. The prepared peptide was mixed with an equal volume of the matrix solution containing α -cyano-4-hydroxy-cinnamic acid and Acetonitrile, and was analyzed on a BRUKER Autoflex MALDI-TOF (Bruker Daltonics Co.). All spectra were obtained with a positive-ion reflector. The resulting PMF, together with the pI and MW values that were estimated from 2-DE gels, were queried against a protein database. This database was based on the available whole genome sequence of *A. ferrooxidans* ATCC 23,270 (www.tigr.org) and was constructed with the mascot (1.9) software tools (www.expasy.ch).

Northern Blotting Analysis

Probes corresponding to each gene were synthesized with the specific primers (Supplementary Table 1) using DIG DNA Labeling Mix (Roche). Total RNA was isolated from cells of *A. ferrooxidans* grown on Fe²⁺ or S⁰ using Trizol Reagent (Invitrogen). A total of 20 μ g of total RNA per sample was used for northern blotting analysis. Hybridization was carried out according to the instruction manual for the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). The quantity of RNA loaded was monitored with 16S rRNA.

Cloning of *IscS* Gene in *A. ferrooxidans*

Genomic DNA was extracted from *A. ferrooxidans* as previously described [13]. The *IscS* gene was amplified with following primers: forward primer, 5'-AAGGATCCA TGCTGCCCTTTCTGACCC-3' with a *Bam*HI site italicized; and reverse primer, 5'-AAAAGCTTTTAGTGCGCG GCCCACTG-3' with a *Hind* III site italicized. The DNA sequence was searched for similarity using BLASTX at the NCBI web servers (<http://www.ncbi.nlm.nih.gov/BLAST>).

Multiple sequence alignment was performed using the CLUSTALW 1.8 program.

Expression of Recombinant IscS Protein in *E. coli*

The *IscS* gene was cloned into pET-His expression vector (Shenzhen Gene Power) after *Bam*HI/*Hind* III digestion. The resulting plasmid was named pET-His-IscS. The *E. coli* BL21 strain (Invitrogen) were transformed with either pET-His-IscS or pET-His, the vector control. The transformed colonies with pET-His-IscS (*E. coli* BL21/pET-His-IscS) were used for expression analysis. Recombinant protein was expressed by induction of 0.1 mM IPTG. Expression of recombinant IscS was analyzed by SDS-PAGE and Western blot as described previously [11].

The Determination of Growth Curve

The growth curves of bacteria in LB medium containing 2.5 % (w/v) sodium thiosulfate were confirmed by comparing growth of cells transformed with pET-His-IscS to those transformed with pET-His. Equal numbers of cells transformed with each plasmid were inoculated in LB medium containing 2.5 % sodium thiosulfate. The OD of the cultures was measured at 600 nm by Bioscreen (Lab-systems). Each group had three parallel samples, and the experiment was done at least three times separately.

Fig. 1 The proteins isolated from *A. ferrooxidans* grown on 9K base medium with added Fe^{2+} (a) or S^0 (b) were analyzed by two-dimensional electrophoresis SDS-PAGE. Each gel had 1 mg of protein loaded. c Histogram showing the expression levels of protein spot 5 (cysteine desulfurase IscS) when *A. ferrooxidans* was grown on Fe^{2+} or S^0 . The results are presented as mean $\pm$ SD from three independent experiments

Results

Global Protein Synthesis Patterns of *A. ferrooxidans* Grown on Elemental Sulfur or Ferrous Iron

The differentially expressed proteins using Fe^{2+} or S^0 as energy sources were characterized by 2-DE of the total-cell proteins from *A. ferrooxidans* grown on S^0 or Fe^{2+} . The total proteins were resolved by 2-DE, and stained with colloidal Coomassie Blue (Fig. 1a, b). The differentially expressed proteins were selected by calculating their expression variation.

Differentially Expressed Proteins of *A. ferrooxidans* Grown on Sulfur or Ferrous Iron

We successfully identified 24 spots that had changed their expression levels by at least 1.5-fold when S^0 and Fe^{2+} were used as energy sources. 16 spots were more abundant when the cells were grown on S^0 , while the expression of eight proteins decreased under that condition or increased when grown on Fe^{2+} . The spots were evaluated by mascot (1.9) software (Table 1).

Confirming of Differentially Expressed Proteins at Transcription Levels

We found that 16 of the proteins were upregulated during growth on S^0 and the other eight were downregulated (Fig. 2), which agrees with the 2-DE analysis, confirming

Table 1 Search results of the PMF of the differentially expressed proteins

Spot no.	AFE	Function/similarity	Mw (kDa)/pI	Peptides matched	Coverage (%)	Regulation (fold change) ^a	Score
<i>Proteins upregulated in ferrous oxidation</i>							
2	3211	Sulfite reductase (NADPH) flavoprotein, alpha component (CysJ)	65.29/5.61	41	65	2.60 ± 0.04	324
9	1261	Phosphoglycolate phosphatase (gph-1)	24.08/5.13	7	43	10.53 ± 3.10	56
11	0345	Glutamyl-tRNA reductase	48.75/5.84	28	77	3.57 ± 0.82	256
15	0007	Oxidoreductase, short-chain dehydrogenase-reductase family	28.92/5.00	7	31	1.66 ± 0.07	279
17	2665	AhpC-TSA family-glutaredoxin domain protein	27.67/5.30	25	80	1.66 ± 0.05	205
22	0365	Major outer membrane protein 40 (omp40)	42.22/4.93	14	23	3.13 ± 0.11	130
23	3096	Conserved hypothetical protein	24.22/5.32	12	35	7.15 ± 1.31	111
24	2062	Antioxidant, AhpC-TSA family	22.54/ 5.69	6	40	2.40 ± 0.04	44
<i>Proteins upregulated in sulfur oxidation</i>							
1	0501	Pyridine nucleotide-disulfide oxidoreductase	42.45/6.19	40	88	2.29 ± 0.37	249
3	1009	Heat shock protein, Hsp20 family	16.92/6.20	23	80	1.80 ± 0.05	163
4	3053	Conserved hypothetical protein	20.36/5.25	9	34	16.48 ± 0.53	106
5	2367	Cysteine desulfurase IscS (IscS-3)	45.29/5.85	28	61	3.21 ± 0.06	179
6	1033	CBS ₁ domain protein	21.90/5.62	20	76	1.93 ± 0.04	129
7	0516	Heterodisulfide reductase subunit B, homolog	50.64/5.95	9	15	1.97 ± 0.02	50
8	2982	Tat (twin-arginine translocation) pathway signal sequence domain protein	29.49/6.55	17	52	2.54 ± 0.03	83
10	1273	Dehydrogenase complex, E1 component, alpha subunit, putative	36.62/5.00	16	49	5.93 ± 0.74	85
12	2657	Thioredoxin-disulfide reductase (trxB)	35.06/5.61	22	68	1.58 ± 0.10	150
13	1283	Fructose-bisphosphate aldolase, putative	33.20/5.77	18	46	13.09 ± 1.83	104
14	0377	Oxidoreductase, short-chain dehydrogenase-reductase family ₂	25.13/5.04	9	24	4.36 ± 0.40	80
16	1165	CBS ₂ domain protein	1632/5.08	12	98	7.24 ± 0.96	193
18	0425	Hypothetical protein	15.80/5.73	10	48	37.60 ± 8.26	69
19	2618	Transaldolase (tal)	39.52/ 5.40	25	75	1.63 ± 0.06	210
20	0774	Hypothetical protein	13.48/5.61	11	55	2.04 ± 0.08	83
21	1070	6-phosphogluconate dehydrogenase family protein	32.84/5.85	12	39	9.11 ± 1.31	72

AFE open reading frame of *A. ferrooxidans* ATCC 23,270 (www.tigr.org)

^a Regulation (fold-change) of differentially expressed proteins is expressed as the mean ± SD of three experiments

the differences in the transcription levels of the genes encoding the differentially expressed proteins.

Cloning and Functionally Characterizing the *A. ferrooxidans* IscS Gene

IscS was identified as upregulated by growth on S⁰ (Table 1). To further understand its function in the sulfur oxidation, we cloned the *IscS* gene of *A. ferrooxidans*. The deduced IscS has the typical structural characteristics of IscS proteins, including an active site (Supplementary Fig. 1, S³⁰³ACTS³⁰⁷) and several conserved motifs (Supplementary Fig. 1, shadowed backgrounds). Until now, a genetic manipulation system was not well established for *A. ferrooxidans*. We therefore used *E. coli* BL21 as a substitutive host. Both SDS-PAGE and

Western blot confirmed the expression of *A. ferrooxidans* IscS in *E. coli* BL21 (data not shown). Analyzing growth curves found that the cells transformed with pET-His-IscS and grown in LB medium with 2.5 % sodium thiosulfate had a faster growth phenotype and an OD₆₀₀ of 0.77 at the stationary phase compared with those transformed with pET-His, which had an OD₆₀₀ of 0.067 at the stationary phase (Fig. 3).

Discussion

The Proteins Upregulated in Sulfur Oxidation or Downregulated in Ferrous Oxidation

When *A. ferrooxidans* utilizes elemental sulfur as its sole energy source, it must first adhere to the sulfur surface

Fig. 2 The spots that were differentially expressed under the tested growth conditions were analyzed by Northern blots. The genes expressed when the cells were grown in 9K base medium with S^0 added are in the S column, and those expressed during growth with supplemental Fe^{2+} are in the Fe column

[14, 15]. Bacterial adhesion is known to mainly depend on such cell envelope layers as lipopolysaccharides (LPS) and exopolysaccharides (EPS) [14]. Here, three proteins related to saccharide metabolism, spot 13, fructose-1,6-bisphosphate aldolase; spot 19, transaldolase; and spot 21, a protein from the 6-phosphogluconate dehydrogenase family (Table 1), were upregulated during growth on S^0 . Fructose-1,6-bisphosphate aldolase catalyzes the aldol condensation of dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate to form fructose-1,6-bisphosphate. Fructose-1,6-bisphosphate can be converted into mannose-6-phosphate by mannose phosphate isomerase, and the latter is an important ingredient for both EPS and LPS [16]. Both transaldolase and 6-phosphogluconate dehydrogenase

are known to be involved in the pentose phosphate pathway, which can lead to the production of D-glycero-D-manno-heptose-7-phosphate, an ingredient of LPS synthesis [17]. The upregulation of the expression of these three proteins during growth on S^0 may facilitate *A. ferrooxidans* adhesion to elemental sulfur by increasing the synthesis of LPS or EPS.

Sulfur activation must occur before oxidation because elemental sulfur consists of a stable octasulfane ring system (S₈ ring) that forms orthorhombic crystals with extremely poor water solubility. Thus the critical step of sulfur metabolism is the opening of the S₈ ring, which is a reaction that the thiol groups of cysteine residues could complete [2, 3]. We identified four proteins that were differentially expressed during growth on S^0 and that can each provide thiol groups from their cysteine residues. Spot 1 protein, pyridine nucleotide-disulfide oxidoreductase, uses the isoalloxazine ring of FAD to shuttle reducing equivalents from NAD(P)H to a Cys residue. That residue is usually a part of a redox-active disulfide bridge that provides the thiol groups needed by the enzyme to oxidize sulfur [12]. Spot 7 enzyme, heterodisulfide reductase subunit B homolog, is a unique disulfide reductase that catalyses the reversible reduction of the mixed disulfide (CoM–S–S–CoB), thus providing the thiol groups of cysteine residues in the mechanism of sulfur oxidation [18]. Spot 12, thioredoxin-disulfide reductase, reduces oxidized thioredoxin in the presence of NADPH to form activated sulfhydryls, which were necessary for sulfur oxidation by *Acidithiobacillus* spp. and *Sulfobacillus thermosulfidooxidans* [19]. Spot 5 enzyme, cysteine desulfurase IscS, is considered to catalyze the conversion of L-cysteine to both L-alanine and sulfane sulfur by forming a protein-bound cysteine persulfide intermediate on a conserved cysteine residue. This intermediate can then open the S₈ ring [20, 21]. Here, four proteins all were upregulated during growth on S^0 , suggesting that *A. ferrooxidans* may provide thiol groups to open the S₈ ring for the sulfur oxidation by different pathways.

To further confirm the function of *A. ferrooxidans* IscS in the sulfur oxidation, we used *E. coli* BL21 as a substitutive host and transformed the recombinant expression vector pET-His-IscS into *E. coli* BL21 cells. The results showed that the cells transformed with pET-His-IscS grew much faster than those transformed with pET-His in LB medium with 2.5 % sodium thiosulfate (Fig. 3), indicating the IscS have a role in the sulfur oxidation of bacteria.

Two bc₁ complexes exist in *A. ferrooxidans* [13, 22]. One bc₁ complex was encoded by the operon *petI*, which functions in the reverse direction by transferring electrons from ferrous iron to NAD(P). Another bc₁ complex, encoded by operon *petII*, functions in the forward direction by transferring electrons from sulfur to oxygen [13]. Spot

Fig. 3 Growth curves were plotted from *E. coli* BL21 cells transformed with pET-His-IscS (filled triangle) and pET-His (filled square) and grown aerobically in LB medium supplemented with 2.5 % sodium thiosulfate

14 is an oxidoreductase of the short-chain dehydrogenase-reductase family whose gene is located in the *petI* operon, which is responsible for the electron transfer from sulfur to oxygen [23]. Meanwhile, spot 15 protein was identified as another oxidoreductase of short-chain dehydrogenase-reductase family, whose gene is located in the *petI* operon [23]. The *petI* operon has been shown to be responsible for the electron transfer from ferrous iron to NAD(P) [13]. Here, spot 14 protein was upregulated when the cells were grown on sulfur, while spot 15 protein was upregulated by growth on ferrous iron (Table 1), suggesting that these two proteins might be involved in the electron transfer during sulfur or ferrous iron oxidation.

The Proteins Upregulated in Ferrous Oxidation or Downregulated in Sulfur Oxidation

The spot 9 protein, 2-phosphoglycolate phosphatase (PGLP), was upregulated more than tenfold when cells were grown in ferrous oxidation. PGLP is one of the core enzymes of plant photosynthetic carbon assimilation. This enzyme converts 2-phosphoglycolate to glycolate in photorespiration [24], and the latter is finally converted to the Calvin cycle intermediate 3-phosphoglycerate by a series of reactions [25]. The expression of Calvin cycle enzymes varies considerably in different chemoautotrophic microorganisms depending on the substrate being oxidized [8]. Two Calvin cycle genes, the small and large subunits of ribulose biphosphate carboxylase, were upregulated when *A. ferrooxidans* was grown on ferrous iron [8, 26]. When *A. ferrooxidans* was grown on ferrous iron, the Calvin cycle was promoted and the required quantity of 3-phosphoglycerate increased. Thus, PGLP was greatly upregulated. This finding suggests that in addition to genes related to the Calvin cycle existing in *A. ferrooxidans*, photorespiration genes may also be present in *A. ferrooxidans*.

The AhpC-TSA family is a ubiquitous family of antioxidant enzymes. The protein spots 17 and 24 are identified as the members of antioxidant AhpC-TSA family. Two antioxidant proteins, thioredoxin peroxidase and superoxide dismutase, had been reported as upregulated on ferrous iron [8]. Here, two antioxidant proteins (spots 17 and 24) were upregulated when cells were grown on Fe^{2+} (Table 1), which might protect cells against strong oxidative stress during ferrous iron oxidation.

Conclusion, we identified 24 proteins that were differentially expressed. Growth on sulfur compounds resulted in the upregulation of 16 of these proteins and the downregulation of the other 8. Some of these proteins may be directly related to the oxidation of S^0 , such as three proteins related to saccharide metabolism, and four proteins involved in the opening of the S8 ring, of which the IscS was confirmed to have a role in the sulfur oxidation. The

eight proteins upregulated during Fe^{2+} oxidation included the photorespiration protein PGLP and oxidoreductase of the short-chain dehydrogenase-reductase family. This study provides new information about the molecular basis of the oxidation of sulfur and ferrous iron. Further research is needed to understand the exact mechanisms for S^0 and Fe^{2+} oxidation by *A. ferrooxidans*.

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