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Differential gene expression in response to copper in *Acidithiobacillus ferrooxidans* strains possessing dissimilar copper resistance

Xueling Wu,* Qi Hu, Dongmei Hou, Bo Miao, and Xueduan Liu

School of Minerals Processing & Bioengineering, Central South University, Changsha 410083, China

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Locus *afe_0454* from *Acidithiobacillus ferrooxidans* (*At.ferrooxidans*) is annotated as related to copper resistance in The Institute for Genomic Research database. In our study, two *At.ferrooxidans* strains, 26[#] and DC, with different levels of copper ion resistance were isolated from acid mine drainages at two major copper mines in China, and their copper-resistance capacity was determined. The 26[#] strain had a copper-tolerance level of 0.22 mol/L, whereas the DC strain had a lower copper-tolerance level of 0.04 mol/L. The mutant 26[#] was generated from strain 26[#], and its copper-tolerance level was 0.25 mol/L. Using real-time quantitative reverse transcription polymerase chain reaction, differential expression of the *afe_0454* gene during copper ion stress of these three strains was investigated. The results showed that the expression of *afe_0454* was increased under copper ion stress, indicating that the *afe_0454* gene is sensitive to copper levels. Furthermore, the *afe_0454* gene expression ratio varied in the different copper-resistant strains. Gene expression was highest in the highest copper-resistant strain. The deduced amino acid sequence of the *afe_0454* gene was 56.87% non-polar, indicating the AFE_0454 protein was hydrophobic. Searching with the AFE_0454 protein in The Institute for Genomic Research database showed that the structure of the copper resistance protein D (CopD), which transports copper ions outside of the cell, had the highest sequence identity (46%). Bioinformatics analysis showed that the AFE_0454 protein has eight transmembrane helices and was predicted to be localized to the plasma membrane. These results strongly suggested that the AFE_0454 protein is likely a transmembrane protein and might be directly involved in copper ion resistance.

Key Words—*Acidithiobacillus ferrooxidans*; copper ion stress; copper resistance; differential gene expression; real-time PCR

Introduction

Presently, bioleaching (Rawlings, 2002) has seen significant development and broad application prospects in mineral research, especially for the process-

ing of low-grade ore. Because of the advantages of bioleaching, including low cost, energy efficiency, ease of operation, and environment friendliness, this method has gained broad consensus throughout the world. Abroad, bioleaching is the leading mineral-processing technique for low-grade copper ore, especially secondary copper sulphide ore (Schrenk and Edwards, 1998). *At.ferrooxidans* is an acid chemoautotrophic bacteria and is the dominant bacteria used in bioleaching to recover metals such as gold and copper (Ingledeu, 1982; Rawlings, 2005). Similar to other bio-

* Address reprint requests to: Dr. Xueling Wu, School of Minerals Processing & Bioengineering, Central South University, Changsha 410083, China.

Tel: +86-731-88804873 Fax: +86-731-88912447

E-mail: xueling0714@yahoo.com.cn

processes, the leaching of ores by bacteria is influenced by various environmental factors, including temperature, pH, composition of the leaching medium, particle size, and surrounding metal concentrations (Huang et al., 2002). At present, one of the most severe limitations in the effort to increase the efficiency of bioleaching is metal toxicity, especially copper ion toxicity. For bacteria, copper is a crucial trace element (Kappler et al., 2008; Waldron et al., 2007), and it acts as a cofactor for the function of various enzymes such as cytochrome *c* oxidase, tyrosinase, superoxide dismutase, etc. However, copper can cause serious damage to cells at high concentrations through radical formation (Finney and O'Halloran, 2003). In the process of bacterial leaching of copper, copper ion concentrations increase significantly in the leaching solution, presenting a major problem if the bacteria cannot tolerate these higher concentrations. As such, much research has been focused on acquiring excellent bioleaching strains that have good traits and a higher copper resistance. Some copper-resistant strains have been obtained (Alvarez and Jerez, 2004); however, little is known about the mechanisms involved in copper resistance. The results of research conducted by Chisholm et al. (1998) and Ahmad et al. (2002) suggest that the copper-resistance genes of *At.ferrooxidans* are chromosomal and not plasmid based, but the role of these genes is still not clear.

Though some studies have focused on copper resistance (Paulino et al., 2002), few genes have been identified that are directly involved in the copper tolerance of *At.ferrooxidans*. In 2007, the *At.ferrooxidans* ATCC23270 genome was sequenced by The Institute for Genomic Research (TIGR), and *afe_0454* was annotated as being related to copper-resistance. Until now, little research about *afe_0454* (1,095 bp) has been reported, and the functionality of this gene remains unknown (Navarro et al., 2009). In our study, we focused on the *afe_0454* gene, which we surmised would encode a protein likely contributing to the copper ion homeostatic mechanisms of the organism and which might be important to the cell's response to copper ion stress. Real-time PCR has become one of the most widely used methods for quantitative gene analysis in recent years (Marisa and Juan, 2005), and has been employed in this study. The purpose of the present study was to determine the differential expression of the *afe_0454* gene in *At.ferrooxidans* strains that possess different copper-tolerance capacities in

an effort to further our understanding of the mechanism(s) influencing copper resistance.

Materials and Methods

Bacterial strains and growth conditions. Two native *At.ferrooxidans* strains were isolated from the Dachang Copper Mine (DC), Guangxi Province, and the Daye Copper Mine (26[#]), Hubei Province, China. The strains were confirmed as *At.ferrooxidans* by 16S rDNA fragment comparison with ATCC 23270 (Yu Yang and Minxi Wan, 2007). The *At.ferrooxidans* strains were grown under aerobic conditions (170 rpm) at 30°C in 9K medium (Silverman and Lundgren, 1959) that contained 3.0 g (NH₄)₂SO₄, 0.1 g KCl, 0.5 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.01 g Ca(NO₃)₂, and 25 g FeSO₄·7H₂O per liter. The pH of the 9K medium was adjusted to 2.0 by 50% volume fraction of H₂SO₄ before the addition of FeSO₄·7H₂O (20.0 g/L) and was then filter sterilized.

Copper-tolerance levels of strains DC and 26[#]. To determine the highest tolerance of the two strains to copper ions, 5 ml of each culture at the logarithmic growth phase was added to 95 ml of autoclaved fresh 9K medium containing different concentrations of copper ions (Hongmei Li, 2000) and incubated in constant-temperature shakers at 170 rpm and 30°C. The ferrous ion concentrations of the 9K medium were tested using the K₂Cr₂O₇ titration method, essentially the same as previously described (Xueling Wu et al., 2007). Briefly, aliquots (2 ml) of culture were added to 20 ml of acid mixture: 98% H₂SO₄ (150 ml) + H₃PO₄ (150 ml) + H₂O (700 ml). It was then titrated with 6 mM K₂Cr₂O₇. One milliliter of 6 mM K₂Cr₂O₇ (0.0018 g) solution was reacted with 0.002 g of ferrous ions. Sodium diphenylamine-4-sulfonate was used as an indicator, and the appearance of a purple color indicated the titration end point. The oxidation rates of ferrous ions in the 9K medium were determined, and copper-tolerance levels of strains DC and 26[#] were compared.

Copper-tolerance levels of mutant 26[#]. In preliminary experiments the mutant 26[#] was generated by inducing mutations using ultraviolet (UV) radiation (Birong Shen and Xueling Wu, 2007). To determine the copper-resistance capacity of 26[#], the cells were incubated in 9K medium containing various concentrations of copper ions under the same conditions as above. The ferrous ion oxidation rates of the mutant were tested and the copper-tolerance capacities were deter-

mined.

Sample collection. Bacteria cells were grown in fresh 9K medium with different concentrations of copper ions. Growth was followed by direct cell counting in a Neubauer chamber (Yarzabal et al., 2004), the oxidation capacities of ferrous ions were determined using the potassium dichromate ($K_2Cr_2O_7$) titration method, and growth curves were generated. Cells were harvested at an early logarithmic phase of cell growth by centrifugation at 4°C for 10 min at 12,000 rpm using a 5804R centrifuge (Eppendorf, Wesbury, NY, USA). The cell pellet was washed three times with diluted H_2SO_4 at pH 2.0. The cells were again collected by centrifugation, and the culture supernatant was removed. Pelleted cells were immediately processed for RNA extraction.

Primer design. Primers used in this study are listed in Table 1. Primers for the *afe_0454* gene were designed using Primer 5. Blast N searches were used to check primer specificity.

Total RNA extraction and purification. Total cellular RNA was isolated using lysozyme reagent (OMEGA) according to the manufacturer's protocol. The purity of the samples was determined via formaldehyde agarose gel electrophoresis by observing the definition of the 23S, 16S, and 5S bands. Total cellular RNA was quantified at OD₂₆₀ (Ki-Hyeong and Julian, 2006) and OD₂₈₀ with a UV-2300 spectrophotometer (Shanghai Tianmei Science Instrument Company, Shanghai, China) to ensure that the extracted RNA was not contaminated with DNA. The purified RNA from each sample served as the template to generate cDNA.

Real-time PCR. Total RNA was used to prepare cDNA by reverse transcription. An RNA sample (15 µl) was mixed with random primers (3 µl, 5 ng/µl). The mixture (18 µl) was incubated at 70°C for 5 min and then placed on ice to cool. After cooling, 6 µl M-MLV 5× Reaction Buffer, 1.5 µl dNTPs, 1.5 µl RNase-Out inhibitor, 1.5 µl DEPC water, and 1.5 µl M-MLV RT were added to each tube. The tubes were incubated at 37°C for 60 min. The cDNA samples were refrigerated at -20°C, and a 1 µl sample of cDNA was used for real-

time PCR.

Real-time PCR was performed on cDNAs generated from RNA samples obtained at the indicated time points. The primers used for real-time PCR are described in Table 1. Each real-time PCR mixture (50 µl) contained 25 µl Green SYBR-490 real-time PCR Master Mix (*Taq* DNA polymerase, dNTPs, $MgCl_2$, SYBR Green I dye), 1 µl forward primer, 1 µl reverse primer, 1 µl cDNA template, and 22 µl nuclease-free water. The real-time PCR was carried out in an iCycler iQ real-time PCR detection system (Bio-Rad).

The expression of each gene was determined from three replicates of a single real-time PCR experiment. The expression ratio was recorded as the fold difference in quantity of real-time PCR product from samples grown at the treatment concentration vs. control concentration. Relative abundance of each mRNA versus a constitutively expressed gene (16S rDNA) was determined.

Results

Copper-tolerance levels of strains DC and 26[#]

In 9K medium, the *At.ferrooxidans* strains DC and 26[#] completely oxidized the ferrous ions within 48 h. When incubated in 9K medium containing 0.02 mol/L copper ions, strain DC completely oxidized the ferrous ions within 96 h (Fig. 1a). As copper ion concentrations reached 0.04 mol/L, DC completely oxidized the ferrous ions within 168 h, indicating that the DC strain was highly inhibited at this copper ion concentration. In contrast, when exposed to 9K medium containing 0.15 mol/L copper ions, the 26[#] strain could completely oxidize the ferrous ions within 72 h (Fig. 1b), indicating that this copper ion concentration did little to hinder the ferrous ion oxidation capacity of 26[#]. Furthermore, when the 26[#] strain was grown under the higher copper ion concentration (0.22 mol/L), the time of complete oxidation was about 168 h, whereas DC oxidized few ferrous ions when the concentration exceeded 0.04 mol/L. These results indicated that the maximum copper ion tolerance concentrations in

Table 1. Primers used for real-time PCR.

Gene	Sequence		Size of amplicon (bp)	Anneal temperature (°C)
	Forward primer (5'-3')	Reverse primer (5'-3')		
<i>afe_0454</i>	GCTGGTCGTGCTGTGGGTC	CAGGCTGCAAGTGCAAAATGA	156	56
16S	AATCCAAGAAGAAGCACCG	CCACTGATGTTCTCCAG	238	52

Fig. 1. Ferrous ion oxidation rates of strains DC and 26[#] when exposed to 9K medium containing different concentrations of copper ions.

(a) *At.ferrooxidans* strain DC; (b) *At.ferrooxidans* strain 26[#].

which strains DC and 26[#] could grow well were about 0.04 mol/L and 0.22 mol/L, respectively. When the copper ion concentrations were 0.05 mol/L and 0.24 mol/L, respectively, the cell growth of DC and 26[#] were completely inhibited. The tolerance level of 26[#] to copper ions was much higher than that of DC.

Copper-tolerance level of mutant 26[#]

Strain 26[#] was mutated using ultraviolet radiation, and the mutant 26[#] was obtained (Birong Shen and Xueling Wu, 2007). In the presence of copper ions (0.22 mol/L), the mutant 26[#] completely oxidized ferrous ions in the 9K medium within 144 h, faster than that of the 26[#] strain. When cultured in 9K medium containing a higher concentration of copper ions (0.25 mol/L), the mutant 26[#] completely oxidized ferrous ions within 192 h, indicating that the mutant 26[#] had a much higher ferrous ion oxidation capacity and copper resistance than 26[#] (Fig. 2).

Growth curves and sample collection

To investigate the function of the *afe_0454* gene and its relationship to copper ions, three *At.ferrooxidans* strains (DC, 26[#], and the mutant 26[#]), which each possessed different copper ion resistances, were assayed in a real-time PCR study. These strains were grown in fresh 9K medium at 30°C in a shaker (170 rpm), and the *afe_0454* gene was induced under different copper ion stresses. Since the DC strain had a lower copper-tolerance capacity (0.04 mol/L), when copper ion concentrations exceeded 0.04 mol/L the hardly any cells could grow. Therefore, we set the copper ion concentration at 0.04 mol/L to investigate and compare *afe_0454* gene expression in the three *At.ferroox-*

Fig. 2. Ferrous ion oxidation rates when the mutant 26[#] was exposed to 9K medium containing different concentrations of copper ions.

idans strains. Furthermore, we set a higher copper ion concentration (0.22 mol/L) to observe the differential gene expression of the mutant 26[#] strain, since it possessed a higher copper ion resistance (0.25 mol/L).

Growth was followed by direct cell counting, and growth curves were generated (Fig. 3). The harvest times for cells in the early logarithmic period were determined from these growth curves. Pelleted cells were immediately processed for RNA extraction. All assays were repeated three times.

The quality of total cellular RNA

The integrity of total cellular RNA was checked by formaldehyde agarose gel electrophoresis and ethidium bromide staining. The definition of 23S, 16S, and 5S bands was very high and the luminance ratio between 23S and 16S was 2/1. The OD₂₆₀/OD₂₈₀, which

Fig. 3. Growth curves of three *At.ferrooxidans* strains (DC, 26[#], and mutant 26[#]) cultured in 9K medium containing different concentrations of copper ions.

Labeled as ①DC Cu²⁺0 mol/L, ②DC Cu²⁺0.04 mol/L, ③26[#] Cu²⁺0 mol/L, ④26[#] Cu²⁺0.04 mol/L, ⑤mutant 26[#] Cu²⁺0 mol/L, ⑥mutant 26[#] Cu²⁺0.04 mol/L, ⑦mutant 26[#] Cu²⁺0.22 mol/L.

was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), was about 1.75–2.0. These results indicated that the purity and integrity of the total cellular RNA was sufficient for this study.

The specificity of PCR products

To determine that the RNA contained no DNA, RNA alone was used in real-time PCR with other samples as a negative control. No fluorescence signal was produced by the negative control, indicating that the RNA wasn't contaminated with DNA. The melt curve had only one peak, so there was no non-specific product or primer dimer in the PCR products.

Response of isolates to different concentrations of copper ions

Real-time PCR was used to analyze changes in the levels of the *afe_0454* transcript induced by copper ion stress, and the *Ct* values were recorded. The expression of the 16S rDNA gene from the three strains, DC, 26[#], and the mutant 26[#], was nearly the same (Fig. 4), indicating that the 16S results could be trusted for normalization. As such and according to the principle of the $2^{-\Delta\Delta Ct}$ method (Kenneth and Thomas, 2001), the expression of *afe_0454* was corrected relative to the reference 16S gene (Table 2).

The copper-resistance capacities of the three strains

Fig. 4. The difference in *afe_0454* gene expression in the three *At.ferrooxidans* strains (DC, 26[#], and mutant 26[#]).

Labeled as ①DC Cu²⁺0 mol/L, ②DC Cu²⁺0.04 mol/L, ③26[#] Cu²⁺0 mol/L, ④26[#] Cu²⁺0.04 mol/L, ⑤mutant 26[#] Cu²⁺0 mol/L, ⑥mutant 26[#] Cu²⁺0.04 mol/L, ⑦mutant 26[#] Cu²⁺0.22 mol/L.

Table 2. Expression ratio of the *afe_0454* gene under different concentrations of copper ions in *At.ferrooxidans*.

Samples	Expression ratio		
	0 mol/L	0.04 mol/L	0.22 mol/L
DC	1.00	2.10	—
26 [#]	1.00	10.34	—
mutant 26 [#]	1.00	11.55	170.07

were determined and the DC strain had the lowest copper resistance (0.04 mol/L) while the 26[#] strain and the mutant 26[#] had higher copper resistances (0.22 mol/L and 0.25 mol/L, respectively). When these three strains were exposed to 9K medium containing 0.04 mol/L copper ions, *afe_0454* expression from strains DC, 26[#], and the mutant 26[#] were up regulated 2.10-fold, 10.34-fold, and 11.55-fold, respectively (Fig. 5), indicating that the *afe_0454* gene was sensitive to copper ion concentrations and implying its function might be related to copper resistance. Furthermore, the *afe_0454* expression ratio differed relative to the different copper-resistance capacities of the strains. In the higher copper-tolerant strains, 26[#] and the mutant 26[#], the gene expression was higher (10.34-fold and 11.55-fold, respectively). In the lower copper-tolerant strain, DC, the gene expression was lower (2.10-fold). This suggested that the *afe_0454* gene might play a role in the tolerance of *At.ferrooxidans* to high copper ion environments.

We further focused on the response of the

At.ferrooxidans mutant, the mutant 26[#], to copper ion stress because of its higher copper ion tolerance. When exposed to 9K medium containing copper ion concentrations of 0.04 mol/L, *afe_0454* expression in the mutant 26[#] was increased 11.55-fold. When exposed to 9K medium containing copper ion concentrations of 0.22 mol/L, *afe_0454* expression was induced at much higher levels (170.07-fold). In contrast, the DC and 26[#] strains were highly inhibited and did not grow well in this environment. These results indicated that *afe_0454* expression was increased under copper ion stress, and that its expression differed greatly in different strains at different concentrations of copper ions. In a highly copper-resistant strain its expression was higher, and when the concentration of copper ions increased its expression ratio increased,

Fig. 5. Comparison of the gene expression difference for *At.ferrooxidans* strains (DC, 26[#], and mutant 26[#]).

Labeled as ①DC Cu²⁺ 0 mol/L, ②DC Cu²⁺ 0.04 mol/L, ③26[#] Cu²⁺ 0 mol/L, ④26[#] Cu²⁺ 0.04 mol/L, ⑤mutant 26[#] Cu²⁺ 0 mol/L, ⑥mutant 26[#] Cu²⁺ 0.04 mol/L, ⑦mutant 26[#] Cu²⁺ 0.22 mol/L.

indicating the *afe_0454* gene might play an important role in the copper resistance of *At.ferrooxidans*.

Protein and amino acid analysis

The nucleotide sequence of *afe_0454* from *At.ferrooxidans* was analyzed and determined to be 1,095 bp long and possibly translates into a 364-amino-acid protein consisting of 207 non-polar, 99 polar, 41 basic, and 17 acidic amino acids. The percentages were calculated as 56.87% non-polar, 27.20% polar, 11.26% basic, and 4.67% acidic. The deduced amino acid sequence of the AFE_0454 protein from *At.ferrooxidans* indicated it was hydrophobic in nature. The transmembrane helices of AFE_0454 were analyzed with the TMHMM v2.0 server (Fig. 6) (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>). The AFE_0454 protein sequence indicated the presence of eight transmembrane helices, and its subcellular localization was predicted to lie in the plasma membrane based on analysis by the PSORTb v2.0 server (<http://www.psort.org/psortb/index.html>). Both of these analyses strongly suggested that the AFE_0454 protein might be a transmembrane protein. The search with the sequence of AFE_0454 in the TIGR database showed that the structure of a copper resistance protein D, CopD, from *Burkholderia* sp. 383 (accession: Bcep18194_B2761) had the highest sequence identity (46%). The copper sequestering activity displayed by some bacteria is determined by CopD together with another copper resistance protein, CopC (Cooksey, 1994). CopD is a member of the copper resistance protein family, and many bacteria encode CopD. This protein functions by combining with copper ions and then transporting them outside of the cell. So, we conjectured that the

Fig. 6. TMHMM posterior probabilities for AFE_0454.

AFE_0454 from *At.ferrooxidans* had similar functions and might be a transmembrane copper transport protein that functions to drive copper ions out the cell.

Discussion

Copper ions are essential nutrients for cell growth. However, copper ions are also well known for their germicidal effect when concentrations are significantly increased. Copper ions combine with enzymes in bacterial cells and increase the bacterial lag phase, making cellular metabolism inactive or disorderly. During the bioleaching process, copper ions are dissolved into solution, increasing the copper ion concentration so as to inhibit the growth of *At.ferrooxidans* and inhibit the bacterial leaching. Therefore, studying the copper-resistance mechanisms of *At.ferrooxidans* is industrially significant in regards to accelerating the bacterial leaching efficiency.

In the present study, three *At.ferrooxidans* strains, DC, 26[#], and the mutant 26[#], each had different copper ion resistances (0.04 mol/L, 0.22 mol/L, and 0.25 mol/L, respectively). Under copper ion stress (0.04 mol/L), the *afe_0454* gene expression increased in all three strains. In the higher copper-tolerant strains, 26[#] and the mutant 26[#], the gene expression ratio was higher (10.34- and 11.55-fold, respectively) relative to the DC strain (2.10-fold), indicating the *afe_0454* gene was most responsive in highly resistant strains. Furthermore, when the copper ion concentration increased from 0.04 to 0.22 mol/L the *afe_0454* gene expression ratio of the mutant 26[#] increased from 11.55-fold to 170.07-fold, indicating that the *afe_0454* gene was highly responsive to copper ion concentrations. These results provided an initial description of the transcriptional responses elicited by *At.ferrooxidans* cells possessing different levels of resistance when challenged with copper ion stress and revealed that the copper ion environment had obvious effects on the expression of *afe_0454* from *At.ferrooxidans*.

The nucleotide sequence of the *afe_0454* gene from *At.ferrooxidans* is 1,095 bp long, and the deduced amino acid sequence has strong hydrophobic properties. Bioinformatic analysis showed that the AFE_0454 protein had eight transmembrane helices. Using the PSORTb v2.0 server, AFE_0454 was predicted to be a plasma membrane protein. Searching using the AFE_0454 sequence in TIGR showed that it had a high sequence identity (46%) with the copper resistance

protein, CopD. The later is a known copper resistance protein, and its function is binding with copper ions and transporting them outside of the cell. These results strongly suggested that the AFE_0454 is likely a transmembrane protein involved in copper ion resistance, and it might play an important role in mechanism(s) influencing copper resistance.

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