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Differential expression of sulfur assimilation pathway genes in *Acidithiobacillus ferrooxidans* under Cd^{2+} stress: evidence from transcriptional, enzymatic, and metabolic profiles

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Abstract *Acidithiobacillus ferrooxidans* is a heavy metal-tolerant acidophilic chemolithotroph found in acidic mine effluent and is used commercially in the bioleaching of sulfide ores. In this work, we investigated the interplay between divalent cadmium (Cd^{2+}) resistance and expression of genes involved in the sulfur assimilation pathway (SAP). We also investigated the response of the thiol-containing metal-chelating metabolites, cysteine and glutathione(GSH), to increasing Cd^{2+} concentrations. During growth in the presence of 30 mM Cd^{2+}, the concentrations of mRNA for 5 genes in the SAP pathway increased more than fourfold: these encode ATP sulfurylase (ATPS), adenosine 5′-phosphosulfate (APS) reductase, sulfite reductase (SiR), serine acetyltransferase (SAT) and O-acetylserine (thiol) lyase (OAS-TL). Increased transcription was also reflected in increased enzyme activities: those of SAT and adenosylphosphosulfate reductase (APR) reached a peak of 26- and 15.8-fold, respectively, compared to the control culture in the presence of 15 mM Cd^{2+}. In contrast, the activity of OAS-TL, which is responsible for the biosynthesis of cysteine, was diminished. At the metabolite level, the intracellular cysteine and GSH contents nearly doubled. These results suggested that Cd^{2+} induced transcription of SAP genes, while directly inhibiting the activities of some enzymes (e.g., OAS-TL). Overall, these results are consistent with a detoxification/resistance mechanism involving enhanced sulfur uptake and sequestration of Cd^{2+} by cysteine and glutathione.

Keywords *Acidithiobacillus ferrooxidans* · Cadmium · Cysteine · Gene expression · Glutathione · Metal resistance · Sulfur assimilation

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Introduction

Acidithiobacillus ferrooxidans is a Gram-negative, chemolithotrophic, acidophilic Proteobacterium that plays a critical economic role in bioleaching of sulfide metal ores. Members of the genus *Acidithiobacillus* derive energy from metabolizing sulfur, sulfide metal ores, and ferrous iron and can be isolated from acidic environments such as mine drainage effluent and acidified sulfate-containing soils. *A. ferrooxidans* is the most studied species within the genera and is widely used for bioleaching, where it obtains energy by oxidation of sulfur and ferrous iron and uses molecular oxygen as the terminal electron acceptor (Vera et al. 2013). These bacteria are also notable for their resistance to high concentrations of heavy metals. *A. ferrooxidans* can grow in the presence of exceptionally high divalent cadmium (Cd^{2+}) concentrations. This property is important for its use in bioleaching processes, where Cd^{2+} and other metal levels can be present in the 5–100 mM range (Baillet et al. 1997). Cadmium is a nonessential metal that is toxic to most organisms even at low concentrations, disrupting

pathways involved in carbon, nitrogen, and sulfur metabolism, as well as cellular redox chemistry (Toppi and Gabbrielli. 1999; Blazhenko et al. 2006). Acidophilic microorganisms found in acidic mine drainage (optimal growth pH < 3) are often highly resistant to heavy metals such as cadmium, with resistance being mediated by multiple mechanisms which are generally more efficient than those found in neutrophiles.

Prokaryotes have evolved a series of general mechanisms to control the cellular levels of heavy metals that are activated when concentrations exceed acceptable levels (Nies 1999), conferring in most organisms a low resistance to Cd^{2+}; for example, *Escherichia coli* has a minimum inhibitory concentration (MIC) of 0.7 mM for Cd^{2+} in Luria–Bertani (LB) medium, while *Ralstonia sp.* CH34 has an MIC of 0.8 mM (Nies 2003, 2000). In Gram-negative bacteria, resistance-nodulation cell division (RND)-driven tripartite protein complexes pump out a wide array of substrates, with the metal transporter CzcA from *Cupriavidus metallidurans* being a typical example (Shamim et al. 2014; Kim et al. 2011; Scherer and Nies 2009). However, these mechanisms only confer resistance at concentrations of approximately 1 mM or less and do not account for the high resistance observed in organisms found in acidic mine drainage effluent, where the concentrations of heavy metals such as cadmium are typically 1 to 2 orders of magnitude higher. A critical question is: what are the mechanisms of heavy metal resistance in highly resistant organisms such as *A. ferrooxidans*? Answering this question will have a profound impact on acidic mine effluent detoxification and human health (Dopson et al. 2003, 2014).

Eukaryotes have evolved distinct resistance mechanisms that enable survival in the presence of toxic heavy metals. One of the most common mechanisms found in yeast, algae, photosynthetic protists, and plants involves intracellular metal chelation by glutathione (GSH), phytochelatins (PCs), and other low molecular weight sulfur-containing peptides derived from cysteine (Cozatl et al. 2005). These peptides bind a variety of metals in the cytosol. Depending on the organism, the metal–PC or metal–GSH complexes are actively transported into vacuoles. Whether such chelating or sequestration mechanisms play a role in *A. ferrooxidans* Cd^{2+} resistance is unknown. Interestingly, all known heavy metal-chelating agents contain sulfur and are therefore products of the sulfur assimilation pathway (SAP) that is present in photosynthetic organisms, fungi, and many bacteria. Thus, the SAP may play a critical role in conferring resistance to heavy metals.

The SAP is present in photosynthetic organisms, fungi, and many bacteria. Sulfate is transported into the cytosol, where it undergoes reduction prior to incorporation into cysteine, GSH, methionine, and a variety of other metabolites (Fig. 1). According to bioinformatic analysis,

a metabolic model was reconstructed for the uptake and assimilation of sulfur in *A. ferrooxidans* (Valdés et al. 2003). In this pathway, ATP sulfurylase (ATPS, encoded by the *cysD* and *cysN* genes) catalyzes the first step of inorganic sulfate assimilation, activating sulfate via an ATP-dependent reaction leading to the formation of adenosine 5′-phosphosulfate (APS) and pyrophosphate. APS is directly reduced to sulfite by APS reductase (encoded by the *cysH* gene) and then the sulfite is reduced to sulfide by NADPH-SiR (encoded by the *cysIJ* gene). The first step in cysteine biosynthesis involves serine acetyltransferase (SAT), which is encoded by *cysE*. CysE catalyzes the reaction of serine and acetyl-coenzyme A to produce OAS. The second step of cysteine synthesis involves an OAS-sulfhydrylase, also called OAS-TL (encoded by the *cysM* gene). OAS-TL converts O-acetylserine (OAS) and sulfide into cysteine and acetate (Fig. 1). Although a SAP has not been characterized in *A. ferrooxidans*, the pathway shown in Fig. 1 is a reasonable framework upon which an understanding of sulfur uptake can be built.

Previous work has shown that in yeast, protists, and plants, expression of SAP genes is up-regulated by exposure to Cd^{2+} (Cozatl et al. 2005). For example, the ATPS gene was induced 13-fold in the model plant species *Arabidopsis thaliana* upon Cd^{2+} (200 μM) exposure (Harada et al. 2002); Cd^{2+} (1 mM) stress also promoted a 6-fold increase in the ATPS protein level in the yeast *Saccharomyces cerevisiae* (Vido et al. 2001). RNA blot analysis of transcript amounts indicated that in 6-week-old *Brassica juncea* plants, the genes for ATPS and APSR were strongly induced by exposure to Cd^{2+} (25 μM) (Heiss et al. 1999). In addition, a DNA microarray analysis of *S. cerevisiae* showed that the GSH synthesis gene (*gsh1*) and almost all transcripts of the enzymes involved in sulfur amino acid metabolism, especially MET14 (APK) and MET17 (PAPSR), were induced at 21- and sixfold, respectively, after exposure to Cd^{2+} (Momose and Iwahashi 2003). Proteomic analysis of PAPSR shows that its concentration is increased fivefold in response to 1 mM Cd^{2+} exposure (Vido et al. 2001). Similarly, the bryophyte *Physcomitrella patens* reacts to elevated Cd^{2+} concentrations by increasing the cellular concentrations of cysteine and GSH (Koprivova et al. 2002). In contrast, recent investigations of Cd^{2+}-stressed bryophytes showed no increase in PC biosynthesis, but instead there was a significant increase in the GSH pool (Helbig K et al. 2008a, b).

Although the genetics of the SAP are well known, little attention has been paid to cytosolic concentrations of cysteine and GSH in response to heavy metal exposure in prokaryotes. Our goal is to elucidate the mechanism of Cd^{2+} resistance in *A. ferrooxidans*. In this work, we investigate gene regulation of the SAP by Cd^{2+} stress in *A. ferrooxidans* and demonstrate a connection between sulfur assimilation and Cd^{2+} tolerance.

Cd^{2+}

SO_4^{2-}

$0.814 \pm 0.041 | 1.992 \pm 0.100 | 7.289 \pm 0.364$ **cysD-1**   ATPS

APS → PAPS   **cysNC**

$0.173 \pm 0.009 | 0.486 \pm 0.024 | 1.125 \pm 0.056$

$1.211 \pm 0.060 | 2.650 \pm 0.132 | 6.576 \pm 0.329$ **cysH**   APR

SO_3^{2-}

$1.235 \pm 0.062 | 1.081 \pm 0.054 | 1.106 \pm 0.055$ **cysJ**   SiR   OAS-TL
$0.977 \pm 0.049 | 1.739 \pm 0.087 | 4.403 \pm 0.220$ **cysI**

S^{2-} → hCys

CT

Ser → SAT → OAS → OAS-TL → Cys → γ-EC → GSH

cysE   **cysM**

$0.730 \pm 0.036 | 1.605 \pm 0.080 | 4.031 \pm 0.202$ $0.638 \pm 0.032 | 1.568 \pm 0.078 | 4.201 \pm 0.210$

Fig. 1 Changes in relative transcript levels of sulfate assimilation biosynthesis enzymes in *A. ferrooxidans* under Cd^{2+} stress, in comparison to an unchallenged control (5 mM Cd^{2+}, 15 mM Cd^{2+}, 30 mM Cd^{2+}). *ATPS* ATP sulfurylase, *APR* adenosylphosphosulfate reductase, *SiR* sulfite reductase, *SAT* serine acetyltransferase, *OAS* o-acetylserine, OAS-TL, and o-acetylserine (thiol) lyase. The induction ratios were determined by comparing the relative transcript cDNA levels from *A. ferrooxidans* grown in the Cd^{2+} concentrations indicated versus those obtained in control cells grown in the absence of the metal and normalized by 16S rRNA expression in each case. Values of standard deviations were obtained with three biological replicates. CT, PCR cycle at which fluorescence first rises above a threshold background fluorescence. The transcript levels were final values ($p < 0.05$)

Materials and methods

Strains, media, and culture conditions

A. ferrooxidans, ATCC 23270, was obtained from the American Type Culture Collection and was routinely grown at 30 °C with shaking (170 rpm) in 9 K medium, containing $(NH_4)_2SO_4$ 3.0 g/L, $MgSO_4$ 0.5 g/L, K_2HPO_4 0.5 g/L, KCl 0.1 g/L, and $Ca(NO_3)_2$ 0.01 g/L with ferrous sulfate (10 g Fe^{2+}/L) as the energy source. The medium was adjusted to pH 2.3 using sulfuric acid before $FeSO_4 \cdot 7H_2O$ was added and then filter sterilized. Bacterial growth was assessed by measuring ferrous iron oxidation rates. The ferrous iron concentration in solution was determined volumetrically by titrating against potassium dichromate ($K_2Cr_2O_7$) using sodium diphenylamine sulfonate (0.2 % wt/vol) as the indicator.

Total RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was isolated using TRIzol® (Invitrogen Corporation, Carlsbad, USA) and purified with the RNeasy® mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. On-column DNase digestion was performed with RNase-free DNase (Qiagen GmbH, Hilden, Germany) to remove genomic DNA. RNA integrity was checked by 1.5 % agarose gel electrophoresis and ethidium bromide staining. Total RNA was quantified at OD_{260} and OD_{280} with a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and then served as the template to synthesize cDNA.

Primer design for real-time PCR

Real-time PCR primers were designed using Primer Premier 5.0 and then synthesized by Sagon Biotech (Sagon, Shanghai, China). Annotations of *A. ferrooxidans* ATCC 23270 genes and their putative cellular functions were obtained from the Institute for Genomic Research website at http://cmr.tigr.org/tigr-scripts/CMRGenomePage, and the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/nuccore/cp001219.1) (Accession: CP001219.1; GI: 218517237). All primer pairs for selected genes that encode putative SAP components are listed in Table 1. Specific fragments were amplified and purified,

Table 1 Primer sequence for transcripts encoding 16S RNA (housekeeping gene) and an enzyme of the SAP used for quantitative reverse transcriptase (RT)-PCR

Gene symbol	Sequence		Size of product (bp)
	Forward primer (5′–3′)	Reverse primer (5′–3′)	
AFE-0139 = AFE_2971 (*cys*NC)	GGGAGTTCCTTGAGGTGTTCG	TGCTGAGGCGTAGCTCTGGT	159
AFE0140 = AFE_2970 (*cys*D-1)	AAGGAGCGCCCAGTCGT	CCAGGGTATCGGCATCG	163
AFE0276 = not number (*cys*M)	GAACCGACCAGCGGGAATA	ACCGTAGGCACGCATCA	126
AFE1579 = AFE_1505 (*cys*E1)	AAGCGTCACCCGACATTG	CCCACGACCGTCATTCC	143
AFE3209 = AFE_3123 (*cys*H)	CCCCATCCAGGTGTATTTC	GTGAGCCGCATCCCATT	232
AFE3210 = AFE_3122 (*cys*I)	ACGCCCGCCTCAAATAC	ATCACCATCGGCAACCC	203
AFE3211 = AFE_3121 (*cys*J)	CTGCTGGGCAAGGTGA	CAGAAAGGGCCATGTGAT	267
AFE0254 = AFE_2854 (*16S rRNA*)	TGGGTTCTAATACAATCTGCTA	CGCATTTCACCGCTACA	244

and were then visualized by 1.5 % agarose gel electrophoresis and ethidium bromide staining to detect whether PCR resulted in single products of the expected sizes.

Real-time PCR detection

The real-time PCR mixture (final volume 50 μL) contained 25 μL of SYBR® Green Real-time PCR Master Mix (Toyobo Co., LTD., Osaka, Japan), 2 μL of a 10 mM solution of sense/anti-sense primer, 10 μL of cDNA template, and 11 μL of nuclease-free water. Real-time PCR was carried out with an iCycler iQ Real-time PCR detection system (Bio-Rad Laboratories, Inc., Irvine, CA, USA): 1 cycle of 95 °C for 30 s, and then 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. At the completion of each run, melting curves for the amplicons were measured by raising the temperature at 0.5 °C intervals from 55 to 95 °C while monitoring fluorescence. The specificity of the PCR amplification was evaluated by examining the derivatized T_m melting curve for symmetry and a lack of non-specific peaks. Amplification of gene transcript amounts for samples from cultures grown in the presence of Cd^{2+} (5, 15, 30 mM) compared to the amounts from control samples was determined by analyses of the respective amounts of PCR products. The results were normalized against 16S rRNA to correct sample-to-sample variation. Each assay was run in triplicate.

Measurements of enzyme activities

Harvested bacterial cells were lysed by sonication (22 kHz) for 5 min with 1 min intervals of cooling and cell debris was removed by centrifugation at 10,000*g* for 10 min. Protein contents of the crude cell-free extracts were measured by the Folin method (Lowry et al. 1951), using bovine serum albumin as the standard. The activity of APS reductase in the extracts was evaluated by

measuring the reduction of ferricyanide in the presence of sulfite and adenosine monophosphate (AMP) (Truper and Rogers 1971). The activity of SAT was estimated by the formation of OAS in the presence of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (Stiller et al. 2007). The activity of SiR was determined by measuring the reduction of NADPH (340 nm) in the presence of sulfite (Ostrowski et al. 1989). The activity of OAS-TL was estimated by measuring the formation of cysteine according to the method of Chronis (Schnell et al. 2007). Assays were performed with two samples for each treatment and each assay was run in triplicate, for a total of six replicates for each sample.

Measurement of cysteine and GSH by colorimetry

The cellular content of cysteine was assayed, by measuring the ability of cell lysates to form a Co^{2+}–cysteine complex which has an optical absorbance maximum at 358 nm. The complex formation reaction was started by adding 3.0 mL NH_4Cl–NH_4OH buffer solution, 1.5 mL $CoCl_2$ solution (100 mg/L Co^{2+}) and 1.0 mL cysteine-containing solution sequentially into 10 mL colorimetric tubes. After 5 min incubation, measure the reactants by a UV spectrophotometer. The cysteine content was estimated by comparison with the absorption of samples to which a standard cysteine solution had been added.

The GSH content in cell lysates was measured by GSH formation of 5-phosphorothioate-2-nitrobenzoic acid with 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). The reaction started by mixing 2.0 mL GSH-containing solution, 2.5 mL of Na_2HPO_4 buffer solution (0.32 mmol/L), and 0.5 mL of DTNB solution in a 10 mL colorimetric tubes. Absorbance was measured at 416 nm within 5 min, at which 5-phosphorothioate-2-nitrobenzoic acid exhibited maximum absorption. The GSH content of cell lysates was estimated by comparison with samples containing a known concentration of GSH.

Assays were performed with two samples for each treatment and each assay was run in triplicate, a total of six replicates for each sample.

Statistical analyses

Student's t test (independent sample t test) was used to determine the statistical significance. The p values were calculated using the null hypothesis. A p value of less than 0.05 indicates that the two sets are statistically different.

Results

Vitality of *A. ferrooxidans* upon Cd^{2+} exposure

To estimate the vitality of *A. ferrooxidans* under Cd^{2+} stress, the effect of increasing Cd^{2+} concentrations on the iron-oxidizing activity of cultures was investigated (Fig. 2). Cd^{2+} inhibits iron-oxidizing activity, as judged by its effect on the rate of iron oxidation in the liquid medium. In the presence of 5 mM Cd^{2+}, *A. ferrooxidans* cell extracts took a slightly longer time to oxidize 50 % of the iron compared with the control, while in the presence of higher Cd^{2+} concentrations the time for 50 % iron oxidation was longer by 6 and 8 min for 15 and 30 mM Cd^{2+}, respectively, compared with the control. In addition, cell densities at the early stationary phase in the presence of 15 and 30 mM Cd^{2+} were smaller than those seen in the absence of the metal or in the presence of 5 mM Cd^{2+} (data not shown). Although *A. ferrooxidans* cells were still able to grow in the presence of 100 mM Cd^{2+}, the culture cell density was very low.

Transcription patterns of genes involved in the SAP upon Cd^{2+} exposure

The transcript concentrations of sulfur assimilation-related genes were measured by real-time PCR to assess how Cd^{2+} affected gene transcription in *A. ferrooxidans* using total RNA extracted from *A. ferrooxidans* cells grown in the presence or absence of Cd^{2+}. Treatment with Cd^{2+} affected transcription of genes coding for enzymes involved in sulfate reduction in a concentration-dependent manner (Fig. 1). In the presence of 5 mM Cd^{2+}, transcript levels of the sulfate assimilation genes of *A. ferrooxidans* were not significantly changed, exhibiting natural fluctuations under the slightly unfavorable growth condition. In the presence of 15 mM Cd^{2+}, most sulfate assimilation genes were up-regulated 1.2- to 3.0-fold, yet there was no observed effect on *cysNC*, a subunit of ATPS, suggesting that the metal concentration may have affected these sulfate assimilation genes differently. In the presence of 30 mM
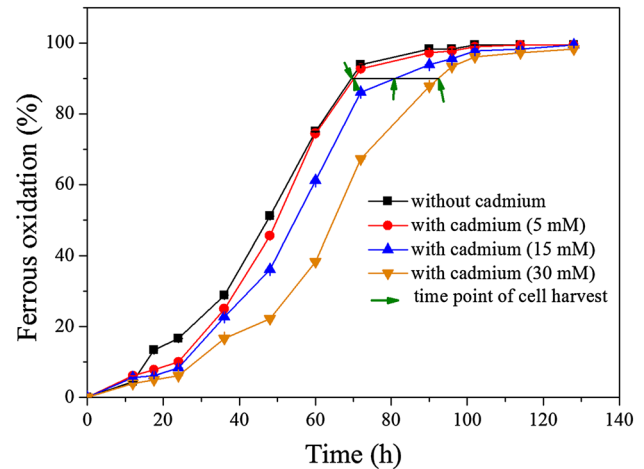
Fig. 2 Ferrous iron oxidation of *A. ferrooxidans* in the absence or presence of cadmium. *Error bars* indicate the standard deviations based on three different cultures

Cd^{2+}, transcription levels of most sulfur assimilation genes were up-regulated, the exception being *cysJ*, which was slightly down-regulated. The increases in mRNA levels for ATPS (*cysD-1*), APR (*cysH*), SiR (*cysI*), SAT (*cysE*), and OAS-TL (*cysM*) were 7.29-, 6.58-, 4.4-, 4.0-, and 4.2-fold, respectively. Overall, exposure to high concentrations of Cd^{2+} had a significant effect on sulfate assimilation gene expression in *A. ferrooxidans*.

Thiol pools are increased upon Cd^{2+} stress

Thiol profiling was carried out to assess whether the observed increases in transcription result in changes in the concentrations of sulfur-containing metabolites. These analyses showed that thiol metabolite concentrations increased in a Cd^{2+} concentration-dependent manner (Table 2). GSH levels varied with Cd^{2+} concentration and exposure time in a more complicated fashion. Prior to 24 h of incubation, Cd^{2+} stress resulted in higher GSH levels compared to the control. At 72 h, GSH levels for most samples were lower than that at 12 h, with the exception being the culture incubated in the presence of 5 mM Cd^{2+}, wherein a maximum concentration of GSH was observed. Finally, at 96 h, all the samples showed very high level of GSH, including the control.

After 12 h, Cd^{2+}-stressed cells contained higher concentrations of cysteine and GSH than control cultures, as determined by UV spectrophotometry. In contrast, only slight changes in cysteine and GSH levels were observed 72 h after Cd^{2+} exposure. After 96 h these metabolites had clearly increased. Most notably, cells exposed to 15 mM Cd^{2+} exhibited significantly enhanced levels of both cysteine and GSH after 96 h of exposure.

Time after Cd^{2+} application (h)	Cd^{2+} concentration			
	0 mM	5 mM	15 mM	30 mM
12				
Cysteine	78.2 ± 1.8	83.3 ± 0.5	86.2 ± 3.8	95.1 ± 5.0
GSH	83.9 ± 0.43	85.2 ± 3.8	108.11 ± 7.8	94.12 ± 1.7
24				
Cysteine	82.5 ± 1.5	84.4 ± 0.5	86.3 ± 4.2	97.6 ± 2.1
GSH	85.6 ± 1.2	115.7 ± 0.2	115.7 ± 0.1	109.4 ± 1.4
72				
Cysteine	66.2 ± 3.4	85.3 ± 2.7	87.2 ± 6.4	116.8 ± 8.7
GSH	66.14 ± 3.8	198.4 ± 13.2	77.5 ± 2.1	57.5 ± 0.3
96				
Cysteine	166.2 ± 2.4	178.2 ± 1.6	206.7 ± 12.4	123.6 ± 2.6
GSH	188.2 ± 10.8	176.4 ± 7.8	194.5 ± 11.6	158.4 ± 1.6

Table 2 Levels of cysteine and glutathione (glutathione) [μmol/L] of *A. ferrooxidans* in absence or presence Cd after 12, 24, 72, and 96 h of application

($p < 0.05$)

Enzyme activities (nmol/mg*min)	Cd^{2+} concentration			
	0 mM	5 mM	15 mM	30 mM
SAT	12.00 ± 0.29	$58.79 \pm 1.13*$	$313.77 \pm 2.73*$	38.08 ± 0.42
APR	3.38 ± 0.01	$9.67 \pm 0.06*$	$53.33 \pm 0.12*$	$28.58 \pm 0.48*$
SiR	8.65 ± 0.43	$4.98 \pm 0.25*$	7.38 ± 0.37	5.38 ± 0.27
OAS-TL	286.93 ± 8.55	589.24 ± 29.46	286.34 ± 2.40	$38.22 \pm 0.01*$

Table 3 Enzyme activities of SAT, APR, SiR, and OAS-TL of *A. ferrooxidans* in absence or presence Cd

* Statistically significant difference compared to 0 mM ($p < 0.05$, student's *t* test)

Activities of cysteine-synthesizing enzymes were differentially affected by Cd^{2+}

To examine whether altered transcription by Cd^{2+} stress is accompanied by changes in enzymatic activities, assays were carried out for key *A. ferrooxidans* SAP enzymes. When cell extracts were analyzed, enzymatic activity levels for various enzymes differed in response to Cd^{2+} concentrations (Table 3). The levels of SAT and APR activities were elevated with increasing Cd^{2+} concentration, showing peaks at 15 mM; however, the activities of SiR and OAS-TL were depressed at high metal concentrations, in spite of the observation that transcript levels of their respective genes were increased. These observations are consistent with direct Cd^{2+} inhibition of SiR and OAS-TL.

Discussion

Previous studies have indicated that the five metal resistance mechanisms identified in neutrophiles are also present in acidophiles, in some cases utilizing homologous proteins. However, a remarkable feature of acidophiles such as *A. ferrooxidans* is their tolerance of much higher concentrations of heavy metals, as judged by observed MIC values (Dopson et al. 2003). Eukaryotes provide a clue

that resistance to heavy metals can be mediated by mechanisms involving chelation/sequestration by cysteine or GSH (Figueira et al. 2005; Gharieb 2004). If such a mechanism is responsible for Cd^{2+} resistance in *A. ferrooxidans*, the SAP would be predicted to be up-regulated by Cd^{2+}, resulting in higher intracellular concentrations of GSH and cysteine (Helbig et al. 2008a, b; Hochgrafe 2007; Latinwo et al. 1998). In this study, we analyzed the effect of Cd^{2+} on the SAP gene transcription, enzymatic activities, and the intracellular levels of GSH and cysteine.

Real-time PCR showed that transcription of most genes in the SAP was up-regulated following exposure to elevated Cd^{2+}. In addition, the thiol pool concentrations, including cysteine and GSH, were increased in the presence of Cd^{2+}. Our findings are thus in agreement with published results for eukaryotes (Cozatl et al. 2005; Dominguez-Solis et al. 2001).

A surprising finding from our study was that elevated transcript levels do not necessarily result in enhanced enzymes activity levels. This phenomenon was observed for *cysI* (coding for SiR subunit) and *cysM* (coding for OAS-TL). The difference between transcription and enzymatic activity for those genes is likely due to Cd^{2+} binding to the enzymes, directly inhibiting their activities, a phenomenon observed for many metal irons (Wang et al. 1996; Bandyopadhyay et al. 1997). The precise mechanism

of this inhibition is currently being investigated in our laboratories.

Another interesting observation made in this study was that two key enzymes in the SAP, SiR and OAS-TL, for synthesis of cysteine were depressed while the level of cysteine was elevated under Cd^{2+} stress. This observation suggests that *A. ferrooxidans* possesses an alternative biosynthetic pathway generating cysteine and GSH to that presented in Fig. 1 (Brzywczy et al. 2002; Marzluf 1997; Ono et al. 1999; Thomas and Surdin-Kerjan 1997).

Conclusion

Overall, the results presented herein clearly demonstrate that exposure to Cd^{2+} results in elevated expression of *A. ferrooxidans* SAP genes, with a concomitant increase in most of the respective enzyme activities. Critically, we show that Cd^{2+} exposure increases cellular concentrations of thiol-containing GSH and cysteine, providing evidence that resistance might be mediated by a mechanism involving heavy metal sequestration. A critical next step will be delineation of the fate of the thiol–metal complexes, and efforts to achieve this are ongoing in our laboratories.

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