Journal of proteome research

Article

pubs.acs.org/jpr

New Copper Resistance Determinants in the Extremophile *Acidithiobacillus ferrooxidans*: A Quantitative Proteomic Analysis

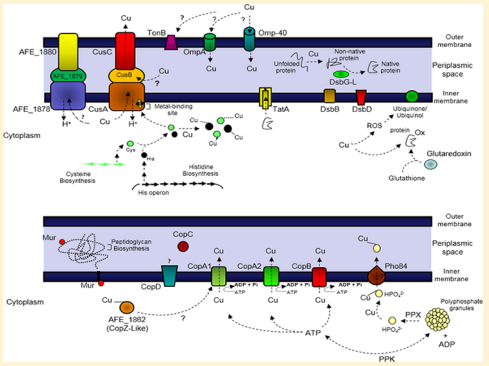
Rodrigo J. Almárcegui,[†] Claudio A. Navarro,[†] Alberto Paradela,[‡] Juan Pablo Albar,[‡] Diego von Bernath,[†] and Carlos A. Jerez*[,†]

[†]Laboratory of Molecular Microbiology and Biotechnology, Department of Biology, Faculty of Sciences, University of Chile, Santiago Casilla 653 Chile

[‡]Proteomics Laboratory, National Biotechnology Center, Madrid 28049, Spain

Supporting Information

ABSTRACT: *Acidithiobacillus ferrooxidans* is an extremophilic bacterium used in biomining processes to recover metals. The presence in *A. ferrooxidans* ATCC 23270 of canonical copper resistance determinants does not entirely explain the extremely high copper concentrations this microorganism is able to stand, suggesting the existence of other efficient copper resistance mechanisms. New possible copper resistance determinants were searched by using 2D-PAGE, real time PCR (qRT-PCR) and quantitative proteomics with isotope-coded protein labeling (ICPL). A total of 594 proteins were identified of which 120 had altered levels in cells grown in the presence of copper. Of this group of proteins, 76 were up-regulated and 44 down-regulated. The up-regulation of RND-type Cus systems and different RND-type efflux pumps was observed in response to copper, suggesting that these proteins may be involved in copper resistance. An overexpression of most of the genes involved in histidine synthesis and several of those annotated as encoding for cysteine production was observed in the presence of copper, suggesting a possible direct role for these metal-binding amino acids in detoxification. Furthermore, the up-regulation of putative periplasmic disulfide isomerases was also seen in the presence of copper, suggesting that they restore copper-damaged disulfide bonds to allow cell survival. Finally, the down-regulation of the major outer membrane porin and some ionic transporters was seen in *A. ferrooxidans* grown in the presence of copper, indicating a general decrease in the influx of the metal and other cations into the cell. Thus, *A. ferrooxidans* most likely uses additional copper resistance strategies in which cell envelope proteins are key components. This knowledge will not only help to understand the mechanism of copper resistance in this extreme acidophile but may help also to select the best fit members of the biomining community to attain more efficient industrial metal leaching processes.



KEYWORDS: *copper resistance, Acidithiobacillus ferrooxidans, isotope-coded protein labelling (ICPL), cell envelope proteins*

1. INTRODUCTION

Industrial biomining processes to extract copper, gold and other metals involve the use of a mixed microbial community that often comprises both autotrophic and heterotrophic acidophilic species capable of oxidizing iron and sulfur. Widely used biomining microorganisms such as *Acidithiobacillus ferrooxidans* can be adapted to grow in the presence of >100 mM copper sulfate.[1] This property is important for the use of this microorganism in biomining processes, where copper levels usually range between 15 and 100 mM $CuSO_4$.[2−4]

The genetic and biochemical mechanisms responsible for metal resistance in acidophilic *Bacteria* and *Archaea* are still largely uncharacterized.[5−8] Current knowledge indicates that key elements involved in *A. ferrooxidans* copper-resistance appear to be the following: a wide repertoire of known copper-resistance determinants and their duplication, the presence of novel copper-chaperones, an abundant reserve of inorganic polyphosphate (polyP) used in a polyP-based copper-resistance system and a defensive response to oxidative stress.[9−11] *A. ferrooxidans* ATCC 23270 can survive high copper concentrations by having in its genome at least 10 genes that are possibly related to copper homeostasis. These include three genes coding for putative ATPases related to the transport of copper (*copA1*$_{Af}$, *copA2*$_{Af}$ and *copB*$_{Af}$), three genes related to a system of the RND family, involved in the extraction of copper from the cell (*cusA*$_{Af}$, *cusB*$_{Af}$, *cusC*$_{Af}$), and two genes coding for periplasmic chaperones for this metal (*cusF*$_{Af}$ and *copC*$_{Af}$). These *A. ferrooxidans* copper-resistance determinants were found to be overexpressed when this bacterium was adapted to $CuSO_4$ in the range of 5−25 mM and conferred a greater resistance to copper in *Escherichia coli* cells expressing the *A. ferrooxidans* genes compared to wild-type cells, supporting their functionality.[12]

Received: September 26, 2013
Published: December 31, 2013

The *A. ferrooxidans* ATCC 53993 genome contains the same copper resistance determinants as those in strain ATCC 23270 plus a 160 kb genomic island (GI), which is absent in the ATCC 23270 type strain. Among other genes, this GI contains several genes coding for an additional putative copper-ATPase and a Cus system. Copper resistance in strain ATCC 53993 is most likely explained by the presence of the additional copper-resistance genes in its GI. As determined by qRT-PCR, it was demonstrated that these genes are overexpressed when *A. ferrooxidans* ATCC 53993 is grown in the presence of copper and the proteins were shown to be functional when synthesized in copper-sensitive *E. coli* mutants.[13] In conclusion, the grounds for resistance to copper of two strains of the same acidophilic microorganism could be determined by slight differences in their genomes, which may not only lead to changes in their capacities to adapt to their environment, but may also indicate which microorganisms are the best for industrial biomining operations.[4]

A recent study utilizing whole-genome DNA microarrays reported genes related to the copper stress response were overexpressed in an *A. ferrooxidans* strain isolated from a copper bioleaching operation in Atacama Desert, Chile. However, as mentioned by the authors this strain apparently corresponds to *A. ferridurans*.[14]

The aim of this work was to search for new possible copper resistance determinants in *A. ferrooxidans* strain ATCC 23270 by using a quantitative proteomic approach. Several proteins were highly up-regulated when the microorganisms were grown in the presence of copper. Some of these macromolecules may have an important role in the adaptability of the microorganisms to their environment.

2. MATERIALS AND METHODS

2.1. Bacterial Strains and Growth Conditions

A. ferrooxidans strain ATCC 23270 was grown at 30 °C in 9K medium containing ferrous sulfate (33.33 g/L) with an initial pH of 1.45−1.5 in the absence or presence of $CuSO_4$ as previously described.[15]

2.2. Preparation of Total Membrane Fraction

A. ferrooxidans ATCC 23270 was adapted to different copper sulfate concentrations (0, 5, 20, or 50 mM) and was grown until the culture reached between 1×10^8 and 5×10^8 cells/mL. Cells were harvested by centrifugation (10 000*g* for 2 min at 25 °C) and were resuspended in sonication buffer (50 mM Tris/HCl pH 8, 1 mM EDTA, 100 mM phenylmethylsulfonyl fluoride [PMSF]). Disruption by sonication was done on ice by using a Misonix XL2020 sonicator (Farmingdale, NY, USA) with 20% intensity and cycles of 15 s on, 30 s off, for a total time of 15 min. Cell debris was removed by centrifugation at 10 000*g* for 10 min at 4 °C. Protein quantitation was performed by using Bio-Rad protein assay system.

The supernatant was centrifuged at 100 000*g* for 1 h at 4 °C to separate the total membrane fraction. This fraction was resuspended in ACA750 buffer (750 mM *n*-aminocaproic acid, 50 mM Bis-Tris/HCl pH 7) and samples were stored at −80 °C.[16] Protein content was determined by using the BCA assay (PIERCE) as the components of the ACA750 buffer interferes with protein determination when using the Bio-Rad system.

2.3. Preparation of Membrane Protein Samples and 2D-PAGE

Approximately 400 μg of proteins from the total membrane fraction was solubilized in ACA750 buffer containing 5% (v/v) *n*-dodecyl β-D-maltoside. Samples were then incubated on ice for 2 h with vortexing every 20 min. Following solubilization, samples were centrifuged at 16 000*g* for 20 min at 4 °C. Proteins in the supernatant were precipitated with the Ready-Prep 2D cleanup Bio-Rad Kit. Precipited proteins were resuspended in 300 μL of isoelectric focusing (IEF) buffer (8 M urea, 2 M thiourea, 0.001% bromophenol blue, 2% *n*-dodecyl β-D-maltoside) followed by the addition of 100 mM dithiothreitol (DTT) and 2% Bio-Rad ampholytes and the sample mix was incubated for 1 h at 25 °C. The entire volume was loaded into the protean IEF focusing Tray (17 cm) using strip pH range 3-10NL (Ready Strip IPG). Focused proteins in the strip were then incubated at 25 °C with gentle agitation for 15 min in equilibrium buffer (6 M urea, 30% glycerol, 2% SDS, 24 mM Tris/HCl pH 6.8) containing 2% DTT followed by a 15 min incubation in equilibrium buffer containing 2.5% iodoacetamide. Finally, the strip was placed onto a 12.5% polyacrylamide gel for the second dimension SDS-PAGE.[17] The gels were stained with silver nitrate as described previously.[18]

2.4. MS Analysis of Proteins Extracted from 2D Gels and Protein Identification

Appropriate copper concentrations to be used in the quantitative proteomic determinations were studied by means of a preliminary 2D-PAGE analysis. Thus, the copper concentrations employed were those in which clearly visible differential changes were observed in triplicate gel runs of samples grown at 0, 5, 20, and 50 mM copper. Under these conditions, a few proteins were not present in the control but were greatly up-regulated (e.g., DsbG) or greatly reduced in the presence of the metal (e.g., Omp40). An image analysis by using the free ImageJ software (http://rsb.info.nih.gov/ij/) was done and a quantitative statistical evaluation of the selected spots of interest was obtained (Table S1a). These proteins were recovered from silver nitrate-stained 2D gels (obtained from three biological replicates in each case) by excising the protein spots. Each spot was identificated by MALDI TOF/TOF analysis as described previously.[17] (See Tables S1b, S3).

2.5. Quantitative Proteomics

2.5.1. Total Protein Extracts Preparation for ICPL Analysis. *A. ferrooxidans* was grown with ferrous iron as substrate until late exponential phase in the absence or presence of 40 mM $CuSO_4$. Cells were harvested by centrifugation (10 000*g* for 2 min) and were first washed three times with dilute sulfuric acid (pH 1.5) by centrifugation at 0 °C as previously described.[15] This was followed by three washes with 50 mM sodium citrate at pH 7.0 by centrifugation at 0 °C to remove any minor ferrous iron remaining and at the same time to neutralize the pH before cell rupture by sonic oscillation. Cells were then resuspended in sonication buffer (50 mM Tris-HCl pH 8, 1 mM EDTA) containing PMSF as protease inhibitor (100 μg/mL) and were disrupted by sonic oscillation during 30 min on ice by using successive 15 s pulses. Finally, the lysate was centrifuged at 10 000*g* for 10 min to remove unbroken cells and cell debris and the total proteins in the cell-free extract were determined.

2.5.2. Sample Preparation for ICPL Determinations. Samples of total protein extracts from three different independent cultures (fifty micrograms of each one) were mixed to obtain a triplicate representative sample of each experimental condition with a total of 150 μg of protein. These mixtures were lyophilized for 48 h at −40 °C. Finally, the dried samples were stored at −20 °C until their isotope-coded protein label (ICPL) labeling.

dx.doi.org/10.1021/pr4009833 | J. Proteome Res. 2014, 13, 946−960

2.5.3. Protein Digestion and ICPL-Labeling. The ICPL-reagent protocol was optimized for labeling of 100 μg of each individual sample per experiment. Thus, 100 μg of total protein extracts was individually dissolved in 8 M urea 25 mM ammonium bicarbonate, reduced and alkylated with iodoacetamide. The urea concentration was reduced to 2 M with 25 mM ammonium bicarbonate and the sample was digested with trypsin (Roche Diagnostics GmbH, Mannheim, Germany) with a ratio of 25:1 overnight at 37 °C. Before ICPL-labeling, salts and urea were removed using high-capacity OMIX C18 tips (Varian, Palo Alto, CA). Sample labeling with the light and heavy versions of the ICPL reagent (Serva Electrophoresis, Heidelberg, Germany) was performed at the peptide level according to the manufacturer's instructions.

2.5.4. 2D-Nano LC ESI−MS/MS Analysis. ICPL-labeled combined samples (200 μg per experiment) were dissolved in 100 μL of 10 mM NH_4OH in water, pH 9.5, and fractionated in a wide-pH range, 5 μm particle size, 100 × 2.1 mm reversed phase XBridge column (Waters) using a Knauer Smartline HPLC system. Gradient elution was performed according to the following scheme: isocratic conditions of 10 mM NH_4OH in water, pH 9.5, for 5 min, a linear increase to 25% B (10 mM NH_4OH in 80% methanol, 20% water, pH 9.5) in 10 min followed by a linear increase to 75% B in 40 min, a linear increase to 100% B in 5 min, isocratic conditions of 100% B for 5 min, and return to initial conditions in 2 min. Flow-rate was 150 μL/min. Injection volume was 100 μL and the wavelength was monitored at 214 nm. Approximately 25−30 individual HPLC fractions (2 min each), were collected in each experiment, dried in a speed-vac and stored at −20 °C until needed. Second dimension of the 2D-nano LC ESI−MS/MS analysis was performed using an Ultimate 3000 nanoHPLC (Dionex, Sunnyvale, CA) coupled with an HCT Ultra ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The analytical column was a silica-based reversed phase column C18 PepMap 75 μm × 15 cm, 3 μm particle size, and 100 Å pore size (Dionex, Sunnyvale, CA). The trap column was a C18 PepMap (Dionex, Sunnyvale, CA), 5 μm particle diameter, 100 Å pore size, switched online with the analytical column. The loading pump delivered a solution of 0.1% trifluoroacetic acid in 98% water/2% acetonitrile (LabScan, Gliwice, Poland) at 30 μL/min. The nanopump provided a flow-rate of 300 nL/min and was operated under gradient elution conditions, using 0.1% formic acid (Fluka, Buchs, Switzerland) in water as mobile phase A, and 0.1% formic acid in 80% acetonitrile/20% water as mobile phase B. Gradient elution was performed according to the following scheme: isocratic conditions of 96% A: 4% B for 5 min, a linear increase to 40% B in 60 min, a linear increase to 95% B in 1 min, isocratic conditions of 95% B for 7 min and return to initial conditions in 10 min. Injection volume was 5 μL and the wavelength was monitored at 214 and 280 nm. The LC system was coupled via a nanospray source (Bruker Daltonics, Bremen, Germany) to a 3D ion trap mass spectrometer operating in positive ion mode with the capillary voltage set at 1400 V. Automatic data-dependent acquisition allowed sequential full scan (m/z 350−1500) MS spectra followed by tandem MS CID spectra of the four most abundant precursor ions. Dynamic exclusion was applied to prevent the same m/z from being isolated for 1 min after its fragmentation.

2.5.5. Protein Identification and Quantitative Analyses. MS and MS/MS data obtained for individual HPLC fractions were merged using the Analysis Combiner tool and subsequently processed as a single experiment using DataAnalysis 3.4 (Bruker Daltonics, Bremen, Germany). For protein identification, MS/MS spectra (in the form of Mascot generic files) were searched against the *A. ferrooxidans* ATCC 23270 UniprotKB forward-reversed database (http://www.uniprot.org) containing 2747 entries and their corresponding reversed sequences. Sequence reversal was done using the program DBToolkit v4.1.5. Database searches were done using a licensed version of Mascot v.2.2.04 (www.matrixscience.com; Matrix Science, London, U.K.). Search parameters were set as follows: tryptic digestion, carbamidomethyl cysteine as fixed modification, oxidized methionine and ICPL-labeling of lysine residues and/or peptide amino termini as variable ones. Peptide mass tolerance was set at 0.6 Da both in MS and MS/MS mode, and 1 missed cleavage was allowed. In most cases, an accuracy of ±0.1−0.2 Da was found both for MS and MS/MS spectra. The False Discovery Rate (FDR) of ≤5% for peptide identification was manually assessed as follows: after database searching, peptide matches were ranked according to their Mascot scores. This list contains peptide sequences matching either forward or reversed database sequences. Then, a subset containing 5% of peptides matching the reversed sequences was extracted, and all the proteins resulted for that FDR were used for further quantitative analysis. Qualitative and quantitative analyses were performed by WARP-LC 1.1 (Bruker Daltonics, Bremen, Germany) using the parameters described above. First, all the peptides were identified, and then, based on a single ICPL-labeled identified peptide, the software calculated the extracted ion chromatogram for the putative ICPL-labeled pair according to: (a) the mass shift defined by the labeling reagent, (b) a mass tolerance of 0.5 Da, and c) a retention time tolerance of 40 s. Relative ratios between light and heavy ICPL-labeled peptides were calculated based on the intensity signals of their corresponding monoisotopic peaks, and according to these individual peptide ratios the software calculates the protein ratio. Finally, ratios were $\log_2$-transformed and normalized by subtracting the median value. Table S4 contains all quantitation data obtained.

2.6. Whole-Genome Alignments

The annotated genome of *A. ferrooxidans* ATCC 23270 (http://cmr.jcvi.org/) was used to perform whole-genome alignment using Mauve software.[19] Whole-genome representations and gene context graphics were carried out using the Geneious PRO 4.7 software.

2.7. Extraction of Total RNA from *A. ferrooxidans* and cDNA Synthesis

To determine the effect of copper on the expression of genes of interest, cells were adapted in batch cultures to grow in the presence of different $CuSO_4$ concentrations (0, 20, 30, and 50 mM) until the late exponential growth phase was reached. At this time, total RNA was extracted standardly from each culture condition by lysing the cells as previously reported,[12,13,20] except that TRIzol (Invitrogen) was used for the extraction. Between three to five biological replicas were used for each experimental condition. Any remaining DNA was eliminated from the RNA preparations by the addition of 4 U of TURBO DNA-*free* DNase (Ambion) following the manufacturer's instructions. For cDNA synthesis, 0.8 μg of total RNA was reverse transcribed for 1 h at 42 °C using ImProm-II (Promega), 0.5 μg of random hexamers (Promega) and 3 mM $MgCl_2$.[13]

dx.doi.org/10.1021/pr4009833 | J. Proteome Res. 2014, 13, 946−960

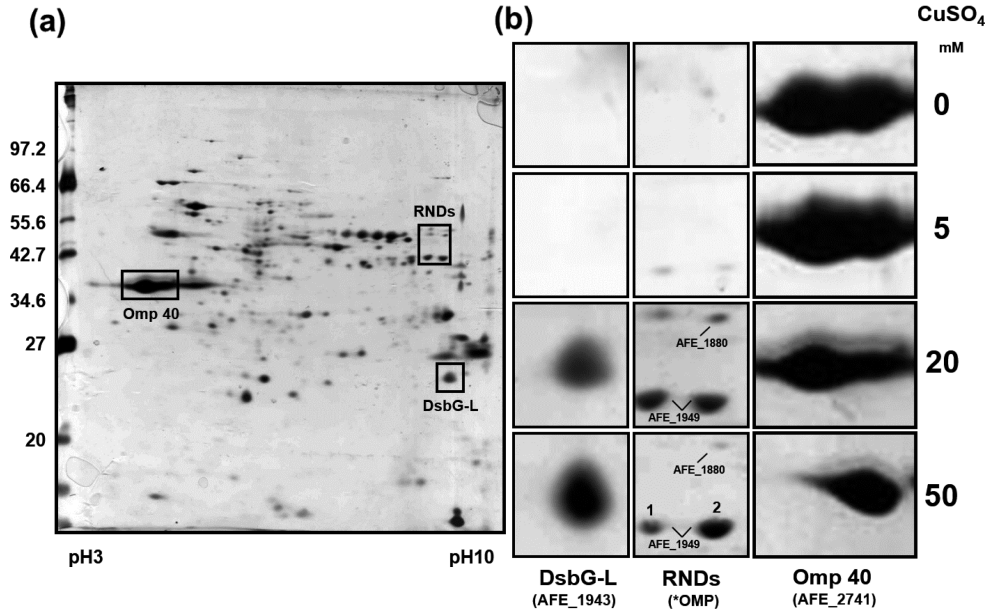
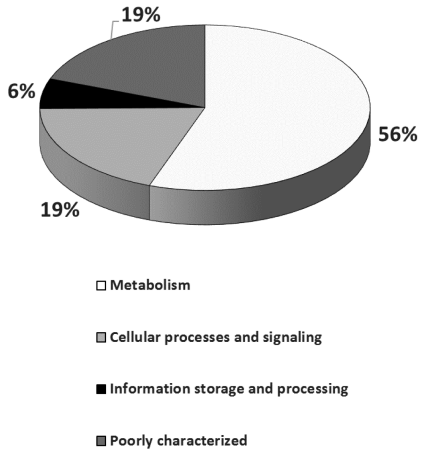


Figure 1. Separation of *A. ferrooxidans* total membrane fraction proteins by 2D-PAGE. Microorganisms were grown in ferrous iron-containing 9K medium at the copper sulfate concentrations indicated. (a) Complete gel with proteins from cells grown in the presence of 20 mM $CuSO_4$. Areas labeled with squares or rectangles indicate some of the protein spots that were differentially synthesized compared with the control in the absence of the metal. Except for Omp-40, these proteins were isolated from the gels for their identification by MS/MS spectrometry (Table S1b). The position of Omp-40 in these type of gels and its N-terminal end amino acid sequence has been previously reported.[22] (b) Amplification of the areas shown in (a) from the corresponding gels in which the membrane proteins analyzed were from cells grown at the indicated increasing $CuSO_4$ concentrations. *OMP (Outer membrane protein).



Functional category	n° prot.	prot. (+)	prot. (-)
Metabolism			
Inorganic ion transport and metabolism	9	7	2
Amino acid transport and metabolism	16	15	1
Carbohydrate transport and metabolism	12	6	6
Nucleotide transport and metabolism	5	2	1
Lipid transport and metabolism	2	1	1
Coenzyme transport and metabolism	4	2	2
Energy production and conversion	20	10	10
Cellular processes and signaling			
Cell motility and secretion	4	3	1
Post-translational modification, protein turnover, and chaperones	13	9	4
Cell wall/membrane/envelope biogenesis	4	3	1
Signal transduction mechanism	2	1	1
Information storage and processing			
Replication, recombination and repair	1	1	0
Transcription	4	1	3
Translation, ribosomal structure and biogenesis	2	0	2
Poorly characterized			
General function prediction only	3	3	0
Function unknown	20	11	9
TOTAL	**120**	**76**	**44**

Figure 2. Functional categories and numbers of *A. ferrooxidans* proteins changing their synthesis levels in cells grown in the presence of copper. The pie chart refers only to the proteins listed in the table.

2.8. Primer Design, Real-Time RT-PCR, and Cloning of *A. ferrooxidans* Genes

Primers for qRT-PCR were designed using the Primer3 software[21] as reported before[12] (Table S2). After separating PCR products by electrophoresis in a 1% agarose gel (0.5× Tris-acetate-EDTA pH 8.0 buffer), no cross-amplification or nonspecific bands were detected.

Copper-resistance related gene expression was analyzed by qRT-PCR with the Corbett Rotor Gene 6000 system as described previously,[12,13] except that the Rotor-gene SYBR green PCR Kit (Qiagen) was used. A housekeeping analysis using three genes was carried out using the Normfinder algorithm.[22] 16S $rRNA_{Af}$ was selected as a reference gene since its expression was found to be the most stable under our experimental conditions.

2.9. Functional Assays of *A. ferrooxidans* Gene AFE-1943 (*DsbG-L*) Expressed in *E. coli*

Since there is currently no efficient and reproducible methodology for the generation of knockouts in *A. ferrooxidans*, the functionality of *DsbG-L* from *A. ferrooxidans* was tested by using heterologous expression in *E. coli*. For this, after PCR amplifying AFE-1943, the resulting DNA fragment was purified and cloned

dx.doi.org/10.1021/pr4009833 | J. Proteome Res. 2014, 13, 946−960

Table 1. Up-Regulation of Proteins in *A. ferrooxidans* ATCC 23270 Grown in Ferrous Iron and in the Presence of 40 mM $CuSO_4$

accession number	AFE	function/similarity	score	coverage (%)	peptide number	fold change (Cu 40 mM/0 mM)
		Metabolism				
		Inorganic ion transport and metabolism				
B7JBW5	1862	Heavy metal-binding protein, putative	353.87	78.38	11	14.78
B7JC44	1948	Heavy metal efflux transporter, MFP subunit, putative ($CusB_{Af}$)	239.96	23.23	16	14.68
B7JC45	1949	Outer membrane heavy metal efflux protein, putative ($CusC_{Af}$)	188.64	16.16	13	11.41
B7JC43	1947	Heavy metal efflux pump, CzcA family ($CusA_{Af}$)	114.37	10.23	12	6.86
B7J3E4	43	Periplasmic solute-binding protein, putative	363.63	31.25	24	5.54
B7J5B8	602	Cation ABC transporter, periplasmic cation-binding protein, putative	43.78	9.18	2	4.97
B7JAJ7	3093	Cation channel protein, putative	95.58	8.81	4	4.39
		Amino acid transport and metabolism				
B7J7F7	981	Glycine dehydrogenase, decarboxylating (GcvP)	45.53	2.68	1	8.86
B7JAM5	3121	Sulfite reductase [NADPH] flavoprotein alpha-component (CysJ)	815.50	37.07	36	6.99
B7JA16	3043	Imidazoleglycerol-phosphate dehydratase (HisB)	70.64	10.55	3	6.15
B7JAH3	3064	Glutamate–cysteine ligase	115.32	10.32	8	5.83
B7JA14	3041	Histidinol dehydrogenase (HisD)	170.77	16.86	9	5.56
B7JA17	3044	Imidazole glycerol phosphate synthase subunit (HisH)	59.14	27.35	5	4.84
B7JAM9	3125	Sulfate adenylyltransferase, large subunit (CysN)	560.76	30.88	26	4.80
B7JAM8	3124	Sulfate adenylyltransferase, small subunit (CysD-2)	189.69	21.79	13	4.68
B7J9U4	2971	Sulfate adenylate transferase, large subunit/adenylylsulfate kinase (CysNC)	72.40	3.47	2	4.60
B7JAM6	3122	Sulfite reductase [NADPH] hemoprotein beta-component (Cysl)	453.95	22.02	23	4.56
B7JAM7	3123	Adenylylsulfate reductase, thioredoxin dependent	72.73	18.78	6	4.18
B7JA15	3042	Histidinol-phosphate aminotransferase (HisC-1)	122.85	7.52	3	4.04
B7JA13	3040	ATP phosphoribosyltransferase (HisG)	65.42	17.67	7	4.04
B7JA19	3046	Imidazole glycerol phosphate synthase subunit (HisF) 1-(5-phosphoribosyl)-5-[(5-	423.10	35.97	13	3.96
B7JA18	3045	phosphoribosylamino)methy hdeneamino] imidazole-4-carboxarnideisomerase (HisA)	200.29	22.31	7	3.96
		Carbohydrate transport and metabolism				
B7J693	2324	Phosphoglucomutase, putative	162.61	12.71	10	7.91
B7JA25	3052	Ribulose bisphosphate carboxylase, small subunit (CbbS-1)	255.94	44.07	10	7.24
B7JA24	3051	Ribulose bisphosphate carboxylase large chain (CbbL-1)	523.76	39.11	37	6.94
B7J9Z1	3018	Phosphoenolpyruvate-protein phosphotransferase (PtsI)	105.99	9.30	7	5.77
B7J9Z6	3023	Phosphoenolpyruvate-dependent sugar PTS system, EIIA component	192.66	37.84	13	4.58
B7JBW0	1857	Glucose 1-dehydrogenase, putative	188.51	15.33	6	4.49
		Nucleotide transport and metabolism				
B7JAG8	3059	Aspartate carbamoyltransferase (PyrB)	104.61	11.62	4	6.39
B7JAG9	3060	PyrR bifunctional protein	49.15	28.57	4	4.40
		Energy production and conversion				
B7J6F2	2388	2-octapreny1-6-methoxyphenol hydroxylase (UbiH-2)	53.51	6.36	2	15.92
B7J7D1	955	Cytochrome *d* ubiquinol oxidase, subunit I (CydA)	136.53	6.83	9	11.01
B7J7D0	954	Cytochrome *d* ubiquinol oxidase, subunit II (CydB)	74.49	7.16	8	6.44
B7JAH4	3065	Citrate synthase II (CitZ)	80.53	15.80	11	5.77
B7JAI8	3079	Nitroreductase family protein	101.32	31.66	8	5.62
B7JBR9	1813	Dehydrogenase complex, E1 component, alpha subunit, putative	134.03	14.97	8	4.62
B7J8A9	2677	3-deoxy-D-manno-octulosonic-acid transferase domain protein	80.05	10.58	2	4.48
B7JAH7	3068	Pyruvate dehydrogenase complex, E2 and E3 components	836.43	21.46	32	4.17
B7JBR4	1808	Phosphate acetyl/butyryl transferase	57.83	9.12	4	4.10
B7JBR7	1811	2-oxo acid dehydrogenase, acyltransferase, putative	103.86	13.27	8	3.86
		Lipid transport and metabolism				
B7J498	2017	PspA/IM30 family	361.96	49.59	32	6.51
		Coenzyme transport and metabolism				
B7J5J4	2206	Nicotinate-nucleotide pyrophosphorylase	43.74	9.09	3	4.14
B7JAC2	1545	Molybdenum-pterin binding protein	335.48	42.65	32	4.07
		Cellular Processes and Signaling				
		Post-translational modification, protein turnover, and chaperones				
B7JC40	1943	DsbG domain protein (DsbG-L)	45.66	10.67	7	15.54
B7JAL7	3113	Cytochrome c-type biogenesis protein (ResC)	87.82	2.55	2	10.11
B7JA11	3038	Glutaredoxin	224.32	40	7	5.84
B7JAL6	3112	Cytochrome c-type biogenesis protein (ResB)	428.45	21.50	19	4.86
B7J3S3	177	Glutathione S-transferase family protein	73.28	11	2	4.71

dx.doi.org/10.1021/pr4009833 | *J. Proteome Res.* 2014, 13, 946−960

Table 1. continued

accession number	AFE	function/similarity	score	coverage (%)	peptide number	fold change (Cu 40 mM/0 mM)
		Post-translational modification, protein turnover, and chaperones				
B7J4U6	2086	Heat shock protein, Hsp20 family	345.71	59.31	23	3.90
B7JAH2	3063	Glutathione synthetase	60.93	8.65	3	3.88
B7J927	2834	SPFH/Band 7 domain protein	49.64	13.14	5	3.84
		Cell wall/membrane/envelope biogenesis				
B7J3 V6	210	UDP-N-acetylmuramoyl-trip eptide–D-alanyl-D-alanine ligase (MurF)	53.47	6.22	2	27.81
B7JA12	3039	UDP-N-acetylglucosamine 1-carboxy vinyltransferase (MurA)	111.45	10.40	5	5.13
B7J7B8	941	Murein transglycosylase, putative	49.34	3.13	2	3.91
		Cell motility and secretion				
B7JA21	3048	Sec-independent protein translocase protein (TatA-1)	218.14	41.66	6	15.00
B7J3D3	32	Inner membrane protein (OxaA)	50.78	6.62	4	12.05
B7J8E2	2710	Pilin, putative	63.91	10.13	1	6.52
		Signal transduction mechanisms				
B7J9Z5	3022	Hpr(Ser) kinase/phosphatase (HprK)	68.64	16.45	6	5.05
		Information Storage and Processing				
		Replication, recombination and repair				
B7JAJ8	3094	DNA polymerase I	152.31	7.89	8	4.91
		Translation, ribosomal structure and biogenesis				
B7J9Z7	3024	Ribosomal subunit interface protein (YfiA)	77.87	28.32	4	4.64
		Poorly Characterized				
		General function prediction only				
B7J4E4	401	Roadblock/LC7 domain protein	40.85	10.29	1	12.04
B7J7R8	3118	Pyridine nucleotide-disulfide oxidoreductase	1369.68	65.69	111	7.81
B7J6R1	846	Alcohol dehydrogenase, zinc-containing	74.22	12.89	10	5.36
B7JAK7	3103	Phosphoribosyl transferase domain protein	53.69	10.81	2	4.83
		Function unknown				
B7J3N6	139	Putative uncharacterized protein	57.69	4.33	1	16.13
B7J3E3	42	Putative uncharacterized protein	83.41	8.89	5	11.24
B7JAM4	3120	Putative uncharacterized protein	40.54	12.50	2	8.55
B7J4M9	2018	Putative uncharacterized protein	188.46	12.41	9	6.98
B7J8K8	2783	Putative uncharacterized protein	164.68	31.37	8	6.83
B7J4N0	2019	Putative uncharacterized protein	68.65	10.76	6	6.42
B7J446	302	Putative uncharacterized protein	88.28	37.63	5	5.32
B7JAN1	3127	Putative uncharacterized protein	124.47	26.49	1	4.86
B7J4 × 2	2114	Putative uncharacterized protein	76.71	10.05	5	4.21
B7JAL8	3114	Putative uncharacterized protein	52.30	7.44	1	4.21
B7JBF1	3273	Putative uncharacterized protein	103.02	31.78	7	3.86

in the expression vector pBAD-TOPO to generate pBAD-TOPO-*DsbG-L*. *E. coli* top10 and the copper sensitive K-12 mutant Δ*copA*/Δ*cusCFBA*/Δ*cueO* were transformed with vector pBAD-TOPO-*DsbG-L*. The heterologous expression of *DsbG-L* was induced or repressed by growing the cells in the presence of L-arabinose or glucose, respectively. The capacity of *DsbG-L* to confer copper resistance to the already mentioned transformants was determined by measuring the inhibition halos in solid medium in the presence of copper-containing discs and by their growth inhibition in medium Luria–Bertani in the presence of 0.6 mM $CuSO_4$.[12]

3. RESULTS AND DISCUSSION

To find the most appropriate conditions to detect proteins responding to copper by means of quantitative proteomics, a preliminary analysis of some envelope proteins from *A. ferrooxidans* ATCC 23270 was done by using 2D-PAGE as shown in Figure 1. Cells were grown in a range of copper between 0 and 50 mM and higher levels of protein DsbG-L (AFE_1943) and RND proteins (AFE_1880 and AFE_1949)[12] was clearly seen at 20 and 50 mM Cu. These proteins may form part of putative RND transport systems and are potentially important *A. ferrooxidans* copper resistance determinants.

On the other hand, at these same Cu concentrations, the major outer membrane protein from *A. ferrooxidans* (Omp40) (AFE_2741)[23,24] began to decrease its levels. Therefore, a Cu concentration of 40 mM was chosen to grow the cells for the ICPL proteomic analysis. Under these conditions, 594 proteins were identified of which 120 changed their levels compared to the control cells grown in the absence of copper. In the group of proteins changing, 76 were up-regulated and 44 down-regulated.

Proteins with different levels were grouped according to functional categories as shown in Figure 2. Metabolism was the functional category with the greatest number of changes (56%). Within the Metabolism category, almost 23% of the proteins belonged to amino acid transport and metabolism and 13% were related to inorganic ion transport and metabolism. Cellular Processes and Signaling was the second functional category most affected by the presence of copper (19%). In this category, 57% of the changes corresponded to post-translational modification, protein turnover, and chaperones. Finally, about 19% of the proteins comprised proteins poorly characterized or with no homologues in databases, being proteins with unknown

dx.doi.org/10.1021/pr4009833 | J. Proteome Res. 2014, 13, 946–960

functions that most likely are unique to *A. ferrooxidans*. Next, we will describe some of the possible identities and putative functions of several differentially synthesized proteins from *A. ferrooxidans* grouped by their functional categories.

3.1. Metabolism

3.1.1. Inorganic Ion Transport and Metabolism. A highly increased level of Cus_{Af}, an RND type efflux system from *A. ferrooxidans*[12] was detected in cells grown in ferrous iron in the presence of copper (Table 1). These tripartite efflux systems are formed by a central RND protein ($CusA_{Af}$) (AFE_1947) working as a proton-substrate antiporter located in the inner membrane, a periplasmic-membrane fusion protein ($CusB_{Af}$) (AFE_1948) and an external membrane factor ($CusC_{Af}$).[25,26] This Cus system would detoxify cells by transporting copper from the periplasm and/or cytoplasm through the outer membrane to the extracelular space. The results presented here together with our previous reports[10,12] strongly suggest that the function of these RND transporters would be especially important for copper resistance in *A. ferrooxidans*.

Previous bioinformatic analysis identified the protein coded by AFE_1949 as a possible component of an RND efflux system involved in metal resistance and similar to the Cus system of *E. coli*. This ORF would form part of a possible transcriptional unit together with ORFs AFE_1948 and AFE_1947.[12] These three ORFs were highly expressed at the transcriptional level in cells subjected to copper as seen in Figure 3, left panel.

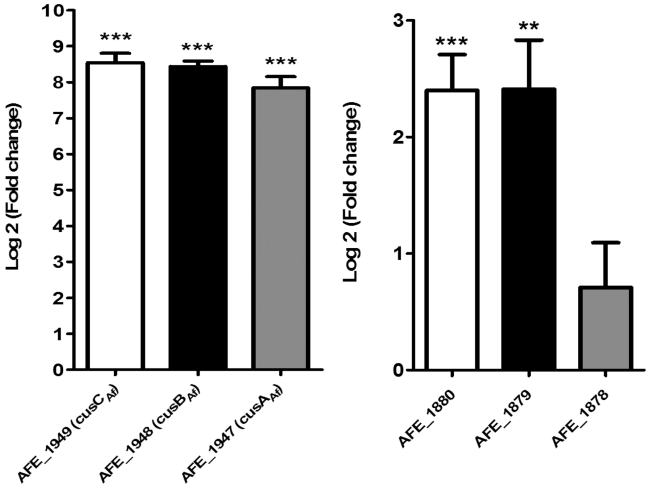


Figure 3. Relative expression of genes putatively encoding to RND type systems. Cells were grown in ferrous iron and in the presence of 50 mM $CuSO_4$. Transcriptional level changes refer to the control condition in the absence of the metal. Data were normalized by using the expression values for the 16S $rRNA_{Af}$ gene. Asterisks indicate $*p < 0.05$, $**p < 0.005$ and $***p < 0.0001$ when compared to the control value in the absence of copper. The error bars indicate the standard deviations based on three to five different experimental values.

In addition, a further membrane protein (AFE_1880) was up-regulated as detected by 2D-PAGE (Figure 1) as well as transcriptionally overexpressed in the presence of copper (Figure 3). The genomic context of this ORF showed as neighbors an ORF (AFE_1879) coding for a membrane fusion protein (MFP) and AFE_1878 coding for a putative membrane transport protein that could form an RND transporter system with an unknown substrate. The genetic context of these genes is similar to that of the Cus system from *E. coli*. The three genes forming this putative RND system were overexpressed at the transcriptional level (Figure 3, right panel) but were not seen up-regulated by the quantitative proteomic analysis.

The RND transport systems are traditionally formed by three different structural proteins coded in an operon.[27] By using RT-PCR it was demonstrated that the putative RND system genes were cotranscribed, strongly suggesting they form a transcriptional unit or possibly an operon (Figure 4). This unit may therefore code for a novel efflux RND system with a possible function in copper detoxification in *A. ferrooxidans*.

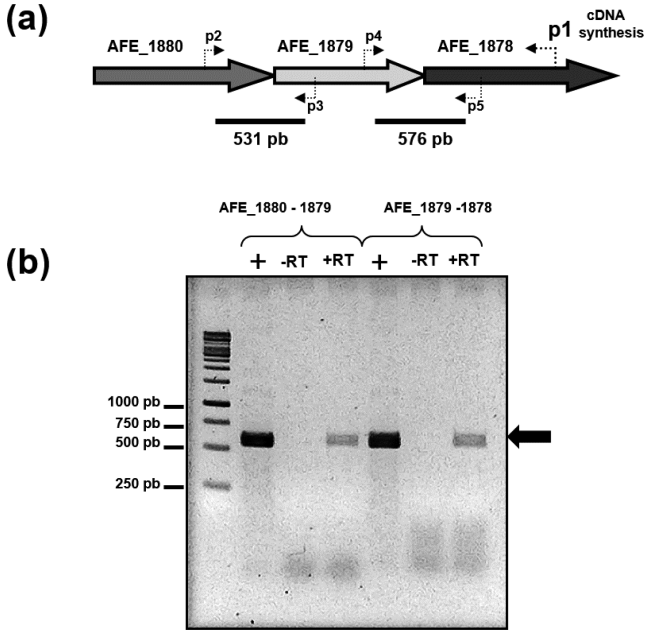


Figure 4. Putative new *A. ferrooxidans* RND type genes are cotranscribed. (a) The relative positions of genes AFE_1880, AFE_1879 and AFE_1878 (arrows in gray), locations for the primers used (dotted arrows) and the intergenic products and sizes expected (black horizontal bars) are indicated. *A. ferrooxidans* total RNA was extracted at the late exponential phase from a culture adapted to grow in 50 mM Cu. cDNA was synthesized with a reverse primer (p1) hybridizing toward the 3′ end of AFE_1878. PCR amplifications of the intergenic regions between AFE_1880−1879 and AFE_1879−1878 were carried out with the cDNA and p2−p3 and p4−p5 pairs of primers, respectively. (b) The results shown were obtained after 35 PCR cycles. PCR reactions were done using cDNA templates synthesized in the presence (+RT) or absence (-RT) of reverse transcriptase to detect the possible presence of genomic DNA contamination. The amplified DNA fragments were separated by agarose gel electrophoresis and are indicated by the black arrow.

In the *E. coli* RND Cus system, the inner membrane protein CusA would be the responsible of substrate specificity by binding copper in a metal-binding site formed by three or four essential methionine residues.[28] However, an alignment of the amino acid sequences between CusA from *E. coli* and the putative RND proteins from *A. ferrooxidans* (AFE_1947 and AFE_1878) (Table 1) indicated that only AFE_1947 (with 48% sequence identity to CusA), contains the metal-binding residues (not shown). On the other hand, AFE_1878 (with 21.8% sequence identity to CusA) had only one of the possible metal-binding methionines. Therefore, at present we can only speculate that the putative RND system from *A. ferrooxidans* is able to bind and transport copper either directly or indirectly. Obviously, further experiments are required to determine the function for this possible transporter.

dx.doi.org/10.1021/pr4009833 | J. Proteome Res. 2014, 13, 946−960

Table 2. Down-Regulation of Proteins in *A. ferrooxidans* ATCC 23270 Grown in Ferrous Iron and in the Presence of 40 mM $CuSO_4$

accession number	AFE	function/similarity	score	coverage (%)	peptide number	fold change (Cu 40 mM/0 mM)
		Metabolism				
		Inorganic ion transport and metabolism				
B7J8H1	2741	Major outer membrane protein 40 (Omp40)	71.95	4.46	4	0.14
B7J4Q1	2040	TonB -dependent receptor	72.83	2.41	2	0.43
		Amino acid transport and metabolism				
B7J4S9	2069	Tryptophan synthase beta chain	42.89	3.01	2	0.94
		Carbohydrate transport and metabolism				
B7J5W5	669	Inositol monophosphate family protein	39.64	16.10	3	0.44
B7JB29	1690	Ribulose bisphosphate carboxylase, small subunit (CbbS-2)	212.65	59.10	9	0.50
B7JB06	1667	Transketolase pyridine binding domain protein	339.27	25.62	33	0.50
B7JB15	1676	Fructose-bisphosphate aldolase class II family protein	211.49	21.12	10	0.55
B7JB30	1691	Ribulose bisphosphate carboxylase large chain (CbbL-2)	730.51	31.50	45	0.58
B7J9Z3	3020	PTS system IIA component, putative	48.53	5.80	2	0.88
		Nucleotide transport and metabolism				
B7J5P8	2261	Phosphoribosylamine-glycine hgase (PurD)	38.44	2.14	1	0.57
		Energy production and conversion				
B7JB01	1662	NADH dehydrogenase, putative	96.42	4.57	2	0.32
B7JB85	3204	ATP synthase gamma chain (AtpG)	44.96	3.47	1	0.33
B7J6S4	861	Flavoprotein with domin globain	75.14	21.57	6	0.40
B7JAQ6	3152	Cytochrome c552 (Cycl)	108.21	22.17	7	0.56
B7J7L8	2551	Iron−sulfur cluster-binding protein	125.10	12.97	9	0.65
B7JAL5	3111	Ubiquinol–cytochrome c reductase, cytochrome c1 subunit (PetC-1)	123.97	14.46	4	0.81
B7JAQ7	3153	Cytochrome c (Cyc2)	146.01	7.42	11	0.85
B7J7L7	2550	Succinate dehydrogenase/fumarate reductase, C subunit (SdhC)	89.56	6.27	4	0.91
B7J7M0	2553	Pyridine nucleotide-disulfide oxidoreductase	391.76	24.50	16	0.92
B7J4H0	427	Succinyl-CoA ligase [ADP-forming] subunit alpha (SucD)	59.35	12.41	3	0.93
		Lipid transport and metabolism				
B7J4G3	420	Enoyl-[acyl-carrier-protein] reductase [NADH] (FabL)	340.52	32.29	10	0.93
		Coenzyme transport and metabolism				
B7JB14	1675	Dethiobiotin synthetase (BioD-1)	72.52	3.98	1	0.20
B7J7N6	2569	Octanoyltransferase	57.81	11.79	2	0.94
		Cellular Processes and Signaling				
		Post-translational modification, protein turnover, and chaperones				
B7J7 × 8	2664	Chaperone protein DnaJ	50.23	4.80	2	0.23
B7J5Z2	696	S-(Hydroxymethyl)glutathione synthase (GfA)	124.38	16.49	4	0.63
B7J5N4	2246	DsbG domain protein	38.44	9.95	2	0.85
B7J789	909	Trigger factor (TiG)	220.62	14.98	6	0.87
		Cell motility and secretion				
B7J4F9	416	Pilin, putative	50.43	10.67	3	0.34
		Cell wall/membrane/envelope biogenesis				
B7J8B7	2685	OmpA family protein	43.09	7.65	2	0.20
		Signal transduction mechanisms				
B7J4Z0	2133	Universal stress family protein	77.39	9.38	1	0.76
		Information Storage and Processing				
		Translation, ribosomal structure and biogenesis				
B7J7G7	991	tRNA (Guanosine-2′-O-)-methyltransferase, putative	40.69	3.53	1	0.52
B7J3F7	56	Asparty1-tRNA synthetase (AspS)	61.18	5.03	4	0.95
		Transcription				
B7JB07	1668	Transcriptional regulator, IclR family	46.96	4.63	1	0.67
B7JB92	3211	ParB family protein	61.65	7.48	2	0.79
B7J5Q8	2271	Transcriptional regulator, putative	60.26	8.82	2	0.92
		Poorly Characterized				
		Function unknown				
B7JC77	1982	Putative uncharacterized protein	115.60	18.24	3	0.31
B7JB19	1680	Putative uncharacterized protein	49.15	5.21	1	0.35
B7JB00	1661	Putative uncharacterized protein	302.50	14.06	24	0.37
B7JB09	1660	Putative uncharacterized protein	200.12	18.42	9	0.48

dx.doi.org/10.1021/pr4009833 | *J. Proteome Res.* 2014, 13, 946−960

Table 2. continued

accession number	AFE	function/similarity	score	coverage (%)	peptide number	fold change (Cu 40 mM/0 mM)
		Function unknown				
B7JAZ9	1670	Putative uncharacterized protein	52.24	8.56	3	0.48
B7JB05	1666	Putative uncharacterized protein	119.48	15.28	8	0.65
B7JAG1	1585	Putative uncharacterized protein	176.36	41.88	14	0.80
B7JAN9	3135	Putative uncharacterized protein	48.14	17.91	4	0.84
B7J8C6	2694	Putative uncharacterized protein	49.75	3.77	1	0.87

The presence of copper in the growth medium of *A. ferrooxidans* greatly stimulated the up-regulation of a putative cation channel protein (AFE_3093) and a putative soluble heavy metal-binding protein (AFE_1862) with a possible heavy metal domain containing a putative CGHC metal-binding site (Table 1). This metal-binding site is similar to that found in CopZ, a copper metallochaperone that transfers the metal to ATPase transmembrane transporters.[29] The function, cellular location and metal specificity of these proteins are currently unknown. Most likely they are involved in binding and transporting cations.

On the other hand, Omp40 (AFE_2741), which is the most abundant porin-like outer membrane protein from *A. ferrooxidans*,[30] was greatly down-regulated when cells were grown in ferrous iron in the presence of copper (Figure 1, Table 2). The positively charged nature of the internal loops of Omp-40 porin has been proposed to be part of a passive mechanism to regulate proton entrance through the outer membrane to avoid excess acid in the *A. ferrooxidans* cytoplasm.[23] The same phenomenon could prevent excess entrance of other cations such as copper. A similar result has been observed in *Pseudomonas aeruginosa*, in which a reduced expression of eight different porins was seen when cells were exposed to copper.[31] Being a porin, Omp40 would play an important role in controlling ion diffusion through the outer membrane.[23,30] Therefore, the lower levels of synthesis of Omp-40 strongly suggests a change in the permeability of the outer membrane to decrease copper ions entrance to the cell and thus to prevent toxicity of the heavy metal.

A similar situation is the down-regulation of a putative OmpA family protein (AFE_2685) in the presence of copper (Table 2). OmpA not only provides structural stability to the cell, but it also generates a diffusion channel that allows a slow penetration of small solutes.[32] Lower levels of OmpA might also reduce copper ions entrance to the cell. This passive mechanism together with the efflux of copper carried out by the RND systems may be key responses to resist the toxic metal. It will be of great interest to establish if this reduced level of porins to avoid metal toxicity is a general phenomenon in bacteria.

Table 2 also shows down-regulation of a TonB-dependent receptor (TBDR; AFE_2040) in the presence of copper. These receptors are outer membrane proteins that form channels permeable to large solutes such as vitamin B_{12} and siderophores.[33,34] In *A. ferrooxidans*, several putative TBDR-like genes and some of the coding proteins have been described,[35,36] although their substrate specificities are unknown. Bioinformatic analysis suggested that this locus codes for the outer membrane receptor CirA involved in iron transport mediated by linear type cathecol siderophores.[37] It is known that cathecols can reduce Cu(II) to Cu(I) generating a stronger stress to cells exposed to the metal since the reduced form of copper is more toxic than the oxidized one.[38,39] Perhaps, AFE_2040 corresponds to a TBDR that is regulated by the presence of copper in *A. ferrooxidans*. In this regard, outer membrane proteins with structural similarities to TBDRs that are strongly repressed in the presence of exogenous copper and derepressed in copper-limiting conditions have been described in *Pseudomonas stutzeri*, *Pseudomonas putida* and *P. aeruginosa* (Schauer et al.[34] and references therein). It is possible that the lower synthesis level of the protein coded by AFE_2040 could also be a defense mechanism against copper toxicity since it might also reduce the income of the metal to the cell via cathecols.

Taken together, these results suggest the existence of an interesting and efficient metal resistance mechanism in *A. ferrooxidans*: to avoid the entrance of excess copper to the cell by decreasing transport and diffusion of the toxic metal. At the same time, the excess copper would be eliminated via RND type efflux pumps.

3.1.2. Energy Production and Conversion. *A. ferrooxidans petI* and *rus* operons contain genes encoding for iron oxidation proteins.[35] The *petI* transcriptional unit encodes the three subunits of the bc_1 complex (PetCAB), a predicted short chain dehydrogenase (Sdr) of unknown function and a cytochrome c_4 that has been suggested to receive electrons from rusticyanin and then pass them to the bc_1 complex (Valdés et al.[35] and references therein). The *rus* operon encodes two c-type cytochromes (Cyc1 and Cyc2), components of the aa_3-type cytochrome oxidase (CoxBACD) and rusticyanin.[40] Cyc2 has been shown to accept electrons directly from Fe(II) and this may be the first step in Fe(II) oxidation (Valdés et al.[35] and references therein).

Proteins ResC and ResB are involved in the maturation of bc_1 complex I (*petI* operon) and showed increased levels in the presence of copper (Table 1). However, reduced amounts of PetC-1 were detected (Table 2). Surprisingly, reduced expression of Cyc2 and Cyc1 was found in cells grown in the presence of copper. An increased time of growth has been observed for *A. ferrooxidans* cells in the presence of copper (data not shown), suggesting that respiration is affected and the oxidation of ferrous iron occurs more slowly in the presence of copper. This may be crucial and could have an impact on bioleaching processes.

Increased amounts of proteins such as CydA and CydB were found in the presence of copper (Table 1). This finding is interesting, since these proteins are components of the cytochrome *bd* terminal oxidase complex (with no heme-copper centers). This is the component of the aerobic respiratory chain in *E. coli* that predominates in cells grown at low aeration. In addition, these cytochrome *bd* oxidases have a higher affinity for oxygen than other cytochrome oxidases. Therefore, it is thought that they are used by microorganisms to continue aerobic respiration under conditions of low oxygen,[41] helping bacteria to survive periods of oxygen limitation. It is becoming increasingly apparent that expression of multiple cytochrome oxidases confers advantages to bacteria in surviving specific growth and stress conditions.[42,43] Thus, *A. ferrooxidans* with a reduced

dx.doi.org/10.1021/pr4009833 | J. Proteome Res. 2014, 13, 946−960

respiration rate due to copper stress might also use an alternative terminal oxidase besides the standard aa_3-type oxidase.

From the results shown in Table 1, it is clear that *A. ferrooxidans* subjected to copper up-regulates several energy-generating metabolic pathways to handle the cellular stress caused by the toxic metal. *A. ferrooxidans* contains an incomplete tricarboxylic acid (TCA) cycle lacking α-ketoglutarate dehydrogenase complex but in which pyruvate can be used as a source to reoxidize NADH and for the formation of citrate and α-ketoglutarate.[44] In this regard, several proteins in the "Energy production and conversion" functional category such as citrate synthase II (CitZ) (AFE_3065), dehydrogenase complex E1 component (AFE_1813) and pyruvate dehydrogenase complex (AFE_3068), which are part of or feed the TCA cycle showed increased levels in *A. ferrooxidans* grown in the presence of copper. Similar findings have been reported in *Ferroplasma acidarmanus* Fer1 subjected to As (III)[5] and in *Exiguobacterium* sp. S17 under arsenic stress.[45]

A putative 2-octaprenyl-6-methoxyphenol hydroxylase (UbiH-2) (AFE_2388) showed a very high up-regulation in *A. ferrooxidans* grown in the presence of copper (Table 1). This enzyme is involved in the biosynthesis of ubiquinone, which is an important component of the respiratory chains in *E. coli* and other bacteria. In addition, the lipid-soluble ubiquinone is known to limit oxidative stress caused by superoxide and peroxide in the *E. coli* cytoplasmic membrane.[46] Since copper generates membrane damaging reactive oxygen species, an increase in the levels of a protein involved in ubiquinone formation in the presence of copper is not unexpected and may contribute to the defense against the toxic metal in *A. ferrooxidans*. The regulation of UbiH-2 has also been reported in *A. ferrooxidans* subjected to a cellular stress such as oxygen limitation.[47]

3.1.3. Carbohydrate and Amino Acid Transport and Metabolism. Related to carbohydrate transport and metabolism category, a putative phosphoglucomutase protein (AFE_2324; Table 1) showed elevated levels in cells grown in the presence of copper. This enzyme generates glucose-6P from glucose-1P, thus producing energy from the former phosphorylated sugar or it could go to the pentose phosphate pathway to generate ribose and reducing power in the form of NADPH.

A. ferrooxidans 23270 fixes CO_2 by means of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)[48] in the Calvin−Benson−Bassham (ccb) reductive pentose phosphate cycle and associated carboxysome formation components. RuBisCO is a key enzyme for a chemolithoautotroph such as *A. ferrooxidans.* The microorganism has two *cbb* operons containing genes coding for RuBisCO.[49] This enzyme is formed by small (CbbS) and large (CbbL) subunits in all species that require to fix CO_2 for their metabolism. The results showed that one of the operons (Cbb1) (AFE_3052)/(AFE_3051) was up-regulated in the presence of copper (Table 1) whereas the other (Cbb2) (AFE_1691)/(AFE_1690) was down-regulated when *A. ferrooxidans* was grown in the presence of the metal (Table 2). The identity between the two kinds of RuBisCO small subunits was 60% and that concerning both large subunits was 84%, suggesting that they do not correspond to duplicate genes.

In this respect, comparing the differential synthesis levels of proteins from *A. ferroxidans* ATCC 23270 grown in ferrous iron versus elemental sulfur, it was found that CbbS-1/CbbL-1 was strongly down-regulated in cells grown in elemental sulfur. On the other hand, CbbS-2/CbbL-2 was not detected among the proteins changing their levels.[50]

There is no current information regarding the effect of copper on the expression of RuBisCO in autotrophic bacteria. However, a down-regulation of RuBisCO was reported in leaves from *Oryza sativa* subjected to copper.[51] On the other hand, when analyzing copper-binding proteins in the roots from *Arabidopsis thaliana* one of the proteins found was the small subunit from RuBisCO.[52] This protein shows 39% and 31% identity to CbbS-1 and CbbS-2 from *A. ferroxidans*, respectively.

Bearing in mind these previous findings, it is plausible that under the excessive ROS production due to the presence of copper, the toxic metal could repress Cbb2 operon. On the other hand, the other *A. ferrooxidans* Cbb1 operon codifies for a protein with high identity to the small RuBisCO subunit from *A. thaliana*, which as mentioned, is a copper-binding protein.[52] The up-regulation of Cbb1 when *A. ferrooxidans* was exposed to copper suggests that this operon not only could participate in carbon fixation but could contribute also to the metal resistance. However, the differential expression could be due to oxidative stress caused by copper or by ferrous iron[49] or by growth under anaerobic conditions.[53]

Surprisingly, seven of the eight putative proteins coded by the histidine operon (HisF, HisA, HisH, HisB, HisC-1, HisD, and HisG) (AFE_3046 to AFE_3040) were highly up-regulated in *A. ferrooxidans* cells grown in the presence of copper (Table 1). The eight proteins of the His operon participate in 10 reactions for the biosynthesis of His,[54] suggesting that the cells require higher amounts of His to respond to the presence of copper. In this connection, it is known that His (−) strains of *Saccharomyces cerevisiae* deficient in the synthesis of His are sensitive to copper, cobalt and nickel. This sensitivity was reverted when the amino acid was added to the culture medium, suggesting that His biosynthesis would confer resistance to Cu, Co and Ni. These studies suggested that intracelular histidine would directly alleviate the toxicity of these heavy metals.[55] Similar results have been described in plants where an up-regulation of the histidine biosynthesis pathway would contribute to nickel resistance possibly by a direct interaction between the metal and the amino acid.[56]

Considering these results, it is temptating to speculate that an increased synthesis of His in *A. ferrooxidans* grown in the presence of copper could contribute to copper resistance of the microorganism perhaps by a direct interaction between the free amino acid and the metal, which obviously remains to be demonstrated.

When *A. ferrooxidans* was grown in the presence of copper, an up-regulation of several proteins involved in cysteine biosynthesis, CysD-2 (AFE_3124), CysJ (AFE_3121), CysI (AFE_3122), CysN (AFE_3125), and Cys NC (AFE_2971), was also seen (Table1). In this connection, *Staphylococcus aureus* has been reported to increase the synthesis of cysteine and glutathione *S*-transferase in the presence of cadmium.[57] In the case of *E. coli*, recent experimental evidence indicated that most of the elements of the cysteine biosynthetic pathway are required to manage tellurite-induced stress in this bacterium and all the Cys regulon genes studied were up-regulated in the presence of this toxic compound.[58] These authors also suggested that this response could be due in part to the depletion of cellular thiols or sulfur-containing molecules such as glutathione.

As in the case of His, Cys also shows a strong affinity for divalent metals. Therefore, both amino acids could eventually protect the cell from the heavy metals in a similar fashion. Obviously, these speculations would have to be proven by

dx.doi.org/10.1021/pr4009833 | J. Proteome Res. 2014, 13, 946−960

determining the levels of free His and Cys in the intracellular space of *A. ferrooxidans* grown in the presence of copper.

Instead, increased biosynthesis of His and Cys in the presence of copper could be due to a higher demand for these amino acids. This situation may arise from the need to express higher levels of proteins involved in copper detoxification, many of them containing these amino acids in their metal binding sites.

3.2. Cellular Process and Signaling

3.2.1. Post-Translational Modification, Protein Turnover, and Chaperones. Within this functional category, a glutaredoxin (AFE_3038) increased its levels of synthesis when *A. ferrooxidans* was grown in the presence of copper (Table 1). Glutaredoxins are enzymes involved in redox reactions and whose main role is to protect cells against oxidative stress. For this reason, they maintain the adequate redox state of sulfhydril groups of celular proteins.[59] Being an oxidative agent, copper would generate disulfide bridges in proteins possessing functional sulfhydryl groups. The increased level of glutaredoxins would contribute to keep the thiol groups reduced in the proteins. The name glutaredoxin arises from the fact that these enzymes use glutathione as a cofactor. Glutathione is synthesized by two independent steps, the second one being catalyzed by the enzyme glutathion synthetase. This enzyme catalyzes the condensation of glycine to the C-terminal of gamma-glutamyl-cysteine.

Table 1 shows that glutathione synthetase (AFE_3063) was up-regulated in *A. ferrooxidans* grown in the presence of copper. This may be the result of a higher need of glutathione which will be used by glutaredoxins and glutathion *S*-transferase (AFE_0177) (also up-regulated in the presence of copper in Table 1) in detoxification processes. In connection with this point, a putative glutamate-cysteine ligase (AFE_3064) involved in glutathione formation was also up-regulated in the presence of the metal. These results are in agreement with previously determined mRNA levels for some of these enzymes in *A. ferrooxidans* subjected to copper[10] and with some of the predicted set of genes in the genome of this microorganism which are needed for nonenzymatic neutralization of reactive oxygen species.[35,44]

In addition, the up-regulation of AFE_1943 annotated as a DsbG domain protein that could correspond to a disulfide bridge isomerase was also seen in *A. ferrooxidans* and it was named DsbG-L. AFE_1943 showed up-regulation in the ICPL analysis (Table 1). Also, as seen in Figure 1, this protein was greatly synthesized when the microorganism was grown in the presence of copper. Furthermore, as determined by qRT-PCR, the transcriptional levels for the gene coding for DsbG-L was highly increased (Figure 5).

DsbG-L could be part of a family of thiol-disulfide oxidoreductases involved in the formation and rearrangement of disulfide bridges and in the correct folding of periplasmic proteins. Specifically, DsbG-L could be a disulfide bridge isomerase protein capable of reordering non-native disulfide bonds to generate the correct ones.[60,61]

As in the case of *E. coli* and other Gram-negative bacteria, *A. ferrooxidans* possesses genes coding for putative thiol-disulfide oxidoreductases which are homologues of DsbA, DsbB and DsbD. However, unlike *E. coli*, the acidophilic bacterium does not have in its genome a DNA sequence coding for the isomerase protein DsbC. On the contrary, *A. ferrooxidans* contains three genes coding for putative disulfide isomerases: AFE_2947 for a DsbG, and genes AFE_1943 and AFE_2246 that would code for

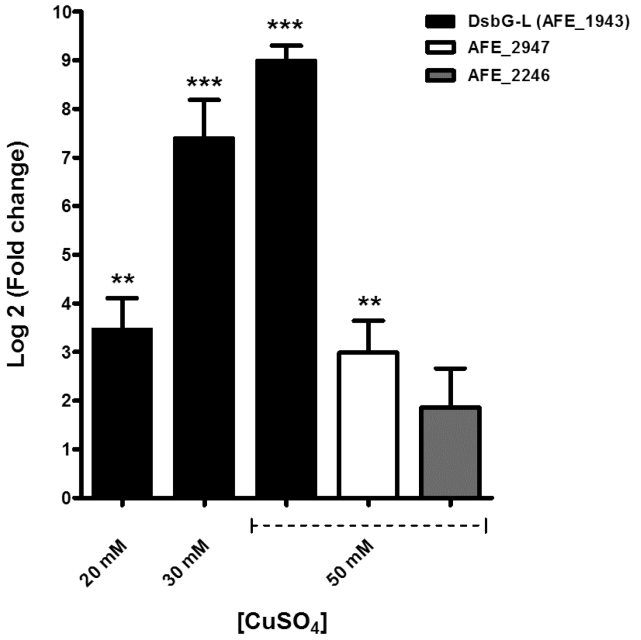


Figure 5. Relative expression of AFE_1943 (dsbG-L), AFE_2947 and AFE_2246 genes of *A. ferrooxidans* ATCC 23270 grown in the presence of copper. Cells were grown in ferrous iron and in the presence of 20, 30, and 50 mM $CuSO_4$. Transcriptional level changes refer to the control condition in the absence of the metal. Data were normalized by using the expression values for the 16 s *rRNA* gene. Asterisks indicate $^{**}p < 0.005$ and $^{***}p < 0.0001$ when compared to the control value in the absence of copper. The error bars indicate the standard deviations based on three to five different experimental values.

disulfide isomerase proteins containing a DsbG domain. This suggests that *A. ferrooxidans* could use extra copies of DsbG type proteins to compensate for the lack of DsbC.

Although *A. ferrooxidans* may therefore contain a complete functional Dsb system, a recent study of this putative system has been reported. It was demonstrated that the putative protein DsbG coded in AFE_2947 showed disulfide bridge isomerase activity although its physiological role still remains unclear.[61] However, overexpression of the gene encoding a protein with a DsbG domain (AFE_2947) seen in Figure 5 also suggests the possible involvement of this protein in repair purposes after metal stress.

Previous studies identified the putative proteins coded in AFE_2947 and AFE_2246 in the periplasm of *A. ferrooxidans* grown in thiosulfate.[36] These results suggested a possible physiological function for these periplasmic proteins. However, in this earlier high-throughput proteomic study. the protein coded by AFE_1943 was not detected since cells were not subjected to copper during growth.

The up-regulation of redox proteins involved in periplasmic folding functions has been previously detected in *P. aeruginosa* adapted to grow at high copper concentrations (DsbD, DsbE and DsbG).[31] In addition, it was demonstrated that in *E. coli* a disulfide bridge isomerase member of the *dsb* system (DsbC) is an important copper resistance determinant for this microorganism.[62]

It has been reported that copper ions catalyze the formation of incorrect disulfide bridges in proteins. This generates inactive proteins that may eventually cause cell death.[62] In this regard, a disulfide isomerase such as DsbG-L may also be an important copper resistance determinant for *A. ferrooxidans* since its up-

dx.doi.org/10.1021/pr4009833 | J. Proteome Res. 2014, 13, 946−960

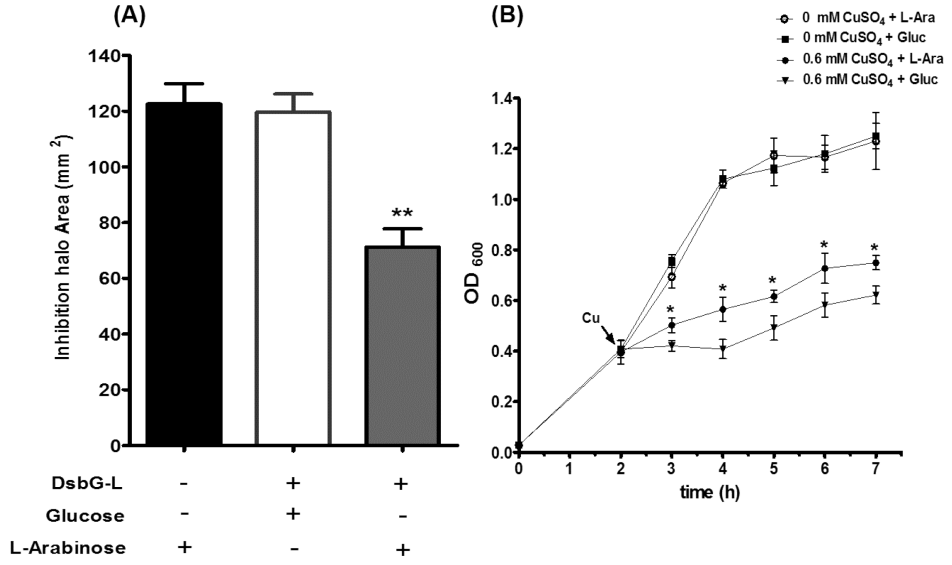


Figure 6. DsbG-L from *A. ferrooxidans* confers higher copper resistance to *E. coli*. (A) *E. coli* top-10 transformed with plasmid pBAD-TOPO containing the heterologous *A. ferrooxidans DsbG-L* gene was grown in the presence of glucose (uninduced, white bar) or in the presence of arabinose (induced expression, gray bar). The control was the same bacterial strain transformed with the plasmid without the inserted *DsbG-L* gene (black bar). The areas of the inhibition halos were determined in each case. (B) Growth curves of *E. coli* strain K-12 Δ*copA*/Δ*cus*CFBA/Δ*cueO* transformed with pBAD-TOPO-*dsbG-L* grown in the presence or absence of 0.6 mM $CuSO_4$ and in the presence of glucose or L-arabinose as indicated. The arrow indicates the time where copper was added to the cultures. Asterisks indicate $*p < 0.05$,$**p < 0.005$ compared to the control in the absence of copper. The error bars indicate the standard deviations based on three to five different experimental values.

regulation could help to restore native disulfide bridges allowing the survival of the acidophile under copper stress. The heterologous expression of *DsbG-L* in *E. coli* conferred increased copper resistance to this bacterium, as determined by the lower size copper inhibition halos and a partial growth recovery of the transformed strain after the addition of copper after 2 h of growth (Figure 6). A possible explanation for the rather limited effect of DsbG-L is that this periplasmic protein would normally function at pH around 2 in the *A. ferrooxidans* periplasm. When synthesized in *E. coli*, it would be confronted to pH 6−7, and therefore, it may not work more efficiently under those conditions.

3.2.2. Cell Wall/Membrane/Envelope Biogenesis. Bacteria possess a cell envelope to maintain cell shape and avoid cell lysis. This structure is rigid due to the presence of a peptidoglycan polymer formed by the two sugar derivatives *N*-acetylglucosamine and *N*-acetylmuramic acid joined together by β1−4 bonds to form a linear chain. In Gram-negative bacteria, this is a thin layer which is localized in the periplasm between the external- and inner-membranes.[63] Peptidoglycan biosynthesis involves several steps where Mur proteins participate.[64]

Table 1 shows that in the presence of copper *A. ferrooxidans* have increased levels of MurA (AFE_3039) and MurF (AFE_0210), which may be involved in peptidoglycan biosynthesis. It has been reported that antibiotics activate the "cell envelope stress stimulon" to overexpress genes associated with peptidoglycan biosynthesis in order to confront cell damage.[65] It can be speculated then, that an increased synthesis of peptidoglycan by MurA and MurF would prevent the possible cell wall damage caused in *A. ferrooxidans* by the presence of copper. Alternatively, peptidoglycan polymers and other surface proteins may sequester metals in part to prevent their toxic effects over sensitive metabolic processes. This happens especially when cells are forming a biofilm.[66]

3.2.3. Cell Motility and Secretion. Table 1 shows that a Sec-independent protein translocase protein (TatA-1) (AFE_3048) is highly up-regulated when *A. ferrooxidans* grows in the presence of copper. This putative protein would be part of twin-arginine translocation pathway in bacteria and other organisms.[67] Although little is known about this pathway in *A. ferrooxidans*, of 131 periplasmic proteins identified previously by using high resolution linear ion trap-FT MS, 5 were predicted to have Tat export signals.[36] The up-regulation of TatA-1 may indicate that an increased export of this kind of proteins takes place to the membrane or periplasm of the bacterium, as illustrated by the high synthesis of the putative uncharacterized protein AFE_0042 in Table 1. Some of these proteins may have a possible role to confront the periplasmic stress caused by copper.

3.2.4. Signal Transduction Mechanisms. Table 1 shows the up-regulation of AFE_3022, a putative Hpr(Ser) kinase/phosphatase (HprK) forming part of a possible phosphotransferase (PTS) system. This putative system has been recently described to be overexpressed in *A. ferrooxidans* grown in a biofilm attached to the mineral pyrite.[68] This PTS system is fundamental for carbohydrate uptake and therefore to form capsular polysaccharides and other molecules.[69] It is a well-known phenomenon that cell membranes and cell walls of microorganisms present in biofilms sequester toxic metals.[66] In the present case, copper might induce the formation of these polymers to prevent part of the toxic metal entrance to the *A. ferrooxidans* cell. Nevertheless, it is not clear yet whether the putative PTS system of this acidophile functions in the same way described for other bacteria.[35]

Several other proteins showed variable levels of synthesis in *A. ferrooxidans* grown in the presence of copper (Tables 1 and 2). However, no plausible explanation for their changing behavior is currently available. Evidently, further studies will be required to shed light on their possible roles if any, during metal toxicity adaptation.

dx.doi.org/10.1021/pr4009833 | *J. Proteome Res.* 2014, 13, 946−960

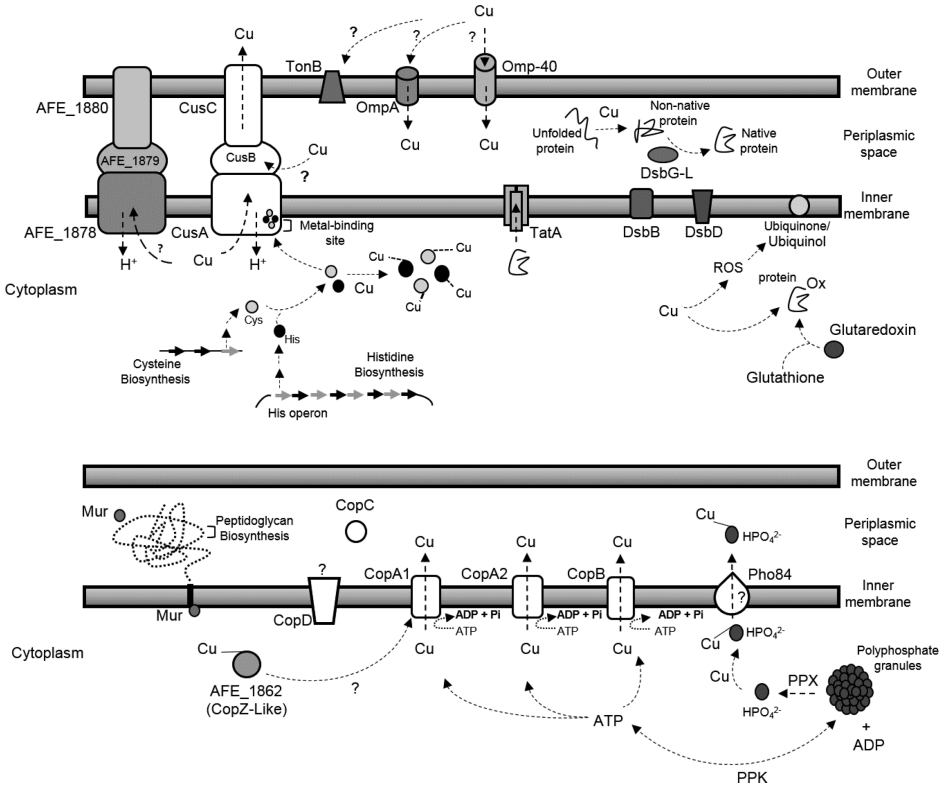


Figure 7. A working scheme showing some selected proteins that change their levels in *A. ferrooxidans* adapted to grow in the presence of copper and their suggested putative functions. Putative proteins shaded in gray were identified in this work. Those in white and the possible role of inorganic polyphosphate (polyP) were identified previously in *A. ferrooxidans* and other microorganisms.[9−12,23,36,70,71] The locations and order in which proteins are illustrated is arbitrary.

3.3. Poorly Characterized

In this category, 20 proteins (11 up-regulated and 9 down-regulated in the presence of copper) were found to be currently annotated as putative uncharacterized proteins (Tables 1 and 2). However, it may be that these proteins are most likely characteristic of *A. ferrooxidans* and may have important functions in metal resistance yet to be defined. The proteomic approach offers an important contribution to the functional annotation for the available genomes of acidophilic biomining microorganisms such as *A. ferrooxidans* for which no genetic systems are easily available to perform functional analysis by homologous recombination and genetic disruption of the genes of interest.

The quantitative proteomics results showed an important number of changes in the levels of proteins from *A. ferrooxidans* due to the presence of copper. A scheme summarizing several of the differentially synthesized proteins some of which may be new copper resistance determinants in *A. ferrooxidans* is shown in Figure 7.

In conclusion, *A. ferrooxidans* would have a wide repertoire of strategies to confront extremely high copper and other metals concentrations in its environment. In addition to previously reported canonical efflux ATPases and RND efflux pumps and their duplications, these are a decrease of copper influx by down-regulation of proteins such as Omp40; increased synthesis of amino acids with high affinity for copper, such as Cysteine and Histidine; an efficient antioxidant defense system; a putative disulfide isomerase DsbG-L system to repair folding damage of periplasmic proteins and an increased synthesis of peptidoglycan to repair the damage caused by copper on the cell envelope. Obviously, these predictions should be experimentally supported in future research.

■ ASSOCIATED CONTENT

Supporting Information

Differences in protein levels and identification of protein spots isolated from 2D-PAGE; primers for qRT-PCR, all quantitation data obtained, Mascot search results. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Carlos A. Jerez, Phone: (56 2) 2 9787376. E-mail: cjerez@uchile.cl.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by grant FONDECYT 1110214 and in part by ICM P-05-001-F project. We also thank CONICYT for a doctoral fellowship to R. Almarcegui and to TIGR for the use of their complete *A. ferrooxidans* ATCC 23270 genome sequence (www.tigr.org/db.shtml). CNB-CSIC Proteomic Facility belongs to ProeoRed-ISCIII.

■ REFERENCES

(1) Das, A.; Modak, J. M.; Natarajan, K. A. Surface chemical studies of *Thiobacillus ferrooxidans* with reference to copper tolerance. *Antonie Van Leeuwenhoek* **1998**, *73*, 215−222.

(2) Watling, H. R. The bioleaching of sulphide minerals with emphasis on copper sulphides-a review. *Hydrometallurgy* **2006**, *84*, 81−108.
(3) Jerez, C. A. Bioleaching and biomining for the industrial recovery of metals. In *Comprehensive Biotechnology*, 2nd ed; Moo-Young, M., Ed.; Elsevier: Boston, MA, 2011, Vol 3 , pp 717−729.
(4) Jerez, C. A. The use of extremophilic microorganisms in industrial recovery of metals. In *Extremophiles: Sustainable Resources and Biotechnological Implications*; Songh, O., Ed/; John Wiley & Sons, Inc.: New York, 2013; pp 319−334.
(5) Baker-Austin, G.; Dopson, M.; Wexler, M.; Sawers, R. G.; Stemmler, A.; Rosen, B. P.; Bond, P. L. Extreme arsenic resistance by the acidophilic archaeon *Ferroplasma acidarmanus* Fer1. *Extremophiles* **2007**, *11*, 425−434.
(6) Franke, S.; Rensing, C. Acidophiles. Mechanisms to tolerate metal and acid toxicity. In: Gerday, C, Glansdorff, N, Eds.; *Physiology and Biochemistry of Extremophiles*; ASM Press: Washington DC, 2007; pp 271−278.
(7) Jerez, C. A. The use of genomics, proteomics and other OMIC technologies for the global understanding of biomining microorganisms. *Hydrometallurgy* **2008**, *94*, 162−169.
(8) Baker-Austin, C.; Dopson, M.; Wexler, M.; Sawers, R. G.; Bond, P. L. Molecular insight into extreme copper resistance in the extremophilic archaeon *'Ferroplasma acidarmanus'* Fer1. *Microbiology* **2005**, *151*, 2637−2646.
(9) Alvarez, S.; Jerez, C. A. Copper ions stimulate polyphosphate degradation and phosphate efflux in *Acidithiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **2004**, *70*, 5177−5182.
(10) Orell, A.; Navarro, C. A.; Arancibia, R.; Mobarec, J. C.; Jerez, C. A. Life in blue: copper resistance mechanisms of bacteria and Archaea used in industrial biomining of minerals. *Biotechnol. Adv.* **2010**, *28*, 839−848.
(11) Orell, A.; Navarro, C. A.; Rivero, M.; Aguilar, J. S.; Jerez, C. A. Inorganic polyphosphates in extremophiles and their possible functions. *Extremophiles* **2012**, *16*, 573−583.
(12) Navarro, C. A.; Orellana, L. H.; Mauriaca, C.; Jerez, C. A. Transcriptional and functional studies of *Acidithiobacillus ferrooxidans* genes related to survival in the presence of copper. *Appl. Environ. Microbiol.* **2009**, *75*, 6102−6109.
(13) Orellana, L. H.; Jerez, C. A. A genomic island provides *Acidithiobacillus ferrooxidans* ATCC 53993 additional copper resistance: a possible competitive advantage. *Appl. Microbiol. Biotechnol.* **2011**, *92*, 761−767.
(14) Salazar, C.; Acosta, M.; Galleguillos, P.; Shmaryahu, A.; Quatrini, R.; Holmes, D. S.; Demergasso, C. Analysis of gene expression in response to copper stress in *Acidithiobacillus ferrooxidans* Strain D2, isolated from a copper bioleaching operation. *Adv. Mater. Res.* **2013**, *825*, 157−161.
(15) Amaro, A. M.; Chamorro, D.; Seeger, R.; Arredondo, R.; Peirano, I.; Jerez, C. A. Effect of external pH perturbations on in vivo protein synthesis by the acidophilic bacterium *Thiobacillus ferrooxidans*. *J. Bacteriol.* **1991**, *173*, 910−915.
(16) Stenberg, F.; Chovanec, P.; Maslen, S. L.; Robinson, C. V.; Ilag, L. L.; Von Heijne, G.; Daley, D. O. Protein complexes of the *Escherichia coli* cell envelope. *J. Biol. Chem.* **2005**, *41*, 34409−34419.
(17) Varela, C.; Mauriaca, C.; Paradela, A.; Albar, J. P.; Jerez, C. A.; Chavez, F. P. New structural and functional defects in polyphosphate deficient bacteria: A cellular and proteomic study. *BMC Microbiol.* **2010**, *10*, 17.
(18) Chevallet, M.; Luche, S.; Rabilloud, T. Silver staining of proteins in polyacrylamide gels. *Nat. Protoc.* **2006**, *1*, 1852−1858.
(19) Darling, A. E.; Mau, B.; Perna, N. T. ProgressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* **2010**, *5*, e11147.
(20) Vera, M.; Pagliai, F.; Guiliani, N.; Jerez, C. A. The chemolithoautotroph *Acidithiobacillus ferrooxidans* can survive under phosphate-limiting conditions by the expression of a C-P lyase operon that allows it to grow on phosphonates. *Appl. Environ. Microbiol.* **2008**, *74*, 1829−1835.
(21) Rozen, S.; Skaletsky, H. J. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* **2000**, *132*, 365−386.
(22) Andersen, C. L.; Jensen, J. J.; Orntoft, T. F. Normalization of real-time quantitative RT-PCR data: a model based variance estimation approach to identify genes suited for normalization applied to bladder and colon-cancer data-sets. *Cancer Res.* **2004**, *64*, 5245−5250.
(23) Guiliani, N.; Jerez, C. A. Molecular cloning, sequencing, and expression of omp-40, the gene coding for the major outer membrane protein from the acidophilic bacterium *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **2000**, *66*, 2318−2324.
(24) Jerez, C. A.; Seeger, M.; Amaro, A. M. Phosphate starvation affects the synthesis of outer membrane proteins in *Thiobacillus ferrooxidans*. *FEMS Microbiol. Lett.* **1992**, *98*, 29−34.
(25) Magnani, D.; Solioz, M. How bacteria handle copper. *Microbiol. Monogr.* **2007**, *6*, 259−285.
(26) Rensing, C.; Grass, G. *Escherichia coli* mechanism of copper homeostasis in a changing environment. *FEMS Microbiol. Rev.* **2003**, *27*, 197−213.
(27) Nikaido, H. Structure and mechanism of RND-type multidrug efflux pumps. *Adv. Enzymol. Relat. Areas Mol. Biol.* **2011**, *77*, 1−60.
(28) Kim, E.-H.; Nies, D. H.; Rensing, C. Switch or funnel: how RND-type transport systems control periplasmic metal homeostasis. *J. Bacteriol.* **2011**, *193*, 2381−2387.
(29) Padilla-Benavides, T.; McCann, C. J.; Argüello, J. M. The mechanism of Cu^+ transport ATPases: interaction with Cu^+ chaperones and the role of transient metal-binding sites. *J. Biol. Chem.* **2013**, *288*, 69−78.
(30) Silva, M.; Ferreira, A.; Rodriguez, M.; Wolff, D. The major *Thiobacillus ferrooxidans* outer membrane protein forms low conductance ion channels in planar lipid bilayers. *FEBS Lett.* **1992**, *296*, 169−173.
(31) Teitzel, G.; Geddie, A.; De Long, S.; Kirisits, M.; Whiteley, M.; Parsek, M. Survival and growth in the presence of elevated copper: Transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. *J. Bacteriol.* **2006**, *188*, 7242−7256.
(32) Sugawara, E.; Nikaido, H. Pore-forming activity of OmpA protein of *Escherichia coli*. *J. Biol. Chem.* **1992**, *2674*, 2507−2511.
(33) Noinaj, N.; Guillier, M.; Barnard, T. J.; Buchanan, S. K. Ton-B dependent transporters: regulation, structure, and function. *Annu. Rev. Microbiol.* **2010**, *64*, 43−60.
(34) Schauer, K.; Rodionov, D. A.; DeReuse, H. New substrates for TonB-dependent transport: do we only see the 'tip of the iceberg'? *Trends Biochem. Sci.* **2008**, *33*, 330−338.
(35) Valdés, J.; Pedroso, I.; Quatrini, R.; Dodson, R. J.; Tettelin, H.; Blake, R.; Eisen, J. A.; Holmes, D. S. *Acidithiobacillus ferrooxidans* metabolism: from genome sequence to industrial applications. *BMC Genomics* **2008**, *9*, 597.
(36) Chi, A.; Valenzuela, L.; Beard, S.; Mackey, A. J.; Shabanowitz, J.; Hunt, D. F.; Jerez, C. A. Periplasmic proteins of the extremophile *Acidithiobacillus ferrooxidans*: a high throughput proteomics analysis. *Mol. Cell. Proteomics* **2007**, *12*, 2239−2251.
(37) Osorio, H.; Martínez, V.; Nieto, P. A.; Holmes, D. S.; Quatrini, R. Microbial iron management mechanisms in extremely acidic environments: comparative genomics evidence for diversity and versatility. *BMC Microbiol.* **2008**, *8*, 203.
(38) Grass, G.; Thakali, K.; Klebba, P. E.; Thieme, D.; Müller, A.; Wildner, G. F.; Rensing, C. Linkage between catecholate siderophores and the multicopper oxidase CueO in *Escherichia coli*. *J. Bacteriol.* **2004**, *186*, 5826−5633.
(39) Chaturvedi, K. S.; Hung, C. S.; Crowley, J. R.; Stapleton, A. E.; Henderson, J. P. The siderophore yersinia bactin binds copper to protect pathogens during infection. *Nat. Chem. Biol.* **2012**, *8*, 731−736.
(40) Appia-Ayme, N.; Guiliani, N.; Ratouchniak, J.; Bonnefoy, V. Characterization of an operon encoding two c-type cytochromes, an aa(3)-type cytochrome oxidase, and rusticyanin in *Thiobacillus ferrooxidans* ATCC 33020. *Appl. Environ. Microbiol.* **1999**, *65*, 4781−4787.
(41) Cook, G. M.; Poole, R. K. Oxidase and periplasmic cytochrome assembly in *Escherichia coli* K-12: CydDC and CcmAB are not required for haem-membrane association. *Microbiology* **2000**, *146*, 527−536.

(42) Jünemann, S. Cytochrome bd terminal oxidase. *Biochim. Biophys. Acta* **1997**, *1321*, 107−127.
(43) VanOrsdel, C. E.; Bhatt, S.; Allen, R. J.; Brenner, E. P.; Hobson, J. J.; Jamil, A.; Haynes, B. M.; Genson, A. M.; Hemm, M. R. The *Escherichia coli* CydX protein is a member of the CydAB cytochrome *bd* oxidase complex and is required for cytochrome *bd* oxidase activity. *J. Bacteriol.* **2013**, *195*, 3640−3650.
(44) Cárdenas, J. P.; Valdés, J.; Quatrini, R.; Duarte, F.; Holmes, D. S. Lessons from the genomes of extremely acidophilic bacteria and archaea with special emphasis on bioleaching microrganisms. *Appl. Microbiol. Biotechnol.* **2010**, *88*, 605−620.
(45) Belfiore, C.; Ordoñez, O. F.; Farías, M. E. Proteomic approach of adaptive response to arsenic stress in *Exiguobacterium* sp. S17, an extremophile strain isolated from a high-altitude Andean Lake stromatolite. *Extremophiles* **2013**, *17*, 421−431.
(46) Soballe, B.; Poole, R. K. Ubiquinone limits oxidative stress in *Escherichia coli*. *Microbiology* **2000**, *146*, 787−796.
(47) Kucera, J.; Bouchal, P.; Cerna, H.; Potesil, D.; Janiczek, O.; Zdrahal, Z.; Mandl, M. Kinetics of anaerobic elemental sulfur oxidation by ferric iron in *Acidithiobacillus ferrooxidans* and protein identification by comparative 2-DE-MS/MS. *Antonie Van Leeuwenhoek* **2012**, *101*, 561−573.
(48) Gale, N. L.; Beck, J. V. Evidence for the Calvin cycle and hexose monophosphate pathway in *Thiobacillus ferrooxidans*. *J. Bacteriol.* **1967**, *94*, 1052−1059.
(49) Esparza, M.; Cárdenas, J. P.; Bowien, B.; Jedlicki, E.; Holmes, D. S. Genes and pathways for CO_2 fixation in the obligate, chemolithoautotrophic acidophile, *Acidithiobacillus ferrooxidans*, carbon fixation, Carbon fixation in *A. ferrooxidans*. *BMC Microbiol.* **2010**, *10*, 229.
(50) Ramírez, P.; Guiliani, N.; Valenzuela, L.; Beard, S.; Jerez, C. A. Differential protein expression during growth of *Acidithiobacillus ferrooxidans* on ferrous iron, sulfur compounds or metal sulfides. *Appl. Environ. Microbiol.* **2004**, *70*, 4491−4498.
(51) Hajduch, M.; Rakwal, R.; Kumar-Awaral, G.; Yonekura, M.; Petrova, A. High resolution two-dimensional electrophoresis separation of proteins from metal stressed rice (*Oryza sativa* L.) leaves: drastic reductions/fragmentation of ribulose 1,5 biphosphate carboxylase/oxygenase and induction of stress related proteins. *Electrophoresis* **2001**, *22*, 2824−2831.
(52) Kung, C. S.; Huang, W. N.; Huang, Y. C.; Yeh, K. C. Proteome survey of copper binding proteins in *Arabidopsis thaliana* by inmobilized metal affinity chromatography and mass spectrometry. *Proteomics* **2006**, *6*, 2746−2758.
(53) Osorio, H.; Mangold, S.; Denis, Y.; Nancucheo, I.; Esparza, M. Anaerobic Sulfur Metabolism Coupled to Dissimilatory Iron Reduction in the Extremophile *Acidithiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **2013**, *79*, 2172−2181.
(54) Alifano, P.; Fani, R.; Lio, P.; Lazcano, A.; Bazzicalupo, M.; Carlomagno, M. S.; Bruni, C. B. Histidine biosynthetic pathway and genes: structure, regulation, and evolution. *Microbiol. Rev.* **1996**, *60*, 44−69.
(55) Pearce, D. A.; Sherman, F. Toxicity of copper, cobalt, and nickel salts is dependent on histidine metabolism in the yeast *Saccharomyces cerevisiae*. *J. Bacteriol.* **1999**, *181*, 4774−4779.
(56) Ingle, R.; Mugford, S.; Rees, J.; Campbell, M.; Smith, A. Constitutively high expression of the histidine biosynthetic pathway contributes to nickel tolerance in hyperaccumulator plants. *Plant Cell* **2005**, *17*, 2089−2106.
(57) Sochor, J.; Zitka, O.; Hynek, O.; Jilkova, E.; Krejcova, L.; Trnkova, L.; Adam, V.; Hubalek, J.; Kynicky, J.; Vrba, R.; Kizek, R. Bio-sensing of Cadmium(II) ions using *Staphylococcus aureus*. *Sensors* **2011**, *11*, 10638−10663.
(58) Fuentes, D. E.; Fuentes, E. L.; Castro, M. E.; Pérez, J. M.; Araya, M. A.; Chasteen, T. G.; Pichuantes, S. E.; Vasquez, C. C. Cysteine metabolism-related genes and bacterial resistance to potassium tellurite. *J. Bacteriol.* **2007**, *189*, 8953−8960.
(59) Lillig, C. H.; Berndt, C.; Holmgren, A. Glutaredoxin systems. *Biochim. Biophys. Acta* **2008**, *1780*, 1304−1317.
(60) Ito, K.; Inaba, K. The disulfide bond formation (Dsb) system. *Curr. Opin. Struct. Biol.* **2008**, *18*, 450−458.
(61) Zhang, C.; Xia, J.; Liu, Y.; He, H.; Qiu, G. The putative thiol-disulphide interchange protein DsbG from *Acidithiobacillus ferrooxidans* has disulphideisomerase activity. *ScienceAsia* **2010**, *36*, 100−104.
(62) Hiniker, A.; Collet, J. F.; Bardwell, J. Copper stress causes an in vivo requirement for the *Escherichia coli* disulfide isomerase DsbC. *J. Biol. Chem.* **2005**, *40*, 33785−33791.
(63) Vollmer, W.; Blanot, D.; De Pedro, M. A. Peptidoglycan structure and architecture. *FEMS Microbiol. Rev.* **2010**, *32*, 149−167.
(64) Barreteau, H.; Kovac, A.; Boniface, A.; Sova, M.; Gobec, S.; Blanot, D. Cytoplasmic steps of peptidoglycan biosynthesis. *FEMS Microbiol. Rev.* **2008**, *32*, 168−207.
(65) Bertsche, U. The polysaccharide peptidoglican and how it is influenced by (antibiotic) stress. In *Bacterial Polysaccharides: Current Innovations and Future Trends*; Ullrich, M., Ed.; Caister Academic Press: Norfolk, U.K., 2009; pp 3−26,
(66) Harrison, J. J.; Ceri, H.; Turner, R. J. Multimetal resistance and tolerance in microbial biofilms. *Nat. Rev. Microbiol.* **2007**, *5*, 928−938.
(67) Fröbel, J.; Rose, P.; Müller, M. Twin-arginine-dependent translocation of folded proteins. *Philos. Trans. R. Soc., B* **2012**, *367*, 1029−1046.
(68) Vera, M.; Krok, B.; Bellenberg, S.; Sand, W.; Poetsch, A. Shotgun proteomics study of early biofilm formation process of *Acidithiobacillus ferrooxidans* ATCC 23270 on pyrite. *Proteomics* **2013**, *13*, 1133−1144.
(69) Deutscher, J.; Francke, C.; Postma, P. W. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 939−1031.
(70) Rao, N. N.; Gómez-García, M. R.; Kornberg, A. Inorganic polyphosphate: essential for growth and survival. *Annu. Rev. Biochem.* **2009**, *78*, 605−647.
(71) Keasling, J. D. Regulation of intracellular toxic metals and other cations by hydrolysis of polyphosphate. *Ann. N.Y. Acad. Sci.* **1997**, *829*, 242−249.