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Global response of *Acidithiobacillus ferrooxidans* ATCC 53993 to high concentrations of copper: A quantitative proteomics approach

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ABSTRACT

Acidithiobacillus ferrooxidans is used in industrial bioleaching of minerals to extract valuable metals. *A. ferrooxidans* strain ATCC 53993 is much more resistant to copper than other strains of this microorganism and it has been proposed that genes present in an exclusive genomic island (GI) of this strain would contribute to its extreme copper tolerance. ICPL (isotope-coded protein labeling) quantitative proteomics was used to study in detail the response of this bacterium to copper. A high overexpression of RND efflux systems and CusF copper chaperones, both present in the genome and the GI of strain ATCC 53993 was found. Also, changes in the levels of the respiratory system proteins such as AcoP and Rus copper binding proteins and several proteins with other predicted functions suggest that numerous metabolic changes are apparently involved in controlling the effects of the toxic metal on this acidophile.

Significance: Using quantitative proteomics we overview the adaptation mechanisms that biomining acidophiles use to stand their harsh environment. The overexpression of several genes present in an exclusive genomic island strongly suggests the importance of the proteins coded in this DNA region in the high tolerance of *A. ferrooxidans* ATCC 53993 to metals.

1. Introduction

The biomining acidophilic bacterium *Acidithiobacillus ferrooxidans* can grow at the high copper concentrations (>100 mM copper sulfate) [1,2] that are usually present in biomining processes [2–4]. Current knowledge indicates that key elements involved in *A. ferrooxidans* copper-resistance are a broad 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 [5–7]. *A. ferrooxidans* ATCC 23270 can survive high copper concentrations by having in its genome at least ten genes that are possibly related to copper homeostasis, such as those coding for CopA efflux ATPases, Cus efflux systems and copper chaperones [8]. *A. ferrooxidans* ATCC 53993 showed a much higher resistance to $CuSO_4$ (>100 mM) than that of strain ATCC 23270 (<25 mM) [1]. Strain ATCC 53993 genome contains the same copper resistance determinants than strain ATCC 23270. The copper resistance determinants shared by the two strains are: three efflux ATPases $CopA1_{Af}$, $CopA2_{Af}$ and $CopB_{Af}$; an RND system: $CusCBA1_{Af}$ and three copper binding chaperones: $CusF1_{Af}$, $CusF2_{Af}$ and $CopC_{Af}$. Strain ATCC 53993 on the other hand has a 160-kb genomic island (GI) in its genome, which is absent in ATCC 23270. This GI contains, among other genes, the following exclusive coper resistance determinants: the efflux ATPase $CopA3_{Af}$, two RND systems $CusCBA2_{Af}$ and $CusCBA3_{Af}$ and the two copper chaperones $CusF3_{Af}$ and $CusF4_{Af}$.

Copper resistance in strain ATCC 53993 could be explained in part by the presence of the additional copper-resistance genes present in its GI. Some of these genes are transcriptionally overexpressed when *A. ferrooxidans* ATCC 53993 is grown in the presence of copper and the coded proteins are functional when expressed in copper-sensitive *Escherichia coli* mutants [1,4].

Due to the current lack of appropriate and reproducible genetic tools in *A. ferrooxidans* it is not possible to study in more detail its copper resistance mechanism. Therefore, a global quantitative proteomic approach was used in the present work to characterize the response of *A. ferrooxidans* strain ATCC 53993 to a high concentration of copper and to further search for new possible copper resistance determinants in this microorganism. In addition to changes in the levels of several proteins in the genome of this strain, several proteins coded only in its GI were highly up-regulated both transcriptionally and translationally when the microorganisms were grown in the presence of copper. Some of these macromolecules apparently have a key role in the adaptation of the microorganisms to their extreme environment, providing it with a much higher copper resistance compared with those strains lacking this genomic element.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

A. ferrooxidans strain ATCC 53993 was grown at 30 °C in liquid 9 K medium containing ferrous sulfate (33.33 g/L) with an initial pH of 1.45 as previously described [9] and in the absence or presence of $CuSO_4$. Initially, cells were adapted to grow in 5 mM copper (a concentration that does not affect its growth) until they reached the stationary phase of growth. These cells were subcultured in the presence of copper by successively growing and transferring them to different increasing $CuSO_4$ concentrations until they were adapted to 40 mM copper.

Once 40 mM concentration was reached, triplicate separate cultures were done. After the cells attained the late exponential growth phase they were collected for their immediate use in different experiments. Bacterial growth was determined by measuring the increase in cell numbers by using an Olympus BX50 optical microscope and a Petroff–Hausser counting chamber.

2.2. Total protein extracts preparation for ICPL analysis

A. ferrooxidans cells grown in the absence or presence of 40 mM $CuSO_4$ were harvested by centrifugation (10,000 × *g* for 2 min). The cell pellets were washed three times with sulfuric acid solution (pH 1.5) by resuspension followed by centrifugation at the same speed and time already mentioned.

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 60 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 total protein concentrations in the cell-free extract was determined as done before [10].

2.3. Sample preparation for ICPL determinations

Samples of total protein extracts from three biological replicates (different independent cultures) were mixed using fifty micrograms of each one to obtain a triplicate representative sample of each experimental condition with a total of 150 μg of protein in each case. 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 as described before [10].

2.4. 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. Further details are as previously described [10].

2.5. 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. Details for gradient elution, flow rate, injection volumes, number of HPCL fractions and second dimension of the 2D-nano LC ESI-MS/MS analysis were as previously reported [10].

2.6. 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). In most cases, an accuracy of ±0.1–0.2 Da was found both for MS and MS/MS spectra. For protein identification, MS/MS spectra (in the form of Mascot generic files) were searched against the *A. ferrooxidans* ATCC 53993 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, UK). Search parameters were as previously described [10]. FDR ≤ 5% for peptide identification were 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.

Qualitative and quantitative analyses were performed by WARP-LC 1.1 (Bruker Daltonics, Bremen, Germany). After peptide identification, the software calculates 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. Protein quantification values based in single (unique) peptides were manually evaluated. Ratios were $\log_2$-transformed and normalized by subtracting the median value.

Only proteins showing $\log_2$ (Cu 40 m/Cu 0 mM) values $R \geq 0.5$ or $R \leq -0.5$ were considered as up- or down-regulated. Protein ratios in the $-0.5 \leq R \leq 0.5$ range ($R = \log_2$ (Cu 40 m/Cu 0 mM)) were considered non-relevant in the context of the biological model studied and their change probably due to artifactual variation (see figure below) associated to the experimental set up used in this case. In addition, to support the consistency of our proteomic data a small and selected subset of proteins considered up or down regulated using this proteomic approach was chosen for further validation using a PCR-based approach.

Proteins changing their levels in the presence of copper were classified according to their functional categories by using the COG (Cluster of Orthologous Groups) database. Those proteins whose ORFs had no associated COGs were classified by using as before [10] the bioinformatics tool COGnitor that allows to assign a COG based on the protein sequence (http://www.ncbi.nlm.nih.gov/COG/old/xognitora.htlm).

2.7. Extraction of total RNA from A. ferrooxidans and cDNA synthesis

To determine the effect of copper on the expression of some genes of interest, cells were adapted in batch cultures to grow in the absence or presence of 40 mM $CuSO_4$ 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 [11], except that TRIzol (Invitrogen) was used for the extraction [1,8]. 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) reverse transcription system, 0.5 μg of random hexamers (Promega) and 3 mM $MgCl_2$ [8].

2.8. Primer design, real-time PCR and cloning of A. ferrooxidans genes

Primers for qRT-PCR were designed using the Primer3 software [12]. After separating PCR products by electrophoresis in a 1% agarose gel (0.5× Tris–acetate–EDTA pH 8.0 buffer), no cross-amplification or non-specific bands were detected. Copper-resistance related gene expression was analyzed by qRT-PCR with the Corbett Rotor Gene 6000

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