Industrial biomining processes to extract copper, gold and other metals involve the use of extremophiles such as the acidophilic Acidithiobacillus ferrooxidans (Bacteria), and the thermoacidophilic Sulfolobus metallicus (Archaea). Together with other extremophiles these microorganisms subsist in habitats where they are exposed to copper concentrations higher than 100 mM. Herein we review the current knowledge on the Cu-resistance mechanisms found in these microorganisms. Recent information suggests that biomining extremophiles respond to extremely high Cu concentrations by using simultaneously all or most of the following key elements: 1) a wide repertoire of Cu-resistance determinants; 2) duplication of some of these Cu-resistance determinants; 3) existence of novel Cu chaperones; 4) a polyP-based Cu-resistance system, and 5) an oxidative stress defense system. Further insight of the biomining community members and their individual response to copper is highly relevant, since this could provide key information to the mining industry. In turn, this information could be used to select the more fit members of the bioleaching community to attain more efficient industrial biomining processes.

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1. Introduction

The use of acidophilic, chemolithoautotrophic microorganisms capable of oxidizing iron and sulfur in industrial processes is a well established biotechnology which enables the recovery of metals from minerals containing copper, gold and uranium (Olson et al., 2003; Rawlings, 2005). Insoluble metal sulfides are oxidized to soluble metal sulfates by the chemical action of ferric iron. Microorganisms play a main role in the reoxidation of the generated ferrous iron back to ferric iron (Rohwerder et al., 2003; Olson et al., 2003; Rawlings, 2005; Watling, 2006).

Many industrial bioleaching operations use mesophilic and thermophilic microorganisms (Lindström et al., 1992; Olson et al., 2003; Rawlings, 2005; Watling, 2006). Biomining is a growing field in the mining sector, since it has distinctive advantages over traditional mining procedures. Perhaps the most important being its economical advantage in the bioleaching of low-grade ores, since biomining makes it cost-effective to leach ores which otherwise would be discarded. Besides, as opposed to roasting and smelting, biomining does not require high amounts of energy nor does it generate harmful
emissions such as sulfur dioxide (Rawlings, 2005). Nevertheless, a major drawback is the risk of acid mine drainage production, which if not properly controlled, pollutes the environment with acid and metals (Olson et al., 2003; Rohwerder et al., 2003).

Microorganisms used in bioleaching are persistently exposed to acid-leaching solutions containing elevated metal concentrations which are toxic to most life. Accordingly and as might be expected, microorganisms that grow in mineral-rich environments are, in most cases, remarkably resistant to a wide range of metal ions (Dopson et al., 2003; Franke and Rensing, 2007). Therefore, microorganisms surviving in acid-leaching environments should possess robust metal resistance mechanisms (Dopson et al., 2003).

Typically, concentrations of Cu in heap or dump leachates are in the range of 2–6 g/l (30–90 mM). Conversely, in agitated tanks, where sulphides concentrations are processed, the concentrations can reach up to 19 g/l (ca. 300 mM) or more (Watkin et al., 2008). Metal tolerance can vary significantly between species and between strains of the same species (Watkin et al., 2008). Given the differences between exhibited metal tolerance and metal concentrations in heap or agitated tanks bioleaching operations, it is important to further understand the mechanisms used by these microorganisms to adapt to and to resist the high concentrations of copper found in their environment.

It has been reported that certain biomining microorganisms, such as *Sulfolobus metallicus*, are greatly affected during tank bioleaching due to high pulp densities and metal toxicity. This in turn, may be the grounds to incomplete metal extraction (Astudillo and Acevedo, 2008; Jones et al., 2009). Activation effects of the mineral particles (Lindström et al., 1993; Edwards et al., 2000). For a recent comprehensive address this matter accordingly (Dopson et al., 2003; Franke and Rensing, 2007; Auernik et al., 2008).

One important point to consider is the difference between biomining bacteria (Hippe 2000; Coram and Rawlings, 2002), in addition, a number of Gram-positive bioleaching bacteria belonging to the genera *Acidimicrobium*, *Ferromicrobium* and *Sulfolobus* have also been described (Clark and Norris, 1996; Hallberg and Johnson, 2001; Schippers, 2007 and references therein) (Table 1). Biomining extremely thermophilic archaeons capable of oxidizing sulfur and iron (II) have been known for many years, and they are mainly from the *Sulfolobus*, *Acidianus*, *Metallosphaera* and *Sulfurisphaera* (Fuchs et al., 1995, 1996; Kurosawa et al., 1998; Norris et al., 1996). Recently, some mesophilic iron (II)-oxidizing archaea belonging to the order *Thermoplasmatales* have been isolated and described: *Ferroplasma acidiphilum* (Golyshina et al., 2000) and *F. acidarmanus* (Edwards et al., 2000). For a recent comprehensive review on microorganisms involved in bioleaching see Schippers, 2007 and references therein.

### Table 1

Copper resistance in microorganisms living in biomining environments.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Copper MIC (mM)</th>
<th>T° optimum (°C)</th>
<th>Oxidative capacity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acidithiobacillus ferrooxidans</em></td>
<td>800</td>
<td>30–35</td>
<td>+</td>
<td>Harvey and Crundwell, 1996</td>
</tr>
<tr>
<td><em>Acidimicrobium ferrooxidans</em> isolate N39-30-03</td>
<td>≥0.786</td>
<td>45–50</td>
<td>+</td>
<td>Watkin et al., 2008</td>
</tr>
<tr>
<td><em>Sulfobacillus thermosulfidooxidans</em> Isolate N19-45-01</td>
<td>786</td>
<td>45–50</td>
<td>+</td>
<td>Watkin et al., 2008</td>
</tr>
<tr>
<td><em>Leptospirillum ferrooxidans</em></td>
<td>393</td>
<td>28–30</td>
<td>–</td>
<td>Hallmann et al., 1993</td>
</tr>
<tr>
<td><em>Ferroplasma acidarmanus</em></td>
<td>312</td>
<td>42</td>
<td>+</td>
<td>Baker-Austin et al., 2005</td>
</tr>
<tr>
<td><em>Sulfobacillus thermosulfidooxidans</em> DSM 9297T</td>
<td>300</td>
<td>45–50</td>
<td>+</td>
<td>Watkin et al., 2008</td>
</tr>
<tr>
<td><em>Sulfobatus metallicus</em></td>
<td>200</td>
<td>65</td>
<td>+</td>
<td>Remonselle et al., 2006</td>
</tr>
<tr>
<td><em>Sulfobacillus montserratensis</em></td>
<td>100</td>
<td>37</td>
<td>+</td>
<td>Schippers, 2007</td>
</tr>
<tr>
<td><em>Acidithiobacillus caldus</em> DSM8587T</td>
<td>24</td>
<td>45</td>
<td>–</td>
<td>Watkin et al., 2008</td>
</tr>
<tr>
<td><em>Thiobacillus prosperus</em></td>
<td>16</td>
<td>33–37</td>
<td>+</td>
<td>Schippers, 2007</td>
</tr>
<tr>
<td><em>Metallosphaera sedula</em></td>
<td>16</td>
<td>75</td>
<td>+</td>
<td>Huber et al., 1989</td>
</tr>
<tr>
<td><em>Acidimicrobium ferrooxidans</em> DSM 10311T</td>
<td>9.4</td>
<td>45–50</td>
<td>+</td>
<td>Watkin et al., 2008</td>
</tr>
<tr>
<td><em>Thiomonas cuprina</em></td>
<td>7.9</td>
<td>30–36</td>
<td>–</td>
<td>Schippers, 2007</td>
</tr>
<tr>
<td><em>Acidithiobacillus thiooxidans</em></td>
<td>ND</td>
<td>28–30</td>
<td>–</td>
<td>Waksman and Joffe, 1922</td>
</tr>
<tr>
<td><em>Leptospirillum ferriphilum</em></td>
<td>ND</td>
<td>30–37</td>
<td>+</td>
<td>Coram and Rawlings, 2002</td>
</tr>
<tr>
<td><em>Acidianus infernus</em></td>
<td>ND</td>
<td>90</td>
<td>+</td>
<td>Segerer et al., 1986</td>
</tr>
</tbody>
</table>

*Archaea, *Bacteria, in columns *, data were taken from Schippers A. (2007), column * references where the MIC values were taken, *sequenced and publically available genome, *genomes for these microorganisms have been sequenced and the data has not been made public, MIC: minimal inhibitory concentration, ND: not determined.
3. General Cu-resistance mechanisms in prokaryotic microorganisms

Copper is an essential element required by all living organisms. Therefore, cells have developed a series of mechanisms to control the levels of free Cu in their compartments. When the concentration of Cu and other metals exceed acceptable levels, they can damage cell membranes as well as nucleic acids structure, enzyme specificity can be altered, and cellular functions can be disrupted in general (Bruins et al., 2000). Therefore, mechanisms of resistance are switched on in order to survive the adverse environment (Magnani and Solioz, 2007; Rensing and Grass, 2003; Silver and Phung, 1996; Teitzel et al., 2006; Waldron and Robinson, 2009).

In Gram-negative bacteria, one of the pathways described for Cu-resistance is the active efflux of the metal from the cytoplasm to the periplasmic space, carried out by ATPases located in the internal membrane of the bacteria. The most studied example of this type of transport is the P-type ATPase CopA from E. coli (Rensing et al., 2000). Additionally, it has also been postulated that some microorganisms are able to pump Cu from the cytoplasm directly to the extracellular space by systems of the RND (resistance nodulation cell division) family of carriers (Franke et al., 2003). The Cus system of E. coli is the best known detoxification organization of this kind (Outton et al., 2001). The capacity of some microorganisms to bind Cu in the periplasmic space has also been reported. The metal is therefore retained by these periplasmic Cu-binding proteins. The Cop system from Pseudomonas syringae pv tomato (Puig et al., 2002) is a well studied case of this type of resistance.

The detoxification systems described here for Gram-negative neutrophilic microorganisms allow them to resist relatively low Cu levels. Although minimal inhibitory concentrations (MIC) values for metals depend on the growth media and methodology used, these values represent a range of tolerance towards the assayed metal for a given microorganism. Interestingly, E. coli has a MIC of 3 mM for Cu (Franke et al., 2003) and P. syringae has a MIC of 8 mM Cu (Puig et al., 2002), which are considered to be low concentrations when compared to Cu concentrations typically found in environments such as mining operations or acid mine drainages. Cu concentrations are one or two orders of magnitude higher in these extreme settings (Table 1). (Dopson et al., 2003). However, widely used biomining microorganisms such as A. ferrooxidans can be adapted to grow in the presence of 800 mM Cu (Harvey and Crundwell, 1996). The archaeon S. metallicus is able to grow in 200 mM Cu (Remonsellez et al., 2006). This enhanced resistance makes these microorganisms suitable to live in such hostile conditions.

4. Cu-resistance in acidophilic biomining microorganisms

In general, some factors have been proposed for acidophiles that would help them resist high external concentrations of metals. One resistance factor is the membrane structure in Archaea, which is different to the structure found in Bacteria, and possesses tetraether-linked monolayer membrane lipids as its main lipid component, making it less sensitive to acid and more impermeable to protons and metals (Baker-Austin and Dopson, 2007; Franke and Rensing, 2007). Nevertheless, this characteristic does not explain the fact that non-biomining acidophilic archaeons (not able to oxidize iron or sulfur or neither) such as S. acidocaldarius (MIC = 1 mM Cu) and S. solfataricus (MIC = 5 mM Cu) are much less resistant to Cu than the biomining archaeons S. metallicus (MIC = 200 mM) or F. acidarmanus (MIC = 312 mM) (Table 1). Supplementary Cu-resistance mechanisms ought to exist in the latter kind of microorganisms that enable them to cope with the high levels of this toxic metal (see below). Moreover, acidophilic bacteria do not possess tetraether-linked monolayer membrane lipids, therefore other factors should be involved in their resistance to metals and acid.

An alternative, yet important contribution to metal resistance in acidophiles appears to be their inside-positive membrane potential (which is opposite to the inside negative of neutrophiles). This seems as an essential adaptation to generate and maintain a large pH gradient (ΔpH), which can reach up to 4 pH units between the exterior and their cytoplasm. The ΔpH generates the proton motive force which is an essential energetic feature for the survival of acidophiles. The large ΔpH would also provide resistance to cation accumulation and it is also believed that it would decrease the required energy to maintain the gradient, since cations would be extruded against their concentration gradient but along their electrogenic gradient (Franke and Rensing, 2007; Baker-Austin and Dopson, 2007).

Overall, the genetic and biochemical mechanisms responsible for metal resistance in acidophilic Bacteria and Archaea are still largely uncharacterized (Baker-Austin and Dopson, 2007; Franke and Rensing, 2007). Thus, it is of great interest to unravel the molecular mechanisms underlying the tolerance and resistance that enable these microorganisms to survive in extreme conditions (Dopson et al., 2003; Rohwerder et al., 2003; Watling, 2006; Jerez, 2008).

Current Cu-resistance studies include, for the most part, Cu tolerance reports (Table 1), (Schippers, 2007). However very few studies related to mechanisms of Cu-resistance in biomining microorganisms have been conducted. In regard of the bioleaching model bacterium A. ferrooxidans, it has been reported that non-identified proteins are expressed on its surface when exposed to copper (Das et al., 1998). In addition, when exposed to this metal, A. ferrooxidans loses extrachromosomal structures, suggesting that if the bacteria possess genes coding for proteins involved in Cu-resistance, they would be present in its genome (Chisholm et al., 1998). Only a small number of genes have been previously identified by RNA arbitrarily primed polymerase chain reaction (RAP-PCR) as being induced or repressed in A. ferrooxidans subjected to Cu. Nevertheless, the role of these genes in the mechanism of Cu-resistance is still unknown, and their expression may be related to indirect metabolic responses to stress (Paulino et al., 2002).

4.1. Efflux ATPases, Cu chaperones and other Cu-resistance determinants in biomining microorganisms

Many heavy metal resistance systems known in neutrophilic bacteria involve either an active efflux or metal ion detoxification through several and diverse transformations (Silver and Phung, 1996). Specifically for Cu, these include intracellular complexation, reduced accumulation and extracellular complexation or sequestration in the periplasm (Rouch et al., 1989; Harwood and Gordon, 1994). But an obvious question is still unresolved: are these systems equivalent and functional in acidophilic biomining extremophiles? The analysis of the genome of A. ferrooxidans ATCC 23270 reveals two ORFs which were suggested as encoding potential Cu-P-type ATPases (Quatrini et al., 2007). Based on their similarities to known genes, these authors proposed that afcopA2 would be most likely involved in Cu efflux and afcopB would be probably involved in Cu import to the cytoplasm. However, the expression of afcopB was increased in A. ferrooxidans by the presence of Cu during growth. Furthermore, the overexpression of afcopB in E. coli also increased the resistance to copper in these bacteria (Navarro et al., 2009). These results strongly suggest that afCopB is an efflux pump rather than an influx transporter.

In addition, by using a more detailed bioinformatic analysis an extra copy of the putative ATPase (afcopA1) was found. Interestingly, these A. ferrooxidans predicted CopA paralogs may contribute to a higher Cu-resistance in A. ferrooxidans compared with neutrophiles such as E. coli which possess just one gene for the CopA protein. AfCopA2 has been recently reported to be expressed in much higher levels than afcopA1 when grown in ferrous iron and in the presence of
Cu as demonstrated by PCR-RFLP, suggesting that \textit{afcopA2} might play a more significant role in Cu homeostasis in \textit{A. ferrooxidans} (Luo et al., 2008). However, it should be noted that the expression of \textit{afcopA1} in \textit{E. coli} also increased its Cu-resistance (Navarro et al., 2009), possibly implying that this protein is also a functional copper determinant in \textit{A. ferrooxidans}.

These ATPases from \textit{A. ferrooxidans} showed several of the conserved characteristic domains and motifs present in these metal transporters (Solioz and Vulpe, 1996; Ward et al., 2008) (Table 2). The heavy metal ATPases are a sub-class of the P-type ATPases called Cpx (Cys-Pro-X)-type ATPases. This name comes from the CPC or CPH transporters (Solioz and Vulpe, 1996; Ward et al., 2008) (Table 2). The conserved characteristic domains and motifs present in these metal transporters are also increased its Cu-resistance (Navarro et al., 2009), possibly implying that this protein is also a functional copper determinant in \textit{A. ferrooxidans}.

After further analysis of the promoter sequences of \textit{copA} and \textit{cueO} from \textit{E. coli}, Outten et al. (2000) found a palindromic region where CueE binds to upregulate the expression of the cue system when this bacterium is exposed to Cu (Rensing and Grass, 2003). An ORF with similarity to \textit{cueO} was not found in \textit{A. ferrooxidans}. On the other hand, in this microorganism an ORF (\textit{afcueO}) coding a protein with 37% identity to the DNA binding domain of \textit{E. coli} CueR was present. However, the nucleotide sequences of the putative promoters present upstream of all the \textit{A. ferrooxidans} ORFs studied did not show the palindromic region present in the \textit{E. coli} promoters, finally suggesting that \textit{A. ferrooxidans} has different regulatory elements. The likelihood of other transcriptional regulators which could possibly control the expression of \textit{A. ferrooxidans} Cu-resistance is expected, but remains to be studied.

Other genes with putative Cu-resistance roles such as those coding for \textit{CopC}, \textit{CopD} and a putative Cus system have also been recently characterized in \textit{A. ferrooxidans}. The genomic contexts of these ORFs showed an organization in possible transcriptional units. The transcriptional expression of most of these ORFs as determined by qRT-PCR was upregulated when \textit{A. ferrooxidans} was exposed to Cu (Navarro et al., 2009). In \textit{P. syringae}, the periplasmic protein \textit{CopC} is a Cu chaperone with two binding sites for the metal (Cha and Cooksey, 1993). In this regard, by using a high throughput proteomic approach \textit{afCopC} was experimentally found in the periplasm of \textit{A. ferrooxidans} grown in the absence of Cu (Chi et al., 2007). The prospective mechanism for this protein is still unknown in \textit{A. ferrooxidans}.

A structural model of \textit{CopC} from \textit{P. syringae} is compared with that obtained for \textit{afCopC} (Fig. 1A). In general, there is a well conserved structure between the two proteins. \textit{CopC} from \textit{P. syringae} contains two Cu-binding sites: one specific for binding Cu (I) and a second one, specific for Cu (II). However, \textit{afCopC} showed only one conserved site for Cu (II). The expected site for Cu (I) does not show the amino acids present in the \textit{P. syringae} protein. Obviously, further experimental evidence would be required to demonstrate the possible existence of a different second copper binding site in \textit{afCopC}.

\textit{A. ferrooxidans} contained a putative operon which included the genes \textit{afcusCBA} which would encode for an RND-like Cu-exporter system (Navarro et al., 2009). On the other hand, \textit{E. coli} has an operon containing in addition of \textit{cusCBA}, a \textit{cusF} gene and a two-component system that regulates the expression of the system (Franke et al., 2003). \textit{CusF} from \textit{E. coli} is a periplasmic protein containing one binding site for Cu. Once the metal is bound, it has been proposed that \textit{CusF} delivers the Cu ion to the Cus system for subsequent efflux to the extracellular medium (Franke et al., 2003). Recent crystal structures of \textit{CusF} from \textit{E. coli} revealed an intriguing Cu-binding site in the motif \textit{HXXXXXXXXWXWXXMXXF} (Loftin et al., 2005) that includes tryptophan. The close proximity of this amino acid to Cu suggested an unusual cation-n interaction between Cu (I) and the aromatic ring of tryptophan (Xue et al., 2008). \textit{A. ferrooxidans} possesses an ORF coding for a protein with ~25% identity to \textit{CusF} from \textit{E. coli} but with a different genomic organization since it is located distantly from \textit{afcusCBA} and divergent from \textit{afcopA2} (Navarro et al., 2009). The amino acid sequence of the putative \textit{afCusF} showed one possible Cu-binding site which differs from that in \textit{E. coli} only in the presence of a methionine instead of the histidine (\textit{MXXXXXXXWXWXXMXXF}) and a signal peptide, suggesting that this protein is also exported, most likely to the periplasmic space. Furthermore, if this putative Cu-binding site was functional in \textit{A. ferrooxidans}, one could predict that \textit{CusF} from the acidophilic bacterium not only contains this newly described and unprecedented type of Cu-binding site but also that it would bind Cu(I) in the periplasm.

The structural models for both \textit{E. coli} and \textit{A. ferrooxidans} \textit{CusF} proteins are compared (Fig. 1B). Clearly, there is not only a strong structural similarity between both proteins, but also a high structural conservation for the Cu-binding site in spite of the replacement of His by Met in the \textit{A. ferrooxidans} protein. It is interesting to speculate that this apparently minor change of a His for a Met in \textit{afCusF} could be an adaptation of the putative Cu chaperone to the acidic environment (pH 2.5) present at the periplasm of \textit{A. ferrooxidans}. \textit{CusF} from \textit{E. coli} functions at around pH 7, a pH value where His would be mostly neutral. If \textit{afCusF} also had a His at the same equivalent position of its Cu-binding site, this amino acid would be protonated at pH 2.5. This additional positive charge would favour the interaction of His with the cation, making it more difficult for \textit{afCusF} to deliver its bound Cu to the putative

---

**Table 2**

<table>
<thead>
<tr>
<th>Domain</th>
<th>MBD Metal Binding</th>
<th>Phosphatase Domain</th>
<th>Translocation Domain</th>
<th>Conserved HP Motif</th>
<th>Conserved GgGgG/A Motif</th>
<th>TCDN Motif</th>
<th>GdGdNdXp Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>CASC...CASC</td>
<td>TGEQ</td>
<td>CPCALGLA</td>
<td>FDRTLT</td>
<td>SSSPL</td>
<td>GLOCVG</td>
<td>TCDN</td>
</tr>
<tr>
<td>E. hiroe</td>
<td>CASC..CASC</td>
<td>TGEQ</td>
<td>CPCALGLA</td>
<td>FDRTLT</td>
<td>SSSPL</td>
<td>GLOCVG</td>
<td>TCDN</td>
</tr>
<tr>
<td>E. hiroe</td>
<td>no</td>
<td>TGES</td>
<td>CPCALGLA</td>
<td>FDRTLT</td>
<td>SSSPL</td>
<td>GLOCVG</td>
<td>TCDN</td>
</tr>
<tr>
<td>A. ferrooxidans</td>
<td>no</td>
<td>TGES</td>
<td>CPCALGLA</td>
<td>FDRTLT</td>
<td>SSSPL</td>
<td>GLOCVG</td>
<td>TCDN</td>
</tr>
<tr>
<td>A. ferrooxidans</td>
<td>no</td>
<td>TGES</td>
<td>CPCALGLA</td>
<td>FDRTLT</td>
<td>SSSPL</td>
<td>GLOCVG</td>
<td>TCDN</td>
</tr>
</tbody>
</table>

Data from neutrophilic bacteria were taken from Toyoshima et al., 2000; Solioz and Vulpe, 1996; Tottey et al., 2001. Data from \textit{A. ferrooxidans} were from Navarro et al., 2009; \textit{S. metallicus}, this work; \textit{S. sulfidiscus} from Ettena et al., 2006; \textit{M. sedula} from Auerink et al., 2007.
afCuSBA efflux system. The replacement of His by Met in the acidophilic bacterium would avoid this situation. Obviously, further experimental work is needed to support this proposal.

Besides, the genome of *A. ferrooxidans* contains other ORFs with the unusual afCuS-type of Cu-binding motifs, suggesting that they may constitute novel extra Cu-resistance determinants in this bacterium.

Regarding the potential cus system from *A. ferrooxidans*, afcusA was upregulated with increasing Cu concentrations, being almost undetectable in the absence of the metal. The same kind of expression pattern was seen for afcusF (Navarro et al., 2009). This phenomenon seems highly relevant, since the cus operon in *E. coli* is induced at high Cu concentrations (i.e. close to its MIC for this metal). Under such conditions, *E. coli* could avoid expensive Cu to the exterior of the cell through the cus complex, avoiding Cu toxicity through its transport to the periplasm, and would also make use of the generated proton motive force in the meantime. Conversely, ΔpH is intrinsically linked to cellular bioenergetics and can be used to generate ATP, which can also be employed if further required for Cu efflux and detoxification. That said, it is of relevance to highlight the fact that the crystal structure of CuSb from *E. coli* was reported recently. These results provide direct evidence that this protein interacts by recognizing and extruding copper as a substrate (Su et al., 2009). Whether afCuSb has a similar structure to its *E. coli* counterpart or not, remains to be unravelled.

Currently there is no efficient and reproducible methodology for the generation of knock-outs of the Cu-resistance genes in most acidophiles. Nevertheless, all *A. ferrooxidans* putative Cu-resistance determinants except for afcopD, conferred a much higher Cu-resistance when expressed in *E. coli* as opposed to the wild type strain, strongly suggesting that they form part of a functionally active mechanism for Cu-resistance in *A. ferrooxidans* (Navarro et al., 2009).

Furthermore, the genomic sequence of *A. ferrooxidans* ATCC 53993 has recently been annotated (http://www.jgi.doe.gov/). This strain contains all the Cu-resistance genes from *A. ferrooxidans* ATCC 23270 that have been experimentally confirmed as being expressed in the presence of Cu. These ORFs are 100% identical to their corresponding DNA sequences. However, *A. ferrooxidans* ATCC 53993 contains several additional putative Cu-resistance determinants such as Lferr_0167, a putative Cu-ATPase and a putative cus system where four ORFs have been described (Lferr_0170 to Lferr_0172 and Lferr_0174). These putative genes form a cluster in a DNA region or genomic island that encodes several different metal resistance ORFs and which is absent in the genome of the *A. ferrooxidans* strain ATCC 23270. It is therefore possible that not only gene duplications but also horizontal gene transfers between biomining microorganisms are key elements to supplementary metal resistance in these extremophiles. When comparing the capacity to grow in the presence of copper of these two *A. ferrooxidans* strains, *A. ferrooxidans* ATCC 53993 had a much higher Cu-resistance than the ATCC 23270 strain (results not shown). This difference may be most likely explained by the fact that additional metal resistance genes are found in the recently sequenced strain. In conclusion, the grounds for resistance to copper of two strains of the same microorganism could underlie slight differences in their genomes, which may lead to differences in their adaptability capacities in a mineral environment. This could also lead to differences in the overall bioleaching capacities of genetically similar organisms.

Related to archaeal Cu-resistance mechanisms, metal efflux pumps have been identified in the sequenced genomes of some members of the *Archaea* domain (Pedone et al., 2004). A Cu-resistance (*cop*) loci has been described in *Archaea*, which includes genes encoding a new type of archaeal transcriptional regulator (*CopT*), a putative metal-binding chaperone (*CopM*) and a putative Cu-transporting P-type
ATPase (CopA) (Ettema et al., 2003). Recently, the same Cu-resistance mechanism was described in Sulfolobus solfataricus P2 and Ferrophosma acidarmanus (Baker-Austin et al., 2005; Ettema et al., 2006). In both microorganisms, the putative metal chaperones and the ATPase are co-transcribed and their transcriptional levels increase significantly in response to Cu2+ ions exposure, suggesting that the transport system is operating for Cu efflux (Baker-Austin et al., 2005; Ettema et al., 2006). Additionally, it has been demonstrated in S. solfataricus that CopT binds to multiple sites in the promoter region of copMA and that Cu modulates the binding of the latter in a negative way. Recently, Villafane et al. (2009) have described the expression of the cluster of genes copR/T/A (copTMA in S. solfataricus P2) from S. solfataricus strain 98/2. Their findings showed that the whole operon was co-transcribed at low levels from the copT promoter under all conditions, whereas increased transcription from the copTA promoter took place in the presence of Cu excess (Villafane et al., 2009). The authors proposed a model for Cu homeostasis in Sulfolobus which relies on Cu efflux and sequestration.

In silico studies have further identified a CPX-ATPase which most likely mediates the efflux of heavy metal cations in the biomining archaeon Metallosphaera sedula (Auernik et al., 2007). This putative protein has significant identity to a P-type ATPase in S. solfataricus (CopA) (Table 2) which has been proposed to be implicated in CuII, and possibly, in cadmium efflux (Ettema et al., 2006). These authors also found that the metal efflux process could involve a putative metallochaperone (CopM) whose gene overlaps 32-bp with copA. In the case of M. sedula, the copMA overlap is limited to only 10 bp, and the location of copT (on the opposite strand) makes the gene organization reminiscent of versions present in S. tokodaii and S. acidocaldarius genomes (Auernik et al., 2007).

We have recently demonstrated the presence of duplicated putative genes encoding for Cu-ATPases (copA1 and copA2) (Table 2) and two putative genes for metallochaperones (copM1 and copM2) in the genomic DNA from S. metallicus. The two Cu-ATPases are expressed when the archaeon is grown either in the presence of Cu or using chloropyrite (CuFeS2) as oxidizable substrates (results not shown).

4.2. Oxidative stress response generated by metals in bioleaching microorganisms

Copper can be a toxic element that induces an oxidative stress response when found in higher than homeostatic levels inside the cells. Whole genome differential transcriptional response to Cu and other heavy metals has been studied in gram negative bacteria such as E. coli (Imlay, 2008 and references therein) and P. aeruginosa (Teitzel et al., 2006). Several genes have been described as essential for the oxidative stress response. A clear example is E. coli, which possess the transcriptional activator OxyR and the two-stage SoxRS system which enables these microorganisms to defend themselves against peroxides and superoxide, respectively. Stress response to copper and iron has barely been studied in biomining microorganisms and it is an important field of study given the high concentrations of these metals these bacteria are exposed to in a bioleaching environment.

There are several genes in non-acidophilic microorganisms that have been previously described as induced by oxidative stress (Imlay, 2008 and references therein). We searched the whole genomic sequence of A. ferrooxidans ATCC 23270 for the presence of putative oxidative stress orthologous genes. These genes were used in the development of a DNA macroarray which further allowed the study of the transcriptional response when A. ferrooxidans was exposed to Cu. When grown in the presence of CuSO4, most of the A. ferrooxidans putative oxidative stress genes increased their expression levels compared to those in bacteria grown in a non-copper containing mineral alone such as pyrite (Fig. 2). The general greater change in gene expression observed when cells were grown in a chloropyrite concentrate compared with that in a pyrite concentrate in the presence of 15 mM Cu initially added does not have an obvious reason. Although these experiments are preliminary, one possible speculation would be that in the case of pyrite in the presence of 15 mM Cu, the bacterial cells would be shocked by high Cu concentration from the beginning of growth, becoming adapted to the metal later when they reach the initial stationary phase where the RNA was obtained for its analysis. Thus, as seen for other microorganisms, a lower oxidative stress response would be expected for adapted cells (Teitzel et al., 2006).

gshB is a gene known to take part in the synthesis of glutathione, while gor is essential in the redox process of glutathione. Glutathione is an antioxidant found in both eukaryotes and prokaryotes that protects the cell from reactive oxygen species (ROS), such as free radicals and peroxide, when its reductive environment is unbalanced. The elevated expression of glutathione synthetase (gshB) and glutathione reductase (gor) genes when bacteria are grown in Cu is a direct response to oxidative stress occurring in the cell. Glutathione is usually found in its reduced state as GSH. However, when exposed to unstable ROS, the glutathione molecule can donate a reductive equivalent, stabilizing ROS. In this process glutathione itself becomes reactive, but given the large concentration of glutathione within the cell, it interacts with a second reactive glutathione forming glutathione disulfide (GSSG). GSSG is rapidly transformed into functional GSH by GshB (Carmel-Harel and Storz, 2000; Imlay, 2008). In agreement with the expression results (Fig. 2), this kind of mechanism should also be a functional one in A. ferrooxidans.

Superoxide dismutase (sod) and alkylhydroperoxidiase (ahpC) were other genes from A. ferrooxidans whose expression greatly augmented in the presence of Cu. Sod catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (Cabiscol et al., 2000). The elevated expression of superoxide dismutase (sod) in A. ferrooxidans (Fig. 2) also supports the fact that copper induces oxidative stress in this biomining microorganism given that both of these proteins are part of the antioxidant defense system found in bacteria.

Sigma factor 32 or heat shock sigma factor is a transcriptional factor known to be highly induced when cells are exposed to oxidative damage, since it activates the transcription of other genes involved in
the stress response as well (Farr and Kogoma, 1991). As expected, the equivalent factor from \textit{A. ferrooxidans} (AFE\textsubscript{1616}), also greatly increased its expression levels in the presence of Cu (Fig. 2).

The mRNA levels of GroES from \textit{A. ferrooxidans} were also increased in the presence of Cu. This was an expected finding, since this chaperone is induced in all microbial cells under a variety of stressing conditions, including oxidative stress. GroEL and GroES have also been described previously as proteins induced by temperature and pH stress in \textit{A. ferrooxidans} (Jerez et al., 1988).

In the non-biomining acidophilic archaean \textit{S. solfataricus}, strain MT4, several proteins largely endowed with the ability to recover from oxidative stress were upregulated when the microorganism was exposed to nickel (Salzano et al., 2007). Furthermore, peroxiredoxins, which are ubiquitous enzymes that are part of the oxidative stress defense system have recently been described in the same microorganism (Limauro et al., 2008). Very recently a ferritin-like antioxidant protein (DPSL) was described in \textit{S. solfataricus} P2 as the most highly regulated species of mRNA and protein in cells of the archaeon subjected to oxidative stress induced by hydrogen peroxide. Furthermore, DPSL together with superoxide dismutase and peroxiredoxin were shown to interact and likely form a novel supramolecular complex for mitigating oxidative damage (Maaty et al., 2009). On the other hand, studies related with oxidative stress response in the presence of Cu have not been reported for biomining microorganisms. Only in the case of \textit{S. metallicus}, some preliminary evidence also suggests the generation of reactive oxygen species (ROS) in slurry of sulfide concentrates (Jones et al., 2009).

The results reviewed in this section clearly indicate that biomining microorganisms growing in Cu-containing minerals are subjected to oxidative stress. This stress response may be important to monitor during industrial bioleaching operations to assess the degree of toxicity generated by the metal ions and its effect on the efficiency of the bioleaching process.

\section*{4.3. Inorganic polyphosphate (polyP)-based Cu-resistance mechanism}

The presence in acidophiles of genes with similarity to most of the Cu-resistance determinants contained in neutrophilic microorganisms does not completely explain the much higher metal resistance of the former acidophiles. As already mentioned, the presence of extra copies of these genes may give them an additional capacity to better resist the metal. Nevertheless, it is possible that multiple systems may contribute simultaneously to provide synergistic Cu-resistance. One of the possible additional mechanisms proposed for metal resistance is the sequestration of metal cations with long polymers of inorganic polyphosphate (polyP) (Kornberg et al., 1999). PolyP is a linear polymer of hundreds of orthophosphate residues linked by phosphoanhydride bonds. Several physiological functions have been attributed to polyP in addition to being a reservoir of phosphate, such as substitute for ATP, source of ATP, chelator of metals and adaptation to stress conditions in the cell (Seufferheld et al., 2008; Rao et al., 2009). The main enzyme involved in the biosynthesis of polyP is the polyphosphate kinase (PPK) that catalyzes the reversible conversion of the terminal phosphate of ATP into polyP (Kornberg et al., 1999). On the other hand, an exopolyphosphatase (PPX) is known to hydrolyze polyP liberating inorganic phosphate (Pi) (Kornberg et al., 1999). These enzymes have been purified from \textit{E. coli} and their genes have been identified in several bacteria, including \textit{A. ferrooxidans} (Vera et al., 2003). These genes show a relatively high degree of sequence conservation (Tzeng and Kornberg, 1998; Cardona et al., 2002).

Several biomining microorganisms have been shown to accumulate electron-dense granules (Fig. 3) composed of polyP as seen in \textit{A. ferrooxidans} and \textit{S. metallicus} (Alvarez and Jerez, 2004; Remonsellez et al., 2006). The presence of polyP in the granules was determined by the electron energy loss spectroscopy (EELS) procedure and quantified by using specific enzymatic methods in \textit{S. acidocaldarius}, \textit{S. solfataricus}, \textit{Acidithiobacillus ferrooxidans}, \textit{A. ferrooxidans}, \textit{A. thiooxidans}, and \textit{A. caldus}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig_3.png}
\caption{Presence of electron-dense granules of polyP in biomining Bacteria and Archaea. Transmission electron microscopy images of unstained and unfixed cells were examined directly for the presence of electron-dense granules. Strains used were \textit{Acidithiobacillus ferrooxidans} ATCC 23270, \textit{Acidithiobacillus thiooxidans} DSM 9463, \textit{Acidithiobacillus caldus} ATCC 51756, \textit{Sulfolobus metallicus} DSM 6482, \textit{Metallosphaera sedula} DSM 5348\textsuperscript{T} and \textit{S. solfataricus} DSM 1616, a non-biomining archaean. Arrows indicate one of the electron-dense bodies (polyP) that all microorganisms shown accumulate, except for \textit{S. solfataricus} DSM 1616.}
\end{figure}
metallicus and S. solfataricus (Remonsellez et al., 2006). All three microorganisms synthesized polyP during growth, but only S. metallicus greatly accumulated polyP granules (Fig. 3). The differences in the capacity to accumulate polyP between these archaea may reflect adaptive responses to their natural environment. Thus, S. metallicus that synthesizes 180 nmol of polyP/mg of protein was able to grow and tolerate up to 200 mM copper sulfate. On the other hand, S. solfataricus (20 nmol of polyP/mg of protein) which does not or barely shows polyP granules (Fig. 3) could not grow in or resist more than 1–5 mM copper sulfate, suggesting an interesting relationship between Cu-resistance and polyP levels found intracellularly.

PolyP granules are also present in A. thiooxidans, M. sedula and A. caldus (Fig. 3). Many other biomining microorganisms may also contain this polymer, but it remains to be demonstrated. Based on this characteristic, a polyP-dependent system for Cu-resistance has been proposed for A. ferrooxidans (Alvarez and Jerez, 2004) and S. metallicus (Remonsellez et al., 2006). Although this proposed mechanism for metal resistance needs to be proven, it may be eventually functional in all polyP-accumulating biomining microorganisms. In the presence of Cu, A. ferrooxidans and S. metallicus cells showed a rapid decrease in polyP levels with a concomitant increase in exopolypophosphatase activity and a stimulation of phosphate efflux. Copper in the μM range greatly stimulated exopolypophosphatase activity in cell-free extracts from both A. ferrooxidans and S. metallicus. In this system polyP was most likely degraded by exopolypophosphatase (PPX) to monomers of inorganic phosphate. These in turn would bind the metal in the periplasmic space (cytoplasm of the microorganism and the metal-phosphate complexes inorganic phosphate. These in turn would bind the metal in the periplasmic space (cytoplasm of the microorganism and the metal-phosphate complexes only in acidic conditions (Fristedt et al., 1999).

Finally, a working model summarizing most of the information reviewed here is presented (Fig. 4). When external Cu concentration increases, all of the Cu-resistance determinants from A. ferrooxidans are expressed in higher levels to eliminate Cu from the periplasm or cytoplasm of the cells (Fig. 4A). This requires high levels of ATP to activate the metal efflux ATPases and the ATPases involved in the removal of protons generated by the cus system to avoid cytoplasmic acidification. The concomitant decrease of polyP in the presence of Cu may be the result of its hydrolysis by PPX to remove Cu-phosphate complexes formed. PolyP is synthesized by PPX in A. ferrooxidans or other bacteria (or by a yet unknown equivalent archaeal enzyme) by using ATP (Fig. 4A). However, in excess of ADP generated by the use of cellular ATP, the reverse reaction of PPX synthesizes more ATP from polyP. In this way, the reserve polyP would also be supplying energy to the metal detoxifying systems. In this regard, a eukaryotic Ca(II)-ATPase has been proposed to use polyP instead of ATP (Reusch et al., 1997).

It is known that a recycling mechanism operating in the efflux of compounds such as lactate in symport with protons can generate a proton motive force (van Veen et al., 1994). Such a mechanism may function in the utilization of polyP (Kornberg et al., 1999). The efflux of a protonated metal chelate of Pi released from polyP creates a proton motive force that may be coupled to the synthesis of ATP (Fig. 4). With respect to the polyP-dependent mechanism proposed for Cu-detoxification in Sulfolobales (Fig. 4B), an ORF with similarity to the PPX previously characterized in S. solfataricus (Cardona et al., 2002) has been reported in M. sedula. Furthermore, as with other acidophiles (Alvarez and Jerez, 2004; Remonsellez et al., 2006), when using the S.
copper-oxidizing as PMs, and the top Pho84-like sequence in A. ferrooxidans as queries, four hits with similarities of 30 to 32% to the major facilitator superfamily of subgroup transporters were found in M. sedula (Auernik et al., 2007). Although it should be confirmed experimentally, the presence of these putative genes and abundant polyP granules (Fig. 3) also suggest the possible existence of a polyP-based Cu-resistance system in M. sedula.

5. Perspective

World copper production has increased steadily in the last twenty years, reaching close to 20 Mt per year. About 20% of that copper is actually produced by hydrometallurgy (Wattling, 2006). Bioleaching is an important part of this production. Although the world demand for copper is growing, mining and metal industries are subjected to boom-and-bust cycles and are confronted with several technical and commercial challenges. Since hydrometallurgy for mine and production will continue to play an important role in mining industries (Brierley, 2009), new opportunities for improved processes will arise. Amongst these, the search for new microorganisms with better capabilities to dissolve minerals at higher temperatures and with higher metal tolerances will be an important area of research.

The key elements in Cu-resistance in biomining extremophiles reviewed here appear to be a wide repertoire of known Cu-resistance determinants; the duplication of many of these Cu-resistance determinants; 3) the presence of novel Cu chaperones; an abundant reserve of PolyP to be used in a polyP-based Cu-resistance system and a defensive response to oxidative stress.

Nevertheless, Cu regulation in these extremophiles may be a more complex process than is currently envisaged. Recently, a coordinated homeostatic response to both iron and Cu mediated by the transcriptional regulator Fur has been suggested for A. ferrooxidans (Quatrini et al., 2007). In future studies, it would be interesting to figure out the relationship between the biomining microorganisms Cu-resistance determinants and their possible polyP-based Cu-detoxification mechanisms.

The continued study of heavy metal toxicity and resistance mechanisms in biomining microorganisms is important, especially in hyperthermophilic biomining archaea such as M. sedula in which tolerance to Cu may require improvement to make it competitive with microorganisms such as S. metallicus and others (Auernik et al., 2008).

The finding of novel putative Cu-resistance determinants in the studied microorganisms will help in the functional annotation of the genes coding for Cu-resistance determinants in the available genomic databases of biomining extremophiles. Furthermore, the characterization of metal resistance determinants will contribute not only to understand these microorganisms’ environmental adaptations but also to have means to monitor their responses to toxic elements. This close type of control of the microbial consortia may eventually improve the biomining processes. The use of different selected or genetically engineered strains with the highest metal resistance yields could play a key role for industrial biomining in the next decades.

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References


From MECESUP to A.O. We thank F.P. Chavez for helpful Acknowledgements could play a key role for industrial biomining in the next decades. This work was supported by grant Fondecyt 1070896 and in part by ICM P-05–001-F project and doctoral fellowships from CONICYT to C.N. and from MECSUP to A.O. We thank F.P. Chavez for helpful discussions and R. Kelly and P. Blum for their kind gift of M. sedula DSM 5348T; N. Giuliani for A. thiooxidans DSM 9463 and A. caldus ATCC 51756. We also thank TIGR for the use of their complete A. ferrooxidans ATCC 23270 genome sequence (www.tigr.org/db/htmt) and the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) for the A. ferrooxidans ATCC 53993 genome sequence.