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Life in blue: Copper resistance mechanisms of bacteria and Archaea used in industrial biomining of minerals

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ABSTRACT

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.

Contents

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; Watling, 2006). 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

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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 concentrates 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).

Nonetheless, in tank bioleaching decreasing the size of the milled concentrate particles has shown improved leaching performance due to increased mineral surface area and mechanical activation effects of the mineral particles (Lindström et al., 1993; Norris et al., 2000; Jones et al., 2009). Furthermore in the process of bioleaching of a mineral such as chalcopyrite iron (II) and copper ions are produced. These two metals are known to generate reactive oxygen species (ROS) in microorganisms through the Fenton reaction (Imlay, 2008 and references therein). Therefore, it is important to identify the extent to which this type of stress together with metal resistance and adaptation may affect the efficiency of industrial biomining operations. It has been pointed out that resistance mechanisms and metal ion fluxes clearly play an important role in defining the capacity of biomining microorganisms to bioleach iron and copper-bearing minerals (Barreto et al., 2003). The recent complete available sequences and ongoing/draft sequences of microbes involved in bioleaching together with different OMICS approaches will greatly help in understanding the molecular and genetic adaptations of these microorganisms in biomining operations and to predict some of the possible interactions between the members of the consortia and their extreme environment (Valenzuela et al., 2006; Jerez, 2008; Siezen and Wilson, 2009 and references therein).

This review will have a special focus in recent advances regarding the study of Cu-resistance mechanisms found in biomining extremophiles. Although knowledge about the resistance to other metals of microorganisms of this kind is also scarce, some recent reviews address this matter accordingly (Dopson et al., 2003; Franke and Rensing, 2007; Auernik et al., 2008).

2. The acidophilic microbial community used in biomining

Population studies demonstrate that at least 11 prokaryotic divisions can survive in acid mine drainage sites (Baker and Banfield, 2003). The *Acidithiobacillus* genus is the most studied of its kind (Kelly and Wood, 2000). *Acidithiobacillus ferrooxidans* and *A. thiooxidans* together with the moderate thermophile *A. caldus* are acidophilic mesophiles, which belong to the Gram-negative γ-proteobacteria (Hallberg and Johnson, 2001; Rohwerder et al., 2003). *A. ferrooxidans* has been considered as a model biomining microorganism (Olson et al., 2003; Rawlings, 2005).

The members of the genus *Leptospirillum* are also considered to be important biomining bacteria (Hippe 2000; Coram and Rawlings, 2002). In addition, a number of Gram-positive bioleaching bacteria belonging to the genera *Acidimicrobium*, *Ferromicrobium* and *Sulfobacillus* 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 *Thermoplasmales* have been isolated and described: *Ferroplasma acidiphilium* (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.

Microorganism	Copper MIC (mM)	T° optimum (°C)[c]	Oxidative capacity[d]		Reference[e]
			Fe^{2+}	S^0	
Acidithiobacillus ferrooxidans [b*]	800	30–35	+	+	Harvey and Crundwell, 1996
Acidimicrobium ferrooxidans[b] islolate N39-30-03	≥786	45–50	+	+	Watkin et al., 2008
Sulfobacillus thermosulfidooxidans Isolate N19-45-01[b]	786	45–50	+	+	Watkin et al., 2008
Leptospirillum ferrooxidans[b**]	393	28–30	+	−	Hallmann et al., 1993
Ferroplasma acidarmanus[a**]	312	42	+	−	Baker-Austin et al., 2005
Sulfobacillus thermosulfidooxidans DSM 9293[Tb]	300	45–50	+	+	Watkin et al., 2008
Sulfolobus metallicus[a]	200	65	+	+	Remonsellez et al., 2006
Sulfobacillus montserratensis[b]	100	37	+	+	Schippers, 2007
Acidithiobacillus caldus DSM8584[Tb**]	24	45	−	+	Watkin et al., 2008
Thiobacillus prosperus[b]	16	33–37	+	+	Schippers, 2007
Metallosphaera sedula[a*]	16	75	+	+	Huber et al., 1989
Acidimicrobium ferrooxidans DSM 10331[Tb]	9.4	45–50	+	+	Watkin et al., 2008
Thiomonas cuprina[b]	7.9	30–36	−	+	Schippers, 2007
Acidithiobacillus thiooxidans[b**]	ND	28–30	−	+	Waksman and Joffe, 1922
Leptospirillum ferriphilum[b**]	ND	30–37	+	−	Coram and Rawlings, 2002
Acidianus infernus[a]	ND	~90	+	+	Segerer et al., 1986

[a]*Archaea*, [b]*Bacteria*, in columns [cd] data were taken from Schippers A. (2007), column [e]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 (*r*esistance *n*odulation cell *d*ivision) family of carriers (Franke et al., 2003). The Cus system of *E. coli* is the best known detoxification organization of this kind (Outten 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). Especifically 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 *afcopA2* might play a more significant role in Cu homeostasis in *A. ferrooxidans* (Luo et al., 2008). However, it should be noted that the expression of *afcopA1* in *E. coli* also increased its Cu-resistance (Navarro et al., 2009), possibly implying that this protein is also a functional copper determinant in *A. ferrooxidans*.

These ATPases from *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 (sometimes also SPC) motif located in the middle of a predicted membrane helix in the most conserved core structure of these ATPases. The amino acids flanking the first proline of this motif (CPC/CPH/SPC) vary between transporters and have been suggested to yield information about the ion specificity. Thus a CPx-type ATPase with a CPCALVIS translation motif is proposed to transport Cd^{2+}, Zn^{2+}, Pb^{2+}, or Hg^{2+}, while in most Cu-translocating ATPases this motif is CPCALGLA (Solioz and Stoyanov, 2003), an amino acid sequence that is also present in the CopA-like proteins from biomining Bacteria and Archaea (Table 2).

In *E. coli* the CopA ATPase is part of the *cue* operon (Cu efflux) together with the oxidase CueO and the transcriptional regulator CueR. After further analysis of the promoter sequences of *copA* and *cueO* from *E. coli*, Outten et al. (2000) found a palindromic region where CueR 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 *cueO* was not found in *A. ferrooxidans*. On the other hand, in this microorganism an ORF (*afcueR*) coding a protein with 37% identity to the DNA binding domain of *E. coli* CueR was present. However, the nucleotide sequences of the putative promoters present upstream of all the *A. ferrooxidans* ORFs studied did not show the palindromic region present in the *E. coli* promoters, finally suggesting that *A. ferrooxidans* has different regulatory elements. The likelihood of other transcriptional regulators which could possibly control the expression of *A. ferrooxidans* Cu-resistance is expected, but remains to be studied.

Other genes with putative Cu-resistance roles such as those coding for CopC, CopD and a putative Cus system have also been recently characterized in *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 *A. ferrooxidans* was exposed to Cu (Navarro et al., 2009). In *P. syringae*, the periplasmic protein 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 afCopC was experimentally found in the periplasm of *A. ferrooxidans* grown in the absence of Cu (Chi et al., 2007). The prospective mechanism for this protein is still unknown in *A. ferrooxidans*.

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

A. ferrooxidans contained a putative operon which included the genes *afcusCBA* which would encode for an RND-like Cu-exporter system (Navarro et al., 2009). On the other hand, *E. coli* has an operon containing in addition of *cusCBA*, a *cusF* gene and a two-component system that regulates the expression of the system (Franke et al., 2003). CusF from *E. coli* is a periplasmic protein containing one binding site for Cu. Once the metal is bound, it has been proposed that CusF delivers the Cu ion to the Cus system for subsequent efflux to the extracellular medium (Franke et al., 2003). Recent crystal structures of CusF from *E. coli* revealed an intriguing Cu-binding site in the motif **H**XXXXXXX**W**XX**M**X**M**X**F** (Loftin et al., 2005) that includes tryptophan. The close proximity of this amino acid to Cu suggested an unusual cation-π interaction between Cu (I) and the aromatic ring of tryptophan (Xue et al., 2008). *A. ferrooxidans* possesses an ORF coding for a protein with ~25% identity to CusF from *E. coli* but with a different genomic organization since it is located distantly from *afcusCBA* and divergent from *afcopA2* (Navarro et al., 2009). The amino acid sequence of the putative afCusF showed one possible Cu-binding site which differs from that in *E. coli* only in the presence of a methionine instead of the histidine (**M**XXXXXXX**W**XX**M**X**M**X**F**) 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 *A. ferrooxidans*, one could predict that 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 *E. coli* and *A. ferrooxidans* 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 *A. ferrooxidans* protein. It is interesting to speculate that this apparently minor change of a His for a Met in afCusF could be an adaptation of the putative Cu chaperone to the acidic environment (pH 2.5) present at the periplasm *of A. ferrooxidans*. CusF from *E. coli* functions at around pH 7, a pH value where His would be mostly neutral. If 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 afCusF to deliver its bound Cu to the putative

Table 2
Alignment of putative Cop proteins from some acidophilic bacteria and archaea with experimentally characterized Cu transporters from neutrophilic bacteria.

	MBD Metal Binding Domain	Phosphatase Domain	6′ Translocation Domain	Phosphorylation Domain	Conserved HP Motif	Conserved GxGxxG/A Motif	TGDN Motif	GDGxNDxP Motif
CopA (*E. coli*)	CASC....CASC	TGEP	CPCALGLA	FDKTGTLT	SSHPL	GLGVSG	TGDN	GDGINAP
CopA (*E. hirae*)	CANC	TGES	CPCALGLA	LDKTGTLT	SEHPL	GAGISG	TGDN	GDGINAP
CopB (*E. hirae*)	no	TGES	CPHALGLA	LDKTGTLT	no	GVGLEA	TGDN	GDGINDAP
CopA1 (*A. fe*)	no	TGES	CPHALGLA	FDKTGTLT	SEHPI	GKGAQA	TGDS	GDGVNDAP
CopA2 (*A. fe*)	no	TGES	CPHALGLA	FDKTGTLT	SEHPI	GKGAQA	TGDS	GDGVNDAP
CopB (A. *fe*)	CASC....CASC	TGEP	CPCAMGLA	LDKTGTLT	SEHPL	GKGVRG	TGDL	GEGINDSP
CopA1 (*S. metallicus*)	CASC	TGES	CPCALGLA	LDKTGTVT	SSHPI	GNGIYG	TGDE	GDGVNDAL
CopA2 (*S. metallicus*)	CATC	TGER	CPCALGLA	LVKTGTVT	SIHPI	GRGIYA	TGDS	GDGINDAI
CopA (*M .sedula*)	CATC	TGEP	CPCGFGLA	LVKTGTVT	SNHPV	NO	TGDS	GDGVNDAQ
CopA (*S. solfataricus*)	CATC	TGEQ	CPCALGLA	LDKTGTVT	SIHPI	GRGIYA	TGDS	GDGINDSI

Data from neutrophilic bacteria were taken from Toyoshima et al., 2000; Solioz and Vulpe, 1996; Tottey et al., 2001. Data from *A. ferrooxidans* were from Navarro et al., 2009; *S. metallicus*, this work; *S. solfataricus* from Ettema et al., 2006; *M. sedula* from Auernik et al., 2007.

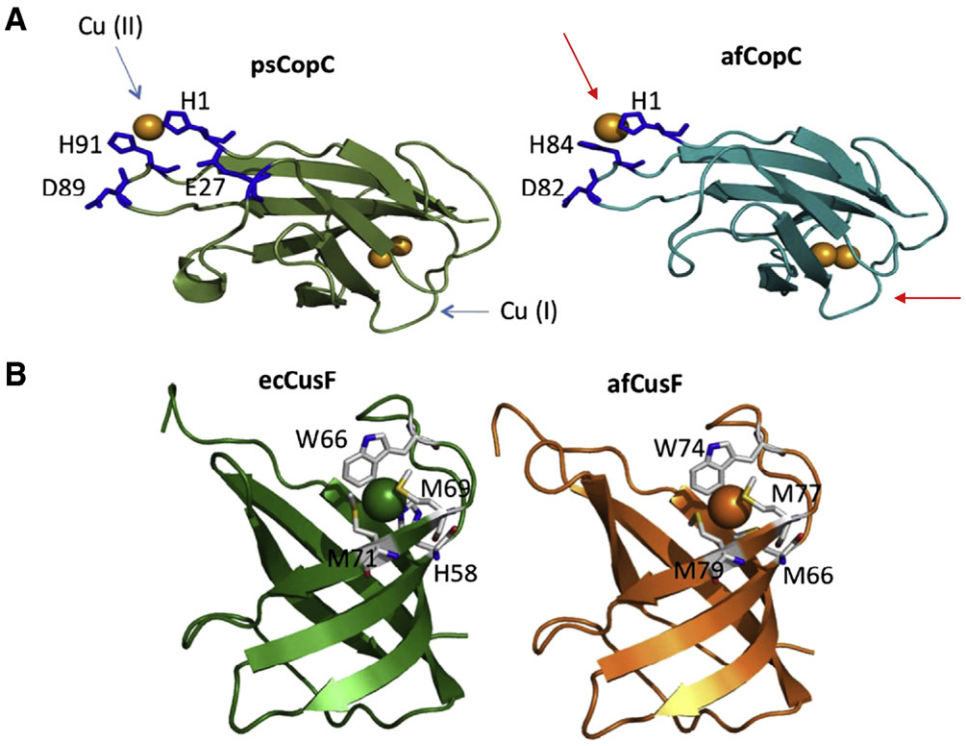

Fig. 1. Structural comparisons between some copper-chaperone proteins from *E. coli*, *P. syringae* and *A. ferrooxidans*. A, Comparison between psCopC and afCopC. The blue arrows indicate the two known Cu-binding sites for psCopC. The two red arrows indicate the expected Cu-binding sites in afCopC. B, Comparison between ecCusF and afCusF. The homology model of *A. ferooxidans* CopC (afCopC) was done based on the crystal structure of *P. syringae* psCopC (PDB entry: 2C9P), resolved at 2.25 Å resolution at pH 4.5 (Zhang et al., 2006) and that of *A. ferooxidans* CusF (afCusF) was based on the high resolution crystal structure of *E. coli* ecCusF (PDB entry: 2QCP), resolved at 1.0 Å resolution (Loftin et al., 2007). In both cases the program MODELLER was used (Sali and Blundell, 1993). One hundred models were built in each case, and each one of the models had the copper ligands modeled as a rigid bodies. From this ensemble of models, we selected the one with the lowest MODELLER objective function (MOF). Their foldings were verified by 3D–1D profiles with Verify-3D showing only positive values (Lüthy et al., 1992).

afCusCBA 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 afCusF-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 directly eliminate Cu to the exterior of the cell through the cus complex, avoiding Cu toxicity through its transportation 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 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 *Ferroplasma 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 Cu^{2+} 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 *copRTA* (*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 *copR* 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 Cu(II), 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 archaeaon is grown either in the presence of Cu or using chalcopyrite ($CuFeS_2$) 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 SoxRsS 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 $CuSO_4$, 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 chalcopyrite 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).

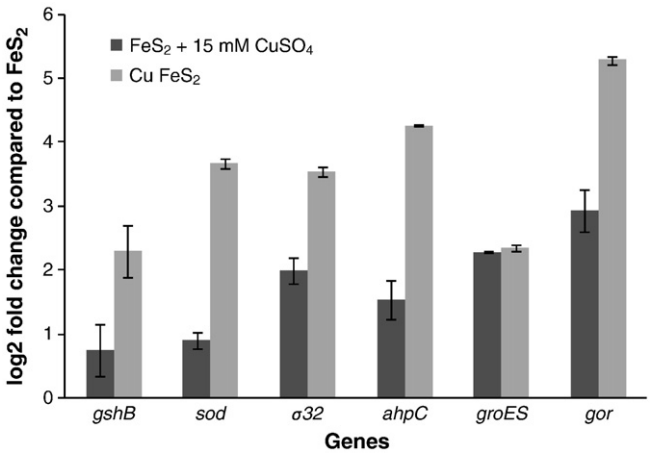

Fig. 2. Oxidative stress genes from *A. ferrooxidans* expressed during its growth in different minerals. *A. ferrooxidans* cultures were grown in shake flasks with 9 K medium except that ferrous iron was replaced by 1% w/v pyrite (FeS_2) as a control, in 1% pyrite in the presence of 15 mM $CuSO_4$ (dark gray bars) or in 1% chalcopyrite ($CuFeS_2$) (light gray bars). Total RNA was extracted from each culture at the stationary phase of growth and was used to determine the expression of the genes indicated by means of DNA macroarrays as described before (Acosta et al., 2005; Valenzuela et al., 2006). Bars indicate the average changes in expression compared to those seen in FeS_2.

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 alkylhydroxyperoxidase (*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). On the other hand, peroxidases catalyze the transformation of hydrogen peroxide, a toxic agent to the cell, into water and oxygen (Chuang et al, 2006). The increased expression of *sod* and *ahpC* 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 *A. ferrooxidans* (AFE_1616), also greatly increased its expression levels in the presence of Cu (Fig. 2).

The mRNA levels of *GroES* from *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 *A. ferrooxidans* (Jerez et al., 1988).

In the non-biomining acidophilic archaeon *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 *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 *S. metallicus*, some preliminary evidence also suggests the generation of reactive oxygen species (ROS) in slurries 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.

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 *E. coli* and their genes have been identified in several bacteria, including *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 *A. ferrooxidans* and *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 quantificated by using specific enzymatic methods in *S. acidocaldarius, S.*

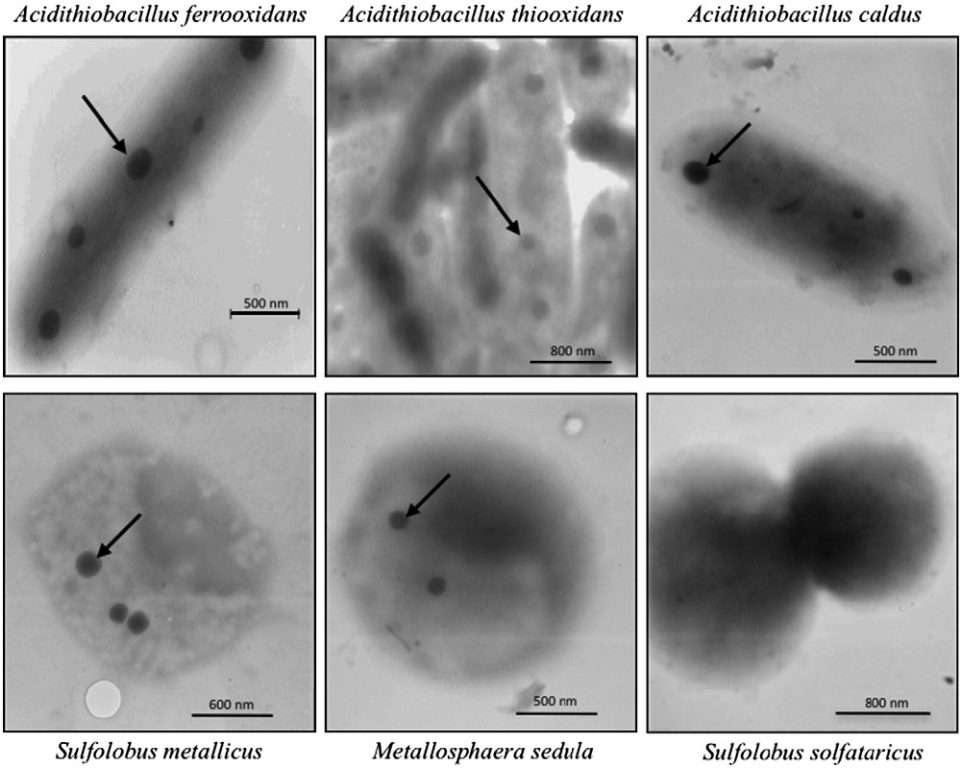

Fig. 3. Presence of electron-dense granules of polyP in biomining *Bacteria* and *Archaea*. Transmission electron microscopy images of unstained and unfixed cells were examined directly for the presence of electron-dense granules. Strains used were *Acidithiobacillus ferrooxidans* ATCC 23270, *Acidithiobacillus thiooxidans* DSM 9463, *Acidithiobacillus caldus* ATCC 51756, *Sulfolobus metallicus* DSM 6482, *Metallosphaera sedula* DSM 5348^T and *S. solfataricus* DSM 1616, a non-biomining archaeaon. Arrows indicate one of the electron-dense bodies (polyP) that all microorganisms shown accumulate, except for *S. solfataricus* DSM 1616.

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 adaptative 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 exopolyphosphatase activity and a stimulation of phosphate efflux. Copper in the μM range greatly stimulated exopolyphosphatase activity in cell-free extracts from both *A. ferrooxidans* and *S. metallicus*. In this system polyP was most likely degraded by exopolyphosphatase (PPX) to monomers of inorganic phosphate. These in turn would bind the metal in the cytoplasm of the microorganism and the metal-phosphate complexes would be pumped out to the periplasmic space (*A. ferrooxidans*) or to the exterior (*S. metallicus*) by means of carriers of inorganic phosphate such as Pit (for Phosphate Inorganic Transport) as proposed for *E. coli*, where the carrier is located in the internal membrane of the bacterium. *A. ferrooxidans*, *S. solfataricus*, *S. acidocaldarius*, *S. tokodaii* and *M. sedula* do not show putative genes for a Pit system in their genomes. However, they have one or more ORFs coding for a putative yeast Pho84 that could act as a phosphate transporter. It has been demonstrated that in *S. cerevisiae* Pho84 can transport $MeHPO_4$ 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 cytoplasm 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 PPK 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 PPK 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.*

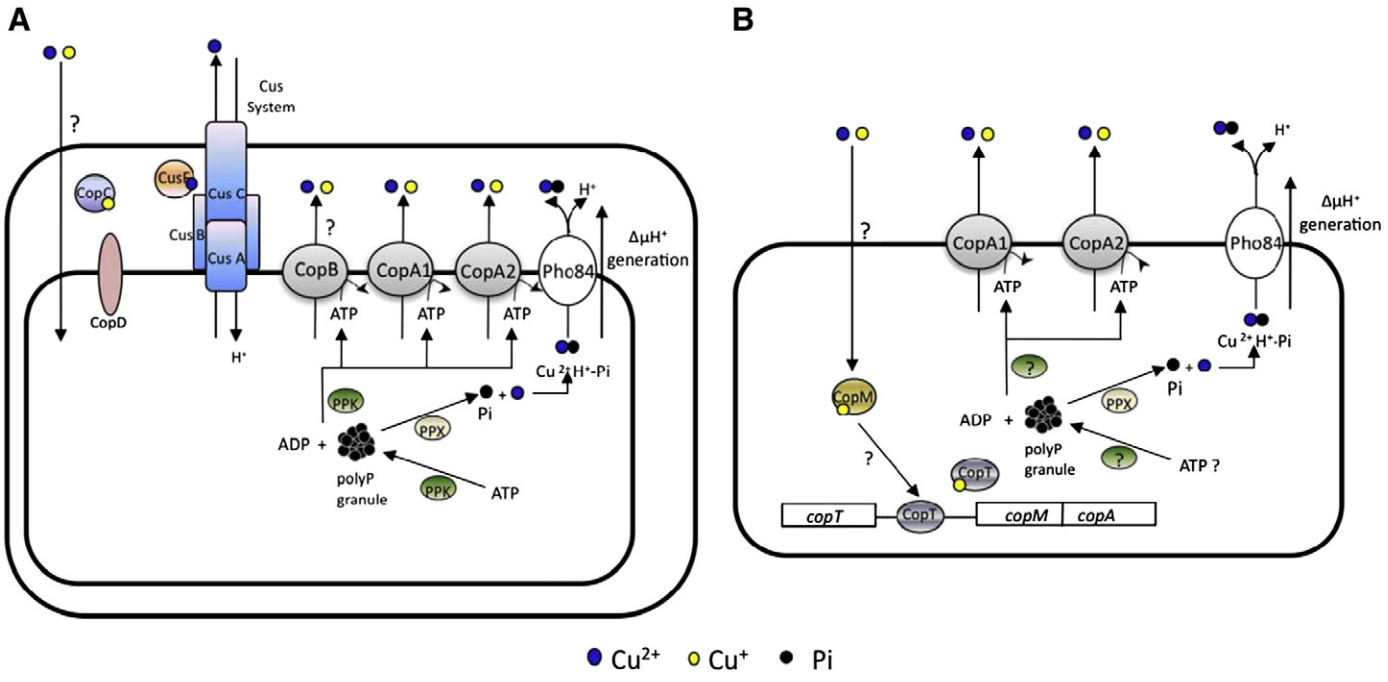

Fig. 4. Qualitative working models summarizing the possible role of Cu-resistance determinants in *A. ferrooxidans* and *Sulfolobales*. A. *A. ferrooxidans*. CopA1, CopA2, CopC, CopB, CopD, CusF, CusA, CusB, CusC are the Cu-resistance determinants from *A. ferrooxidans* ATCC 23270 whose expression has been demonstrated to increase in the presence of Cu. PPK is the polyphosphate kinase enzyme that synthezises polyP and PPX is the exopolyphosphatase that hydrolyzes the polymer. Pho84 is the putative phosphate transporter that *A. ferrooxidans* could use instead of the lacking Pit system. B. *Sulfolobales*. CopA, CopT, CopM, are the Cu-resistance determinants described in several archaea. PPK The polyphosphate kinase enzyme that synthezises polyP is not known in Archaea and is represented by an oval (?) and PPX is the exopolyphosphatase that hydrolyzes the polymer. Pho84 is the putative phosphate transporter that members of the *Sulfolobales* contain in their genomes and could use instead of the absent Pit system. This working model is based in part in the results of the following authors: Van Veen et al., 1994; Keasling, 1997; Outten et al., 2001; Cardona et al., 2002; Alvarez and Jerez, 2004; Remonsellez et al., 2006; Auernik et al., 2008; Ettema et al., 2006 and Navarro et al., 2009.

cerevisiae Pho84 sequence 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 substrate 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 (Watling, 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 biohydrometallurgy 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 sequences 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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