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Transcriptional and Functional Studies of *Acidithiobacillus ferrooxidans* Genes Related to Survival in the Presence of Copper[▽]

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The acidophilic *Acidithiobacillus ferrooxidans* can resist exceptionally high copper (Cu) concentrations. This property is important for its use in biomining processes, where Cu and other metal levels range usually between 15 and 100 mM. To learn about the mechanisms that allow *A. ferrooxidans* cells to survive in this environment, a bioinformatic search of its genome showed the presence of at least 10 genes that are possibly related to Cu homeostasis. Among them are three genes coding for putative ATPases related to the transport of Cu (*A. ferrooxidans copA1* [$copA1_{Af}$], $copA2_{Af}$, and $copB_{Af}$), three genes related to a system of the resistance nodulation cell division family involved in the extraction of Cu from the cell ($cusA_{Af}$, $cusB_{Af}$, and $cusC_{Af}$), and two genes coding for periplasmic chaperones for this metal ($cusF_{Af}$ and $copC_{Af}$). The expression of most of these open reading frames was studied by real-time reverse transcriptase PCR using *A. ferrooxidans* cells adapted for growth in the presence of high concentrations of Cu. The putative *A. ferrooxidans* Cu resistance determinants were found to be upregulated when this bacterium was exposed to Cu in the range of 5 to 25 mM. These *A. ferrooxidans* genes conferred to *Escherichia coli* a greater Cu resistance than wild-type cells, supporting their functionality. The results reported here and previously published data strongly suggest that the high resistance of the extremophilic *A. ferrooxidans* to Cu may be due to part or all of the following key elements: (i) a wide repertoire of Cu resistance determinants, (ii) the duplication of some of these Cu resistance determinants, (iii) the existence of novel Cu chaperones, and (iv) a polyP-based Cu resistance system.

Cells in general have developed a series of mechanisms to control the levels of free Cu in their compartments. Thus, when the concentration of copper exceeds acceptable levels, mechanisms of resistance are activated in order to survive in the adverse environment (17, 26, 29, 31). In gram-negative bacteria, one of the pathways described for Cu resistance is an active efflux of this 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 *Escherichia coli* (27). It has also been postulated that microorganisms may pump the metal from both the cytoplasm and the periplasm to the extracellular space by systems of the resistance nodulation cell division family of carriers, the Cus system of *E. coli* being the best-known detoxification organization of this kind (19, 26). The capacity of some species to bind the metal in the periplasmic space has also been reported (21). Copper is therefore retained by these periplasmic proteins through their Cu-binding sites. An example of this mechanism is the CopABCD system from *Pseudomonas syringae* pv. tomato, in which proteins involved in the binding of Cu (CopB and CopC) and a multicopper oxidase (CopA) are present. Most likely, the activity of CopA is responsible for Cu resistance (21). A detailed DNA microarray transcriptional profiling of Cu-adapted and Cu-shocked *Pseudomonas aeruginosa* cells with similar metal tolerance systems has also been recently reported (31). These systems from gram-negative microorganisms allow them to tolerate relatively low Cu levels. Thus, *E. coli* has a MIC of 3 mM for Cu in LB medium (9), and in *P. syringae*, a MIC of 8 mM was reported (21). These are very low concentrations compared to those present in environments such as mining operations or acid mine drainages, where the concentrations of heavy metals, especially Cu, are 1 or 2 orders of magnitude higher (7). *Acidithiobacillus ferrooxidans* is a gram-negative, acidophilic chemolithoautotroph that can use ferrous iron, reduced sulfur species, or metal sulfides as energy sources (11, 24, 28, 33). This microorganism can also be adapted to grow in the presence of 800 mM Cu (5). These abilities make this bacterium and others with similar properties potentially valuable in the extraction and recovery of metals such as copper or gold by means of biomining processes (36). Therefore, it is very interesting to determine the molecular mechanisms that allow these microorganisms to resist extremely high concentrations of metals in their environment. Very little is known about the mechanisms that acidophiles use to tolerate metal and acid toxicity (8). When *A. ferrooxidans* is exposed to Cu, its surface changes and proteins yet to be identified appear on the surface (5). In addition, when this bacterium is exposed to Cu, it loses extrachromosomal structures, suggesting that if the bacterium possesses genes coding for proteins involved in Cu resistance, they would be present on its genome (4). Hence, it is reasonable to assume that *A. ferrooxidans* expresses surface resistance determinants when exposed to Cu.

Only few genes have been previously identified by RNA arbitrarily primed 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 unclear, and their expression may be related to indirect metabolic responses

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to stress (20). Novo et al. (18) also studied changes in some proteins from *A. ferrooxidans* when subjected to Cu and other metals, but the proteins were not identified.

Very recently, by using PCR-restriction fragment length polymorphism, the expression of genes Afe0663 (*A. ferrooxidans copA1* [$copA1_{Af}$]) and Afe0329 ($copA2_{Af}$) from *A. ferrooxidans* has been reported, and the authors of the report suggested that $copA2_{Af}$ might be more important for Cu homeostasis in this bacterium (16).

An additional polyP-dependent system for Cu resistance has been suggested for polyP-accumulating *A. ferrooxidans* (1) that would be similar to that previously proposed for *E. coli* (12, 14). In this system, polyP is degraded by exopolyphosphatase (PPX) to inorganic phosphate monomers, which bind the metal in the cytoplasm of the bacterium, and then metal-phosphate complexes are thought to be pumped out to the periplasmic space by means of inorganic phosphate carriers. It was found that in the presence of Cu, *A. ferrooxidans* degrades polyP with the concomitant efflux of phosphate (1). A similar phenomenon has also been observed in the mineral sulfide-oxidizing acidophilic archaeon *Sulfolobus metallicus*, which is also a polyP-accumulating microorganism highly resistant to Cu (25).

The purpose of the present work was to characterize in detail several of the putative Cu resistance genes present in the genome of *A. ferrooxidans* ATCC 23270 and to study their transcriptional expression under the Cu concentrations these microorganisms normally encounter in their environment. The expression of these open reading frames (ORFs) with Cu resistance roles was analyzed, and the majority of them were upregulated when *A. ferrooxidans* was exposed to different extracellular Cu concentrations. Finally, most of the *A. ferrooxidans* putative Cu resistance determinants conferred a higher Cu tolerance to *E. coli* Δ*copA* and Δ*cusCFBA* Δ*cueO* mutants, strongly suggesting that they are part of at least one of the functional mechanisms for Cu resistance in *A. ferrooxidans*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The type strain of *A. ferrooxidans* (ATCC 23270) was grown at 30°C in ferrous sulfate-containing 9K medium at pH 1.5 as described before (23). Growth was monitored by determining cell numbers under a phase-contrast Olympus BX50 microscope with a Petroff-Hauser counting chamber. *E. coli* K-12, Δ*copA* and Δ*cusCFBA* Δ*cueO* mutants of K-12, and all transformants of these strains were grown at 37°C in Luria-Bertani (LB) medium supplemented with the required compounds as indicated for the different experiments.

Extraction of total RNA from *A. ferrooxidans*. To determine the effect of different Cu concentrations on the expression of the genes of interest, cells were adapted to grow continuously in the presence of the concentrations of Cu sulfate indicated in the figures until they reached between 9×10^7 and 1×10^8 cells per ml, the time at which total RNA was extracted from each culture condition. To minimize cell damage and RNA degradation during storage, bacteria harvested by centrifugation were immediately processed for RNA extraction and reverse transcriptase PCR (RT-PCR) and real-time RT-PCR transcriptional expression determinations. Total RNA was prepared from *A. ferrooxidans* cultures after lysing the cells as previously reported (34) except that TRIzol (Invitrogen) was used for the extraction. DNA was eliminated by the addition of 4 U of RNase-free RQ1 DNase (Promega).

Cotranscriptional analysis by RT-PCR. The expression of adjacent genes in some of the putative operons of interest was studied by means of cotranscriptional experiments. cDNA was synthesized by using reverse primers hybridizing to $cusA_{Af}$ or $copD_{Af}$ and 0.8 μg of total RNA from an *A. ferrooxidans* culture grown in the presence of 5 mM Cu. The primers used for the upstream and downstream genes are shown in Table 1. PCR amplifications were performed with 1 μl of a 1/10 dilution of the cDNA and 25 pmol of each primer. Amplification conditions included an initial 3 min of denaturation at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 1.5 min at 72°C and finished by 10 min at 72°C.

TABLE 1. Oligonucleotides used in this work

Primer	Oligonucleotide sequence	Use(s)[a]
afcopA1 fwex	5′ CATATGAAACGTTTTTATCTCACCC 3′	3
afcopA1 rvex	5′ CTAGGCGGTGAGCCGAACCC 3′	3
afcopB fw	5′ GCGGATGTGGGTATGGCTCT 3′	1
afcopB rev	5′ GCTGCCTGAATGGCGGTAAT 3′	1
afcopB fwex	5′ CATATGGTAGATGTGGAGATAGG 3′	3
afcopB rvex	5′ TCAGGGTGCTGCCGCCCAGGGCT 3′	3
afcopC fw	5′ AAGAATGCCCAAGGTGCGGT 3′	1, 2
afcopC rev	5′ ACGACGCTCCAGTACACGAA 3′	1, 2
afcopC fwex	5′ CATATGACCAAGAAAGCCAT 3′	3
afcopC rvex	5′ TTACTTCACCGCAAATGACC 3′	3
afcopD fw	5′ AGGGCTTGGGAAGGCTGATG 3′	1, 2
afcopD rev	5′ TCCAGATGTCCAGTGTCGGA 3′	1, 2
afcopD fwex	5′ CATATGAAACCACTGTTTGT 3′	3
afcopD rvex	5′ TTACCCCTGGTGAATAGTCAC 3′	3
afcusA fw	5′ GCCCTGCTCCTTTACTTCAA 3′	1, 2
afcusA rev	5′ ACCGCCACCGATAACTTGTA 3′	1, 2
afcusA2 rev	5′ GCCAGGGACTCTTCCAGCAA 3′	2
afcusB fw	5′ CTGGCGTCAACAACTCCAAT 3′	1, 2
afcusB rev	5′ AGGCATACCCATACTGTCCT 3′	1, 2
afcusC fw	5′ ACTGAAAGGACAACAGGCGG 3′	1, 2
afcusC rev	5′ TCTTCGGCATAGAGGGCTTG 3′	1, 2
afcusB2 fw	5′ GTGGTCGAGACCCTCCATGT 3′	2
afcusF fw	5′ GGGATTCATGCCGTTGATTT 3′	1
afcusF rev	5′ TATCTCACCCTCATCCTGGC 3′	1
afcusF fwex	5′ CATATGAAACGTTTTTATCTCACCC 3′	3
afcusF rvex	5′ TCATTGTCTGACCGGCGTGA 3′	3
pBAD fw	5′ ATGCCATAGCATTTTTATCC 3′	3
16S rRNA fw	5′ TGGTGCCTAGCGTACTGAGTG 3′	1
16S rRNA rev	5′ CCGAAGGGCACTTCCGCA 3′	1

[a] 1, real-time RT-PCR; 2, coexpression analyses; 3, cloning fragments in different vectors.

Real-time RT-PCR. Primers for real-time RT-PCR were designed with the software Light Cycler Probe Design (Roche). For cDNA synthesis, 0.8 μg of total RNA were reverse transcribed for 1 h at 42°C by using ImProm-II (Promega), 0.5 μg of random hexamers (Promega), and 3 mM $MgCl_2$. PCR was carried out by using the Corbett Rotor Gene 6000 system following the manufacturer's instructions by using SYBR green master mix (Roche). Thermal cycling conditions were an initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 5 s, 55 to 64°C for 5 s, and 72°C for 16 s. Fluorescence measurements were recorded at the end of each extension step. Mean values and standard deviations were obtained by analyzing cDNA obtained from three independently grown cultures for each data series. Each mRNA expression value was normalized against 16S rRNA gene expression.

Cloning *A. ferrooxidans* genes in an expression vector. *A. ferrooxidans* genes of interest were cloned in the commercial vector pBAD-TOPO (Invitrogen) by following the manufacturer's instructions. For the ligation reaction, 25 ng of the DNA to be cloned and 10 ng of the vector DNA were used, and incubation was for 30 min at room temperature. Two microliters of this reaction was used to transform TOP10 cells (Invitrogen). The clones obtained were analyzed for the correct orientation and the expected sizes of the PCR fragments. This was done by PCRs using GoTaq DNA polymerase (Promega). The forward primer (pBAD fw) hybridized with the promoter of the vector, and the following reverse primers hybridized with the 3′ end of the cloned PCR products: afcopA1 rvex for $copA1_{Af}$, afcopB rvex for $copB_{Af}$, afcopC rvex for $copC_{Af}$, afcopD rvex for $copD_{Af}$, and afcusF rvex for $cusF_{Af}$ (Table 1). The amplification conditions included an initial 2 min of denaturation at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 50°C, and 2 min at 72°C and finished by 10 min at 72°C. The nucleotidic sequence of all the inserts was also checked by DNA sequencing.

Generation of the *E. coli* Δ*copA* and Δ*cusCFBA* Δ*cueO* deletion mutants. Deletions of the *copA* or *cueO* and *cusCFBA* genes from *E. coli* K-12 were performed according to the method of Datsenko and Wanner (6). Briefly, PCR primers (60 nucleotides [nt]) were designed with homology to 40 nt of the *E. coli*

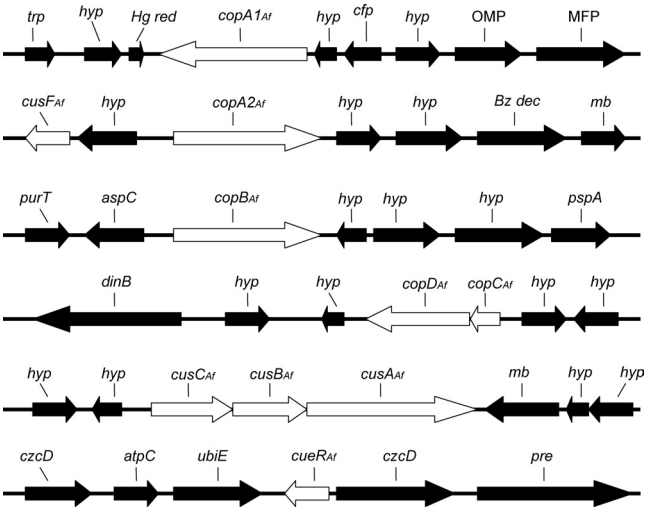

FIG. 1. Genomic contexts of the genes coding for putative Cu resistance determinants in *A. ferrooxidans* ATCC 23270. Unfilled arrows represent all the ORFs predicted to be related to Cu homeostasis. Their equivalent annotations in the genome (www.tigr.org/db.shtml) are the following: $copA1_{Af}$ (AFE0663), $copA2_{Af}$ (AFE0329), $copB_{Af}$ (AFE1073), $copC_{Af}$ (nonannotated), $copD_{Af}$ (AFE0454), $cusA_{Af}$ (AFE1145), $cusB_{Af}$ (AFE1144), $cusC_{Af}$ (AFE1143), $cusF_{Af}$ (AFE0326), and $cueR_{Af}$ (AFE0681).

K-12 genes to be deleted and 20 nt of the plasmid pKD4 or pKD3. Plasmids pKD3 and pKD4 contain an FLP recombination target-flanked chloramphenicol or kanamycin resistance cassette, respectively, and they are used as templates for the PCRs. In this context, the primers used (nucleotides corresponding to the target gene are underlined) were as follows: *copA* (fw), 5′AATACCGATACTGTTGTAGATAAACGCACCGAGCAGGTTCTGTAGGCTGGAGCTGCTTCG3′; *copA* (rev), 5′GGATGTGTCTATCACTGAAGCGCACGTTACCGGGACTGCCCATATGAATATCCTCCTTAG3′; *cueO* (fw), 5′CTTAAAATATTCCGTCGCGCTGGGTGTGGCTTCGGCTTTGCTGTAGGCTGGAGCTGCTTCG3′; *cueO* (rev), 5′CTGGCGGTTTGCCATTTTCTGCACCGCTACGGAACTGCGTCATATGAATATCCTCCTTAG3′; *cus* operon (fw), 5′CAGAACGGCCTGGTTAACGCAGCAGATAACTATCAGAACGCTGTAGGCTGGAGCTGCTTCG3′; and *cus* operon (rev), 5′CTGAACCAGCCCCCGTTCCCCACAGAATCGGCAGCAGACCCATATGAATATCCTCCTTAG3′.

DNA products were obtained by PCRs using GoTaq DNA polymerase (Promega) and plasmid pKD4 or pKD3 as DNA templates (6). The deletion of each gene of interest was obtained by using the corresponding primers described above. Amplification conditions included an initial 2 min of denaturation at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 50°C, and 2 min at 72°C and finished by 10 min at 72°C. *E. coli* K-12/pKD46 cells (6) grown at 30°C in LB medium supplemented with 10 mM L-arabinose and 100 mg liter^{-1} ampicillin were then transformed by electroporation with the purified DNA fragments obtained by PCR as already described.

Bacteria were plated on LB agar plates (1.5% agar) supplemented with kanamycin or chloramphenicol and were incubated at 37°C to select for the allelic exchange that took place and to cure the microorganisms from plasmid pKD46. The presence of the mutant alleles was confirmed by PCR amplification by using primers flanking the substitution sites.

Copper resistance and functional assays. MICs were determined by using the *E. coli* K-12 Δ*copA* and Δ*cusCFBA* Δ*cueO* generated mutants transformed with the different putative Cu resistance genes from *A. ferrooxidans*. Overnight-grown cultures of each transformant were diluted 1:1,000 in LB medium supplemented with ampicillin (100 mg liter^{-1}), 0.1% (wt/vol) arabinose or glucose (to either induce or repress the cloned genes, respectively), and Cu ranging from 0.2 to 4 mM. Cultures were incubated at 37°C with shaking for 10 h. Finally, the lowest Cu concentration inhibiting growth by 50% (monitored by reading the optical density at 600 nm) relative to that of the control cells (not exposed to Cu) was considered the MIC for the tested transformant.

RESULTS AND DISCUSSION

Putative Cu resistance genes present in the genome of *A. ferrooxidans* ATCC 23270 and their possible organization. In the annotated *A. ferrooxidans* ATCC 23270 genome sequence, several ORFs have been proposed to code for putative proteins related to Cu resistance. Figure 1 shows the genomic contexts of all of these putative *A. ferrooxidans* genes. Based on their similarities to known genes, two ORFs encoding potential Cu P-type ATPases have been suggested (22). $copA2_{Af}$ would be involved in Cu efflux, and $copB_{Af}$ in Cu import to the cytoplasm, as it has been proposed for *copA* from *Enterococcus hirae* (17). Additionally, we found a third possible Cu P-type ATPase ($copA1_{Af}$) as shown in Fig. 1 and Table 2. Curiously, these *A. ferrooxidans* predicted paralog ATPases ($copA1_{Af}$ and $copA2_{Af}$) may contribute to a higher Cu resistance in *A. ferrooxidans* than neutrophils such as *E. coli* that have only one type of CopA protein. These ATPases from *A. ferrooxidans* showed several of the conserved characteristic domains and motifs present in these metal transporters (30, 35) (Table 2). The heavy metal ATPases are a subclass of the P-type ATPases called CPx (Cys-Pro-X)-type ATPases. This name arises 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 (30). $CopA1_{Af}$ and $CopA2_{Af}$ contained the motif CPHALGLA present in *E. hirae* CopB, whereas $CopB_{Af}$ contained CPCAMGLA (Table 2). The $copA1_{Af}$ and $copA2_{Af}$ nucleotide sequences show high identity (94.5%) with one another (16). However, when the upstream sequence present in

TABLE 2. Alignment of putative Cop proteins from *A. ferrooxidans* with experimentally characterized Cu transporters from other bacteria[a]

Protein	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
$CopA1_{Af}$	No	TGES	CPHALGLA	FDKTGTLT	SEHPI	GKGAQA	TGDS	GDGVNDAP
$CopA2_{Af}$	No	TGES	CPHALGLA	FDKTGTLT	SEHPI	GKGAQA	TGDS	GDGVNDAP
$CopB_{Af}$	CASC. . . .CASC	TGEP	CPCAMGLA	LDKTGTLT	SEHPL	GKGVRG	TGDL	GEGINDSP

[a] Some data was taken from previously published work (30, 32, 35). Ellipses indicate separation between two existing metal binding domains.

the DNA coding for these two ORFs was analyzed, no potential common regulatory elements were found (results not shown). This strongly suggests that the expression of these genes may have a different kind of regulation.

E. coli CopA ATPase is part of the Cue (Cu efflux) system, together with the oxidase CueO and the transcriptional regulator CueR. Analyzing the promoter sequences of *copA* and *cueO* from *E. coli*, Outten et al. (19) noticed a palindromic region on them where CueR binds to upregulate the expression of the Cue system when this bacterium is exposed to Cu. An ORF with homology to *cueO* was not found in *A. ferrooxidans*. On the other hand, an ORF in this microorganism (putative $cueR_{Af}$) coding for 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 studied *A. ferrooxidans* ORFs did not show the palindromic region present in the *E. coli* promoters, suggesting that *A. ferrooxidans* has different regulatory elements.

In the genetic context of $cueR_{Af}$ (Fig. 1), there was an ORF, *czcD*, that might encode for a protein of the cation diffusion facilitator family, related to the efflux of cadmium, zinc, and cobalt but not Cu. This genetic organization suggests that $CueR_{Af}$ might be involved in the regulation of the expression of *czcD* and not necessarily in those genes related to Cu resistance. Obviously, the existence of other transcriptional regulators controlling the expression of *A. ferrooxidans* Cu resistance is expected.

The genomic context of some of the *A. ferrooxidans* ORFs potentially related to Cu resistance in Fig. 1 showed an organization in possible transcriptional units, such as $copC_{Af}$ and $copD_{Af}$. These ORFs from *A. ferrooxidans* encoded putative proteins showing 32% and 30% identities to CopC and CopD from *P. syringae*, respectively, which are part of the *copABCD* operon in this microorganism (2). However, *A. ferrooxidans* did not show genes with significant homology to *copA* and *copB* from *P. syringae*. In the latter microorganism, the periplasmic CopC is a Cu chaperone with two binding sites for the metal (2). In this regard, protein $CopC_{Af}$ was experimentally found in the periplasm of *A. ferrooxidans* (3), but it only holds the site for Cu(II) conserved (not shown).

Figure 1 also shows that *A. ferrooxidans* contained a possible operon formed by the genes $cusCBA_{Af}$. On the other hand, *E. coli* has an operon that contains *cusCBA* and a *cusF* gene. The expression of these genes is regulated by a two-component system (CusRS) (9). *E. coli* CusF is a periplasmic protein containing one binding site for Cu. Once the metal is bound, CusF is thought to deliver it to the Cus system for its efflux to the extracellular medium (9). Recent crystal structures of *E. coli* CusF revealed an intriguing Cu-binding site, HXXXXXX XWXXMXMXF (15), 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 (37). Figure 1 shows the presence in *A. ferrooxidans* of 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 $cusCBA_{Af}$ and divergently from $copA2_{Af}$. The amino acid sequence of the putative $CusF_{Af}$ showed one possible Cu-binding site differing from that in *E. coli* only in the presence of a methionine instead of histidine (MXXXXX XXWXXMXMXF) and a signal peptide, suggesting that it is also an exported protein most likely being delivered to the periplasmic space. Furthermore, if this putative Cu-binding site was functional in *A. ferrooxidans*, one could predict that $CusF_{Af}$ might not only contain this newly described and unprecedented type of Cu-binding site but also that it would bind Cu(I) in the periplasm. Interestingly, a second *cusF*-like gene is present in the genome of *A. ferrooxidans* ATCC 23270, having the same Cu-binding motif of the $CusF_{Af}$ gene reported here. Its genomic context contains at least three annotated proteins possibly related to metal resistance: a copper-translocating P-type ATPase, an efflux transporter of the resistance nodulation cell division family, and a heavy metal efflux pump of the CzcA family (not shown).

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 confirmed experimentally as being expressed in the presence of Cu (see below). These ORFs present in both *A. ferrooxidans* strains show 100% identity between their corresponding DNA sequences. However, strain ATCC 53993 contains several additional putative Cu resistance determinants, such as Lferr_0167, a putative Cu ATPase, and a putative Cus system with four ORFs (Lferr_0170 to Lferr_0172). These putative genes are clustered in a short DNA region coding for several different metal resistance ORFs, a region which is absent in the genome of strain ATCC 23270. It is therefore possible that gene duplications are a key element to metal resistance in these extremophiles. It will be very interesting to determine experimentally whether *A. ferrooxidans* ATCC 53993 has a higher Cu resistance than the ATCC 23270 strain due to the presence of these additional genes.

Adaptation of *A. ferrooxidans* ATCC 23270 to different Cu concentrations. To study the differential expression of the putative Cu resistance genes from *A. ferrooxidans* when the microorganism was subjected to different concentrations of the metal, it was first necessary to determine the appropriate concentrations of Cu and their effect on the growth of the acidophile. It has been reported that a strain of *A. ferrooxidans* was affected in its iron-oxidizing activity by high concentrations of Cu when the cells were not previously adapted to grow in the presence of the metal (5).

A. ferrooxidans cells were adapted to grow for at least three subcultures in the presence of 5, 25, and 100 mM Cu. Figure 2 shows the growth curves of these adapted cells. In the presence of 25 mM and 100 mM Cu, there was a greater initial growth lag and a lower growth rate than in 5 mM Cu or in the untreated culture. In addition, cell numbers obtained at the early stationary phase in the presence of 25 and 100 mM Cu were fairly smaller than those seen in the absence of the metal or in the presence of 5 mM Cu (Fig. 2). Although *A. ferrooxidans* cells were still able to grow in 100 mM Cu, total RNA was extracted from *A. ferrooxidans* cells grown in the presence or absence of 5 or 25 mM Cu for most of the experiments.

Several genes coding for putative Cu resistance determinants are cotranscribed in *A. ferrooxidans*. Fig. 1 shows the possible existence of two operons: $copC_{Af}$-$copD_{Af}$ and $cusC_{Af}$-$cusB_{Af}$-$cusA_{Af}$. Our previous results acquired through Northern blot experiments (not shown) suggested that the sizes of the $copC_{Af}$ and $copD_{Af}$ transcripts (about 1.5 kb) were identical

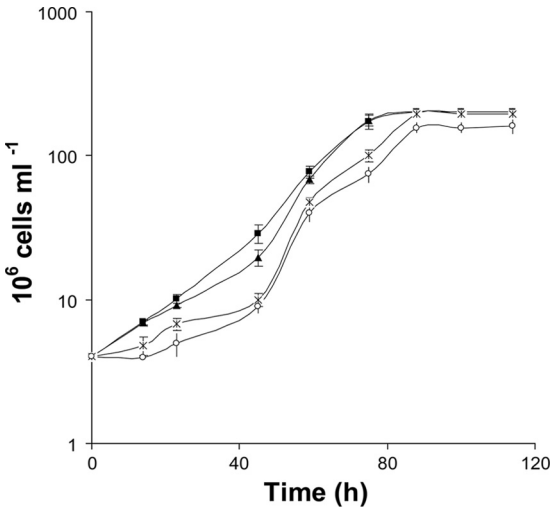

FIG. 2. Growth curves of cultures used for transcriptional studies. *A. ferrooxidans* ATCC 23270 was previously adapted to grow for at least three subcultures in the presence of 5 mM, 25 mM, or 100 mM Cu. These cells were then inoculated in the same fresh medium in the presence of their respective metal concentrations as follows: 5 mM Cu (filled triangles), 25 mM Cu (asterisks), or 100 mM Cu (unfilled circles). A control culture was done with cells never exposed to Cu (filled squares). Growth curves were determined in each case. Error bars indicate the standard deviations based on three different cultures.

when using their respective probes and corresponded to the sum of the sizes of each gene (375 bp for $copC_{Af}$ and 1,072 bp for $copD_{Af}$), strongly suggesting that both ORFs were cotranscribed. This was confirmed by carrying out cotranscription experiments (Fig. 3) in which the cDNAs were obtained by using RNA extracted from a culture grown in the presence of 5 mM Cu by using a reverse primer hybridizing with the $cusA_{Af}$ (Fig. 3A) or $copD_{Af}$ (Fig. 3B) gene. PCR amplifications were carried out by using the corresponding cDNAs as templates and each pair of primers lying in adjacent genes. The presence of an amplicon of the expected size in each case indicated the adjacent genes were part of polycistronic messengers. These results clearly show that *A. ferrooxidans* genes coding for $CusC_{Af}$, $CusB_{Af}$, and $CusA_{Af}$ and those for $CopD_{Af}$ and $CopC_{Af}$ were expressed in the form of transcriptional units. The first was composed of three genes ($cusC_{Af}$-$cusB_{Af}$-$cusA_{Af}$) and the latter composed of two ($copC_{Af}$-$copD_{Af}$).

The transcription of most putative Cu resistance genes is upregulated in *A. ferrooxidans* grown in the presence of Cu. Real-time RT-PCR experiments showed that the transcription of all of the Cu resistance genes took place in the absence of Cu but some of them at low relative copy numbers (Table 3), indicating the existence of a minimum variable basal level of expression under these conditions. $copA2_{Af}$ has recently been reported to be expressed in higher levels than $copA1_{Af}$ when grown in ferrous iron and in the presence of Cu by using PCR-restriction fragment length polymorphism, suggesting that $copA2_{Af}$ might be more important than $copA1_{Af}$ for Cu homeostasis in *A. ferrooxidans* (16). Since $copA1_{Af}$ and $copA2_{Af}$ have 94.5% nucleotide sequence identity, it is unlikely that their differential expression can be determined by using real-time RT-PCR. Therefore, we did not include them in this analysis.

$CopB_{Af}$ has been predicted to be similar to *E. hirae* CopA

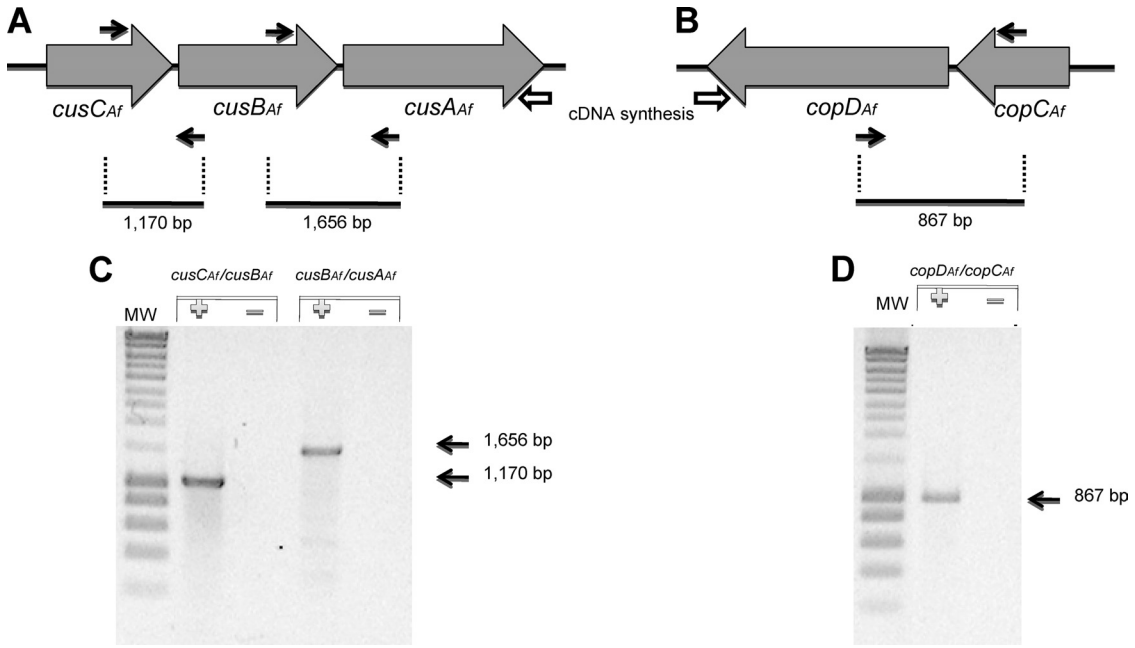

FIG. 3. Two sets of Cu resistance genes are cotranscribed in *A. ferrooxidans*. cDNA was synthesized with a reverse primer (unfilled arrows) hybridizing toward the 3′ end of $cusA_{Af}$ (A) or $copD_{Af}$ (B). *A. ferrooxidans* total RNA was extracted at the late exponential phase from a culture adapted to grow in 5 mM Cu. PCR amplifications were carried out with these cDNAs and each corresponding primer pair (black arrows). Determination of the cotranscription for each pair of adjacent genes was carried out by using primers labeled fw (upstream genes) and rev (downstream genes) in Table 1. The results shown (C, D) were obtained after 35 PCR cycles. PCRs were done using cDNA templates synthesized in the presence (+) or absence (−) of reverse transcriptase to detect the possible presence of genomic DNA contamination.

TABLE 3. Induction of transcript levels for Cu resistance genes from *A. ferrooxidans* ATCC 23270 as determined by real-time RT-PCR[a]

Gene	0 mM Cu		5 mM Cu		Induction ratio (5 mM/0 mM)	25 mM Cu		Induction ratio (25 mM/0 mM)
	CT	Relative copy no.	CT	Relative copy no.		CT	Relative copy no.	
$copB_{Af}$	31.7	$4.2 \times 10^2 \pm 6.7 \times 10^1$	24.8	$2.2 \times 10^4 \pm 3.9 \times 10^3$	39.6	24.5	$2.7 \times 10^4 \pm 5.8 \times 10^3$	89.5
$copC_{Af}$	22.5	$1.8 \times 10^4 \pm 2.3 \times 10^3$	21.3	$4.0 \times 10^4 \pm 4.2 \times 10^3$	1.7	20.0	$1.0 \times 10^5 \pm 2.2 \times 10^4$	8.3
$copD_{Af}$	21.7	$3.3 \times 10^4 \pm 6.5 \times 10^3$	20.6	$7.7 \times 10^4 \pm 1.3 \times 10^4$	1.7	20.6	$8.0 \times 10^4 \pm 1.7 \times 10^4$	3.5
$cusC_{Af}$	20.3	$5.8 \times 10^3 \pm 1.4 \times 10^3$	15.1	$4.2 \times 10^5 \pm 9.9 \times 10^4$	53.8	14.7	$5.9 \times 10^5 \pm 4.9 \times 10^4$	143.9
$cusB_{Af}$	23.2	$8.3 \times 10^2 \pm 1.2 \times 10^1$	17.2	$5.1 \times 10^4 \pm 7.7 \times 10^3$	45.3	15.3	$2.0 \times 10^5 \pm 2.1 \times 10^4$	332.3
$cusA_{Af}$	27.1	$3.4 \times 10^2 \pm 4.6 \times 10^1$	21.2	$1.2 \times 10^4 \pm 3.2 \times 10^2$	25.8	20.0	$2.4 \times 10^4 \pm 3.5 \times 10^3$	100.4
$cusF_{Af}$	28.3	$1.6 \times 10^3 \pm 3.0 \times 10^2$	22.2	$5.7 \times 10^4 \pm 1.1 \times 10^4$	26.0	20.9	$1.3 \times 10^5 \pm 2.8 \times 10^4$	108.3
16S rRNA	12.5	$3.5 \times 10^7 \pm 7.6 \times 10^6$	12.0	$4.6 \times 10^7 \pm 9.3 \times 10^6$	1.0	13.0	$2.4 \times 10^7 \pm 1.9 \times 10^6$	1.0

[a] The induction ratios were determined by comparing the relative transcript cDNA levels from *A. ferrooxidans* grown in the Cu concentrations indicated versus those obtained in control cells grown in the absence of the metal and normalized by 16S rRNA expression in each case. Values of standard deviations were obtained with three biological replicates. CT, PCR cycle at which fluorescence first rises above a threshold background fluorescence.

ATPase (22) and showed a canonical metal-binding site (Table 2). Although a downregulation of the expression of $copB_{Af}$ would be expected in the presence of a high concentration of Cu, $copB_{Af}$ was also induced by the presence of Cu during growth (Table 3). In order to find out about the possible function of $copB_{Af}$, a functional analysis would be required (see below).

The relative copy numbers of $copC_{Af}$ in the absence of Cu was rather high (Table 3), in agreement with the detection of $CopC_{Af}$ in the periplasm of *A. ferrooxidans* under the same conditions (3). In the presence of 25 mM Cu, $copC_{Af}$ was induced about eightfold, strongly suggesting that this protein is involved in Cu resistance in this acidophilic bacterium. $copD_{Af}$ was also induced in the presence of Cu, albeit at a lower level.

Table 3 shows that the genes coding for the potential efflux Cus system from *A. ferrooxidans* were those with the highest induction ratios, reaching values between 100- and 300-fold. This strongly suggests their importance in the Cu resistance mechanism of this extremophile. These results are in agreement with those reported for *E. coli* (13).

The *E. coli* Cus operon is induced at high Cu concentrations (i.e., close to its MIC for this metal). Under these conditions, *E. coli* could directly eliminate Cu to the cell's exterior through the Cus complex, avoiding Cu toxicity on the periplasm and at the same time using its proton motive force. This proton motive force is a very important feature in the acidophilic *A. ferrooxidans*, given that the ΔpH between the cytoplasm and the exterior can be up to 4 pH units.

Cu resistance genes from *A. ferrooxidans* confer higher resistance to the metal when expressed in *E. coli*. Having established the expression of the putative *A. ferrooxidans* Cu resistance determinants under different Cu concentrations made it necessary to search for their functionality. Currently, there is no efficient and reproducible methodology for the generation of knockouts for these genes in this acidophile. Therefore, to ascertain that the *A. ferrooxidans* genes characterized conferred Cu resistance to a heterologous host, they were expressed in *E. coli*. Several of these genes were cloned in the pBAD-TOPO expression vector under the control of a promoter induced by arabinose and repressed by glucose. After these plasmids were used to transform an *E. coli* Δ*copA* mutant, their ability to give this microorganism more resistance to Cu was studied by determining their MICs as described in Materials and Methods. As seen in Fig. 4A, except for $copD_{Af}$, all of the *A. ferrooxidans* putative Cu resistance determinants conferred resistance to Cu when expressed in *E. coli*. On the contrary, $copD_{Af}$ generated a slight Cu sensitivity in *E. coli*. This was a predictable result given that it has been described that when *copD* is expressed in a *P. syringae* mutant lacking the complete *cop* operon, this mutant is more sensitive to Cu than the wild type (2). Nonetheless, the mechanism concerning the functionality of $CopC_{Af}$ and $CopD_{Af}$ in *A. ferrooxidans* is presently unknown.

Luo et al. suggested that $copA1_{Af}$ (AFE0663) was the least important of the two *copA* genes for Cu homeostasis in *A. ferrooxidans* (16). However, when this gene was expressed in the *E. coli* Δ*copA* mutant, it also conferred to the bacteria higher Cu tolerance as seen in Fig. 4A. Therefore, the importance of $copA1_{Af}$ should probably not be underestimated as a Cu resistance determinant in *A. ferrooxidans*.

$copB_{Af}$ also conferred to *E. coli* Δ*copA* a greater resistance to Cu. The results in Fig. 4A suggest that $copB_{Af}$ is more probably related to a Cu efflux protein rather than to a Cu import protein, as had been previously proposed through a bioinformatic prediction (22). Obviously, the exact functional role for $CopB_{Af}$ remains to be proven.

Figure 4B clearly shows that when $cusF_{Af}$ and $copC_{Af}$ are expressed in the *E. coli* Δ*cusCFBA* Δ*cueO* mutant, the MICs for Cu are increased in the bacterium. Although this *E. coli* construction showed low Cu MICs, as reported before (10), it was possible to detect an increased Cu resistance when the two periplasmic Cu chaperones from *A. ferrooxidans* were expressed in it. It is unknown whether $CusF_{Af}$ is able to interact with the *E. coli* Cus system or not. However, it should be highlighted that the Δ*cusCFBA* Δ*cueO* mutant cannot bind $CusF_{Af}$. As a consequence, the observed increase in Cu resistance when both $cusF_{Af}$ and $copC_{Af}$ were expressed (Fig. 4B) could most likely be explained by the capacity of $CusF_{Af}$ and $CopC_{Af}$ to bind the toxic metal.

If any, a fairly smaller increase in Cu resistance was apparent when the entire $cusCBA_{Af}$ operon was expressed in *E. coli* Δ*cusCFBA* Δ*cueO* (Fig. 4B). When the expression of the plasmid containing $cusCBA_{Af}$ was induced by the addition of arabinose to the medium, the mutant cells grew considerably more slowly, most certainly due to a toxic effect caused by the *A. ferrooxidans* proteins. An alternative explanation would be that the $cusCBA_{Af}$ operon only partially complemented the *E. coli* double mutant, perhaps because of the great differences in

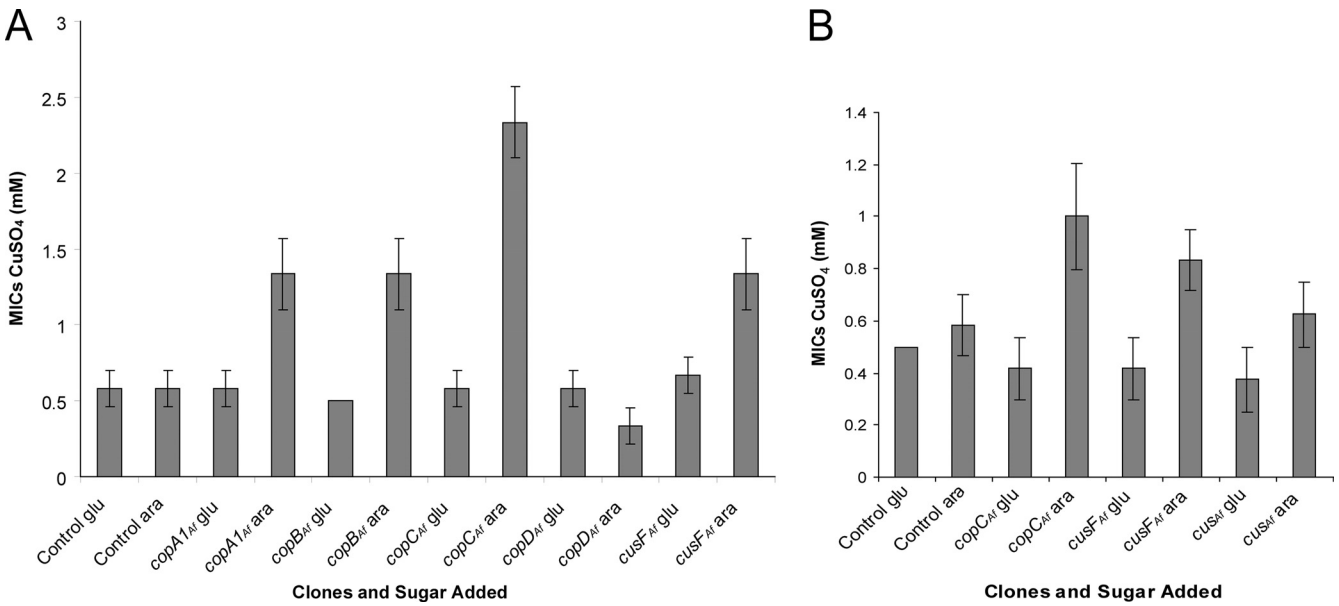

FIG. 4. Heterologous functional analysis of *A. ferrooxidans* genes in *E. coli*. Most of the *A. ferrooxidans* genes predicted to code for Cu resistance determinants were cloned in the vector pBAD-TOPO, and these constructions were used to transform *E. coli* Δ*copA* (A) or Δ*cusCFBA* Δ*cueO* (B) cells. The expression of these genes was induced by the addition of 0.1% arabinose (ara) or repressed by the presence of 0.1% glucose (glu) in the growth medium. The ability of each of these *E. coli* constructions to resist Cu was determined by measuring the MICs. The error bars indicate the standard deviations based on three different experimental values.

periplasmic pH between the neutrophilic and acidophilic microorganisms which may have made it difficult for the *E. coli* mutant to form a functional Cus complex when complemented with the *A. ferrooxidans* proteins.

The results presented here strongly support the functionality of the Cu resistance determinants from *A. ferrooxidans* and will also contribute to the functional annotation of the genes coding for Cu resistance determinants in *A. ferrooxidans*.

The working model in Fig. 5 speculates on the possible relationship between the *A. ferrooxidans* Cu resistance deter-

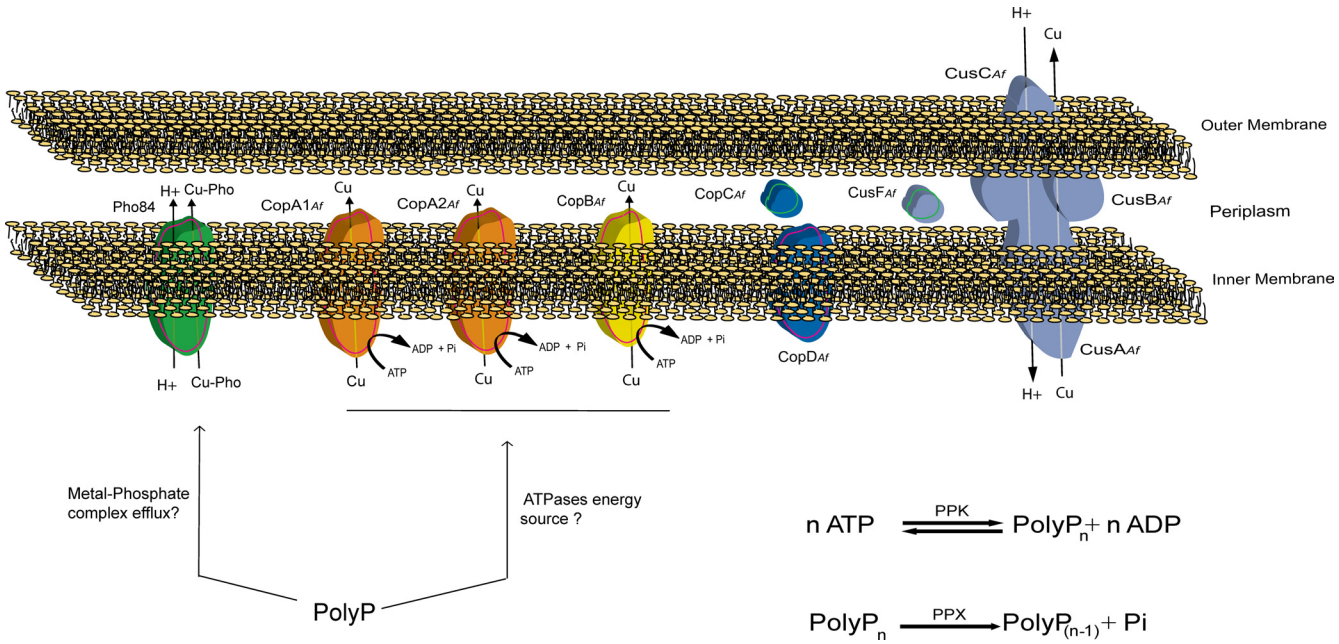

FIG. 5. Scheme summarizing the possible role of Cu resistance determinants in *A. ferrooxidans*. This proposal is based on the expression results presented in this work and previously published results from our laboratory and other laboratories (1, 2, 12, 14, 19). PPK is the polyphosphate kinase enzyme that synthesizes 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 P_i transport system.

minants studied in this work and polyP. When external Cu concentration increases, all of the Cu resistance determinants would be induced in order to eliminate Cu from the periplasm or cytoplasm of the cells. This requires high levels of ATP to activate the metal efflux ATPases. The concomitant decrease of polyP in the presence of Cu (1) may be the result of its hydrolysis by PPX in order to remove Cu-phosphate complexes that arise. Alternatively, the decrease of polyP might be a consequence of its use by the cell in order to regenerate ATP. PolyP is synthesized by PPK by using ATP. However, when there is an 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 supply energy to the metal detoxifying systems as well.

In summary, the current experimental evidence indicates that the high resistance of *A. ferrooxidans* to Cu may be due to part or all of the following key elements: (i) a wide repertoire of Cu resistance determinants, (ii) the duplication of some of these Cu resistance determinants, (iii) the existence of novel Cu chaperones, and (iv) a polyP-based Cu resistance system.

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