Molecular Microbiology (2010) 78(5), 1246–1258 ■
doi:10.1111/j.1365-2958.2010.07402.x
First published online 6 October 2010

Distinct functional roles of homologous Cu^+ efflux ATPases in *Pseudomonas aeruginosa*

Manuel González-Guerrero,† Daniel Raimunda,† Xin Cheng and José M. Argüello*
Department of Chemistry and Biochemistry, Worcester Polytechnic Institute, Worcester, MA 01609, USA.

Summary

In bacteria, most Cu^+-ATPases confer tolerance to Cu by driving cytoplasmic metal efflux. However, many bacterial genomes contain several genes coding for these enzymes suggesting alternative roles. *Pseudomonas aeruginosa* has two structurally similar Cu^+-ATPases, CopA1 and CopA2. Both proteins are essential for virulence. Expressed in response to high Cu, CopA1 maintains the cellular Cu quota and provides tolerance to this metal. CopA2 belongs to a subgroup of ATPases that are expressed in association with cytochrome oxidase subunits. Mutation of *copA2* has no effect on Cu toxicity nor intracellular Cu levels; but it leads to higher H_2O_2 sensitivity and reduced cytochrome oxidase activity. Mutation of both genes does not exacerbate the phenotypes produced by single-gene mutations. CopA1 does not complement the *copA2* mutant strain and vice versa, even when promoter regions are exchanged. CopA1 but not CopA2 complements an *Escherichia coli* strain lacking the endogenous CopA. Nevertheless, transport assays show that both enzymes catalyse cytoplasmic Cu^+ efflux into the periplasm, albeit CopA2 at a significantly lower rate. We hypothesize that their distinct cellular functions could be based on the intrinsic differences in transport kinetic or the likely requirement of periplasmic partner Cu-chaperone proteins specific for each Cu^+-ATPase.

Introduction

Copper is an essential micronutrient required as enzyme co-factor in biological processes such as oxidative respiration (cytochrome oxidases) and free radical protection (Cu,Zn superoxide dismutases) (Desideri and Falconi, 2003; Brunori *et al.*, 2005; Osman and Cavet, 2008). However, excess Cu is detrimental to cells because it produces DNA damaging free radicals (Osman and Cavet, 2008). Cu^+-ATPases are responsible for cytoplasmic Cu^+ efflux in bacterial systems. These constitute the P_{1B-1} subgroup of the P-type ATPases family of transporters (Argüello, 2003; Argüello *et al.*, 2007; Lutsenko *et al.*, 2007). Metal efflux occurs in the presence of ATP when cytoplasmic Cu^+-chaperones deliver the ion to the two-transmembrane Cu^+ transport sites (González-Guerrero and Argüello, 2008; González-Guerrero *et al.*, 2008; 2009). Following the catalytic phosphorylation of the enzyme, Cu^+ is translocated to the periplasmic or extracellular milieus. After the metal is released, the enzyme returns to the initial conformation completing the classical E1/E2 Albers-Post cycle that describes the transport mechanism of all P-type ATPases (Argüello *et al.*, 2007; Lutsenko *et al.*, 2007).

Most archaeal and bacterial genomes encode at least one Cu^+-ATPase that is transcribed in an operon with its Cu^+-chaperone, if present, and a Cu-dependent transcriptional regulator (Osman and Cavet, 2008; Solioz *et al.*, 2010). These particular ATPases are associated with a well-described role in cytoplasmic Cu^+ detoxification (Argüello *et al.*, 2007; Osman and Cavet, 2008; Solioz *et al.*, 2010). In addition, the requirement of these transporters for bacterial virulence has been documented (Francis and Thomas, 1997; Schwan *et al.*, 2005; Zhang and Rainey, 2007; White *et al.*, 2009). However, extra copies of Cu^+-ATPases – up to five – are frequently present in the genomes of pathogenic/symbiotic bacteria (Preisig *et al.*, 1996; Francis and Thomas, 1997; Argüello, 2003; Agranoff and Krishna, 2004; Schwan *et al.*, 2005; Pontel *et al.*, 2007). Characterization of rhizobial *fixI* genes (Kahn *et al.*, 1989; Preisig *et al.*, 1996), *Rhodobacter capsulatus ccoI* (Koch *et al.*, 2000) and recently *Rubrivivax gelatinosus ctpA* (Hassani *et al.*, 2010) has shown that the extra Cu^+-ATPase genes are in some cases associated with cytochrome *c* oxidases coding regions. Deletion of *fixI*, *ccoI* or *ctpA* leads to impaired oxidase activity, a phenotype that is reversed by supplementation of the media with Cu^{2+} (Kahn *et al.*, 1989; Preisig *et al.*, 1996; Koch *et al.*, 2000; Hassani *et al.*, 2010). To explain these phenotypes, the authors proposed that these particular ATPases are involved in Cu^+

Accepted 14 September, 2010. *For correspondence. E-mail arguello@wpi.edu; Tel. (+1) 508 831 5326; Fax (+1) 508 831 4116. †These authors contributed equally to this work.

influx, rather than Cu^+ efflux. However, it is challenging to consider molecular mechanisms compatible with alternative transport directions given the high structural homologies and the full conservation of key functional residues among all Cu^+-ATPases.

Toes *et al.* recently pointed out the phylogenetic proximity among FixI-like Cu^+-ATPases while studying a subset of ATPases from *Shewanella* and *Vibrio* species (Toes *et al.*, 2008). We extended a similar analysis to all available bacterial genomes (180 genomes as of December 2009) and identified 56 sequences in a 'FixI/CopA2-like Cu^+-ATPases' subgroup (Fig. 1 and Table S1). These are present in almost 30% of the sequenced bacteria and accounted for 17% of the sequences included in the analysis. Interestingly, branches close to this subgroup contain *Enterococcus hirae copA*, and *Synechocystis* PCC6803 and *Synechococcus* PCC7942 *ctaA*s. Mutation of these genes does not lead to increased Cu sensitivity; on the contrary, it might result in reduced intracellular Cu levels or increased Cu tolerance (Odermatt *et al.*, 1993; Phung *et al.*, 1994; Tottey *et al.*, 2001). These data are consistent with these ATPases driving Cu^+ influx as proposed for FixI/CopA2-like proteins.

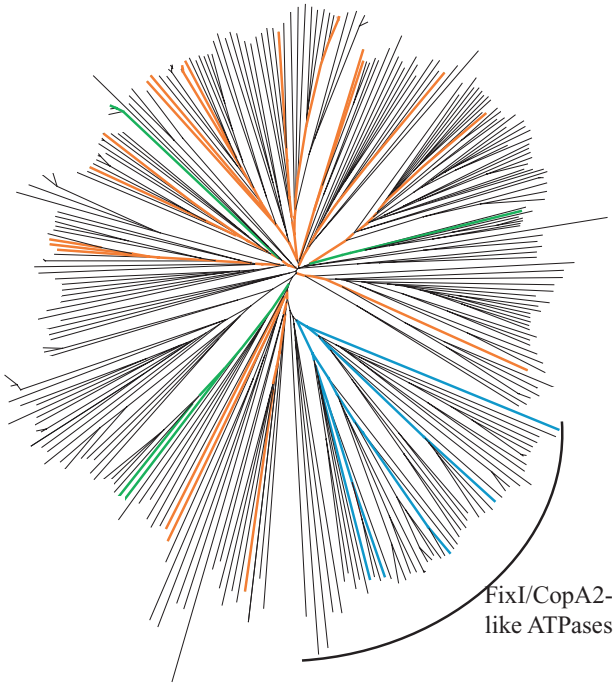

Fig. 1. Unrooted tree of Cu^+-ATPases from all sequenced bacteria. FixI/CopA2-like sequences are listed in Table S1. Characterized Cu^+-ATPases in this subgroup are indicated in blue (*R. capsulatus* CcoI, *B. japonicum* FixI, *S. meliloti* FixI, *R. gelatinosus* CtpA, *P. aeruginosa* CopA2). Characterized Cu^+-ATPases involved in Cu^+ detoxification are indicated in orange and listed in Table S2. *E. hirae* CopA, *L. monocytogenes* CtpA, *Synechocystic PCC6803* CtaA and *Synechococcus elongatus* PCC7942 CtaA are indicated in green.

Considering their evident structural similarities and identical key functional elements, we hypothesize that the homologous Cu^+-ATPases present in a given organism perform a similar biochemical work; i.e. they drive cytoplasmic Cu^+ efflux. However, because of different transcriptional regulation, kinetic characteristics or other factors, they play distinct cellular functions. To test these ideas and explain phenotypical observations within the structural and mechanistic constrains of P-type ATPases, we studied the Cu^+-ATPases present in *Pseudomonas aeruginosa* PAO1. This opportunistic pathogen of plants and animals appears to be a parsimonious model. It only has two Cu^+-ATPases, CopA1 (CueA)[1] (PA3920) and CopA2 (PA1549), each representing the two referred ATPase subtypes. Both proteins are highly homologous (50% similarity, 35% identity). They have the same membrane topology, identical transmembrane Cu^+ binding sites and two cytoplasmic metal binding domains in their N-terminus (N-MBDs). CopA1 is expressed in response to Cu^{2+} in the culture medium (Teitzel *et al.*, 2006; Thaden *et al.*, 2010) and mutation of *copA1* leads to the lack of Cu tolerance and virulence (Schwan *et al.*, 2005; Teitzel *et al.*, 2006). Although CopA2 has not been characterized, it does not appear to be expressed in response to extracellular Cu^{2+} (Teitzel *et al.*, 2006).

Results

Cellular roles of CopA1 and CopA2

To understand the biochemical basis for the distinct roles that homologous Cu^+-ATPases play in *P. aeruginosa*, phenotypes of a *copA1* mutant strain were compared with those of the *copA2* mutant under identical experimental conditions. This was initiated by monitoring the response of the mutant strains to Cu^{2+} exposure. Figure 2A shows that, as expected, *copA1* mutant strain did not grow in the presence of high Cu^{2+} levels. The wild-type phenotype was restored by reintroducing the *copA1* gene under the control of 500 bp up stream fragment containing its promoter region. Consistent with these observations, *copA1* expression was activated by the metal (Fig. 2B) and the strain lacking the CopA1 function accumulated more Cu than either wild-type or *copA2* mutant strains when exposed to the metal (Fig. 2C). These results are similar to those previously reported for deletion of this and others classical Cu^+-ATPases associated with Cu^+ detoxification (Schwan *et al.*, 2005; Teitzel *et al.*, 2006; Osman and Cavet, 2008; Solioz *et al.*, 2010). In contrast, the *copA2* mutant strain

[1] Cu^+-ATPases have previously received an array of names: CopA, PacS, CueA, SilP, CtaA, ActP, CtpA, CtpB, CtpV, FixI, CcoI, etc. Following the nomenclature given to funding members of this family, we have chosen to refer the ubiquitous Cu^+-ATPase as CopA1 and the second form as CopA2.

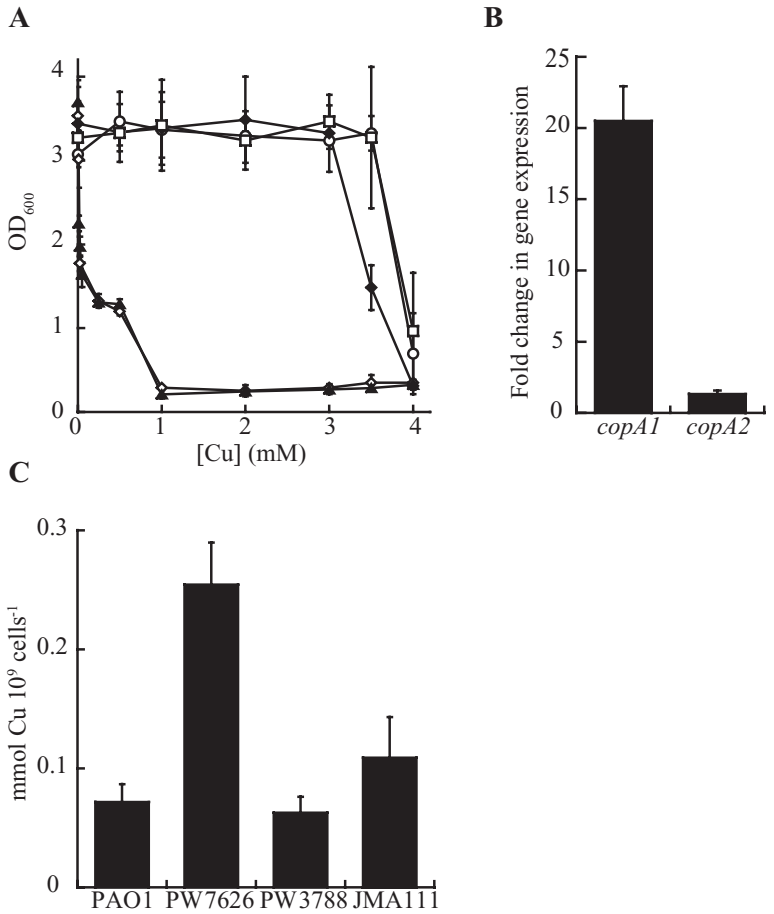

Fig. 2. Roles of CopA1 and CopA2 in Cu tolerance.
A. Effects of increasing concentrations of Cu^{2+} on the growth of *P. aeruginosa* PAO1 (wild-type, ○), PW7626 (*copA1* mutant, ◇), PW3788 (*copA2* mutant, □), JMA0 (*copA1, copA2* double mutant, ▲) and JMA111 (*copA1* mutant transformed with *copA1* under control of its own promoter, ◆). Turbidity (OD_{600}) was determined after 16 h of growth in LB liquid medium at 37°C.
B. Cu-dependent regulation of *copA1* and *copA2* expression. Gene expression levels were determined by qPCR on RNA extracted from cultures grown in the presence of Cu^{2+} and standardized to the expression levels of *proC*.
C. Cu content of *P. aeruginosa* strains exposed to 0.2 mM $CuSO_4$. Data are the mean ± SE of three independent experiments.

was not susceptible to high Cu^{2+} concentrations and its expression was not modified by the presence of the metal (Fig. 2A and B). Interestingly, this strain also showed a small but consistent decrease in intracellular Cu level after a relatively short exposure to the metal (Fig. 2C). Experiments testing the sensitivity of the *copA2* mutant strain to other metals showed no significant defects (data not shown).

It has been proposed that FixI/CopA2-like ATPases are involved in assembly of cytochrome *c* oxidase, a copper requiring redox enzyme (Kahn *et al.*, 1989; Preisig *et al.*, 1996; Koch *et al.*, 2000; Hassani *et al.*, 2010). *P. aeruginosa copA2* is located downstream two sets of cytochrome oxidase subunits coding genes (*cbb$_3$-1* and *cbb$_3$-2*) (Comolli and Donohue, 2004). The co-transcription of these genes was verified by RT-PCRs of the intergenic regions between contiguous open reading frames (ORFs) (Fig. S1). Testing the role of CopA2, the response of the mutant strains to oxidative stressors and their cellular oxidase activity was measured. The *copA2* mutant strain did not grow in the presence of H_2O_2, while the *copA1* mutant was insensitive to peroxide (Fig. 3A). Similar results were observed when paraquat was included in the culture medium (data not shown). However, neither exposure for 30 min to 30 mM H_2O_2 nor overnight growth in the presence of 400 μM paraquat induced expression of *copA1* or *copA2* in the wild-type strain (data not shown). The requirement of CopA2 for cytochrome oxidase functionality was shown by the decrease in cellular oxidase activity observed in the *copA2* mutant strain (PW3788) (Fig. 3B). On the contrary, the *copA1* mutant strain showed a modest increase in oxidase activity as compared with the wild-type strain. The reduced activity in the *copA2* mutant was corrected by including 10 μM Cu^{2+} in the growth media. A similar reversal of this phenotype by high extracellular Cu^{2+} was previously observed for *R. capsulatus* strains where the Cu^{+}-ATPase *ccoI* was mutated (Koch *et al.*, 2000). Reintroducing *copA2* under the control of its operon promoter region (500 bp downstream of PA1557, see Fig. S1) restored the wild-type phenotype (Fig. 3).

Considering the high structural homology of CopA1 and CopA2, and assuming that they perform the same biochemical work (i.e. cytoplasmic Cu^{+} efflux), partial redundancy in the cellular functions of these proteins could be assumed. However, the double mutant strain (JMA0, lacking functional *copA1* and *copA2*) behaved as the *copA1* mutant with respect to Cu^{+} tolerance (Fig. 2A, note similarities in the 0–1 mM Cu^{+} range) and as the *copA2*

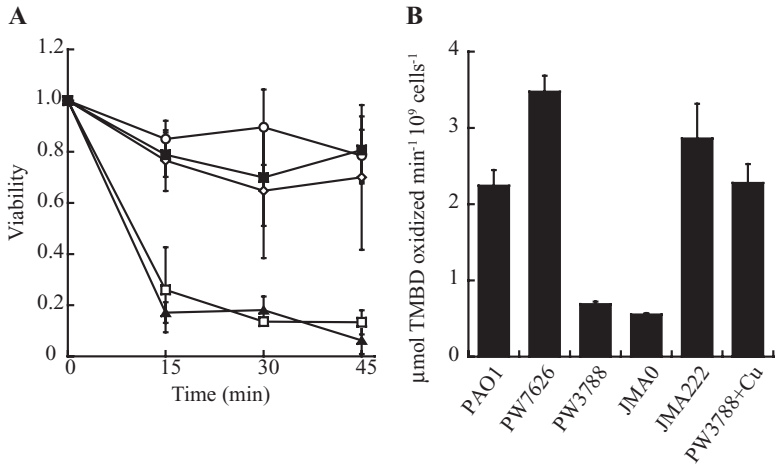

Fig. 3. Roles of CopA1 and CopA2 in oxidative stress tolerance.
A. Cell survival of *P. aeruginosa* PAO1 (○), PW7626 (*copA1* mutant, ◇), PW3788 (*copA2* mutant, □), JMA0 (*copA1, copA2* double mutant, ▲) and JMA222 (*copA2* mutant transformed with *copA2* under its own promoter, ■) after treatment with 30 mM H_2O_2 in LB medium for various lengths of time. Cell viability is shown as the ratio of cfu of treated/untreated cultures.
B. TMPD oxidase activity of whole cells of *P. aeruginosa* strains. Cu^{2+} (10 μM) was included in the media when indicated. Data are the mean ± SE of three independent experiments.

mutant when the oxidase activity was assessed (Fig. 3). These observations suggest that the proteins cannot compensate, even partially, for each other.

Importance of CopA1 and CopA2 for P. aeruginosa *virulence*

It was previously reported that Cu^+-ATPases responsible for Cu^+ detoxification, including *P. aeruginosa* CopA1, play a crucial role in bacterial virulence (Francis and Thomas, 1997; Schwan *et al.*, 2005; Zhang and Rainey, 2007; White *et al.*, 2009). Considering that bacterial oxidases with high O_2 affinity are likely required in the host microaerobic environments (Kahn *et al.*, 1989; Preisig *et al.*, 1996; Myllykallio and Liebl, 2000) and that the hypersensitive response in plants produces free radicals to eliminate the invading pathogenic bacteria (Levine *et al.*, 1994; Jabs *et al.*, 1997), the relevance of FixI/CopA2-like ATPases such as *copA2* for pathogenicity was explored. A simple assay that takes advantage of *P. aeruginosa* capability to infect plants (Starkey and Rahme, 2009) was used. Figure 4A shows that indeed both *P. aeruginosa* Cu^+-ATPases were required for host infection. Moreover, expression of both proteins was greatly stimulated by the host environment (Fig. 4B). Complementation with the wild-type genes reverted the lack of virulent phenotypes.

Biochemical function of P. aeruginosa *Cu⁺-ATPases*

The observed lack of redundancy in the cellular function of these ATPases might be associated with their different transcriptional regulation. This possibility was previously postulated to explain phenotypical effects of corresponding mutants (Hassani *et al.*, 2010). Testing this

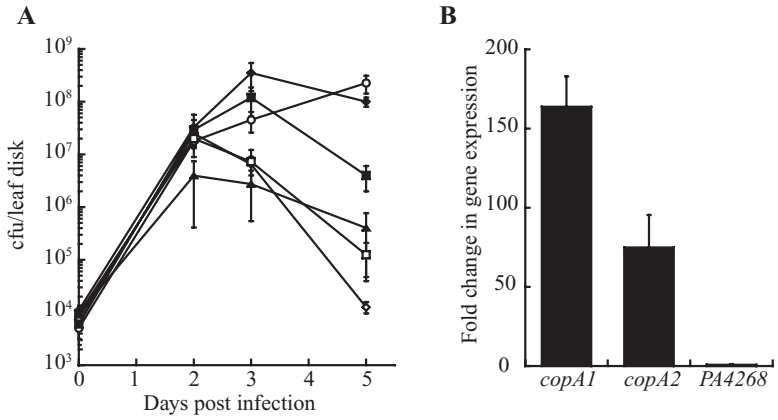

Fig. 4. Role of CopA1 and CopA2 in infection.
A. Growth of *P. aeruginosa* PAO1 (○), PW7626 (*copA1* mutant, ◇), PW3788 (*copA2* mutant, □), JMA0 (*copA1, copA2* double mutant, ▲), JMA111 (*copA1* mutant transformed with *copA1* under control of its own promoter, ◆) and JMA222 (*copA2* mutant transformed with *copA2* under its own promoter, ■) in *A. thaliana* leaves.
B. *copA1*, *copA2* and *PA4268* (30S ribosomal protein S12, constitutive gene) expression in *A. thaliana* leaves compared with bacteria grown for 16 h in LB (free living conditions). Gene expression levels were standardized to the expression levels of *proC*. Data are the mean ± SE of three independent experiments.

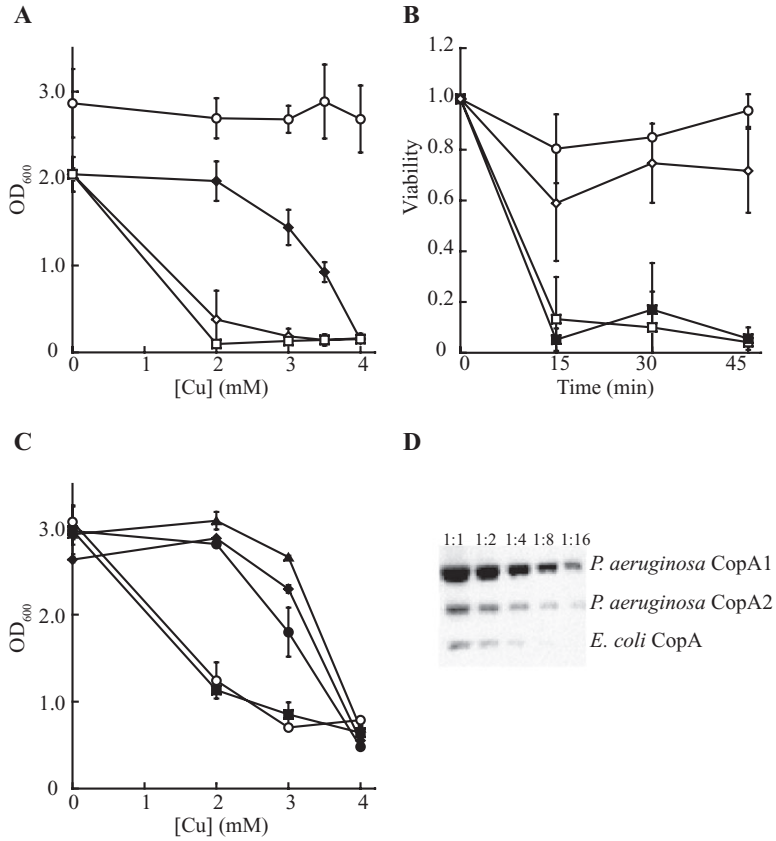

Fig. 5. A. Cu^{2+} sensitivity of *P. aeruginosa* PAO1 (○), PW7626 (*copA1* mutant, ◇), JMA121 (*copA1* mutant transformed with *copA1* under the *copA2* promoter, ◆) and JMA112 (*copA1* mutant transformed with *copA2* under the *copA1* promoter, □) in LB liquid medium. Turbidity (OD_{600}) was determined after 16 h of growth at 37°C.
B. Cell survival of *P. aeruginosa* PAO1 (○), PW3788 (*copA2* mutant, □), JMA221 (*copA2* mutant transformed with *copA1* under the *copA2* promoter, ■) and JMA212 (*copA2* mutant transformed with *copA2* under the *copA1* promoter, ◇), after treatment with 30 mM H_2O_2 in LB medium for the indicated times. Cell viability is shown as the ratio of cfu of treated/untreated cultures.
C. Cu^{2+} sensitivity of *E. coli* LMG194 (wild-type, ▲), DC194 (Δ*copA*, ○), DC194-EcCopA (Δ*copA* heterologously expressing *E. coli copA,* ●), DC194-CopA1 (Δ*copA* expressing *P. aeruginosa copA1,* ◆) and DC194-CopA2 (Δ*copA* expressing *P. aeruginosa copA2,* ■) in LB liquid medium. Turbidity (OD_{600}) was determined after 16 h of growth at 37°C. Data are the mean ± SE of three independent experiments.
D. Protein expression levels in *E. coli* DC194 expressing *P. aeruginosa copA1*, *copA2* or *E. coli copA*. Identical amounts of whole-cell proteins from each strain were subject to serial dilution, separated in SDS-PAGE and the corresponding Western blot immunostained with anti-His-tag antibody. Dilution factors are indicated above the figure.

hypothesis, the complementation of the *copA1* mutant strain (PW7626) by *copA2* under the control of the *copA1* promoter region (JMA112 strain) was attempted. For comparison, complementation of the *copA1* mutant by *copA1* under the control of *copA2* promoter (JMA121) was performed. Conversely, complementation of the *copA2* mutant (PW3788) by *copA1* under the control of the *copA2* promoter region (JMA221) and *copA2* under the control of the *copA1* promoter (JMA212) was also analysed. Figure 5A and B show the results of challenging these strains with either high Cu^{2+} or H_2O_2. Significantly, neither protein could complement the absence of the other independently of the driving promoter. This is, the *copA1* mutant strain JMA112 was sensitive to high Cu^{2+}, while the JMA221 was unable to growth in the presence of H_2O_2. It is also interesting that the proteins could partially complement their respective mutants even when under the control of the alternate promoter (JMA121 and JMA212), suggesting that the basal expression under these conditions was sufficient to satisfy physiological requirements.

Escherichia coli single Cu^+-ATPase, CopA, drives cytoplasmic Cu^+ efflux. Then, analysis of the capability of *P. aeruginosa* ATPases to complement the Δ*copA E. coli* DC194 strain provided an opportunity to explore their functions under a common arabinose-inducible promoter. In this system, while *copA1* complemented the lack of endogenous enzyme, *copA2* did not (Fig. 5C). A key factor in these experiments was the relative expression of the heterologous enzymes since a lack of complementation might have been due to low expression. Since the three tested protein constructs had a His-tag, Western blots with the corresponding antibody showed that both *P. aeruginosa* CopA1 and CopA2 were expressed at much higher levels than *E. coli* CopA under the same promoter (Fig. 5D).

Based on phenotypes similar to those described here, it has been suggested that FixI/CopA2-like ATPases are Cu^+ importers (Preisig *et al.*, 1996; Koch *et al.*, 2000; Osman and Cavet, 2008; Hassani *et al.*, 2010). To determine the direction of transport of CopA1 and CopA2, sealed everted vesicles were prepared from Δ*copA E. coli* strains expressing these proteins and their Cu^+ transport capability was measured. This system is composed of mixed vesicle populations: an 'inside-out' pool where the cytoplasmic side of the membrane faces the outside of the vesicle; and a 'right-side-out' population where the periplasmic side of the membrane faces outside. However, because ATP is present in the uptake media (not inside the vesicles) only those ATPase molecules present in

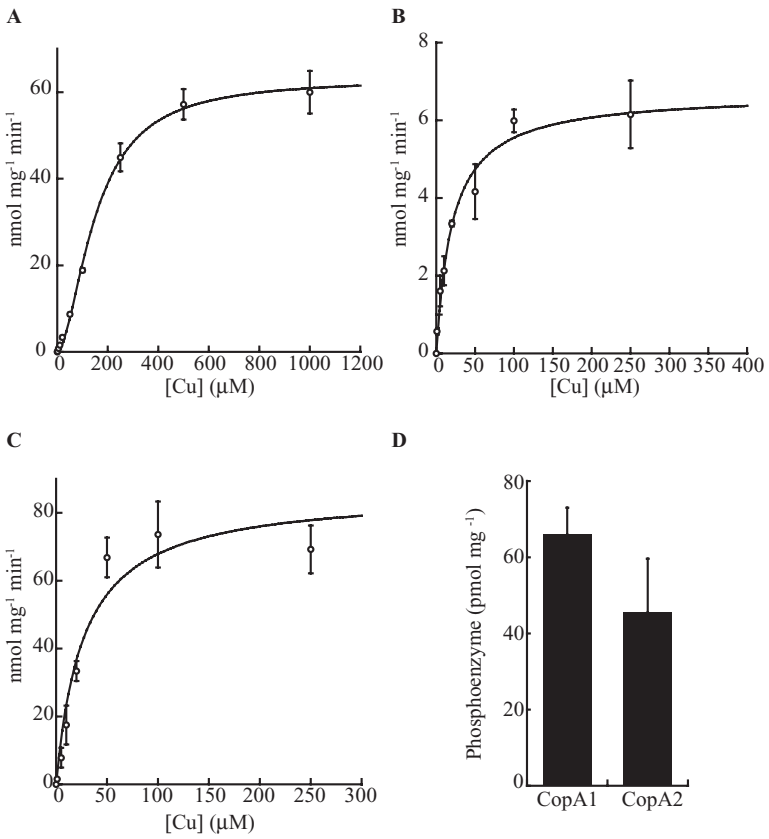

Fig. 6. Cu^+ transport kinetics in *E. coli* DC194 everted vesicles expressing *P. aeruginosa* CopA1 (A), *P. aeruginosa* CopA2 (B) and *E. coli* CopA (C). Data of Cu^+ transport by *P. aeruginosa* CopA1 were fitted by using $K_{1/2} = 152.6 \pm 7.9$ μM and $V_{max} = 63.1 \pm 1.5$ nmol mg^{-1} h^{-1}. Data of Cu^+ transport by *P. aeruginosa* CopA2 were fitted by using $K_{1/2} = 20.7 \pm 3.7$ μM and $V_{max} = 6.7 \pm 0.4$ nmol mg^{-1} h^{-1}. Data of Cu^+ transport by *E. coli* CopA were fitted by using $K_{1/2} = 27.2 \pm 8.5$ μM and $V_{max} = 86.3 \pm 8.4$ nmol mg^{-1} h^{-1}. In all cases, Cu^+ uptake by vesicles from empty vector transformed *E. coli* was subtracted prior to data analysis. (D) Cu^+-dependent phosphorylation of CopA1 and CopA2. These were calculated as the difference between phosphorylation levels in the presence and the absence of Cu^+. Data are the mean ± SE of three independent experiments.

inside-out vesicles are functional during the assays (Mana-Capelli *et al.*, 2003; Yang *et al.*, 2007). Figure 6A and B show that vesicles containing *P. aeruginosa* CopA1 or CopA2 were able to uptake Cu^+ in the presence of ATP, suggesting that both enzymes drive Cu^+ efflux from the cytoplasm. For comparison, Fig. 6C shows the uptake observed in similarly prepared vesicles containing *E. coli* CopA. Vanadate, a classical inhibitor of P-type ATPases, inhibited the $^{64}Cu^+$ transport by these enzymes (Table S4). Vesicles prepared from Δ*copA E. coli* transformed with empty vector did not show any significant amount of $^{64}Cu^+$ transport (Fig. S2). Similarly, no transport of $^{64}Cu^{2+}$ was observed when these experiments were performed in the absence of reducing agents (data not shown).

Figure 6 shows important differences in the transport kinetics of these proteins, particularly the significantly slower turnover rate and smaller $K_{1/2}$ for Cu^+ activation of CopA2. While these parameters might explain the observed phenotypes (see the Discussion section), it could still be argued that in addition to the observed small efflux, CopA2 might drive a significant periplasmic Cu^+ influx responsible for its cellular role. Although considered unlikely, this possibility was tested. Vesicles were loaded with Cu^+ in the presence of ATP during 60 min, subsequently washed to remove external Cu^+ and ATP, and further incubated in the presence and in the absence of ATP. In these conditions, if CopA2 were a Cu^+ importer, Cu^+ release from the inside of the vesicle (equivalent to the periplasm) to the outside (cytoplasm) should be detected. However, Cu^+ efflux from loaded vesicles was not detected after following the reaction for up to 20 min (Table S4). In a similar experiment, the possibility of periplasmic Cu^{2+} influx through CopA2 was tested. After Cu^+ loading in the presence of ATP, vesicles were washed to remove external Cu^+, ATP and reducing agents. Cu^+-loaded vesicles were incubated (20 min) with permeable (paraquat, diamide, H_2O_2) and impermeable ($KMnO_4$) oxidizing reagents. After this treatment vesicles were washed and Cu^{2+} transport (from the periplasmic to the cytoplasmic side) tested by the addition of ATP to the media. Again, no flux was observed after monitoring the reaction for 20 min (Table S4).

The structural coupling between ATP hydrolysis and substrate transport is a hallmark of P-type ATPases (Kaplan, 2002; Toyoshima and Inesi, 2004). In these, ATP hydrolysis and enzyme phosphorylation require the binding of the outwardly transported substrate to the cytoplasmic facing transmembrane sites. On the other hand, enzyme dephosphorylation is associated with substrate binding sites opening towards the extracellular/organellar compartments (Kaplan, 2002). Figure 6D shows the Cu^+-dependent phosphorylation of CopA1 and CopA2,

further supporting the hypothesis that Cu^+ is outwardly transported. It should be noted that both proteins are phosphorylated even in the absence of Cu^+. This accounted for 50–60% of the total activity in the presence of Cu^+. Although unconventional, this is not unique to *P. aeruginosa* Cu^+-ATPases. Inesi and his collaborators have characterized in detail a similar phenomenon in *Thermotoga maritima* Cu^+-ATPase (Hatori *et al*., 2008).

Discussion

Previous studies have shown the frequent presence of two or more Cu^+-ATPase genes in bacterial genomes (Preisig *et al*., 1996; Francis and Thomas, 1997; Argüello, 2003; Agranoff and Krishna, 2004; Schwan *et al*., 2005; Pontel *et al*., 2007; Toes *et al*., 2008). Bioinformatics analysis of the bacterial Cu^+-ATPases shows that as a result of gene duplication, at least one new subfamily with a different function has appeared, the FixI/CopA2-like ATPases. The acquisition of *copA2* probably occurred early in bacterial evolution since it is distributed in various bacterial phyla and within somewhat different genetic environments. Data presented in this manuscript show that homologous Cu^+-ATPases perform their canonical function of transporting cytoplasmic Cu^+ into the periplasmic/extracelullar compartments, although they might be involved in quite different cellular roles (Cu^+ tolerance and cuproprotein assembly).

P. aeruginosa *Cu^+-ATPases are not functionally equivalent*

Transcription of *copA1* was upregulated by Cu. Mutation of this gene resulted in lower tolerance to this metal, as well as increased cytoplasmic Cu levels. CopA1 was also able to restore the Cu^+ tolerance in a Δ*copA E. coli* strain. These predictable observations are clearly linked to the role of CopA1 driving cytoplasmic Cu^+ efflux across the plasma membrane. On the other hand, mutation of the homologous *copA2* had no effect on *P. aeruginosa* Cu sensitivity not in cytoplasmic Cu levels. Transcription of the gene was not upregulated by the metal nor it complemented Δ*copA E. coli*. Thus, CopA2 appears irrelevant for the control of the cytoplasmic Cu level. In fact, removal of *copA2* function in the background of the *copA1* mutant did not exacerbate the phenotypes observed in single mutants. *copA2* was co-transcribed with cytochrome *c* oxidase subunits, and its mutation led to a deficiency in the activity of this cuproprotein. Therefore, CopA2 seems involved in Cu loading during assembly of the oxidase. Interestingly, this phenotype was reversed by increasing extracellular Cu^{2+}. Following the assumption that under this condition cytoplasmic Cu^+ level was increased, it was proposed that the FixI/CopA2 proteins drive influx of periplasmic Cu^+ into the cell (Preisig *et al*., 1996; Koch *et al*., 2000; Osman and Cavet, 2008; Hassani *et al*., 2010). This indirectly implies that Cu should access the cytochrome *c* oxidase from the cytoplasmic side. However, there is no experimental evidence for this mechanism. Most importantly, since an increase of extracellular Cu^{2+} would raise both periplasmic and cytoplasmic Cu pools, it could be argued that the described experimental data are inconclusive on the direction of CopA2 transport or whether Cu is loaded into the oxidase from the periplasmic or cytoplasmic compartments.

Involvement of Cu^+-ATPases in virulence

Previous works have indicated that CopA1-like ATPases are involved in infectivity (Francis and Thomas, 1997; Schwan *et al*., 2005; Zhang and Rainey, 2007; White *et al*., 2009). It has been recently shown that the macrophage loads Cu^+ into the phagosome as part of its bactericide strategies (White *et al*., 2009). Consequently, a role of Cu^+ efflux in bacterial virulence is immediately apparent. Our results support this mechanism and extend it to plant systems. On the other hand, the role of CopA2 in virulence had not been explored. We observed that this protein was also required for survival in the host environment, although this does not mean a common role for both ATPases. CopA2 by participating in the synthesis of cytochrome *c* oxidases would be probably involved in obtaining energy in the relatively micro-anaerobic host tissues and overcoming the oxidative stress. An oxidative burst is a well-described response to pathogens (Shepherd, 1986; Torres *et al*., 2006).

The different physiological roles of Cu^+-ATPases are not due to transcriptional control

It has been reasonably argued that the distinct physiological roles of Cu^+-ATPases might be the result of a different gene regulation (Hassani *et al*., 2010). This possibility was tested by exchanging promoter regions, i.e. *copA1* regulated by *copA2* promoter and vice versa, in mutant strain complementation experiments. In these, CopA1 did not complement the *copA2* mutant, nor did CopA2 restore the *copA1* phenotype. This indicates that although the genes are transcriptionally regulated, their functional roles cannot be interchanged and these are determined by structural/functional characteristics intrinsic to each of these proteins. Nevertheless, it might be hypothesized that if expression levels were sufficiently increased, complementation by the non-mutated gene could perhaps be achieved. The experiments performed on a Δ*copA E. coli* strain, in which both *copA1* and *copA2* are expressed at high levels under an arabinose-inducible promoter, suggest that this is not the case. Even though CopA2 was

produced at much higher levels than *E. coli* CopA, this was not enough to even partially restore the wild-type tolerance to Cu.

Both Cu^+-ATPases drive cytoplasmic Cu^+ efflux

To explain the phenotypes resulting from mutating FixI/CopA2-like ATPases, it has been postulated that these ATPases are Cu^+ importers (Preisig *et al.*, 1996; Koch *et al.*, 2000; Osman and Cavet, 2008; Hassani *et al.*, 2010). If this were the case, the influx of periplasmic Cu^+ would be coupled to the hydrolysis of cytoplasmic ATP. Contrary to this, everted vesicles containing either CopA1 or CopA2 were loaded with Cu^+ when both ATP and Cu^+ were present in the assay media, i.e. the cytoplasmic side of the membranes. These observations are supported by a number of control experiments confirming the identity of the transporter (either P-type ATPase), the transported substrate (Cu^+) and the direction of transport (cytoplasmic Cu^+ efflux). Further supporting a similar transport mechanism and direction, the phosphorylation of CopA1 and CopA2 by ATP was stimulated by Cu^+. Classical studies of P-type ATPases, such us the Na,K-ATPase, Ca-ATPase or H,K-ATPase, have shown that the outwardly transported substrates stimulate enzyme phosphorylation by binding to the inwardly facing transmembrane binding sites (Kaplan, 2002; Toyoshima and Inesi, 2004; Argüello *et al.*, 2007). Alternatively, for instance in the Na,K- and H,K-ATPases, inwardly transported substrates inhibit phosphorylation by ATP (Kaplan, 2002).

While both CopA1 and CopA2 carried Cu^+ from the cytosol to the periplasm, they did this with distinct kinetic characteristics. These might explain the different physiological roles of these pumps. CopA1 has a lower apparent affinity but a much faster transport rate than CopA2. Then, it could be proposed that CopA2 cannot compensate for the high transport rate of Cu^+ detoxifying ATPases (CopA1 or *E. coli* CopA) and therefore it is incapable to function in this role. Similarly, a role in cuproproteins biosynthesis might not require a high rate of transport but rather a high affinity for the substrate that ensures its supply to the target protein. However, in this analysis we must keep in mind that contrary to the transport experiment conditions, under physiological conditions Cu^+ accesses the transport sites bound to its corresponding chaperone (González-Guerrero and Argüello, 2008). Then, the observed Cu^+ $K_{1/2}$ might not accurately represent the CopA1 and CopA2 relative affinities for Cu^+.

Although the kinetic characteristics might be a key element in the cellular roles of these enzymes, some of the observed results hint at the presence of additional component/s in these systems. Consider for instance that while the increase of Cu^+ in the media could compensate for the lack of functional CopA2, expression of CopA1 under the *copA2* promoter region did not. Although cytoplasmic Cu^+ transfer to Cu^+-ATPases is relatively well understood, very little is known on how Cu^+ is released to the periplasm/exterior of the cell. Since Cu^+ chaperones exist in the periplasm, it could be hypothesized that the Cu^+-ATPases specifically interact and transfer Cu^+ to these proteins rather than simply releasing the metal into the compartment. Then, it is tempting to speculate that the accepting periplasmic chaperone would be different for either CopA1 or CopA2. For instance, a key role of periplasmic Cu^+ binding proteins, SenC/SCO1 and Cox11, in synthesis of bacterial cytochrome oxidases has already been postulated (Frangipani and Haas, 2009; Thompson *et al.*, 2010). It is interesting to note that the *senC* deletion mutant showed similar decrease in cell cytochrome oxidase activity as *copA2* mutant and that in both cases activities could be rescued by adding Cu to the growth media.

In summary, our results show that FixI/CopA2-like proteins constitute a distinct subgroup of Cu^+-ATPases. As previously characterized Cu^+-ATPases, they drive the efflux of cytoplasmic Cu^+. However, they present distinct kinetic characteristics in tone with their specific function in cuproprotein assembly.

Experimental procedures

Bacterial strains and growth conditions

Pseudomonas aeruginosa PAO1 wild-type strain, PW7626 (PA3920/*copA1* insertional mutant) and PW3788 (PA1549/*copA2* insertional mutant) were obtained from the Comprehensive *P. aeruginosa* Transposon Mutant Library at the University of Washington Genome Center (Jacobs *et al.*, 2003) (Table 1). The *E. coli* strains CC118 (λpir) (pTNS1) and HB101 (pRK2013) were provided by H.P. Schweizer (Colorado State University) (Choi and Schweizer, 2006). LMG194 and DC194 (LMG194 Δ*copA*) were a gift of B.P. Rosen (Florida International University) (Rensing *et al.*, 2000). Double mutant strain JMA0 was generated from parental strain *P. aeruginosa* PW7626. An internal fragment corresponding to 600 bp of the gene PA1549 was cloned into pCHESIΩKm, between KpnI and XbaI sites (Llamas *et al.*, 2003). Subsequently, this plasmid was mobilized from *E. coli* DH5α cells into *P. aeruginosa* PW7626 by triparental conjugation using *E. coli* HB101 (pRK2013) strain as helper. Transconjugants were selected on selective Luria–Bertani (LB) agar plates. JMA111-JMA222 strains were obtained by cloning 500 bp of the promoter region regulating either *copA1* or *copA2* with the ORF of either *copA1* or *copA2* in the HindIII–SpeI sites of pUC18T-miniTn7T-Gm (Choi and Schweizer, 2006). Since *copA2* is part of a polycistronic operon, the promoter region was first identified by successive RT-PCRs of the intergenic regions between contiguous ORFs (Fig S1). The megaprimer-PCR method was employed to obtain the fusion of *copA2* with its promoter, as well as, its fusion with the *copA1* promoter (Maniatis *et al.*, 1989). The primers used in this and other procedures are listed in

Table 1. Strains used in this study.

	Relevant characteristics	Reference
P. aeruginosa strains		
PAO1	Wild-type	
PW3788	IS*phoA* insertion in *copA2* (PA1549)	Jacobs *et al.* (2003)
PW7626	IS*phoA* insertion in *copA1* (PA3920)	Jacobs *et al.* (2003)
JMA0	IS*phoA* insertion in *copA1*, *copA2*::pCHESIΩ-Km	This study
JMA111[a]	PW7626::mini-Tn7T-P_{copA1}-*copA1*[b]	This study
JMA222	PW3788::mini-Tn7T-P_{copA2}-*copA2*	This study
JMA121	PW7626::mini-Tn7T-P_{copA2}-*copA1*	This study
JMA112	PW7626::mini-Tn7T-P_{copA1}-*copA2*	This study
JMA221	PW3788::mini-Tn7T-P_{copA2}-*copA1*	This study
JMA212	PW3788::mini-Tn7T-P_{copA1}-*copA2*	This study
E. coli strains		
CC118(λpir)	Helper strain for *P. aeruginosa* transformation	Choi and Schweizer (2006)
HB101	Helper strain for *P. aeruginosa* transformation	Choi and Schweizer (2006)
DH5α	*supE44* Δ*lac*U169 (φ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *rel*A1	Invitrogen, Carlsbad, CA, USA
LMG194	Wild-type	Invitrogen, Carlsbad, CA, USA
DC194	LMG194 Δ*copA*	Rensing *et al.* (2000)
DC194-EcCopA	LMG194 Δ*copA* transformed with pBAD-*E. coli copA*	This study
DC194-CopA1	LMG194 Δ*copA* transformed with pBAD-*P. aeruginosa copA1*	This study
DC194-CopA2	LMG194 Δ*copA* transformed with pBAD-*P. aeruginosa copA2*	This study

a. In this nomenclature the first digit correspond to the mutated gene (CopA1 or CopA2), the second to the promoter driving expression (P_{copA1} and P_{copA2}) of the introduced gene, and the third numeral to the Cu^+-ATPase gene introduced into the original mutant strain.

Table S3. The resulting vectors were inserted in the genome of *P. aeruginosa* by tetraparental mating conjugation method as described (Choi and Schweizer, 2006). All constructs and mutants were confirmed by sequencing. Cells were grown at 37°C in LB medium, supplemented with irgasan (25 μg ml^{-1}), tetracycline (60 μg ml^{-1}), gentamicin (30 μg ml^{-1}) or kanamycin (35–50 μg ml^{-1}) as required.

Heterologous expression of P. aeruginosa *Cu^+-ATPases in* E. coli *DC194*

copA1 and copA2 cDNAs were obtained by PCR of *P. aeruginosa* genomic DNA. *E. coli copA* cDNA was also obtained in a similar manner. Amplicons were cloned into the pBAD-TOPO/His vector (Invitrogen, Carlsbad, CA, USA). DNA sequences were confirmed by automated sequencing. DC194 cells transformed with these constructs were grown at 37°C in ZYP-505 media supplemented with 0.05% arabinose, 100 μg ml^{-1} ampicillin, 50 μg ml^{-1} kanamycin (Studier, 2005). Cells were harvested at 24 h post inoculation, washed with 25 mM Tris, pH 7.0, 100 mM KCl and stored at −70°C.

Bioinformatics analyses

Cu^+-ATPase protein sequences present in most bacterial genomes were obtained from the TransportDB (http://www.membranetransport.org) (Ren *et al.*, 2007). Additional sequences were retrieved by BLAST searches at the Comprehensive Microbial Resource (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi). The identity of these Cu^+-ATPases was confirmed by the conserved membrane topology and the presence the signature amino acids: CPC motif in helix 6, YN in helix 7 and MXXS in helix 8 (González-Guerrero *et al.*, 2008). Transmembrane segments were determined by topology modelling using SOSUI (Hirokawa *et al.*, 1998) and TopPred2 (von Heijne, 1992). Sequences were aligned using ClustalW2 (Thompson *et al.*, 1994) and cladogram visualized with TreeView (Page, 1996).

Metal sensitivity tests

The LB liquid cultures were inoculated at OD_{600} of 0.1 from overnight cultures and supplemented with the desired $CuSO_4$ concentration as indicated in the figures. Cells were grown 16 h and OD_{600} was measured.

H_2O_2 sensitivity test

The LB liquid cultures were inoculated at OD_{600} of 0.1 from overnight cultures. When an OD_{600} value of 0.6 was reached, cultures were split, a half was supplemented with 30 mM H_2O_2 and the other left unamended. Cells were counted at 0, 15, 30 and 45 min after adding H_2O_2. Survival rate was estimated as the ratio of number of cells in the presence and the absence H_2O_2.

Infectivity assay

Arabidopsis thaliana ecotype Columbia was infected with *P. aeruginosa* as described by Starkey and Rahme (2009). Briefly, 3-week-old plants were inoculated by leaf infiltration with approximately 2×10^6 colony-forming units (cfu) per millilitre (OD_{600} 0.002). Two leaf discs were taken from each infected plant at 0, 2, 3 and 5 days post infection and homogenized in 10 mM $MgSO_4$. Serial dilutions of this homogenate were plated in selected media and cfu were counted after 16 h growth at 37°C. Four plants were infected per each bacterial strain.

Gene expression determinations

P. aeruginosa PAO1 wild-type cells from 5 ml LB liquid cultures (supplemented with 2 mM $CuSO_4$, 30 mM H_2O_2 or 0.4 mM paraquat) were harvested. RNA was stabilized with RNA Protect Bacteria reagent (Qiagen, Valencia, CA, USA) and isolated with RNeasy Minikit (Qiagen). In the case of RNA extraction from infected Arabidopsis, 3 days post infection total RNA was extracted from two leaves of each of four independently infected plants using plant RNA purification reagent (Invitrogen).

Gene expression was studied by quantitative real-time RT-PCR (iCycler iQ, Bio-Rad, Hercules, CA, USA). The primers used are indicated in Table S1. cDNAs was obtained with SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. qPCR reactions were carried out with iQ SYBR Green Supermix (Bio-Rad) in 25 μl final volume. The qPCR program consisted of a 5 min incubation at 95°C, followed by 35 cycles of 30 s at 95°C, 45 s at 60°C and 45 s at 72°C. The specificity of the PCR amplification was verified with a heat dissociation protocol (from 70°C to 100°C) after the final cycle of the PCR. The efficiency of primer sets was evaluated by performing real-time PCR on dilutions of cDNA. The results were normalized to the pyrroline-5-carboxylate reductase (*proC*) rRNA levels (Savli *et al*., 2003). As an additional control, the expression of the invariant 30S ribosomal protein S12 (PA4268) was monitored. RT-PCR determinations were carried out with RNA extracted from three independent biological samples, with the threshold cycle (C_t) determined in triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). A different primer set (Table S1) was used to avoid cross-hybridization with *Arabidopsis* genes in qPCR of cDNA obtained from *Arabidopsis*-infecting *P. aeruginosa*. In all experiments, a control sample not treated with reverse transcriptase was included to detect possible DNA contamination.

Cytochrome oxidase activity measurement

Determination of cytochrome oxidase activity in whole cells was performed as described (Frangipani and Haas, 2009) with the following modifications. Cells were grown for 16 h at 37°C in LB media, 60 μg ml^{-1} tetracycline, 30 μg ml^{-1} gentamicin. $CuSO_4$ (10 μM) was added when indicated. Cells were harvested, washed twice with 0.9% NaCl solution and resuspended at OD_{600} ~1 in 1.4 ml 50 mM HEPES, 200 mM NaCl, pH 7.0. The reaction was initiated by adding 5 μl 0.54 M N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) and cytochrome oxidase activity was monitored at 520 nm.

Membrane and everted vesicles preparations

Membranes from *E. coli* cells expressing each of the studied Cu^+-ATPases were prepared as described (Mandal *et al*., 2002). Membranes were resuspended in 25 mM Tris-HCl, pH 7.0, 100 mM sucrose at a protein concentration of 10–15 mg protein ml^{-1} and stored at –70°C. Sealed everted vesicles were prepared as described by Yang *et al*. (2007). Vesicles were resuspended in 50 mM MOPS, pH 7.0, 250 mM sucrose, 200 mM KCl and 10 mM $MgSO_4$, and stored at 4°C. Protein was measured in accordance to Bradford (Bradford, 1976). Heterologous proteins were detected by electroblotting SDS-PAGE Tris-glycine gels onto nitrocellulose membranes and immunostaining with primary rabbit anti-His-tag antibody and secondary goat anti-rabbit IgG horseradish peroxidase conjugated antibody (GenScript, Piscataway, NJ, USA).

Cu^+ transport assays

Cu^+ transport into the everted vesicles was measured using ^{64}Cu (Mallinckrodt Institute of Radiology, Washington University Medical School). Vesicles (1 mg protein ml^{-1}) were assayed in uptake media containing 50 mM MOPS, pH 7.3, 3 mM $MgCl_2$, 400 mM NaCl, 20 mM cysteine, 2.5 mM DTT and 1–1000 μM [^{64}Cu]$CuSO_4$ at 37°C as described (Mana-Capelli *et al*., 2003). Tubes were pre-incubated at 37°C for 1 min and the reactions were started by addition of 3 mM ATP. After 10 min (*P. aeruginosa* CopA1 and *E. coli* CopA) and 20 min (*P. aeruginosa* CopA2) incubation, aliquots were filtered onto 0.2 μm nitrocellulose filters. Filters were then washed with ice-cold 25 mM Tris-HCl, pH 7.0, 200 mM KCl, 250 mM sucrose, 0.1 mM $CuSO_4$ and radioactivity was measured in a scintillation counter. Cu uptake was estimated as the difference between values obtained in presence and absence of ATP. In addition, for each condition, control experiments were run in parallel with everted membrane vesicles made from *E. coli* DC194 cells transformed with the empty vector (Fig. S2). These values were discounted accordingly. However, error values were added in all cases.

The specific inhibitor vanadate (1.5 mM) was added to the uptake media in order to confirm P-ATPase-dependent Cu uptake activity. Cu^{2+} uptake was evaluated assaying vesicles in conditions where DTT and cysteine were absent in the uptake media. Cu efflux from everted membranes was measured by Cu^+ loading of the vesicles in a media containing 50 mM MOPS, pH 7.3, 3 mM $MgCl_2$, 400 mM NaCl, 3 mM ATP, 2.5 mM DTT, 20 mM cysteine and 100 μM [^{64}Cu]$CuSO_4$. DTT and cysteine were replaced by 10 mM ascorbic in experiments designed to test Cu^{2+} efflux. After 1 h incubation at room temperature vesicles were washed by dilution in media containing 50 mM MOPS, pH 7.3, 3 mM $MgCl_2$, 400 mM NaCl, 100 μM $CuSO_4$ and centrifugation for 15 min at 100 000 *g*. After two washes where $CuSO_4$ was excluded from the media, vesicles were resuspended in 50 mM MOPS, pH 7.3, 3 mM $MgCl_2$, 400 mM NaCl. Aliquot was filtered through a 0.22 μm nitrocellulose filter, washed as described above, and ^{64}Cu radioactivity measured. To reduce or oxidize the Cu loaded into the vesicles, the Cu-loaded vesicles preparations were pre-incubated for 20 min at room temperature in presence of 2.5 mM DTT (reducing agent), 0.5 mM H_2O_2, 1 mM diamide, 1 mM paraquat or 10 mM $KMnO_4$ (oxidizing agents). After this period, aliquots were filtered, washed, and ^{64}Cu radioactivity measured as described. ATP-driven Cu efflux from the treated vesicles was then initiated by adding 3 mM ATP. After 20 min of incubation at 37°C in the presence of ATP, the reaction was stopped by filtration and radioactivity measured as indicated above.

Phosphorylation assays

Previous to these assays, membranes were incubated with 1 mM bathocuproinedisulphonic acid (BCS) for 10 min at 20°C and then collected by centrifugation at 20 000 *g*. This step was repeated twice and membranes were resuspended in phosphorylation buffer without BCS. Enzyme phosphorylation with ATP was carried out in a medium containing 50 mM Tris, pH 6.0, 1 mM $MgCl_2$, 25 μM [γ-^{32}P]-ATP, 0.5 mM EDTA, 20 mM cysteine, 0.01% asolectin, 0.01% dodecyl maltoside, 50 mM NaCl, 20% DMSO, 1 mg ml^{-1} membrane protein, 2.5 mM DTT and either 100 μM Cu_2SO_4 or 1 mM BCS as indicated. Reactions were initiated by the addition of [γ-^{32}P]-ATP and stopped after 20 s incubation at 37°C with five volumes of ice-cold 1 mM P_i in 10% trichloroacetic acid. Samples were centrifuged at 14 000 *g* for 10 min, resuspended in acidic SDS-PAGE loading buffer and resolved by SDS-PAGE in an 8% acidic gel (Sarkadi *et al*., 1986). Gels were dried and radioactivity was monitored in a phosphoimager. In alternative experiments, samples were filtered through nitrocellulose 0.45 μm filters (Millipore, Billerica, MA, USA), washed five times with acid stopping solution, and radioactivity was measured in a scintillation counter. Cu^+-dependent phosphorylation was calculated as the difference between values obtained in the presence (100 μM Cu^+) or the absence (1 mM BCS) of Cu^+. Error values were added in all cases.

Copper accumulation in P. aeruginosa *cells*

Thirty millilitres of liquid LB cultures of wild-type and mutant strains in late exponential phase was supplemented with 0.2 mM $CuSO_4$ and incubated for 1 h. After this incubation, OD_{600} was determined, cells were harvested, and washed with 0.9% NaCl. Pellets were acid digested with 1.25 ml NO_3H (trace metal grade) for 1 h at 80°C and then overnight at 20°C. Digestions were concluded after addition of 0.25 ml of 30% H_2O_2 and dilution to 10 ml with water. Copper content in digested samples was measured by atomic absorption spectroscopy (AAnalyst 300; Perkin-Elmer, Foster City, CA, USA).

Acknowledgements

This work was supported by NIH grant 1R21AI082484-01 (J.M.A.) and NSF grant MCB-0743901 (J.M.A). We thank Mrs Encarnación Guerrero-Román for her kind help downloading and processing ATPases sequences for bioinformatics analysis, Dr Melissa Starkey and Dr. Laurence Rahme for their assistance in the plant infection assays, Dr Reeta Rao for her critical reading of the manuscript, and Mr Don Pellegrino for his valuable assistance with AAS determinations.

References

Agranoff, D., and Krishna, S. (2004) Metal ion transport and regulation in *Mycobacterium tuberculosis*. *Front Biosci* **9:** 2996–3006.

Argüello, J.M. (2003) Identification of ion-selectivity determinants in heavy-metal transport P-1B-type ATPases. *J Membr Biol* **195:** 93–108.

Argüello, J.M., Eren, E., and González-Guerrero, M. (2007) The structure and function of heavy metal transport P-1B-ATPases. *Biometals* **20:** 233–248.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254.

Brunori, M., Giuffre, A., and Sarti, P. (2005) Cytochrome c oxidase, ligands and electrons. *J Inorg Biochem* **99:** 324–336.

Choi, K.H., and Schweizer, H.P. (2006) mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. *Nat Protoc* **1:** 153–161.

Comolli, J.C., and Donohue, T.J. (2004) Differences in two *Pseudomonas aeruginosa cbb3* cytochrome oxidases. *Mol Microbiol* **51:** 1193–1203.

Desideri, A., and Falconi, M. (2003) Prokaryotic Cu,Zn superoxidies dismutases. *Biochem Soc Trans* **31:** 1322–1325.

Francis, M.S., and Thomas, C.J. (1997) The *Listeria monocytogenes* gene *ctpA* encodes a putative P-type ATPase involved in copper transport. *Mol Gen Genet* **253:** 484–491.

Frangipani, E., and Haas, D. (2009) Copper acquisition by the SenC protein regulates aerobic respiration in *Pseudomonas aeruginosa* PAO1. *FEMS Microbiol Lett* **298:** 234–240.

González-Guerrero, M., and Argüello, J.M. (2008) Mechanism of Cu+-transporting ATPases: soluble Cu+ chaperones directly transfer Cu+ to transmembrane transport sites. *Proc Nat Acad Sci USA* **105:** 5992–5997.

González-Guerrero, M., Eren, E., Rawat, S., Stemmler, T.L., and Argüello, J.M. (2008) Structure of the two transmembrane Cu+ transport sites of the Cu+-ATPases. *J Biol Chem* **283:** 29753–29759.

González-Guerrero, M., Hong, D., and Argüello, J.M. (2009) Chaperone-mediated Cu+ delivery to Cu+ transport ATPases. Requirement of nucleotide binding. *J Biol Chem* **284:** 20804–20811.

Hassani, B.K., Astier, C., Nitschke, W., and Ouchane, S. (2010) CtpA, a copper-translocating P-type ATPase involved in the biogenesis of multiple copper-requiring enzymes. *J Biol Chem* **285:** 19330–19337.

Hatori, Y., Hirata, A., Toyoshima, C., Lewis, D., Pilankatta, R., and Inesi, G. (2008) Intermediate phosphorylation reactions in the mechanism of ATP utilization by the copper ATPase (CopA) of *Thermotoga maritima*. *J Biol Chem* **283:** 22541–22549.

von Heijne, G. (1992) Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *J Mol Biol* **225:** 487–494.

Hirokawa, T., Boon-Chieng, S., and Mitaku, S. (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* **14:** 378–379.

Jabs, T., Tschope, M., Colling, C., Hahlbrock, K., and Scheel, D. (1997) Elicitor-stimulated ion fluxes and O-2(-) from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proc Nat Acad Sci USA* **94:** 4800–4805.

Jacobs, M.A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., *et al*. (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Nat Acad Sci USA* **100:** 14339–14344.

Kahn, D., David, M., Domergue, O., Daveran, M.L., Ghai, J., Hirsch, P.R., and Batut, J. (1989) *Rhizobium meliloti* fixGHI sequence predicts involvement of a specific cation pump in symbiotic nitrogen fixation. *J Bacteriol* **171:** 929–939.

Kaplan, J.H. (2002) Biochemistry of Na,K-ATPase. *Annu Rev Biochem* **71:** 511–535.

Koch, H.G., Winterstein, C., Saribas, A.S., Alben, J.O., and Daldal, F. (2000) Roles of the *ccoGHIS* gene products in the biogenesis of the *cbb*(3)-type cytochrome c oxidase. *J Mol Biol* **297:** 49–65.

Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. (1994) H2O2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **79:** 583–593.

Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25:** 402–408.

Llamas, M.A., Rodríguez-Herva, J.J., Hancock, R.E., Bitter, W., Tommassen, J., and Ramos, J.L. (2003) Role of *Pseudomonas putida* tol-oprL gene products in uptake of solutes through the cytoplasmic membrane. *J Bacteriol* **185:** 4707–4716.

Lutsenko, S., Barnes, N.L., Bartee, M.Y., and Dmitriev, O.Y. (2007) Function and regulation of human copper-transporting ATPases. *Physiol Rev* **87:** 1011–1046.

Mana-Capelli, S., Mandal, A.K., and Argüello, J.M. (2003) *Archeoglobus fulgidus* CopB is a thermophilic Cu^{2+}-ATPase – functional role of its histidine-rich N-terminal metal binding domain. *J Biol Chem* **278:** 40534–40541.

Mandal, A.K., Cheung, W.D., and Argüello, J.M. (2002) Characterization of a thermophilic P-type Ag+/Cu+-ATPase from the extremophile *Archaeoglobus fulgidus*. *J Biol Chem* **277:** 7201–7208.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, p. 545.

Myllykallio, H., and Liebl, U. (2000) Dual role for cytochrome cbb3 oxidase in clinically relevant proteobacteria? *Trends Microbiol* **8:** 542–543.

Odermatt, A., Suter, H., Krapf, R., and Solioz, M. (1993) Primary structure of two P-type ATPases involved in copper homeostasis in *Enterococcus hirae*. *J Biol Chem* **268:** 12775–12779.

Osman, D., and Cavet, J.S. (2008) Copper homeostasis in bacteria. *Adv Appl Microbiol* **65:** 217–247.

Page, R.D. (1996) TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12:** 357–358.

Phung, L.T., Ajlani, G., and Haselkorn, R. (1994) P-type ATPase from the cyanobacterium *Synechococcus* 7942 related to the human Menkes and Wilson disease gene products. *Proc Natl Acad Sci USA* **91:** 9651–9654.

Pontel, L.B., Audero, M.E.P., Espariz, M., Checa, S.K., and Soncini, F.C. (2007) GolS controls the response to gold by the hierarchical induction of *Salmonella*-specific genes that include a CBA efflux-coding operon. *Mol Microbiol* **66:** 814–825.

Preisig, O., Zufferey, R., and Hennecke, H. (1996) The *Bradyrhizobium japonicum* fixGHIS genes are required for the formation of the high-affinity *cbb*(3)-type cytochrome oxidase. *Arch Microbiol* **165:** 297–305.

Ren, Q., Chen, K., and Paulsen, I.T. (2007) TransportDB: a comprehensive database resource for cytoplasmic membrane transport systems and outer membrane channels. *Nucleic Acids Res* **35:** D274–D279.

Rensing, C., Fan, B., Sharma, R., Mitra, B., and Rosen, B.P. (2000) CopA: an *Escherichia coli* Cu(I)-translocating P-type ATPase. *Proc Natl Acad Sci USA* **97:** 652–656.

Sarkadi, B., Enyedi, A., Foldes-Papp, Z., and Gardos, G. (1986) Molecular characterization of the in situ red cell membrane calcium pump by limited proteolysis. *J Biol Chem* **261:** 9552–9557.

Savli, H., Karadenizli, A., Kolayli, F., Gundes, S., Ozbek, U., and Vahaboglu, H. (2003) Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. *J Med Microbiol* **52:** 403–408.

Schwan, W.R., Warrener, P., Keunz, E., Stover, C.K., and Folger, K.R. (2005) Mutations in the cueA gene encoding a copper homeostasis P-type ATPase reduce the pathogenicity of *Pseudomonas aeruginosa* in mice. *Int J Med Microbiol* **295:** 237–242.

Shepherd, V.L. (1986) The role of the respiratory burst of phagocytes in host defense. *Semin Respir Infect* **1:** 99–106.

Solioz, M., Abicht, H.K., Mermod, M., and Mancini, S. (2010) Response of gram-positive bacteria to copper stress. *J Biol Inorg Chem* **15:** 3–14.

Starkey, M., and Rahme, L.G. (2009) Modeling *Pseudomonas aeruginosa* pathogenesis in plant hosts. *Nat Protoc* **4:** 117–124.

Studier, F.W. (2005) Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* **41:** 207–234.

Teitzel, G.M., Geddie, A., De Long, S.K., Kirisits, M.J., Whiteley, M., and Parsek, M.R. (2006) Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. *J Bacteriol* **188:** 7242–7256.

Thaden, J.T., Lory, S., and Gardner, T.S. (2010) Quorum-sensing regulation of a copper toxicity system in *Pseudomonas aeruginosa*. *J Bacteriol* **192:** 2557–2568.

Thompson, A.K., Smith, D., Gray, J., Carr, H.S., Liu, A.M., Winge, D.R., and Hosler, J.P. (2010) Mutagenic analysis of Cox11 of *Rhodobacter sphaeroides*: insights into the assembly of Cu-B of cytochrome c oxidase. *Biochemistry* **49:** 5651–5661.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22:** 4673–4680.

Toes, A.C.M., Daleke, M.H., Kuenen, J.G., and Muyzer, G. (2008) Expression of *copA* and *cusA* in *Shewanella* during copper stress. *Microbiology* **154:** 2709–2718.

Torres, M.A., Jones, J.D., and Dangl, J.L. (2006) Reactive oxygen species signaling in response to pathogens. *Plant Physiol* **141:** 373–378.

Tottey, S., Rich, P.R., Rondet, S.A.M., and Robinson, N.J. (2001) Two Menkes-type ATPases supply copper for photosynthesis in *Synechocystis* PCC 6803. *J Biol Chem* **276:** 19999–20004.

Toyoshima, C., and Inesi, G. (2004) Structural basis of ion pumping by Ca^{2+}-ATPase of sarcoplasmic reticulum. *Ann Rev Biochem* **73:** 269–292.

White, C., Lee, J., Kambe, T., Fritsche, K., and Petris, M.J. (2009) A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. *J Biol Chem* **284:** 33949–33956.

Yang, Y., Mandal, A.K., Bredeston, L.M., González-Flecha, F.L., and Argüello, J.M. (2007) Activation of *Archaeoglobus fulgidus* Cu^{+}-ATPase CopA by cysteine. *Biochim Biophys Acta-Biomembranes* **1768:** 495–501.

Zhang, X.X., and Rainey, P.B. (2007) The role of a P1-type ATPase from *Pseudomonas fluorescens* SBW25 in copper homeostasis and plant colonization. *Mol Plant Microbe Interact* **20:** 581–588.

Supporting information

Additional supporting information may be found in the online version of this article.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.