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Periplasmic response upon disruption of transmembrane Cu transport in *Pseudomonas aeruginosa*†

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Pseudomonas aeruginosa, an opportunistic pathogen, has two transmembrane Cu^+ transport ATPases, CopA1 and CopA2. Both proteins export cytoplasmic Cu^+ into the periplasm and mutation of either gene leads to attenuation of virulence. CopA1 is required for maintaining cytoplasmic copper levels, while CopA2 provides copper for cytochrome *c* oxidase assembly. We hypothesized that transported Cu^+ ions would be directed to their destination *via* specific periplasmic partners and disruption of transport should affect the periplasmic copper homeostasis. Supporting this, mutation of either ATPase gene led to large increments in periplasmic cuproprotein levels. Toward identifying the proteins participating in this cellular response the periplasmic metalloproteome was resolved in non-denaturing bidimensional gel electrophoresis, followed by X-ray fluorescence visualization and identification by mass-spectrometry. A single spot containing the electron shuttle protein azurin was responsible for the observed increments in cuproprotein contents. In agreement, lack of either Cu^+-ATPase induced an increase in *azu* transcription. This is associated with an increase in the expression of *anr* and *rpoS* oxidative stress response regulators, rather than *cueR*, a copper sensing regulator. We propose that azurin overexpression and accumulation in the periplasm is part of the cellular response to cytoplasmic oxidative stress in *P. aeruginosa*.

Introduction

Copper is an essential trace element required for central cellular functions in all living organisms.[1] Due to the high reactivity of both ion species ($Cu^{+/2+}$), cells have acquired different strategies to maintain intracellular copper quota under restricted space and concentration limits, minimizing in this way possible toxic effects.[2] Compartmentalization of copper requirements, Cu^+ sensing transcription factors and specific protein–protein interactions required to establish Cu^+ traffic contribute to these goals.[3] Perhaps one of the better characterized mechanisms involved in copper homeostasis includes cytosolic chaperones (Atx1-like) that sequester, traffic and deliver Cu^+ to membrane P_{IB-1}-ATPases for the subsequent Cu^+ transport to another cellular compartment.[3a,4] In prokaryotes, Cu^+-ATPases are involved in Cu^+ efflux, conferring copper resistance,[3b,5] and cuproprotein assembly.[6] These distinct functional roles were observed for the two homologous Cu^+-ATPases, CopA1 and CopA2, present in *Pseudomonas aeruginosa.*[7] CopA1 was expressed in response to high Cu,[7,8] and its deletion induced copper sensitivity[4a,8,9] and cytosolic copper accumulation.[7] Further biochemical characterization showed that CopA1 presents a comparatively lower affinity for Cu^+ and a high rate of transport. These attributes support its role in the control of cytoplasmic Cu^+. CopA2 belongs to the FixI-like subgroup of P_{IB-1}-ATPases.[7] This transporter was co-expressed with cytochrome *c* oxidase subunits and the *copA2* mutant strain showed reduced oxidase activity of the cbb_3 complex. Transport studies indicated that CopA2 has a higher affinity for Cu^+ and a low turnover rate, consistent with its proposed role enabling copper loading into cytochrome *c* oxidase.[7] Pointing to the

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relevance of these ATPase specific roles in the pathogen capability to overcome the host innate immunity mechanisms,[10] reduced virulence was observed in both Cu^+-ATPase mutant strains when tested in a model plant infection assay.[7,9]

While both enzymes export cytoplasmic Cu^+ into the periplasm, their different functional roles might be explained by the intrinsic differences in transport kinetics, as well as by the presence of putative periplasmic partners acting as copper chaperones. That is, specific chaperones would handle the metal after its release by either CopA1 or CopA2 and direct it to the client proteins. In this model, the unidirectional metal transfer would be mediated by protein–protein interaction among a unique transporter–chaperone pair.

Regarding possible periplasmic chaperones partnering with CopA1, transcriptional analysis has shown that several *P. aeruginosa* genes codifying for secreted proteins are associated with copper resistance/stress response.[8] Among those up-regulated by the presence of copper in the growth media, two multicopper oxidases (*PA2064* and *PA2065*) and two periplasmic Cu-binding proteins (*PA2807* and *PA2808*) were induced at least 50-fold. In *Escherichia coli*, the CusCBA Cu-export system is associated with CusF, a periplasmic metal chaperone that carries Cu^+ and Ag^+ to the efflux pump.[11] However, a CusF homolog seems to be missing in *P. aeruginosa* and an alternative chaperone likely transfers Cu^+ to CusA in this organism. For instance, studies have linked the periplasmic protein PtrA (*PA2808*) with Cu resistance, although the precise mechanism is still unknown.[12] *P. putida* KT2440 CinA, a *PA2807* ortholog, has been shown to be expressed under copper stress and proposed to act as an azurin-like electron shuttle probably required in the Cu-oxidation process by multicopper oxidases.[13] However, the mutation of this cuproprotein bearing a type-1 Cu site did not lead to Cu sensitivity in *P. putida*[13] and a subtle Cu-sensitive phenotype was found in the *P. aeruginosa* Δ*PA2807* mutant strain.[8]

Considering possible CopA2 partner proteins, the aerobic respiration of *P. aeruginosa* relies strongly on two heme–Cu containing oxidases (cbb_3-1 and cbb_3-2).[14] These terminal oxidases appear to require a Cu chaperone from the SCO1/SenC family in order to be assembled and functional. In *S. cerevisiae*, SCO1 (*s*ynthesis of *c*ytochrome *c* *o*xidase) delivers Cu to the dinuclear CuA site in cytochrome *c* oxidase.[15] In *Rhodobacter capsulatus*, SenC promotes optimal activity of the cytochrome cbb_3 presumably by sequestering and delivering Cu to the active site of this oxidase.[16] Similarly, mutation of *P. aeruginosa senC* induces poor growth in low Cu media as well as low cytochrome cbb_3-type oxidase activity. Consequently, it has been proposed that periplasmic SenC could deliver copper ions to the cbb_3 terminal oxidases.[17]

We hypothesized that analysis of the periplasmic metalloproteome would reveal the role that CopA1 and CopA2 play in periplasmic copper homeostasis. To this end, we used a novel approach combining non-denaturing bidimensional gel electrophoresis (2D-GE), metalloprotein visualization by X-ray fluorescence (XRF) and protein identification by mass spectroscopy.[18] We observe that mutation of the coding genes (Δ*copA1* and Δ*copA2* strains) led to largely increased periplasmic copper levels. However, this was not associated with putative Cu^+-ATPase partner proteins but with significant increases in azurin levels, suggesting a common cellular response to cytoplasmic copper overload, oxidative stress, and energetic deficiency.

Materials and methods

Cell culture and *P. aeruginosa* strains

P. aeruginosa PAO1 WT, Δ*copA1*, and Δ*copA2* insertional mutation strains were obtained from the Comprehensive *P. aeruginosa* Transposon Mutant Library at the University of Washington Genome Center.[19] Cells were grown in aerobiosis at 37 °C, 200 rpm in LB medium (1 μM Cu), and supplemented with tetracycline (60 μg ml^{-1}) as required. Complemented strains were obtained as described previously[7] and grown in LB supplemented with gentamycin (30 μg ml^{-1}).

Preparation of subcellular fractions

Cells cultured in 125 ml of LB were harvested at the early stationary phase by centrifugation at 4000 × *g*, 4 °C for 10 min. Proteins in the supernatant were concentrated (5–10 mg ml^{-1}) using 3 kDa-Centricon and considered the secreted protein fraction. Pelleted cells were resuspended and washed with 30 ml of ice-cold 50 mM Tris–HCl, pH 7.5, 0.3 M sorbitol (buffer S). Cells were recovered by centrifugation at 9000 × *g*, 4 °C for 10 min, resuspended in 10 μM EDTA buffer S, and incubated for 20 min at room temperature. A third wash was done in buffer S to remove remnant EDTA. Finally, cells were resuspended in ice-cold MilliQ H_2O, incubated for 5 min and centrifuged at 14 000 × *g*, 4 °C for 30 min. The supernatant was collected, centrifuged to remove possible remnant cellular contamination, concentrated (5–10 mg ml^{-1}) using 3 kDa-Centricon filters and considered the periplasmic protein fraction. The pelleted cells were resuspended in 50 mM Tris–Cl, pH 7.4 and passed through a French press at 20 000 psi. The membrane fraction was recovered after centrifugation at 100 000 × *g* for 1 h at 4 °C and resuspended in the same buffer. Cytosolic proteins were obtained following previously established protocols to purify the different fractions from *P. aeruginosa* with minimal cross contamination.[20] Briefly, periplasmic proteins were released by harvesting the cells at 25 °C and resuspending the pellets at 1 g per 30 ml in medium at 30 °C containing 0.5 M sucrose, 4 mM Na_2EDTA and 40 mM Tris–Cl, pH 8.0, plus lysozyme 0.25 mg ml^{-1}. The solution was left at 30 °C for 30 min and centrifuged at 15 000 × *g* for 20 min. After separation of the supernatant and washing of pelleted spheroplasts, cytoplasmic proteins were released from the spheroplasts by resuspension in 10 mM Tris–Cl at the same concentration as before. After a few minutes it was centrifuged to yield a supernatant of cytoplasmic proteins and a pellet of lysed membranes. All fractions were analyzed immediately to minimize metal equilibration and potential metal transfer.

Copper content determinations

Protein fractions were acid digested with concentrated nitric acid overnight at room temperature. Following digestion,

samples were treated with 1.5% H_2O_2. The copper concentration in each sample was measured by furnace Atomic Absorption Spectrometry (AAS, Varian SpectrAA 880/GTA 100, Santa Clara, CA) as previously described.[21]

Metalloproteomic analysis

Analysis of periplasmic metalloproteins of *P. aeruginosa* WT, Δ*copA1* and Δ*copA2* strains was performed as previously described using 2D-GE/XRF coupled to LC-MS/MS.[18] Briefly, periplasmic proteins (100 μg) were resolved by isoelectric focusing using 1.5 mm polyacrylamide tubes (6 cm long) containing 5% (w/v) acrylamide, 3% (v/v) ampholytes pI range 3–10, 6% (v/v) ampholytes pI range 5–7 and 15% (v/v) glycerol. Second dimension electrophoresis was performed in 10% polyacrylamide gels (PAGE). Denaturing (SDS, DTT, *etc.*) agents were not used in either electrophoresis. Gels were blotted onto PVDF membranes using a wet transfer system with Tris–glycine running buffer for 1.5 h at 350 mA and 4 °C and blots were analyzed using XRF at beamline 8-BM-B of Sector 8, at the Advanced Photon Source (APS). Hard X-rays (10.2 keV) were monochromatized and passed through a pinhole (spot diameter on samples: 0.5 mm). Full X-ray fluorescence spectra were collected at each raster-scan step using a four element silicon drift detector (Vortex ME4, SII NanoTechnology). Spectra were fitted against NIST standards NBS1832 and NBS1833 or AXO standards (Blake Industries, Inc.) using per-pixel peak fitting algorithms with MAPS software.[22] Images obtained using XRF were superimposed onto the PVDF membranes using fiduciary marks etched into the edges of the blot, and the spots corresponding to the metal signal were excised for tryptic digestion and LC-MS/MS identification; MS/MS determinations were performed at the University of Massachusetts – Medical School, Proteomics and Mass Spectrometry Facility. Total copper quantification in the regions of interest (ROIs) was estimated using the quantification tool in MAPS. ROIs were defined arbitrarily as the circumscribing region of the Cu spots with values <3SD. Normalized spectrum counts of azurin in Cu spots analyzed by MS/MS were obtained with Scaffold_3.5.1 (Proteome software Inc.).

Gene expression determinations

P. aeruginosa PAO1 WT, Δ*copA1* or Δ*copA2* cells from 5 ml LB liquid cultures were harvested. RNA was stabilized with the RNA Protect Bacteria reagent (Qiagen, Valencia, CA, USA) and isolated with an RNeasy Minikit (Qiagen). Gene expression was studied by semi-quantitative RT-PCR. The cDNA sequences were obtained with SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Primers 5′-CGCTGCGGTATCCCTGCTGT-3′ and 5′-CGACGGTGATGGCATTGGTG-3′ were used to evaluate *azu* (*PA4922*), 5′-CATGTGGTCAAGGAGCTCAA-3′ and 5′-CTCGACAGGCCATTCTTCTC-3′ for *rpoS* (*PA3622*), 5′-AAGACATGGATTCGCTGGAC-3′ and 5′-ACAGGTTGACCAGGAAGGTG-3′ for *anr* (*PA1544*), 5′-AATGCCTGTATTGCCTCCAT-3′ and 5′-ATCATGCCCAGGCAGAAC-3′ for *PA0526*, 5′-GCTGAAAACGAACGAAGAGG-3′ and 5′-AACGACGGGTTGAAGATCAC-3′ for *cioA* (*PA3930*), 5′-GGCATGCTCTATCCGTTCTT-3′ and 5′-TCTTCATGATCAGCCAGGTG-3′ for *cioB* (*PA3929*), 5′-TCCGTTACTACGAGTCCATCG-3′ and 5′-GTGCTGAGCTCCTCGATCTT-3′ for *cueR* (*PA4778*) transcripts. PCRs were carried out following standard procedures (2 min incubation at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 58 °C and 45 s at 72 °C). The specificity of the PCR amplification was confirmed by analyzing the amplification product by ethidium bromide–agarose gel electrophoresis, and quantification was performed on digital images by densitometry using Quantity One v4.6.3 (Bio-Rad). The results were normalized to the pyrroline-5-carboxylate reductase (*proC*) rRNA levels.[7]

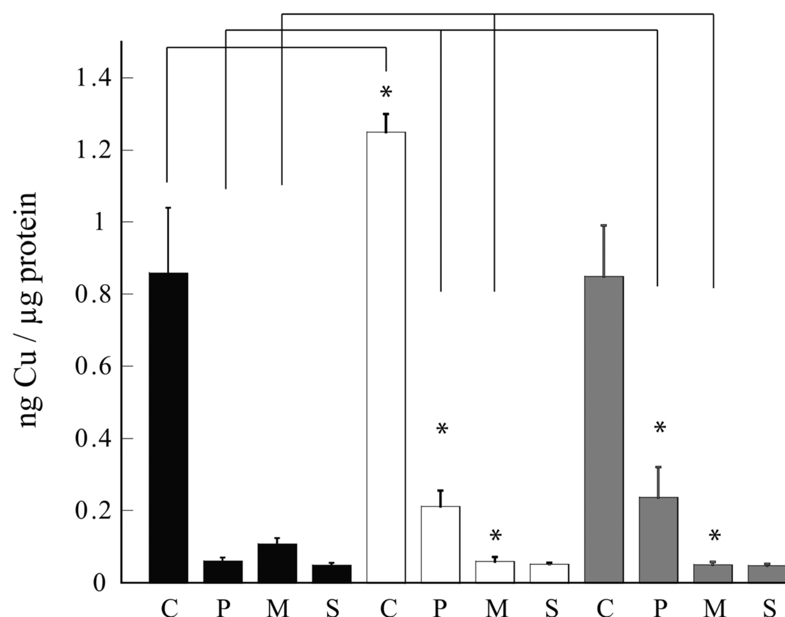

Fig. 1 Cu content in cytosolic (C), periplasmic (P), membrane (M), and secreted (S) protein fractions of *P. aeruginosa* PAO1 WT (black bars), Δ*copA1* (white bars) and Δ*copA2* (gray bars) mutant strains. Cu was determined by furnace AAS and normalized to protein content in the sample. The data represent mean ± SE (n = 3). *p < 0.05 *vs.* WT.

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Results

Copper homeostasis is altered in Δ*copA1* and Δ*copA2* mutant strains

P. aeruginosa CopA1 and CopA2 export intracellular Cu^+ to the periplasm with different transport rates and apparent affinities adapted to their functional roles in copper homeostasis and protein assembly respectively.[7] These unique kinetic parameters might be further affected by the release/delivery rate of the exported substrate to periplasmic chaperones. In this model, specific protein–protein interactions between the Cu^+-ATPase and a periplasmic chaperone would affect Cu^+ transport, while ensuring its transit and delivery to periplasmic targets. In a first approach to locate these hypothetical partners and analyze the influence of both Cu^+-ATPases on Cu^+ homeostasis, the protein-bound copper content was measured in cytosolic, membrane, secreted and periplasmic fractions of WT, Δ*copA1* and Δ*copA2* strains obtained from cells grown till the stationary phase in LB media (Fig. 1). No differences were observed in the protein-bound copper content in the secreted fractions. Membrane fractions from Cu^+-ATPase mutant strains showed a lower copper content compared with similar samples from wild-type strains. An increase in cytosolic copper levels was observed in the Δ*copA1* mutant. These findings correlate with the lack of CopA1 functionality and the decrease in oxidase activity observed in the Δ*copA2* strain.[7] In contrast to these, periplasmic fractions from Δ*copA1* and Δ*copA2* strains showed unexpectedly higher copper levels. This surprising result suggests significant changes in periplasmic copper homeostasis; perhaps modifying the status of protein components in this compartment. Importantly, gene complementation restored the copper levels in the periplasmic fractions of both mutant strains to those observed in WT cells (data not shown). In this direction,

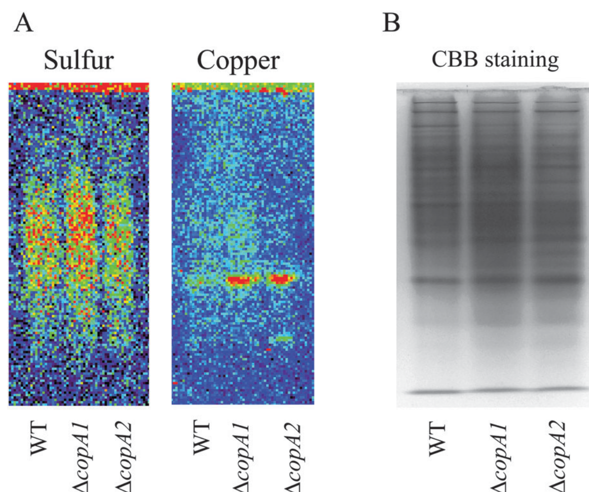

Fig. 2 Analysis of periplasmic protein fractions of *P. aeruginosa* PAO1 WT, Δ*copA1*, and Δ*copA2* mutant strains by non-denaturing PAGE. (A) XRF analysis showing sulfur and copper signals from samples run on 4–20% PAGE transferred to the PVDF membrane. (B) CBB staining of samples run on 10% PAGE.

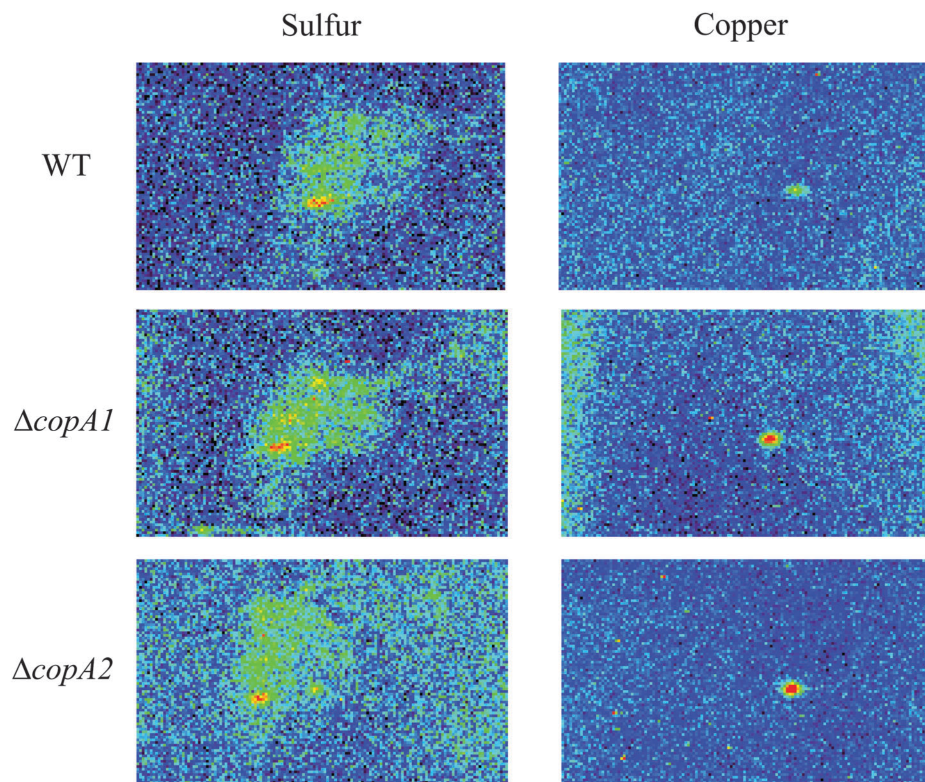

Fig. 3 XRF analysis of periplasmic proteins extracted from *P. aeruginosa* PAO1 WT, Δ*copA1*, and Δ*copA2* mutant strains separated by non-denaturing 2D-GE. Sulfur signal is shown on the left and copper on the right column.

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an XRF image obtained from samples resolved in a non-denaturing 4–20% gradient PAGE system showed a significant increase in Cu signal from apparently a single band of the fraction from mutant strains (Fig. 2A). However, the periplasmic protein profile showed no major differences among samples of these strains (Fig. 2B) suggesting that the protein synthesis and the secretion/export machinery are largely unaffected.

High periplasmic Cu levels are associated with azurin in Δ*copA1* and Δ*copA2* mutant strains

To identify the protein/s associated with periplasmic copper and likely involved in the increased cuproprotein levels observed for Δ*copA1* and Δ*copA2* strains, periplasmic fractions were resolved using 2D-GE/XRF (Fig. 3). This method has been previously used to identify *P. aeruginosa* periplasmic zinc chaperones.[18] In this analysis, the sulfur signal provided a control for protein load during electrophoresis and transfer to the PVDF membranes. Analysis of copper signals revealed a single cuproprotein spot for the three strains: WT, Δ*copA1*, and Δ*copA2*. Interestingly, the spots for mutant samples were more intense (*i.e.*, high copper levels) than that for the WT (Fig. 4A). These spots were excised from the membranes and analyzed by MS/MS. Several proteins were identified in these samples (Table S1, ESI†). However, only one, azurin (*PA4922*), was a periplasmic cuproprotein.[23] Moreover, among the identified proteins azurin was the only one showing mass levels matching those of copper in the three compared sample sets (Fig. 4A and B).

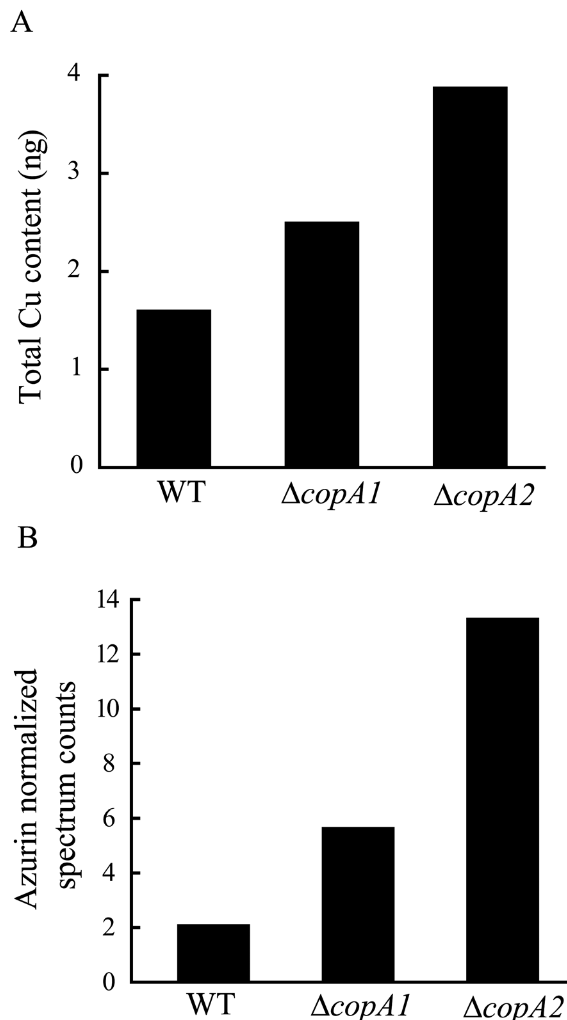

Fig. 4 Representative data of (A) quantitative analysis of total Cu content in ROIs using MAPS software, and (B) azurin normalized spectrum count from MS/MS data of Cu spots in WT, Δ*copA1*, and Δ*copA2* mutant strains. The experiment was performed in triplicate and similar patterns were observed in all cases.

Azurin is overexpressed in Δ*copA1* and Δ*copA2* mutant strains *via* Anr

It is known that the regulation of *azu* is controlled by means of Anr and RpoS transcriptional regulators,[24] which sense oxygen concentration and cellular stress respectively. To test if the copper accumulation observed in the periplasmic protein fraction was linked to overexpression of *azu*, mRNA was obtained, retrotranscribed and used for semiquantitative PCR experiments. Images were analyzed using densitometry and the *azu* transcripts were normalized to those of *proC*. The analysis showed that the level of *azu* transcripts was significantly higher in Δ*copA1* and Δ*copA2* compared to WT (1.5 and 1.9-fold increase respectively) (Fig. 5A). Toward dissecting the pathway leading to *azu* induction, the expression of *rpoS* and *anr* genes was measured. Fig. 5B and C show that *anr* was up-regulated in both strains and that *rpoS* induction was increased only in the Δ*copA1* mutant. Supporting the idea that these are significant inductions, genes from the *anr* regulon such as *cioA*, *cioB* and *PA0526* were up-regulated in both strains (Fig. 5D and E). Surprisingly, *cueR*, the copper responsive regulator, was not induced in any of the mutant strains (Fig. 5F).

Discussion

The alternative roles of Cu^+-ATPases maintaining cytoplasmic copper levels and participating in the assembly of cytochrome *c* oxidases were previously shown.[7] Continuing these studies, the effects of mutating their coding genes on periplasmic copper homeostasis were characterized. A significant increase in the levels of azurin-bound copper was observed along with increased *azu* transcription. We propose that the increase in azurin levels is a general cellular response to copper imbalances or energy deficiency leading to oxidative stress.

The initial goal of these studies was the identification of periplasmic chaperones that specifically partner with each Cu^+-ATPase to deliver the metal to client metalloenzymes or Cu^+-transport pathways. To this end, we employed a novel metalloproteomic approach that was proved to be successful in identifying periplasmic zinc metallochaperones.[18] This methodology, although highly sensitive, was unable to reveal the presence of plausible metallochaperone proteins. This might have been due to small pools of these proteins in the wild type and mutant strains under the tested conditions. Alternatively, some of these proteins might be membrane associated and therefore are not present in the characterized periplasmic fraction. Nevertheless, the analysis enabled the

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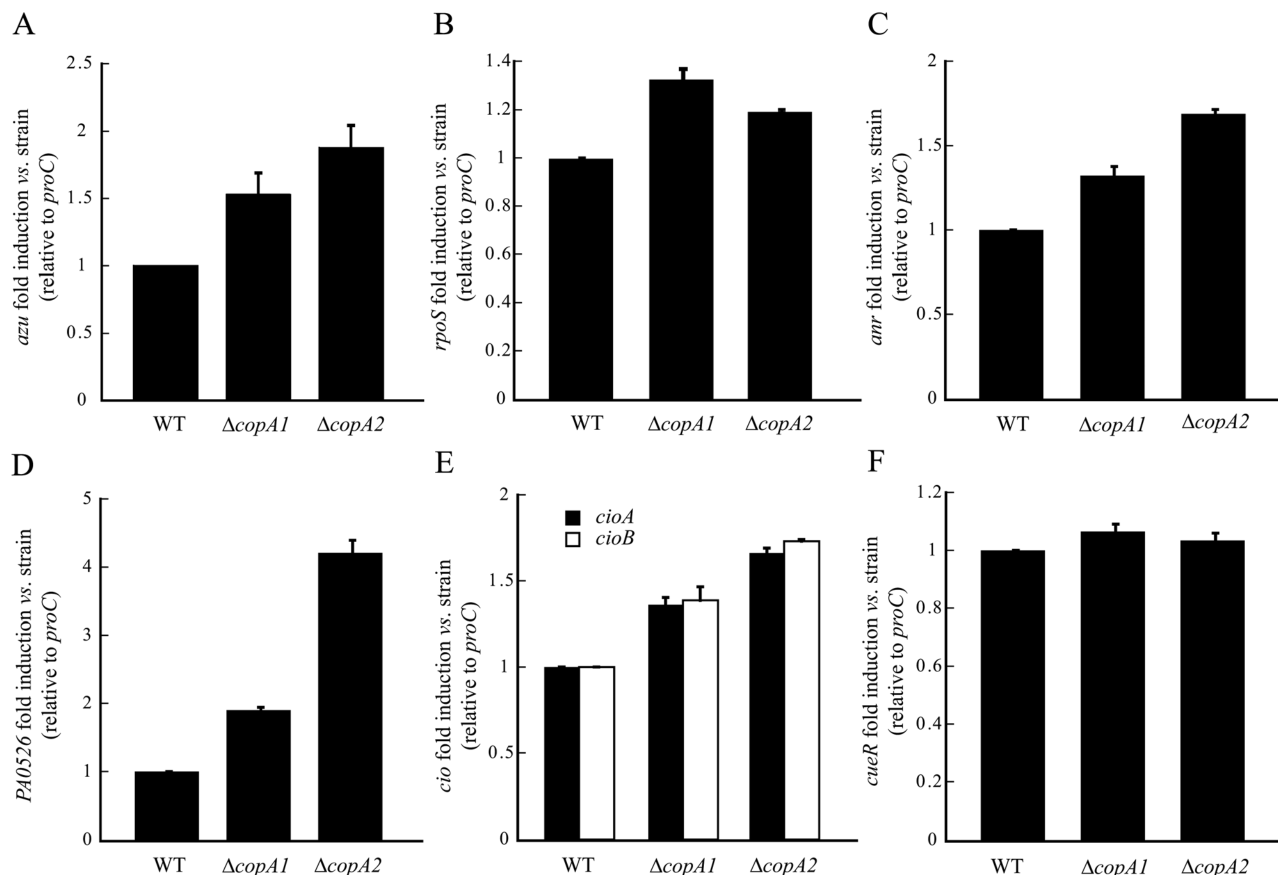

Fig. 5 Semiquantitative RT-PCR of *azu* (A), *rpoS* (B), *anr* (C), *PA0526* (D), *cioA-B* (E), and *cueR (F)* in *P. aeruginosa* PAO1 WT, Δ*copA1*, and Δ*copA2* mutant strains. Expression was normalized to *proC* transcript levels. The data represent mean ± SE (*n* = 3).

identification of azurin as the major cuproprotein in the *P. aeruginosa* periplasm.

Copper presence in periplasmic space is essential as it participates as a protein co-factor in aerobic respiration and for redox stress related proteins.[6b] However, we expected that removal of cytoplasmic copper efflux ATPases would lead to reduced periplasmic copper levels. In contrast to this, a surprisingly large increase in this pool was observed upon mutation of *P. aeruginosa* Cu^+-ATPases; even though they play different roles. Moreover, in both cases a similar response was observed; *i.e.*, increase in the azurin levels. As a model cuproprotein, the azurin structure and chemistry of its copper site have been extensively characterized.[25,26] Still, the physiological role of azurin is somewhat uncertain. It is very unlikely that azurin could participate in copper trafficking by contributing a side chain ligand for Cu coordination, as the type-1 Cu site is considered a static Cu binding site (low k_{off}). However, it is particularly interesting that Cu atoms have been encountered in the proximity of the two exposed Cys in crystal structures (PDB: 3NP3, 3FPY). On the other hand, *in vitro* evidence supports its participation in anaerobic respiration in the presence of nitrite or nitrate together with cytochrome *c551*, although an essential role in the denitrification pathway has been questioned by *in vivo* studies.[24] Nevertheless, the azurin copper site appears to be directly involved in electron transfer. Interestingly, it has been proposed that a disulfide bridge between residues Cys3 and Cys26 is also a redox-active center.[27] Consequently, a redox response function appears to be the likely role of this periplasmic protein.

How can the azurin accumulation in the periplasm of Δ*copA1* and Δ*copA2* strains be explained? Disruption of *copA1* and *copA2* genes leads to different stress conditions with similar consequences in terms of copper homeostasis in the periplasm. CopA1 is considered the housekeeper in bacterial intracellular Cu^+ homeostasis even at low copper concentrations in the media.[5] The cytosolic copper accumulation observed for the Δ*copA1* mutant even under the basal tested conditions supports this idea (Fig. 1). Previous characterization of the *E. coli* response to copper overload showed expression of the SoxRS, OxyR and RpoS regulon genes, such as *sod*, *katG* and *katE*.[2b] It is known that in *P. aeruginosa azu* is under the regulation of RpoS.[24] The *anr* transcripts, together with *rpoS*, were induced in the Δ*copA1* mutant and the Δ*copA2* showed high levels of *anr* messengers (Fig. 5B and C). Other genes from the *anr* regulon were induced in both mutant strains pointing to the likely Anr participation in the *azu* induction. These observations explain the up-regulation of *azu* in our system. In addition, Macomber *et al.*[2b] observed that in *E. coli* periplasmic copper increase might protect DNA oxidation when cells are exposed to H_2O_2. Interestingly, it has been shown that deletion of

azu augments *P. aeruginosa* sensitivity towards H_2O_2 *in vitro*.[24] Considering the similar observation in the Δ*copA2* strain, we have previously shown that cytochrome *c* oxidase activity is compromised in these mutant cells.[7] The lack of cytochrome *c* oxidase function induces an anaerobic-like response leading to de-repression of genes in the Anr regulon, specifically of those participating in energy metabolism like *azu*.[14a] However, it must also be considered that *P. aeruginosa* relies on alternative terminal oxidases like cytochrome *aa3*, *bo3* and the quinol oxidase CIO[14b] and that *cbb*$_3$-1 deletion leads to their up-regulation.[14a]

In summary, using a novel metalloproteomic approach we have identified the cuproprotein azurin as an important element in the cellular response to alterations in the periplasmic copper homeostasis of *P. aeruginosa*. This appears to be a general ameliorating response to the oxidative and energetic stress generated by copper imbalances.

Acknowledgements

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