Functional diversity of five homologous Cu\(^+\)-ATPases present in *Sinorhizobium meliloti*

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Copper is an important element in host–microbe interactions, acting both as a catalyst in enzymes and as a potential toxin. Cu\(^+\)-ATPases drive cytoplasmic Cu\(^+\) efflux and protect bacteria against metal overload. Many pathogenic and symbiotic bacteria contain multiple Cu\(^+\)-ATPase genes within particular genetic environments, suggesting alternative roles for each resulting protein. This hypothesis was tested by characterizing five homologous Cu\(^+\)-ATPases present in the symbiotic organism *Sinorhizobium meliloti*. Mutation of each gene led to different phenotypes and abnormal nodule development in the alfalfa host. Distinct responses were detected in free-living *S. meliloti* mutant strains exposed to metal and redox stresses. Differential gene expression was detected under Cu\(^+\), oxygen or nitrosative stress. These observations suggest that CopA1a maintains the cytoplasmic Cu\(^+\) quota and its expression is controlled by Cu\(^+\) levels. CopA1b is also regulated by Cu\(^+\) concentrations and is required during symbiosis for bacteroid maturation. CopA2-like proteins, FixI1 and FixI2, are necessary for the assembly of two different cytochrome c oxidases at different stages of bacterial life. CopA3 is a phylogenetically distinct Cu\(^+\)-ATPase that does not contribute to Cu\(^+\) tolerance. It is regulated by redox stress and required during symbiosis. We postulated a model where non-redundant homologous Cu\(^+\)-ATPases, operating under distinct regulation, transport Cu\(^+\) to different target proteins.

**INTRODUCTION**

The influence of Cu\(^+\) distribution in the outcome of host–bacteria interactions is becoming increasingly evident (Argüello *et al.*, 2011; Hodgkinson & Petris, 2012; Samanovic *et al.*, 2012). Toward preventing bacterial proliferation, compartmental ‘flooding’ of potentially deleterious metals such as Cu\(^+\) has been observed in infected phagosomes and in the xylem of plants (Hodgkinson & Petris, 2012; Wagner *et al.*, 2005; Yuan *et al.*, 2010). Cu\(^+\) toxicity through Fenton-like reactions, and as a competitor at cognate metals sites in proteins or affecting Fe–S clusters of hydratases and isomerases, is well established (Macomber & Imlay, 2009; Stohs & Bagchi, 1995). As a consequence, to manage elevated cytoplasmic Cu\(^+\), infectious bacteria have evolved sophisticated mechanisms of metal sensing and detoxification. These consist of at least a Cu\(^+\)-responsive transcription factor, a Cu\(^+\)-binding chaperone and a transmembrane (TM) Cu\(^+\) transporting P-type ATPase (Dupont *et al.*, 2011; Rademacher & Masepohl, 2012). Copper is also required for metallation of various cuproenzymes, such as cytochrome c oxidases (Coxs), Cu/Zn superoxide dismutases (Cu/Zn-Sods), laccases, tyrosinases, nitrite reductases, methane monooxygenases, NADH dehydrogenase 2, Cu\(^+\)-dependent amine oxidases and Cu\(^+\)-dependent polysaccharide oxidogenases (Argüello *et al.*, 2013; Rensing & McDevitt, 2013; Ridge *et al.*, 2008). Some of these enzymes are necessary for virulence, as they either prevent the toxicity of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Lynch & Kuramitsu, 2000; Philippot, 2005), or are part of the respiratory electron transport chain (Preisig *et al.*, 1996b). Given, then, that these proteins are located in distinct compartments (plasma membrane, periplasm or secreted into the media), Cu\(^+\) distribution for metalloprotein assembly also appears critical for bacterial survival (Argüello *et al.*, 2011; Waldron & Robinson, 2009).

Bioinformatics studies have shown that genomes of many pathogenic/symbiotic bacteria contain a diverse array of genes involved in Cu\(^+\) homeostasis (Hernández-Montes *et al.*, 2012). In particular, the presence of multiple Cu\(^+\)-ATPase-coding genes is notable (Argüello, 2003; Argüello *et al.*, 2011). These P\(_{1B}\)-type ATPases are membrane-spanning proteins that couple cytoplasmic Cu\(^+\) efflux to ATP hydrolysis. Amongst their structural features is the presence of signature residues (two Cys in TM6, a Tyr and
an Asp in TM7, and a Met and a Ser in TM8) constituting two well-characterized TM metal-binding sites (TM-MBSs) required for Cu\(^{+}\) transport (Fig. 1a) (González-Guerrero & Argüello, 2008; Mandal et al., 2004). In addition, one or two regulatory Cu\(^{-}\)-binding domains are present in their cytoplasmic N- and C-terminal domains (Argüello, 2003; Argüello et al., 2007). Enzymatic studies have shown the common transport mechanism, kinetic features, substrate-binding stoichiometry and direction of transport, i.e. cytoplasmic Cu\(^{+}\) efflux (Argüello et al., 2007, 2011). The high-resolution (3.2 Å) crystal structure of the *Legionella pneumophila* Cu\(^{+}\)-ATPase CopA revealed the architecture of TMs, the chaperone-docking platform and ligand-exchanging-invariant residues at the cytoplasmic interphase (Gourdon et al., 2011; Padilla-Benavides et al., 2013). From a physiological point of view, prokaryotic Cu\(^{+}\)-ATPases have been associated typically with maintaining low intracellular Cu\(^{+}\) levels (Argüello et al., 2007; Osman & Cavet, 2008; Solioz et al., 2010). However, the presence of multiple Cu\(^{+}\)-ATPases in a given organism challenges the idea of a singular and redundant function for these enzymes (Argüello et al., 2011), albeit the possibility of alternative function does appear counterintuitive considering their unifying structural and biochemical characteristics (Argüello, 2003; Argüello et al., 2007; Raimunda et al., 2011).

Testing these ideas our laboratory has shown that in the opportunistic pathogen *Pseudomonas aeruginosa*, two Cu\(^{+}\)-ATPases play distinct functional roles (González-Guerrero et al., 2010). CopA1 is the ‘classical’ Cu\(^{+}\)-ATPase driving Cu\(^{+}\) efflux in response to elevated cytoplasmic copper levels. Mutation of *copA1*-like-coding genes leads to well-described phenotypes: cytoplasmic accumulation and increased sensitivity to external Cu\(^{+}\) (Argüello et al., 2007; Osman & Cavet, 2008; Solioz et al., 2010). The second Cu\(^{+}\)-ATPase, CopA2, is encoded in an operon together with Cox subunits. Phylogenetically, CopA2-like proteins cluster with ‘FixI ATPases’ in reference to the founding member of this family: *Sinorhizobium meliloti* FixI1 (Kahn et al., 1989) (Fig. 1b). CopA2 drives cytoplasmic Cu\(^{+}\) efflux similar to CopA1, although at a slow rate (González-Guerrero et al., 2010). Mutation of the *copA2*-like-coding gene does not result in sensitivity to Cu\(^{+}\) or cytoplasmic metal accumulation. Rather, it leads to a significant decrease in oxidase activity and sensitivity to oxidative stress (González-Guerrero et al., 2010). This defect in respiratory activity is due to the participation of CopA2 in Cox assembly by transporting Cu\(^{+}\) into the periplasm where it is likely trafficked by a SenC homologue to the FixIN/CooN subunit of the enzyme (Koch et al., 2000; Lohmeyer et al., 2012).

The possibility of additional novel roles is supported by the presence of multiple Cu\(^{+}\)-ATPase-coding genes in various organisms such as *Agrobacterium tumefaciens*, *Mycobacterium tuberculosis*, *Methyllococcus capsulatus* and *L. pneumophila*, among others. The *S. meliloti* genome encodes five Cu\(^{+}\)-ATPase genes (*Sma1013/copA1a*, *Smb21578/copA1b*, *Sma1209/fixI1*, *Sma0621/fixI2* and *Sma1087/copA3*; Fig. 1a, b). A variety of names have been assigned to Cu\(^{+}\)-ATPases in different studies (CopA, PacS, CueA, SilP, ActP, CtpV, etc.). Here, we have chosen to refer to CopA2-like *S. meliloti* Cu\(^{+}\)-ATPases as named previously (FixI1 and FixI2), and name the three novel proteins as CopA1a, CopA1b and CopA3. *S. meliloti* lives both as a free-living organism in soil and as a symbiotic partner of legumes. Although the relationship between rhizobia and plants is ultimately a mutualistic one, the initial infection is in many ways similar to a pathogenic interaction. *S. meliloti* enters the root tissue via infection threads leading to the development of a novel structure in the plant: the nodule (Gage, 2004; Spaínk, 2000). As a response, the host plant cells trigger different defensive mechanisms known as the hypersensitive response, which includes the generation of ROS and RNS (Deledonne et al., 2003; Santos et al., 2001). Therefore, the ability of *S. meliloti* to cope with these stresses is vital to generate an effective symbiotic interaction. The frontline defence mechanism of the bacteria is the secretion of bacterial metalloenzymes such as catalase and Cu/Zn-Sod (Jamet et al., 2003). Following the infection, *S. meliloti* cells differentiate into nitrogenase-producing bacteroids that fix atmospheric N\(_2\) in the form of NH\(_3\). The plant incorporates the NH\(_3\) into amino acids, whilst providing carbohydrates to the bacteria (Freiberg et al., 1997).

What are the distinct functions of *S. meliloti* Cu\(^{+}\)-ATPases? Analysis of their genetic environment provides clues to their likely functions. In *S. meliloti*, all the homologous Cu\(^{+}\)-ATPase genes are located on the symbiotic plasmids, pSymA and pSymB (Fig. 1c), suggesting their importance for bacterial interaction with legumes. CopA1a and CopA1b are highly homologous to CopA1-like ATPases (Fig. 1b). *copA1a* is encoded in the same operon with a metal-responsive transcription factor from the MerR family of regulators, *hmrR* (Fig. 1c). *copA1b* is the only Cu\(^{+}\)-ATPase-encoded gene on pSymB and it is likely transcribed along with another predicted metal-responsive transcription factor named *hmrR2* (Fig. 1c). CopA1a and CopA1b are homologous to ActP from *Sinorhizobium medicae* strain WSM419, which prevents low-pH-induced Cu\(^{+}\) toxicity (Reeve et al., 2002). However, deletion of ActP apparently does not affect the nodulation process (Reeve et al., 2002). The first question that arises from this similar genetic background is whether both CopA1a and CopA1b are required for controlling the cytoplasmic Cu\(^{+}\) levels. Do they resemble the situation in *Salmonella enterica* sv. Typhimurium, where two CopA1-like Cu\(^{+}\)-ATPases are present (Osman & Cavet, 2008; Pontel et al., 2007)?

Early mutagenesis and bioinformatics studies of the *S. meliloti* *fixGH1* operon pointed out the importance of *FixI1* in symbiotic N\(_2\) fixation (Kahn et al., 1989). Analysis of similar operons in *Bradyrhizobium japonicum*, *Rhodobacter capsulatus* and *P. aeruginosa* has shown the role of CopA2-like Cu\(^{+}\)-ATPases in Cox assembly and their capability to drive cytoplasmic Cu\(^{+}\) efflux (González-Guerrero et al., 2010; Koch et al., 2000; Preisig et al., 1996a). In this context, *fixI1* and *fixI2* are located downstream of different *cbb*-type Cox subunits (Fig. 1c). The presence of two CopA2-like ATPases is also observed in the genomes of *Mesorhizobium*.
Fig. 1. Genomic characteristics of *S. meliloti* Cu⁺-ATPases. (a) Partial alignment of the five homologous Cu⁺-ATPase sequences. Lp, *L. pneumophila*. Black boxes indicate sequences corresponding to Cu⁺-binding sites in N-terminal MBDs (first and second panel), as well as conserved TM-MBSs in TM6 (third panel), TM7 and TM8 (bottom panel). (b) Unrooted tree of Cu⁺-ATPases from sequenced bacteria (Gonzalez-Guerrero et al., 2010). Characterized CopA1-like Cu⁺-ATPases are indicated in light blue, including *S. meliloti* CopA1a and CopA1b in dark blue. Characterized CopA2/FixI-like Cu⁺-ATPases are indicated in orange, including *S. meliloti* FixI1 and FixI2 in red. *S. meliloti* CopA3 is indicated in pink. Bar, evolutionary distance as amino acid substitutions per position. (c) Genetic environment of *S. meliloti* Cu⁺-ATPases and their localization in symbiotic plasmids pSymA and pSymB. Arrows, putative transcriptional regulator-binding sites.
loti, Mesorhizobium opportunost WSM2075, Rhizobium leguminosarum WSM1325, Sinorhizobium fredii and S. medicae, which are all closely related organisms that encounter similar environments (soil and plant roots) during their life. Consequently, it can be hypothesized that the associated cbb3-type Cox has distinct properties that fit better with these alternative environments. The presence of a FixK-binding site upstream of fixI suggests a distinctive gene regulation as well. FixK, under the control of the two-component regulatory system FixJL, is induced under microaerobic conditions and has been shown to regulate genes required for nitrogen fixation (Batut et al., 1989). Moreover, FixK-binding sites are homologous to Fnr (fumarate and nitrate reductase)-binding sites, which are responsive to the nitrosative stress response in different rhizobial species (Mesa et al., 2006). However, no putative binding site for transcription regulators has been identified upstream of fixI2 (Fig. 1c). Finally, copA3 encodes a unique Cu\(^{+}\)-ATPase transcribed in a putative operon with hypothetical protein-coding genes Sma1089 and Sma1090. CopA3 belongs to a novel early divergent branch in the CopA3-like subgroup have yet been characterized. Structurally, S. meliloti Cu\(^{+}\)-ATPases are quite similar. For instance, CopA1a and CopA1b share the highest homology with 80% sequence similarity. FixI1/FixI2, share 24% homology. They all contain the archetypical structural features and invariant residues on their TM-MBSs that confer Cu\(^{+}\) selectivity (Argüello, 2003) (Fig. 1a). However, it is notable that CopA1a and CopA1b present two regulatory MBDs, whilst FixI1, FixI2 and CopA3 have only one MBD (Fig. 1a).

To test whether Cu\(^{+}\)-ATPases in S. meliloti have distinct functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance, we explored the phenotypical consequences of mutating each of the coding genes, their functions and importance.

**METHODS**

**Bioinformatics analysis.** S. meliloti Cu\(^{+}\)-ATPases protein sequences were aligned with MUSCLE (Edgar, 2004) and visualized using ESPript software (Gouet et al., 1999). The whole set of bacterial Cu\(^{+}\)-ATPase sequences was aligned using CLUSTALW2 (Thompson et al., 1994) and the phylogenetic tree visualized with FigTree (tree.bio.ed.ac.uk/software/figtree/). The genetic environment of each gene was obtained from RhizoBase (http://genome.microbedb.jp/rhizobase).

**Bacterial strains and culture conditions.** All S. meliloti strains used in this study are listed in Table S1 (available in the online Supplementary Material). S. meliloti (WT2011) and transposon mutant fixI1::mTn5 strains were generously provided by Dr Jacques Batut (University of Toulouse, France). S. meliloti mutant strains copA1b::mTn5, fixI2::mTn5 and copA3::mTn5 were obtained from Dr Anke Becker (Center for Biotechnology, University of Bielefeld, Germany). S. meliloti copA1a::mTn5 mutant strain was obtained by plasmid insertion mutagenesis. S. meliloti strains were grown at 30 °C in either tryptone yeast (TY) medium or yeast extract mannitol agar (YEMA) medium (Beringer, 1974). Media were supplemented with Congo red to assist in the recognition of rhizobia (Vincent, 1970). E. coli strains were grown on LB broth at 37 °C. Media were supplemented with the following antibiotics as required: 100 µg ampicillin ml\(^{-1}\), 20 µg chloramphenicol ml\(^{-1}\), 50 µg gentamicin ml\(^{-1}\), 20 µg kanamycin ml\(^{-1}\), 100 µg neomycin ml\(^{-1}\), 400 µg streptomycin ml\(^{-1}\) or 20 µg tetracycline ml\(^{-1}\) (Table S1).

For microaerobic growth, S. meliloti strains were grown initially in 5 ml TY medium in aerated shaken tubes to late exponential phase. Cultures were diluted to OD\(_{600}\) 0.3 in TY medium and incubated in the GasPak EZ Campy gas-generating pouch system (BD Biosciences). Cells were grown at 30 °C with constant shaking at 220 r.p.m.

**Plasmid insertion mutagenesis.** Suicide plasmid pK19mob2HMB with an internal fragment (~0.3 kb) of the copA1a gene was provided from Dr Anke Becker (Center for Biotechnology, University of Bielefeld, Germany). This plasmid was transferred by conjugation from the mobilizing E. coli S17-1 strain to S. meliloti WT strain for integration into the target gene by single crossover. Colonies were selected in media containing streptomycin and neomycin. Plasmid insertion into the target gene was confirmed by PCR using the pK19mob-Rev primer that annealed to the suicide plasmid and P-CopA1a-For primer that annealed in the promoter region of the copA1a gene (Table S2).

**Verification of single-transposon insertion.** Single-transposon insertion was confirmed by Southern blot analysis. Briefly, genomic DNA was isolated from S. meliloti WT and mutant strains using phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) extraction. Approximately 5 µg of each DNA was digested with EcoRV-HF (New England Biolabs) for 6–8 h at 37 °C. DNA fragments were separated by electrophoresis on 0.8% agarose gel and transferred onto a positively charged nylon membrane (Roche) by capillary transfer using 20 × saline sodium citrate buffer. The neomycin resistance gene, nptII, was amplified using the pK19mob2HMB plasmid as template and NptII-For and NptII-Rev primers (Table S2). DIG-labelled DNA fragments were prepared using the nptII amplicon as a template and DIG High Prime DNA Starter Kit II containing Klenow polymerase (Roche). Resulting amplicons were employed as hybridization probes for Southern blot analysis. Membranes were stained using an alkaline-phosphatase-conjugated anti-DIG antibody and the chemiluminescent substrate disodium 3-(4-methoxyphenyl)-1,2-dioxetane-3.3.1.13,7-decan-1,5-diyl)phenyl phosphate reagent (CSPD; Roche).

**Preparation of complemented strains.** Cu\(^{+}\)-ATPase full-length genes were obtained by PCR using genomic DNA from WT S. meliloti as template. Primers used coded for appropriate cloning restriction sites (Table S2). The DNA fragment was fused downstream from the lac promoter in pBBR1MC3-3, using the plasmids pCOM1013 (lac::copA1a), pCOM21578 (lac::copA1b), pCOM1209 (lac::fixI2), pCOM0621 (lac::fixI2) and pCOM1087 (lac::copA3). It has been
reported that the lac promoter acts as constitutive promoter in S. meliloti (Bittner & Oke, 2006; Stuurman et al., 2000). Plasmids were introduced into the corresponding mutant strains by triparental conjugation using E. coli pRK600 as helper strain (Simon et al., 1983). Stationary-phase cultures of donor, acceptor and helper strains were rinsed, mixed in equal amounts, spotted on 0.2 µm sterile filter discs on LB plates, and incubated overnight at 30 °C. Then, discs were scraped and immersed in liquid TY medium without antibiotics for overnight growth. The mating mixture was spread on TY plates containing neomycin and tetracycline for transconjugant selection. All constructs were verified by automated DNA sequencing, and the presence of the genes on the mutant strains was confirmed by PCR.

Heterologous expression of S. meliloti Cu⁺-ATPases in ΔcopA E. coli. E. coli WT (LMGI94) and ΔcopA strains were provided generously by Dr Barry P. Rosen (Florida International University, Miami, FL, USA) (Rensing et al., 2000). Full-length genes of S. meliloti Cu⁺-ATPases (copA1a, copA1b, fixI1, fixI2 and copA3) were obtained by PCR using S. meliloti genomic DNA as template. Amplicons were cloned into the pBAD TOPO/His vector that adds a C-terminal His6-tag sequence (Invitrogen). DNA sequences were verified by automated sequencing. The ΔcopA E. coli strain was transformed with the resulting constructs and a pBAD-lacZ vector lacking ATPase genes. These clones were selected on LB plates containing ampicillin and kanamycin. Transformed ΔcopA E. coli clones were grown at 37 °C in LB and expression of Cu⁺-ATPases was induced with 0.2 % arabinose when cells reached OD600 0.7. To evaluate protein expression, cells were harvested, washed with 50 mM Tris, pH 7.4, and sonicated in 25 mM Tris, pH 7.4, 100 mM sucrose and 20 % glycerol solution (Buffer A). Cell debris was removed by centrifugation at 11 000 g for 20 min. The supernatant was cleared by centrifugation at 163 000 g for 1 h and the collected membrane fraction was solubilized in Buffer A containing 1 % SDS. Protein (4 µg) from membrane fractions of cells transformed with the Cu⁺-ATPases and soluble fractions of cells transformed with pBAD-lacZ were analysed by 10 % SDS-PAGE following by blotting onto nitrocellulose membranes, and staining using an anti-His6-tag rabbit antibody and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (GenScript).

Plant growth and nodulation assays. Medicago sativa (alfalfa) seeds were surface-sterilized with 90 % ethanol and 15 % bleach followed by three washes with sterile deionized H₂O. The sterilized seeds were stored at 4 °C overnight and allowed to germinate at room temperature for 48 h. Germinated seeds were planted in nitrogen-free agar Murashige and Skoog media containing 1 mM CaCl₂, 1.1 mM K₂HPO₄, 0.17 mM KH₂PO₄, 12.5 µM iron citrate, 0.25 mM MgSO₄, 0.25 mM K₂SO₄, 1 µM MnSO₄, 0.4 µM ZnSO₄, 1.5 mM H₂BO₃, 0.2 µM CuSO₄, 0.025 µM CoSO₄, and 0.05 µM Na₂MoO₄. pH 6.6–6.9 (Murashige & Skoog, 1962) in clear glass culture tubes under sterile conditions. The plants were cultured in a growth chamber at 25 °C, with a 16 h photoperiod and photosynthetic radiation rate of 164 mmol s⁻¹ m⁻² (Lagares et al., 1992). Seedling roots (3 days old) were inoculated with 0.5 ml of either WT or mutant S. meliloti strains grown to OD₆₀₀ 1.5. Plants were monitored daily for emergence of the first nodule. The total number of nodules per plant was counted at 10 and 30 days post-infection (p.i.).

Bacterial viable count after nodulation. Medicago sativa plants were grown and infected as described above. Mature nodules (20 days p.i.) from a single plant were harvested, pooled and homogenized in 25 % YEMA media. The whole bacteria and bacteroids mixture was serially diluted and plated on YEMA-Congo red plates with streptomycin (WT) or streptomycin and neomycin (mutant strains) (Somasegaran, 1985). Plates were incubated at 30 °C for 3 days and c.f.u. were counted.

Light microscopy of plant nodules. Medicago sativa plants were grown and infected as described above, and nodules were collected after 21 days p.i. Intact nodules were imaged using a stereoscopic microscope Nikon SMZ-U (Nikon) and spot 5.1 software (Diagnostic Instrument). Nodules were fixed in buffer containing 4 % paraformaldehyde, 0.25 % glutaraldehyde and 2.5 % sucrose in 50 mM potassium phosphate buffer, pH 7.4, and incubated overnight at 4 °C with gentle agitation (Rodriguez-Haas et al., 2013). Dehydration was carried out using a graded ethanol series (0, 30, 50, 70, 85, 90, 95, 100 %) at 4 °C. Subsequently, nodules were infiltrated with LR-White Resin (London Resin) overnight at 4 °C. Embedded nodules were transferred to gelatine capsules and polymerized in fresh LR-White resin for 16 h at 60 °C. Sections (1 µm) were obtained using a Leica EM UC6 ultramicrotome (Leica Microsystems). Semi-thin sections were stained with 0.1 % toluidine blue solution for 3 min and then observed under a Leica upright fluorescence microscope DMLB2 equipped with Leica Application Suite V3 (Leica Microsystems).

Copper sensitivity assays. TY liquid cultures supplemented with different CuSO₄ concentrations were inoculated with overnight cultures of S. meliloti strains at OD₆₀₀ 0.1 (aerobiosis) or 0.3 (microaerobiosis). Cells were grown for either 24 or 40 h under aerobic or microaerobic conditions and OD₆₀₀ was measured.

Nitrosative stress sensitivity assays. TY liquid cultures were inoculated with overnight cultures of WT and mutant S. meliloti strains at OD₆₀₀ 0.2 and supplemented with increasing concentrations of freshly prepared spermine NONOate (SpNN; Cayman Chemicals) in 0.1 M sodium phosphate buffer, pH 6.9 (Meihooc et al., 2010). Cells were grown for 14 h and OD₆₀₀ was measured.

Copper accumulation assays. Copper accumulation was measured as described previously with minor modifications (Raimunda et al., 2011). Aliquots of 5 ml TY liquid cultures of WT and mutant S. meliloti strains grown to late exponential phase were supplemented with 0.5 mM CuSO₄ and incubated at 30 °C for 2 h. Cells were harvested and total protein determined (Bradford, 1976). Cells were acid-digested with 0.5 ml NO₃H (trace metal grade) for 1 h at 80 °C and incubated overnight at room temperature. Digestions were concluded after addition of 0.2 ml 30 % H₂O₂ and diluted to 1 ml with water. Metal content was measured by furnace atomic absorption spectroscopy (Varian SpectrAA 880/GTA 100).

Gene expression analysis. WT S. meliloti cells grown to exponential phase were supplemented with 2 mM CuSO₄, 0.3 mM CoCl₂, 0.1 mM ZnSO₄, 1 mM FeCl₃, 25 µM SpNN, 20 µM pparaquat or 30 mM H₂O₂ and incubated for 2 h at 30 °C (except H₂O₂ for 15 min). Alternatively, cells were exposed to microaerobiosis as described, with or without 2 mM CuSO₄, and incubated for 4 h at 30 °C. Following these treatments, cells were harvested and RNA was stabilized with RNA Protect Bacteria reagent (Qiagen) and isolated with the RNeasy Mini Kit (Qiagen). Gene expression from three independent samples was analysed by quantitative (q) real-time PCR using an iCycler iQ system (Bio-Rad) as described previously (Gonzalez-Guerrero et al., 2010). Results were normalized to the 16S ribosomal unit levels and relative levels of transcription were calculated using the 2-ΔΔCt method (Livak & Schmittgen, 2001). Primer pairs used yielded qPCR efficiencies >90 % and single bands upon gel analysis (not shown). In all experiments, control samples not treated with reverse transcriptase were included to detect possible DNA contamination.
RESULTS

Deletion of Cu\(^+\)-ATPases results in distinct phenotypes during symbiosis

Several rhizobial species, *S. meliloti* among them, encode multiple Cu\(^+\)-ATPases (Fig. 1) (Argüello, 2003; González-Guerrero et al., 2010). As these genes are frequently located on symbiotic plasmids, it is likely that they are important for the establishment or persistence of the symbiotic interaction. Transposon insertion mutants (mTn5) of each of the five ATPases were used to explore their role during the nodulation process. The presence of single-transposon insertions in the target gene sites was confirmed by PCR and Southern blot (Fig. S1).

Seedlings of *Medicago sativa*, the host plant, were infected with either *S. meliloti* WT or mutant strains and different parameters of the symbiotic interaction were evaluated. A change in the timing of the appearance of the first nodule is a simple indication of alterations in the initial infection steps. Monitoring this parameter, it was observed that deletion of the fixI1 gene led to a delay of 2 days for the beginning of nodulation (5.2 ± 0.5 days p.i. WT; 7.13 ± 0.03 days p.i. fixI1::mTn5), whilst mutations copA1a::mTn5, copA1b::mTn5, fixI2::mTn5 and copA3::mTn5 did not affect the time of nodule appearance (Table S3). These data suggested that FixI1 may be important during the early stages of nodulation.

The formation of new nodules, although dependent on the presence of rhizobia, is a plant-controlled process (Schauer et al., 1999). As the symbiotic interaction progresses, the plant typically increases the number of nodules to keep up with the growing demand for fixed nitrogen. Conversely, if bacteroids are not efficiently fixing N\(_2\), the plant will cut off carbohydrate supply or decrease O\(_2\) supply to terminate the non-functioning nodules (Kiers et al., 2003). Fig. 2(a) shows that after 10 days p.i. (grey bars) the fixI2::mTn5 strain generated almost twice as many nodules per plant (8 ± 1) as the WT (3.8 ± 0.6) than those infected with the WT strain (Fig. 2a, white bars). Plants infected with the copA3::mTn5 strain formed significantly fewer nodules per plant (5.7 ± 0.6) than those infected with the WT strain at 30 days p.i. (Fig. 2a). These plants did not appear to develop additional nodules beyond those originated after 10 days p.i. These results suggested FixI2 and CopA3 contribute distinctly to the function of mature nodules.

During symbiosis, two different populations of rhizobia are established in the nodule. One corresponds to a small pool of undifferentiated viable bacteria, capable of reproduction and growth in free-living conditions. The second population is the well-defined, differentiated N\(_2\)-fixing bacteroids unable to divide or revert back to the free-living form (Oke & Long, 1999). We hypothesized that fixI2 and copA3 mutations, which altered nodule formation, might have altered levels of rhizobia/differentiated bacteroids. Fig. 2(b) shows that the number of undifferentiated bacteria contained in nodules derived from the fixI2::mTn5 and copA3::mTn5 strains was higher than those infected with the WT strain. An even larger increase was observed in the number of viable bacteria in the copA1b::mTn5 strain-induced nodules, suggesting that CopA1b, FixI2 and CopA3 are necessary for rhizobia to differentiate into bacteroids.

Morphological characterization of nodules produced by *S. meliloti* Cu\(^+\)-ATPase mutant strains further exposed the different roles of each transporter. A temporal development of the symbiosis can be studied by observing spatial differentiation along an indeterminate nodule. Four zones have been defined in the mature nodule: zone I corresponds to the apical meristem in the distal portion of the nodule; zone II is characterized by few infected plant cells where rhizobia are released into the host cells and differentiate; zone III is an intermediate large N\(_2\)-fixing zone constituted by infected cells, called symbiosomes, which are larger than the uninfected cells and contain a dense population of bacteroids; and zone IV is the senescent zone (Franssen et al., 1992). This tissue organization (zonation) and the bacterial infection of the nodules were analysed by histochemical staining and light microscopy. After 21 days p.i., WT-induced nodules (Fig. 3a1, a2) were elongated mono- or bi-lobed. Their pink colour, due to the presence of leghemoglobin, a plant protein induced during N\(_2\) fixation, is an indication of healthy nodules (Banba et al., 2001). Histological analyses of semi-thin sections of alfalfa nodules produced by the WT strain showed the typical zone distribution associated with the indeterminate nodule: meristem (I), bacterial differentiation (II) and N\(_2\) fixation (III) zones (Fig. 3a3). The senescence zone (IV) was not distinguishable at this maturation stage (21 days p.i.). Microscopic examination at higher magnification of symbiotic cells in WT nodules revealed the endocytic uptake of the rhizobia. Typical, large symbiosomes (~50 µm) are fully packed with elongated bacteroids (8–10 µm; Fig. 3a4) (Mergaert et al., 2006; Vasse et al., 1990). Other features such as the vascular bundles located below the inner cortex, nodule parenchyma and infection threads were also observed.

Although the pink coloration of the nodules produced by the copA1a::mTn5 strain (Fig. 3b1, b2) is similar to the WT strain, microscopic analysis of semi-thin sections showed fewer infected cells in zone III (Fig. 3b3) and larger vacuoles in symbiosomes (Fig. 3b4). The copA1b::mTn5 strain produced nodules with a characteristic larger zone II (Fig. 3c3). This suggests that, in agreement with the observation of a higher viable count, apparently these bacteria are not undergoing differentiation into bacteroids (Fig. 2b). Interestingly, higher magnification analysis revealed smaller symbiosomes with more vacuoles and lower density of bacteroids than in WT-derived nodules (Fig. 3c4). The fixI1::mTn5-derived nodules were white, suggesting a decrease in leghemoglobin (Fig. 3d2). The main difference observed in these nodules was a smaller zone III and number of symbiosomes (~30 µm) (Fig. 3d3,
Participation of two different mechanisms of nodule necrosis. It has been shown that CopA2-like Cu\(^{2+}\)-ATPases are induced by ROS. For instance, P. aeruginosa copA2 expression is induced by H\(_2\)O\(_2\) and the copA2 mutant strain is sensitive to the presence of H\(_2\)O\(_2\) in the media (data not shown). Nevertheless, the fixI1 mutant strain showed a slight but detectable growth defect at early exponential phase (Fig. S2). As symbiosis occurs in a microaerobic environment (Gage, 2004; Spaink, 2000), it was then surprising also to observe normal growth for copA1a, copA1b, fixI2 or copA3 mutant strains under microaerobiosis (data not shown). On the contrary, and in agreement with the regulation of the fixNOQP1 operon by FixK (Fig. 1c), fixI1 showed a notable slower growth rate under microaerobiosis (Fig. 4a). This pointed to the particular requirement of its associated oxidase during symbiosis.

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**Participation of S. meliloti Cu\(^{2+}\)-ATPases in stress tolerance**

In order to consider the effects of Cu\(^{2+}\)-ATPase mutations on symbiosis (Figs 2 and 3), bacterial growth under stress conditions was examined. As a starting point, it was established that mutation of S. meliloti copA1a, copA1b, fixI2 or copA3 did not affect growth under aerobiosis in TY media (data not shown). Nevertheless, the fixI1 mutant strain showed a slight but detectable growth defect at early exponential phase (Fig. S2). As symbiosis occurs in a microaerobic environment (Gage, 2004; Spaink, 2000), it was then surprising also to observe normal growth for copA1a, copA1b, fixI2 or copA3 mutant strains under microaerobiosis (data not shown). On the contrary, and in agreement with the regulation of the fixNOQP1 operon by FixK (Fig. 1c), fixI1 showed a notable slower growth rate under microaerobiosis (Fig. 4a). This pointed to the particular requirement of its associated oxidase during symbiosis.

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meliloti Cu\(^+\)-ATPases in this function was evaluated by measuring the growth rate in the presence of increasing metal concentrations. Fig. 5(a) shows that copA1a::mTn5 is sensitive to Cu\(^+\) concentrations as low as 0.5 mM. Its Cu\(^+\) tolerance was restored partially by reintroducing the copA1a gene under the control of a constitutive lac
The role of CopA1b and CopA3 maintaining Cu$^+$ sensitivity (data not shown). Similar results were observed under microaerobiosis with increasing Cu$^+$ concentrations (Fig. 5b). Interestingly, the described 'rescue' of the copA2-like ATPase deletion phenotype by external Cu$^+$ was observed in the fixI1::mTn5 strain, which grew at rates comparable with WT in the presence of high Cu$^+$ under microaerobiosis (Fig. 5b) (Hassani et al., 2010; Koch et al., 2000; Preisig et al., 1996a).

Consistent with a role in controlling cytoplasmic Cu$^+$, increased Cu$^+$ levels are observed when expression of functional CopA1-like Cu$^+$-ATPases is disrupted (Arguillo et al., 2011; Osman & Cavet, 2008; Solioz et al., 2010). Fig. 6 shows that under basal conditions (black bars), no significant differences in cytoplasmic Cu$^+$ levels were observed between mutant and WT strains. However, when the media were supplemented with 0.5 mM Cu$^{2+}$, a significant increase in the cytoplasmic Cu$^+$ of the copA1a::mTn5 mutant was detected (Fig. 6, white bars). No alterations were observed in the Cu$^+$ levels of other S. meliloti mutant strains.

Results presented in Figs 5 and 6 are in agreement with our early description of CopA1-like (CopA1a) and CopA2-like (FixI1 and FixI2) ATPases (González-Guerrero et al., 2010; Raimunda et al., 2011). However, they are inconclusive on the role of CopA1b and CopA3 maintaining Cu$^+$ quotas. To explore this, the capability of S. meliloti Cu$^+$-ATPases to complement the ΔcopA E. coli strain was tested. As expected, both predicted CopA1-like enzymes, CopA1a and CopA1b, complemented the Cu$^+$-sensitive phenotype of the ΔcopA E. coli mutant strain (Fig. 7a). However, fixI1-,

fixI2- or copA3-complemented strains showed the Cu$^+$-sensitive phenotype of ΔcopA E. coli cells (Fig. 7a). Thus, we hypothesized that CopA3 might, as with CopA2-like proteins, participate in the metallation of periplasmic/secreted proteins, rather than maintaining the cytoplasmic Cu$^+$ quota. Controls were performed to verify that similar levels of heterologous proteins were present in the membranes of ΔcopA E. coli cells (Fig. 7b).
Differential gene expression of \( S. \) \( meliloti \) \( Cu^{+} \)-ATPases

The functional diversity of \( S. \) \( meliloti \) \( Cu^{+} \)-ATPases might be associated with a differential control of expression. That is, although performing similar cellular function, evolutionarily advantages have been gained by the distinct regulation of highly homologous and functionally equivalent sets of genes. This appears to be the case with \( fixI1 \) and \( fixI2 \). However, the presence of homologous metal-responsive transcription factors co-transcribed with \( copA1a \) and \( copA1b \) does not necessarily imply redundant functions (Humbert et al., 2013) (Fig. 1c). Thus, the gene expression profile of \( Cu^{+} \)-ATPases in response to various redox conditions, \( Cu^{+} \) and \( O_2 \) levels was analysed.

DISCUSSION

Previous studies have described the presence of up to five \( Cu^{+} \)-ATPase genes within bacterial genomes (Argüello, 2003). We have shown that most of these transporters fit within two major subgroups (Argüello et al., 2011; González-Guerrero et al., 2010). The \( CopA1 \)-like enzymes, whose expression is controlled by cytoplasmic \( Cu^{+} \) sensors, are responsible primarily for maintaining low cytoplasmic metal levels. The subgroup of \( CopA2 \)-like (\( FixI \)) \( Cu^{+} \)-ATPases includes transporters required for the assembly of \( Cox \). However, we hypothesized that additional physiological roles might be attributed to \( Cu^{+} \)-ATPases to explain the presence of multiple homologous proteins performing the same basic biochemical function (i.e. the \( ATP \)-dependent efflux of cytoplasmic \( Cu^{+} \) towards the periplasm or the extracellular milieu). In order to test this hypothesis, the five \( Cu^{+} \)-ATPases present in \( S. \) \( meliloti \) were studied. Their phylogenetic relation (Fig. 1b) and genetic environment (Fig. 1c) suggest that two of them, \( CopA1a \) and \( CopA1b \), are \( CopA1 \)-like transporters; two
others, FixI1 and FixI2, are CopA2-like proteins; and the fifth protein, CopA3, belongs to a novel uncharacterized subgroup. Thus, *S. meliloti* appears to be an excellent model not only to study a new Cu⁺-ATPase subgroup, but also to explore the significance of the presence of two CopA1-like or two CopA2-like ATPases in a given organism. We observed that mutation of each of the coding genes led to a distinct phenotype. Moreover, each gene has a unique expression profile in response to different stresses. The data support the presence of a novel CopA3-like subgroup of Cu⁺-ATPases and a model where duplications of Cu⁺-ATPases within a given subgroup provide the advantage of distinct regulatory mechanisms adapted to alternative environments.

**CopA1a is required for maintenance of cytoplasmic Cu⁺ levels**

CopA1a appears as the housekeeping ATPase responsible for maintaining cytoplasmic Cu⁺ levels. The *S. meliloti* copA1a::mTn5 mutant strain is sensitive to and accumulates Cu⁺. Interestingly, these phenotypes are not masked by the presence of the similar CopA1b (see below). Likely regulated by *hmrR*, copA1a expression is induced in the presence of Cu⁺. These characteristics are evident under both aerobic and microaerobic conditions. Moreover, CopA1a is a typical CopA1-like transporter able to complement the *E. coli* CopA function, conferring Cu⁺ tolerance to the ΔcopA *E. coli* mutant strain. The role of CopA1a during symbiosis is less clear. No alterations in nodulation were reported for nodules generated by *S. medicae* carrying a mutation of the CopA1-like ActP Cu⁺-ATPase (Reeve et al., 2002). Expression of the *S. meliloti* copA1a gene is not induced during symbiosis (Capela et al., 2005) (Fig. S4). However, we observed a decrease in the amount of infected cells in the N₂ fixation zone (III) in nodules generated by the copA1a mutant strain, suggesting a defective N₂ fixation process.

**CopA1b is required for bacterial differentiation**

CopA1b is highly homologous to CopA1-like ATPases and, like copA1a, is co-transcribed with a putative Cu⁺ responsive transcription factor (*hmrR2*). Along this line,
**copA1b** is induced by the presence of $\text{Cu}^+$, although to a lesser extent than **copA1a**. Furthermore, **copA1b** restored $\text{Cu}^+$ tolerance of the $\Delta$**copA E. coli** mutant strain, suggesting that this ATPase might have a $\text{Cu}^+$ transport rate similar to that reported for $\text{CopA1-like ATPases (Raimunda et al., 2011)}$. However, mutation of $S. meliloti$ **copA1b** does not lead to changes in $\text{Cu}^+$ tolerance or increased cytoplasmic $\text{Cu}^+$ levels, most likely because these effects are masked by the presence of the functional **CopA1a**. Importantly, the $\text{copA1b}::\text{mTn5}$ strain showed a singular phenotype during symbiosis. The differentiation of the mutant strain into bacteroids seems altered, since an increase of undifferentiated cells (zone II) was observed and a significantly higher number of viable bacteria were measured in these nodules. **copA1b** is encoded within the $S. meliloti$ *pSymB* (Fig. 1c). Based on genomic parameters, it was proposed that *pSymB* was acquired by an ancestral $S. meliloti$ before *pSymA* (Finan et al., 2001; Galibert et al., 2001). $S. meliloti$ *pSymA* contains genes essential for nodulation, nitrogen fixation and catabolism of plant compounds, whereas *pSymB* encodes numerous genes involved in the transport of micronutrients (Glazebrook & Walker, 1989; Renalier et al., 1987). For instance, 20% of the *pSymB* sequence corresponds to uptake systems, most of them members of the ATP-binding cassette family (Bardin et al., 1996; Finan et al., 2001; Watson et al., 1988). Genes required for polysaccharide and exopolysaccharide synthesis correspond to 14% of the *pSymB* sequence, whilst genes for thiamine synthesis/catabolism and utilization of atypical carbon sources such as phosphonates are also present (Finan et al., 2001; Gage & Long, 1998). Thus, *pSymB*-encoded genes seem to participate in the saprophytic competence of the bacteria in the soil environment. It is noteworthy that the presence of two **CopA1-like enzymes is not unique to $S. meliloti$**. This is also observed in the genomes of other symbiotic/pathogenic bacterial species like $B. japonicum$, *Rhizobium leguminosarum*, *A. tumefaciens*, *Corynebacterium efficiens*, *Corynebacterium diptheriae*, *Corynebacterium glutamicum*, *Campylobacter jejuni*, *Nocardia farcinica*, *Synechococcus* sp. and *Salmonella enterica* sv. *Typhimurium*. A common characteristic among most of these species is the capability to survive in saprophytic environments as well. Thus, it is tempting to hypothesize that **CopA1b** may represent an isolated ATPase that is required mainly for controlling cytoplasmic $\text{Cu}^+$ levels under particular soil conditions or, as suggested by our observations, during bacterial differentiation in the nodule.

**FixI1 and FixI2 are required at different stages of $S. meliloti$ life**

**CopA2-like (FixI) ATPases** are co-transcribed with $\text{cbb}_3$-type Cox subunits and their mutation results in a deficiency in the activity of these cuproproteins (González-Guerrero et al., 2010; Hassani et al., 2010; Koch et al., 2000; Preisig et al., 1996a). Previous reports have shown that whilst these ATPases drive the efflux of cytoplasmic $\text{Cu}^+$, their slow transport rate is not sufficient to confer $\text{Cu}^+$ tolerance (Argiuello et al., 2011; Raimunda et al., 2011). In agreement with this observation, neither **FixI1** nor **FixI2** was able to complement the $\text{Cu}^+$ sensitivity of the $\Delta$**copA E. coli** mutant strain. Both $S. meliloti$ **fixI1** and **fixI2** genes are downstream of a $\text{cbb}_3$-type Cox-coding operon. $\text{Cu}^+$ did not induce their expression; nor do they confer $\text{Cu}^+$ tolerance. What is their particular role? The presence of two **CopA2-like ATPases** is not uncommon. Various *Rhizobiales* (*Mesorhizobium ciceri*, *Mesorhizobium loti*, *Mesorhizobium opportunistic*, *Rhizobium leguminosarum*, *S. fredii*, *S. medicae* and *S. meliloti*) have at least two operons coding for $\text{cbb}_3$-type Cox and, consequently, two associated **CopA2-like ATPases**. We observed that in $S. meliloti$ only **fixI1** is induced by microaerobiosis and nitrosative stress. The **fixI1** mutant has partial sensitivity to SpNN, and **fixI1**, rather than **fixI2**, is predominantly expressed during nodulation (Capela et al., 2005) (Fig. S4). The latter observation is consistent with the bioinformatics analysis showing a FixK recognition site in the **fixI1** promoter region. Moreover, *Medicago sativa* plants infected with the $\text{fixI1}::\text{mTn5}$ strain showed a significant delay in appearance of the first nodule and morphological alterations of the nodules suggesting that **fixI1** and the **fixNOPQI** operon may be predominantly involved in respiration under microaerobiosis during symbiosis. Interestingly, studies of *B. japonicum* electron chain terminal oxidases suggest that $\text{aa}_3$-type Cox is active during aerobic respiration, whereas microaerobic respiration is maintained by a $\text{cbb}_3$-type Cox (Preisig et al., 1996a). Showing the distinct role of each FixI-like ATPase, the **fixI2** mutation does not alter $S. meliloti$ growth under microaerobiosis or nitrosative stress conditions. Moreover, **fixI2** expression remains constant under varying oxygen levels and nitrosative stress. However, the **fixI2**::**mTn5** strain generates abnormal nodules and is apparently unable to differentiate into bacteroids, pointing to a role in the **fixNOPQ** operon and the associated **FixI2** during symbiosis. We hypothesize that **FixI2** is the housekeeping $\text{Cu}^+$-ATPase involved in the metallation of a constitutive Cox required for respiration during all steps of bacterial life.

**CopA3 is probably required for secreted protein metallation in $S. meliloti$**

**CopA3** appears as a novel singular ATPase. The lack of sensitivity to $\text{Cu}^+$ or accumulation in the **copA3**:**mTn5** mutant strain suggests that this ATPase is not required for controlling the cytoplasmic $\text{Cu}^+$ levels. Unlike **CopA1-like ATPases**, heterologously expressed **CopA3** is not able to complement $\text{Cu}^+$ sensitivity in the $\Delta$**copA E. coli** mutant strain. However, the $S. meliloti$ **copA3**:**mTn5** mutant strain is sensitive to SpNN, suggesting an important role of **CopA3** in coping with RNS upon infection. Moreover, the presence of a FixK-binding site upstream of **copA3** appears to upregulate this gene under microaerobiosis and nitrosative stress. In agreement with these observations, **CopA3** is important for the nodulation process. Mature nodules formed by the **copA3**:**mTn5** strain were small and

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presented a quite altered morphology. Moreover, an increase in viable cells was observed, perhaps as an attempt to compensate for poorly functional bacteroids. Upon establishment of the interaction between S. melloti and Medicago sativa, the bacteria must cope with the plant hypersensitive response (Delledonne et al., 2003). As CopA3 is not involved in maintaining cytoplasmic Cu\(^{2+}\) levels, we propose that copA3 is one such ATPase necessary for metatallation of periplasmic/secreted proteins required to overcome the plant immune response. Whilst candidates for final acceptor cuproproteins have been proposed (Argüello et al., 2013; Rensing & McDevitt, 2013; Ridge et al., 2008), studies to identify them are beyond the scope of this report.

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