Copper homeostasis in cyanobacteria.

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The CopRS two-component system is responsible for resistance to copper in the cyanobacterium *Synechocystis* sp. PCC 6803.

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ABSTRACT

Photosynthetic organisms need copper for cytochrome oxidase and for plastocyanin in the fundamental processes of respiration and photosynthesis. However, excess of free copper is detrimental inside the cells and therefore organisms have developed homeostatic mechanisms to tightly regulate its acquisition, sequestration and efflux. Herein we show that the CopRS two-component system (also known as Hik31-Rre34) is essential for copper resistance in *Synechocystis* sp. PCC 6803. It regulates expression of a putative HME-RND type copper efflux system (encoded by *copBAC*) as well as its own expression (in the *copMRS* operon) in response to the presence of copper in the media. Mutants in this two-component system or the efflux system render cells more sensitive to the presence of copper in the media and accumulate more intracellular copper than the WT. Furthermore, CopS periplasmic domain is able to bind copper suggesting that CopS could be able to detect copper directly. Both operons (*copMRS* and *copBAC*) are also induced by the photosynthetic inhibitor DBMIB but this induction requires the presence of copper in the media. The reduced response of two mutant strains to copper, one lacking plastocyanin and a second one impaired in copper transport to the thylakoid, due to the absence of the P_I-type ATPases PacS and CtaA, suggests that CopS can detect intracellular copper. In addition, a tagged version of CopS with a triple HA epitope localizes to both the plasma and the thylakoid membranes, suggesting that CopS could involved in copper detection in both the periplasm and the thylakoid lumen.

INTRODUCTION

Copper is an element required for essential biological processes such as respiration, through the cytochrome oxidase, or in photosynthesis through the electron transfer protein plastocyanin in plants, some algae and cyanobacteria. It is also used as a metal cofactor of different enzymes including oxidases, monooxygenases, dioxygenases and superoxide dismutases. The ability of copper to alternate between its cuprous Cu(I) and cupric Cu(II) oxidation states makes it an excellent biological cofactor. However, when unbound within a cell redox cycling means copper is toxic, largely due to its ability to catalyze Fenton-like reaction, causing the production of highly reactive hydroxyl radicals that damage biomolecules such as DNA, proteins, and lipids (Imlay, 2003). An alternative copper toxicity mechanism has been also demonstrated in some bacteria in which copper interferes with the formation of catalytic Fe-S clusters, damaging essential enzymatic activities and also leading to the generation of reactive oxygen species (Macomber and Imlay, 2009; Chillappagari *et al.*, 2010; Tottey *et al.*, 2012). As a result, microorganisms have developed diverse mechanisms for the control of copper homeostasis.

Copper homeostasis is a complex process involving acquisition, sequestration, and efflux of the metal ion. In bacteria, active efflux is one of the key mechanisms for copper resistance and three non-related families of export system have been implicated in copper resistance and homeostasis: P_I-type ATPases, such as *Escherichia coli* CopA (Rensing *et al.*, 2000; Grass and Rensing, 2001; Rensing and Grass, 2003), Heavy-metal Eflux-Resistance Nodulation and Division (HME-RND) efflux system, such as CusBAC (Grass and Rensing, 2001), and membrane proteins such as CopB and CopD from *Pseudomonas syringae* (Mills *et al.*, 1993; Osman and Cavet, 2008). Periplasmic copper metabolism has also an important role in copper homeostasis, since most copper containing proteins are periplasmic or plasma membrane proteins. In fact, copper homeostasis systems usually contains periplasmic copper binding proteins, and in some cases, copper oxidases, that oxidizes Cu(I) to the less toxic Cu(II) (Osman and Cavet, 2008; Kim *et al.*,

2010). In addition some bacteria contain intracellular copper chaperones, which deliver intracellular copper to target proteins (Robinson and Winge, 2010). These copper resistance systems are in general regulated by metalloregulatory proteins able to bind the metal. Two unrelated families of copper responsive repressors have been described: CopY, a winged helix DNA binding protein, and CsoR, that belongs to new family of transcriptional repressors (Solioz *et al.*, 2010). Whereas, two other regulatory systems that work as activators have been also described: CueR, a MerR family copper dependent activator (Outten *et al.*, 2000), and CopRS, a two component copper responsive system (Osman and Cavet, 2008). CueR, CopY and CsoR detect cytoplasmic copper levels while CopRS is thought to detect periplasmic copper.

Photosynthetic organisms have high intracellular copper requirements mainly for the photosynthetic electron transfer protein plastocyanin, and they have adapted to accommodate to variable copper concentrations in the environment. In plants, copper import requires the action of several transporters at different locations in the plant. The import of copper in the roots is mediated by CTR and ZIP family of transporters while the P_I-type ATPases PAA1 and PAA2 are involved in copper transport into the chloroplast (Pilon *et al.*, 2006; Pilon *et al.*, 2009; Puig and Penarrubia, 2009). Copper transport system from roots to shoots is much less characterized (Puig and Penarrubia, 2009). As in other organisms copper chaperones assist the trafficking and loading of copper to proteins in the cytosol (ATX1, CCH1, CCS1), the mitochondria (COX17) or the chloroplast (CCS1) (Puig *et al.*, 2007). Most of these genes are regulated at the transcriptional level after copper excess. Thus, transporters such as COPT1-2 and COPT4; ZIP2 and 4; PAA1, PAA2 and HMA1 are down-regulated, while copper chaperones are induced (del Pozo *et al.*, 2010). Under copper deficiency conditions, photosynthetic organisms express alternative isoenzymes that use different metal cofactors to copper and also induce copper import proteins (Yamasaki *et al.*, 2009; Castruita *et al.*, 2011; Bernal *et al.*, 2012), in order to save copper for plastocyanin which is strictly required for photosynthesis in plants (Puig *et al.*, 2007). Some algae and cyanobacteria can also express an alternative electron transfer protein, a heme

containing cytochrome c_6 (Merchant and Bogorad, 1986; Zhang *et al.*, 1992; Merchant *et al.*, 2006). This response is regulated by homologous transcriptional factors in eukaryotic photosynthetic organisms: CRR1 in *Chlamydomonas reinhardtii* (Kropat *et al.*, 2005) and SPL7 in *Arabidopsis thaliana* (Yamasaki *et al.*, 2009; Bernal *et al.*, 2012). In contrast, very little is known about copper gene regulation in cyanobacteria despite the early discovery of the switch in gene expression between plastocyanin (encoded by *petE*) and cytochrome c_6 (encoded by *petJ*) depending on copper availability (Zhang *et al.*, 1992). In cyanobacteria copper metabolism has been analyzed mainly in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*). Copper import is mediated by two P_I-type ATPases, CtaA and PacS, a small soluble copper metallochaperone Atx1 (SynAtx1) (Tottey *et al.*, 2002) and a periplasmic iron containing protein FutA2 (Waldron *et al.*, 2007). These proteins are required for normal photosynthetic electron transfer via plastocyanin and for the activity of a second thylakoid-located copper protein, a caa_3 type cytochrome oxidase (Tottey *et al.*, 2001; Tottey *et al.*, 2002; Waldron *et al.*, 2007; Tottey *et al.*, 2012), although the exact role of the periplasmic protein FutA2 is not completely clear (Waldron *et al.*, 2007). Copper is imported inside the cell by CtaA which delivers it to SynAtx1 which is then thought to transfer it to PacS that transports copper to the thylakoid lumen. Recently, glutathione has been shown to cooperate with SynAtx1 to buffer cytoplasmic copper levels preventing deleterious side reactions (Tottey *et al.*, 2012).

Here we present evidence that the Hik31/Rre34 two-component system (designated CopRS here) is involved in copper resistance in *Synechocystis* by directly regulating a HME-RND export system (CopBAC; encoded by ORFs *slr6042*, *slr6043 and slr6044*) and a protein of unknown function CopM (encoded by ORFs *sll0788* and *slr6039)*. Although responding to copper, CopRS is neither involved in the regulation of copper import system nor in the switch between *petE* and *petJ*. Furthermore, using a combination of different genetic and molecular biology approaches we show that CopS is able to bind copper and partially

localizes to thylakoid membranes. *copMRS* is also induced by conditions that alter the electron transport rate around PSI, which indicates that these genes are under redox control. Under these conditions plastocyanin protein levels decrease and this mirrors *copMRS* induction. This induction strictly requires the presence of copper in the media and CopRS. Furthermore, induction of *copMRS* after low copper addition is diminished in mutants with reduced levels of plastocyanin, suggesting that part of the signal detected by CopS needs copper to be incorporated into plastocyanin.

RESULTS

CopRS is involved in copper resistance.

A gene cluster involved in metal resistance in *Synechocystis* was previously characterized (Thelwell *et al.*, 1998; Rutherford *et al.*, 1999; Garcia-Dominguez *et al.*, 2000). The two component system *hik31-rre34 (sll0789* and *sll0790)* is located next to the metal resistance cluster, downstream to *ziaA* (Fig. 1A), and code for the closest homolog to the NrsRS two-component system in *Synechocystis* (46 % identity; 64% similarity). Upstream of these two genes there is an additional ORF (*sll0788*) that contains two DUF305 domains of unknown function and likely forms an operon with them. These three genes are repeated in one of the *Synechocystis* endogenous plasmids (Kaneko *et al.*, 2003), pSYSX (*slr6039*, *slr6040* and *slr6041* with a 93% identity at the nucleotide level, including 71 pb before the starting GTG for *sll0788* and *slr6039*, and 95% at the amino acid sequence level). We have named these genes *copMRS* and *pcopMRS*, respectively. Their location and homology led us to study its putative role in metal resistance. As a first step we have analyzed their expression in response to different metals in the media. We analyzed expression of both *copMRS* and *pcopMRS* since their high sequence homology did not allow us to distinguish between them (therefore we will refer to both copies simply as *copMRS* when analyzing gene expression). As shown is Fig 1B *copM* expression was induced in the presence of an excess of copper (3 µM $CuSO_4$), but induction by other metals was negligible (Fig 1B). Furthermore, northern and RT-PCR analysis confirmed that *copM* was co-transcribed with *copR*

and *copS* and therefore the three genes form an operon (Fig S1; and Summerfield *et al.*, 2011). To further study their role in metal homeostasis we analyzed growth of mutant strains lacking one or both copies of these genes (Table S1) in the presence of different metals in the media. Mutants lacking a functional copy of *copMRS* (GCOP strain) or *pcopMRS* (PCOP strain; Fig. 1C) are indistinguishable from WT. In contrast, double mutants lacking functional copies of both *copR* and *pcopR* (COPR strain), *copS* and *pcopS* (COPS strain) or carrying a mutation in the catalytic histidine ($COPS_{H227A}$) showed reduced growth at 0.75 µM of copper and failed to grow at 1 µM of the metal (Fig. 1C) showing that this two component system is essential for copper resistance, but not to other metals (Fig. S2). Moreover COPR cells accumulate about twice the amount of copper than WT cells (576±43 vs 339±14 µg Cu mg^{-1} dry weight), after a 5 hour exposure to 3 µM of copper, suggesting that CopRS controls a copper resistance system.

Two-component systems are often auto-regulated in a positive feedback-loop and in order to test if CopRS regulated its own expression we analyzed *copM* expression in COPR, COPS and $COPS_{H227A}$ strains. *copM* mRNA levels increased (75-fold induction) at least during the first two hours after addition of 3 µM of copper in WT cells but this induction was completely lost in COPR, COPS and $COPS_{H227A}$ strains (Fig. 1D), suggesting that CopRS controls its own induction in response to copper.

CopRS controls the expression of a HME-RND efflux system involved in copper resistance.

Downstream of *pcopMRS,* in the plasmid pSYSX, there are three ORFs (*slr6042*, *slr6043* and *slr6044*) that code for a putative HME-RND transport system (Fig. 1A). These three ORFs code for proteins with homology to a membrane fusion protein (MFP), a RND protein and an outer membrane protein (OMP), respectively. We have designated these three genes as *copB*, *copA* and *copC*. In order to test if *copBAC* were involved in metal resistance, we analyzed its expression in response

to the presence of different metals in the media. *copB* was induced in response to the presence of copper, and, to a lesser extent, zinc, while induction by other metals was negligible (Fig. 2A). Northern and RT-PCR analysis showed that *copA* and *copC* were also induced by copper composing a single transcriptional unit with *copB* (Fig. S3). Since they were induced by copper, we wanted to test if they were regulated by the CopRS system. *copBAC* expression increased (14-fold induction) after addition of 3 µM of copper during at least the first four hours, although with delayed kinetics when compared to *copMRS*. This induction was lost in COPR strain (Fig. 2B), showing that CopRS is involved in *copBAC* induction in response to copper. To further clarify their role in metal homeostasis we constructed mutants in all three genes (table S1) and tested their sensitivity to different metals. These strains were sensitive to the presence of copper, but its tolerance to other metals was not drastically different from WT (Fig. S2). COPB and COPA strains presented growth defects in the presence of 3.5 µM or higher copper concentrations (Fig. 2C). However, COPC strain showed lower sensitivity to copper, being able to grow on 3.5 µM of copper and only at 5 µM of copper its growth was fully inhibited (Fig. 2C). We have also analyzed the copper content of COPB cells (which lacks expression of *copBAC*) in liquid media and these cells also accumulate 20% more intracellular copper than WT cells (400±8 vs 339±14 µg Cu mg^{-1} dry weight), when challenged with 3 µM of copper for 5 h, although to lesser extent that COPR cells, which is agreement to a lower sensitivity of COPB cells to copper in our plate assay.

CopR binds to copMRS and copBAC promoters.

The transcription start-points were determined by primer extension to establish the location of *copMRS* and *copBAC* promoters. Both *copMRS* and *pcopMRS* transcripts start 27 nt upstream the predicted *copM* or *pcopM* starting codon (Fig. 3A), since these sequences are identical and we could not distinguish between them. *copBAC* transcript start 19 nt upstream of the putative *copB* starting codon (Fig. 3B). No consensus -10 and -35 boxes could be identified in these promoters but two repeats, in the form of TTTCAT separated by 5 bp, are present in both

promoters replacing -35 boxes (Fig. 3C). CopR belongs to the OmpR family of response regulators which binds to direct repeats around the -35 boxes in promoters to activate transcription (Kenney, 2002; Blanco *et al.*, 2011). To test if CopR binds to these promoters we purified a truncated version lacking the amino terminal receiver domain fused to GST (CopRΔN; as we were unable to obtain a soluble full length protein preparation) and used it in electrophoretic mobility shift assays. CopRΔN was able to bind to probes containing *copM* and *copB* promoters (Fig. 3D and E) and therefore, the repeated sequences found in *copMRS* and *copBAC* promoters are likely to be CopR binding sites to regulate their transcription

CopS periplasmic domain binds metals.

CopS is composed by two protein domains: a carboxy-terminal domain containing the histidine kinase catalytic site and amino-terminal sensor domain. This sensor domain contains two putative transmembrane segments (residues 15-37 and 185-207) and a putative periplasmic region. In order to test if the periplasmic region was able to bind metals, we have expressed and purified the region between the transmembrane segments (expanding from residue 38 to 183) fused to a strep-tag to facilitate its purification ($CopS_{38-183}$). We tested whether $CopS_{38-183}$ was able to bind metals using metal chromatography. The protein was retained by beads charged with 0.5 mM of Cu^{2+} but not by Zn^{2+}, Ni^{2+} and Co^{2+} charged beads (Fig. 4A and B). To further analyze $CopS_{38-183}$ interaction with copper we used ligand competition with apo-4-(2-pyridylazo)-resorcinol (PAR). $CopS_{38-183}$ was able to extract one equivalent of Cu^{2+} from PAR, suggesting that one atom of copper binds to one molecule of $CopS_{38-183}$ (Fig. 4C). Titration of Cu^{2+} loaded PAR with increasing amounts of $CopS_{38-183}$ revealed a concentration dependent decrease in PAR-Cu^{2+} concentration (Fig. 4D and S4), and allowed us to calculate an apparent dissociation constant (K_{Dapp}) for $CopS_{38-183}$ of $2.3 \cdot 10^{-19}$ after calibration of the assay with EDTA (Fig. 4D and E). These data demonstrated that CopS periplasmic region is able to bind copper with high affinity *in vitro*.

Redox induction of copMRS depends on the presence of copper.

Previous microarray studies have shown that *copMRS* operon is highly induced by 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB, which blocks electron transfer from the plastoquinone pool to the cytochrome b_6f), but not by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; which blocks electron transfer from PSII to the platoquinone pool) (Hihara *et al.*, 2003), suggesting that these genes were controlled by the redox state of the plastoquinone pool. Having established that *copMRS* had a role in copper homeostasis we wanted to investigate if there was any interaction between copper metabolism and DBMIB induction of *copMRS*. First, we confirmed that addition of 10 µM of DBMIB to a *Synechocystis* culture induced expression of *copMRS* (Fig. 5A and S5A) and *copBAC* (Fig. S5A), but these genes were not induced by the addition of 10 µM DCMU (Fig. S6). Second, when the DBMIB treatment was performed in medium without copper (BG11C-Cu) plus bathocuproinedisulfonic acid (BCSA), a copper chelator to avoid any residual copper in the media (Duran *et al.*, 2004), neither *copMRS* nor *copBAC* operons were induced, as determined by *copM* and *copB* expression (Fig. 5A and S5A). DBIMB treatment in the COPR strain was also ineffective at inducing the expression of both *copM* and *copB* (Fig. S7). However *sll0528*, another gene induced by DBMIB in the microarray analysis (Hihara *et al.*, 2003), was still fully induced in both cases (Fig. 5A, S5 and S6). These results suggested that induction after DBMIB treatment of both *copMRS* and *copBAC* was related to copper metabolism, rather than a direct effect of the redox state of the plastoquinone pool, and that it was dependent on CopRS.

The response of CopS to plastocyanin protein levels.

Photosynthetic electron transport between cytochrome b_6f complex and PSI is mediated by plastocyanin or cytochrome c_6 depending on the availability of copper in *Synechocystis* (Zhang *et al.*, 1992; Waldron *et al.*, 2007). DBMIB blocks the electron transfer between the plastoquinone pool and cytochrome b_6f, and therefore impairs the plastocyanin and cytochrome c_6 reduction, causing their accumulation in the oxidized form (Trebst, 2007). Plastocyanin is the main copper containing protein in *Synechocystis* cells and it is confined to the thylakoid lumen

(Waldron *et al.*, 2007). In order to test if DBMIB treatment induces plastocyanin degradation, we analyzed plastocyanin protein levels by western blot. As shown in Fig. 5C the amount of plastocyanin rapidly declined after DBMIB treatment. To test if reduction of plastocyanin levels were responsible of *copM* induction, lincomycin, a protein synthesis inhibitor was added to *Synechocystis* cells growing in copper containing medium. Induction of *copM* and *copB* and the decrease in plastocyanin levels occurred in parallel after lincomycin treatment (Fig. 5B and D; figure S5), but with delayed time-course respect to the DBMIB treatment (Fig. 5). In agreement to this, plastocyanin half-life was three time longer in lincomycin treated cells ($t_{1/2}$=182 min.) compared to DBMIB treated cells ($t_{1/2}$=59 min.; Fig. 5F). Similarly to the DBMIB treatment, no induction of *copM* and *copB* expression was observed when lincomycin was added to cells growing in medium without Cu + BCSA (Fig. S5B). Furthermore, we analyzed if plastocyanin was required for *copM* and *copB* induction. For that a *Synechocystis* mutant lacking plastocyanin was constructed (PETE) and *copM* induction was followed after addition of 200 nM of copper, since higher copper concentrations were toxic to the PETE strain. As shown in Fig. 6, *copM* expression was lower in the PETE strain (about 60% of the WT induction), although it followed the same kinetics of the WT strain (Fig. 6B), suggesting that part of the signal sensed by CopS depends on the presence of plastocyanin in the thylakoid lumen. Copper is delivered to plastocyanin by the sequential action of two P_I-type ATPases: CtaA and PacS, and mutants strains lacking these genes have reduced levels of plastocyanin (Tottey *et al.*, 2001; Tottey *et al.*, 2012). We have constructed a double mutant lacking both ATPases (SAS strain) in order to test if copper import was needed for CopS activation. After addition of 200 nM of copper to the SAS strain *copM* induction was also lower (about 50% of the WT induction), similar to the PETE strain behavior, and with the same kinetics of the WT strain (Fig. 6B). Although the behavior of both strains was similar, they accumulate different amounts of intracellular copper after this treatment: the PETE strain accumulates only 60% of the WT copper (42.7±1.8 vs 70±14 μg mg^{-1} dry weight), while the SAS strain accumulates the same amount of the WT (71.4±9.1 μg mg^{-1} dry weight). Even more, the SAS strain failed to do the switch from *petJ* to *petE*

expression after this low copper addition, unlike the WT and PETE strains (Fig. 6). Single mutants in these two ATPases have been shown to accumulate similar Cu contents but reduced copper loaded plastocyanin (Tottey *et al.*, 2001; Tottey *et al.*, 2012), and our double mutant (SAS strain) does not express *petE,* reinforcing that copper loading into plastocyanin is needed for activation of CopS.

CopS is localised to both plasma and thylakoid membranes.

All the aforementioned results pointed out that CopS could detect signals both at the periplasmic space and at the thylakoid lumen (where plastocyanin is located). In that way CopS would need to be inserted into both the plasma and thylakoid membranes. With the aim of determining the subcellular localization of CopS, we constructed a strain (COPSHA) that express CopS fused to a triple HA epitope (CopS-3HA) under control of *nrsBACD* promoter that is induced by nickel (Garcia-Dominguez *et al.*, 2000; Lopez-Maury *et al.*, 2002). After the addition of 2 µM of nickel for 4 hours to the COPSHA strain, thylakoid and plasma membrane fractions were prepared by sucrose density gradient centrifugation and aqueous polymer two-phase partitioning (Norling *et al.*, 1998). As shown in Fig. 7, a single protein band of the corresponding molecular mass of CopS-3HA (56 kDa) was detected in both thylakoid, about 25% of total fraction, and plasma membranes, while marker proteins PsaC (a PSI protein (Kruip *et al.*, 1997)) and NtrA (a plasma membrane attached protein (Norling *et al.*, 1998)) were exclusively detected in thylakoid fraction and plasma membrane fraction, respectively. This result shows that CopS is localized to both thylakoid and plasma membranes and therefore could perceive signals in both compartments.

DISCUSSION.

This work shows the existence of a copper resistance system in *Synechocystis* comprising a two-component system (CopRS), a HME-RND transport system (CopBAC) and a protein of an unknown function, CopM. CopRS is essential for the expression of both *copBAC* and *copMRS* operons. The system responds specifically to the presence of copper but not to other metals (Fig. 1 and 2). Mutant

strains affecting the regulatory system (COPR, COPS, COPSH227A) are more sensitive to the presence of copper in the media than strains lacking components of the CopBAC transport system (Fig. 1C and 2C), suggesting that CopRS might control more genes involved in copper homeostasis. These strains lack expression of both *copBAC* and *copM* (Fig. 1D and 2B) and therefore the more likely candidate to be involved in copper resistance is CopM. CopM contains an uncharacterized Duf305 domain that is present in conserved proteins in several other cyanobacteria (Nagarajan *et al.*, 2012) and bacteria, but function of proteins containing the Duf305 domain has not been reported. CopM contained an elevated number of methionine and histidine residues and a signal peptide that will target it to the periplasmic and/or thylakoid compartment. In other copper resistance system, periplasmic proteins with an elevated number of these residues work as copper chaperones acting either as buffer and/or transferring periplasmic copper to RND transport systems (Loftin *et al.*, 2005; Bagai *et al.*, 2008; Chong *et al.*, 2009; Mealman *et al.*, 2011), that efflux it outside the cell. Attempts to delete *copM* without affecting *copRS* expression have been unsuccessful, and for that reason, we could not determine the contribution of CopM to copper resistance. The fact that COPR strains accumulate more copper than WT or COPB cells suggest that either CopM contributes to copper extrusion or that CopRS controls other genes involved in copper transport. Other obvious candidates to be controlled by CopRS are genes that code for proteins required for copper import (*ctaA*, *pacS*, *atx1*), cytochrome c_6 (*petJ*) and plastocyanin (*petE*) all of which are regulated by the presence of copper in the media. We have tested if the expression of these genes was under CopRS control but they behaved similarly in WT and COPR strains (Fig. S8). On the other hand, mutants strains in *copB* or *copA* tolerate up to 3.5 µM of copper, while mutant strain in *copC* resist up to 5 µM. *copC* codes for an outer membrane protein, which in other HME-RND system connects the RND protein to the outer membrane and allows extrusion of metals outside the cells. In this regard, recent structural and functional studies show that the *E. coli* CusBA complex could be able to transport copper from the cytosol to the periplasm in the absence of

CusC, the homolog of *Synechocystis* CopC (Franke *et al.*, 2003; Su *et al.*, 2011), where it could be buffered by CopM.

The CopRS two-component system (previously known as Hik31-Rre34) was reported as affecting *Synechocystis* cells growth under mixotrophic and heterotrophic conditions (Kahlon *et al.*, 2006; Nagarajan *et al.*, 2012), and also in the regulation of the response to low oxygen conditions (Summerfield *et al.*, 2011). Even more, their mutants lack the expression of *icfG*, a gene essential for glucose metabolism (Kahlon *et al.*, 2006). In our hands the COPR strain is able to grow in the presence of glucose and expresses the *icfG* gene to similar levels of the WT, both in the presence and absence of glucose (Fig. S9). It has been previously shown that differences in strain genetic background affect glucose sensitivity in *Synechocystis* (Kahlon *et al.*, 2006) and this could explain these discrepancies. *Nagarajan et al.* also showed that their single and double mutants of the *copMRS* genes presented different metal sensitivities to Ni, Co, Zn and Cd (Nagarajan *et al.*, 2012), but our mutants in both *copRS* or *copBAC* were as resistant as the WT to all metals except copper (Fig 1, 2 and S2).

In contrast to most bacteria, cyanobacteria have high intracellular copper requirements in the form of the electron transfer protein plastocyanin (Waldron *et al.*, 2007). This protein is localized to the thylakoid lumen and is essential for electron transfer reaction during photosynthesis in copper containing media (Duran *et al.*, 2004). *copMRS* have been described to be highly induced by different conditions, all of which alter the photosynthetic electron transport, such as treatment with DBMIB (Fig. 5; (Hihara *et al.*, 2003)), nitrogen starvation (Fig. S10; (Osanai *et al.*, 2006)) and sulphur starvation (Zhang *et al.*, 2008). We have shown here that induction in DBMIB treated cells (Fig. 5) and nitrogen starved cells (Fig. S9) is dependent on the presence of copper in the media, thus establishing that this induction is related to copper metabolism and not to other factors. All these conditions have in common a general decrease in photosynthetic electron flux (or a complete blockage in the case of DBMIB) that will probably lead to accumulation of oxidized plastocyanin. We have shown that under these conditions plastocyanin

protein levels are reduced *in vivo* (Fig. 5 and S10). This reduction in plastocyanin protein levels leads to activation of CopS (Fig 5). Further support for this comes from the induction of *copM* and *copB* after treatment with the translation inhibitor lincomycin, which also causes a reduction in plastocyanin protein levels (Fig. 5D). In both cases induction of these genes correlates with plastocyanin degradation, although the response is maintained after lincomycin treatment since it blocks completely translation and therefore cells are not able to respond to this treatment. Furthermore, these results are reinforced with our genetic data about *copM* and *copB* induction using the PETE and SAS strains (Fig. 6). Both of these mutants lack copper-plastocyanin in the thylakoid lumen (Fig. 6 and (Waldron *et al.*, 2007; Tottey *et al.*, 2012)) and showed a reduced induction of the *copMRS* operon, even if they accumulate less (PETE mutant) or the same amount copper (SAS mutant) that WT strain. These data strongly suggest that copper needs to be incorporated into plastocyanin to be detected by CopS. Plastocyanin degradation will probably release copper into the thylakoid lumen and this copper could be detected by CopS. In addition we have shown that CopS periplasmic domain is able to bind one atom of Cu^{2+} with comparable affinity to recently described MAP kinase (Turski *et al.*, 2012), supporting that CopS detects copper directly (Fig. 4). All these data, together with the localization of CopS to both plasma and thylakoid membranes (Fig. 7), pointed that this protein responds to copper (probably by direct binding to it) both in the periplasm and the thylakoid lumen (Fig. 8). Since plastocyanin levels have been estimated to be in the high micromolar range inside the thylakoid (Duran *et al.*, 2004; Finazzi *et al.*, 2005), thus even a small decrease in plastocyanin levels could generate large amount of free copper ions in the thylakoid lumen. This copper could be enough to activate CopS, even if it is present at low levels in the thylakoid membrane (Fig. 7). Why is CopS detecting thylakoid copper levels? The thylakoid lumen contains numerous proteins that are highly sensitive to oxidative damage (Nishiyama *et al.*, 2001) and therefore copper will be highly toxic in this compartment. CopS activation will induce *copMRS* and *copBAC*. CopBAC efflux system is unlikely to work detoxifying copper from the thylakoid lumen, but it will at least export the surplus of copper that could be

accumulated in the periplasm and the cytosol, creating a positive concentration gradient for copper efflux from the thylakoid. In addition, CopM could have an unidentified role in detoxifying thylakoid copper, preventing damage in this compartment. Finally, we cannot rule out that CopRS controls other unknown genes involved in copper homeostasis.

Whether the responses described here are conserved in photosynthetic eukaryotes is unknown, but copper trafficking in the chloroplast is also mediated by P_I-type ATPases, homologous to CtaA and PacS, and copper chaperones (Puig *et al.*, 2007). Therefore, it seems reasonable to expect that drastic reduction in the photosynthetic electron flux that lead to accumulation of oxidized plastocyanin could lead to its degradation, releasing free copper in the thylakoid lumen. It is also anticipated that this excess of free copper could be detected and a response similar the one observed here launched in order to detoxify this copper. The proteins studied here are only conserved in some cyanobacteria ((Nagarajan *et al.*, 2012) and our unpublished observations) and therefore the response in photosynthetic eukaryotes is likely mediated by a different set of regulatory proteins and effectors, in the same way the *petE* to *petJ* switch is conserved between cyanobacteria and *Chlamydomonas* but the regulatory mechanism are not (Merchant and Bogorad, 1986; Zhang *et al.*, 1992; Merchant *et al.*, 2006).

CONCLUSION.

In summary, we have shown that CopRS two-component system is essential for copper resistance in *Synechocystis* by regulating expression of *copMRS* and *copBAC* operons in respond to copper. CopS is probably detecting copper directly, as its putative periplasmic sensor domain is able to bind copper *in vitro.* We also present evidence that redox induction of *copMRS* is strictly dependent on the presence of copper and that this induction is probably related to plastocyanin degradation. Furthermore, we show that CopS localized to both plasma and thylakoid membranes and therefore could respond to copper both in the periplasm and in the thylakoid lumen. Whether CopRS controls additional mechanism involved in thylakoid copper detoxification remains to be elucidated. To our

knowledge CopS is the first histidine kinase detecting events directly inside the thylakoid lumen in cyanobacteria, despite the extensive regulation mediated by changes that occurs in this compartment in photosynthetic organisms.

MATERIALS AND METHODS

Strains and culture conditions.

Synechocystis cells were grown photoautotrophically on BG11C, BG11C-Cu (lacking $CuSO_4$) and BG11C-N (lacking $NaNO_3$) medium (Rippka *et al.*, 1979) at 30 ºC under continuous illumination (50 µE m^{-2} s^{-1}) and bubbled with a stream of 1% (v/v) CO_2 in air. For figures 5, S3, S4 and S7, BG11C-Cu or BG11C-Cu-N was supplemented with 300 µM bathocuproinedisulfonic acid (BCSA) as a chelating agent to eliminate any traces of copper (Duran *et al.*, 2004). For plate cultures, medium were supplemented with 1% (wt/vol) agar. Kanamycin, chloramphenicol and spectinomycin were added to a final concentration of 50 µg mL^{-1}, 20 µg mL^{-1} and 5 µg mL^{-1}, respectively. BG11C-Cu medium was supplemented with different concentrations of $CuSO_4$, $NiSO_4$, $ZnSO_4$, $CdCl_2$ and $CoCl_2$ when indicated. Experiments were performed using cultures from the mid-logarithmic phase (3 to 5 µg chlorophyll mL^{-1}). Glucose, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and lincomycin were added to a final concentration of 5 mM, 10 µM, 10 µM and 250 µg mL^{-1}, respectively. *Synechocystis* strains and their relevant genotypes are described in table S1. *E. coli* DH5α or BL21 cells were grown in Luria broth medium and supplemented with 100 µg mL^{-1} ampicillin, 50 µg mL^{-1} kanamycin, 20 µg mL^{-1} chloramphenicol and 100 µg mL^{-1} spectinomycin when required.

Construction of Synechocystis strains.

Synechocystis cells were transformed as described in (Ferino and Chauvat, 1989). Plasmid construction is detailed in supplementary material. All the oligonucleotides used in this work are described in Table S2.

RNA isolation and Northen Blot Analysis.

Total RNA was isolated from 30 mL samples of *Synechocystis* cultures in the mid-exponential growth phase (3 to 5 µg chlorophyll mL^{-1}). Extractions were performed by vortexing cells in the presence of phenol-chloroform and acid-washed baked glass beads (0.25-0.3 mm diameter) as previously described (Garcia-Dominguez and Florencio, 1997). 5 µg of total RNA was loaded per lane and electrophoresed in 1.2 % agarose denaturing formaldehyde gels (Sambrook *et al.*, 1989) and transferred to nylon membranes (Hybond N-Plus; Amersham). Prehybridization, hybridization, and washes were in accordance with Amersham instruction manuals. All probes were synthesized by PCR and oligonucleotides pairs used are described in table S3. Hybridization signals were quantified with a Cyclone Phosphor System (Packard).

Determination of cellular copper content.

The cellular copper contents were determined from 800 mL of exponentially growing cells that were treated with 200 nM of copper for 1 h (WT, SAS and PETE strains) or 3 µM of copper for 5h (WT, COPR and COPB strains). Cells were centrifuged at 5000 *g*, washed twice with BG11C-Cu and dried overnight in an oven at 85 ºC. 100 mg of dried cells were microwave digested, dissolved in suprapure HNO_3 and analyzed by ICP in an ICP-OES Varian ICP 720-ES (Tottey *et al.*, 2001; Andres-Colas *et al.*, 2006). Data shown represent the average ± standard error.

Primer extension analysis of copMRS and copBAC transcripts.

Oligonucleotides NIY3 and COPA3, end-labeled with T4 polynucleotide kinase and [γ-32P]-dATP (3000 Ci $mmol^{-1}$) following standard procedures (Sambrook *et al.*, 1989), were used for primer extension analysis of *copMRS* or *copBAC* promoters respectively. For annealing, a 10 µl mixture containing 0.15 M KCl, 10 mM Tris HCl pH 8.0, 1 mM EDTA, 20 µg of total RNA and about 2 pmoles of oligonucleotides (10^6 cpm) were prepared. The annealing mixture was heated for 2 min at 90 ºC in a water bath and cooled slowly to 50 ºC. For extension, a 10 µl mixture was prepared with half of the annealing mixture: 10 mM DTT, 0.5 mM each dNTP, 2 mg mL^{-1} of Actinomycin D, 50 mM Tris HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 100 U of Superscript™ II RNase H-Reverse Transcriptase (Invitrogen). The mixture was incubated for 45 min at 45 ºC, and the reaction was stopped by adding 4 µl of formamide-loading buffer. Half of the reaction was electrophoresed on a 6% polyacrylamide sequencing gel together with a sequencing reaction of the *copMRS* or *copBAC* promoter regions using the same oligonucleotides.

Cloning and purification of CopRΔN.

The complete DNA binding domain from *copR* was cloned from *Synechocystis* DNA after PCR amplification with oligonucleotides COPR3 and NIY2 and cloned into *Bam*HI-*Sal*I pGEX6P. GST-CopRΔN fusion protein was expressed in *E. coli* DH5α. 200 mL of culture was grown in Luria broth medium to an optical density at 600 nm of 0.6, induced with 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG) for 2.5 h, harvested by centrifugation, and resuspended in 5 mL of PBS buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 4 mM phenylmethylsulfonyl fluoride, 7 mM β-mercaptoethanol) supplemented with 0.1% Triton X-100. Cells were broken by sonication on ice, and insoluble debris were pelleted by centrifugation. Extracts were mixed with 1 mL of glutathione agarose beads (Amersham) and incubated for 2 h at 4 ºC with gentle agitation. Then beads were transferred to a column and washed extensively with PBS buffer until no more protein was eluted from the column. GST fusion proteins were eluted with 3 mL of 50 mM Tris HCl (pH 8) containing 10 mM of reduced glutathione

Gel retardation assays.

Probes were PCR-synthesized using oligonucleotides NIY4-NIY5, for *copMRS* promoter, and COPA4 and COPA5 for *copBAC* promoter, which introduce an *Nco*I restriction sites in both cases. The resulting DNA was digested with *Nco*I and end-labelled with [α-^{32}P]-dCTP (3000 Ci mmol^{-1}) using Klenow fragment. The binding reaction was carried out in a final volume of 25 µl containing 4 ng of labelled DNA and 4 µg salmon sperm DNA in 20 mM Tris HCl (pH 8.0), 150 mM KCl, 10 mM spermidine, 10 mM DTT, 1 mM EDTA, 10 % glycerol and different amounts (from 0.2 µg to 1 µg) of partially purified GST-CopRΔN. The mixtures were incubated for 25 min at 4ºC and loaded on a non-denaturing 6% polyacrylamide gel. Electrophoresis was carried out at 4ºC and 200V in 0.25X TBE. Gels were transferred to a Whatman 3 MM paper, dried and autoradiographed.

Cloning, purification and metal binding assays of CopS periplasmic domain ($CopS_{38\text{-}183}$*).*

A 462 pb band coding for the CopS periplasmic domain was PCR amplified from genomic DNA with oligonucleotides CopSperiF2-CopSperiR2, digested with *BamH*I and *Sac*I and cloned into pET51 digested with the same enzymes. $CopS_{38\text{-}183}$ was expressed in *E. coli* BL21. 1.5 L of culture were grown in Luria broth medium to an optical density at 600 nm of 0.6, induced with 0.2 mM IPTG and incubated for 6 h at 25 ºC, cells were harvested by centrifugation and frozen at –20 ºC. Frozen pellets were resuspended in 40 mL of 100 mM Tris HCl (pH 8), 150 mM NaCl, 1 mM BCSA, 1 mM EDTA and 2 mM TCEP (buffer S) and broken by sonication. The suspension was centrifuged 30 minutes at 30000 *g* at 4 ºC and the supernatant was loaded into a 5 mL streptavidin beads (IBA GmbH) column equilibrated in buffer S. Beads were washed with 50 mL of buffer S and $CopS_{38\text{-}183}$ was eluted with 10 mL of 1X Strep-Tag elution buffer (IBA GmbH). $CopS_{38\text{-}183}$ was further purified by gel filtration in a Hi-Load Superdex 75 (GE-Healthcare) column equilibrated with 20 mM Tris HCl (pH 8), 150 mM NaCl. The purified protein was concentrated using a 3K Vivaspin concentrator.

Interaction of $CopS_{38-183}$ with Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} was investigated by inmobilized metal ion affinity chromatography. A 100 µl aliquot of His-bind resin (Novagen) was loaded with 0.5 mL of 0.5 mM of $CuSO_4$, $NiSO_4$, $ZnSO_4$ or $CoCl_2$ in water and then equilibrated in 25 mM Tris HCl (pH 8), 500 mM NaCl (buffer A). About 10 µg of purified $CopS_{38-183}$ were applied to the columns. Unbound proteins were removed by washing with 2 mL of buffer A. Bound proteins were eluted with 100 µl of 0.4 M imidazole in buffer A. 15 µl of the imidazol eluted and flowthrough fractions were analyzed by SDS-PAGE and Coomassie blue staining. Quantities of bound and unbound proteins were determined by densitometry.

Analysis of $CopS_{38-183}$ Cu^{2+} binding was obtained via colorimetric titration similar to described previously with the divalent metal ligand Apo-4-(2-pyridilazo)-resorcinol (PAR) (Tottey *et al.*, 2008). PAR (10 µM) in 20 mM Tris HCl (pH 7.5), 50 mM NaCl (buffer B) was titrated against copper (0-20 µM) measuring absorbance in the 600-350 nm range. Absorbance of PAR (410 nm) and Cu^{2+}-PAR (500 nm) were plotted against [Cu^{2+}]. Titration was repeated in the same way but with the addition of 10 µM apo-$CopS_{38-183}$. The apparent dissociation constant (K_D) of $CopS_{38-183}$ for Cu^{2+} was estimated using competition experiments as described previously (Turski *et al.*, 2012). The quantitative release of the 1:1 Cu^{2+}/PAR complex upon titration of apo$CopS_{38-183}$ was monitored spectrophotometrically at 500 nm in buffer B. The samples were equilibrated for 5 min at room temperature before the measure. The affinity of Cu^{2+}-PAR complex (formation constant [β]) is 3.2×10^{17}, and the Cu^{2+} binding affinity was calibrated using a spectroscopically silent ligand EDTA, with a known affinity for Cu^{2+} of 1.6×10^{-19} (Turski *et al.*, 2012)

Membrane fractionation and Western blotting.

Thylakoid and plasma membranes were prepared from *Synechocystis* as described previously (Norling *et al.*, 1998). For western blot analysis, proteins were fractionated on SDS PAGE and immunoblotted (Sambrook *et al.*, 1989) with antibodies against: HA (1:1,000; Sigma catalog number H9658), NrtA (1:10,000; (Omata *et al.*, 1989)), PsaC (1:3,000; (Mata-Cabana *et al.*, 2007)), plastocyanin

(1:12,000; (Duran *et al.*, 2004)), or *Synechococcus* sp. PCC 6301 Glutamine synthetase I (1:20,000; (Merida *et al.*, 1990)). The ECL Plus immunoblotting system (Amersham) was used to detect the different antigens with anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (1:10,000). Films were scanned and quantified using Image J software.

Supplemental data.

The following materials are available in the online version of this article.

Supplemental material and metods

Figure S1. *copMRS* are expressed as a single transcriptional unit.

Figure S2. Mutants in *cop* genes are not differentially affected respect to the WT strain by Ni^{2+}, Co^{2+} and Zn^{2+}.

Figure S3. *copBAC* are expressed as a single transcriptional unit.

Figure S4. Spectral changes of the Cu^{2+}-PAR complex on the $CopS_{(38-183)}$ titration.

Figure S5. Redox induction of *copMRS* and *copBAC* expression depends on the presence of copper in the medium.

Figure S6. *copMRS* and *copBAC* expression is not induced after DCMU treatment.

Figure S7. *copM* and *copB* induction depends on CopR after DBMIB treatment.

Figure S8. CopRS do not control copper related genes.

Figure S9. Growth of COPR is not affected by glucose.

Figure S10. Nitrogen starvation leads to *copM, copB* induction and plastocyanin degradation.

Supplemental Table S1. *Synechocystis* strains used in this work

Supplemental Table S2. Oligonucleotides used in this work.

Supplemental Table S3. Oligonucleotides pairs used to synthesize probes used for Northen blot analysis.

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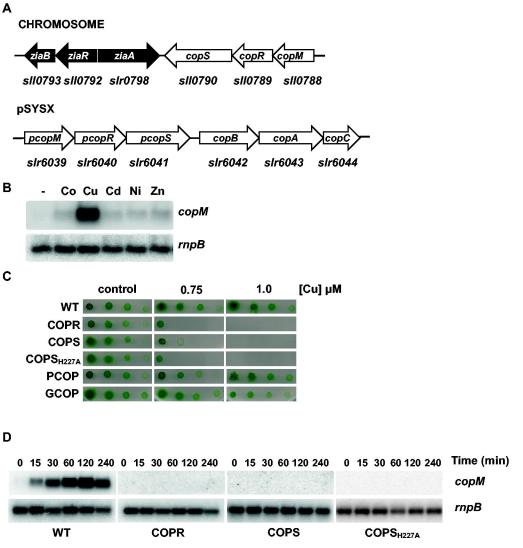

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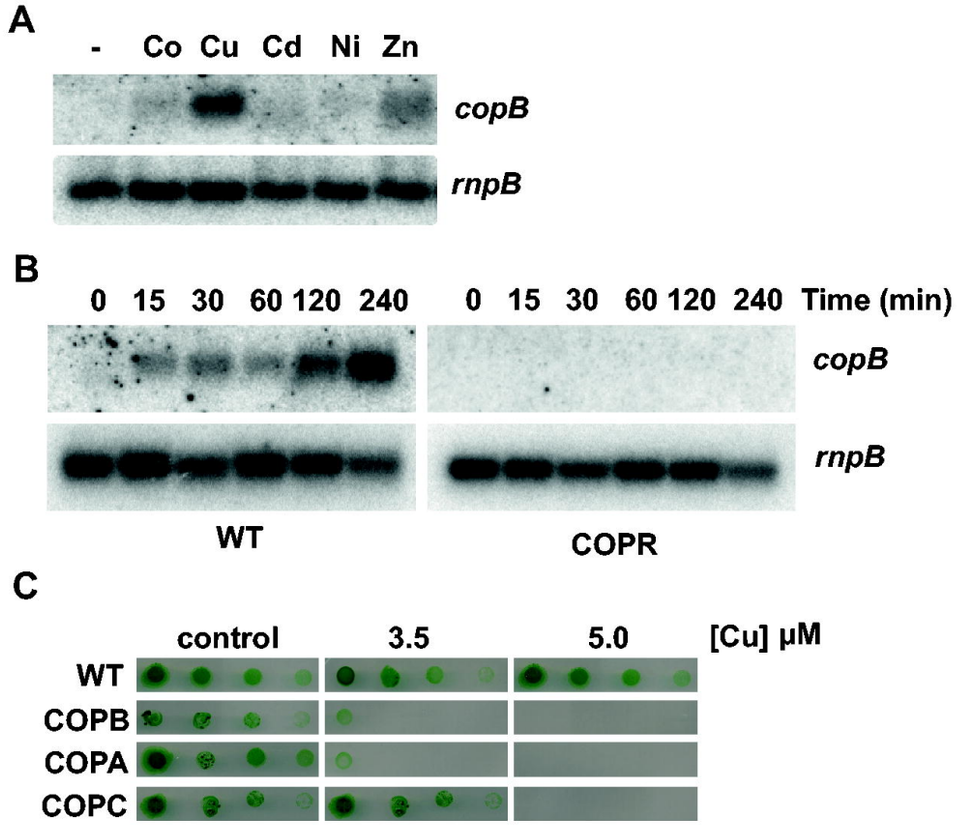

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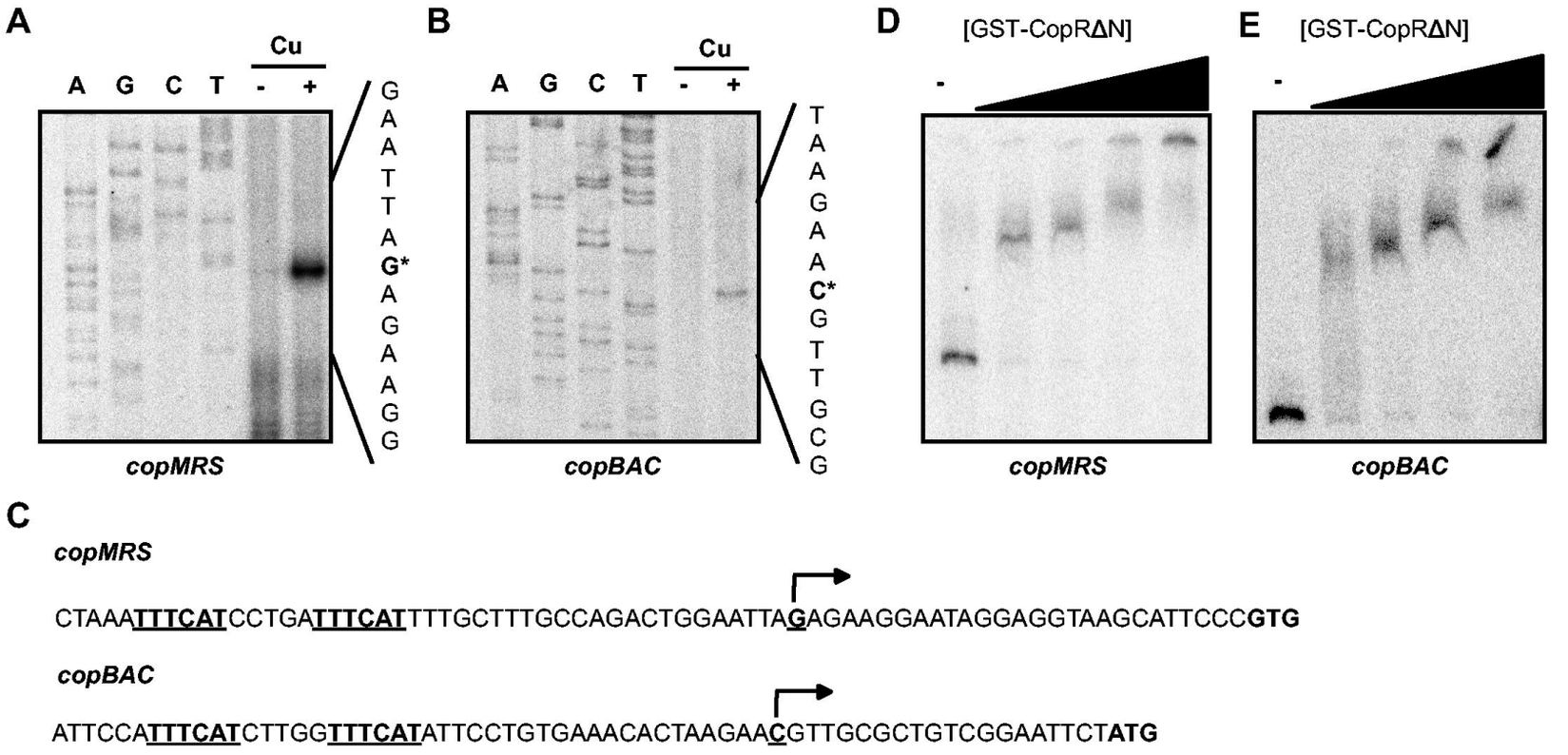

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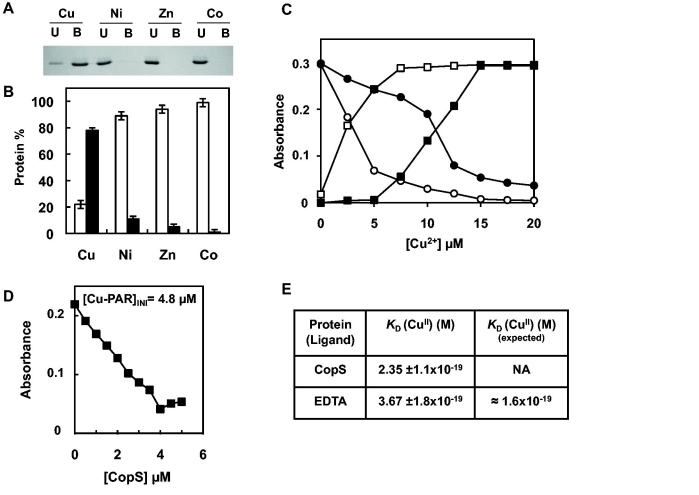

E

Protein (Ligand)	K_D (Cu^{II}) (M)	K_D (Cu^{II}) (M) (expected)
CopS	$2.35 \pm 1.1 \times 10^{-19}$	NA
EDTA	$3.67 \pm 1.8 \times 10^{-19}$	$\approx 1.6 \times 10^{-19}$

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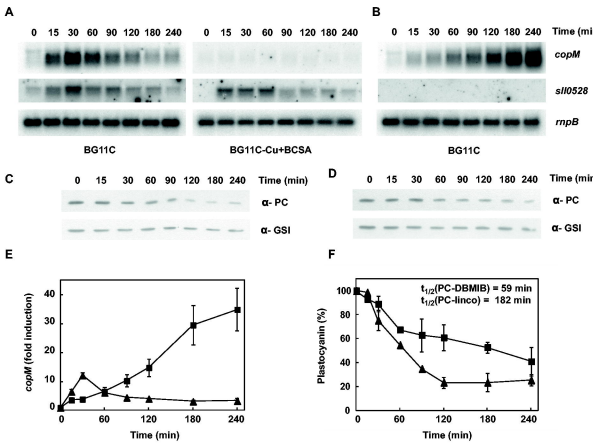

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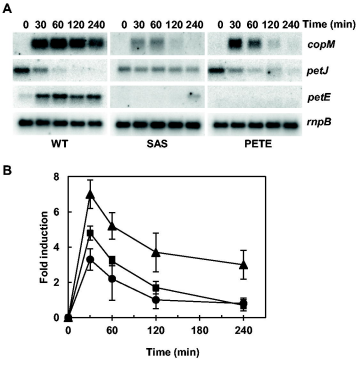

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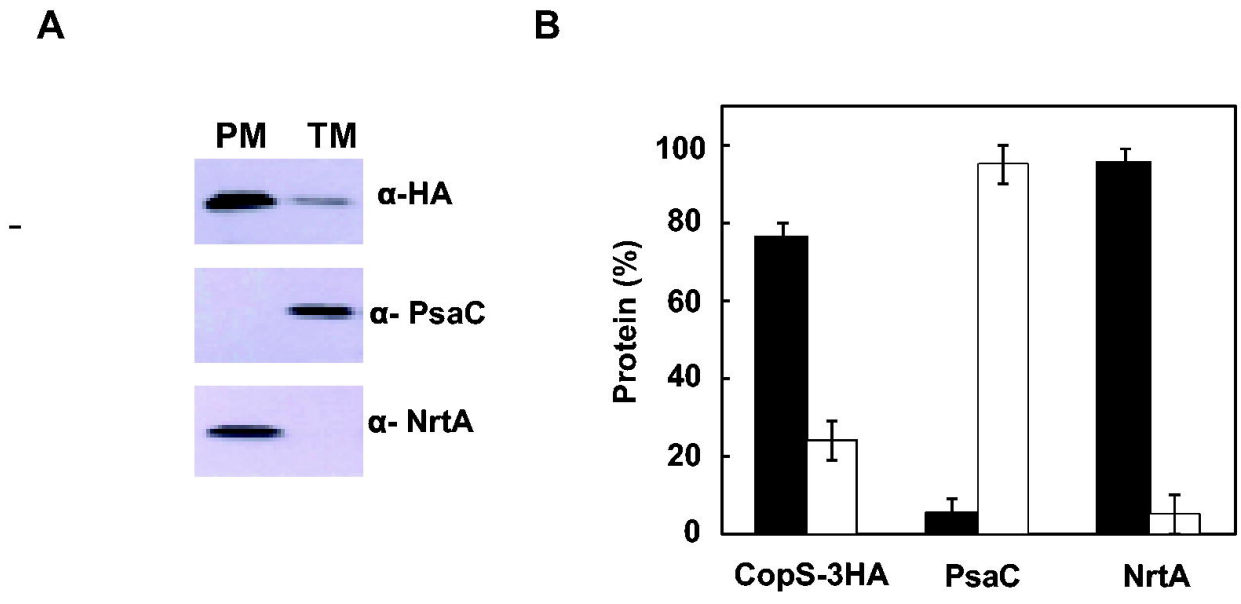

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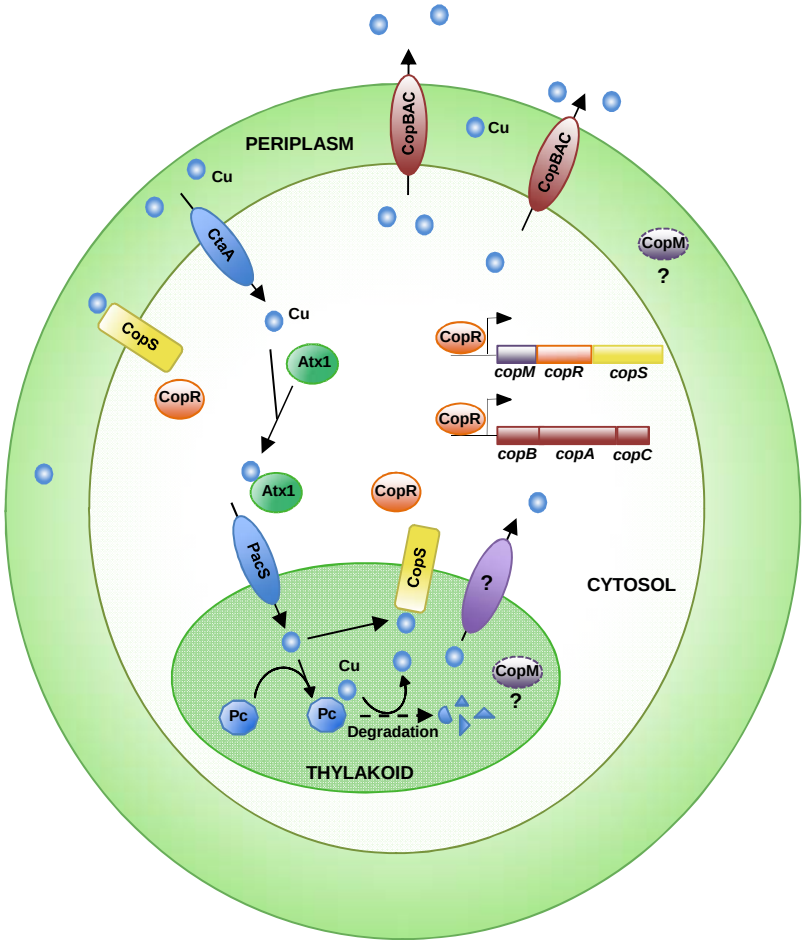

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