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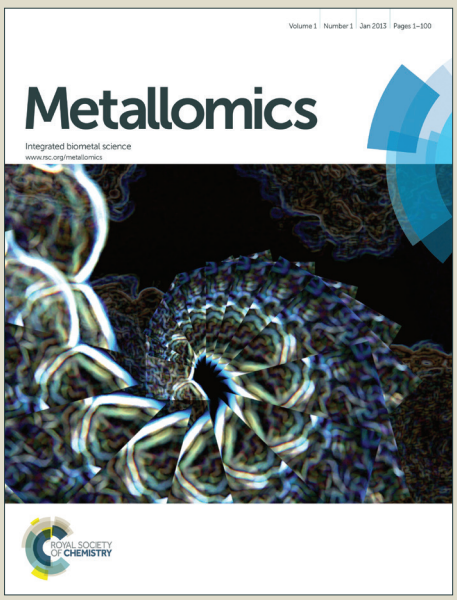

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Cu binding by the *Escherichia coli* metal-efflux accessory protein RcnB

Camille Blériot [1,5 §], Manon Gault [1 §], Erwan Gueguen [1], Pascal Arnoux [2,3,4], David Pignol [2,3,4], Marie-Andrée Mandrand-Berthelot [1] and Agnès Rodrigue [1 *]

[1] Microbiologie, Adaptation et Pathogénie, UMR5240 CNRS INSA Lyon Université Lyon 1, F-69621 Villeurbanne, France.

[2] CEA, DSV, IBEB, Lab Bioenerget Cellulaire, Saint-Paul-lez-Durance, F-13108, France.

[3] CNRS, UMR Biol Veget & Microbiol Environ, Saint-Paul-lez-Durance, F-13108, France.

[4] Aix-Marseille Université, Saint-Paul-lez-Durance, F-13108, France.

[5] Present address : Institut Pasteur, Inserm U1117, 75724 Paris Cedex 15 France

[§] contributed equally to this work

* For correspondence:
Agnès Rodrigue
MAP UMR 5240 CNRS- INSA Lyon-UCBL
Bat Lwoff – 10 rue Dubois
F- 69622 Villeurbanne Cedex
Phone : (+33) 472447980 - Fax : (+33) 472431584
e-mail : agnes.rodrigue@insa-lyon.fr

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Abstract

Divalent cations play fundamental roles in biological systems where they act as structural and reactive determinants. Their high reactivity with biomolecules have forced living cells to evolve specific pathways for their *in vivo* handling. For instance the excess of metal can be expelled by dedicated efflux systems. The *E. coli* RcnA efflux pump expels both Ni and Co. This pump functions together with the periplasmic protein RcnB to maintain metal ion homeostasis. To gain insights into the efflux mechanism, metal binding properties of RcnB were investigated. Initial screening of metal ions by fluorescence quenching elicited Cu as a potential ligand for RcnB. Non-denaturing mass spectrometry and ITC experiments revealed the binding of one Cu ion per monomer with a micromolar affinity. This set of *in vitro* techniques was broadened by *in vivo* experiments that showed the accuracy of Cu binding by RcnB. RcnB implication in Cu detoxification was questioned and growth experiments as well as transcriptional analysis excluded a role for RcnB in Cu adaptation. Finally a mutant in a conserved Methionine residue (Met86) displayed altered Cu binding. This mutant protein when tested for its Ni and Co resistance capacity was unable to complement an *rcn* mutant. Taken together these data show that RcnB is a new Cu-binding protein that is strikingly involved in a Ni/Co efflux system.

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Introduction

Metal ions are essential components of all living cells including bacteria. Among metals, cobalt (Co), either as a co-factor or associated with vitamin B12, is required for many biological functions[1]. Nickel (Ni) is a catalytic cofactor of several prokaryotic enzymes, the best characterized being hydrogenases and ureases [2]. Copper (Cu) is mainly involved in redox reactions permitting, by cycling between the cupric Cu(II) and cuprous Cu(I) forms, to oxidize a wide variety of substrates [3].

However, like many other transition metals, Ni, Co or Cu are toxic when present in excess, causing growth arrest and cell death. Toxicity can result from the non specific interactions between metal ions and proteins or DNA [4]. Another mechanism of toxicity is the oxidative stress, which can lead to the creation of hazardous reactive oxygen species [5-6]. Furthermore, an excess of Co impairs the biogenesis of Fe/S clusters by a direct competition with iron [7].

The most widespread mechanism bacteria use to overcome this toxicity is the efflux of these cations out of cells. Ni and Co are often expelled by the same efflux pumps. In *Escherichia coli*, the RcnA efflux pump is the only known system responsible for the cytoplasmic detoxification of Ni and Co. RcnA is an inner membrane protein, widespread in bacteria, thought to function as a secondary transporter [8]. The expression of *rcnA* is specifically induced *in vivo* by Ni and Co and not by other divalent cations as shown by using reporter gene fusion [8,9].

The metallo-regulator RcnR represses the expression of *rcnA* [9] as well as its own expression through binding on the shared operator site of these two divergently transcribed genes [10]. *In vitro*, Ni and Co are able to dissociate RcnR from its cognate DNA binding site whereas Zn or Cu display the same effect for concentrations 4 to 8 times higher respectively [10]. RcnR, like most of the metallo-regulators, is involved in maintaining metal homeostasis by controlling the expression of the metal transporter. We have shown recently that a second protein, RcnB, is essential to control Ni and Co intracellular levels in *E. coli* [11]. In contrast to RcnR, RcnB is periplasmic and its mode of action which is actually unclear, does not seem to reside in a regulatory event [11]. Still RcnB is thought to act as a modulator of the efflux mediated by the RcnA pump and is required to maintain proper metal homeostasis [11].

In this work, in the course of identifying ligands for RcnB, we detected Cu as being a co-factor of RcnB. Cu-binding proteins described so far are either involved in redox reactions, in maintaining Cu homeostasis, or act as chaperones [12]. As Cu can undergo redox cycling

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between Cu(I) and Cu(II) oxidation states, it can act as an electron acceptor/ donor like in cytochrome oxidase or is present in redox active enzymes like superoxide dismutase. It is noteworthy that all bacterial Cu-requiring enzymes are extracytoplasmic, with the exception of cyanobacteria that contain Cu-enzymes within internal membrane-bound compartments [3]. Cu is highly toxic in the cytoplasm [13] and cells require systems to overcome this danger. In *E. coli* the inner membrane P-type ATPase CopA constitutes the core element of Cu resistance. *copA* is inducible by Cu in both anaerobic and aerobic conditions [14]. A second resistance system is the periplasmic multi-Cu oxidase CueO that oxidizes Cu(I) ions in less toxic Cu(II) ions [15]. Since the enzyme activity is dependent on oxygen CueO works strictly under aerobic conditions. Briefly, CueO contains Cu active sites that can oxidize several substrates, one of them being Cu(I) ions [16]**.** The last system, Cus*,* is composed of a RND-driven tripartite efflux complex, CusCBA, and a soluble periplasmic Cu-binding protein, CusF [17]. The molecular mechanism is not fully understood yet but it is thought to correspond to a switch model where Cu primary binds to CusF that transfers it to CusB. This step induces a conformational change that allows periplasmic Cu ions to be taken in charge by CusA and expelled via CusC [18]. The Cus system is predominantly active under anaerobic conditions and a mutant strain has been shown to be Cu sensitive [19].

Here we show that RcnB, which does not bind Ni and Co ions directly [11], is able to bind copper. Using a combination of biophysical methods, the *in vitro* binding properties of Cu by RcnB were characterized. This binding was further investigated and shown to occur *in vivo*. As RcnB is a periplasmic protein, its implication in Cu homeostasis was assessed. Physiological experiments clearly demonstrated that RcnB is not a partner of systems controlling cellular Cu level and that RcnB plays no role in Cu tolerance. Finally the use of copper as a co-factor for RcnB activity was examined. Mutagenesis of the motif ($H_{82}W_{83}XXM_{86}$) conserved among RcnB homologs was carried out. Interestingly, mutant M86A RcnB showed altered Cu-binding and lost the ability to control Ni and Co homeostasis. As a conclusion, RcnB is a novel copper-binding protein and copper is essential for the activity of RcnB.

Materials and Methods

Bacterial strains, plasmids and culture conditions

Strains and plasmids used in this work are summarized in Table 1 and are all derivatives of *E. coli* K-12. Bacterial cells were grown in LB medium and cultures were performed at 37°C unless otherwise stated. Anaerobic conditions were obtained by placing

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Petri dishes in a GasPak jar, according to the manufacturer's instructions (BD). Where required, antibiotics purchased from Sigma were used at the following concentrations: chloramphenicol (Cam) at 20 μg/mL, kanamycin (Kan) at 50 μg/mL, ampicillin (Amp) at 100 μg/mL. *cusF*, *copA* and *cueO* mutant strains were obtained from the KEIO collection [20]. Mutant genes were further transferred from strain BW25113 to strain W3110 or WRCB1, via generalized phage transduction using P1 vir, as described by Miller [21]. The Δ*rcnA*Δ*rcnB* mutant was constructed by deletion of the corresponding genes. For that purpose, a 0.5-kb fragment corresponding to the immediately upstream region of *rcnA* was amplified using oligos pair L28 (CGGCCGCGGACCGGATGCGATGATAAATCGCAGAG) and L29 (GCTTTTTTTAGCGATGTTCGGTCATGATAATAATTCTTAG). A 0.5-kb fragment corresponding to the immediately downstream region of *rcnB* was amplified using oligos pair L30 (TATTATCATGACCGAACATCGCTAAAAAAAGCCCCCTC) and L31 (GGCCGCTCTAGAGGACAGCCACAGGTAACAAAGCA). Both PCR-amplified DNA fragments were directly cloned into *Bam*HI-linearized gene replacement pKO3 vector [22], using the SLIC cloning method [23]. The *E. coli* W3110 Δ*rcnAB* in-frame deletion mutant was then constructed following the method described previously [22]. Site directed mutagenesis of *rcnB* was performed using the QuikChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene). The sequence of the plasmid was verified subsequently.

Ni and Cu susceptibility testing

Plate assay metal-sensitivity assays were conducted as follows: first, bacteria were grown until mid-log phase in LB medium. A 10-fold serial dilution of the cultures in M63 medium was performed and 5 μL were spotted onto 0.4% glucose M63 minimal medium plates containing increasing concentrations of metal. The plates were incubated at 37°C for 24-48 h, under aerobic conditions, or for 48-72 h under anaerobic conditions. Susceptibility testing in liquid cultures was conducted in M63 medium supplemented with 0.4% glucose. Cultures were incubated at 37°C for 16h and the number of bacteria estimated using OD_{600nm}. The RcnAB efflux system was previously shown to be specific for Ni or Co [8, 11], in this study Ni was employed as the cognate metal of the system. As the *in vitro* experiments performed here demonstrated an interaction between RcnB and Cu, exposition to this metal was also tested at the physiological level.

Overexpression and purification of the RcnB protein

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Overexpression and purification of RcnB were performed as previously described [11]. The purification process is illustrated on supplementary Figure 1. Strain BL21, harboring the pETRCB plasmid, was grown to mid-log phase in LB medium. Next, 1 mM isopropyl β-D thiogalactopyranoside (IPTG) was added to induce the expression of RcnB. After 4 h at 30°C, cells were harvested by centrifugation and either frozen at -80°C for later use or diluted in 50 mM Tris-HCl, pH 7, supplemented with 1.5 M ammonium sulfate and lysed by 3 passages through a French press in the presence of Halt protease inhibitor (Thermo). The supernatant was filtered through a 0.45 µm filter and loaded onto a phenyl HP column (HiTrap, GE Healthcare). Elution followed a linear gradient, from 1.5 M to 0 M ammonium sulfate in 50 mM Tris-HCl, pH 7, and fractions containing the RcnB protein (0.7 M ammonium sulfate) were pooled and dialyzed against 50 mM Tris-HCl, pH 7 to remove the ammonium sulfate. The fraction containing RcnB was then loaded onto an anion-exchange UnoQ1 column (Bio-Rad). Under these conditions, RcnB was not retained on the column, in contrast to the contaminating proteins present in the loaded fraction. The purity of RcnB was verified on a 15% Tris-Tricine polyacrylamide gel. When necessary, purified RcnB was concentrated using 6-kDa cut-off Vivaspin 6 concentrators (Sartorius). RcnB was quantified either by measuring OD_{278nm} using an ε of 21430 $M^{-1}cm^{-1}$ or using the Bradford protein assay kit (Bio-Rad). Typically, the purification process yielded 20 mg of pure RcnB per liter of culture.

Circular dichroism Spectroscopic Analysis

RcnB protein was used at a final concentration of 25 µM in 50 mM sodium phosphate buffer (pH 7.0). Spectra were recorded on a spectrometer Chirascan (Applied Photophysics Ltd) between 195 and 260 nm. Ni^{2+} and Cu^{2+} ions were added at the same concentration of 25 µM and incubated 5 minutes at 25°C before the analysis. Denaturation was performed by increasing gradually (1°C.min^{-1}) the temperature to 90°C and circular dichroism at 220 nm was monitored in order to follow the unfolding process. Then, renaturation was accomplished by decreasing the temperature to 25°C (10°C.min^{-1}). Algorithmic deconvolution was performed by using methods SELCON3, CONTIN, CDSSTR and K2D available on the on-line server Dichroweb (dichroweb.cryst.bbk.ac.uk) [24].

Fluorescence spectroscopy

Fluorescence quenching of RcnB protein was monitored between 300 and 400 nm with an excitation wavelength of 280 nm. Protein was dissolved in 100 mM Bis-Tris propane buffer (pH 6.0) to a final concentration of 5 µM. At each step, a ten-fold excess of metal ions solution was titrated into the protein solution with a volume variation below 1 % and

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incubated for 5 min at room temperature before the analysis. Cu(II) ions were given by a solution of $CuSO_4$.

Isothermal Titration Calorimetry (ITC)

ITC measurements were performed on a Microcal (Northampton, MA) ITC200 microcalorimeter at 30 °C in 10 mM Pipes buffer (pH 7.0). For a titration experiment, approximately 250 µL of protein (150 µM) was placed in a reaction cell and injected with the Cu(II) solution (1.5 mM). The first injection was 2 µL, and all subsequent injections were 4 µL. A total of 14 injections were made with 2 min intervals between each injection. To ensure adequate mixing, the reaction cell was continuously stirred at 1000 rpm. The heat due to dilution, mechanical effects, and other nonspecific effects was identified by averaging the last three points of the titration and subtracting that value from all data points. The enthalpy changes observed in the control titrations of Cu(II) into buffer and of buffer into apo-RcnB were not significant (data not shown) but were subtracted to obtain the results shown on the Fig. 1C. A single-site binding model was used to fit the data using the ORIGIN software (Microcal). The software uses a nonlinear least-squares algorithm and the concentrations of the species to fit the enthalpy change per injection to an equilibrium binding equation. The binding enthalpy change (ΔH), association constant (K_a), and binding stoichiometry (n) were permitted to float during the least-squares minimization process and taken as the best-fit values.

Mass spectrometry

5 µM of apo-RcnB were reconstituted with or without a ten-fold excess of Cu^{2+} ions in 20 mM ammonium acetate buffer (pH 7.0). The mass measurements were performed in a positive ion mode using ESI-Q-TOF. All the parameters were systematically optimized in denaturing conditions in the presence of 0.2 % formic acid. Data acquisition and analysis were performed using the instrument's Analyst software.

RT-PCR assays

Total RNA was isolated from liquid cultures of WT *E. coli* cells with the RNeasy kit (Qiagen) and treated twice with DNase (Ambion) for 30 min at 37°C. The absence of DNA contamination was verified by direct PCR. RNA was quantified by measuring the optical density at 260 nm, and its integrity was confirmed by agarose gel electrophoresis. RNA was reverse transcribed using the Access RT-PCR kit (Promega). The following primers were used: YohN1RQ (CACCGTCGTAGGCTTTAATG) and YohN2FQ (GGCAGGAAAGATATGCGACT); YoNup (CTTCAGCCCCATATGATTCTTAAATCAGC) and Yodwn2

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(CCACCATATAGGTCCAG) ; CusFup (TGACTTTTAACTCCAGGAGAG) and CusFdwn (TTTTCATCTCATTAAACCTGGG); and CueOup (GTTTGATTTTGTTTCGCCTGC) and CueOdwn (CGGGCATATTTCCGAATACG) for the amplification of the $rcnB_{int}$, $rcnB_{ext}$, *cusF* and *cueO* transcripts, respectively. Controls were treated as samples, but reverse transcriptase (RT) was omitted. PCR and RT-PCR products were visualized on a 2% agarose gel.

UV-Visible spectroscopy

UV-visible (UV-vis) absorption spectra were obtained at 25°C on a Nicolet Evolution 100 spectrophotometer. Purified apo-RcnB was diluted in 50 mM Tris-HCl, pH 7.0 buffer at a final concentration of 130 µM. Equivalents of Cu(II) (donor solution : $CuSO_4$) were added with a volume variation below 1 % step by step and absorption spectra were recorded from 350 to 850 nm after 5 minutes of equilibration.

Results

RcnB is a Cu-binding protein

RcnB was previously shown to be essential to control Ni and Co homeostasis [11]. Some direct and indirect experiments were performed but failed to show interactions between purified RcnB and Ni or Co ions. RcnB possesses two tryptophan residues in its mature form and can generate a strong fluorescence emission signal after an excitation at λ = 280 nm (Fig. 1A). This signal was not altered upon addition of 100 µM (i.e. 20 metal equivalents) Ni and Co ions or other divalent cations like Zn or Mg (data not shown). In contrast, addition of increasing amounts of Cu ions quenched gradually this signal showing that this metal altered the environment of at least one tryptophan residue (Fig. 1A). These results suggest that Cu is a ligand of RcnB, at least *in vitro*. However, the binding isotherm indicated saturation for 10 equivalents of Cu or more. This could reflect a global weak affinity of RcnB for Cu or this could be due to the presence of several binding sites, some having a weak affinity for Cu.

To characterize the *in vitro* metal-protein stoichiometry, mass spectroscopy was used. RcnB was incubated or not with a ten-fold excess of Cu(II) ions and the metal-protein complexes were analyzed by ESI-Q-TOF. In the absence of Cu, one peak was recorded corresponding to the monomeric form of RcnB (Fig. 1B). In the presence of Cu, this peak was displaced from a mass corresponding exactly to one atom of Cu (63 Da) (Fig. 1B). No other peaks were observed on the whole spectrum indicating that Cu did not modify the oligomeric

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state of RcnB. This observation was strengthened by a size-exclusion chromatography experiment that revealed the same elution volume for the apo and the Cu-bound forms of RcnB (data not shown). Taken together, these results indicate that RcnB binds one atom of Cu per monomer.

Isothermal titration calorimetry was employed in order to determine the dissociation constant of the RcnB-Cu complex. The titration of Cu(II) into a solution of apo-RcnB in 10 mM Pipes buffer pH 7.0 showed significant changes in enthalpy (Fig. 1C). A one-site binding model was used to fit the data, yielding a K_D value of 1.8 µM (+/- 0.8) and a stoichiometry of Cu to RcnB of 0.81 (+/- 0.01). The experiment was repeated in different buffers (10 mM cacodylate, pH 7.0; 10 mM Hepes pH 7.0), using concentrations of RcnB ranging from 50 to 300 µM, resulting in similar results (K_D= 1.8 - 6 µM, N=0.8-0.9) (data not shown). Taken together, these results show that RcnB binds one atom of Cu per monomer with a micromolar affinity.

ITC was also performed in the same conditions using Ni or Co and not changes in enthalpy were observed suggesting that in these cases the K_D is significantly higher than 100 µM (data not shown).

Cu acts on RcnB stability

Native RcnB displayed a far-ultraviolet circular dichroism spectrum with a positive peak at 230 nm probably due to aromatic residues [25] and a negative peak around 210 nm (Fig. 2A). A bioinformatic deconvolution of the spectrum of the apo-protein predicted mainly beta sheets as secondary structures, about 40 percent of the total protein, whereas the other half was randomly folded and helical structures were largely under-represented with circa 5%. RcnB shares 71 % identity with YohN of *Klebsiella pneumoniae* whose structure has been solved by NMR in the absence of any ligand (PDB :2L1S, Wahab A., Serrano P., Geralt M. and Wuthrich K.). The CD data obtained here for RcnB are compatible with this structure which contains 15 % helical and 36 % beta sheet secondary structures. When recorded in the presence of 1 equivalent of Cu or Ni, the spectra superimposed perfectly indicating that these two metals did not induce major secondary structure rearrangements (not shown). The apo-RcnB protein was denaturated by increasing the temperature to a maximum of 90°C and then renaturated by decreasing the temperature to 25°C. Spectra before and after thermal denaturation of apo-RcnB superimposed indicating that the process was fully reversible (Fig. 2A). The thermal denaturation was followed after addition of one equivalent of Ni or Cu ions (Fig. 2B). In the presence of Ni the denaturation process was similar to the one obtained for

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the apo-protein adding an evidence of the absence of any interaction between RcnB and these ions (Fig. 2B). In contrast, the protein folding was modified when denaturation occurred in the presence of Cu ions showing that Cu ions modulate RcnB stability. The Tm value (50 % of unfolded form) was 75°C in the presence of Cu whereas it was 86°C for apo-RcnB.

Cu binding by RcnB is physiological

We have shown that RcnB is able to bind Cu *in vitro*. To investigate the hypothesis of a physiological incidence of this interaction, as for instance the participation of RcnB to Cu resistance in *E. coli*, the resistance against Cu of a strain deleted for the *rcnB* gene was studied. After aerobic culture, growth of the wild-type strain was observed only for the most concentrated bacterial dilutions when 15 µM Cu was present in the medium (Fig. 3, upper panel). The *rcnB* mutant showed exactly the same phenotype indicating that the absence of *rcnB* does not increase the cell sensitivity to Cu. In contrast, the metal resistance phenotype increased when the *rcnB* mutant was complemented by a plasmid-borne copy of the gene. In this case, the growth of the strain was not altered in the presence of metal throughout the dilution range, showing enhanced Cu resistance of the bacteria. Under anaerobic growth conditions, similar profiles were obtained but the resistance of all the strains was lower (Fig. 3, lower panel). This increase in Cu toxicity in the absence of oxygen has been previously described [26]. Therefore, an *rcnB* mutant strain is not more sensitive to Cu but an overproduction of the protein can increase significantly the resistance profile.

One explanation for the increased resistance of the complemented strain could be the reduced accumulation of free hazardous Cu ions in bacteria. Metal quantification by ICP-OES was performed on whole, dried bacteria after growth in LB medium supplemented with 0.5 mM Cu, a sub-inhibitory concentration. In the presence of Cu, the Cu content in the *rcnB* mutant was slightly lower but not significantly different of the level measured in the wild-type strain (Table 2). These results are consistent with those of the metal susceptibility test. In contrast, complementation of the *rcnB* mutant by *rcnB* hosted on the pUC18 plasmid increased twice the cellular Cu content. These results show that RcnB is able to bind Cu *in vivo* and strongly suggest that the increased Cu resistance of the strain overexpressing RcnB, may be linked to the Cu-binding capacity of RcnB which lowers the free Cu content present in the periplasm.

RcnB is not directly involved in Cu homeostasis

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To gain insights into the mode of action of RcnB, we investigated the consequences of changes in intracellular Cu levels. To achieve this, we studied the connection between *rcnB* and the others genetic systems known to be involved in Cu homeostasis in *E. coli*. More precisely we studied the periplasmic detoxification systems. Firstly, the consequence of an *rcnB* mutation in a *cusF* background was examined. A Δ*cusF* mutant was shown to be slightly more sensitive to Cu than a wild type strain [17]. We have determined if RcnB could have a similar role as CusF or substitute for CusF in the absence of this protein. We confirmed that the Δ*cusF* mutant is more sensitive to Cu than the parental wild type strain (Fig. 4A). When assayed, the Δ*cusF* Δ*rcnB* double mutant showed a similar Cu resistance profile. It indicates that RcnB is unrelated to the *cus* system and cannot substitute for CusF as a metallo-chaperone working with CusCBA. So, RcnB is a soluble periplasmic Cu-binding protein, like CusF, but with a distinct function.

The other periplasmic Cu detoxification system in *E. coli* is the multi-Cu oxidase CueO that is thought to oxidize Cu(I) into its less toxic form Cu(II) [26]. The MIC of a Δ*cueO* strain was determined in liquid cultures. As previously shown [26], the Δ*cueO* strain was more sensitive to Cu than the wild-type strain (Fig. 4B). The Δ*rcnB* mutant displayed a resistance pattern similar to the wild-type strain consistent with the assay on Petri dishes (Fig. 3). Finally, the Δ*rcnB* Δ*cueO* double mutant had a growth phenotype similar to the single Δ*cueO* mutant as Cu increased (Fig. 4B). These results confirm that *rcnB* is not involved in Cu homeostasis and that *rcnB* is not required by the *cue* system for Cu detoxification. We analyzed further the *in vivo* Cu binding capacities of RcnB in a *cueO* mutant. When complemented by *rcnB*, borne on a high copy plasmid, the Cu resistance of the Δ*cueO* strain was restored (Fig. 4C). This can be interpreted, in the light of our previous results (Fig. 3 and Table 2), as the consequence of Cu binding by RcnB in the periplasm. Since the expression of the *cueO* gene was previously shown to be inducible by silver [27] we investigated the behavior of the strains in the presence of this metal. We confirmed that the Δ*cueO* mutant was slightly more sensitive to silver than the wild-type strain (Fig. 4C). However, complementation of the Δ*cueO* mutant by *rcnB* hosted on the high-copy plasmid pUC18 did not restore the wild-type phenotype towards Ag resistance, indicating that RcnB response is specific to Cu.

To gain insight into the role of *rcnB*, its expression was followed using RT-PCR. When using a primer annealing 162 bp upstream of the ATG of *rcnB* and a primer complementary to the end of *rcnB,* transcripts were detected only after growth in the presence of Ni or Co (Fig. 4, $rcnB_{ext}$). When primers internal to *rcnB* were used, transcripts were

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detected after growth in LB medium or supplemented with Ni or Co or Cu with more transcripts detected after growth in the presence of Ni or Co (Fig. 4, $rcnB_{int}$). It has been previously shown that *rcnB* is transcribed from the promoter of *rcnA* and that this transcription is specifically induced by Ni and Co [11]. This regulation of expression is observed with primers external to *rcnB*. When using primers internal to *rcnB*, transcripts are detected in all growth conditions and in particular in the absence of the cognate inducers Ni and Co. This demonstrates that *rcnB* is constitutively expressed and suggests that a second promoter might exist upstream of *rcnB*, the 200 pb *rcnA-rcnB* intergenic region being compatible with this hypothesis. In contrast, expression of the *cusF* gene was detected only after growth in LB supplemented with Cu (Fig. 4D). As for *cueO*, its expression was detected after growth under all the tested conditions, but it was strongly induced by Cu (Fig. 4D). These results show that, in contrast to genes related to copper metabolism, *rcnB* is not induced by copper, and dismiss *rcnB* as being involved in controlling copper homeostasis in *E. coli*.

Mutant RcnB-M86A deficient in Cu binding is deficient in controlling Ni and Co homeostasis

When Cu was added to purified RcnB, the solution turned blue. The metallation of RcnB by Cu was monitored by following the spectral changes between 350 and 850 nm. Addition of increasing amount of Cu lead to the appearance of an absorption band at 610 nm (Fig. 5). The signal increased linearly up to 15 equivalents of Cu, no saturation was observed. Above 15 equivalents, the protein precipitated. In reason of the absence of saturation of RcnB by Cu, the extinction coefficient could not be deduced from the spectra. The metallation of RcnB was carried out in the presence of competitors: EDTA, a strong affinity divalent cations chelator, or glycine, a weaker affinity chelator (Kd of Cu(II) for glycine is *circa* 500 nM at pH 7.5 [28]) and followed by UV-visible spectroscopy. In neither case RcnB was able to compete with the chelators (not shown), confirming that the dissociation constant (K_D) of Cu for RcnB is significantly higher than 500 nM.

The extinction of fluorescence of RcnB upon the addition of Cu (Fig. 1A) suggests that at least one of the two tryptophans present in RcnB is close to the Cu-binding site. Alignment of the sequences of homologs of RcnB highlighted the presence of a conserved motif in enterobacteria: $H_{82}W_{83}XXM_{86}$. Methionine and histidine residues being potent Cu ligands, H82 and M86 mutants were constructed by site directed mutagenesis in which His_{82} or Met_{86} were substituted with Ala. The mutant proteins were over-expressed and purified using the same protocol as for the wild type protein. Mutant H82A RcnB appeared to be

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highly unstable, prone to degradation, preventing its purification and further analysis. The binding of Cu to the M86A mutant protein was monitored by UV-visible spectroscopy. Addition of up to 10 equivalents of Cu did not trigger the appearance of the absorption band centered at 610 nm as observed for the wild type protein (Fig. 5 inset). In fact no changes of the spectra could be detected when Cu was added, indicating that M86A RcnB had lost the Cu center detected in the wild type protein, and in turn that the Cu center giving rise to the 610nm absorption band involves Cu liganding by M_{86}.

As we have shown previously [11], the biological function of RcnB is to participate in the efflux of Ni and Co ions mediated by the efflux pump RcnA. All the data presented here show that RcnB is a Cu-binding protein, that it is not involved in maintaining Cu homeostasis. It can be hypothesized that the binding of Cu by RcnB is required for its biological function i.e. maintaining Ni and Co homeostasis. To address this question, the gene encoding mutant M86A RcnB was introduced into an expression vector and its function was determined by measuring the susceptibility of the tested strain to Ni or Co toxicity. To ensure stoichiometric amounts of RcnR, RcnA and RcnB, we used a plasmid containing *rcnR-rcnAB* in a pUC18 backbone (pAR123 [8]). The same plasmid was subjected to site directed mutagenesis to encode for an M86A RcnB variant (pMG10). These plasmids were introduced into strain EGE119 a Δ*rcnA*Δ*rcnB* double deletion mutant. As expected the Δ*rcnAB* mutant was more sensitive to Ni and Co than the WT strain indicating a default in Ni and Co efflux and complementation by pAR123 restored the wild type phenotype (Fig. 6A). Most interestingly, when the mutation M86A was introduced into *rcnB*, the complementation phenotype was lost, indicating that the RcnAB efflux system was no longer functional. Western blot using antibodies directed against RcnB was performed to monitor the stability of M86A RcnB. As expected, in crude extracts, both the precursor and mature forms of RcnB were detected (Fig. 6B). The mature form corresponding to the periplasmic form of RcnB was equally detected in extracts expressing the wild-type or the M86A variant of RcnB showing that the mutation did not alter the stability of the protein.

RcnB shares 71 % identity with YohN of *Klebsiella pneumoniae* whose structure has been solved by NMR in the absence of any ligand (PDB :2L1S, Wahab A., Serrano P., Geralt M. and Wuthrich K., unpublished) (Supplementary Figure 2). The overall structure is in agreement with CD spectra obtained in this study for RcnB of *E. coli*. The surface representation shows that the potential Cu coordinating residue M86 is accessible to the solvent (Supplementary Fig. 2, B). The modeled structure of YohN was used to examine the PDB in order to retrieve structural neighbors using PDBefold [32]. No protein structure

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similarity was detected, emphasizing the fact that the Cu-binding site present in RcnB is novel.

Discussion

The RcnB protein was previously shown to be a periplasmic accessory protein involved in Ni and Co efflux in conjunction with the RcnA pump. However it lacks affinity for Ni and Co ions [11]. In the present work we demonstrate that RcnB is a Cu-binding protein. The first evidence came from tryptophan fluorescence quenching experiments where Cu was the only tested divalent cation able to quench tryptophan fluorescence (Fig. 1). Cu binding was further evidenced by UV-visible spectroscopy (Fig. 5). The binding stoichiometry determined by mass spectrometry and ITC led to the conclusion that RcnB binds one Cu per monomer, with a dissociation constant in the micromolar range (Fig. 1). This affinity is relevant for a biological function, however it is weak when compared to known periplasmic copper-binding proteins participating in mechanisms of Cu cell detoxification. For instance *copH* encoded by the large plasmid pMOL30 of *Cupriavidus metallidurans* is part of the *cop* cluster involved in detoxification of copper. CopH is a periplasmic protein shown to bind Cu(II) on a high affinity site with a K_D of 36 nM and on a low affinity site with a K_D of 2.5 μM [29].

As the RcnA efflux pump was shown to play a role in Ni or Co but not in Cu resistance [8], RcnB was not expected to bind copper and the certainty of *in vivo* Cu binding was questioned. To address this point, *rcnB* was expressed from a high copy plasmid. In these conditions, *rcnB* conferred the cells enhanced Cu resistance combined with increased Cu accumulation in cells (Fig. 3 and Table 2). These experiments clearly showed that RcnB binds Cu *in vivo*. All together the *in vitro* and *in vivo* experiments demonstrate that RcnB is a new copper protein as it shares no homology to previously described proteins.

E. coli possesses two chromosomally encoded periplasmic Cu-proteins, CueO and CusF. CueO is a multicopper oxidase able to oxidize Cu(I) in its less toxic form Cu(II) [30]. CusF is the periplasmic metallochaperone of the RND Cu-efflux system CusCBA [18]. Both CueO and CusF are involved in Cu detoxification. While the transcription of *cueO* is induced by copper via the cytoplasmic metallo-regulator CueR, the copper induction of the *cusCFBA* operon is under the control of the two-components system CusSR able to sense periplasmic copper concentration [26]. A possible function of RcnB would also be to participate in copper detoxification. The CMI of the *rcnB* mutant towards Cu was the same as the wild type strain (Fig. 4). Since *E. coli* contains multiple Cu detoxification systems, the *rcnB* mutation can

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remain silent in reason of compensation by the other systems. However *rcnBcusF* and *rcnBcueO* double mutants displayed no enhanced Cu sensitivity compared to *cusF* and *cueO* mutants respectively (Fig. 4). Moreover, in contrast to *cueO* or *cusF*, the expression of *rcnB* was not induced by Cu. We show here that *rcnB* is expressed on two transcripts: a long transcript arising from transcription at the *rcnA* promoter specifically induced by Ni or Co (10, 12) and a smaller one expressed in all growth conditions including that without metal. Therefore, Cu is not an inducer of the expression of *rcnB* and *rcnB* is likely to be transcribed from its own promoter in harmony with the wide 218 bp *rcnA-rcnB* intergenic region. Sequence analysis of RcnB homologs among proteobacteria revealed that it can be encoded by an orphan gene, which suggests that RcnB-like proteins could also play a role unrelated to Ni and Co efflux and resistance. Besides CueR or CusR binding sites were not retrieved in the promoter region. Moreover transcriptomic studies of Cu-stressed *E. coli* cells did not identify *rcnB* among the Cu-regulated genes [31]. Taken together our results rule out the possibility that *rcnB* is part of a Cu detoxification system. Interestingly enough, when expressed on a high copy plasmid under the control of the *lac* promoter, *rcnB* complemented a *cueO* deficient strain (Fig. 4C). Thus, overproduced RcnB is able to substitute periplasmic CueO, the enzyme that detoxifies Cu as it can bind Cu in the same cellular compartment. This complementation was not observed for *cus* deficient strains (data not shown).

At the sequence level, RcnB does not contain any previously described Cu binding site. Still RcnB contains 2 methionines, 3 histidines, 11 acidic residues but no cysteine in its mature form as potent Cu ligands. It has a potential metal-binding motif $H_{82}W_{83}XXM_{86,}$ well conserved among RcnB homologs found in proteobacteria, that we investigated by site-directed mutagenesis. While the H82A mutant protein appeared to be unstable when overexpressed, purification of the M86A mutant protein was successful. This mutant lost the spectroscopic signature of the wild type protein characterized by a broad band centered at 610 nm upon Cu addition (Fig. 5). It can be concluded that Cu binding by RcnB involves at least one methionine residue. RcnB shares 71 % identity with YohN of *Klebsiella pneumoniae* whose structure has been solved by NMR in the absence of any ligand (PDB :2L1S, Wahab A., Serrano P., Geralt M. and Wuthrich K.). The CD data obtained here for RcnB are compatible with this structure. No change of the spectrum was detected on addition of Cu or Ni indicating that the global fold of the protein was preserved with metal. Protein Data Bank search using the structure of YohN or using models of RcnB based on this latter were performed using PDBefold [32] and failed to retrieve any structural neighbors.

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At the physiological level, mutation M86A rendered the RcnAB efflux system ineffective with respect to Ni and Co resistance (Fig. 6). This suggests that copper binding by RcnB is essential for its function. In light of the CD experiments it can be hypothesized that Cu plays a structural role as it was shown to destabilize RcnB leading to precipitation of the protein when subjected to thermal denaturation (Fig. 2). One can also suggest that RcnB requires Cu to bind Ni or Co as free ions or in complex with prosthetic groups, It has been shown at least for Ni that NikA, the periplasmic binding protein of the Ni ABC importer NikABCDE is unable to bind Ni directly but requires an organic chelator as a nickelophore. Small organic ligands [33] as well as (L-His)2 [34 35] have been identified as nickelophores. In the case of RcnB, Cu could favor such interactions or could favor the binding of Co or Ni ions. Still, Ni binding on a Cu-loaded form of RcnB or Cu loading in the presence of Ni were assayed and monitored by fluorescence and UV-visible spectroscopy and no differences were observed when compared to the metallation assays performed in the presence of Cu only (data not shown). Further work aiming at determining the structure of RcnB in interaction with metals will help in better understanding its function.

Acknowledgements

This work was supported by CNRS, INSA Lyon BQR. C.B. and M.G. were supported by a grant from the French Ministry of Research. We thank R. Montserret of IBCP for supervision of the CD and ITC experiments and G. Effantin for technical assistance.

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Figure legends

Figure 1

RcnB is a Cu binding protein. (A) Fluorescence quenching after the addition of increasing amounts of $CuSO_4$. Protein was dissolved in 100 mM Bis-Tris propane buffer (pH 6.0) to a final concentration of 5 µM. At each step, a ten-fold excess of metal ions solution was titrated into the protein solution Fluorescence intensity was monitored between 300 and 400 nm after

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an excitation at λ = 280 nm. Intensity is expressed in arbitrary units. (B**)** Mass spectra of RcnB (5 μM) reconstituted with or without a ten-fold excess of Cu^{2+} ions in 20 mM ammonium acetate buffer (pH 7.0). The mass spectrum of apo-RcnB (upper panel) or after the addition of 10 equivalents of Cu^{2+} (panel below) is shown. (C) Isothermal titration calorimetry of RcnB. Cu(II)SO_4 (1.5 mM) titrated into apo-RcnB (150 μM) in 10 mM Pipes buffer (pH 7) at 30°C. Top, raw data. Bottom, plot of integrated heats versus Cu/RcnB ratio. The solid line represents the best fit for a one-site binding model.

Figure 2

Cu acts on RcnB stability. (A) CD spectra of RcnB (25 μM) in 50 mM mM sodium phosphate buffer (pH 7.0) at 25°C before (solid line) or after (dashed line) thermal denaturation at 90°C. **B.** CD-monitored thermal denaturation of apo-RcnB (solid line), RcnB in the presence of Ni (dotted and dashed line) or RcnB in the presence of Cu (dashed line). The value of CD at 220 nm is used to estimate the fraction of unfolded RcnB.

Figure 3

Cu binding by RcnB is physiological. The wild-type strain (W3110), the Δ*rcnB* isogenic mutant (WRCB1), WRCB1 pUC18, and WRCB1 pUCRCB (*rcnB* in pUC18) were grown in LB medium to mid-log phase. A 10-fold serial dilution of the cultures was performed, and 5 μl was spotted on M63 minimal medium supplemented with 0.4% glucose (G), 0.4% glucose plus 10 or 15 μM $CuSO_4$ and grown aerobically (top) or anaerobically (bottom) at 37°C in Petri dishes. Spots corresponding to 10^7 (rightmost column) to 10^4 (leftmost column) bacteria are shown.

Figure 4

RcnB is not directly involved in Cu homeostasis. (A). The wild-type strain (W3110), Δ*rcnB,* Δ*cusF* and Δ*rcnB* Δ*cusF* isogenic mutants were grown in LB medium to mid-log phase. A 10-fold serial dilution of the cultures was performed, and 5 μl was spotted on M63 minimal medium supplemented with 0.4% glucose (G), 0.4% glucose plus 2 μM $CuSO_4$ and grown anaerobically on Petri dishes. Spots corresponding to 10^7 (rightmost column) to 10^4 (leftmost column) bacteria are shown. (B) The wild-type strain (W3110), Δ*rcnB,* Δ*cueO* and Δ*rcnB* Δ*cueO* isogenic mutants were grown in M63 minimal medium supplemented with 0.4% glucose (G) plus increasing amounts of $CuSO_4$. OD_{600nm} was recorded after 16 hours aerobic incubation at 37°C. (C) The wild-type strain (W3110), Δ*cueO* isogenic mutant

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complemented or not with pUCRCB were grown as in (A) and 5 µl was spotted on M63 minimal medium supplemented with 0.4% glucose (G), or 0.4% glucose plus 10 µM $CuSO_4$ or 1 µM $AgSO_4$ and grown aerobically on Petri dishes. Spots corresponding to 10^7 (rightmost column) to 10^4 (leftmost column) bacteria are shown. (D). Total RNA was extracted from WT bacteria grown in LB medium alone or supplemented with non-inhibitory amounts of $NiSO_4$, $CoCl_2$ or $CuSO_4$. RNA were PCR amplified without (RTase -) or with (RTase +) a reverse transcription step. The amplification was performed by using specific primers for *rcnB*, *cusF* or *cueO* genes. A representative agarose gel is shown.

Figure 5

Methionine 86 is involved in Cu binding. (A). UV-visible Cu titration of 130 µM RcnB or 80 µM RcnBM86A (inset) in 50 mM Tris-HCl buffer (pH 7.0) with $CuSO_4$ at 25°C. The differential spectra corresponding to the subtraction of the spectrum of the apo protein are shown.

Figure 6

Methionine 86 mutants are deficient in controlling Ni and Co homeostasis (A) The wild-type strain W3110 (closed symbols) and EGE119 Δ*rcnA*Δ*rcnB* isogenic mutant (open symbols) complemented with pUC18 (vector) or pAR123 (*rcnR-rcnAB*) or pMG10 (as pAR123 but M86A RcnB) were grown in LB medium to mid-log phase. A 10-fold serial dilution of the cultures was performed, and 5 µl was spotted on M63 minimal medium supplemented with 0.4% glucose (G), 0.4% glucose plus 40 µM $NiSO_4$ or 0.4% glucose plus 5 µM $CoCl_2$ and grown aerobically on Petri dishes. Spots corresponding to 10^2 (rightmost column) to 10^7 (leftmost column) bacteria are shown. (B) Western blot against RcnB. Lane 1 : BL21/pETRCB, lane 2 : BL21/pET30, lane 3: BL21/pETRBM. RcnB-p : precursor form, RcnB-m : mature form.

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631 **Table 1 : Strains and plasmids used in this study**

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Strains or plasmids	Genotype or description	Source, reference
strains		
BW25113	*lacI*q *rrnB*T14 _*lacZ*WJ16 *hsdR514* _*araBAD*AH33 _*rhaBAD*LD78	[20]
JW0119	BW25113, Δ*cueO*::*kanR*	[20]
JW0562	BW25113, Δ*cusF*::*kanR*	[20]
W3110	Wild type	Laboratory stock
WRCB1	W3110, Δ*rcnB*::*cm*	[11]
WCUF1	W3110, Δ*cusF*::*kanR*	This study
WCUE1	W3110, Δ*cueO*::*kanR*	This study
WRBC1	WRCB1, *ΔcueO*::*kanR*	This study
WRBF1	WRCB1, Δ*cusF*::*kanR*	This study
EGE119	W3110 Δ*rcnA*Δ*rcnB*	This study
BL21	F- *ompT gal* [*dcm*] [*lon*] *hsd*SB (rB- mB-) (DE3)	[36]
plasmids		
pKO3	*repA*(ts) Cm^R M13ori *sacB*	[22]
pEGL16	PCR fragment Δ*rcnAB* in pKO3	This study
pET30	Overexpression vector	Novagen
pETRCB	pET30 containing *rcnB* coding sequence	[11]
pETRBM	As pETRCB with a point mutation in *rcnB* leading to M86A -RcnB	This study
pUC18	expression vector, AmpR	[37]
pAR123	*rcnR-rcnAB* in pUC18	[8]
pMG10	As pAR123 with point mutations in *rcnB* leading to M86A -RcnB	This study
pUCRCB	pUC18 containing *rcnB* coding sequence	[11]

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Table 2 Cu content in bacteria

Strain	WT	Δ*rcnB*	Δ*rcnB*/*rcnB*
Cu (µg/g)	75 ± 6	67 ± 1	144 ± 5

The wild-type strain (W3110), the Δ*rcnB* isogenic mutant (WRCB1), and the Δ*rcnB* mutant complemented by *rcnB* (WRCB1/pUCRCB) were grown in LB medium supplemented with 500 µM $CuSO_4$. Values (µg/g bacterial dry weight) are the means of at least three independent ICP-OES measurements performed on independent cultures.

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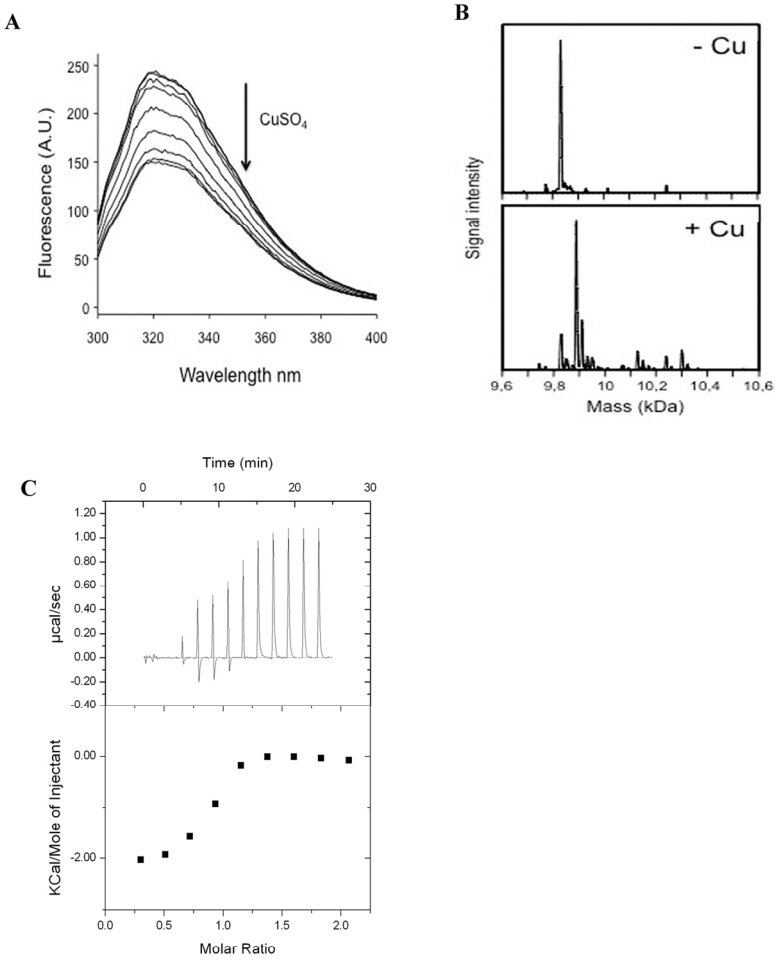

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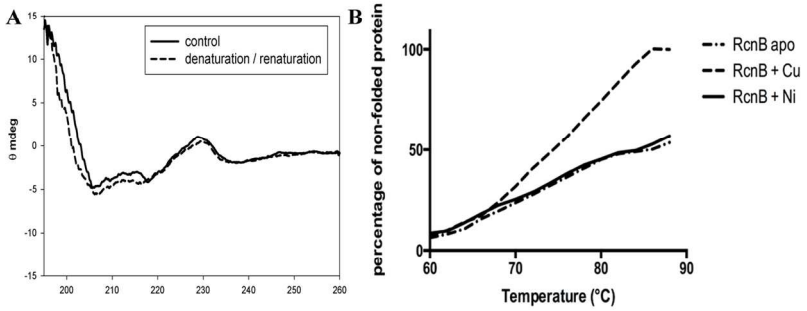

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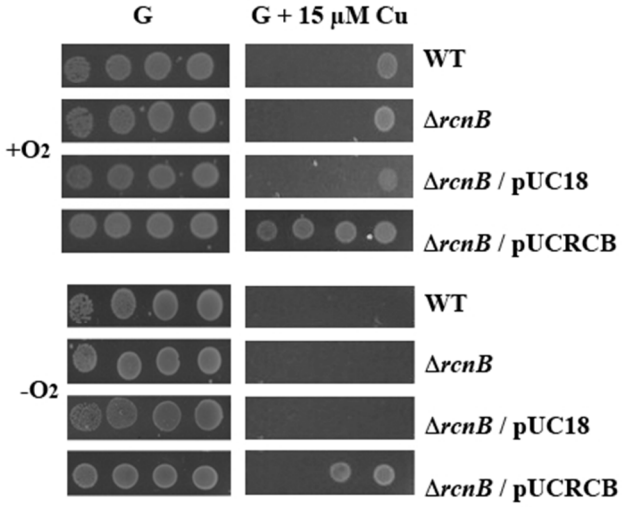

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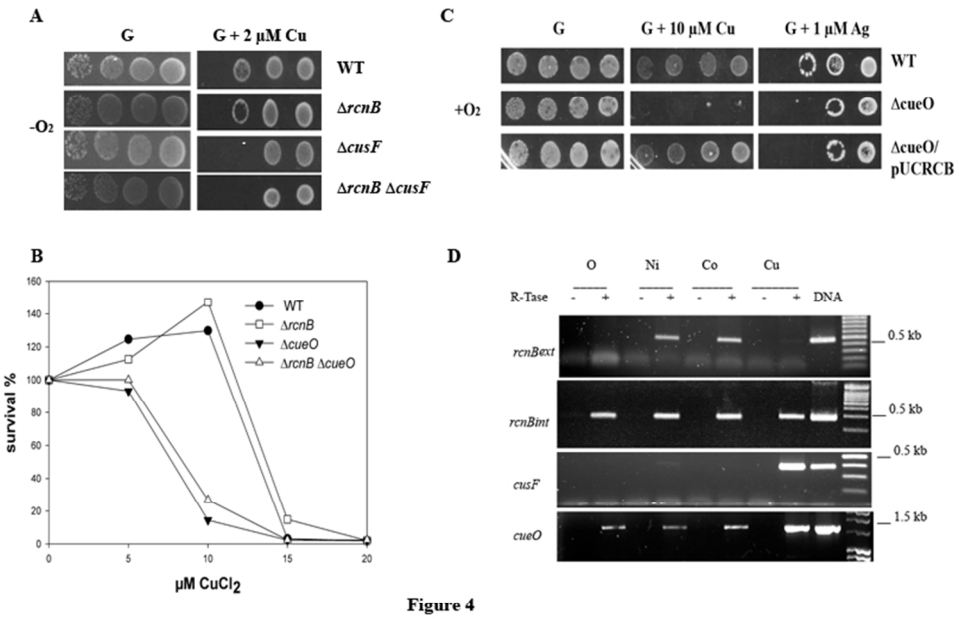

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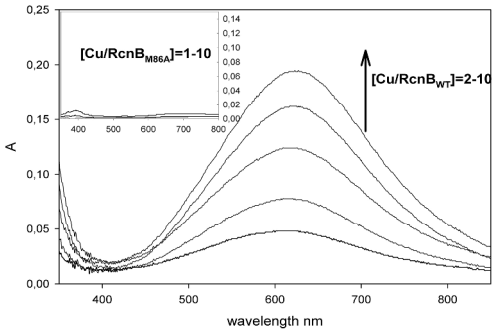

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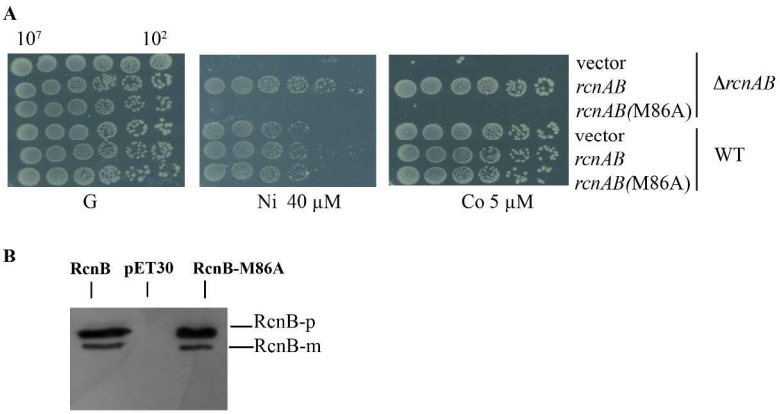

Figure 6

Methionine 86 mutants are deficient in controlling Ni and Co homeostasis (A) The wild-type strain W3110 (closed symbols) and EGE119 ΔrcnAΔrcnB isogenic mutant (open symbols) complemented with pUC18 (vector) or pAR123 (rcnR-rcnAB) or pMG10 (as pAR123 but M86A RcnB) were grown in LB medium to mid-log phase. A 10-fold serial dilution of the cultures was performed, and 5 µl was spotted on M63 minimal medium supplemented with 0.4% glucose (G), 0.4% glucose plus 40 µM NiSO4 or 0.4% glucose plus 5 µM CoCl2 and grown aerobically on Petri dishes. Spots corresponding to 102 (rightmost column) to 107 (leftmost column) bacteria are shown. (B) Western blot against RcnB. Lane 1 : BL21/pETRCB, lane 2 : BL21/pET30, lane 3: BL21/pETRBM. RcnB-p : precursor form, RcnB-m : mature form.
209x297mm (300 x 300 DPI)

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