Supporting Information for

The interplay of the metallosensor CueR with two distinct CopZ chaperones defines copper homeostasis in *Pseudomonas aeruginosa*

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Running title: Cytoplasmic Cu^{+} distribution in *P. aeruginosa*

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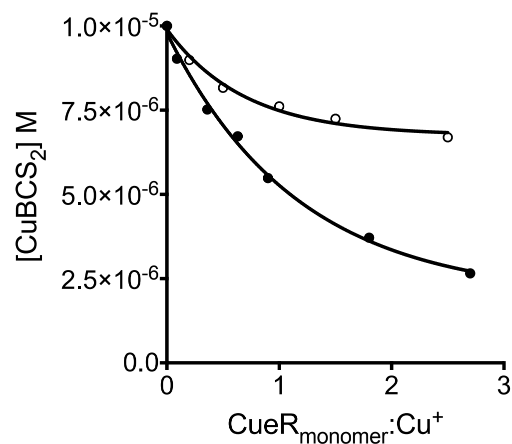

Fig. S1. CueR-Cu^+ dissociation constant K_D determined using a BCS competition assay (2). Reaction mixtures were prepared in buffer H with constant concentrations of Cu^+ (10 µM) and BCS (25 or 100 µM) and varying concentrations of CueR (1-30 µM monomer). After 10 min equilibration, $CuBCS_2$ concentrations were estimated using A_{483} (β_2 $10^{19.8}$ M^{-2}, ε_{483} 13000 $M^{-1}cm^{-1}$). CueR-Cu^+ K_D was calculated by fitting the experimental data to the equilibrium binding equation (2,3). As CueR dimers bind two Cu^+ (4,5), $[P]_{total}$ = [CueR monomer]. K_D values determined using 25 µM $CuBCS_2$ (black circles) and 100 µM $CuBCS_2$ (open circles) were curve fitted to $K_D = 3.1 \pm 0.2 \times 10^{-16}$ M and $K_D = 7.9 \pm 0.9 \times 10^{-17}$ M, respectively. An average $K_D = 2.5 \pm 1.0 \times 10^{-16}$ M was calculated based on 6 independent experiments.

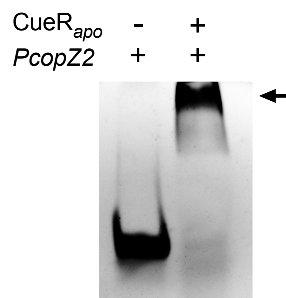

Fig. S2. Interaction CueR$_{apo}$-*PcopZ2* examined by Electrophoretic Mobility Shift Assay. 10 pmol of 50-bp unlabeled *PcopZ2* (Table S1) were incubated 30 min at room temperature with 500 pmol CueR dimer in 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 5% glycerol, 0.01 mg/mL BSA. The electrophoretic mobility of *PcopZ2* in the absence (left) and the presence of CueR$_{apo}$ (right), was observed in 10% polyacrylamide/TAE gels stained with ethidium bromide. The arrow highlights the mobility shift of *PcopZ2* in the presence of CueR.

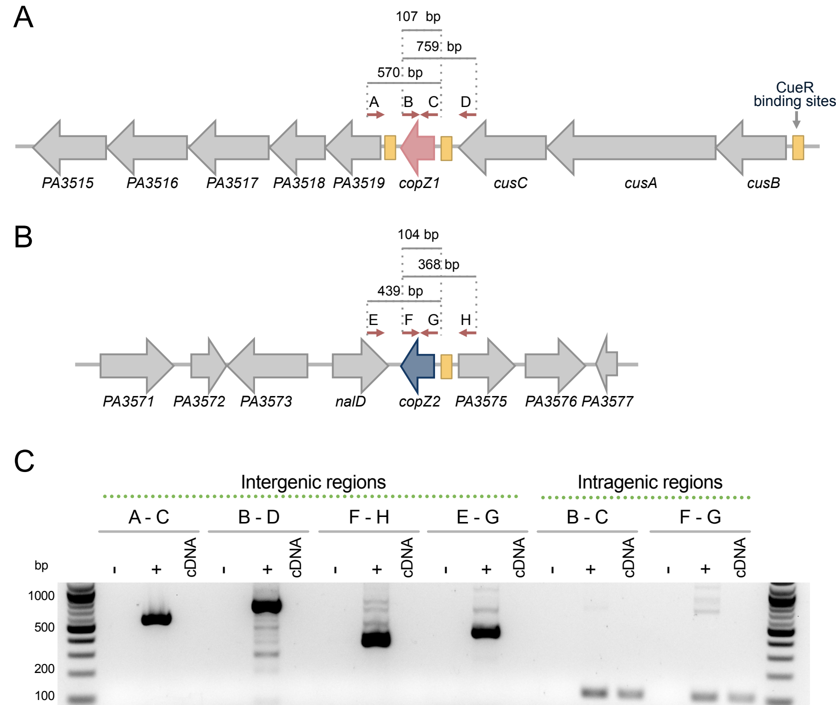

Fig. S3. *P. aeruginosa copZ1* and *copZ2* operon mapping. Total RNA from *P. aeruginosa* PAO1 in exponential growth phase was reverse transcribed and the cDNA used as template for PCR. Primers were designed in order to amplify the junctions between adjacent genes. Primers in intragenic regions were included as controls (Table S1). Schematic representation of the position of primers designed for co-transcription analysis of the gene cluster of (A) *copZ1* and (B) *copZ2* operons. (C) Each panel is constituted by three lanes: negative control, H_2O; gDNA lane, which contains amplified products from genomic DNA and serves as positive control; and the cDNA lane, that contains the RT-PCR products.

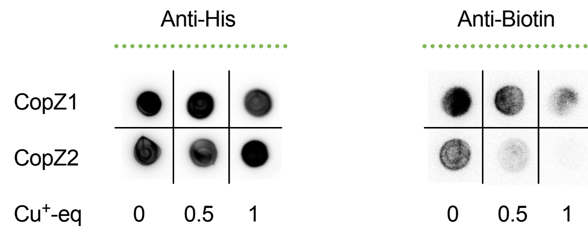

Fig. S4. Cu^{+} protection of Cys residues at the metal binding sites from biotin-maleimide alkylation. Pure extracts of His-tagged CopZ1 and CopZ2 were separately alkylated with biotin-maleimide in the presence of 0, 0.5, and 1 eq of Cu^{+}. After removing excess of alkylating agent by Ni-NTA affinity chromatography, proteins were dot-blotted and immune stained either with anti-His antibodies or anti-Biotin antibodies. The dividing lines indicate where the images have been spliced; signals were from an identical original image and have not been altered. A decrease in anti-biotin staining indicated that Cu^{+} protects Cys against chemical modification.

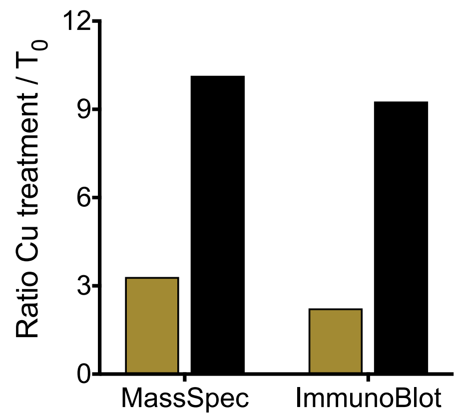

Fig. S5. Levels of CopZ1 (ochre) and CopZ2 (black) in the presence of 0.5 mM $CuSO_4$ (10 min) relative to those in the absence of metal, determined by MS/MS and immunostaining. Relative ratios in MS/MS were generated by spectral counts (total spectra). In both approaches, samples were normalized prior to the experiment (equal cell counts).

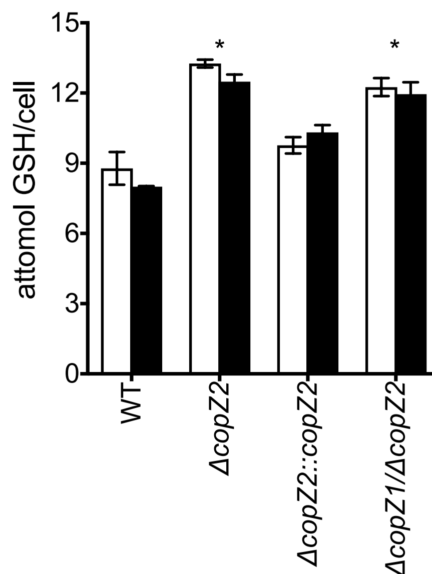

Fig. S6. Levels of GSH in WT, Δ*copZ2*, Δ*copZ1/ΔcopZ2* mutant strains and *copZ2* complemented strain upon 0.5 mM $CuSO_4$ treatment (10 min). The amounts of total GSH in the absence (white) and the presence of copper (black) were measured spectrophotometrically using a kinetic assay of DNTB reduction coupled to GSSG recycling. Cells were incubated in 0.5 mM $CuSO_4$ in antibiotic-free LB medium. 2 mL aliquots were taken before and after 10 min of $CuSO_4$ addition, harvested by centrifugation, resuspended in 150 µL 5% sulfosalicylic acid, sonicated 30 sec in a dry-ice/ethanol bath, and incubated for 10 min on ice. Cellular debris and proteins were removed by centrifugation at 10,000 rpm for 10 min, and supernatants used for total GSH quantification. 20 uL samples were pre-incubated 5 min with 78 µM DTNB, 0.115 units/mL GSH reductase, 100 mM Na_2HPO_4/Na_2HPO_4 (pH 7), 1 mM EDTA. Upon addition of 46 µM NADPH, A412 was measured at 1 min intervals for 7 minutes. 0-200 µM GSH in 5% sulfosalicylic acid were used as standards. Data are the mean ± SE of three independent experiments. Significant differences from the WT as determined by paired two-tailed Student's t-test are $*P < 0.05$.

Table S1. Bacterial strains, plasmids and primers used in this study

Strains	Relevant features	Reference
Pseudomonas aeruginosa		
PAO1	Wild type	(6)
Δ*copZ1*	*copZ1* gene replaced by a *BamH*I site	(7)
Δ*copZ1*::*copZ1*	*copZ1* complemented gene, C-terminal TEV_His-tag, under endogenous promoter, Gm^R	This study
Δ*copZ2*	*copZ2* gene interrupted by a *Pvu*II site and a stop codon	This study
Δ*copZ2*::*copZ2*	*copZ2* complemented gene, C-terminal TEV_His-tag, under endogenous promoter, Gm^R	This study
Δ*copZ1*/Δ*copZ2*	*copZ2* gene interrupted by a *Pvu*II site and a stop codon in the Δ*copZ1* background	This study
Δ*copA1*	PW7626, *copA1*(PA3920)::ISphoA::Tet^R	(8)
Δ*cueR*	PW9026, *cueR*(PA4778)-B07::ISphoA/hah::Tet^R	(8)
Escherichia coli		
BL21(DE3)	F^- *ompT gal dcm lon* $hsdS_B(r_B^- m_B^-)$ *λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB*$^+$*]*$_{K\text{-}12}$*(λ*^{S}*)*	Novagen
BL21(DE3)pLysS	BL21(DE3) strain *pLysS[T7p20* ori_{p15A}*](Cm*^{R}*)*	Novagen
Top10	F^- *mcrA Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str*^{R}*) endA1 nupG*	Invitrogen
S17.1	*Tp*^{R} *Sm*^{R} *recA, thi, pro, hsdR*$^-$*M*$^+$*RP4- 2 Tc:Mu::Kan::Tn7/λpir.*	(9)

Plasmids	Relevant features	Reference
pET-30b+	T7 promoter, *lac* operator, 6xHis tag, *lac*I, Kan^R	Novagen
copZ1::pET-30b+	*PA3520*::pET-30b+, Kan^R	(2)
copZ2::pET-30b+	*PA3574.1*::pET-30b+, Kan^R	(2)
pBADtopo	*araBAD* promoter, V5 epitope tag, 6xHis tag, Amp^R	Invitrogen
*cueR*strep::pBAD	*PA4778*_strep::pBAD, Amp^R	This study
pDONRPEX18Gm	Gateway allelic exchange vector, Gm^R	(10)
pUC18-mini-Tn7-Gm	Suicide delivery vector, Gm^R	(11)
pTNS2	Helper plasmid (TnsABCD site), Amp^R	(12)

Primers	Sequence (5'-3')
copZ2 mutant	
CopZ2upF-GWB1	ggggacaagtttgtacaaaaaagcaggctacatcagcaccgcgtacagcag
CopZ2upR01	cagctgtcaggcgtggccggcggtcatgccctgaaccttgaaa
CopZ2downF01	gccggccacgcctgacagctggtacgggcgatcacccatgc
CopZ2downR-GWB2	ggggaccactttgtacaagaaagctgggtagcgccgtctactggcacttc
Mutant complementation	
Comp_CopZ1_F	ttttactagtctggcgcggatcactcgtc
Comp_CopZ1_R	ttttggtacctcaatgatgatgatgatgatgggactgaaaatacaggttttcgccgctgctacggccctccggataaccgg
Comp_CopZ2_F	ttttactagtcggcatgacgatcagcaccg
Comp_CopZ2_R	ttttggtacctcaatgatgatgatgatgatgggactgaaaatacaggttttcgccgctgctggccagctcggcgccgta
copZ2 promoter	
*copZ2*prom_F	tatttcgaggattgaccttgacaccatgtcaaggtcgaaaatcgccccat
*copZ2*prom_R	atggggcgattttcgaccttgacatggtgtcaaggtcaatcctcgaaata
Operon mapping	
OW_*PA3519*_to_*copZ1* (A)	tgtaacggatttgaagtcat
OW_*PA3521*_to_*copZ1* (D)	agcgcgacagtcgctcctga
OW_*PA3575*_to_*copZ2* (H)	gaatagcggcggatggacat
OW_*PA3574*_to_*copZ2* (E)	gcgccgcatcctgaccatcc
qPCR	
qPA4268F	gcaaaactgcccgcaacgtc
qPA4268R	tacacgaccgccacggatca
qCopZ1F (C)	aagactgtcacccgtatcct
qCopZ1R (B)	aatgcgtgcttgtccaga
qCopZ2F (G)	ggtgcaggcgaaggatt
qCopZ2R (F)	ggatcgcctcgagtacct
qCopA1F	gaaacggtgctggcgaagat
qCopA1R	ttaaccagggcctgctccag

Table S2. Docking studies of CopZ1 and CopZ2 with CueR.

$CueR_{holo}$ – CopZ1								
Cluster		**HADDOCK**	**RMSD**	**vdW**	**Electrostatic**	**Desolvation**	**Z**
No.	Size	a.u.	Å	kcal/mol	kcal/mol	kcal/mol	
1	56	-82 ± 1	3.9 ± 0.2	-29 ± 2	-324 ± 8	12 ± 2	-2.0
2	41	-71 ± 3	2.0 ± 0.1	-37 ± 4	-195 ± 59	4 ± 10	-1.0
3	20	-61 ± 2	2.6 ± 0.2	-28 ± 3	-179 ± 34	3 ± 8	-0.1
4	16	-62 ± 2	3.6 ± 1.1	-27 ± 5	-231 ± 30	11 ± 3	-0.2
5	15	-47 ± 1	7.1 ± 0.5	-28 ± 5	-132 ± 18	7 ± 6	1.1
6	8	-49 ± 7	5.3 ± 0.1	-38 ± 5	-96 ± 19	7 ± 3	1.0
7	5	-45 ± 5	6.2 ± 0.5	-28 ± 4	-106 ± 23	4 ± 4	1.3
8	5	-58 ± 15	1.1 ± 0.7	-34 ± 4	-179 ± 43	12 ± 8	0.1
9	4	-63 ± 4	4.8 ± 0.2	-27 ± 5	-268 ± 34	11 ± 7	-0.3
Average cluster size ± SD = 18.9 ± 18.1							

$CueR_{holo}$ – CopZ2							
Cluster		**HADDOCK**	**RMSD**	**vdW**	**Electrostatic**	**Desolvation**	**Z**
No.	Size	a.u.	Å	kcal/mol	kcal/mol	kcal/mol	
10	113	-73 ± 1	7.4 ± 0.1	-32 ± 7	-183 ± 22	-5 ± 4	-1.4
11	14	-71 ± 6	0.1 ± 0.3	-34 ± 4	-242 ± 70	9 ± 8	-1.2
12	13	-53 ± 5	5.7 ± 0.1	-29 ± 4	-81 ± 24	-8 ± 5	0.5
13	13	-54 ± 3	8.6 ± 0.1	-30 ± 4	-152 ± 25	6 ± 7	0.5
14	9	-45 ± 2	4.3 ± 0.1	-35 ± 3	-44 ± 10	-1 ± 5	1.3
15	6	-65 ± 11	6.5 ± 0.1	-35 ± 2	-215 ± 30	10 ± 6	-0.6
16	6	-49 ± 8	6.0 ± 0.1	-26 ± 3	-186 ± 28	12 ± 4	0.9
Average cluster size ± SD = 24.9 ± 39.0							

HADDOCK: HADDOCK score in a.u. (arbitrary units); **RMSD**: Root-mean-square deviation from the overall lowest-energy structure; **vdW**: van der Waals; **Z**: Z-Score indicates how many SD from the average the cluster is located in terms of score (negative values indicate stronger simulation). Significant conformational clusters are highlighted in grey.

Table S3. Interacting residues of CopZ1 and CopZ2 with CueR

CueR$_{holo}$ – CopZ1					
Hydrogen bond			**Salt bridges**		
CueR	Distance (Å)	CopZ1	CueR	Distance (Å)	CopZ1
Asp107	1.7	Lys16	Asp107	2.6	Lys16
Glu110	1.7	Lys16	Glu110	4.0	Lys16
His111	2.0	Thr17	Glu110	2.7	Lys16
Gln113	2.2	Cys11	Asp125	2.8	Arg20
Gln113	2.2	Cys14	Asp125	3.6	Arg20
Arg117	2.0	Tyr60	Asp125	3.0	Arg20
Lys124	1.7	Glu57	Asp125	3.4	Arg20
Asp125	1.9	Arg20			
Asp125	2.2	Arg20			
Cys131	3.0	Glu57			

CueR$_{holo}$ – CopZ2					
Hydrogen bond			**Salt bridges**		
CueR	Distance (Å)	CopZ2	CueR	Distance (Å)	CopZ2
Glu110	2.2	Cys11	Lys124	3.8	Glu58
Arg117	2.1	Gly8	Lys124	2.6	Glu58
Arg117	2.4	Thr10			
Arg117	1.8	Thr10			
Asp119	1.7	Tyr60			
Lys124	1.6	Glu58			
Cys131	1.7	Glu58			

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