Electronic Supplementary Material (ESI) for Metallomics.

For *Metallomics*

Supporting Information

The S2 Cu(I) site in CupA from *Streptococcus pneumoniae* is required for cellular copper resistance

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This file contains **Supporting Tables S1-S2** and **Supporting Figures S1-S6.**

Supporting Table S1. Bacterial strains used in this study

***S. pneumoniae* strains**			
Strain number	Genotype (description)[a]	Antibiotic resistance[b]	Reference or source
K643	D39 Δ*cps* Δ*copA*:: P_c-[*kan*^{R}-*rpsL*$^+$] (IU1945 transformed with Δ*copA* :: P_c-[*kan*^{R}-*rpsL*$^+$] amplicon)	Str^S Kan^R	[1]
K645	D39 Δ*cps* Δ*cupA*:: P_c-[*kan*^{R}-*rpsL*$^+$] (IU1945 transformed with Δ*cupA* :: P_c-[*kan*^{R}-*rpsL*$^+$] amplicon)	Str^S Kan^R	[1]
IU1690	D39 (Single colony isolate of serotype 2 strain encapsulated D39 NCTC 7466)	None	[2]
IU1781	D39 *rpsL1* (IU1690 transformed with *pulA-rpsL1-rpsG-fusA* amplicon)	Str^R	[3]
IU1945	D39 Δ*cps*	None	[2]
IU5921	D39 *rpsL1* Δ*cupA*:: P_c-[*kan*^{R}-*rpsL*$^+$] (IU1781 transformed with Δ*cupA* :: P_c-[*kan*^{R}-*rpsL*$^+$] amplicon from K645)	Str^S Kan^R	[1]
IU5923	D39 *rpsL1* Δ*copA*:: P_c-[*kan*^{R}-*rpsL*$^+$] (IU1781 transformed with Δ*copA* :: P_c-[*kan*^{R}-*rpsL*$^+$] amplicon from K643)	Str^S Kan^R	[1]
IU5971	D39 *rpsL1* Δ*cupA* (IU5921 transformed with Δ*cupA* amplicon)	Str^R	[1]
IU5975	D39 *rpsL1* Δ*copA* (IU5923 transformed with Δ*copA* amplicon)	Str^R	[1]
IU6041	D39 *rpsL1 cupA*-(C)-FLAG (IU5921 transformed with *cupA*-(C)-FLAG amplicon)	Str^R	[1]
IU6084	D39 *rpsL1 cupA* (Δ(2-28)) (IU5921 transformed with *cupA* Δ(2-28) amplicon)	Str^R	[1]
IU6242	D39 *rpsL1 copA* (C49S) (IU5923 transformed with *copA* (C49S) amplicon)	Str^R	This study
IU6458	D39 *rpsL1 copA* (C49S M172A E216A D347A) ≡ *copA* MBS (IU5924 transformed with *copA* MBS fusion amplicon)	Str^R	This study
IU6585	D39 *rpsL1 cupA* (M113A; M115A) ≡ *cupA* (2A) (IU5921 transformed with *cupA* (2A) amplicon)	Str^R	[1]
IU6618	D39 *rpsL1 copA* (K155E K166E K167E) (IU5924 transformed with *copA* P Helix charge revertant fusion amplicon	Str^R	This study
IU7391	D39 *rpsL1 copA* (C49S K155E K166E K167E) (IU5923 transformed with *copA* C49S K155E K166E K167E fusion amplicon)	Str^R	This study
IU7487	D39 *rpsL1* $cupA_{(aa28\text{-}123)}$::$copZ_{Bsu\ (aa2\text{-}69)}$-(C)-FLAG (IU5921 *cupA::kanrpsL1* transformed with $cupA_{(aa1\text{-}27)}$-$copZ_{Bsu(NC\_000964\ aa2\text{-}69)}$-FLAG fusion amplicon)	Str^R	This study
IU7620	D39 *rpsL1 cupA* C111S (IU5921 D39 *rpsL1* 1781 Δ*cupA*::*kanrpsL* transformed with *cupA* C111S fusion amplicon)	Str^R	This study

IU9405	D39 *rpsL1 cupA* C111S-(C)-FLAG (IU5921 D39 *rpsL1* 1781 Δ*cupA*::*kanrpsL* transformed with *cupA* C111S-(C)-FLAG fusion amplicon)	Str^R	This study
IU9584	D39 *rpsL1 cupA* H117A-FLAG (IU5921 D39 *rpsL1 cupA::kanrpsL1* transformed with *cupA* H117A-FLAG fusion amplicon)	Str^R	This study
IU10134	D39 *rpsL1 copA* ΔMBD-FLAG (Δaa2-98) (IU5923 D39 *rpsL1 copA::kanrpsL1* transformed with *copA* ΔMBD-FLAG fusion amplicon)	Str^R	This study
IU10148	D39 *rpsL1 copA* WF-to-AA-FLAG (W162A/F165A) (IU5923 D39 *rpsL1 copA::kanrpsL1* transformed with *copA* WF-to-AA-FLAG fusion amplicon)	Str^R	This study
IU10316	D39 *rpsL1 copA* WF-to-LL-FLAG (W162L/F165L) (IU5923 D39 *rpsL1 copA::kanrpsL1* transformed with *copA* WF-to-LL-FLAG fusion amplicon)	Str^R	This study

[a]Primers used to synthesize fusion amplicons for this study are listed in Supplemental Table S2. All FLAG-tagged fusions ((C)-FLAG) were made to the carboxyl end (C) of reading frames. The amino acid sequence for the FLAG epitope is DYKDDDDK[3]. aa = amino acids. [b]Antibiotic resistance markers: Kan^R, kanamycin; Str^R, streptomycin, Concentrations of antibiotics used for *S. pneumoniae* strains: 250 µg Str per mL (Sigma S6501) and 250 µg Kan per mL (Sigma K0254).

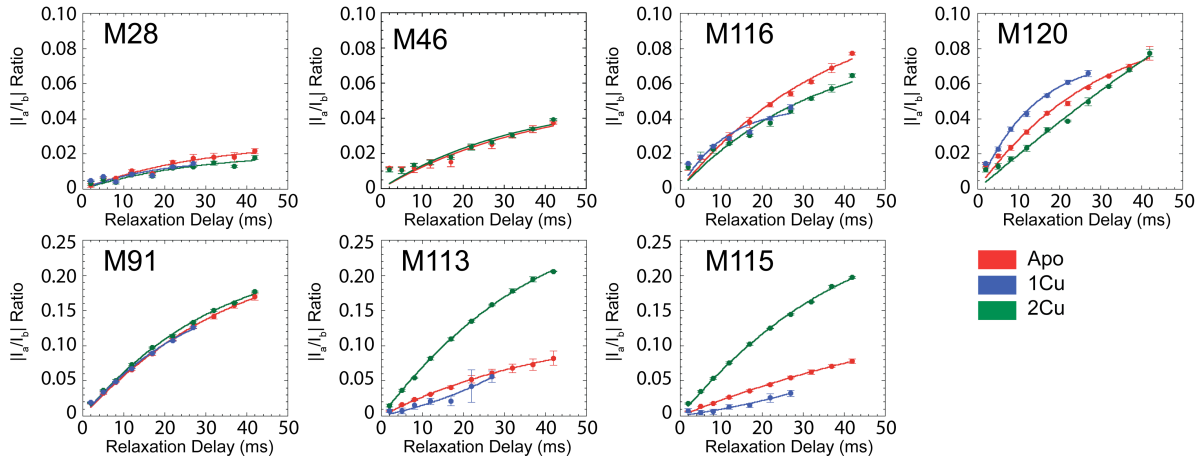
Supporting Table S2. Oligonucleotide primers used in this study

Primer	Sequence (5' to 3')[a]	Template[b]	Amplicon Product
For construction of IU6242 (*copA* (C49S))			
P1360	TTCGACTGGTCCAAGTCAACGGTTCAGACA	D39	5' flanking fragment
TT331	CTTCTTCAAACAGAATTTCCTTATAAGAGTTTGAAG GAGTAGCACGATGAAAG		
TT332	CGTGCTACTCCTTCAAACTCTTATAAGGAAATTCTG TTTGAAGAAGAAGGTAT	D39	3' flanking fragment with *copA*-FLAG[C]
P1361	TCGTTCAAAGCAGGAGCGATGAATGAGCGT		
For construction of IU6458 (*copA* (C49S M172A E216A D347A)) ≡ *copA* MBS			
P1360	TTCGACTGGTCCAAGTCAACGGTTCA	IU 6242 (CopA: C49S)	5' flanking fragment
KB29	CCAGGGCAACCAAGGTATCAGCGTTGGCATTGTGC TTCTTAAAACTGG		
KB30	TTTTAAGAAGCACAATGCCAACGCTGATACCTTGG TTGCCCTGGGAAC	IU 6242 (CopA: C49S)	MBS mid 1
KB31	TGAGACGTATTTTTTCTCATTTTTTCAGCAAAAACT GCTCCCAAAAGAACG		
KB32	GTTCTTTTGGGAGCAGTTTTTGCTGAAAAAATGAGA AAAAATACGTCTCAAGC	IU 6242 (CopA: C49S)	MBS mid 2
KB33	GGACAAAAATCCCTGAAATCTTAGCTGTCAAGTCC TGAATCGGCG		

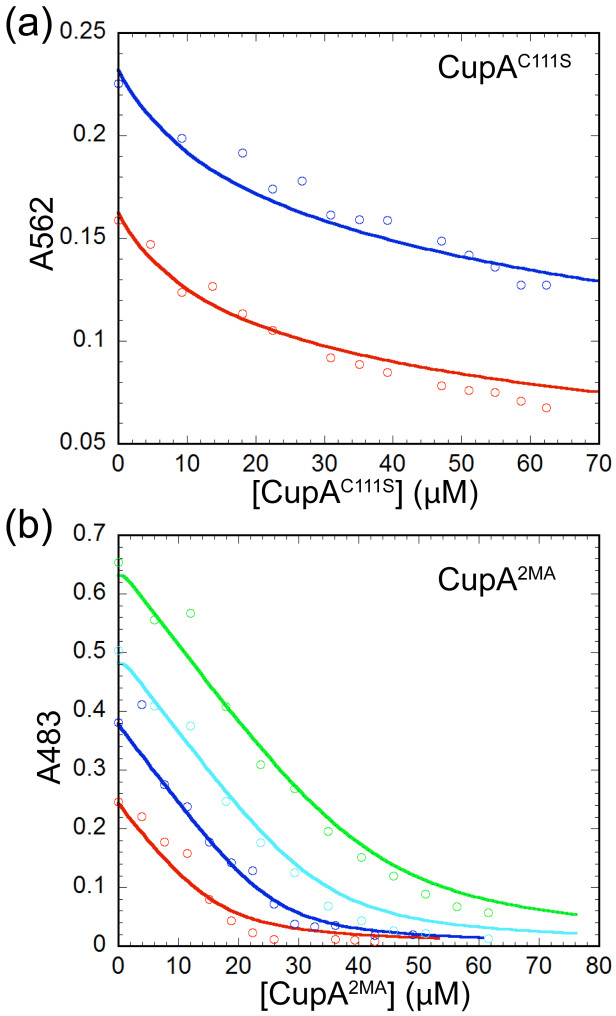
KB34	GCCGATTCAGGACTTGACAGCTAAGATTTCAGGGATTTTTGTCCCAG	IU 6242 (CopA: C49S)	3' flanking fragment
P1361	TCGTTCAAAGCAGGAGCGATGAATGA		
For construction of IU6618 (*copA* C49S K155E K166E K167E) = *copA* P-helix			
P1360	TTCGACTGGTCCAAGTCAACGGTTCA	D39 genomic	5' flanking fragment
KB69	CCCAAGCACTCTGGATATATGGCTCACCTGCAACTAACATAATAGGCGTTG		
KB70	CGCCTATTATGTTAGTTGCAGGTGAGCCATATATCCAGAGTGCTTGGGC	D39 genomic	Mid fragment
KB71	AGGTATCCATGTTGGCATTGTGCTCCTCAAAACTGGCCCAAGCACTCTGG		
KB72	GAGTGCTTGGGCCAGTTTTGAGGAGCACAATGCCAACATGGATACCTTGG	D39 genomic	3' flanking fragment
P1361	TCGTTCAAAGCAGGAGCGATGAATGA		
For construction of IU7391 (*copA* C49S K155E K166E K167E)			
P1356	AGTCCCTGCAATGGTCAAAGCACCGG	IU6618 copA K155E K166E K167E	5' flanking fragment
TT331	CTTCTTCAAACAGAATTTCCTTATAAGAGTTTGAAGGAGTAGCACGATGAAAG		
TT332	CGTGCTACTCCTTCAAACTCTTATAAGGAAATTCTGTTTGAAGAAGAAGGTAT		3' flanking fragment
P1361	TCGTTCAAAGCAGGAGCGATGAATGA		
For construction of IU7487 (*cupA*$_{(aa28-123)}$::*copZ*$_{Bsu\ (aa2-69)}$-(C)-FLAG)			
P1356	AGTCCCTGCAATGGTCAAAGCACCGG	IU6041 cupA-FLAG	5' flanking fragment
KB208	TCAACTTGCAATGTTTTTTGTTCTTTTTCAGGCTTTTTGAAAAACCAAA		
KB207	TTTCAAAAAGCCTGAAAAAGAACAAAAAACATTGCAAGTTGAAGGAA	Plasmid with copZ$_{Bsu}$	Mid fragment
KB209	TATTTATCATCATCATCTTTATAATCCTTGGCTACGTCATAGCCCTGATCT		
KB210	AGGGCTATGACGTAGCCAAGGATTATAAAGATGATGATGATAAATAGGTGGAGA	IU6041 cupA-FLAG	3' flanking fragment
P1357	AGGTCGCCTACCTTGACTTGTTCCAA		
For construction of IU7620 (*cupA* C111S)			
P1356	AGTCCCTGCAATGGTCAAAGCACCGG	D39	5' flanking fragment
KB271	CATACCAGAAGCAAAGCTAAACTCTCCAG		
KB270	CTTTGCTTCTGGTATGAACATGATGCAC		3' flanking fragment
P1357	AGGTCGCCTACCTTGACTTGTTCCAA		
For construction of IU9405 (*cupA* C111S-(C)-FLAG)			
P1356	AGTCCCTGCAATGGTCAAAGCACCGG	IU6041 cupA-FLAG	5' flanking fragment
KB271	CATACCAGAAGCAAAGCTAAACTCTCCAG		
KB270	CTTTGCTTCTGGTATGAACATGATGCAC		3' flanking

P1357	AGGTCGCCTACCTTGACTTGTTCCAA		fragment
For construction of IU9584 (*cupA* H117A-(C)-FLAG)			
P1356	AGTCCCTGCAATGGTCAAAGCACCGG	IU6041 cupA-FLAG	5' flanking fragment
KB358	CTCTACAATCATCTTGCCAGCCATCATGTTCATACC ACAAGCAAAGC		
KB359	CTTGTGGTATGAACATGATGGCTGGCAAGATGATT GTAGAGGATTATAAAGATG		3' flanking fragment
P1357	AGGTCGCCTACCTTGACTTGTTCCAA		
For construction of IU10134 (*copA* ΔMBD-(C)-FLAG)			
P1360	TTCGACTGGTCCAAGTCAACGGTTCA	IU6044 copA-FLAG	5' flanking fragment
KB387	GATTTTCGAGTCTTCATAGTCTCCACCTACTCTACA ATCATCTTGCCGTG		
KB388	GATTTTCGAGTCTTCATAGTCTCCACCTATTTATCA TCATCATCTTTATAATCCTCTAC		3' flanking fragment
P1361	TCGTTCAAAGCAGGAGCGATGAATGA		
For construction of IU10148 (*copA* WF-to-AA-(C)-FLAG)			
P1360	TTCGACTGGTCCAAGTCAACGGTTCA	IU6044 copA-FLAG	5' flanking fragment
KB389	CATGTTGGCATTGTGCTTTTTAGCACTGGCAGCAGC ACTCTGGATATATGGCTTACCTGC		
KB390	AAGCCATATATCCAGAGTGCTGCTGCCAGTGCTAA AAAGCACAATGCCAACATGGATACC		3' flanking fragment
P1361	TCGTTCAAAGCAGGAGCGATGAATGA		
For construction of IU10316 (*copA* WF-to-LL-(C)-FLAG)			
P1360	TTCGACTGGTCCAAGTCAACGGTTCA	IU6044 copA-FLAG	5' flanking fragment
KB391	CATGTTGGCATTGTGCTTTTTAAGACTGGCCAAAGC ACTCTGGATATATGGCTTACCTGC		
KB392	AAGCCATATATCCAGAGTGCTTTGGCCAGTCTTAA AAAGCACAATGCCAACATGGATACC		3' flanking fragment
P1361	TCGTTCAAAGCAGGAGCGATGAATGA		

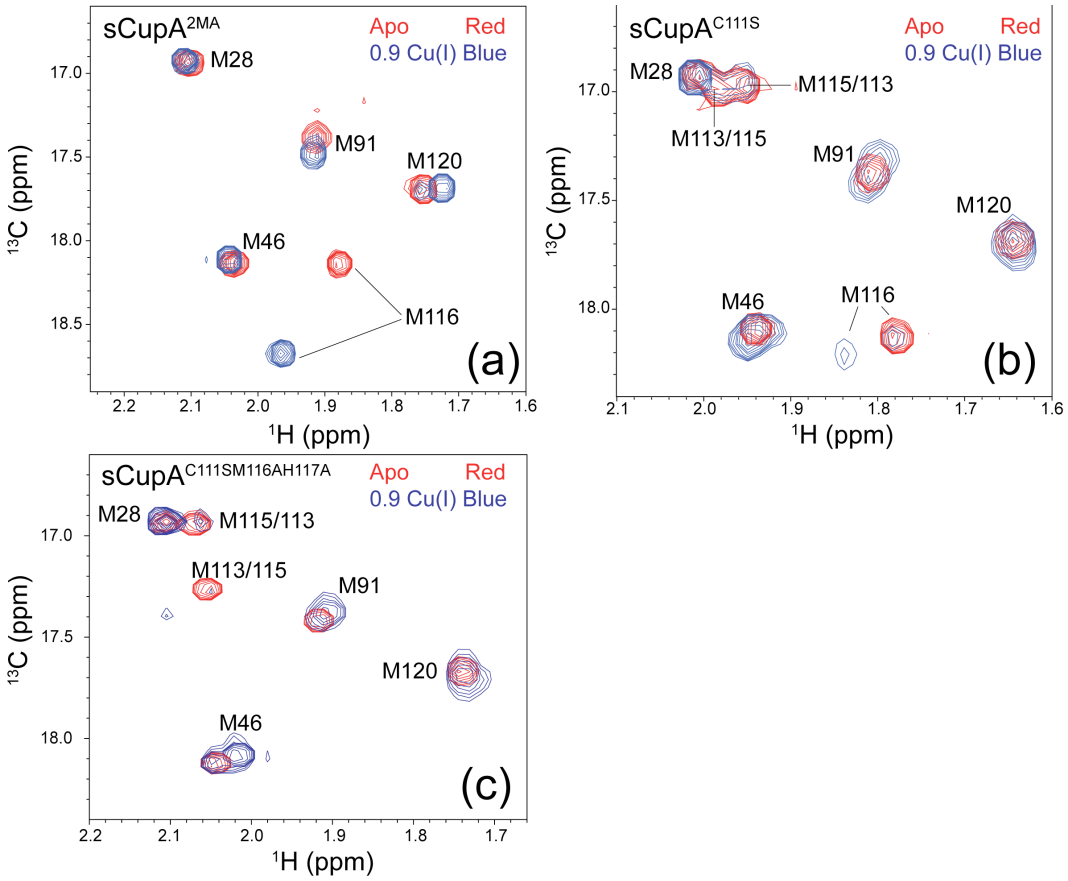
[a]Sequences in italic and bold letters represent FLAG tag. Underlined sequences indicate sequence changes to produce the desired mutation. [b]Genomic DNA of the indicated *S. pneumoniae* strains was used as templates for PCR reactions. aa = amino acids.



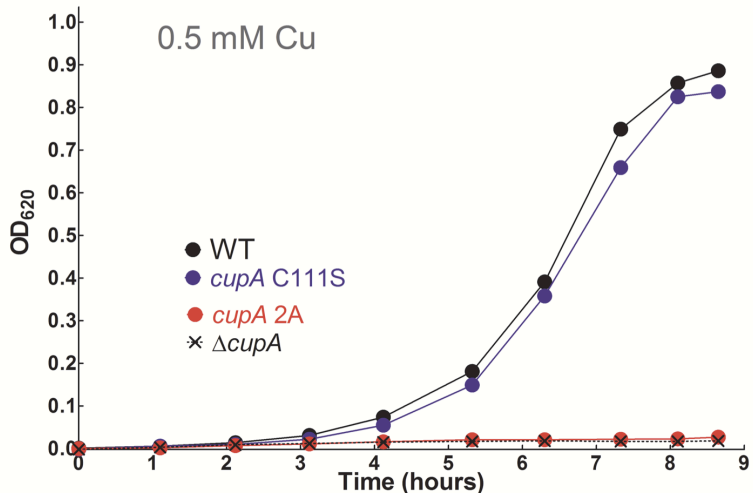
Supporting Figure S1. Build-up curves as a function of intensity ratio (I_a/I_b) (see Methods, main text) used to determine the intramethyl ^{1}H-^{1}H dipole-dipole cross-correlated relaxation rate (η) for each of the seven Met εCH_3 groups in sCupA in each of three ligation states, apo (*red*), 1 Cu bound to the S1 sites (*blue*), and both S1 and S2 sites filled (*green*). η is related to the axial order parameter, S^2_{axis} and the molecular rotational correlation time, τ_c (see **Figure 4**, main text). Data for Met46 in the Cu_1 state were obscured by resonance overlap and are therefore not shown.



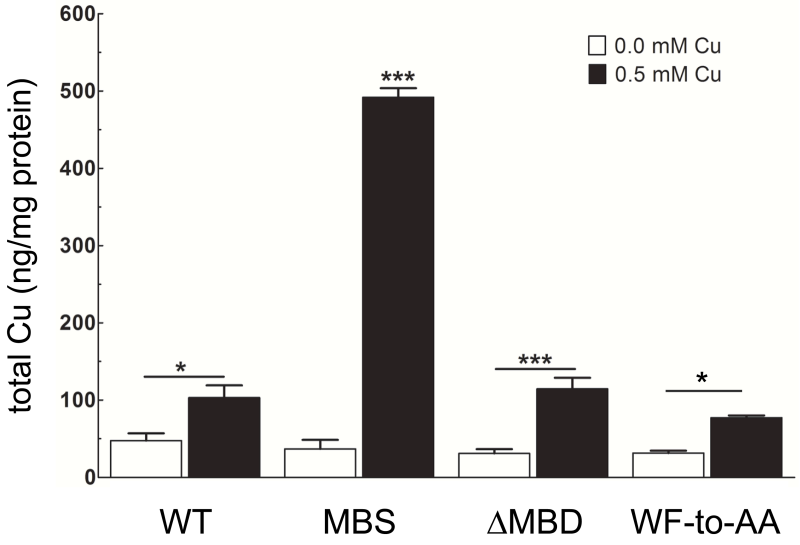
Supporting Figure S2. Cu(I) binding affinity titrations obtained for (A) C111S sCupA and (B) sCupA^{2MA}. (A) For C111S sCupA, 21 or 30 μM CuCl was mixed with 50 μM (*red*) or 70 μM (*blue*) BCA and protein was titrated into this mixture. The absorbance of BCA:Cu(I) complex was monitored at 562 nm. (B) For CupA^{2MA}, 18, 28, 37 or 48 μM CuCl was mixed with 44 μM (*red*), 60 μM (*blue*), 71 μM (*cyan*) or 93 μM (*green*) BCS and protein was titrated into this mixture. The absorbance of BCS:Cu(I) complex was monitored at 483 nm. Lines in each panel represent the results of a global fit of a 1:1 binding model as described previously.[1]



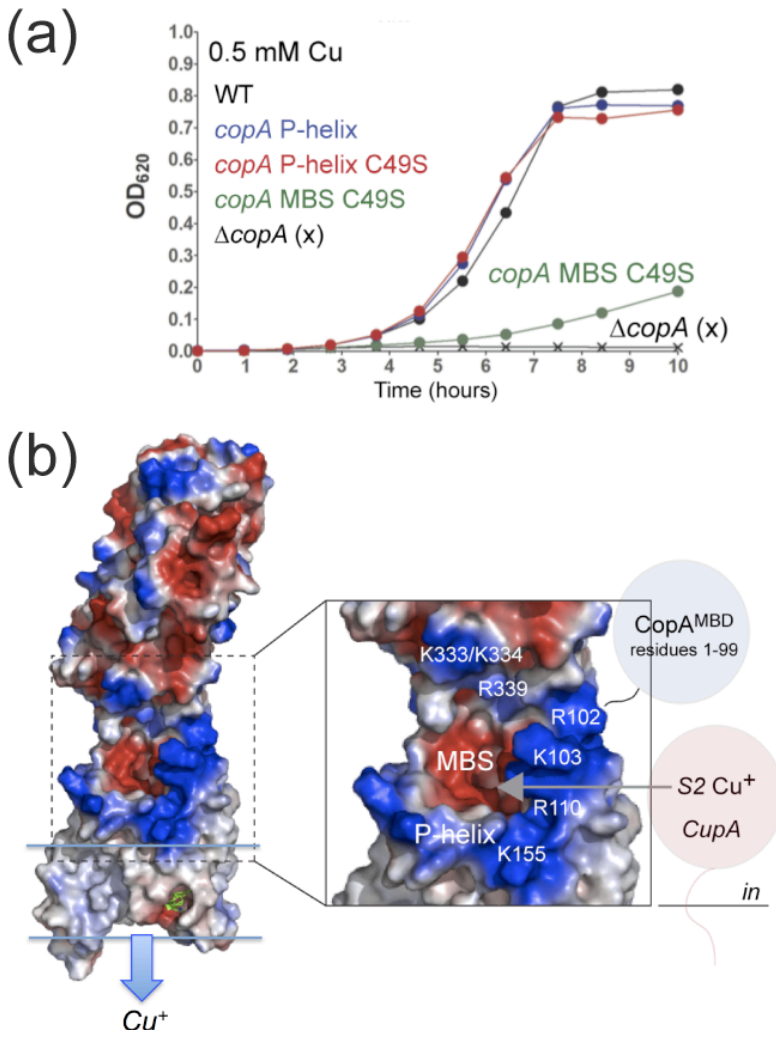
Supporting Figure S3. $^{1}H,^{13}C$ HSQC spectra showing 1H-13C crosspeaks of the εCH_3 Met methyl groups recorded for sCupA^{2MA} (A), C111S sCupA (B) and the triple substitution mutant C111S/M116A/H117A (C) in the absence (apo, *red* crosspeaks) and presence (*blue* crosspeaks) of 0.9 mol equivalents Cu(I). These data reveal that all mutant proteins are folded and that the C111S substitution introduces considerable chemical exchange broadening into the S2 Cu-binding site region that is not quenched by Ala substitution of immediately adjacent, potential non-native Cu(I)-ligating residues M116 and H117. The methyl groups of S2 Cu(I)-ligating M113 and M115 are significantly broadened in panels B and C when Cu(I) is added. No εCH_3 Met resonances outside of this spectral window were observed, in contrast to the wild-type protein.[1]



Supporting Figure S4. Microaerophilic growth rate analysis of *cupA*^{C111S} and *cupA*^{2MA} strains compared to parent isogenic Δ*cupA* and wild-type (WT) strains in the presence of 0.5 mM Cu. A *cupA*^{2MA} mutant strain (*red* symbols) exhibits a growth phenotype indistinguishable from a Δ*cupA* strain (X), with while a *cupA*^{C111S} mutant (*blue* symbols) grows like WT (*black* filled circles) when 0.5 mM Cu is added to the BHI growth medium at *t*=0.



Supporting Figure S5. Total [Cu] (ng Cu/mg protein) in the indicated strains as measured by ICP-MS in the absence (*open* bars) or presence (*filled* bars) of 0.5 mM Cu added to cultures at OD_{620}≈0.03. These data represent mean ± S.E. from three biological replicates. Statistical significance was determined using one-way ANOVA with Tukey post-test where ***p<0.0001; *, p<0.05. WT, wild-type strain; MBS, M172A/E216A/D347A *copA* strain; ΔMBD, *copA* lacking the N-terminal metal binding domain (MBD; residues 1-99), WF-to-AA, W162A/F165A *copA* strain. The MBS strain also carries a $copA^{C49S}$ substitution which has no detectable growth phenotype.[1]



Supporting Figure S6. (A) Representative growth curves (BHI + 0.5 mM Cu) obtained for wild-type (WT) (*black* filled circles), a *copA* strain expressing a triple mutant (K155E/K166E/K167E) CopA (P-helix) (*blue* filled circles), a *copA* strain expressing a quadruple mutant (C49S/K155E/K166E/K167E) CopA (P-helix C49S) (*red* filled circles), a *copA* strain expressing the triple MBS mutant in a C49S background (MBS C49S) (*green* filled circles), compared to a Δ*copA* strain (x) which fails to grow under these conditions. The $copA^{C49S}$ strain (not shown) grows like WT under these conditions.[1] (B) Space filling model of the MBS region in a homology model of *S. pneumoniae* CopA threaded through *Lp* apo-CopA[4] with basic residues surrounding the MBS and the platform helix (P-helix) highlighted. Surface electrostatic charge is represented by *blue* (positive potential) and *red* (negative potential). The approximate placement of the $CopA^{MBD}$ of known structure[1] within the CopA model is indicated; the work presented here is consistent with a model in which the S2 Cu(I) of CupA delivers Cu(I) directly to the MBS as indicated by the *grey* arrow (*right*). The direction of the Cu transport is indicated by the *light blue* arrow.

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