Supporting Information

Macomber and Imlay 10.1073/pnas.0812808106

SI Methods

Strain Constructions. Mutations were introduced into strains by P1 transduction and selected with the appropriate antibiotic. λ(*copA-lacZ*) lysogens in W3110 (WT) were selected for kanamycin resistance. λ(*copA-lacZ*) lysogens in LEM33 (*copA*::*kan cueO cusCFBA*) were picked from turbid plaques. The lysogen copy number was tested by PCR analysis (1).

In Vivo Repair of Fumarase A. W3110 (WT) were grown aerobically in glucose/alanine medium to an OD_{500} of ≈0.1 and then exposed to 80 μM copper for 1 h. Chloramphenicol (150 μg/mL) was then added, and cells were centrifuged, washed twice, and resuspended, all in medium containing chloramphenicol. The resuspended culture was incubated at 37 °C aerobically, and at intervals aliquots were removed for assay of fumarase activity.

EPR Analysis. Fumarase A (4 μM) was inactivated to 15% of its initial activity by a 3-min exposure to 30 μM Cu(I). EPR signals were measured with a Varian Century E-112 X-band spectrophotometer equipped with a Varian TE102 cavity and temperature controller. The spectra were measured at temperatures of 5, 8, and 15 K with the following settings: field center, 2,500 G; field sweep, 5,000 G; modulation frequency, 100 kHz; modulation amplitude, 8 G; time constant, 0.032; receiver gain, 12,500; and power, 1 mW. In control experiments, 4 μM fumarase A was exposed to 10 μM H_2O_2 for 3 min, which led to a 99% inactivation of the enzyme and a large $[^3Fe\text{-}^4S]^+$ signal. The subsequent addition of 100 μM dithionite, but not of ascorbate (350 μM), silenced the EPR signal by reducing the cluster to $[^3Fe\text{-}^4S]^0$. Unfortunately, the further addition of 1 mM hydrogen peroxide failed to regenerate the $[^3Fe\text{-}^4S]^+$ EPR signal (data not shown), and addition of 1 mM ferricyanide regenerated only a small fraction of the original signal (data not shown). Therefore, we were unable to definitively test for the presence of a $[^3Fe\text{-}^4S]^0$ cluster in copper-treated enzyme by this method.

Succinate Dehydratase Assay. Cells were lysed using a French press. Membrane vesicles were isolated by centrifugation at 100,000 × *g* for 2 h at 4 °C. Succinate dehydrogenase activity was determined by measuring succinate-dependent (4 mM) oxygen consumption. The oxygen concentration was measured using an oxygen-electrode (model 10 digital oxygen system; Micrometrix).

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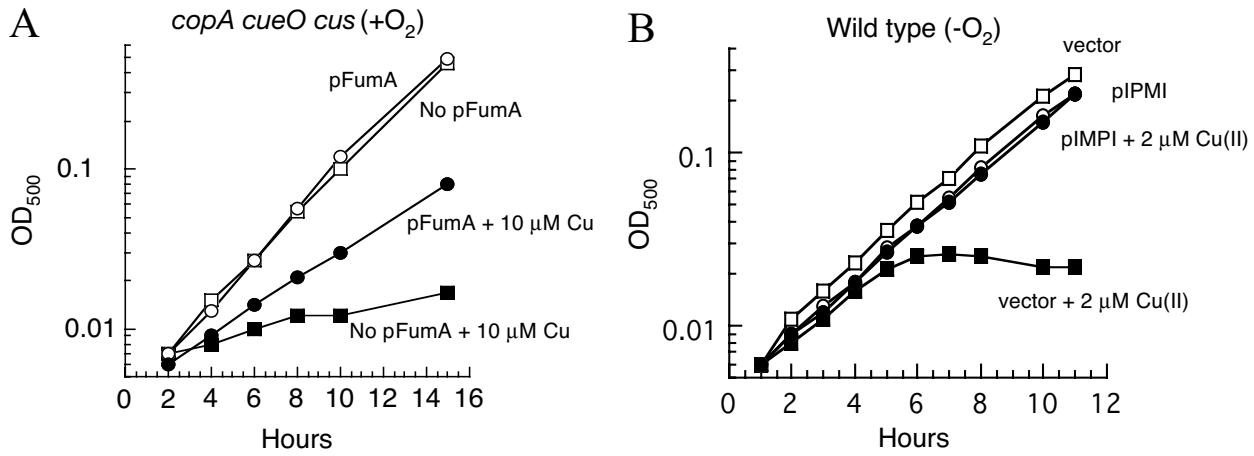

Fig. S1. The overexpression of dehydratases restores growth in the presence of copper. (*A*) Lem33 (*copA cueO cusCFBA*) cells containing pGS97 (fumarase A expressed from its own promoter; circles) or cells with normal levels of fumarase A (squares) were grown aerobically at 37 °C in succinate/ILV medium and supplemented with 0 μM (open symbols) or 10 μM $CuSO_4$ (closed symbols). (*B*) W3110 (WT) cells containing pLEUCD2 (overexpressing IPMI; circles) or the vector alone (squares) were grown anaerobically at 37 °C in lactose/alanine medium and supplemented with 0 μM (open symbols) or 2 μM $CuSO_4$ (closed symbols). The data are representative of 3 independent experiments.

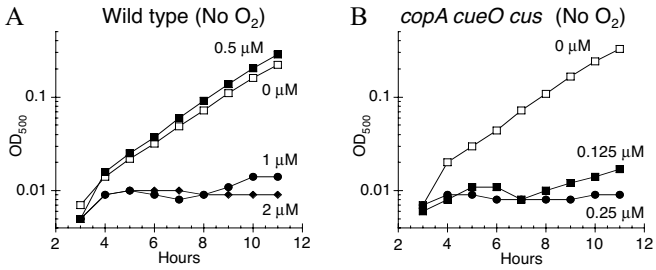

Fig. S2. Copper is highly toxic in the absence of oxygen. *E. coli* cultures were grown anaerobically at 37 °C in glucose medium supplemented with $CuSO_4$. (*A*) W3110 (WT) cultures were challenged with 0 μM (open square), 0.5 μM (closed squares), 1 μM (closed circles), or 2 μM $CuSO_4$ (closed diamonds). (*B*) LEM33 (*copA cueO cusCFBA*) cultures were challenged with 0 μM (open squares), 0.125 μM (closed squares), or 0.25 μM $CuSO_4$ (closed circles). The data are representative of 3 independent experiments.

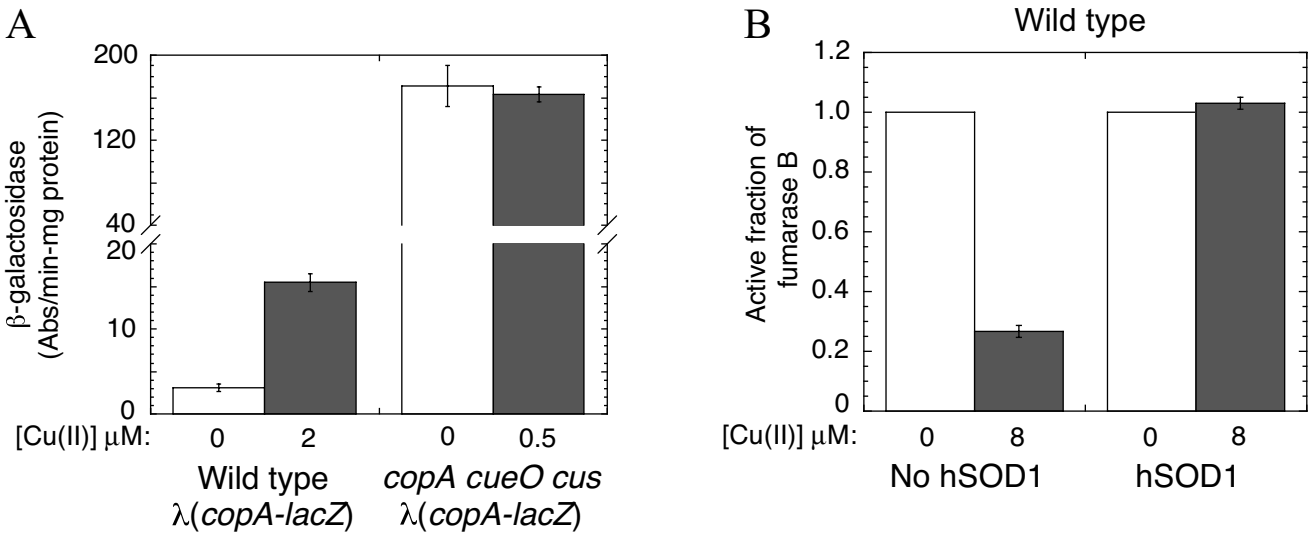

Fig. S3. Excess copper is sensed by cytoplasmic CueR, and a heterologous cytoplasmic copper-binding protein protects dehydratases from copper-dependent damage. (*A*) LEM179 [λ(*copA-lacZ*)] and LEM180 [*copA cueO cus* λ(*copA-lacZ*)] were grown anaerobically in glucose/ILV medium at 37 °C to an OD_{500} ≈0.1. Cultures were then challenged with (gray bars) or without copper (white bars) for 1 h. (*B*) W3110 (WT) with or without pNco5A (human Cu, ZnSOD, or hSOD1) was grown anaerobically in glucose/alanine medium to an OD_{500} of ≈0.1, and then 1 mM IPTG was added to induce hSOD1 expression for 1 h. Cultures were then challenged with 0 μM (open bars) or 8 μM $CuSO_4$ (gray bars) for 30 min. The data are the averages of 3 independent experiments, and the error bars represent SD.

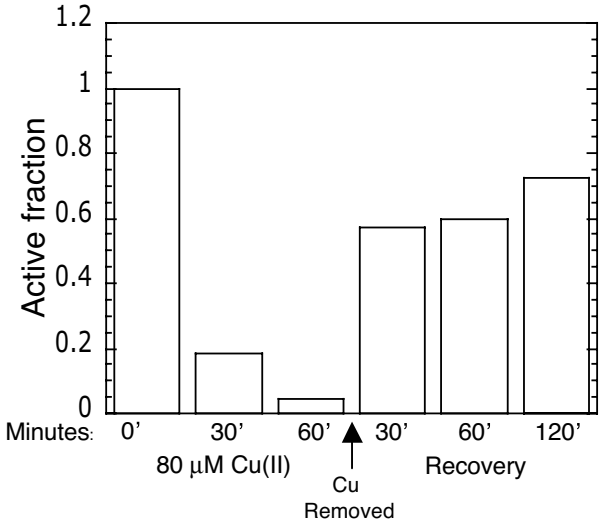

Fig. S4. Copper-damaged fumarase is repaired in vivo. W3110 (WT) cells were grown aerobically at 37 °C in glucose/alanine medium to an OD_{500} of ≈0.1 and then challenged with 80 μM $CuSO_4$. After 60 min, chloramphenicol (150 μg/mL) was added to stop protein synthesis, and the copper was removed by washing. Cells were resuspended in medium supplemented with chloramphenicol and incubated aerobically. The fumarase activities were measured as described. The data are representative of 3 independent experiments.

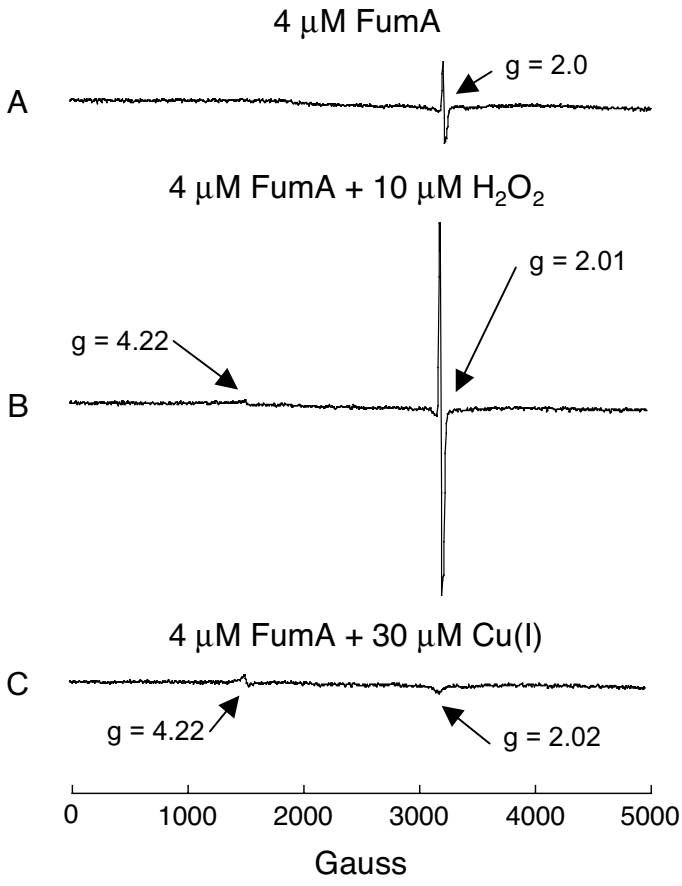

Fig. S5. Copper-damaged fumarase A did not present an EPR spectrum. (*A*) Fumarase A (4 μM) in 50 mM Tris-HCl/10 mM $MgCl_2$, pH 8, contains a small $[^3Fe\text{-}^4S]^+$ iron-sulfur cluster signal at g = 2.0. (*B*) Fumarase A (4 μM) treated anaerobically at 27 °C with 10 μM H_2O_2 for 3 min contains a $[^3Fe\text{-}^4S]^+$ iron-sulfur cluster signal at g = 2.01 and a weak free-iron signal at g = 4.22. (*C*) Fumarase A (4 μM) treated anaerobically at 27 °C with 30 μM Cu(I) for 3 min contains a weak signal at g = 2.02 and a free-iron signal at g = 4.22. The data were collected at a temperature of 8 K and are representative of 3 independent experiments.

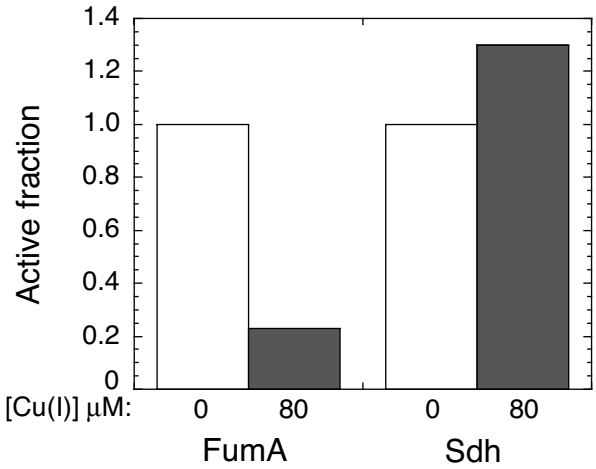

Fig. S6. Copper does not damage succinate dehydrogenase. Fumarase A was mixed with membrane vesicles containing succinate dehydrogenase (*Sdh*), and the enzyme mixture was then challenged anaerobically with 0 μM (open bars) or 80 μM Cu(I) (gray bars) for 3 min at 27 °C. Neocuproine (100 μM) was added to the samples to chelate copper before measuring enzyme activities. The data are representative of 3 independent experiments.

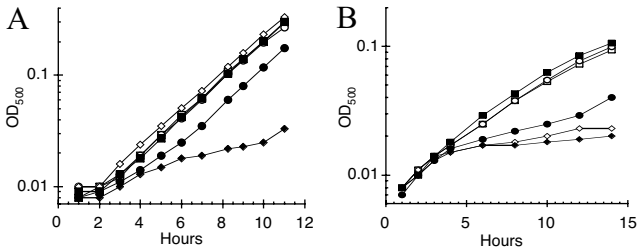

Fig. S7. Glutathione and the Suf iron-sulfur cluster assembly system contribute to copper resistance in vivo. (*A*) W3110 (WT; open symbols) and LEM122 (*gshA*; closed symbols) cells were grown anaerobically at 37 °C in glucose/alanine medium in the presence of 0 μM (squares), 0.5 μM (circles), or 1 μM $CuSO_4$ (diamonds). (*B*) W3110 (WT) (open symbols) and LEM146 (*sufABCDSE*; closed symbols) cells were grown anaerobically at 37 °C in glycerol/nitrate/ILV medium in the presence of 0 μM (squares), 1 μM (circles), or 2 μM $CuSO_4$ (diamonds). The data are representative of 3 independent experiments.

Table S1. Strains

Strains, plasmids, and phage	Genotype	Source (SI ref. no.)
Strains		
AB1157	F$^-$ λ$^-$ *thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY1 galK2 rpsL supE44 ara-14 xyl-15 mtl-1 tsx-33*	2
CSH7	*lacY rpsL thi-1*	2
JI360	As MG1655 plus *katE12*::*Tn10*	3
JI364	As MG1655 plus Δ(*katG17*::Tn10)*1*	3
JS126	As CSH7 plus Δ*ahpCF1*::*cat*	Lab strain
LEM33	As W3110 plus *copA*::*kan* Δ*cueO* Δ*cusCFBA*	4
LEM122	As W3110 plus *gshA*::*kan srlC300*::Tn10	4
LEM133	As W3110 plus *copA*::*kan* Δ*cueO* Δ*cusCFBA* Δ*ahpCF1*::*cat* Δ(*katG17*::Tn10)*1* Δ(*katE12*::Tn10)*1*	Present study
LEM146	As W3110 plus Δ*sufABCDSE*::*kan*~*zdi57*::Tn10	Present study
LEM179	As W3110 plus λ(*copA-lacZ*)	Present study
LEM180	As W3110 plus λ(*copA-lacZ*) *copA*::*kan* Δ*cueO* Δ*cusCFBA*	Present study
MG1655	F$^-$ λ$^-$ WT	2
OD500	As MG1655 plus Δ*sufABCDSE19*::*kan*~*zdi57*::Tn10	Lab strain
PD401	As AB1157 plus *srlC300*::Tn10 *cysJ93*	Lab strain
SJ50	As MG1655 plus Δ(*katG17*::Tn10)*1* Δ(*katE12*::Tn10)*1* Δ(*ahpC-ahpF'*) *kan*::*'ahpF* Δ*fumAC* Δ*fumB*::*cm*	5
W3110	F$^-$ λ$^-$ IN(rrnD-rrnE)1 rph-1 WT	2
Plasmids		
pBR322	Cloning vector	6, 7
pCKR101	P_{lac}-lacI^q P_{tac} polylinker Am^r	8
pFumA	As pCKR101 plus *fumA*	5
pGS97	As pBR322 plus *fumA* under its own promoter	9
pJRS102	As pBR322 plus *cysJIH cysG*	10
pLEUCD2	As pWKS30 plus *leuCD* insert	5
pNco5A	As pBR322 plus pcHSOD1	11
pWKS30	P_{lac} polylinker Am^r	12
Phage		
λ(*copA-lacZ*)	(*copA-lacZ*) kan^r	13