Supporting Information

Antimicrobial action of copper is amplified *via* inhibition of heme biosynthesis

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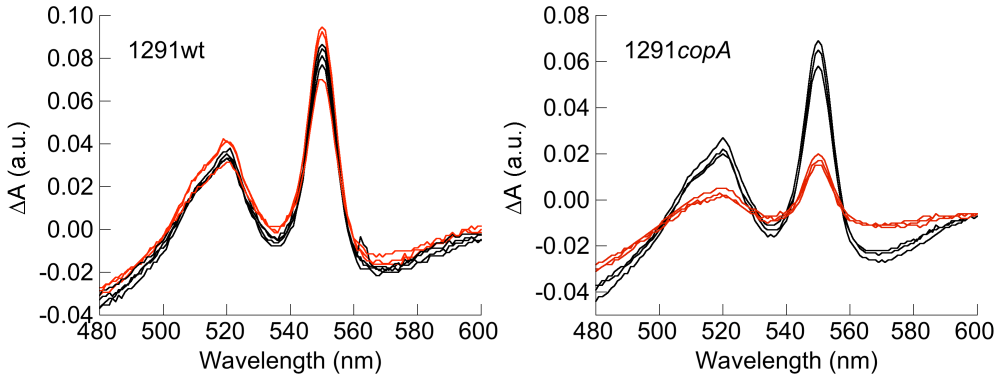
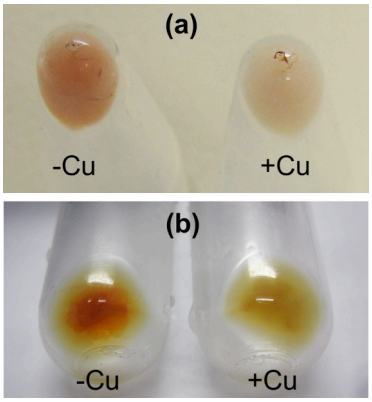
Supplementary Figure 1. Cu treatment led to loss of heme during growth in aerated liquid cultures. Difference pyridine hemochrome spectra (reduced *minus* oxidised) of whole gonococci after growth in the absence (black lines) and presence (red lines) of 30 μM $Cu(NO_3)_2$. Three independent replicates are shown for each sample.



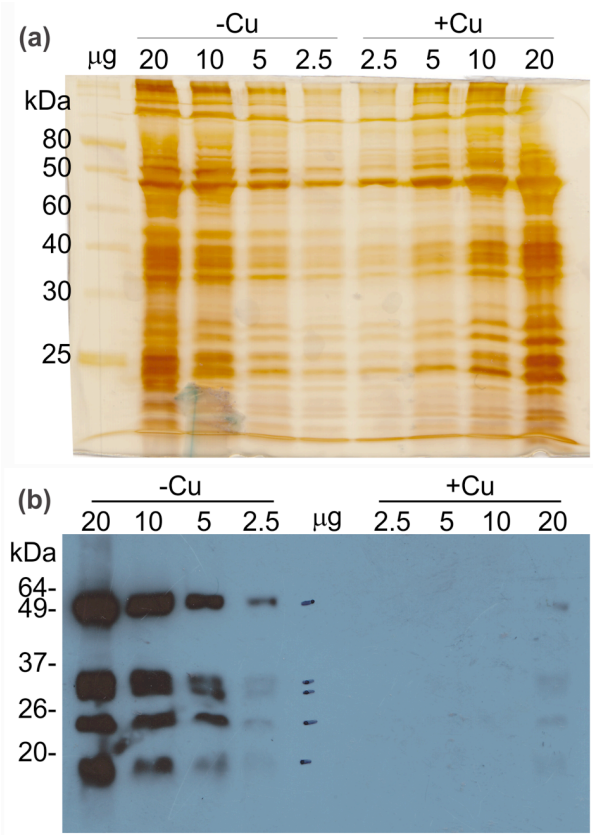
Supplementary Figure 2. Cu treatment led to bleaching of the characteristic orange-red pigment of *N. gonorrhoeae*. 1291*copA* was grown in aerated liquid cultures for 5 hours in the absence (-Cu) and presence (+Cu) of 30 μM $Cu(NO_3)_2$. a) Whole gonococcal pellets were harvested by centrifugation and washed 3 times with PBS before the picture was taken under ambient light conditions. b) Cytoplasmic membrane proteins were isolated as described below and the picture was taken under ambient light conditions.



Isolation of cytoplasmic membranes

Gonococci were grown in large aerated broth cultures (500 mL in 2.5 L flasks) with and without 30 μM $Cu(NO_3)_2$ for 5 hours. Cells were collected by centrifugation and washed with PBS (2 x 50 mL). The pellet was resuspended in PBS (15 mL) and disrupted by passage through a French Press cell (3 x 15,000 psi). Insoluble cell debris was removed by low-speed centrifugation (4000x*g*, 30 min, 4 ^{o}C). The supernatant was collected and filtered through a 0.45 μm polyethlynesulfonate filtration units (MilliPore). Cytoplasmic membrane proteins were separated by high-speed centrifugation (150000x*g*, 3 h, 4 ^{o}C), homogenised in PBS (1 mL), and stored at 4 ^{o}C until further analyses. All analyses were performed within 2 days of preparations.

Supplementary Figure 3. Cu treatment led to loss of heme from cytochromes. a) Protein expression profiles of isolated cytoplasmic membranes from 1291*copA* after 5 hours of growth in the absence (-Cu) and presence (+Cu) of 30 μM $Cu(NO_3)_2$. No obvious difference was detected in the banding pattern. b) The gel from panel a) was stained for heme-dependent peroxidase activity as described below. A global loss of the heme cofactor was detected from all cytochrome subunits. (a-b) Each lane contained between 2.5-20 μg of proteins as indicated. Approximate positions of size standards are shown in kDa.

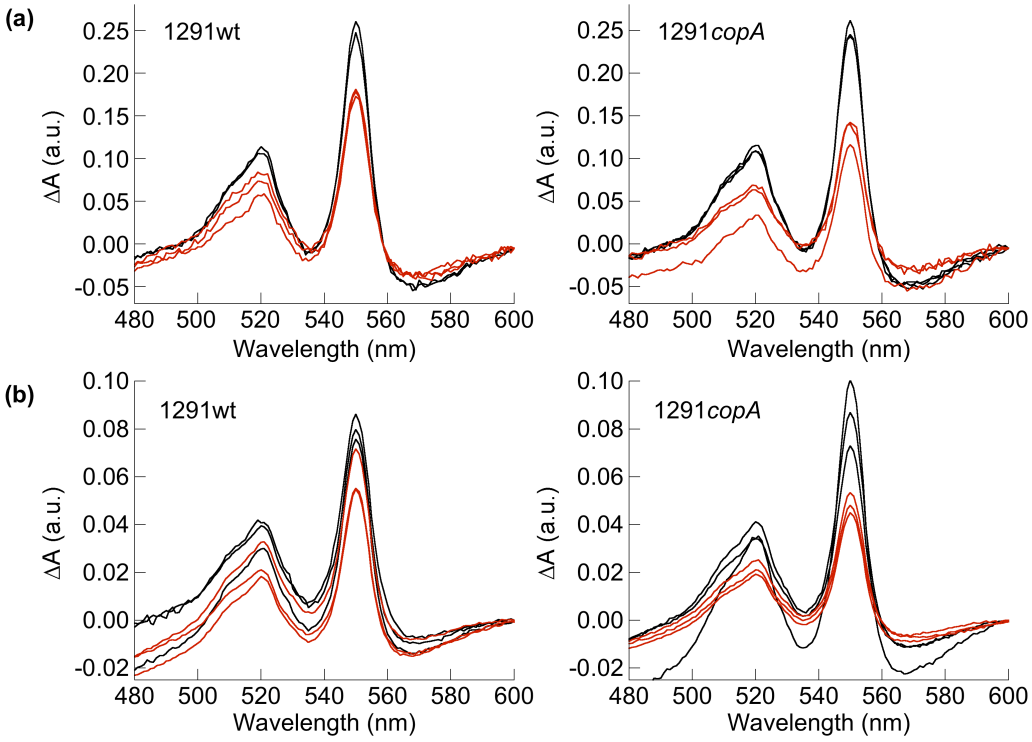


Heme-dependent peroxidase activity stain

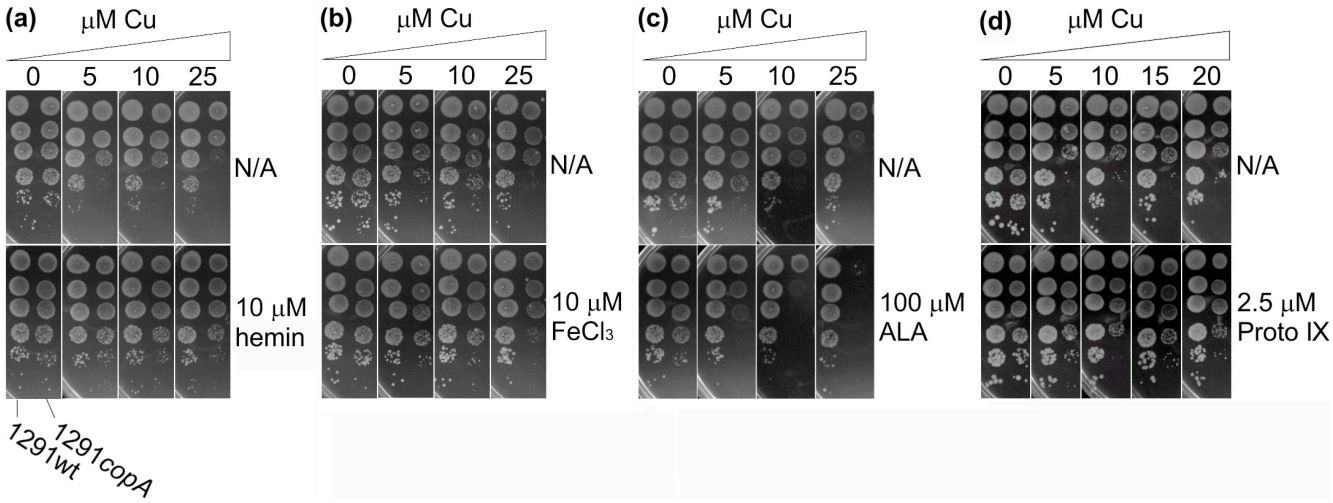
Homogenised cytoplasmic membranes were separated on a 12% SDS PAGE gel under non-reducing conditions (no added DTT). Between 2.5-20 μg of membranes were loaded on each lane as indicated. All electrophoresis steps were performed using standard Tris-Glycine buffer (100 V, 4 ^{o}C). Gels were pre-run for ca. 1 h to remove gel casting contaminants. Proteins were resolved for ca. 3

hours until the dye front reached the bottom of the gel. Gels were developed by silver staining following standard procedures. For heme-dependent peroxidase activity staining, proteins were transferred to a nitrocellulose membrane (20 V, 4 ^{o}C, 16 hours). The membrane was immersed in SuperSignal West Femto® chemiluminescent substrate (Pierce) and visualised immediately by exposure to film (Fuji Film).

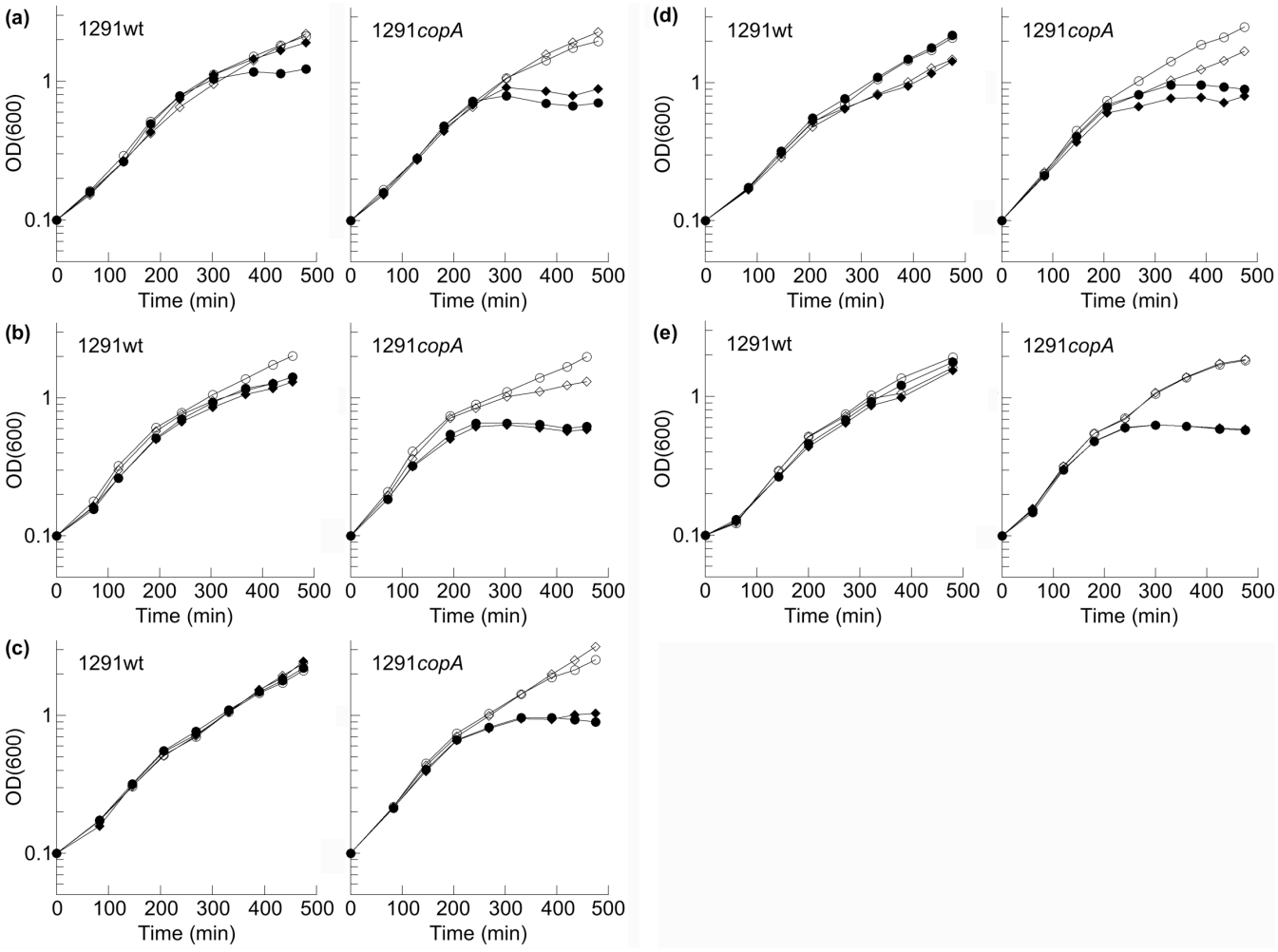
Supplementary Figure 4. Cu treatment led to loss of heme during a) microaerobic growth in liquid cultures and b) on solid media. a) Difference pyridine hemochrome spectra (reduced *minus* oxidised) of whole gonococci after microaerobic growth in the absence (black lines) and presence (red lines) of 5 μM $Cu(NO_3)_2$. Three independent replicates are shown for each sample. b) Gonococcal pre-cultures were re-suspended in PBS to an OD_{600} of 0.4. Approximately 1 mL of this suspension was spread on solid GC (Oxoid) media containing 0 μM (black lines) or 20 μM $Cu(NO_3)_2$ (red lines). Any excess liquid was removed by pipetting. Bacteria were incubated at 37 ^{o}C and 5% CO_2 for 24 hours. Bacteria were harvested and washed with PBS (2 x 20 mL) prior to heme extraction. Difference pyridine hemochrome spectra (reduced *minus* oxidised) are presented below. Three independent replicates are shown for each sample.



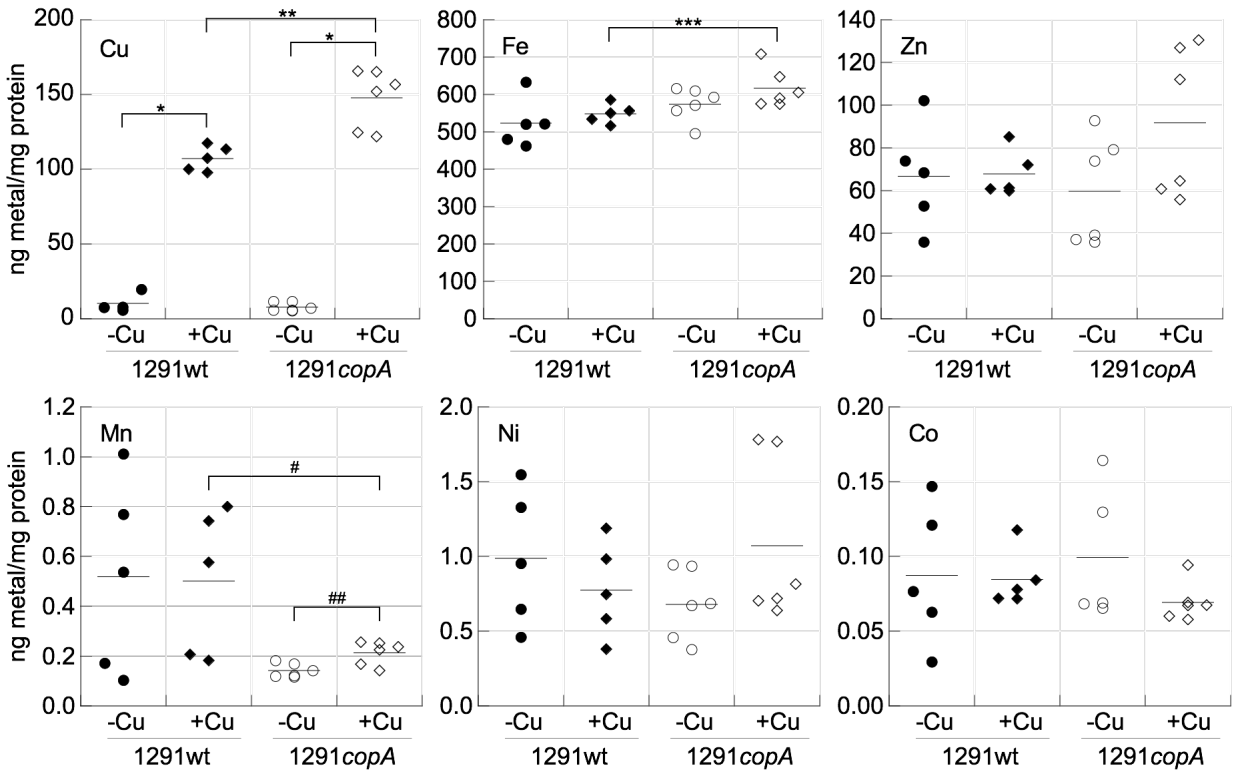
Supplementary Figure 5. Effects of various treatments on Cu toxicity during growth on solid media. Gonococcal pre-cultures were resuspended in PBS to an OD_{600} of 0.4 (approximately 1 x 10^8 colony forming units/mL) and serially diluted (1:10 steps) in the same buffer. Exactly 5 µL of each dilution was dropped on fresh GC solid media (Oxoid) containing the various concentrations of $Cu(NO_3)_2$ and a) 10 µM hemin; b) 10 µM $FeCl_3$; c) 100 µM ALA; and d) 2.5 µM Proto IX as indicated. Bacteria were incubated for 24 h at 37 ^oC/5% CO_2 until colonies were visible.



Supplementary Figure 6. Effects of various treatments on Cu toxicity during growth in aerated liquid cultures. Growth of gonococci without (empty icons ○ and ◇) and with (filled icons ● and ◆) 30 μM $Cu(NO_3)_2$ in the absence (circles ○ and ●) and presence (diamonds ◇ and ◆) of a) 1 μM hemin, b) 0 μM added $FeCl_3$ (Fe-depleted), c) 40 μM added $FeCl_3$ (Fe-enriched), d) 100 μM ALA, or e) 1 μM Proto IX.



Supplementary Figure 7. Effects of Cu overloading on the intracellular levels of Cu, Fe, Zn, Mn, Ni, or Co. Intracellular concentrations of various transition metals in gonococci after 5 hours of aerobic growth in the absence (-Cu) and presence (+Cu) of 30 μM $Cu(NO_3)_2$. Six independent replicates are shown for each sample. **P* = 0.0001, ***P* = 0.002, ****P* = 0.03, $^{\#}P$ = 0.04, $^{\#\#}P$ = 0.01 by two-tailed Student's *t*-test. All others had *P* > 0.1.



Supplementary Figure 8. Cu treatment led to the accumulation of Cop III. Gonococci were grown aerobically for 5 hours in the absence (-Cu) and presence (+Cu) of 30 μM $Cu(NO_3)_2$. a) Orange-red fluorescence of whole gonococci under UV light. (b-e) Effects of various growth conditions on Cop III production. Fluorescence of acetone/HCl extracts. Bacteria were grown aerobically without any additional treatment (-) or with: b) 40 μM $FeCl_3$ (+Fe) or 1 μM hemin (+h); c) 0 μM $FeCl_3$ (-Fe), d) 1 μM Proto IX (PPIX); or e) 100 μM ALA.