The *copB & mco* genes promote hypertolerance to copper and protect *Staphylococcus aureus* from killing by host phagocytes

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1. Background

- **All *Staphylococcus aureus* strains possess a conserved chromosomal operon, *copA-copZ* conferring low level resistance to copper.**
- **The *copB-mco* operon, encoding a copper efflux pump (CopB) and a multicopper oxidase (Mco), is prevalent among invasive isolates from across Europe, and among three successful clonal lineages of *S. aureus* (clonal complex 22, 30 & 398) (Zapotoczna *et al*., 2018. mBio 9(5). pii: e00550-18). The *copB-mco* operon is carried on mobile genetic elements (e.g. on plasmid pSCBU).**
- **Here we investigate the role of copper-hypertolerance in conferring resistance to host immune defences to *S. aureus*.**

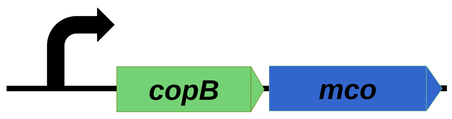

Figure 1. Schematic representation of the *copB-mco* operon

2. Copper hypertolerance allows growth of *S. aureus* at subinhibitory concentrations of copper

- **Isogenic *copB* and *mco* mutants were generated in strain CC22 strain 14-2533T (pSCBU).**
- **To determine if the *copB* and *mco* genes had an impact on bacterial growth under copper stress, we monitored the growth of cultures in broth containing a concentration of copper below the minimum inhibitory concentration for all strains and mutants (Fig. 2).**
- **14-2533T lacking the pSCBU plasmid had reduced growth in copper-supplemented broth as did isogenic *copB* or *mco* deletion mutants.**

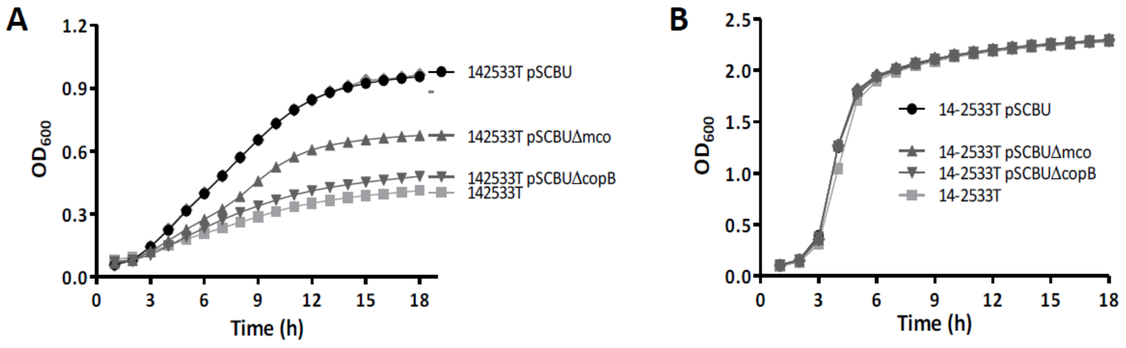

Figure 2. Enhanced growth in subinhibitory concentrations of copper chloride requires copper hypertolerance genes. Growth of *S. aureus* was measured in TSB supplemented with sub-inhibitory (4 mM) concentrations of copper chloride (A) or TSB broth alone (B). Growth curves representing data obtained from at least three independent experiments are presented.

3. The *mco* gene promotes survival of *S. aureus* in copper chloride

- **The contribution of *mco* to copper tolerance in *S. aureus* was also examined in CC30 strain MRSA252.**
- **An isogenic *mco* deletion mutant of MRSA252 grew more slowly and to a lower OD_{600} than wild-type MRSA252 in broth containing a sub-inhibitory concentration of $CuCl_2$ (Fig. 3A).**
- **We next investigated how carriage of the *mco* gene affected the ability of MRSA252 to survive incubation in a solution of $CuCl_2$ (2.5 mM) at 22°C.**
- **Viable counts recovered for MRSA252Δ*mco* were significantly lower than viable counts of the wild-type 2h and 4h post-inoculation (Fig. 3B) showing that the *mco* gene increases the ability of *S. aureus* to survive copper toxicity.**

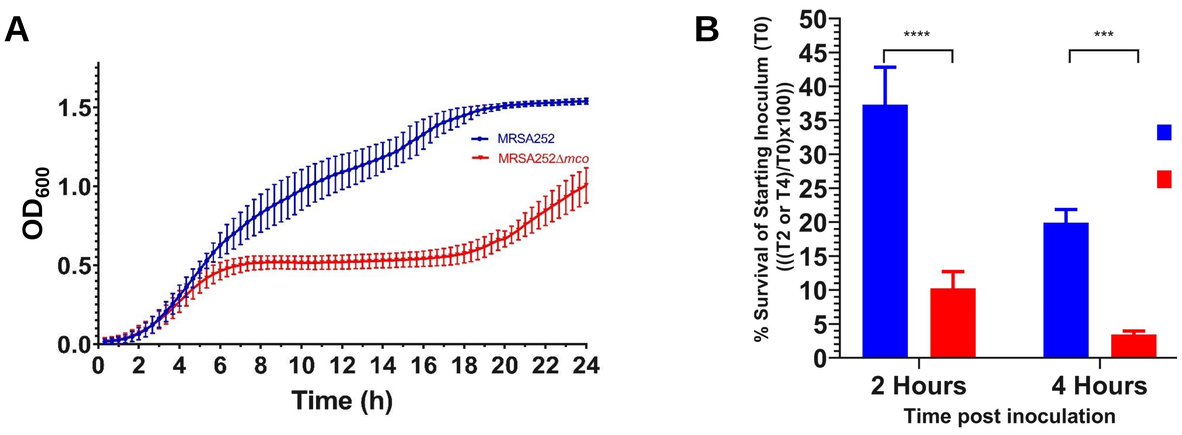

Figure 3. A) Growth of *S. aureus* was measured in TSB supplemented with sub-inhibitory (2 mM) concentrations of $CuCl_2$. *B) S. aureus* was incubated in MilliQ water containing $CuCl_2$ (2.5 mM) for 4 h. Total viable counts were determined for the initial inoculum (T0) and samples 2 h and 4 h post-inoculation. The percentage of bacterial survival was determined by dividing the T2 or T4 count by the T0 count x 100. Statistical significance was determined using 2 way ANOVA and Sidaks Multiple Comparisons Test ****, P<0.0001, ****, P<0.0004. Control experiments indicated that there was no reduction in viability of MRSA252 or MRSA252Δ*mco* in MilliQ water without copper during the 4 hincubation period (data not shown).

4. Copper hypertolerance genes increase resistance of *S. aureus* to macrophage killing

- **We investigated if bacterial tolerance to copper might influence the outcome for *S. aureus* following phagocytosis by macrophages.**
- **The 14-2533T (pSCBU) strain survived inside RAW264.7 murine macrophages at significantly higher levels than 14-2533T without the plasmid (Fig. 4A).**
- **The *copB* and *mco* mutants had a survival defect, suggesting that copper tolerance in *S. aureus* prevents killing by macrophages (Fig. 4A).**

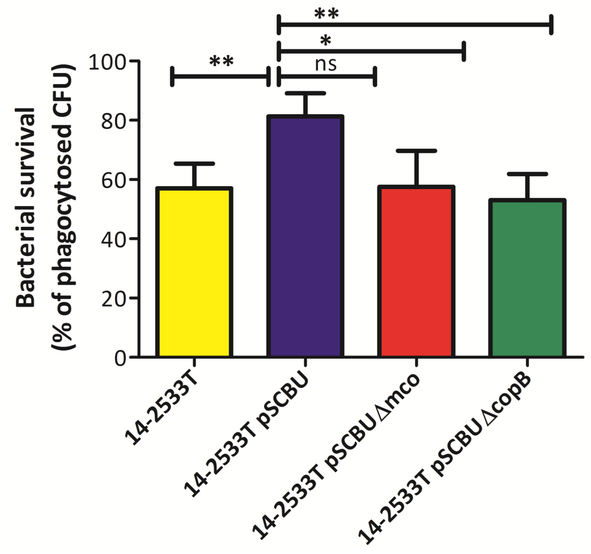

Figure 4. Hypertolerance to copper increases resistance of *S. aureus* to macrophage killing. Murine RAW264.7 macrophages were suspended in DMEM supplemented with mouse IFNγ (40 ng/ml) and Cu_2SO_4 (40 μM) and cultured for 18 h at 37°C in 5% CO_2. Bacteria were grown overnight and then added at an MOI of 10 allowing phagocytosis for 30 min followed by killing of extracellular bacteria with gentamycin/lysostaphin for 30 min. Macrophages were then lysed and viable counts were used to determine the levels of bacterial survival. Bars represent means ± SD of three independent experiments. Statistical significance is indicated, ***P<0.005 * P<0.05.* ns, not significant.

5. Copper hypertolerance genes promote survival of *S. aureus* in blood

- **To determine if *copB-mco* is of relevance to infection in humans, *ex vivo* infection studies were performed with whole human blood.**
- **Copper-hypertolerant *S. aureus* 14-2533T (pSCBU) had higher survive in whole human blood compared to 14-2533T without the plasmid (Fig. 5A).**
- **The *mco* and *copB* mutants had a significant defect in whole blood survival compared to the wild-type (Fig. 5B).**
- **Wild-type and mutants had similar growth rates in plasma showing that copper hypertolerance is important for *S. aureus* to resist cellular killing in blood.**

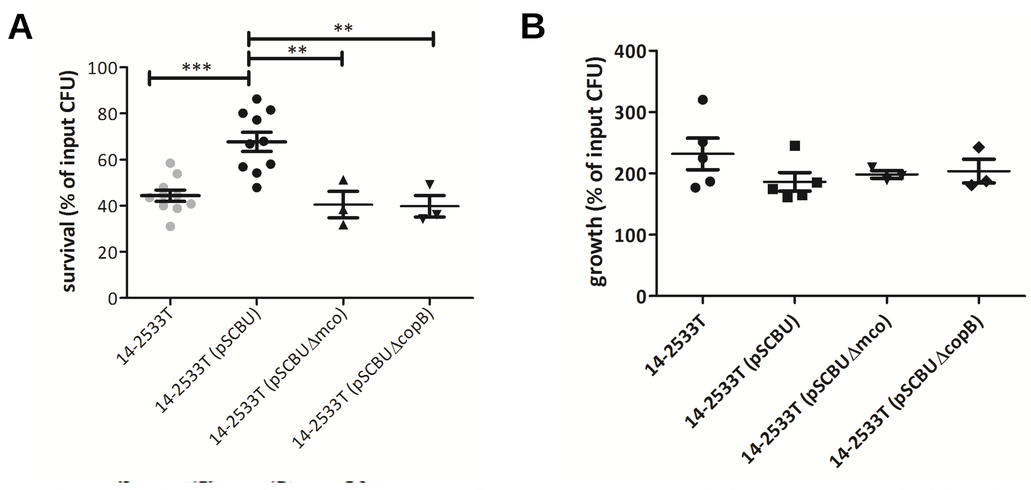

Figure 5. Increased survival of copper hypertolerant *S. aureus* in human blood. *S. aureus* (ca. 1×10^4 CFU/ml) was inoculated into freshly drawn human blood (A) or plasma (B) and incubated at 37˚C. The CFU after 3 h is expressed as a percentage of the original input CFU at 0 h. Lines represent the means ± SD of >3 independent experiments. Statistical significance was determined by ANOVA following Dunnett's multiple-comparison test **, P 0.01; ***, P 0.001