Copper stress perturbs zinc and manganese homeostasis in *Streptococcus pyogenes*.

Running title: Cu stress perturbs Zn and Mn homeostasis in GAS

Young J Hong\(^1\)*, Eilidh S Mackenzie\(^2\)*, Samantha J Firth\(^1\)*, Jack FR Bolton\(^1\)*, Louisa J Stewart\(^1\)*,

Kevin J Waldron\(^2\), and Karrera Y Djoko\(^1\)

\(^1\)Department of Biosciences, Durham University, Durham DH1 3LE, United Kingdom

\(^2\)Biosciences Institute, Newcastle University, Newcastle upon Tyne NE2 4HH, United Kingdom

*These authors contributed equally to the work. Author order was decided by an on-line list randomiser.

*Corresponding author: karrera.djoko@durham.ac.uk

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ABSTRACT

All bacteria possess homeostatic mechanisms that control the availability of micronutrient metals within the cell. Regulatory cross-talks between different metal homeostasis pathways within the same bacterial organism have been reported widely. In addition, there have been previous suggestions that some metal uptake transporters can promote adventitious uptake of the wrong metal. This work describes the cross-talk between the Cu homeostasis pathway and the Zn, Mn, and Fe pathways in Group A Streptococcus (GAS). Using a ΔcopA mutant strain that lacks the primary Cu efflux pump and thus traps excess Cu in the cytoplasm, we show that Cu stress leads to strong downregulation of Zn and Mn uptake genes, and mild downregulation of Fe uptake genes. This effect is associated with depletion of cellular Zn levels, but not those of Mn or Fe. Co-supplementation of the culture medium with Zn and, to a lesser extent, Mn, but not Fe alleviates key Cu stress phenotypes, namely bacterial growth and production of the fermentation end-product lactate. However, neither Zn nor Mn treatment influences cellular Cu levels or Cu availability in Cu-stressed cells. In addition, we show that the Zn and Mn uptake transporters in GAS do not promote Cu uptake. Taken together, the results strengthen and extend our previous proposal that mis-repression of Zn uptake genes and cellular Zn depletion are key mechanisms of Cu stress in GAS. By comparison, although Mn homeostasis in GAS is perturbed during Cu stress, the relationship between the two metals is yet to be defined.
INTRODUCTION

In general, metal homeostasis systems are specific for their particular metals. Each metal sensor, importer, exporter, storage protein, and metallochaperone is specialised to manage the cellular availability of their cognate metal ion, and is typically inefficient in managing non-cognate metal ions. However, regulatory cross-talks between different metal homeostasis systems can occur. Perturbations to a metal homeostasis system, whether as a result of exposure to an excess of the cognate metal ion, depletion of that metal ion, or genetic manipulation of a component of that system, can lead to the accumulation or depletion of a different metal ion in the cell, and/or the transcriptional activation or suppression of another metal homeostasis system. In prokaryotes, cross-talks between Cu and Fe homeostasis systems, Cu and Zn, Mn and Zn, Fe and Zn, and Mn and Fe have been widely described.

The molecular mechanisms behind such cross-talks and their corresponding cellular outcomes vary. Some metals play direct roles in the homeostasis of a different metal. For example, the CopY Cu sensor from streptococci is Zn-dependent. CopY derepresses expression of Cu efflux genes in response to increases in cellular Cu availability. Conversely, it represses expression in response to decreases in Cu availability. However, Zn is required to stabilise the repressor form of this sensor. Thus, Zn supplementation suppresses expression of the CopY regulon, while Zn limitation upregulates it, even without additional exposure to Cu.

Metals can also bind adventitiously to non-cognate metal homeostasis proteins and interfere directly with the function of these proteins. In Streptococcus pneumoniae, an excess of Zn can bind adventitiously to the Mn-binding site of the Mn uptake protein PsaA, preventing uptake of Mn via the PsaABC Mn-importing ABC transporter, limiting cellular Mn, and subsequently inducing expression of Mn uptake genes. An excess of Zn is also thought to bind adventitiously to the Mn-sensing site of the Mn sensor PsaR and promote inadvertent derepression of Mn uptake genes.

Adventitious binding of a metal to non-cognate sites can indirectly influence cellular handling of another metal. Bacillus subtilis responds to excess Cu by increasing expression of Fe uptake genes. In this organism, excess Cu mismetalates Fe-S clusters and thus inactivates Fe-S cluster-dependent enzymes as well as Fe-S cluster assembly machineries. The displaced Fe atoms should have increased cellular Fe availability and thus suppressed (rather than induced) expression of Fe.
uptake genes via the Fe sensor Fur. However, the loss of functional Fe-S clusters transcriptionally induces expression of more cluster assembly machineries. This generates a cellular Fe sink, lowers cellular Fe availability, and thus induces (rather than suppresses) expression of Fe uptake genes.

We previously identified a potential regulatory cross-talk between Cu stress and Zn, Mn, and Fe homeostasis in the Gram-positive bacterium *Streptococcus pyogenes* (Group A Streptococcus, GAS). Like other streptococci, GAS employs a single system for Cu homeostasis and efflux, controlled by the CopY Cu sensor (Figure 1). This organism is not known to import, use, or store nutrient Cu. When cellular Cu availability rises, CopY transcriptionally derepresses expression of the Cu-effluxing P$_{1B}$-type ATPase CopA, the Cu-binding metallochaperone CopZ, and a membrane protein of unknown function named CopX. Zn homeostasis in GAS is composed of two systems, one each for Zn uptake and Zn efflux, which are controlled by the AdcR and GczA Zn sensors, respectively. Under conditions of low cellular Zn availability, AdcR transcriptionally derepresses expression of the Zn-importing AdcAl/AdcAll-AdcBC ABC transporter (Figure 1), along with accessory proteins such as the poly-His triad protein Pht. Under conditions of high cellular Zn availability, GczA transcriptionally derepresses expression of the Zn-effluxing cation diffusion facilitator CzcD. The uptake of Mn and Fe in GAS is controlled by the dual Mn/Fe sensor MtsR. In response to low cellular Mn availability, MtsR transcriptionally derepresses expression of the Mn-importing MtsABC ABC transporter (Figure 1). In response to low cellular Fe availability, this sensor derepresses expression of a variety of Fe uptake systems, including the ferrichrome-importing FhuADBG and heme-importing SiaABC ABC transporters (Figure 1). Finally, GAS also employs the cation diffusion facilitator MntE to efflux Mn, and the P$_{1B}$-type ATPase PmtA to efflux Fe, although neither transporter is known to be directly regulated by a Mn- or Fe-sensing transcriptional regulator.

Our previous work shows that Cu stress does not perturb transcription of *czcD*, *mntE*, or *pmtA*, but it does lead to mis-repression of the *adc*, *mts*, *fhu*, and *sia* genes. In addition, Cu stress induces upregulation of a *cadDC* operon, whose protein products are predicted to efflux and sense Cd (and, therefore, potentially Zn) but are yet to be characterised experimentally. Therefore, the goal of this study was to further investigate the potential cross-talks between Cu stress and the AdcR and MtsR regulons in GAS.
Figure 1. Cu, Zn, Mn, and Fe metal homeostasis systems in GAS. Only those components that are directly relevant to this work are shown. The metallosensor responsible for regulating the organism’s transcriptional responses to each metal is shown, along with the direction of transcriptional regulation. Transporters responsible for the efflux of Cu or uptake of Zn, Mn, and Fe are also shown.

METHODS

Data presentation. Except for growth curves, individual data points from independent experiments are plotted, with lines or shaded columns representing the means, and error bars representing standard deviations. Growth curves show the means of independent experiments, with shaded regions representing standard deviations. The number of independent experiments (N) is stated clearly in each figure legend.

Reagents. All reagents were of analytical grade and obtained from Sigma or Melford Chemicals unless otherwise indicated. The sulfate and chloride salts of Cu, Zn, Mn, or Fe were used interchangeably. All reagents were prepared in deionised water.

Strains and culture conditions. GAS M1T1 5448 strains were propagated from frozen glycerol stocks onto solid THY medium without any antibiotics. Unless otherwise indicated, liquid cultures were prepared in a chemically defined medium containing glucose as the carbon source (CDM-glucose). All solid and liquid growth media contained catalase (50 µg/mL).

Bacterial growth. Growth was assessed at 37 °C in flat-bottomed 96-well plates using an automated microplate shaker and reader. Each well contained 200 µL of culture. Each plate was sealed with a gas permeable, optically clear membrane (Diversified Biotech). OD₆₀₀ values were
measured every 20 min. The plates were shaken at 200 rpm for 1 min in the double orbital mode immediately before each reading. OD\textsubscript{600} values were not corrected for path length (ca. 0.58 cm for a 200 µL culture).

**ICP MS analyses.** GAS was cultured in 40 mL of CDM-Glucose. At the desired time points, cultures were harvested (5,000 x g, 4°C, 10 min), washed once with Tris-HCl buffer (50 mM, pH 8.0) containing D-Sorbitol (1 M), MgCl\textsubscript{2} (10 mM), and EDTA (1 mM), and twice with ice-cold PBS. The pellet was resuspended in ice-cold PBS (1 mL). An aliquot was collected for the measurement of total protein content. The remaining suspension was re-centrifuged. The final pellet was dissolved in conc. nitric acid (150 µL, 80 °C, 1 h) and diluted to 3.5 mL with deionised water. Total metal levels in these samples were determined by inductively-coupled plasma mass spectrometry (ICP MS). The results were normalised to total cellular protein content.

**Secreted lactate levels.** GAS was cultured in 96-well plates as described earlier for growth analysis. At the desired time points, samples were centrifuged (5,000 x g, 4°C, 10 min) and concentrations of lactate in the supernatants were determined using K-LATE kit (Megazyme).

**GapA activity.** Bacteria were cultured in 40 mL of CDM-glucose. At the desired time points, bacteria were harvested (5,000 x g, 4°C, 10 min), washed once with Tris-HCl buffer (50 mM, pH 8.0) containing D-Sorbitol (1 M), MgCl\textsubscript{2} (10 mM), and EDTA (1 mM), and twice with ice-cold PBS. Bacterial pellets were resuspended in a buffer containing sodium phosphate (100 mM) and triethanolamine (80 mM) at pH 7.4, transferred to a tube containing Lysing Matrix B (MP Biomedicals), and lysed in a FastPrep 24G instrument (MP Biomedicals, 10 m/s, 20 s, 2 cycles). Intact cells and cell debris were removed by centrifugation (20,000 x g, 1 min) and cell-free lysate supernatants were kept on ice and used immediately.

To determine GapA activity, the reaction mixture contained NAD\textsuperscript{+} (4 mM), DL-glyceraldehyde-3-phosphate (G3P, 0.3 mg/mL), sodium phosphate (100 mM), DTT (1 mM), and triethanolamine (80 mM) at pH 7.4. Each reaction (100 µL) was initiated by addition of cell-free lysate supernatants (10 µL). Absorbance values at 340 nm were monitored for up to 10 min at 37 °C. Initial rates of reaction were normalised to protein content in the cell-free lysate supernatants. Control reactions without any substrate (G3P for GapA) were always performed in parallel. One unit of activity was defined as follows: 1000 nmol NAD\textsuperscript{+} oxidised min\textsuperscript{-1} mg protein\textsuperscript{-1} for GapA.
**SodA activity.** SodA activity was assessed qualitatively using a gel-based assay. First, bacteria were cultured and pelleted as described above for measurements of GapA activity. Cell-free lysate supernatants were also prepared as above, but using Tris-HCl (50 mM, pH 8.0) containing NaCl (150 mM) as the buffer. Protein content in the cell-free lysate supernatants was determined and 8 µg of proteins were resolved on 15% native polyacrylamide gels. The gels were incubated in buffer containing potassium phosphate (50 mM, pH 7.8), EDTA (1 mM), nitro blue tetrazolium chloride (0.25 mM), and riboflavin (0.05 mM), then exposed to light to detect SodA activity. Purified recombinant Fe-loaded and Mn-loaded SodA from *S. pyogenes* (metal-verified by ICP MS; 0.25 µg each) were loaded in parallel as controls. Incubation of replica gels with InstantBlue® Coomassie Protein Stain (Abcam) was performed to assess sample loading. All gels were imaged using a ChemiDoc™ imaging system (Bio-Rad), using the same settings for all gels compared within a single experiment.

**Protein content.** Total protein content in all cell extracts was determined using the QuantiPro BCA Assay Kit (Sigma).

**RNA extraction.** Bacteria were cultured in 1.6 mL of CDM-glucose. At the desired time points, cultures were centrifuged (4,000 x g, 4°C, 5 min). Bacterial pellets were resuspended immediately in 500 µL of RNAPro Solution (MP Biomedicals) and stored at -80°C until further use. Bacteria were lysed in Lysing Matrix B and total RNA was extracted following the manufacturer’s protocol (MP Biomedicals). RNA extracts were treated with RNase-Free DNase I enzyme (New England Biolabs). Complete removal of gDNA was confirmed by PCR using gapA-check-F/R primers (Supporting Table 1). gDNA-free RNA was purified using RNeasy Mini Kit (QIAGEN) and visualised on an agarose gel.

**qRT-PCR analyses.** cDNA was generated from 1 µg of RNA using the SuperScript® IV First-Strand Synthesis System (Invitrogen). qPCR was performed in 20 µL reactions using Luna qPCR Universal qPCR Master Mix (New England Biolabs), 5 ng of cDNA as template, and 0.4 µM of the appropriate primer pairs (Supporting Table 1). Each sample was analysed in technical duplicates. Amplicons were detected in a CFXConnect Real-Time PCR Instrument (Bio-Rad Laboratories). \( C_q \) values were calculated using LinRegPCR after correcting for amplicon efficiency. \( C_q \) values of technical duplicates were typically within ± 0.25 of each other. *holB*, which encodes DNA polymerase III, was used as the reference gene.
RESULTS

Cu treatment leads to downregulation of Zn, Mn, and Fe uptake genes.

Our previous work showed that growth in a metal-limited, chemically defined medium in the presence of supplemental Cu led to aberrant regulation of metal homeostasis in the GAS 5448 ΔcopA mutant strain\(^\text{15}\). Specifically, Cu supplementation led to downregulation of genes under the control of AdcR and MtsR. This effect appeared in the late exponential growth phase (after 4 h of growth) and coincided with cessation of bacterial growth \(i.e.\) Cu stress.

This work confirmed that the AdcR-regulated gene \(adcAI\) and the MtsR-regulated gene \(mtsC\) are strongly downregulated in Cu-stressed Δ\(\text{copA}\) mutant cells, \(i.e.\) after 6 and 8 h of growth in the presence of supplemental Cu (Figures 2A-B). As observed previously, the MtsR-regulated gene \(siaA\) was only mildly downregulated (Figure 2C). As the control, expression of the CopY-regulated gene \(\text{copZ}\) was upregulated (Figure 2D), consistent with the expected increase in Cu availability in these Cu-stressed cells.

**Figure 2. Effects of Cu supplementation on expression of metal homeostasis genes.** The GAS 5448Δ\(\text{copA}\) mutant strain was cultured with or without added Cu (5 µM) for \(t = 4, 6,\) or \(8\) h \((N = 3)\). Levels of (A) \(adcAI\), (B) \(mtsC\), (C) \(siaA\), and (D) \(\text{copZ}\) mRNA in Cu-supplemented cultures (+Cu) were determined by qRT-PCR and normalised to those in the corresponding unsupplemented samples (-Cu) that were cultured for the same time periods. Dotted horizontal lines represent the sensitivity limit of the assay \((\log_2\text{FC} = \pm 0.5)\).
Cu treatment leads to cellular Zn, but not Mn or Fe, limitation.

The protein products of \( \text{adcAI} \), \( \text{mtsC} \), and \( \text{siaA} \) contribute to Zn, Mn, and Fe (heme) uptake, respectively\(^{26–29}\). Thus, downregulation of these genes by excess Cu may lower cellular Zn, Mn, or Fe. Our previous work showed that growth in the presence of supplemental Cu does not affect total cellular Zn, Mn, or Fe levels in \( \Delta \text{copA} \) cells at the onset of Cu stress and gene repression\(^{18}\). These results were reproduced here using cells that were sampled after 4 h of growth in the presence of supplemental Cu (Supporting Figure 1). By contrast, when cells were sampled after 8 h, \( i.e. \) after a period of sustained Cu stress and gene repression, there was a marked decrease in cellular Zn levels (Figure 3A), consistent with the strong downregulation of \( \text{adcAI} \) at these later time points (\( cf. \) Figure 2A). Levels of cellular Mn were well below detection limit (Figure 3B). Levels of cellular Fe remained largely unchanged (Figure 3C), consistent with only a minor downregulation of \( \text{siaA} \) (see Figure 2C). As controls, levels of cellular Cu were confirmed to increase in these Cu-stressed \( \Delta \text{copA} \) cells (Figure 3D).

**Figure 3. Effects of Cu supplementation on cellular levels of (A) Zn, (B) Mn, (C) Fe, and (D) Cu.**

The GAS 5448\( \Delta \text{copA} \) mutant strain was cultured with supplemental Cu (0, 1, or 5 \( \mu \)M) for \( t = 8 \) h (\( N = 3 \)). Total cellular levels of all metals were measured by ICP MS and normalised to total protein content.

As an alternative assessment of cellular Mn levels, which were below the detection limit of our assay (\( cf. \) Figure 3B), we measured the activity of the superoxide dismutase SodA in \( \Delta \text{copA} \) cell-free extracts. SodA from \( S. \text{pyogenes} \) is active with either Mn or Fe in the catalytic site, although enzyme activity with Mn is much higher\(^{30}\). Previous studies with Mn-deficient \( \Delta \text{mtsABC} \) mutant strains of GAS...
indicated that loss of cellular Mn is associated with decreased SodA activity. However, growth in the presence of Cu for ~8 h does not reduce SodA activity in ∆copA cells (Figure 4). Therefore, despite strong downregulation of mtsC, there is currently no evidence that Cu-stressed cells are Mn-limited.

Figure 4. Effects of Cu supplementation on SodA activity. The GAS 5448ΔcopA mutant strain was cultured with supplemental Cu (0, 1, 5, or 10 µM) with or without supplemental Mn (0 or 5 µM) for t = 8 h. SodA activity was evaluated using an in-gel assay and total protein was evaluated with Coomassie staining. A representative gel from N = 3 independent replicates is shown. The activity of purified SodA loaded with either Mn (SpSodA-Mn) or Fe (SpSodA-Fe) was measured in parallel as control.

Supplementation with Zn and Mn, but not Fe, partially alleviates Cu stress.

The decrease in cellular Zn levels may promote cellular Zn limitation and contribute to the observed growth defect or other phenotypes associated with Cu stress. These phenotypes include a decrease in the extracellular (secreted) levels of the fermentation end-product lactate after 24 h. Consistent with this hypothesis, co-supplementation with Zn restored cellular Zn to normal (Figure 5A), partially reversed the growth defect (Figure 6A), and partially restored extracellular lactate levels (Figure 7A) in Cu-supplemented ΔcopA cultures.

Likewise, co-supplementation with Mn increased cellular Mn levels multiple folds such that they were now detectable (Figure 5B). Interestingly, less cellular Mn was detected in cells that were co-supplemented with low Mn (0.5 µM) but grown in the presence of high Cu (5 µM) when compared
with those grown in the presence of low Cu (1 µM) (Figure 5B). Thus, even though Cu stress did not appear to promote cellular Mn limitation and perturb SodA function (cf. Figure 4), it did nonetheless suppress accumulation of cellular Mn. Co-supplementation with Mn also weakly improved growth (Figure 6B) and restored extracellular lactate levels (Figure 7B) in ∆copA cultures.

Co-supplementation with Fe had no detectable effect on growth (Figure 6C) or on secreted lactate levels (Figure 7C) in ∆copA cultures. Given these results, the minor downregulation of siaA in Cu-stressed cells (cf. Figure 2C), and the lack of an observable change in cellular Fe levels (cf. Figure 3C), Cu stress in the ∆copA mutant strain is not likely linked to Fe and thus was not investigated further.

Figure 5. Effects of Zn and Mn co-supplementation on cellular levels of (A) Cu, (B) Zn, and (C) Mn. The GAS 5448∆copA mutant strain was cultured with added Cu (0, 1, or 5 µM), with or without Zn or Mn (0, 0.5, or 5 µM) for $t = 8$ h ($N = 3$). Total cellular levels of all metals were measured by ICP MS and normalised to total cellular protein content. Note that H₂O data are also shown in Figure 3.
Figure 6. Effects of (A) Zn, (B) Mn, and (C) Fe co-supplementation on bacterial growth. The GAS 5448ΔcopA mutant strain was cultured with added Cu (0, 1, or 5 µM), with or without added Zn, Mn, or Fe (0, 0.5, or 5 µM) for t = 8 h (N = 3).

Figure 7. Effects of (A) Zn, (B) Mn, and (C) Fe co-supplementation on homolactic fermentation. The GAS 5448ΔcopA mutant strain was cultured with added Cu (0, 1, or 5 µM), with or without added...
Zn, Mn, or Fe (0, 0.5, or 5 µM) for $t = 24$ h ($N = 3$). Amounts of secreted lactate were measured in the spent culture medium.

**Supplementation with Zn or Mn does not suppress cellular Cu levels and availability.**

Cu stress in GAS ΔcopA cultures is linked with a reduction in the activity of the central glycolytic enzyme, namely the phosphorylating, ATP-generating, GAPDH enzyme, GapA. This enzyme is likely mismetalated by excess cytoplasmic Cu ions, which may bind to the side chains of the catalytic Cys and a nearby His. The observed improvement in bacterial growth and lactate secretion in response to Zn or Mn co-supplementation (cf. Figures 6 and 7) hints at an improvement in GapA activity. However, Figure 8 clearly shows that Zn or Mn co-supplementation does not improve the activity of GapA in Cu-supplemented ΔcopA cultures. Therefore, the putative link between Cu stress and Zn or Mn homeostasis is likely independent of GapA.

**Figure 8. Effects of (A) Zn and (B) Mn co-supplementation on GapA activity.** The GAS 5448ΔcopA mutant strain was cultured with added Cu (0, 1, 2, or 3 µM), with or without added Zn or Mn (5 µM each) for $t = 8$ h ($N = 3$). GapA activities were determined in cell-free extracts.

The observations that: (i) GapA remained inhibited and (ii) Cu levels in Cu-treated cells remained unchanged (cf. Figure 5), despite co-supplementation with Zn or Mn, suggest that neither Zn nor Mn exerts a protective effect by suppressing Cu uptake. To more sensitively measure cellular Cu availability, we assessed expression of the copZ gene. This gene was upregulated in a dose-dependent manner during growth in the presence of supplemental Cu (Figure 9). However, co-
supplementation with Zn or Mn did not appear to perturb this Cu-dependent expression of copZ
(Figure 9). These results strengthen our proposal that cellular Cu availability remains high and that Zn
or Mn does not exert a protective effect by suppressing Cu uptake.

Figure 9. Effects of Zn or Mn supplementation on Cu-dependent expression of the copZ gene.
The GAS 5448ΔcopA mutant strain was cultured with added Cu (0 – 5000 nM), with or without added
Zn or Mn (5 µM each) for \( t = 8 \) h \( (N = 3) \). Levels of copZ mRNA in these samples were determined by
qRT-PCR and normalised to expression of holB as the control.

MtsABC or AdcAI/AdcBC does not promote uptake of Cu into GAS.

Our results appear to differ from those reported recently in Staphylococcus aureus. In S.
aureus, MntABC, a Mn-importing ABC transporter, is thought to promote uptake of Cu into the
cytoplasm\(^2\). Expression of mntABC in S. aureus is controlled transcriptionally by the Mn-sensing
derepressor MntR. A decrease in MntABC production (and activity), whether by deletion of the mntA
gene or by transcriptional repression of the mntABC operon in response to Mn co-supplementation,
leads to a reduction in cellular Cu levels\(^2\).

If the MtsABC transporter in GAS and, by extension, the AdcAI/II-AdcBC transporter takes up
Cu into the GAS cytoplasm, then supplemental Mn or Zn may alleviate Cu stress by repressing the
mtsABC or adcAI/II-adcBC genes and suppressing Cu uptake via their protein products. Similarly,
mis-repression of mtsABC or adcAI/II-adcBC genes by excess Cu potentially suppresses further Cu 
uptake and self-limits the toxicity of this metal.

Under our experimental conditions (5 µM each of supplemental Zn or Mn), supplemental Zn 
was confirmed to strongly repress the expression of adcAI, even in the absence of Cu (Supporting 
Figure 2A). However, supplemental Mn did not repress the expression of mtsC in the absence of Cu 
(Supporting Figure 2B). Therefore, to better evaluate the potential role of these ABC transporters in 
Cu uptake, knockout mutant strains lacking functional AdcAI and AdcAII, AdcBC, or MtsABC were 
assessed. According to the above model, these mutant strains would be more resistant to Cu stress 
as a result of decreased Cu uptake.

Contrary to our model, none of the mutant strains was any more resistant to growth inhibition 
by supplemental Cu when compared with the wild-type parent strain (Figure 10). In fact, the ΔmtsABC 
mutant strain was marginally, but reproducibly, less resistant to growth inhibition by supplemental Cu 
when compared with the wild type. Complementation of this mutant in cis with a functional copy of the 
mtsABC operon restored the wild-type phenotype (Supporting Figure 3).

Figure 10. Effects of Cu supplementation on growth of various mutant strains. The GAS 5448 
wild-type (WT, filled circles) and ΔcopA (crosses), ΔadcAI/II (triangles), ΔadcBC (diamonds), and 
ΔmtsABC (circles) mutant strains were cultured with added Cu (0, 100, 200, 300, or 400 µM) for t = 12 
h (N = 3).

The presence of a functional CopA efflux pump in all the strains used in Figure 10 may mask 
the inhibitory effects of Cu on bacterial growth. However, despite screening thousands of 
transformants, the double mutant strains ΔcopAΔadcAI/II, ΔcopAΔadcBC, and ΔcopAΔmtsABC were 
not obtained. Therefore, to assess the potential role of AdcAI/II-AdcBC or MtsABC in Cu uptake, we
assessed whether the loss of these transporters reduce Cu availability in the cytoplasm, specifically by examining the expression of *copA* in the single knockout mutant strains generated earlier.

In this final experiment, the wild-type and mutant strains were cultured for 8 h in the absence of Cu. Each culture was subsequently exposed to 500 nM Cu and sampled at 10 min intervals for up to 30 min. If the ABC transporters take up Cu as hypothesised, then we would observe a decrease and/or a time-dependent delay in *copA* expression in knockout mutant strains that lack these ABC transporters when compared with the wild type. However, Figure 11 shows that there was negligible difference in the expression profile of *copA* in the various strains. Therefore, there is currently no experimental evidence that supports the uptake of Cu via the MtsABC or AdcAI/II-AdcBC transporter in GAS.

**Figure 11. Effects of ABC transporters on cellular Cu availability.** The GAS 5448 wild-type (WT, filled circles), Δ adcAI/II (triangles), Δ adcBC (diamonds), and Δ mtsABC (circles) were cultured with added Cu (0 or 500 nM), with or without added Zn or Mn (5 µM each), for *t* = 8 h (*N* = 3). Levels of *copA* mRNA in these samples were determined by qRT-PCR and normalised to expression of *holB* as the control.

**DISCUSSION**

**Cu stress in GAS perturbs Zn and Mn homeostasis**

This work strengthens and extends our previous observation that growth in the presence of excess Cu leads to mis-regulation of cellular Zn and Mn in GAS\(^6\). In the case of Zn, Cu treatment
leads to mis-repression of Zn uptake genes and, subsequently, cellular Zn depletion in Cu-stressed cells. Cu treatment also leads to mis-regulation of Mn uptake genes. However, there is currently little evidence that Cu-stressed cultures are also Mn-limited. Thus, the relationship between Cu stress and Mn homeostasis in GAS is less defined.

Cross-talks between Cu stress and Zn homeostasis have been reported in other bacteria, although the molecular details differ. These differences may reflect inherent differences in the biochemistry of the different metal homeostasis systems in different organisms. Alternatively, they may reflect differences in experimental design and setup, leading to different degrees of Cu stress.

For instance, in S. pneumoniae, supplemental Zn exacerbates (rather than alleviates) Cu stress in a ∆copA mutant strain and in a ∆czcD mutant strain lacking the primary Zn efflux transporter. Here, excess Zn in the cytoplasm is thought to bind to the allosteric sensing site of the Cu sensor CopY, stabilise the repressor form of this sensor, and thus suppress transcriptional sensing of Cu. Based on the patterns of copZ expression in Figure 9, there is no evidence that supplemental Zn perturbs transcriptional Cu sensing in GAS, at least under the experimental conditions employed here.

As another example, Cu treatment in Salmonella leads to upregulation (and not downregulation) of Zn uptake genes under the control of the Zn sensor Zur. Whether Cu treatment also perturbs Zn levels in this organism has not been reported. In Acinetobacter baumanii, supplemental Cu does not perturb Zn levels in wild-type or ∆copA mutant strains. However, supplemental Zn does lead to a slight decrease in cellular Cu levels in the wild-type strain. The molecular mechanism is unclear, but several putative metal transporter genes are differentially regulated in response to Zn, potentially leading to increased efflux or decreased uptake of Cu from the cytoplasm. This scenario would resemble that reported in Escherichia coli. Supplemental Zn alleviates Cu stress and decreases cellular Cu levels in the E. coli wild type and ∆cueO mutant strains. In this case, Zn supplementation leads to mis-activation of the cusCFBA operon encoding an RND-family Cu efflux transporter, and thus a lowering of cellular Cu. As stated earlier, there is no evidence that supplemental Zn perturbs transcription of Cu homeostasis genes in GAS.

This work further shows that supplemental Mn partially alleviates Cu stress in the GAS ∆copA mutant strain, even though Cu-stressed cells do not appear to be Mn-limited. Similar to our findings, growth of Cu-stressed ∆copA mutant strains of S. pneumoniae is improved by co-supplementation with Mn. The excess Cu in this organism is hypothesised to inhibit the Mn-dependent ribonucleotide
reductase NrdF. Therefore, co-supplementation with Mn is hypothesised to restore NrdF activity\textsuperscript{33}. Cu may similarly inhibit NrdF in GAS. However, loss of NrdF activity is likely only a minor component of Cu stress in GAS, since co-supplementation with Mn is less protective than co-supplementation with Zn (cf. Figure 6).

In contrast with our studies, exposure to Cu leads to upregulation (instead of downregulation) of the mtsABC operon in a wild-type strain of \textit{S. agalactiae} and an increase in cellular Mn levels in a \textit{ΔcopA} mutant strain\textsuperscript{39}. The mechanism behind this observation is yet to be determined. Mn is often involved in the response against oxidative stress. This metal is a cofactor for the superoxide dismutase SodA. The rise in Mn levels in \textit{S. agalactiae} may lead to an increase in SodA activity, though this hypothesis is yet to be tested. In \textit{E. coli}, Cu treatment does indeed lead to an increase in \textit{sodA} expression and other genes under the control of the superoxide sensor SoxRS\textsuperscript{40,41}. In this case, the excess Cu is thought to catalyse formation of superoxide in the periplasmic (but not cytoplasmic) space\textsuperscript{42}. The \textit{sodA} gene in GAS is transcriptionally upregulated by the peroxide sensor PerR in response to peroxide challenge\textsuperscript{30}. However, we have found no evidence that Cu stress induces a change in \textit{sodA} expression\textsuperscript{18} or SodA activity in GAS (cf. Figure 4).

\textbf{What are the likely mechanisms of Cu stress in GAS?}

Aberrant regulation of Zn or Mn homeostasis genes by excess Cu is consistent with our previous model in which the excess Cu outcompetes Zn or Mn from their binding sites in proteins in GAS, which leads to the increase in the cellular availability of these metal ions, subsequent binding of Zn to AdcR or Mn to MtsR, and, finally, repression of Zn or Mn uptake genes. Alternatively, excess cellular Cu may bind directly to AdcR or MtsR, which leads to mis-sensing of metals and mis-repression of target genes by Cu, the wrong metal. There is currently insufficient biochemical data to distinguish between the two models.

Interestingly, this work further suggests that, contrary to our previous proposal\textsuperscript{18}, inactivation of GapA is not a key mechanism of Cu stress in GAS. Growth in the presence of supplemental Cu was confirmed here to promote a reduction in GapA activity in the \textit{ΔcopA} mutant strain. However, co-supplementation with Zn did not rescue GapA activity (cf. Figure 8), even though it rescued bacterial growth (cf. Figure 6) and production of the fermentation end-product lactate (cf. Figure 7).
apparent uncoupling between GapA activity and Cu stress suggests that a loss in GapA activity may
be a symptom but not a cause of Cu stress (in the same manner that an increase in copZ expression
is a symptom but not a cause of Cu stress).

Instead, this work suggests that depletion in cellular Zn is a more likely mechanism of Cu
stress in GAS. This proposal is consistent with the observation that supplemental Zn restores cellular
Zn levels (cf. Figure 5) and rescues bacterial growth (cf. Figure 6). The loss in cellular Zn may lead to
specific inactivation of one key Zn-dependent enzyme and/or global inactivation of multiple Zn-
dependent enzyme(s). Given that mis-repression of Zn uptake genes and the associated decrease in
cellular Zn levels are observed only at the end of the exponential growth phase, we speculate that
likely target enzymes are those that specifically promote cellular metabolism and survival during the
transition to and/or during the stationary phase of growth. Efforts to identify these targets are currently
ongoing.

Do ABC transporters promote Cu uptake into GAS?

Our work further suggests that neither the Zn-importing AdcAI/II-AdcBC transporter nor the
Mn-importing MtsABC transporter promotes uptake of Cu into GAS. To take up a metal ion, the
extracytoplasmic solute binding protein (SBP) domain captures its cognate metal ion and
subsequently releases it to the metal-binding site in the permease domain. In turn, the permease
internalises the metal ion into the cytoplasm and this action is powered by ATP hydrolysis by the
nucleotide-binding domain. Unpublished studies in our laboratory suggest that AdcAI, the Zn-binding
SBP from GAS, binds Cu(II) more tightly than it binds Zn(II). Although the AdcAll SBP from GAS has
not been biochemically characterised, the homologue from *S. pneumoniae* has also been reported to
bind Cu(II)<sup>43</sup>. Likewise, the Mn-binding MtsA SBP from GAS binds Cu(II)<sup>44</sup>, as does PsaA, the MtsA
homologue from *S. pneumoniae*<sup>45</sup>. Our data suggest that the bound Cu(II) in any of the above SBPs is not transferred to the
metal-coordinating site in the partner permease and subsequently internalised into the cytoplasm.
There is evidence that an SBP does not load the permease with non-cognate metal ions, a result of
incompatible coordination chemistry between the partners. For example, extracellular Zn
competitively inhibits Mn uptake via PsaABC in *S. pneumoniae*<sup>8,9</sup>. The permeases PsaB (which
imports Mn) and AdcB (which imports Zn) in this organism possess the same, conserved metal coordination site, suggesting that PsaB should be competent to receive Zn from PsaA. However, while PsaC efficiently releases the bound Mn to PsaB, it does not release bound Zn. However, whether Zn can access the metal-binding site in PsaB directly, without the PsaA SBP, is unknown. Similarly, whether excess extracellular Cu can bind directly to the metal-binding site in the Zn-importing permease AdcB or the Mn-importing permease MtsC and become subsequently internalised into the cytoplasm is unknown. Our data do not support this hypothesis, but direct biochemical support, for instance via metal transport assays of purified transporters, remains to be obtained.

**AUTHOR CONTRIBUTIONS**

KD initiated and designed the research. KD also had overall responsibility for the conceptualisation and coordination of the programme. KD and LS measured bacterial growth, GapA activity, and cellular metal levels. JB and KD measured gene expression levels. EM and KW measured SodA activity. SF and YH carried out literature review and preliminary experiments leading to the final work shown here. KD drafted the manuscript with input from SF and YH. KD, YH, and EM prepared the figures. KD, SF, YH, EM, and KW edited the manuscript. All authors approved the final version.

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GRAPHICAL ABSTRACT