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

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

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Keywords: copper, zinc, streptococcus, metal toxicity, metal homeostasis, metal uptake

ABSTRACT

All bacteria possess homeostastic 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 Δ*copA* 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.

37 **INTRODUCTION**

38

39 In general, metal homeostasis systems are specific for their particular metals. Each metal
40 sensor, importer, exporter, storage protein, and metallochaperone is specialised to manage the
41 cellular availability of their cognate metal ion, and is typically inefficient in managing non-cognate
42 metal ions. However, regulatory cross-talks between different metal homeostasis systems can occur.
43 Perturbations to a metal homeostasis system, whether as a result of exposure to an excess of the
44 cognate metal ion, depletion of that metal ion, or genetic manipulation of a component of that system,
45 can lead to the accumulation or depletion of a *different* metal ion in the cell, and/or the transcriptional
46 activation or suppression of *another* metal homeostasis system. In prokaryotes, cross-talks between
47 Cu and Fe homeostasis systems[1–5], Cu and Zn[4,6,7], Mn and Zn[8,9], Fe and Zn[10], and Mn and Fe[11–14]
48 have been widely described.

49 The molecular mechanisms behind such cross-talks and their corresponding cellular
50 outcomes vary. Some metals play direct roles in the homeostasis of a *different* metal. For example,
51 the CopY Cu sensor from streptococci is Zn-dependent. CopY derepresses expression of Cu efflux
52 genes in response to increases in cellular Cu availability[15]. Conversely, it represses expression in
53 response to decreases in Cu availability. However, Zn is required to stabilise the repressor form of
54 this sensor[16]. Thus, Zn supplementation suppresses expression of the CopY regulon[15], while Zn
55 limitation upregulates it, even without additional exposure to Cu[7].

56 Metals can also bind adventitiously to non-cognate metal homeostasis proteins and interfere
57 directly with the function of these proteins. In *Streptococcus pneumoniae*, an excess of Zn can bind
58 adventitiously to the Mn-binding site of the Mn uptake protein PsaA, preventing uptake of Mn *via* the
59 PsaABC Mn-importing ABC transporter, limiting cellular Mn, and subsequently inducing expression of
60 Mn uptake genes[8,9]. An excess of Zn is also thought to bind adventitiously to the Mn-sensing site of
61 the Mn sensor PsaR and promote inadvertent derepression of Mn uptake genes[17].

62 Adventitious binding of a metal to non-cognate sites can indirectly influence cellular handling
63 of *another* metal. *Bacillus subtilis* responds to excess Cu by increasing expression of Fe uptake
64 genes[1,5]. In this organism, excess Cu mismetalates Fe-S clusters and thus inactivates Fe-S cluster-
65 dependent enzymes as well as Fe-S cluster assembly machineries[5]. The displaced Fe atoms should
66 have increased cellular Fe availability and thus suppressed (rather than induced) expression of Fe

67 uptake genes *via* the Fe sensor Fur. However, the loss of functional Fe-S clusters transcriptionally
68 induces expression of more cluster assembly machineries[5]. This generates a cellular Fe sink, lowers
69 cellular Fe availability, and thus induces (rather than suppresses) expression of Fe uptake genes.
70 We previously identified a potential regulatory cross-talk between Cu stress and Zn, Mn, and
71 Fe homeostasis in the Gram-positive bacterium *Streptococcus pyogenes* (Group A Streptococcus,
72 GAS)[18]. Like other streptococci, GAS employs a single system for Cu homeostasis and efflux,
73 controlled by the CopY Cu sensor[19] (Figure 1). This organism is not known to import, use, or store
74 nutrient Cu. When cellular Cu availability rises, CopY transcriptionally derepresses expression of the
75 Cu-effluxing P_{1B}-type ATPase CopA, the Cu-binding metallochaperone CopZ, and a membrane
76 protein of unknown function named CopX[18,19]. Zn homeostasis in GAS is composed of two systems,
77 one each for Zn uptake and Zn efflux, which are controlled by the AdcR and GczA Zn sensors,
78 respectively[20,21]. Under conditions of low cellular Zn availability, AdcR transcriptionally derepresses
79 expression of the Zn-importing AdcAI/AdcAII-AdcBC ABC transporter (Figure 1), along with accessory
80 proteins such as the poly-His triad protein Pht. Under conditions of high cellular Zn availability, GczA
81 transcriptionally derepresses expression of the Zn-effluxing cation diffusion facilitator CzcD. The
82 uptake of Mn and Fe in GAS is controlled by the dual Mn/Fe sensor MtsR[22,23]. In response to low
83 cellular Mn availability, MtsR transcriptionally derepresses expression of the Mn-importing MtsABC
84 ABC transporter (Figure 1). In response to low cellular Fe availability, this sensor derepresses
85 expression of a variety of Fe uptake systems, including the ferrichrome-importing FhuADBG and
86 heme-importing SiaABC ABC transporters (Figure 1). Finally, GAS also employs the cation diffusion
87 facilitator MntE to efflux Mn[24] and the P_{1B}-type ATPase PmtA to efflux Fe[25], although neither
88 transporter is known to be directly regulated by a Mn- or Fe-sensing transcriptional regulator.
89 Our previous work shows that Cu stress does not perturb transcription of *czcD*, *mntE*, or
90 *pmtA*, but it does lead to mis-repression of the *adc*, *mts*, *fhu*, and *sia* genes[18]. In addition, Cu stress
91 induces upregulation of a *cadDC* operon, whose protein products are predicted to efflux and sense
92 Cd (and, therefore, potentially Zn) but are yet to be characterised experimentally. Therefore, the goal
93 of this study was to further investigate the potential cross-talks between Cu stress and the AdcR and
94 MtsR regulons in GAS.

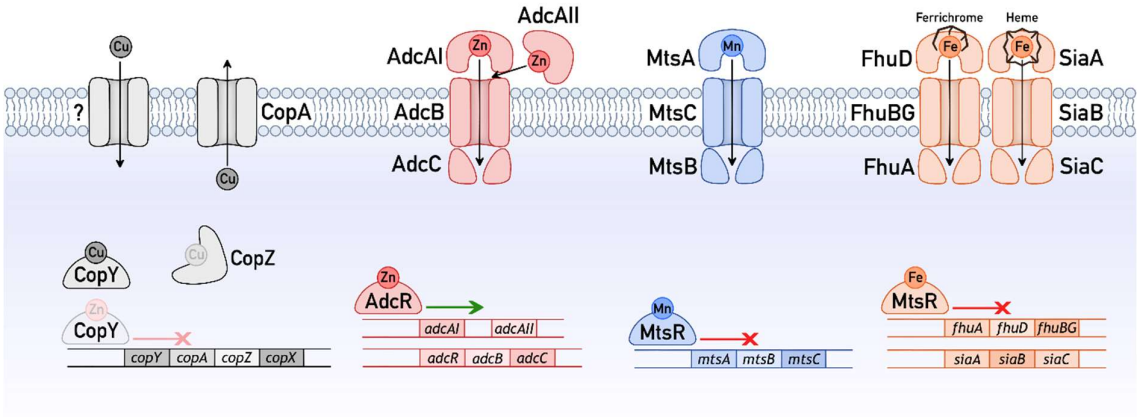


Figure 1. Cu, Zn, Mn, and Fe metal homeostasis systems in GAS. Only those components that are directly relevant to this work are shown. The metallosensor responsible for regulating the organism's transcriptional responses to each metal is shown, along with the direction of transcriptional regulation. 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[18]). 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). OD_{600} values were

118 measured every 20 min. The plates were shaken at 200 rpm for 1 min in the double orbital mode
119 immediately before each reading. OD_{600} values were not corrected for path length (*ca*. 0.58 cm for a
120 200 µL culture).

121 **ICP MS analyses.** GAS was cultured in 40 mL of CDM-Glucose. At the desired time points,
122 cultures were harvested (5,000 x *g*, 4°C, 10 min), washed once with Tris-HCl buffer (50 mM, pH 8.0)
123 containing *D*-Sorbitol (1 M), $MgCl_2$ (10 mM), and EDTA (1 mM), and twice with ice-cold PBS. The
124 pellet was resuspended in ice-cold PBS (1 mL). An aliquot was collected for the measurement of total
125 protein content. The remaining suspension was re-centrifuged. The final pellet was dissolved in conc.
126 nitric acid (150 µL, 80 °C, 1 h) and diluted to 3.5 mL with deionised water. Total metal levels in these
127 samples were determined by inductively-coupled plasma mass spectrometry (ICP MS). The results
128 were normalised to total cellular protein content.

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

132 **GapA activity.** Bacteria were cultured in 40 mL of CDM-glucose. At the desired time points,
133 bacteria were harvested (5,000 x *g*, 4°C, 10 min), washed once with Tris-HCl buffer (50 mM, pH 8.0)
134 containing *D*-Sorbitol (1 M), $MgCl_2$ (10 mM), and EDTA (1 mM), and twice with ice-cold PBS. Bacterial
135 pellets were resuspended in a buffer containing sodium phosphate (100 mM) and triethanolamine (80
136 mM) at pH 7.4, transferred to a tube containing Lysing Matrix B (MP Biomedicals), and lysed in a
137 FastPrep 24G instrument (MP Biomedicals, 10 m/s, 20 s, 2 cycles). Intact cells and cell debris were
138 removed by centrifugation (20,000 x *g*, 1 min) and cell-free lysate supernatants were kept on ice and
139 used immediately.

140 To determine GapA activity, the reaction mixture contained NAD^+ (4 mM), *DL*-glyceraldehyde-
141 3-phosphate (G3P, 0.3 mg/mL), sodium phosphate (100 mM), DTT (1 mM), and triethanolamine (80
142 mM) at pH 7.4. Each reaction (100 µL) was initiated by addition of cell-free lysate supernatants (10
143 µL). Absorbance values at 340 nm were monitored for up to 10 min at 37 °C. Initial rates of reaction
144 were normalised to protein content in the cell-free lysate supernatants. Control reactions without any
145 substrate (G3P for GapA) were always performed in parallel. One unit of activity was defined as
146 follows: 1000 nmol NAD^+ oxidised min^{-1} mg $protein^{-1}$ for GapA.

147 **SodA activity.** SodA activity was assessed qualitatively using a gel-based assay. First,
148 bacteria were cultured and pelleted as described above for measurements of GapA activity. Cell-free
149 lysate supernatants were also prepared as above, but using Tris-HCl (50 mM, pH 8.0) containing
150 NaCl (150 mM) as the buffer. Protein content in the cell-free lysate supernatants was determined and
151 8 µg of proteins were resolved on 15% native polyacrylamide gels. The gels were incubated in buffer
152 containing potassium phosphate (50 mM, pH 7.8), EDTA (1 mM), nitro blue tetrazolium chloride (0.25
153 mM), and riboflavin (0.05 mM), then exposed to light to detect SodA activity. Purified recombinant Fe-
154 loaded and Mn-loaded SodA from *S. pyogenes* (metal-verified by ICP MS; 0.25 µg each) were loaded
155 in parallel as controls. Incubation of replica gels with InstantBlue® Coomassie Protein Stain (Abcam)
156 was performed to assess sample loading. All gels were imaged using a ChemiDoc™ imaging system
157 (Bio-Rad), using the same settings for all gels compared within a single experiment.
158 **Protein content.** Total protein content in all cell extracts was determined using the QuantiPro
159 BCA Assay Kit (Sigma).
160 **RNA extraction.** Bacteria were cultured in 1.6 mL of CDM-glucose. At the desired time
161 points, cultures were centrifuged (4,000 x *g*, 4°C, 5 min). Bacterial pellets were resuspended
162 immediately in 500 µL of RNAPro Solution (MP Biomedicals) and stored at -80°C until further use.
163 Bacteria were lysed in Lysing Matrix B and total RNA was extracted following the manufacturer's
164 protocol (MP Biomedicals). RNA extracts were treated with RNase-Free DNase I enzyme (New
165 England Biolabs). Complete removal of gDNA was confirmed by PCR using gapA-check-F/R primers
166 (Supporting Table 1). gDNA-free RNA was purified using RNeasy Mini Kit (QIAGEN) and visualised
167 on an agarose gel.
168 **qRT-PCR analyses.** cDNA was generated from 1 µg of RNA using the SuperScript® IV First-
169 Strand Synthesis System (Invitrogen). qPCR was performed in 20 µL reactions using Luna qPCR
170 Universal qPCR Master Mix (New England Biolabs), 5 ng of cDNA as template, and 0.4 µM of the
171 appropriate primer pairs (Supporting Table 1). Each sample was analysed in technical duplicates.
172 Amplicons were detected in a CFXConnect Real-Time PCR Instrument (Bio-Rad Laboratories). C_q
173 values were calculated using LinRegPCR after correcting for amplicon efficiency. C_q values of
174 technical duplicates were typically within ± 0.25 of each other. *holB*, which encodes DNA polymerase
175 III, was used as the reference gene.
176

177 **RESULTS**

178

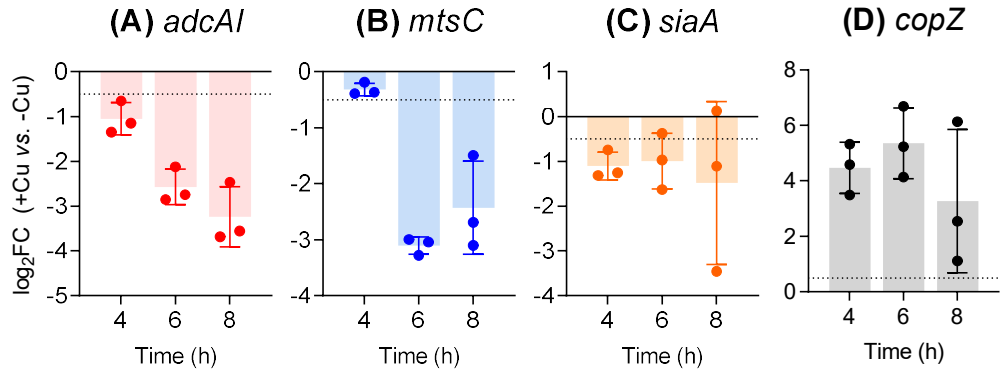
179 **Cu treatment leads to downregulation of Zn, Mn, and Fe uptake genes.**

180

181 Our previous work showed that growth in a metal-limited, chemically defined medium in the
182 presence of supplemental Cu led to aberrant regulation of metal homeostasis in the GAS 5448 Δ*copA*
183 mutant strain[18]. Specifically, Cu supplementation led to downregulation of genes under the control of
184 AdcR and MtsR. This effect appeared in the late exponential growth phase (after 4 h of growth) and
185 coincided with cessation of bacterial growth (*i.e.* Cu stress).

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

192



193

194 **Figure 2. Effects of Cu supplementation on expression of metal homeostasis genes.** The GAS
195 5448Δ*copA* mutant strain was cultured with or without added Cu (5 μM) for *t* = 4, 6, or 8 h (*N* = 3).
196 Levels of **(A)** *adcAI*, **(B)** *mtsC*, **(C)** *siaA*, and **(D)** *copZ* mRNA in Cu-supplemented cultures (+Cu) were
197 determined by qRT-PCR and normalised to those in the corresponding unsupplemented samples
198 (-Cu) that were cultured for the same time periods. Dotted horizontal lines represent the sensitivity
199 limit of the assay ($\log_2$FC = ± 0.5).

200

201 **Cu treatment leads to cellular Zn, but not Mn or Fe, limitation.**

202

203 The protein products of *adcAI*, *mtsC*, and *siaA* contribute to Zn, Mn, and Fe (heme) uptake,
204 respectively[26–29]. Thus, downregulation of these genes by excess Cu may lower cellular Zn, Mn, or
205 Fe. Our previous work showed that growth in the presence of supplemental Cu does not affect total
206 cellular Zn, Mn, or Fe levels in Δ*copA* cells at the *onset* of Cu stress and gene repression[18]. These
207 results were reproduced here using cells that were sampled after 4 h of growth in the presence of
208 supplemental Cu (Supporting Figure 1). By contrast, when cells were sampled after 8 h, *i.e.* after a
209 period of sustained Cu stress and gene repression, there was a marked decrease in cellular Zn levels
210 (Figure 3A), consistent with the strong downregulation of *adcAI* at these later time points (*cf.* Figure
211 2A). Levels of cellular Mn were well below detection limit (Figure 3B). Levels of cellular Fe remained
212 largely unchanged (Figure 3C), consistent with only a minor downregulation of *siaA* (see Figure 2C).
213 As controls, levels of cellular Cu were confirmed to increase in these Cu-stressed Δ*copA* cells (Figure
214 3D).

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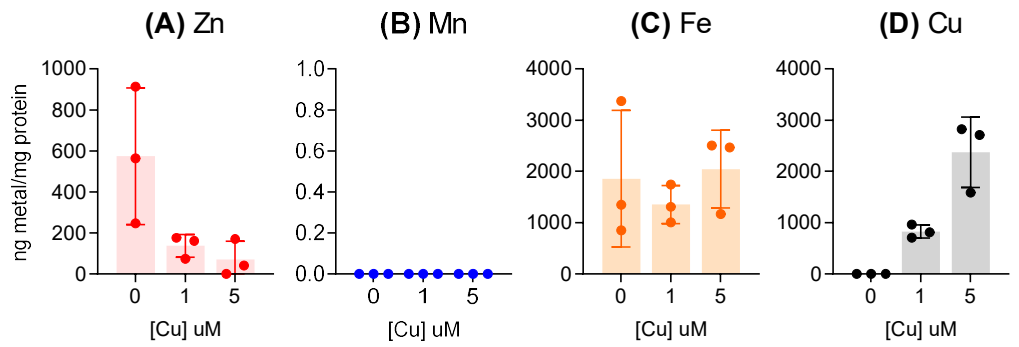

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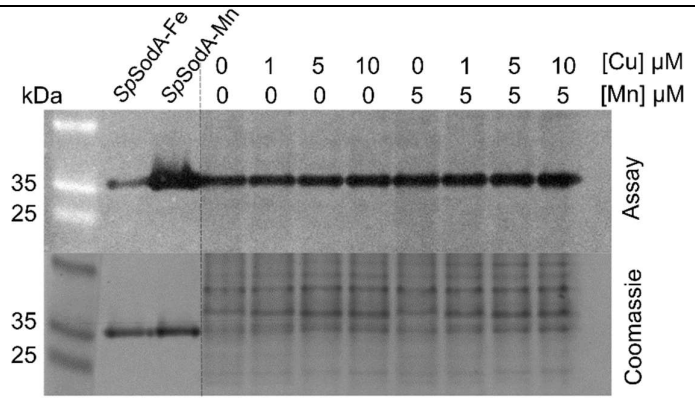
217 **Figure 3. Effects of Cu supplementation on cellular levels of (A) Zn, (B) Mn, (C) Fe, and (D) Cu.**
218 The GAS 5448Δ*copA* mutant strain was cultured with supplemental Cu (0, 1, or 5 μM) for t = 8 h (N =
219 3). Total cellular levels of all metals were measured by ICP MS and normalised to total protein
220 content.

221

222 As an alternative assessment of cellular Mn levels, which were below the detection limit of our
223 assay (*cf.* Figure 3B), we measured the activity of the superoxide dismutase SodA in Δ*copA* cell-free
224 extracts. SodA from *S. pyogenes* is active with either Mn or Fe in the catalytic site, although enzyme
225 activity with Mn is much higher[30]. Previous studies with Mn-deficient Δ*mtsABC* mutant strains of GAS

226 indicated that loss of cellular Mn is associated with decreased SodA activity[27,30]. However, growth in
227 the presence of Cu for ~8 h does not reduce SodA activity in Δ*copA* cells (Figure 4). Therefore,
228 despite strong downregulation of *mtsC*, there is currently no evidence that Cu-stressed cells are Mn-
229 limited.
230



231
232 **Figure 4. Effects of Cu supplementation on SodA activity.** The GAS 5448Δ*copA* mutant strain
233 was cultured with supplemental Cu (0, 1, 5, or 10 μM) with or without supplemental Mn (0 or 5 μM) for
234 *t* = 8 h. SodA activity was evaluated using an in-gel assay and total protein was evaluated with
235 Coomassie staining. A representative gel from *N* = 3 independent replicates is shown. The activity of
236 purified SodA loaded with either Mn (SpSodA-Mn) or Fe (SpSodA-Fe) was measured in parallel as
237 control.
238
239 **Supplementation with Zn and Mn, but not Fe, partially alleviates Cu stress.**
240
241 The decrease in cellular Zn levels may promote cellular Zn limitation and contribute to the
242 observed growth defect or other phenotypes associated with Cu stress. These phenotypes include a
243 decrease in the extracellular (secreted) levels of the fermentation end-product lactate after 24 h[18].
244 Consistent with this hypothesis, co-supplementation with Zn restored cellular Zn to normal (Figure
245 5A), partially reversed the growth defect (Figure 6A), and partially restored extracellular lactate levels
246 (Figure 7A) in Cu-supplemented Δ*copA* cultures.
247 Likewise, co-supplementation with Mn increased cellular Mn levels multiple folds such that
248 they were now detectable (Figure 5B). Interestingly, less cellular Mn was detected in cells that were
249 co-supplemented with low Mn (0.5 μM) but grown in the presence of high Cu (5 μM) when compared

250 with those grown in the presence of low Cu (1 μM) (Figure 5B). Thus, even though Cu stress did not
251 appear to promote cellular Mn limitation and perturb SodA function (*cf.* Figure 4), it did nonetheless
252 suppress accumulation of cellular Mn. Co-supplementation with Mn also weakly improved growth
253 (Figure 6B) and restored extracellular lactate levels (Figure 7B) in Δ*copA* cultures.
254 Co-supplementation with Fe had no detectable effect on growth (Figure 6C) or on secreted
255 lactate levels (Figure 7C) in Δ*copA* cultures. Given these results, the minor downregulation of *siaA* in
256 Cu-stressed cells (*cf.* Figure 2C), and the lack of an observable change in cellular Fe levels (*cf.* Figure
257 3C), Cu stress in the Δ*copA* mutant strain is not likely linked to Fe and thus was not investigated
258 further.
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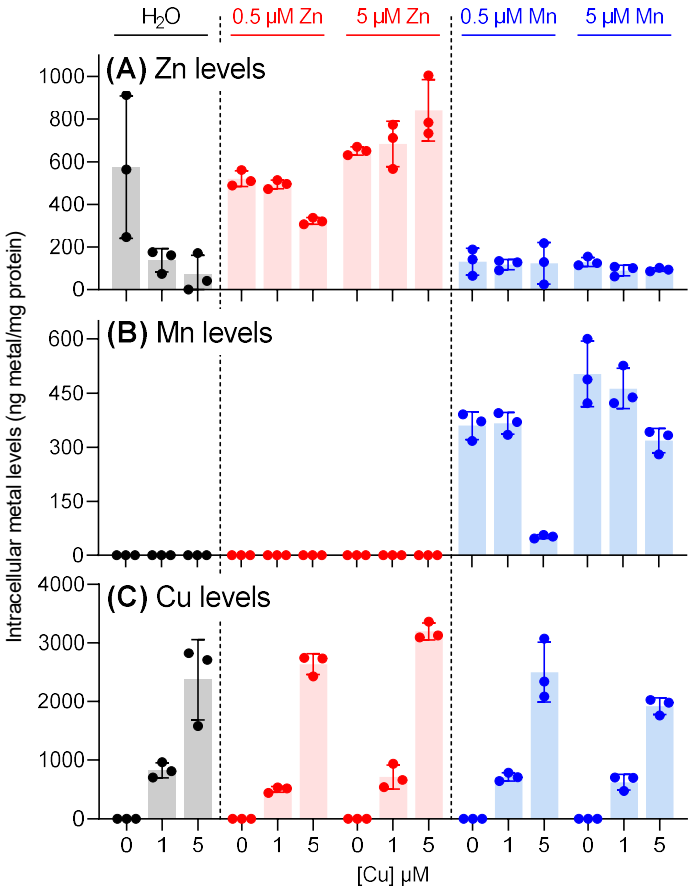

260
261 **Figure 5. Effects of Zn and Mn co-supplementation on cellular levels of (A) Cu, (B) Zn, and (C)**
262 **Mn.** The GAS 5448Δ*copA* mutant strain was cultured with added Cu (0, 1, or 5 μM), with or without Zn
263 or Mn (0, 0.5, or 5 μM) for t = 8 h (N = 3). Total cellular levels of all metals were measured by ICP MS
264 and normalised to total cellular protein content. Note that H_2O data are also shown in Figure 3.

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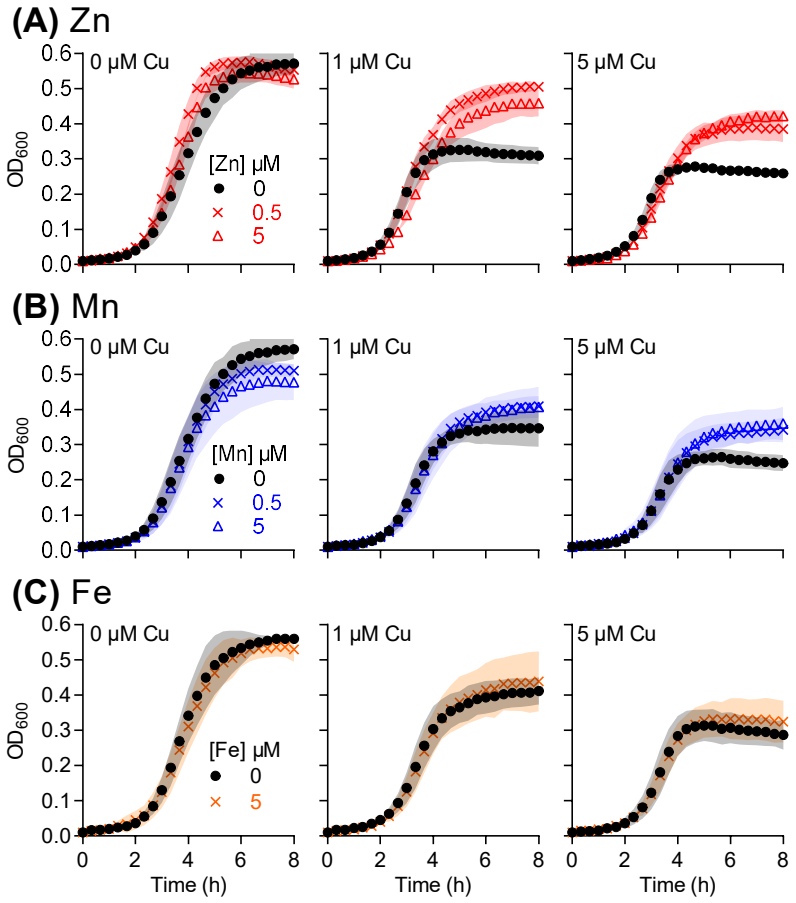

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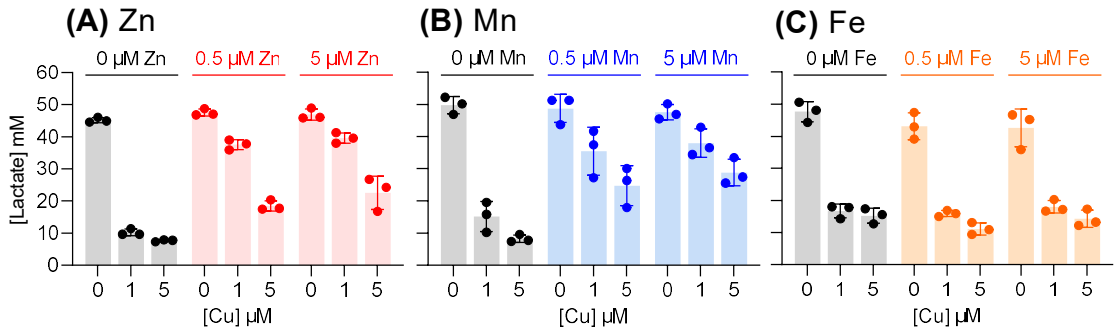
267 **Figure 6. Effects of (A) Zn, (B) Mn, and (C) Fe co-supplementation on bacterial growth.** The

268 GAS 5448Δ*copA* mutant strain was cultured with added Cu (0, 1, or 5 μM), with or without added Zn,

269 Mn, or Fe (0, 0.5, or 5 μM) for t = 8 h (N = 3).

270



271

272 **Figure 7. Effects of (A) Zn, (B) Mn, and (C) Fe co-supplementation on homolactic fermentation.**

273 The GAS 5448Δ*copA* mutant strain was cultured with added Cu (0, 1, or 5 μM), with or without added

274 Zn, Mn, or Fe (0, 0.5, or 5 μM) for *t* = 24 h (*N* = 3). Amounts of secreted lactate were measured in the
275 spent culture medium.
276
277 **Supplementation with Zn or Mn does not suppress cellular Cu levels and availability.**
278
279 Cu stress in GAS Δ*copA* cultures is linked with a reduction in the activity of the central
280 glycolytic enzyme, namely the phosphorylating, ATP-generating, GAPDH enzyme, GapA[18]. This
281 enzyme is likely mismetalated by excess cytoplasmic Cu ions, which may bind to the side chains of
282 the catalytic Cys and a nearby His[31]. The observed improvement in bacterial growth and lactate
283 secretion in response to Zn or Mn co-supplementation (*cf.* Figures 6 and 7) hints at an improvement
284 in GapA activity. However, Figure 8 clearly shows that Zn or Mn co-supplementation does not
285 improve the activity of GapA in Cu-supplemented Δ*copA* cultures. Therefore, the putative link
286 between Cu stress and Zn or Mn homeostasis is likely independent of GapA.
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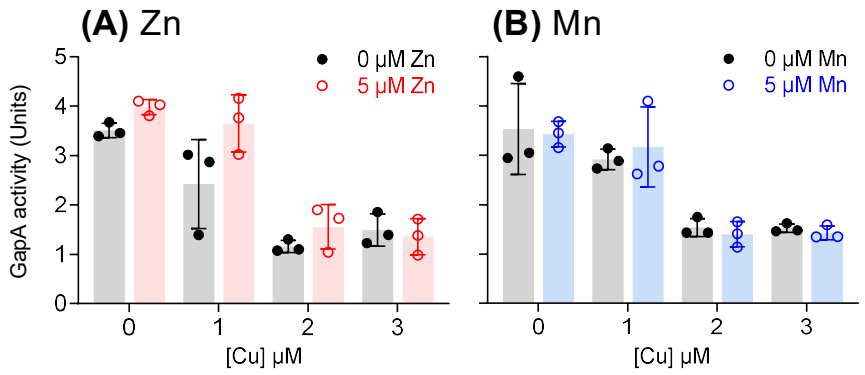

288
289 **Figure 8. Effects of (A) Zn and (B) Mn co-supplementation on GapA activity.** The GAS
290 5448Δ*copA* mutant strain was cultured with added Cu (0, 1, 2, or 3 μM), with or without added Zn or
291 Mn (5 μM each) for *t* = 8 h (*N* = 3). GapA activities were determined in cell-free extracts.
292
293 The observations that: (i) GapA remained inhibited and (ii) Cu levels in Cu-treated cells
294 remained unchanged (*cf.* Figure 5), despite co-supplementation with Zn or Mn, suggest that neither
295 Zn nor Mn exerts a protective effect by suppressing Cu uptake. To more sensitively measure cellular
296 Cu availability, we assessed expression of the *copZ* gene. This gene was upregulated in a dose-
297 dependent manner during growth in the presence of supplemental Cu (Figure 9). However, co-

298 supplementation with Zn or Mn did not appear to perturb this Cu-dependent expression of *copZ*
299 (Figure 9). These results strengthen our proposal that cellular Cu availability remains high and that Zn
300 or Mn does not exert a protective effect by suppressing Cu uptake.
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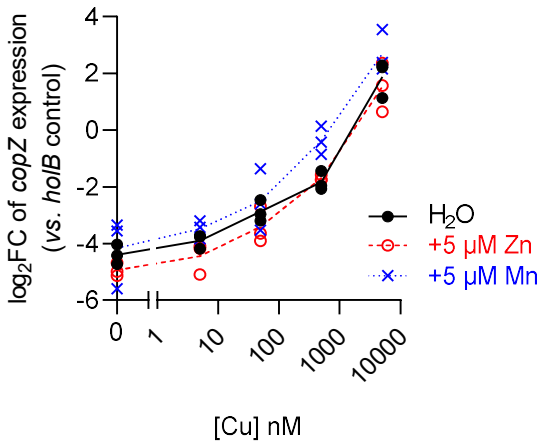


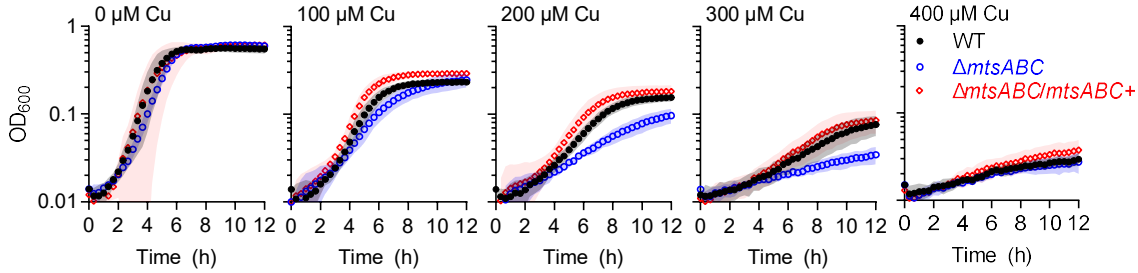
302
303 **Figure 9. Effects of Zn or Mn supplementation on Cu-dependent expression of the *copZ* gene.**
304 The GAS 5448Δ*copA* mutant strain was cultured with added Cu (0 – 5000 nM), with or without added
305 Zn or Mn (5 μM each) for t = 8 h (N = 3). Levels of *copZ* mRNA in these samples were determined by
306 qRT-PCR and normalised to expression of *holB* as the control.
307
308 **MtsABC or AdcAI/AdcBC does not promote uptake of Cu into GAS.**
309
310 Our results appear to differ from those reported recently in *Staphylococcus aureus*. In *S.*
311 *aureus*, MntABC, a Mn-importing ABC transporter, is thought to promote uptake of Cu into the
312 cytoplasm[32]. Expression of *mntABC* in *S. aureus* is controlled transcriptionally by the Mn-sensing
313 derepressor MntR. A decrease in MntABC production (and activity), whether by deletion of the *mntA*
314 gene or by transcriptional repression of the *mntABC* operon in response to Mn co-supplementation,
315 leads to a reduction in cellular Cu levels[32].
316 If the MtsABC transporter in GAS and, by extension, the AdcAI/II-AdcBC transporter takes up
317 Cu into the GAS cytoplasm, then supplemental Mn or Zn may alleviate Cu stress by repressing the
318 *mtsABC* or *adcAI/II-adcBC* genes and suppressing Cu uptake *via* their protein products. Similarly,

319 mis-repression of *mtsABC* or *adcAI/II-adcBC* genes by excess Cu potentially suppresses further Cu
320 uptake and self-limits the toxicity of this metal.

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

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

333



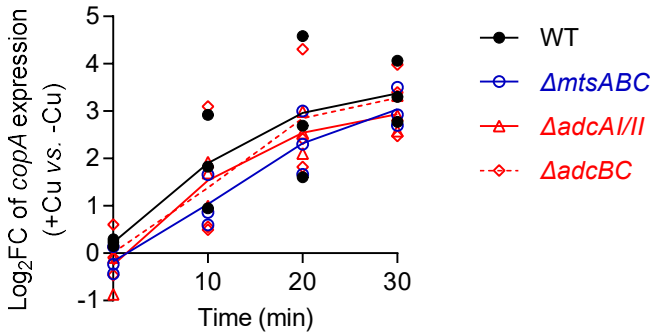
334

335 **Figure 10. Effects of Cu supplementation on growth of various mutant strains.** The GAS 5448
336 wild-type (WT, filled circles) and Δ*copA* (crosses), Δ*adcAI/II* (triangles), Δ*adcBC* (diamonds), and
337 Δ*mtsABC* (circles) mutant strains were cultured with added Cu (0, 100, 200, 300, or 400 μM) for t = 12
338 h (N = 3).

339

340 The presence of a functional CopA efflux pump in all the strains used in Figure 10 may mask
341 the inhibitory effects of Cu on bacterial growth. However, despite screening thousands of
342 transformants, the double mutant strains Δ*copA*Δ*adcAI/II*, Δ*copA*Δ*adcBC*, and Δ*copA*Δ*mtsABC* were
343 not obtained. Therefore, to assess the potential role of AdcAI/II-AdcBC or MtsABC in Cu uptake, we

344 assessed whether the loss of these transporters reduce Cu availability in the cytoplasm, specifically
345 by examining the expression of *copA* in the single knockout mutant strains generated earlier.
346 In this final experiment, the wild-type and mutant strains were cultured for 8 h in the absence
347 of Cu. Each culture was subsequently exposed to 500 nM Cu and sampled at 10 min intervals for up
348 to 30 min. If the ABC transporters take up Cu as hypothesised, then we would observe a decrease
349 and/or a time-dependent delay in *copA* expression in knockout mutant strains that lack these ABC
350 transporters when compared with the wild type. However, Figure 11 shows that there was negligible
351 difference in the expression profile of *copA* in the various strains. Therefore, there is currently no
352 experimental evidence that supports the uptake of Cu *via* the MtsABC or AdcAI/II-AdcBC transporter
353 in GAS.
354



355
356 **Figure 11. Effects of ABC transporters on cellular Cu availability.** The GAS 5448 wild-type (WT,
357 filled circles), Δ*adcAI/II* (triangles), Δ*adcBC* (diamonds), and Δ*mtsABC* (circles) were cultured with
358 added Cu (0 or 500 nM), with or without added Zn or Mn (5 μM each), for t = 8 h (N = 3). Levels of
359 *copA* mRNA in these samples were determined by qRT-PCR and normalised to expression of *holB* as
360 the control.
361
362 **DISCUSSION**
363
364 **Cu stress in GAS perturbs Zn and Mn homeostasis**
365
366 This work strengthens and extends our previous observation that growth in the presence of
367 excess Cu leads to mis-regulation of cellular Zn and Mn in GAS[18]. In the case of Zn, Cu treatment

368 leads to mis-repression of Zn uptake genes and, subsequently, cellular Zn depletion in Cu-stressed
369 cells. Cu treatment also leads to mis-regulation of Mn uptake genes. However, there is currently little
370 evidence that Cu-stressed cultures are also Mn-limited. Thus, the relationship between Cu stress and
371 Mn homeostasis in GAS is less defined.

372 Cross-talks between Cu stress and Zn homeostasis have been reported in other bacteria,
373 although the molecular details differ. These differences may reflect inherent differences in the
374 biochemistry of the different metal homeostasis systems in different organisms. Alternatively, they
375 may reflect differences in experimental design and setup, leading to different degrees of Cu stress.
376 For instance, in *S. pneumoniae*, supplemental Zn exacerbates (rather than alleviates) Cu stress in a
377 Δ*copA* mutant strain[33] and in a Δ*czcD* mutant strain lacking the primary Zn efflux transporter[34]. Here,
378 excess Zn in the cytoplasm is thought to bind to the allosteric sensing site of the Cu sensor CopY,
379 stabilise the repressor form of this sensor, and thus suppress transcriptional sensing of Cu[16]. Based
380 on the patterns of *copZ* expression in Figure 9, there is no evidence that supplemental Zn perturbs
381 transcriptional Cu sensing in GAS, at least under the experimental conditions employed here.

382 As another example, Cu treatment in *Salmonella* leads to upregulation (and not
383 downregulation) of Zn uptake genes under the control of the Zn sensor Zur[4]. Whether Cu treatment
384 also perturbs Zn levels in this organism has not been reported. In *Acinetobacter baumanii*,
385 supplemental Cu does not perturb Zn levels in wild-type or Δ*copA* mutant strains[35]. However,
386 supplemental Zn does lead to a slight decrease in cellular Cu levels in the wild-type strain[36]. The
387 molecular mechanism is unclear, but several putative metal transporter genes are differentially
388 regulated in response to Zn, potentially leading to increased efflux or decreased uptake of Cu from
389 the cytoplasm. This scenario would resemble that reported in *Escherichia coli*. Supplemental Zn
390 alleviates Cu stress and decreases cellular Cu levels in the *E. coli* wild type and Δ*cueO* mutant
391 strains[37,38]. In this case, Zn supplementation leads to mis-*activation* of the *cusCFBA* operon encoding
392 an RND-family Cu efflux transporter, and thus a lowering of cellular Cu[38]. As stated earlier, there is no
393 evidence that supplemental Zn perturbs transcription of Cu homeostasis genes in GAS.

394 This work further shows that supplemental Mn partially alleviates Cu stress in the GAS Δ*copA*
395 mutant strain, even though Cu-stressed cells do not appear to be Mn-limited. Similar to our findings,
396 growth of Cu-stressed Δ*copA* mutant strains of *S. pneumoniae* is improved by co-supplementation
397 with Mn[33]. The excess Cu in this organism is hypothesised to inhibit the Mn-dependent ribonucleotide

398 reductase NrdF. Therefore, co-supplementation with Mn is hypothesised to restore NrdF activity[33]. Cu
399 may similarly inhibit NrdF in GAS. However, loss of NrdF activity is likely only a minor component of
400 Cu stress in GAS, since co-supplementation with Mn is less protective than co-supplementation with
401 Zn (*cf.* Figure 6).

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

413

414 **What are the likely mechanisms of Cu stress in GAS?**

415

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

423 Interestingly, this work further suggests that, contrary to our previous proposal[18], inactivation
424 of GapA is not a key mechanism of Cu stress in GAS. Growth in the presence of supplemental Cu
425 was confirmed here to promote a reduction in GapA activity in the Δ*copA* mutant strain. However, co-
426 supplementation with Zn did not rescue GapA activity (*cf.* Figure 8), even though it rescued bacterial
427 growth (*cf.* Figure 6) and production of the fermentation end-product lactate (*cf.* Figure 7). This

428 apparent uncoupling between GapA activity and Cu stress suggests that a loss in GapA activity may
429 be a symptom but not a cause of Cu stress (in the same manner that an increase in *copZ* expression
430 is a symptom but not a cause of Cu stress).

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

440

441 **Do ABC transporters promote Cu uptake into GAS?**

442

443 Our work further suggests that neither the Zn-importing AdcAI/II-AdcBC transporter nor the
444 Mn-importing MtsABC transporter promotes uptake of Cu into GAS. To take up a metal ion, the
445 extracytoplasmic solute binding protein (SBP) domain captures its cognate metal ion and
446 subsequently releases it to the metal-binding site in the permease domain. In turn, the permease
447 internalises the metal ion into the cytoplasm and this action is powered by ATP hydrolysis by the
448 nucleotide-binding domain. Unpublished studies in our laboratory suggest that AdcAI, the Zn-binding
449 SBP from GAS, binds Cu(II) more tightly than it binds Zn(II). Although the AdcAII SBP from GAS has
450 not been biochemically characterised, the homologue from *S. pneumoniae* has also been reported to
451 bind Cu(II)[43]. Likewise, the Mn-binding MtsA SBP from GAS binds Cu(II)[44], as does PsaA, the MtsA
452 homologue from *S. pneumoniae*[45].

453 Our data suggest that the bound Cu(II) in any of the above SBPs is not transferred to the
454 metal-coordinating site in the partner permease and subsequently internalised into the cytoplasm.
455 There is evidence that an SBP does not load the permease with non-cognate metal ions, a result of
456 incompatible coordination chemistry between the partners. For example, extracellular Zn
457 competitively inhibits Mn uptake *via* PsaABC in *S. pneumoniae*[8,9]. The permeases PsaB (which

458 imports Mn) and AdcB (which imports Zn) in this organism possess the same, conserved metal
459 coordination site[46], suggesting that PsaB should be competent to receive Zn from PsaA. However,
460 while PsaC efficiently releases the bound Mn to PsaB, it does not release bound Zn[45]. However,
461 whether Zn can access the metal-binding site in PsaB directly, without the PsaA SBP, is unknown.
462 Similarly, whether excess extracellular Cu can bind directly to the metal-binding site in the Zn-
463 importing permease AdcB or the Mn-importing permease MtsC and become subsequently
464 internalised into the cytoplasm is unknown. Our data do not support this hypothesis, but direct
465 biochemical support, for instance *via* metal transport assays of purified transporters, remains to be
466 obtained.
467
468 **AUTHOR CONTRIBUTIONS**
469
470 KD initiated and designed the research. KD also had overall responsibility for the
471 conceptualisation and coordination of the programme. KD and LS measured bacterial growth, GapA
472 activity, and cellular metal levels. JB and KD measured gene expression levels. EM and KW
473 measured SodA activity. SF and YH carried out literature review and preliminary experiments leading
474 to the final work shown here. KD drafted the manuscript with input from SF and YH. KD, YH, and EM
475 prepared the figures. KD, SF, YH, EM, and KW edited the manuscript. All authors approved the final
476 version.
477
478 **ACKNOWLEDGEMENT**
479
480 We thank Dr Peter Chivers (Department of Biosciences, Durham University) for helpful
481 discussions related to this project. Dr Christopher Ottley (Department of Earth Sciences, Durham
482 University) provided technical assistance with ICP MS. The Δ*mtsABC*, Δ*adcAI/II*, and Δ*adcBC* mutant
483 strains are kind gifts from Dr Andrew Turner, Dr Cheryl-lynn Ong, Prof. Alastair McEwan, and Prof.
484 Mark Walker (School of Chemistry and Molecular Biosciences, The University of Queensland).
485
486 **FUNDING SOURCES**
487

488 LS was funded by a Wellcome Trust Seed Award (214930/Z/18/Z) to KD. Preliminary work
489 leading to this study was financially supported by a Royal Society Research Grant (RSG\R1\180044)
490 and a Department of Biosciences (Durham University) Start-up Funds to KD. JB, SF, EM were
491 supported by studentships from the Biotechnology and Biological Sciences Research Council
492 Newcastle-Liverpool-Durham Training Partnership.
493
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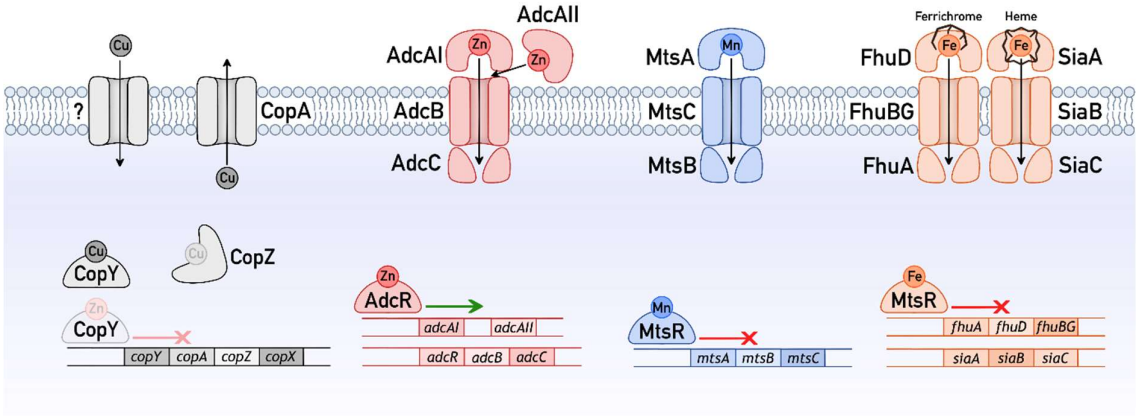
607

608 **GRAPHICAL ABSTRACT**



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