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1 **Copper intoxication in group B *Streptococcus* triggers transcriptional activation
2 of the *cop* operon that contributes to enhanced virulence during acute infection**

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10

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22 **Abstract**

23 Bacteria require Copper (Cu) as an essential trace element to support cell processes;
24 however, excess Cu can intoxicate bacteria. Here, we characterize the *cop* operon in
25 group B *Streptococcus* (GBS), and establish its role in evasion of Cu intoxication and
26 the response to Cu stress on virulence. Growth of GBS mutants deficient in either the
27 *copA* Cu exporter, or the *copY* repressor, were severely compromised in Cu-stress
28 conditions. GBS survival of Cu stress reflected a mechanism of CopY activation of the
29 CopA efflux system. However, neither mutant was attenuated for intracellular survival in
30 macrophages. Analysis of global transcriptional responses to Cu by RNA-sequencing
31 revealed a stress signature encompassing homeostasis of multiple metals. Genes
32 induced by Cu stress included putative metal transporters for manganese import,
33 whereas a system for iron export was repressed. In addition, *copA* promoted the ability
34 of GBS to colonize the blood, liver and spleen of mice following disseminated infection.
35 Together, these findings show that GBS *copA* mediates resistance to Cu intoxication,
36 via regulation by the Cu-sensing transcriptional repressor, *copY*. Cu stress responses in
37 GBS reflect a transcriptional signature that heightens virulence and represents an
38 important part of the bacteria's ability to survive in different environments.

39

40 **Keywords:** Metallobiology; group B *Streptococcus*; Metal ions; Copper efflux; Bacterial
41 pathogenesis

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42 **Introduction**

43 Copper (Cu) is the most reactive of the biologically-relevant first-row *d*-block transition
44 metals. It can readily displace other metals, including Zinc (Zn), Nickel (Ni), Cobalt (Co),
45 Iron (Fe) and Manganese (Mn) from metalloproteins in which these are bound (1, 2). In
46 cells across all Kingdoms of life, Cu-dependent enzymes are pivotal to many essential
47 processes that underpin physiologic biochemical reactions, owing to its ability to redox
48 cycle between Cu(I) and Cu(II) states (3). When present in excess, however, Cu is
49 hazardous to cellular processes and macromolecules due to effects of localized free-
50 radical damage (4). A key role for Cu in bacterial cell biology encompasses the host-
51 pathogen interface where a balance between Cu usage and avoidance of Cu
52 intoxication resulting from host antimicrobial responses must be attained for bacteria to
53 survive, as reviewed elsewhere (5). At this interface, human immune cells can mobilize
54 Cu in a defence response to infection that culminates in exposure of intracellular
55 bacteria to antimicrobial levels of Cu that kill the invading pathogen (6-9). Thus,
56 bacterial responses to Cu stress and mechanisms of resistance to metal-ion intoxication
57 have emerged as important facets of bacterial disease pathogenesis (10).
58
59 There are several mechanisms that enable bacteria to tolerate excess extracellular Cu,
60 and these have been characterized in several pathogenic species. These mechanisms,
61 which represent survival strategies against Cu intoxication (10) typically involve Cu-
62 transporting P-type ATPases, exemplified by CopA, which detoxify Cu by exporting it
63 from the bacterial cytosol (11-13). This mechanism has been described in gram-

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64 negative and gram-positive bacteria, and among *Streptococcaceae* (14-16), including
65 pneumococci and *Streptococcus pyogenes* (17).
66
67 An opportunistic streptococcal pathogen of humans and animals for which resistance to
68 excess extracellular Cu has not been described is *Streptococcus agalactiae*, also known
69 as group B streptococcus (GBS). This organism, unlike other *Streptococcaceae*, is
70 associated with a comparatively broad host-range that encompasses humans, cattle
71 and fish (18). In humans, GBS is a major cause of invasive infection in infants <3
72 months of age (19), and causes a more diverse range of disease aetiologies compared
73 to other streptococci, including meningitis, pneumonia, skin and soft-tissue infections,
74 sepsis, arthritis, osteomyelitis, urinary tract infection, and endocarditis (19). GBS has a
75 several virulence factors that enable survival in cytotoxic environments, such as acid
76 stress, oxidative stress, and during host colonization, as reviewed elsewhere (20).
77 Cellular tolerance to Cu stress and mechanisms of responding to and surviving Cu
78 intoxication have not been defined in GBS.
79
80 In this study, we characterise a system that enables Cu efflux in GBS, encompassing a
81 *copA*-homologue, which we show mediates control of Cu efflux in the bacteria. We
82 show this system affects survival, growth and virulence of GBS in the mammalian host.
83

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84 **Results**

85 ***Transcriptomic profiling of GBS responses to extracellular Cu***

86 To dissect the global response of GBS to extracellular Cu stress we performed RNA
87 sequencing (RNASeq) to define the complete primary transcriptional response to Cu.
88 RNASeq analysis of mid-log phase cells of GBS strain 874391 exposed to 0.5 mM Cu
89 (a sub-inhibitory level; below) compared to non-exposed controls revealed a surprisingly
90 modest Cu-responsive transcriptome. The response comprised 18 transcriptional
91 responses, defined as significant based on $-2 \leq$ FC ≥ 2 (FDR < 0.05, n=4 biological
92 replicates), of 11 up-regulated transcripts and 7 down-regulated transcripts (Figure 1,
93 Table 1). The most significantly up-regulated were homologues of the *copY-copA-copZ*
94 gene cluster (3.6-4.0-fold), which encode the putative CopY (Cu-binding transcriptional
95 repressor), CopA (P-type ATPase Cu-efflux system) and CopZ (Cu-chaperone) proteins.
96 To validate the expression levels of selected targets identified in RNASeq, we targeted
97 *pcl1*, *hvgA* and *copY* in qRT-PCR assays, confirming near-identical fold-change values
98 to RNASeq analyses (Figure 1B). The products of *copY*, *copA* and *copZ* in GBS (Figure
99 2A) are moderately conserved compared to other Lactobacillales, including *S. mutans,*
100 *S. thermophilus, S. pyogenes, S. pneumoniae*, and *Enterococcus* (Figure 2B, ranked by
101 CopA similarity) for which a homologous Cu management system is defined (9, 13-15).
102 Together, these findings provide transcriptional and comparative insights that support a
103 proposed model of Cu efflux in GBS (Figure 2C).
104
105 In addition to *copYAZ*, GBS up-regulated putative metal transporters for Mn import
106 (*mtsABC*, *mntH2*), and down-regulated an iron (Fe) export system *(fetAB;* Figure 1,

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107 Table 1). In addition, we detected significant down-regulation of *pcl1*, that encodes a
108 putative membrane protein, and the gene for hypervirulence-associated factor, *hvgA*.
109

110 ***Roles of copA and copY in Cu-resistance in GBS***

111 To provide molecular functional characterization of two of the major elements of the Cu-
112 responsive transcriptome in GBS, we targeted *copA* and *copY* by generating isogenic
113 deletion mutants and performing comparative growth assays with the WT. In a nutrient-
114 rich medium (Todd Hewitt Broth; THB) supplemented with increasing amounts of Cu,
115 we detected no significant attenuation effect of high Cu levels (up to 1.5 mM) on the
116 growth of WT GBS; the lag phases, growth rates, and final biomass yields were
117 equivalent for the WT exposed to different levels of Cu (Figure 3). In contrast, Δ*copA*
118 GBS exhibited a major attenuation for growth in conditions of Cu stress ≥ 1 mM.
119 Deletion of *copY*, encoding a putative Cu-dependent repressor of *copA*, had no effect
120 on the growth rate or lag phase in THB supplement with Cu (Figure 3). Taken together,
121 these data establish that *copA* confers cellular resistance to Cu stress in GBS in a
122 manner that supports bacterial growth in nutritive conditions.
123

124 ***Temporal- and Concentration-Dependent Bactericidal effects of Cu towards GBS***

125 To more precisely define the toxicity effects of Cu towards GBS, we performed time-kill
126 curves using 1-5 x 10^6 CFU/mL exposed to Cu concentrations ranging between 50 μM
127 and 1 mM. We used a minimal Chemically-Defined Medium (CDM) in the context of prior
128 studies of antibacterial activity in minimal media (21, 22), that antibacterial activity in such
129 assays can be strongly dependent on the culture media (23, 24), and to minimize cell-

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130 protective effects that can result from some buffering agents, such as glutathione (15).
131 We found potent killing effects that depended on both concentration and time with ≥ 0.5
132 mM Cu significantly killing WT GBS after 6h of exposure (Figure 4, WT); after 24h, there
133 was a ~230-fold reduction in CFU/mL at 0.5 mM Cu (0 mM Cu = 5.6 Log_{10} CFU/mL, 0.5
134 mM CFU/mL = 3.2 Log_{10} CFU/mL), and no viable GBS remained in cultures exposure to
135 1 mM Cu. The Δ*copA* strain was significantly more susceptible to Cu toxicity compared
136 to the WT; Cu levels above ≥ 0.5 mM significantly reduced the number of viable Δ*copA*
137 GBS beginning as early as 1h exposure (Figure 4, Δ*copA*); the degree of bacterial killing
138 was dramatic at the 24h timepoint for ≥ 0.5 mM Cu (Figure 4). Complementation of the
139 *copA* mutation *in trans* restored the phenotype to WT levels of growth (Supplementary
140 Figure S1). Interestingly, at the 6h timepoint, high Cu (≥ 0.5 mM) did not affect viability
141 of Δ*copY* GBS, compared to the WT strain (Figure 4, Δ*copY*), a hyper-resistance
142 phenotype that was not apparent at the 24 h timepoint. Together, these findings are
143 consistent with a role for CopA in resisting Cu stress and CopY as a repressor of Cu
144 resistance.
145
146 ***Regulation of Cu efflux by CopY, and accumulation of metals during Cu stress***
147 We next sought to identify regulatory mechanisms controlling the Cu-response in GBS
148 at the genetic level. The capacity of Cu stress to induce expression of *copA* for Cu
149 export was examined by analyzing *copA* expression by qRTPCR in GBS exposed to Cu
150 Cu concentrations ranging from 0.25-1.5 mM Cu in THB. GBS significantly upregulated
151 *copA* in response to Cu (3.7-fold – 14.2-fold) in a manner that was titratable with the Cu
152 concentration (Figure 5A). To ascertain the role of CopY as a putative Cu-responsive

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153 repressor of *copA* expression in GBS, we quantified *copA* mRNA transcripts in the
154 Δ*copY* mutant exposed to 0.5 mM Cu. This level of Cu was carefully chosen as sub-
155 inhibitory to enable comparisons independent of metabolic state and therefore limiting
156 any potential bias from possible discordant Cu stress between WT and mutants with
157 varied resistance phenotypes. Deletion of *copY* impacted final biomass yield of cultures
158 compared to WT (Figure 5B), and resulted in severe de-regulation of *copA* expression,
159 causing a ~207±45-fold increase of *copA* transcripts in the *copY* background (Figure
160 5C). Thus, *copA* responds transcriptionally to extracellular Cu, and GBS *copY* functions
161 to repress *copA* in the absence of Cu.
162
163 To examine the impact of *copA* and *copY* mutations on accumulation of Cu within the
164 cell we used the equivalent conditions per RNASeq and qPCR assays, exposing GBS
165 to 0.5 mM Cu in THB and measuring total Cu content of cells compared to non-exposed
166 controls. Inductively coupled optical emission spectroscopy (ICP-OES) demonstrated
167 that standard THB contained 0.2±0.08 μM Cu, reflecting trace amounts in the medium.
168 In the absence of supplemental Cu, WT GBS limited intracellular Cu content such that
169 only 0.8±0.1 μg Cu. g dry weight^{-1} were detected in cultures grown in THB. However,
170 exposure of WT GBS to 0.5 mM Cu resulted in a dramatic increase in intracellular Cu to
171 31.9±3.1 μg Cu. g dry weight^{-1} (Figure 5D). Strikingly, Δ*copY* GBS exhibited significantly
172 less cellular Cu upon exposure to Cu (6.7±0.4 μg Cu. g dry weight^{-1}), consistent with the
173 observation that transcription of the Cu-exporter CopA is significantly elevated in this
174 mutant. In addition, we noted massive accumulation of cellular Cu in the Δ*copA* strain
175 (109.6±1.9 μg Cu. g dry weight^{-1}), confirming a Cu-efflux role for *copA*. Interestingly, we

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176 noted no changes in other metals, including Zn, Mn and Fe (data not shown) in WT
177 versus Δ*copA* GBS, consistent with a report in *S. pneumoniae* (14).
178
179 ***Role of CopA in macrophage killing of GBS***
180 To examine whether the CopA Cu efflux system in GBS supports survival of the bacteria
181 in phagocytes we performed antibiotic protection assays with murine macrophages and
182 human monocyte-derived macrophage-like cells. Macrophages were infected with WT
183 or Δ*copA* GBS for 1h, and antibiotics were added to kill extracellular bacteria. Viable
184 intracellular GBS were quantified at 1h post-antibiotic addition, and at 24h and 48h.
185 These assays demonstrated significant reduction in the numbers of viable GBS over the
186 time course (24 to 48h) in both human and murine macrophages; however, there were
187 no significant differences detected between the numbers of WT and Δ*copA* GBS
188 recovered from macrophages at any time point (Figure 6). Thus, under these conditions,
189 *copA* does not contribute to the intracellular survival of GBS in macrophages.
190
191 ***GBS CopA contributes to virulence* in vivo**
192 To examine the contribution of Cu efflux to GBS virulence, we used a murine model of
193 disseminated infection (25). In mice challenged with 10^7 GBS, we detected significantly
194 fewer Δ*copA* mutant in the liver (median of 3.5 vs 4.2 $\log_{10}$ CFU.g tissue^{-1}; P= 0.005),
195 spleen (median of 3.8 vs 4.1 $\log_{10}$ CFU.g tissue^{-1} P= 0.013) and blood (median of 1.4 vs
196 1.9 $\log_{10}$ CFU.mL^{-1}; P= 0.044) compared to the WT at 24h post-inoculation (Figure 7).
197 No differences were observed between counts of the WT and Δ*copA* mutant in the
198 brain, heart, lungs, or kidneys (data not shown). These data support a modest but

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199 statistically significant role for cellular management of Cu via *copA* in supporting
200 disseminated infection *in vivo*.
201

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202 ## Materials and Methods

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203 **Bacterial strains, plasmids and growth conditions**

204 GBS, *E. coli* and plasmids used are listed in Table 2. GBS was routinely grown in Todd-
205 Hewitt Broth (THB) or on TH agar (1.5% w/v). *E. coli* was grown in Lysogeny Broth (LB)
206 or on LB agar. Media were supplemented with antibiotics (spectinomycin (Sp)
207 100µg/mL; chloramphenicol (Cm) 10 µg/mL), as indicated. Growth assays used 200µL
208 culture volumes in 96-well plates (Greneir) sealed using Breathe-Easy® membranes
209 (Sigma Aldrich) and measured attenuance (*D*, at 600nm) using a ClarioSTAR
210 multimode plate reader (BMG Labtech) in Well Scan mode using a 3mm 5x5 scan
211 matrix with 5 flashes per scan point and path length correction of 5.88mm, with agitation
212 at 300rpm and recordings taken every 30min. Media for growth assays were THB and a
213 modified Chemically-Defined Medium (CDM) (26) (with 1g/L glucose, 0.11g/L pyruvate
214 and 50µg/L L-cysteine), supplemented with Cu (supplied as $CuSO_4$) as indicated. For
215 attenuance baseline correction, control wells without bacteria were included for Cu in
216 media alone.

217 **DNA extraction and genetic modification of GBS**

218 Plasmid DNA was isolated using miniprep kits (QIAGEN), with modifications for GBS as
219 described elsewhere (27). Deletions in *copA* (CHF17_00507 / CHF17_RS02570) and
220 *copY* (CHF17_00506 / CHF17_RS02565) *were* constructed by markerless allelic
221 exchange using pHY304aad9 as described previously (28). Plasmids and primers are
222 listed in Table 2 and Supplementary Table S1, respectively. Mutants were validated by
223 PCR using primers external to the mutation site and DNA sequencing.

224 **RNA extraction, qRTPCR**

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225 For Cu exposure experiments, 1mL overnight THB cultures were back-diluted 1/100 in
226 100mL of THB (prewarmed at 37°C in 250mL Erlenmeyer flasks) supplemented with
227 0.25, 0.5, 1.0 or 1.5mM Cu. Cultures were grown shaking (200rpm) at 37°C; after
228 exactly 2.5h, 10-50mL volumes containing approximately 500 million mid-log bacteria
229 were harvested; RNA was preserved and isolated as described previously (29). RNA
230 quality was analysed by RNA LabChip using GX Touch (Perkin Elmer). RNA (1000ng)
231 was reverse-transcribed using Superscript IV according to manufacturer's instructions
232 (Life Technologies) and cDNA was diluted 1:50 in water prior to qPCR. Primers
233 (Supplementary Table S1) were designed using Primer3 Plus (30, 31) to quantify
234 transcripts using Universal SYBR Green Supermix (Bio-Rad) using a Quantstudio 6
235 Flex (Applied Biosystems) system in accordance with MIQE guidelines (32). Standard
236 curves were generated using five-point serial dilutions of genomic DNA (5-fold) from WT
237 GBS 874391 (33). Expression ratios were calculated using C_T values and primer
238 efficiencies as described elsewhere (34) using *dnaN*, encoding DNA polymerase III β-
239 subunit as housekeeper.

240 **Whole bacterial cell metal content determination**

241 Metal content in cells was determined as described (35) with minor modifications.
242 Cultures were prepared essentially as described for *RNA extraction, qRTPCR* with the
243 following modifications; THB medium was supplemented with 0.5 mM $CuSO_4$ or not
244 supplemented (Ctrl), and following exposure for 2.5h, bacteria were harvested by
245 centrifugation at 4122 x g at 4°C. Cell pellets were washed 3 times in PBS + 5mM
246 EDTA to remove extracellular metals, followed by 3 washes in PBS. Pelleted cells were
247 dried overnight at 80°C and resuspended in 1mL of 32.5% nitric acid and incubated at

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248 95°C for 1h. The metal ion containing supernatant was collected by centrifugation
249 (14,000 x g, 30min) and diluted to a final concentration of 3.25% nitric acid for metal
250 content determination using inductively coupled plasma optical emission spectroscopy
251 (ICP-OES). ICP-OES was carried out on an Agilent 720 ICP-OES with axial torch,
252 OneNeb concentric nebulizer and Agilent single pass glass cyclone spray chamber. The
253 power was 1.4kW with 0.75L/min nebulizer gas, 15L/min plasma gas and 1.5L/min
254 auxiliary gas flow. Cu was analyzed at 324.75nm, Zn at 213.85nm, Fe at 259.94nm and
255 Mn at 257.61nm with detection limits at <1.1ppm. The final quantity of each metal was
256 normalised using dry weight biomass of the cell pellet prior to nitric acid digestion,
257 expressed as $\mu g.g^{-1}$dry weight. Baseline concentrations were determined to be 0.2 ±
258 0.08 μM Cu in THB medium, and 40 ± 4 nM Cu in CDM medium from at least three
259 independent assays.

260 **RNA sequencing and bioinformatics**

261 Cultures were prepared as described above for *RNA extraction, qRTPCR* to compare
262 mid-log phase cells grown in THB + 0.5 mM Cu to THB without added Cu. RNase-free
263 DNase-treated RNA that passed Bioanalyzer 2100 (Agilent) analysis was used for RNA
264 sequencing (RNA-seq) using the Illumina NextSeq 500 platform. We used TruSeq
265 library generation kits (Illumina, San Diego, California). Library construction consisted of
266 random fragmentation of the poly(A) mRNA, followed by cDNA production using random
267 primers. The ends of the cDNA were repaired and A-tailed, and adaptors were ligated
268 for indexing (with up to 12 different barcodes per lane) during the sequencing runs. The
269 cDNA libraries were quantitated using qPCR in a Roche LightCycler 480 with the Kapa
270 Biosystems kit (Kapa Biosystems, Woburn, Massachusetts) prior to cluster generation.

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271 Clusters were generated to yield approximately 725K–825K clusters/mm^2. Cluster
272 density and quality was determined during the run after the first base addition
273 parameters were assessed. We ran paired-end 2 × 75–bp sequencing runs to align the
274 cDNA sequences to the reference genome. For data preprocessing and bioinformatics,
275 STAR (version 2.7.3a) was used to align the raw RNA sequencing fastq reads to the
276 WT GBS 874391 reference genome (33). HTSeq-count, version 0.11.1, was used to
277 estimate transcript abundances (36). DESeq2 was then used to normalized and test for
278 differential expression and regulation. Genes that met certain criteria (i.e. fold change of
279 > ±2.0, q value (false discovery rate, FDR of <0.05) were accepted as significantly
280 altered (37). Raw and processed data were deposited in Gene Expression Omnibus
281 (accession no. GSE161127).

282 **Mammalian cell culture**

283 J774A.1 murine macrophages or U937 human monocyte-derived macrophages (MDMs)
284 were grown in RPMI and seeded (10^5) into the wells of a 96-well tissue culture-treated
285 plate (Falcon) essentially as described elsewhere (38, 39), except that U937 MDMs
286 were differentiated by exposure to 30ng/mL phorbol 12-myristate 13-acetate (PMA) for
287 48h and cells subsequently rested in media without PMA for 72h to enhance
288 morphological and phenotypic markers of MDMs (40). A multiplicity of infection (MOI) of
289 100 bacteria: macrophage for 1h was used in RPMI without antibiotics. Non-adherent
290 bacteria were removed by five washes of 200µL PBS using a Well Wash Versa (Thermo
291 Scientific). RPMI containing 250U/mL penicillin, streptomycin (Gibco) and 50µg/mL
292 gentamicin (Sigma-Aldrich) were used for antibiotic protection assays to kill extracellular
293 bacteria as described previously by incubating for 1h at 37°C in 5% CO_2 (39). Samples

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294 were processed after 1h (time zero), 24h or 48h after infection, monolayers were
295 washed five times with 200µL PBS and lysed by brief exposure to 50µL of 2% trypsin
296 and 0.02% Triton-X-100 (10min) prior to dilution with 150µL PBS and estimation of
297 CFU/mL by serial dilution and plate counts on agar. Relative CFU (rCFU) was
298 determined as described previously (41) as follows; rCFU= CFU.mL^{-1} at 24h or 48h /
299 CFU.mL^{-1} at time zero.

300 **Animals and Ethics statement**

301 Virulence was tested using a mouse model of disseminated infection based on
302 intravenous challenge with 10^7 GBS (WT or ΔcopA) as described elsewhere (25). This
303 study was carried out in accordance with the guidelines of the Australian National
304 Health and Medical Research Council. The Griffith University Animal Ethics Committee
305 reviewed and approved all experimental protocols for animal usage according to the
306 guidelines of the National Health and Medical Research Council (approval:
307 MSC/01/18/AEC).

308 **Statistical methods**

309 All statistical analyses used GraphPad Prism V8 and are defined in respective Figure
310 Legends. Statistical significance was accepted at P values of ≤0.05.

311

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312 **Discussion**

313 Transcriptional and cellular responses of bacterial pathogens to metal ions including Cu
314 can influence host-pathogen interactions and thereby play a role in disease pathogenesis
315 (4). The role Cu homeostasis and detoxification in the biology of GBS have not hitherto
316 been characterized, and no reports of a Cu stress response in this important human and
317 animal pathogen are published. The principal finding of this study is the establishment
318 of a transcriptional and cellular connection between the response to Cu stress in GBS
319 and survival of the bacteria in conditions of Cu toxicity; this connection is mediated
320 through *copA* and controlled through *copY,* and enables the bacteria to resist killing via
321 Cu-mediated intoxication. Additionally, this study establishes that the connection
322 between Cu stress responses in GBS and bacterial survival promotes virulence in the
323 host during systemic, disseminated infection. The new insights into gene function in
324 GBS viewed through the lens of the Cu stress transcriptome, combined with the findings
325 of enhanced virulence elucidate molecular mechanisms that underpin GBS survival of
326 intoxicating conditions, including those likely to be encountered in the host.
327
328 The transcriptional remodelling that occurs in GBS in response to Cu stress, as defined
329 in this study on a global level, comprises an intriguingly constrained subset of genes.
330 These findings indicate a tightly controlled system of transcriptional responses to Cu in
331 GBS. Interestingly, equivalent low numbers of target genes were identified in previous
332 transcriptional analyses of other streptococci exposed to Cu stress (14-16). Our findings
333 are consistent with these prior reports, and support the notion that these transcriptional
334 responses function in housekeeping or homeostasis to set a low limit of Cu availability

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335 in the cytoplasm (15). In GBS, *copA* is among the most strongly activated genes in the
336 transcriptional response to Cu stress, and the mutational analysis performed in this
337 study shows that *copA* is crucial for the bacteria to attain an essential Cu efflux response
338 during Cu stress. However, *copA* is only one of an assembly of genes engaged by GBS
339 during Cu stress and it is likely that other genes in the transcriptome contribute to Cu
340 detoxification via additional means. For example, we detected up-regulation of putative
341 metal transporters for Mn import (*mtsABC*, *mntH2*) along with concurrent down-
342 regulation of a system that encodes Fe export machinery *(fetAB)*. These transcriptional
343 insights are interesting because they hint at additional stress response mechanisms that
344 occur during Cu stress in GBS, which extend beyond CopA and which need elucidation.
345
346 Transcription of metal ion-import and export genes, including those above-mentioned, is
347 typically controlled by metal-dependent regulatory proteins termed metalloregulators that
348 sense metal ion-bioavailability and work to maintain cellular metal homeostasis (42). In
349 our study, we detected no major changes in the expression of genes predicted to encode
350 metalloregulators, such as for transport of Mn (*mntR/mtsR*), Fe (*fur*), Zn (*adcR/sczA*) and
351 for the sensing of peroxide (*perR*) in GBS undergoing Cu stress (42, 43). It would be of
352 interest to elucidate whether such regulators undergo mis-metallation in GBS during Cu
353 stress, which might occur due to excess Cu likely outcompeting Mn and Fe for binding
354 sites in proteins; however, precisely if and how metalloregulators might respond to mis-
355 metallation in GBS, if this actually occurs, will need to be elucidated by further study.
356

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357 A previous study of *S. pneumoniae* reported a role for Mn in rescuing Cu toxicity in the
358 bacteria (14). The authors suggested that the mechanism of rescue related to
359 modulated expression of aerobic and anaerobic dNTP synthesis pathways, encoded by
360 *nrdF* and *nrdD*, respectively. If we consider that Mn rescues Cu-mediated inhibition of
361 dNTP synthesis, it is conceivable that Mn import may be linked to Cu stress in GBS.
362 Our transcriptional assays of WT GBS showed no response of *nrdD* expression to Cu
363 stress, consistent with the findings of Johnson *et al.* Nevertheless, we observed a highly
364 statistically significant, but modestly up-regulated response of the *nrdFIA* genes,
365 encoding the aerobic dNTP synthesis system (1.7-fold, FDR < 0.001), to Cu stress (data
366 not shown). The role of *nrdFIA* genes in the GBS response to Cu stress requires further
367 study, noting that NrdD encodes an iron-containing enzyme, whereas NrdF encodes a
368 Mn-dependent pathway, further hinting at a role for Mn in the GBS Cu stress response.
369

370 In *S. pyogenes*, Mn uptake is facilitated by *mtsABC* and is protective against peroxide
371 induced stress (43). Expression of *mtsABC* is up-regulated by Mn deficiency, and is
372 controlled by the MtsR regulator (43, 44). Mn import and Fe efflux are co-ordinated in
373 order to control the metalation of superoxide dismutase (43), which can use Mn or Fe at
374 its catalytic site in streptococci; noting that Mn uptake can be disrupted by Zn (35).
375 Recently, the Cu-sensing transcription factor Mac1p in *Candida* was shown to regulate
376 the cells response to Cu starvation by controlling Cu import, and control reactive oxygen
377 species homeostasis by repressing a Cu-containing superoxide dismutase and inducing
378 Mn-containing SOD3 (45). A system for the dual import of Mn and Fe in GBS is
379 encoded by *mntH* that is regulated by pH (46). In some strains of GBS, including the

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380 hypervirulent ST17 lineage used in this study, two homologues of MntH exist, encoded
381 by *mntH* and *mntH2*. This study demonstrates up-regulation of *mntH2*, but not *mntH*, in
382 response to Cu, however the role of *mntH2* is undefined in GBS. We also note that
383 *mtsABC* and *mntH2* are also up-regulated in concert with down-regulation of Fe-
384 transporting *fetAB* in response to Zn stress in GBS (47). Together, our data indicate that
385 modulation of *mtsABC*, *mntH2* and *fetAB* expression forms parts of a transcriptional
386 signature of GBS to Zn stress.
387
388 It is notable that modulation of putative Mn (*mtsABC*) and Fe (*fetAB*) transporters, as
389 well as other proteins predicted to localise to the membrane (*pcl1*) or cell surface (*hvgA*)
390 in GBS in response to Cu stress takes place in the absence of altered cellular Mn or Fe
391 content. This leads us to suggest that examining the roles of targets identified in this
392 study in the context of Cu transport is needed in concert with other metals (*e.g.,* Mn).
393 Indeed, the 'dearth of information' on precisely how Cu is transported into bacterial cells
394 from the external environment represents a central mystery for Cu trafficking in bacteria
395 (48). Perhaps a system for Cu import is inversely regulated to a *bona fide* Cu export
396 system, in a fashion that parallels the identified acquisition and efflux systems for Zinc
397 described in streptococci (49).
398
399 Our assays of bacterial growth *in vitro* in conditions of Cu stress demonstrate that GBS
400 deficient in *copA* cannot grow as efficiently compared to the WT in conditions of elevated
401 extracellular Cu. The differences noted in GBS growth between nutritionally rich (THB)
402 and limited (CDM) media possibly reflect relative quantities of compounds that confer a

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403 protective advantage for survival during Cu stress, such as glutathione (15) or other
404 thiol-containing amino acids that may interact with free Cu ions in solution. Glutathione is
405 not included in CDM as a separate chemical constituent but the quantities of
406 methionine, cystine and cysteine are 30, 62.6 and 50 mg.L^{-1}. It is also possible that the
407 levels of Cu utilized in our assays are distinct to those in host niches. Importantly,
408 however, Cu exposure assays *in vitro* are almost certainly influenced by the compounds
409 present in the medium that likely affect levels of Cu that become inhibitory (15). Thus,
410 the Cu exposure assays used in this study are beneficial in establishing *bona fide* gene
411 function and revealing bacterial responses to a defined stress condition.
412
413 Our analysis of Cu content in GBS cells exposed to Cu stress in defined conditions
414 shows that GBS maintains low steady-state levels of cellular Cu, in the absence of
415 excess extracellular Cu. In supplying extracellular Cu in excess, we show the level of
416 cellular Cu content is increased. Our finding that *copY* functions to repress *copA* in the
417 absence of Cu is consistent with previous reports in other bacteria (50, 51). Disrupting
418 the genetic systems for Cu efflux in GBS, via mutation of the CopA exporter, or the
419 CopY regulator, reveals divergent phenotypes that stem from loss of export or
420 regulatory function, resulting in accumulation (*copA*$^{-}$) or reduction (*copY*$^{-}$) of cellular Cu.
421 These phenotypes will be of interest to dissect in terms of the role of CopY in GBS
422 biology in other models of infection and disease in the future.
423
424 Historically, studies have demonstrated increased Cu levels in the blood of humans
425 during bacterial infection (52, 53) but most insight into Cu-driven antimicrobial

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426 responses is derived from *in vitro* studies of mammalian cells infected experimentally. In
427 macrophages, bioavailability of Cu correlates with antibacterial activities (54), and Cu 'hot
428 spot' formation mediates antimicrobial responses against intracellular bacteria (7). The
429 cellular consequences of Cu stress to GBS, which likely encounters such stress in the
430 host, remains undefined. Our findings based on *in vitro* infection macrophages showed
431 no attenuation of GBS devoid of CopA in survival in host cells, which was surprising
432 given the important role of Cu management in survival of other bacteria inside
433 macrophages (6), and epithelial cells (12). Notably, however, intracellular survival of
434 *Salmonella* deficient in *cueO*, which encodes an enzyme required for resistance to Cu
435 ions, was not impaired in murine macrophages in a previous study (55), leading the
436 authors to suggest multiple host factors are involved in clearance of the bacteria. Our
437 findings are consistent with these hypotheses. Other researchers have described the
438 limitations of *in vitro* tissue culture monolayer assays for determining intracellular survival
439 of bacteria in the context of Cu homeostasis (56).
440
441 Despite negative findings in macrophage monolayer assays *in vitro*, systemic infection of
442 mice exposed a connection between the ability of GBS to generate a Cu management
443 response via CopA and bacterial virulence *in vivo*. Here, *copA* was essential for GBS to
444 fully colonize and survive in the blood, as well as in other tissues. In demonstrating a
445 significant attenuation of GBS deficient in CopA to be fully virulent in mice, we suggest
446 that Cu toxicity may represent a form of stress experienced by the bacteria *in vivo* during
447 systemic infection. In other bacteria, including *Pseudomonas aeruginosa* and *Listeria*
448 *monocytogenes*, compromised Cu transport leads to attenuation for colonization in

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449 various infection models (56, 57). Attenuation of GBS for colonization of the blood, liver
450 and spleen indicates that Cu management in the bacterial cell is essential not only for
451 efficient survival of the bacteria in the bloodstream but also for colonization of highly
452 immunologically active tissues; *i.e.,* Kupffer cells and splenic lymphocytes for innate and
453 adaptive immune responses, respectively. It would be of interest to analyze the effect of
454 Cu transport deficiency in GBS in other relevant models of infection, including in vaginal
455 colonization (58). Additional to defining the effects of metal homeostasis in GBS on the
456 nature of infection and disease caused by this organism, small-molecules probes might
457 hold promise for the identification of other molecular mechanisms of metal homeostasis
458 in GBS, as reported for Gram-positive bacteria (59).
459
460 In summary, this study shows that management of Cu export in GBS is essential for the
461 bacteria to survive in environments of Cu stress. Cu intoxication in GBS generates a
462 transcriptional signature that includes activation of the *cop* operon to confer bacterial
463 survival and virulence in stressful environments. The exact role for Cu ions as an
464 antibacterial response against GBS warrants further investigation.
465

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659 **TABLE 1. Transcriptional signature of Cu intoxication in GBS**

Locus_tag†	Genbank accession	Label	Annotation	FC	FDR
02565	ASZ00809.1	*copY*	CopY/TcrY family copper transport repressor	4.1	1.09E-12
02575	ASZ00811.1	*copZ*	carbonate dehydratase	4.0	1.02E-37
02570	ASZ00810.1	*copA*	copper-translocating P-type ATPase	3.7	2.25E-35
10970	ASZ02392.1		peptidoglycan-binding protein LysM	3.4	6.13E-13
07965	ASZ01821.1	*mtsA*	metal ABC transporter substrate-binding protein	2.9	2.15E-12
07960	ASZ01820.1	*mtsB*	metal ABC transporter ATP-binding protein	2.6	1.02E-15
10190	ASZ02237.1	*mntH2*	divalent metal cation transporter	2.4	6.34E-09
10965	ASZ02391.1		transglycosylase	2.2	1.87E-06
09125	ASZ02032.1		CHAP domain-containing protein	2.1	1.58E-09
07955	ASZ01819.1	*mtsC*	metal ABC transporter permease	2.1	2.62E-10
10305	ASZ02261.1		PAP2 family protein	2.0	6.13E-13
08045	ASZ01834.1		glycosyltransferase family 2 protein	-2.0	9.51E-06
08200	ASZ01861.1		branched-chain amino acid ABC transporter substrate-binding protein	-2.3	1.98E-04
10430	ASZ02284.1	*hvgA*	pathogenicity protein	-2.3	2.18E-07
04720	ASZ01199.1	*pcl1*	membrane protein	-4.3	1.38E-09
04185	ASZ01095.1	*fetB*	iron export ABC transporter permease subunit, FetB	-4.9	1.39E-08
04180	ASZ01094.1	*fetA*	ABC transporter ATP-binding protein FetA	-4.9	4.58E-11

660 † denotes locus tag of *S. agalactiae* 874391, preceded by CHF17_RS

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661 **TABLE 2. Bacterial strains and Plasmids**

662

Bacteria		
Strains	**Characteristics**	**Source**
E. coli DH5α	*huA2 lac(Δ)U169 phoA glnV44 Φ80' lacZ(Δ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Bethesda Research Labs
S. agalactiae 874391	Wild type, Sequence type-17, Serotype III strain, Vaginal isolate (Japan)	(60)
S. agalactiae GU2691	874391Δ*copA* (*copA*$^{-}$ mutant) Locus tag: CHF17_RS02570	This work
S. agalactiae GU2857	874391Δ*copY* (*copY*$^{-}$ mutant) Locus tag: CHF17_RS02565	This work
S. agalactiae GU3121	GU2857 (*ΔcopA*) containing *copYAZ* complement construct pGU3112; Sp	This work
Plasmids		
pHY304aad9	*ori* (Ts); temperature-sensitive shuttle vector; Sp	(28)
pGU2650	pHY304*aad9*-derivative *copA*Δ construct; Sp	This work
pGU2847	pHY304*aad9*-derivative *copY*Δ construct; Sp	This work
pGU3112	pDL278 containing cloned *copYAZ* operon; Sp	This work

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663 **FIG 1.** Global transcriptomic analysis of GBS in response to Cu stress. Volcano plot
664 showing data from RNASeq analysis of WT GBS cultures exposed to 0.5 mM Cu
665 compared to non-exposed controls. Transcripts detected as up- or down-regulated in
666 response to Cu (*n*=4, >± 2-fold, FDR <0.05) are highlighted in red and blue,
667 respectively. Dotted lines show False discovery rate (FDR; q-value) and fold-change
668 cut-offs. Grey points indicate genes that were not significant changed in expression,
669 according to these analysis cut-offs. Selected genes are identified with black lines. B,
670 Validation of RNASeq data. Expression ratio (Fold-change) of *pcl1*, *hvgA* and *copY*
671 quantified by qRTPCR in THB medium containing 0.5 mM Cu compared to THB without
672 Cu. Ratios for qRTPCR were normalized using housekeeping *dnaN* and RNASeq ratios
673 were calculated using DESeq2. Bars show means and S.E.M from 4 independent
674 experiments.
675
676 **FIG 2.** Organisation of the *copY-copA-copZ* locus in GBS. A, *copY-copA-copZ* are
677 adjacent in the GBS genome and likely controlled by the promoter-proximal *copY,*
678 encoding a putative Cu-sensing repressor. Locus tags from the GBS 874391 genome
679 are indicated. B, Distribution of homologous *cop* genes in other Lactobacillales,
680 arranged by percentage identity of amino acid sequence to CopA of *S. agalactiae*. C,
681 Model of Cu efflux in GBS highlighting CopA as transmembrane Cu exporter,
682 transcriptionally repressed by CopY in the absence of Cu, which is likely inhibited by the
683 Cu-binding chaperone protein CopZ. Figure based on previous studies in other
684 lactobacillales (51, 61).

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685 **FIG 3.** Growth curve analyses of GBS cultured in nutrient-rich THB medium (A), or in
686 THB supplemented with 0.5 mM Cu (B), 1.0 mM Cu (C) or 1.5 mM Cu (D), comparing
687 WT, Δ*copA* or Δ*copY* strains as indicated in panel A. Points and bars show mean and
688 S.E.M of 3 independent experiments monitoring attenuance at 600nm.

689

690 **FIG 4.** Bactericidal effect of Cu on GBS viability. Time-kill assays comparing WT, Δ*copA*
691 or Δ*copY* GBS, incubated in CDM or in CDM supplemented with 0.05, 0.1, 0.2, 0.5 or 1
692 mM Cu. Viable cells were quantified at 1h, 3h, 6h and 24h post inoculation. † Viable Cell
693 counts of 0 CFU/mL were assigned a value of 1 to enable visualisation on $\log_{10}$ y-axes.
694 Points and bars show mean and S.E.M of 4 independent experiments. Data were
695 analysed by One-way ANOVA with Holm Sidak Multiple comparisons, comparing Cu-
696 exposed conditions to non-exposed controls (0 mM Cu) at each time-point, for each
697 strain (* $P < 0.05$, ** $P < 0.01$. *** $P < 0.001$).

698

699 **FIG 5.** Expression analysis of *copA* and intracellular Cu content in GBS strains. A,
700 Expression ratio (Fold-change) of *copA* quantified by qRT-PCR in THB medium
701 containing 0.25, 0.5, 1.0 and 1.5 mM Cu, compared to THB without Cu. B, Relative
702 *copA* transcripts were quantified in WT and Δ*copY* strains with and without Cu
703 supplementation (0.5 mM) to demonstrate de-regulation of *copA* expression in the
704 Δ*copY* background. C, Final biomass yield comparisons of WT, Δ*copA* and Δ*copY*
705 strains following growth for 18h in THB with 1.5 mM Cu. D, Intracellular accumulation of
706 Cu was compared with and without Cu supplementation (0.5 mM) in WT, Δ*copA* and
707 Δ*copY* strains. Ratios in A were calculated as described previously (34) using C_T

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708 values, primer efficiencies and housekeeping *dnaN*. Bars show means and S.E.M from
709 3-4 independent experiments and compared by One-way ANOVA with Holm-Sidak
710 multiple comparisons (*** P <0.001).
711
712 **FIG 6.** Interactions of GBS with macrophages of mouse and human origin. Gentamicin
713 protection assays with WT and Δ*copA* strains and mouse (J774A.1) or human (U937
714 monocyte-derived macrophage-like) macrophages. Surviving bacteria are expressed as
715 rCFU, indicating the difference between the number of initial intracellular bacteria (1h
716 after antibiotic treatment) and the number of intracellular bacteria at 24h or 48h post
717 infection (h.p.i). Data are means and S.E.M of 4-5 independent experiments.
718
719 **FIG 7.** Virulence of WT (grey circles) or Δ*copA* (blue diamonds) GBS (orange triangles)
720 in a mouse model of disseminated infection. C57BL/6 mice (6-8 weeks old) were
721 intravenously injected with 10^7 bacteria; bacteremia and disseminated spread to liver
722 and spleen were monitored at 24h post infection. CFU were enumerated and counts
723 were normalized using tissue mass in g. Lines and bars show median and interquartile
724 ranges and data are pooled from 2 independent experiments each containing n=10
725 mice compared using Mann-Whitney U-tests (*P < 0.05, **P < 0.01).
726
727 **Supplementary FIG S1.** Complementation of *copA* mutation restores growth of GBS to
728 WT levels under Cu stress. Growth curve analyses of GBS cultured in nutrient-rich THB
729 medium (A), or in THB supplemented with 0.5 mM Cu (B), 1.0 mM Cu (C) or 1.5 mM Cu
730 (D), comparing WT, Δ*copA::copA* strains.

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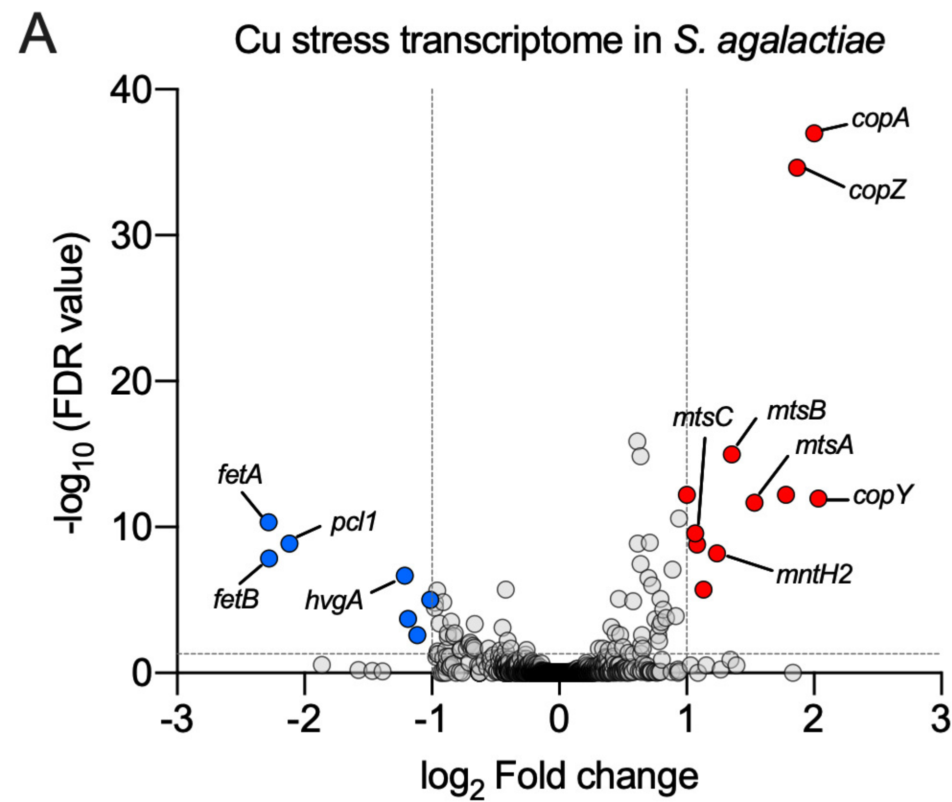

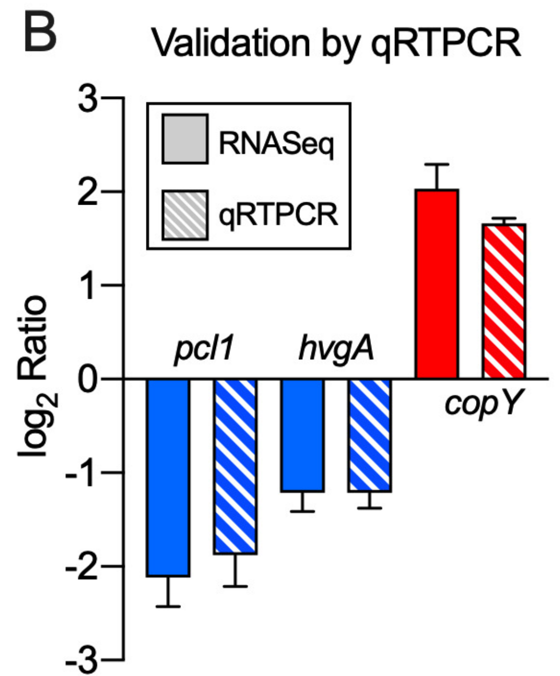

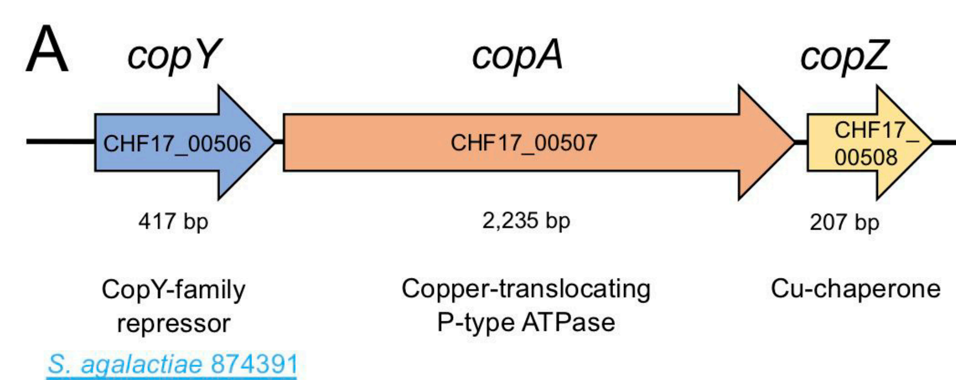
A
copY
copA
copZ
CHF17_00506
CHF17_00507
CHF17_
00508
417 bp
2,235 bp
207 bp
CopY-family
repressor
Copper-translocating
P-type ATPase
Cu-chaperone
S. agalactiae 874391
B
50%
60%
45%
copY
copA
copZ
S. mutans UA159
46%
58%
54%
copY
copA
copZ
S. thermophilus LMG 18311
40%
56%
52%
copY
copA
copZ
copX
S. pyogenes M1 GAS
45%
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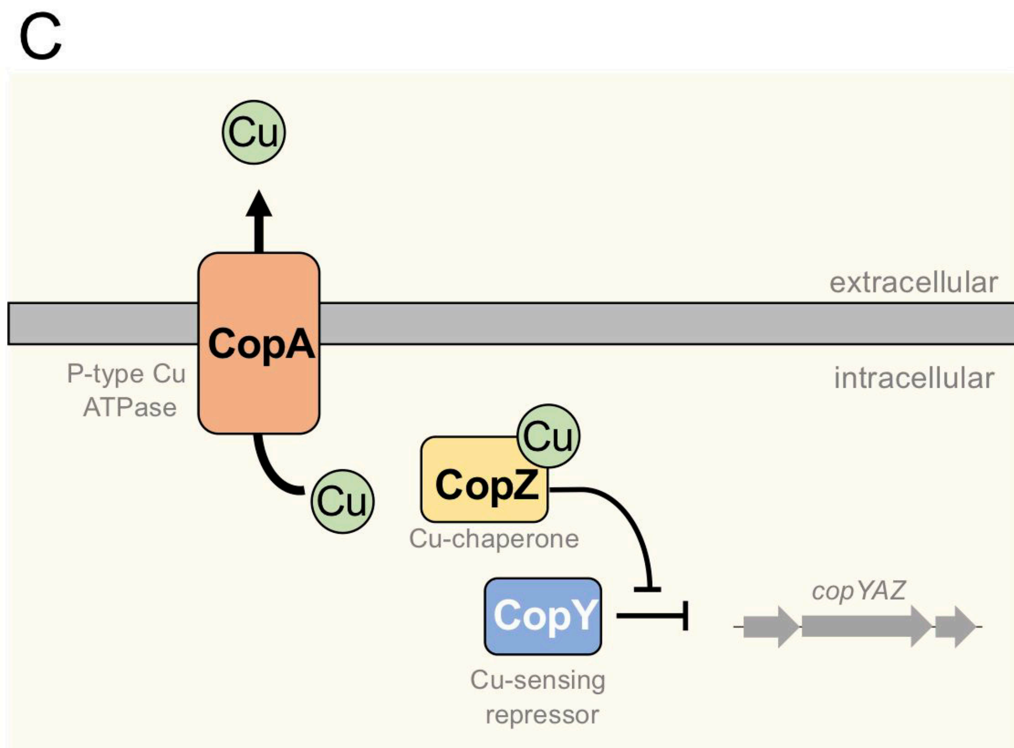
Enterococcus faecalis OG1RF
37%
44%
copY
cupA
copA
S. pneumoniae R6


C
Cu
extracellular
CopA
P-type Cu
ATPase
intracellular
Cu
CopZ
Cu
Cu-chaperone
copYAZ
CopY
Cu-sensing
repressor

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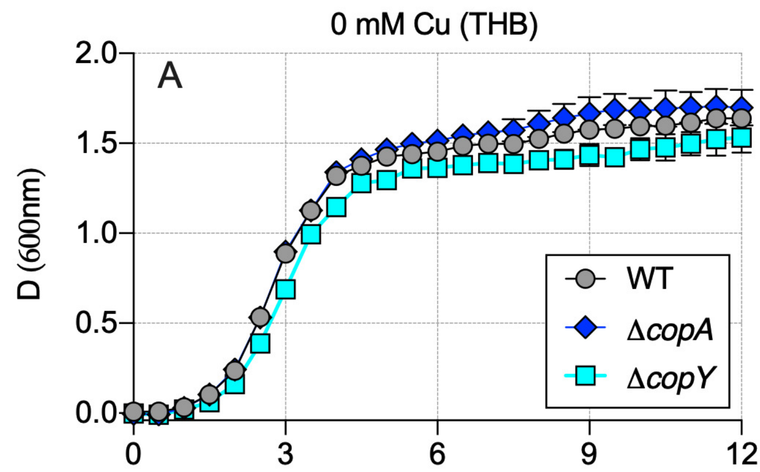

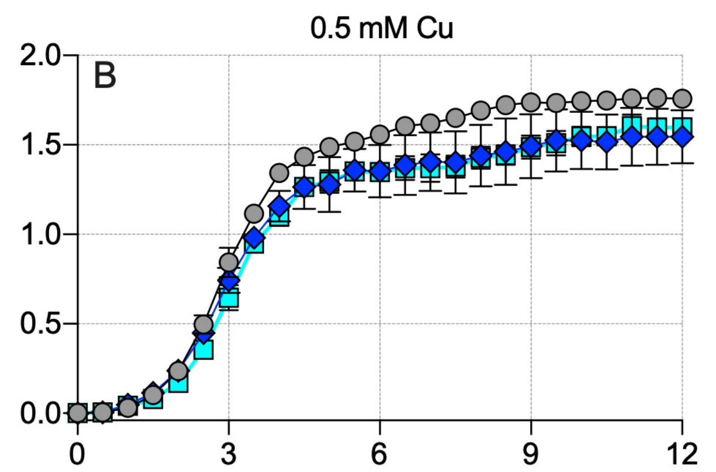

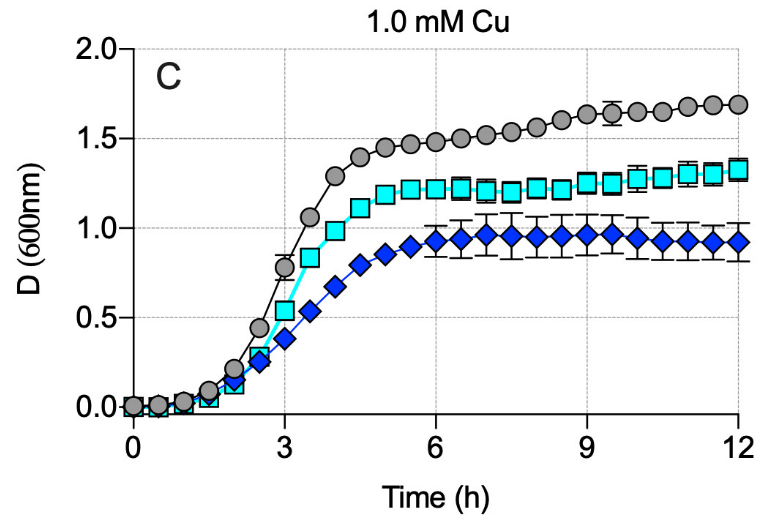

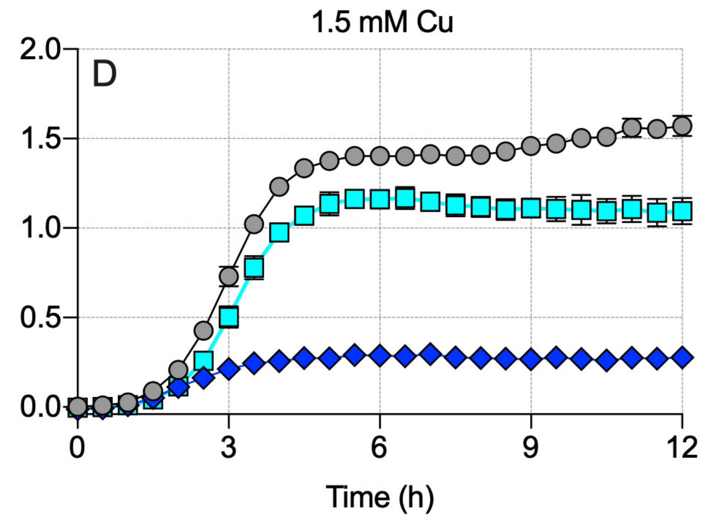

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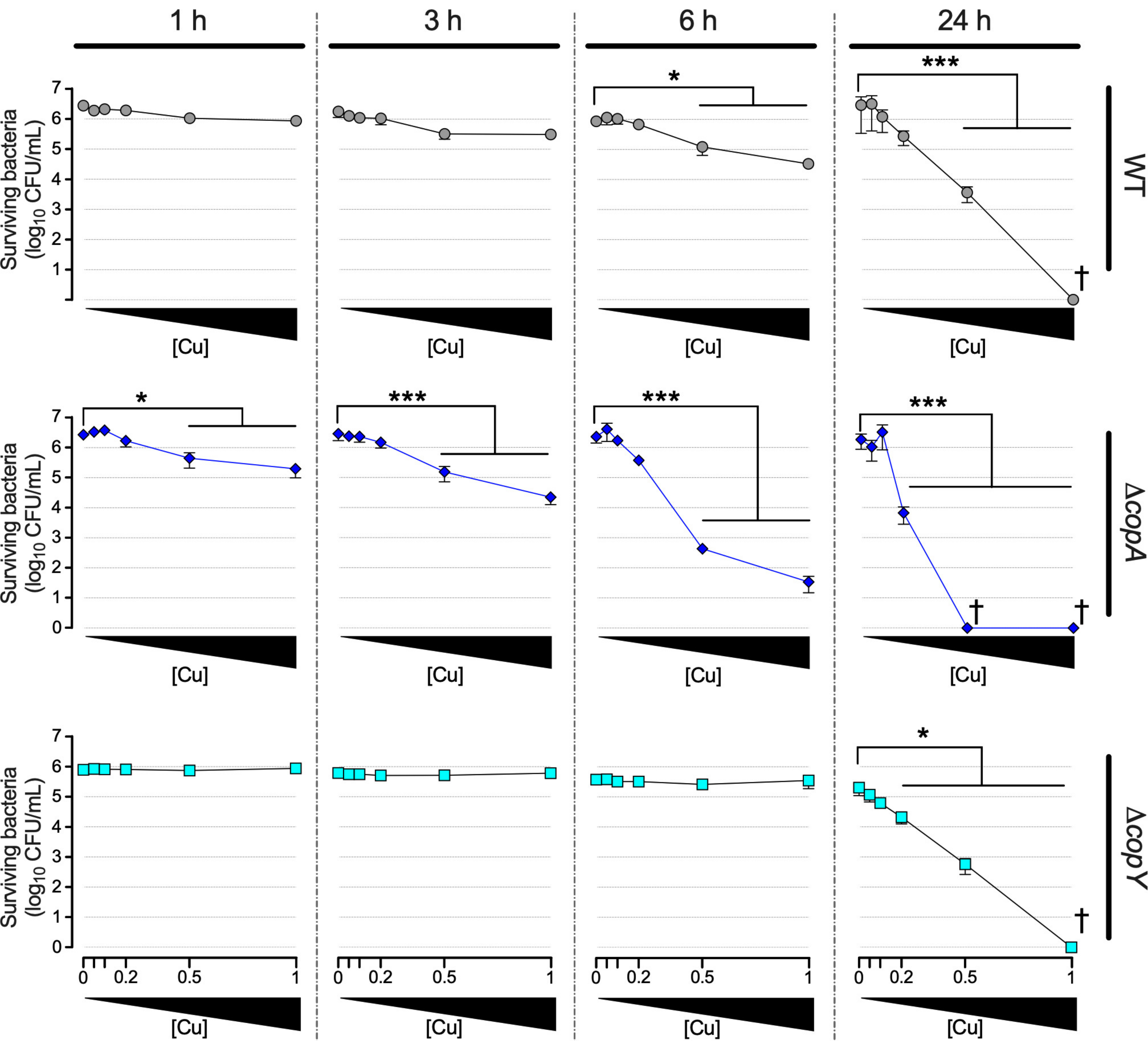

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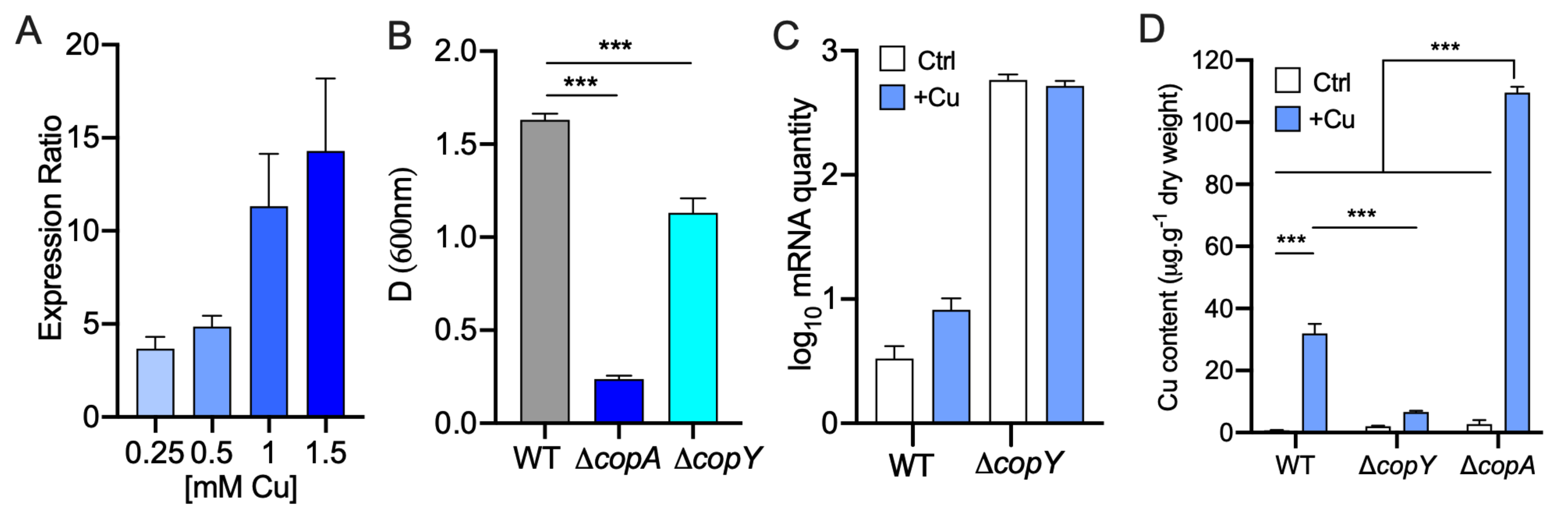

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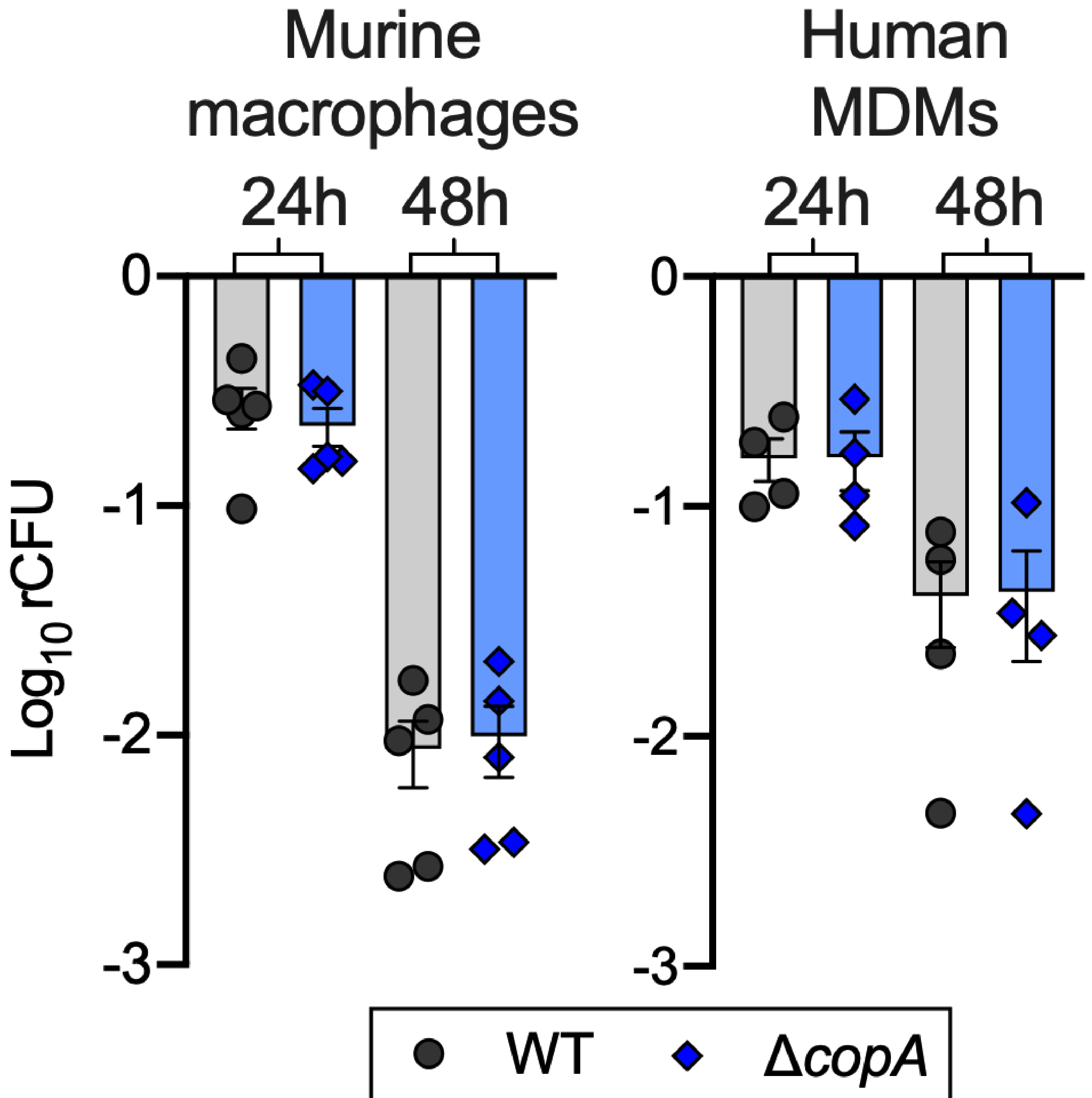

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