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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22 **Abstract**

23 Bacteria can utilize Copper (Cu) as a trace element to support cellular 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 de-repression of
29 the CopA efflux system. However, neither mutant was attenuated for intracellular
30 survival in macrophages. Analysis of global transcriptional responses to Cu by RNA-
31 sequencing revealed a stress signature encompassing homeostasis of multiple metals.
32 Genes 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

42 **Importance**

43 Understanding how bacteria manage cellular levels of metal ions, such as copper, helps
44 to explain how microbial cells can survive in different stressful environments. We show
45 how the opportunistic pathogen group B Streptococcus (GBS) achieves homeostasis of
46 intracellular copper through the activities of the genes that comprise the cop operon,
47 and describe how this helps GBS survive in stressful environments, including in the
48 mammalian host during systemic disseminated infection.

49

50 Introduction

51 Copper (Cu) is the most reactive of the biologically-relevant first-row *d*-block transition
52 metals. It can readily displace other metals, including Zinc (Zn), Nickel (Ni), Cobalt (Co),
53 Iron (Fe) and Manganese (Mn) from metalloproteins in which these are bound (1, 2). In
54 cells across all Kingdoms of life, Cu-dependent enzymes are pivotal to many essential
55 processes that underpin physiologic biochemical reactions, owing to its ability to redox
56 cycle between Cu(I) and Cu(II) states (3). When present in excess, however, Cu is
57 hazardous to cellular processes and macromolecules due to effects of localized free-
58 radical damage (4). A key role for Cu in bacterial cell biology encompasses the host-
59 pathogen interface where a balance between Cu usage and avoidance of Cu
60 intoxication resulting from host antimicrobial responses must be attained for bacteria to
61 survive, as reviewed elsewhere (5). At this interface, human immune cells can mobilize
62 Cu in a defence response to infection that culminates in exposure of intracellular
63 bacteria to antimicrobial levels of Cu that kill the invading pathogen (6-9). Thus,
64 bacterial responses to Cu stress and mechanisms of resistance to metal-ion intoxication
65 have emerged as important facets of bacterial disease pathogenesis (10).

66

67 There are several mechanisms that enable bacteria to tolerate excess extracellular Cu,
68 and these have been characterized in several pathogenic species. These mechanisms,
69 which represent survival strategies against Cu intoxication (10) typically involve Cu-
70 transporting P-type ATPases, exemplified by CopA, which detoxify Cu by exporting it
71 from the bacterial cytosol (11-13). This mechanism has been described in gram-

72 negative and gram-positive bacteria, and among *Streptococcaceae* (14-16), including
73 pneumococci and *Streptococcus pyogenes* (17).

74

75 An opportunistic streptococcal pathogen of humans and animals for which resistance to
76 excess extracellular Cu has not been described is *Streptococcus agalactiae*, also known
77 as group B streptococcus (GBS). This organism, unlike other *Streptococcaceae*, is
78 associated with a comparatively broad host-range that encompasses humans, cattle
79 and fish (18). In humans, GBS is a major cause of invasive infection in infants <3
80 months of age (19), and causes a more diverse range of disease aetiologies compared
81 to other streptococci, including meningitis, pneumonia, skin and soft-tissue infections,
82 sepsis, arthritis, osteomyelitis, urinary tract infection, and endocarditis (19). GBS has
83 several virulence factors that enable survival in cytotoxic environments, such as acid
84 stress, oxidative stress, and during host colonization, as reviewed elsewhere (20).
85 Cellular tolerance to Cu stress and mechanisms of responding to and surviving Cu
86 intoxication have not been defined in GBS.

87

88 In this study, we characterise a system that enables Cu efflux in GBS, encompassing a
89 *copA*-homologue, which we show mediates control of Cu efflux in the bacteria. We
90 show this system affects survival, growth and virulence of GBS in the mammalian host.

91

92 Results

93 *Transcriptomic profiling of GBS responses to extracellular Cu*

94 To dissect the global response of GBS to extracellular Cu stress we performed RNA
95 sequencing (RNASeq) to define the complete primary transcriptional response to Cu.
96 RNASeq analysis of mid-log phase cells of GBS strain 874391 grown in the presence of
97 0.5 mM Cu (a sub-inhibitory level; see below) for 2.5h (compared to control cultures that
98 were grown without supplemental Cu) revealed a surprisingly modest Cu-responsive
99 transcriptome. The response comprised 18 transcriptional responses, defined as
100 significant based on $-2 \geq \text{FC} \geq 2$ (FDR < 0.05, $n=4$ biological replicates), of 11 up-
101 regulated transcripts and 7 down-regulated transcripts (Figure 1, Table 1). The most
102 significantly up-regulated were homologues of the *copY-copA-copZ* gene cluster (3.6-
103 4.0-fold), which encode the putative CopY (Cu-binding transcriptional repressor), CopA
104 (P-type ATPase Cu-efflux system) and CopZ (Cu-chaperone) proteins. To validate the
105 expression levels of selected targets identified in RNASeq, we targeted *pcl1*, *hvgA* and
106 *copY* in qRT-PCR assays, confirming near-identical fold-change values to RNASeq
107 analyses (Figure 1B). The products of *copY*, *copA* and *copZ* in GBS (Figure 2A) are
108 moderately conserved compared to other Lactobacillales, including *S. mutans*, *S.*
109 *thermophilus*, *S. pyogenes*, *S. pneumoniae*, and *Enterococcus* (Figure 2B, ranked by
110 CopA similarity) for which a homologous Cu management system is defined (9, 13-15).
111 Together, these findings provide transcriptional and comparative insights that support a
112 proposed model of Cu efflux in GBS (Figure 2C).

113

114 In addition to *copYAZ*, GBS up-regulated putative metal transporters for Mn import
115 (*mtsABC*, *mntH2*), and down-regulated an iron (Fe) export system (*fetAB*; Figure 1,
116 Table 1). In addition, we detected significant down-regulation of *pcl1*, that encodes a
117 putative membrane protein, and the gene for hypervirulence-associated factor, *hvgA*.
118

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

120 To functionally characterize two major elements of the Cu-responsive transcriptome in
121 GBS, we targeted *copA* and *copY* by generating isogenic deletion mutants and
122 comparing growth of these with the WT. In a nutrient-rich medium (Todd Hewitt Broth;
123 THB) supplemented with increasing amounts of Cu, we observed no growth inhibitory
124 effects of high Cu (up to 1.5 mM) for WT GBS; the lag phases, growth rates, and final
125 biomass yields were equivalent for the WT comparing different levels of Cu (Figure 3).
126 In contrast, growth of $\Delta copA$ GBS was severely attenuated by Cu stress ≥ 1 mM;
127 complementation of the *copA* mutation restored the phenotype to WT (Figure 3).
128 Deletion of *copY*, encoding a putative Cu-dependent repressor of *copA*, had no effect
129 on the growth rate or lag phase in THB supplemented with Cu but significantly affected
130 final biomass yield of cultures (*D* at 600nm at 18h; Supplementary Figure S1). Together,
131 this shows that *copA* confers cellular resistance to Cu stress in GBS in a manner that
132 supports bacterial growth in nutritive conditions.
133

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

135 We examined the effects of Cu towards survival of GBS in nutrient limited conditions by
136 using a minimal Chemically-Defined Medium (CDM), in consideration of prior studies of

137 antibacterial activity in minimal media (21, 22), the influence of culture media (23, 24),
138 and possible cell-protective effects from buffering agents, e.g. glutathione (15). We
139 observed that the level of Cu required to inhibit GBS growth in CDM was markedly
140 lower than THB (Supplementary Figure S2); for example, the growth of $\Delta copA$ GBS was
141 significantly attenuated in CDM in the presence of 0.5 mM supplemental Cu, a level that
142 caused only slight inhibition of growth in THB (Figure 3). To more precisely define the
143 bactericidal effects of Cu towards GBS in CDM, we performed time-kill curves using $1-5$
144 $\times 10^6$ CFU/mL exposed to Cu concentrations ranging between 50 μ M and 1 mM. We
145 observed potent killing effects that depended on both Cu concentration and time with $\geq$
146 0.5 mM Cu significantly killing WT GBS after 3h of exposure (Figure 4, WT); after 24h,
147 there was a ~230-fold reduction in CFU/mL at 0.5 mM Cu (0 mM Cu = 5.6 Log₁₀
148 CFU/mL, 0.5 mM CFU/mL = 3.2 Log₁₀ CFU/mL), and no viable GBS remained in
149 cultures exposure to 1 mM Cu. The $\Delta copA$ strain was significantly more susceptible to
150 Cu-induced toxicity compared to the WT; Cu levels above $\geq$ 0.5 mM significantly
151 reduced the number of viable $\Delta copA$ GBS beginning as early as 1h exposure (Figure 4,
152 $\Delta copA$); the degree of bacterial killing was significant at the 24h timepoint for $\geq$ 0.5 mM
153 Cu (Figure 4). Interestingly, at the 6h timepoint, Cu at $\geq$ 0.5 mM did not affect viability of
154 $\Delta copY$ GBS, compared to the WT strain (Figure 4, $\Delta copY$), a hyper-resistance
155 phenotype that was not apparent at the 24 h timepoint. Together, these findings are
156 consistent with a role for CopA in resisting Cu stress, and CopY as Cu-dependent
157 repressor of *copA*.

158

159 ***Regulation of Cu efflux by CopY, and accumulation of metals during Cu stress***

160 We next sought to examine the control of Cu-responses in GBS at the transcriptional
161 level. The capacity of Cu stress to induce expression of *copA* for Cu export was
162 examined by analysing *copA* expression by qRT-PCR in GBS exposed to Cu
163 concentrations ranging from 0.25-1.5 mM Cu in THB. GBS significantly upregulated
164 *copA* in response to Cu (3.7-fold – 14.2-fold) in a manner that was titratable with the Cu
165 concentration (Figure 5A). To ascertain the role of CopY as a putative Cu-responsive
166 repressor of *copA* expression in GBS, we quantified *copA* mRNA transcripts in the
167 $\Delta copY$ mutant exposed to 0.5 mM Cu. This level of Cu was carefully chosen as sub-
168 inhibitory to enable comparisons independent of metabolic state and therefore limiting
169 any potential bias from possible discordant Cu stress between WT and mutants with
170 varied resistance phenotypes. Deletion of *copY* resulted in severe de-regulation of *copA*
171 expression, causing a $\sim 207 \pm 45$ -fold increase of *copA* transcripts in the *copY*
172 background (Figure 5B). Thus, these data establish that GBS *copY* represses *copA* in
173 the absence of Cu, and are consistent with a model of CopY-mediated regulation of
174 *copA* that is responsive to intracellular accumulation of Cu.

175

176 To examine the impact of *copA* and *copY* mutations on accumulation of Cu within the
177 cell we used the equivalent conditions per RNASeq and qPCR assays, exposing GBS
178 to 0.5 mM Cu in THB and measuring total Cu content of cells compared to non-exposed
179 controls (Ctrl). Inductively coupled optical emission spectroscopy (ICP-OES)
180 demonstrated that standard THB contained $0.2 \pm 0.08 \mu\text{M}$ Cu, reflecting trace amounts in
181 the medium. In the absence of supplemental Cu, WT GBS limited intracellular Cu
182 content such that only $0.8 \pm 0.1 \mu\text{g Cu. g dry weight}^{-1}$ were detected in cultures grown in

183 THB. However, exposure of WT GBS to 0.5 mM Cu resulted in a significant increase in
184 intracellular Cu to $31.9 \pm 3.1 \mu\text{g Cu. g dry weight}^{-1}$ (Figure 5C). Strikingly, ΔcopY GBS
185 exhibited significantly less cellular Cu upon exposure to Cu ($6.7 \pm 0.4 \mu\text{g Cu. g dry}$
186 weight^{-1}), consistent with the observation that transcription of the Cu-exporter CopA is
187 significantly elevated in this mutant. In addition, we noted significant accumulation of
188 cellular Cu in the ΔcopA strain ($109.6 \pm 1.9 \mu\text{g Cu. g dry weight}^{-1}$), confirming a Cu-efflux
189 role for *copA*. Interestingly, we noted modest but significant increases in Mn and Fe in
190 the ΔcopA strain, and Zn in the ΔcopY strain due to Cu stress (Figure 5D-F).

191

192 To extend our findings comparing mutant strains to WT at a level of Cu that is sub-
193 inhibitory to the ΔcopA strain, we next undertook experiments to investigate the impact
194 of severe Cu stress on WT GBS; by repeating metal accumulation analyses using cells
195 grown in 1.5 mM Cu. We detected modest but statistically significant accumulation of Fe
196 inside GBS cells exposed to 1.5 mM Cu, co-occurring with significantly higher Cu levels;
197 however, no difference in Mn or Zn content (Supplementary Figure S3). Fe status has
198 been reported to influence bacterial resistance to peroxide stress (25) and therefore we
199 also undertook experiments to investigate the consequence of Cu toxicity on resistance
200 to oxidative stress using assays of susceptibility to hydrogen peroxide (H_2O_2). These
201 experiments demonstrated a significant reduction in the viability of ΔcopA GBS exposed
202 to H_2O_2 , which was dependent on pre-exposure to Cu (Supplementary Figure S4).
203 Thus, GBS cells undergoing Cu stress are rendered significantly more susceptible to
204 oxidative stress.

205

206 ***Role of CopA in macrophage killing of GBS***

207 To examine whether the CopA Cu efflux system in GBS supports survival of the bacteria
208 in phagocytes we performed antibiotic protection assays with human monocyte-derived
209 macrophage-like cells. Macrophages were infected with WT, $\Delta copA$ GBS or its
210 complemented strain for 1h, and antibiotics were added to kill extracellular bacteria.
211 Viable intracellular GBS were quantified at 1h post-antibiotic addition, and at 24h and
212 48h to assess intracellular survival. We performed assays comparing culture conditions
213 of standard RPMI medium to medium that was supplemented with 20 μ M Cu to ensure
214 adequate availability of Cu for host cellular responses. These assays demonstrated
215 significant reductions in the numbers of viable GBS over the time course (24 to 48h);
216 however, we did not detect any significant differences between the numbers of WT and
217 $\Delta copA$ GBS recovered from the host cells at any time point (Figure 6). The numbers of
218 bacteria recovered comparing the WT and $\Delta copA$ mutant were equivalent regardless of
219 the presence of supplemental Cu in the medium. Thus, under these conditions, *copA*
220 does not contribute to the intracellular survival of GBS in human macrophage-like cells.

221

222 ***GBS CopA contributes to virulence in vivo***

223 To examine the contribution of Cu efflux to GBS virulence, we used a murine model of
224 disseminated infection (26). In mice challenged with 10^7 GBS, we detected significantly
225 fewer $\Delta copA$ mutant in the liver (median of 3.5 vs 4.2 $\log_{10}$ CFU.g tissue⁻¹; P= 0.005),
226 spleen (median of 3.8 vs 4.1 $\log_{10}$ CFU.g tissue⁻¹ P= 0.013) and blood (median of 1.4 vs
227 1.9 $\log_{10}$ CFU.mL⁻¹; P= 0.044) compared to the WT at 24h post-inoculation (Figure 7).
228 However, no significant differences were observed between the counts of the WT and

229 $\Delta copA$ mutant in several other tissues, including the brain, heart, lungs, and kidneys
230 (Supplementary Figure S5). These data support a modest but significant role for cellular
231 management of Cu via *copA* in GBS in supporting disseminated infection *in vivo*.
232

233 **Materials and Methods**

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

235 GBS, *E. coli* and plasmids used are listed in Table 2. GBS was routinely grown in Todd-
236 Hewitt Broth (THB) or on TH agar (1.5% w/v). *E. coli* was grown in Lysogeny Broth (LB)
237 or on LB agar. Routine retrospective colony counts were performed by plating dilutions
238 of bacteria on tryptone soya agar containing 5% defibrinated horse blood (Thermo
239 Fisher Scientific). Media were supplemented with antibiotics (spectinomycin (Sp)
240 100µg/mL; chloramphenicol (Cm) 10 µg/mL), as indicated. Growth assays used 200µL
241 culture volumes in 96-well plates (Greiner) sealed using Breathe-Easy® membranes
242 (Sigma-Aldrich) and measured attenuance (*D*, at 600nm) using a ClarioSTAR
243 multimode plate reader (BMG Labtech) in Well Scan mode using a 3mm 5x5 scan
244 matrix with 5 flashes per scan point and path length correction of 5.88mm, with agitation
245 at 300rpm and recordings taken every 30min. Media for growth assays were THB and a
246 modified Chemically-Defined Medium (CDM) (27) (with 1g/L glucose, 0.11g/L pyruvate
247 and 50mg/L L-cysteine), supplemented with Cu (supplied as CuSO₄) as indicated. For
248 attenuance baseline correction, control wells without bacteria were included for Cu in
249 media alone.

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

251 Plasmid DNA was isolated using miniprep kits (QIAGEN), with modifications for GBS as
252 described elsewhere (28). Deletions in *copA* (CHF17_00507 / CHF17_RS02570) and
253 *copY* (CHF17_00506 / CHF17_RS02565) were constructed by markerless allelic
254 exchange using pHY304aad9 as described previously (29). Plasmids and primers are

55 listed in Table 2 and Supplementary Table 1, respectively. Mutants were validated by
256 PCR using primers external to the mutation site and DNA sequencing.

257 **RNA extraction, qRT-PCR**

258 For Cu exposure experiments, 1mL of overnight THB cultures were back-diluted 1/100
259 in 100mL of THB (prewarmed at 37°C in 250mL Erlenmeyer flasks) supplemented with
260 0.25, 0.5, 1.0 or 1.5mM Cu. Cultures were grown shaking (200rpm) at 37°C; after
261 exactly 2.5h, 10-50mL volumes containing approximately 500 million mid-log bacteria
262 were harvested; RNA was preserved and isolated as described previously (30). RNA
263 quality was analysed by RNA LabChip using GX Touch (Perkin Elmer). RNA (1000ng)
264 was reverse-transcribed using Superscript IV according to manufacturer's instructions
265 (Life Technologies) and cDNA was diluted 1:50 in water prior to qPCR. Primers (Table
266 3) were designed using Primer3 Plus (31, 32) to quantify transcripts using Universal
267 SYBR Green Supermix (Bio-Rad) using a Quantstudio 6 Flex (Applied Biosystems)
268 system in accordance with MIQE guidelines (33). Standard curves were generated
269 using five-point serial dilutions of genomic DNA (5-fold) from WT GBS 874391 (34).
270 Expression ratios were calculated using C_T values and primer efficiencies as described
271 elsewhere (35) using *dnaN*, encoding DNA polymerase III β -subunit as housekeeper.

272 **Whole bacterial cell metal content determination**

273 Metal content in cells was determined as described (36) with minor modifications.
274 Cultures were prepared essentially as described for *RNA extraction, qRT-PCR* with the
275 following modifications; THB medium was supplemented with 0.5 mM CuSO₄ or not
276 supplemented (Ctrl), and following exposure for 2.5h, bacteria were harvested by
277 centrifugation at 4122 x g at 4°C. Cell pellets were washed 3 times in PBS + 5mM

278 EDTA to remove extracellular metals, followed by 3 washes in PBS. Pelleted cells were
279 dried overnight at 80°C and resuspended in 1mL of 32.5% nitric acid and incubated at
280 95°C for 1h. The metal ion containing supernatant was collected by centrifugation
281 (14,000 x g, 30min) and diluted to a final concentration of 3.25% nitric acid for metal
282 content determination using inductively coupled plasma optical emission spectroscopy
283 (ICP-OES). ICP-OES was carried out on an Agilent 720 ICP-OES with axial torch,
284 OneNeb concentric nebulizer and Agilent single pass glass cyclone spray chamber. The
285 power was 1.4kW with 0.75L/min nebulizer gas, 15L/min plasma gas and 1.5L/min
286 auxiliary gas flow. Cu was analysed at 324.75nm, Zn at 213.85nm, Fe at 259.94nm and
287 Mn at 257.61nm with detection limits at <1.1ppm. The final quantity of each metal was
288 normalised using dry weight biomass of the cell pellet prior to nitric acid digestion,
289 expressed as $\mu\text{g.g}^{-1}$ dry weight. Baseline concentrations were determined to be $0.2 \pm$
290 $0.08 \mu\text{M}$ Cu in THB medium, and $40 \pm 4 \text{ nM}$ Cu in CDM medium from at least three
291 independent assays.

292 **Hydrogen Peroxide assay**

293 Peroxide survival assays were based on prior studies (37, 38) with minor modifications
294 to encompass using GBS cells that had been pre-grown in conditions of Cu stress.
295 Overnight cultures of WT and mutants were grown in THB, cultures were back-diluted
296 1/100 into either fresh THB or THB supplemented with 0.5mM Cu and grown for exactly
297 2.5 h at 37°C with 200 rpm agitation. Bacteria were harvested by centrifugation at 4122
298 x g and washed twice in PBS and resuspended in assay buffer (0.1M sodium phosphate
299 buffer pH 7.5). This was prepared by combining 41 mL of 0.2M dibasic sodium
300 phosphate and 9 mL of 0.2M monobasic sodium phosphate in a volume of 200 mL.

301 GBS cells were diluted in assay buffer alone, or assay buffer containing 5 mM H₂O₂
302 (Sigma-Aldrich H1009), to a final concentration of ~5x10⁶ CFU/mL. Cell suspensions +/-
303 H₂O₂ were then incubated for 1h at 37°C and survival was monitored by serial dilution
304 and plating for CFU/mL counts.

305 **RNA sequencing and bioinformatics**

306 Cultures were prepared as described above for *RNA extraction*, *qRT-PCR* to compare
307 mid-log phase cells grown in THB + 0.5 mM Cu to THB without added Cu. RNase-free
308 DNase-treated RNA that passed Bioanalyzer 2100 (Agilent) analysis was used for RNA
309 sequencing (RNA-seq) using the Illumina NextSeq 500 platform. We used a Bacterial
310 Ribosomal RNA (rRNA) Depletion kit (Invitrogen) prior to library construction, and
311 TruSeq library generation kits (Illumina, San Diego, California). Library construction
312 consisted of random fragmentation of the RNA, and cDNA production using random
313 primers. The ends of the cDNA were repaired and A-tailed, and adaptors were ligated
314 for indexing (with up to 12 different barcodes per lane) during the sequencing runs. The
315 cDNA libraries were quantitated using qPCR in a Roche LightCycler 480 with the Kapa
316 Biosystems kit (Kapa Biosystems, Woburn, Massachusetts) prior to cluster generation.
317 Clusters were generated to yield approximately 725K–825K clusters/mm². Cluster
318 density and quality was determined during the run after the first base addition
319 parameters were assessed. We ran single-end 75–bp sequencing runs to align the
320 cDNA sequences to the reference genome. For data preprocessing and bioinformatics,
321 STAR (version 2.7.3a) was used (parameters used: --outReadsUnmapped Fastx --
322 outSAMtype BAM SortedByCoordinate --outSAMattributes All) to align the raw RNA
323 sequencing fastq reads to the WT *S. agalactiae* 874391 reference genome (34).

324 HTSeq-count, version 0.11.1 (parameters used: -r pos -t exon -i gene_id -a 10 -s no -f
325 bam), was used to estimate transcript abundances (39). DESeq2 was then used to
326 normalized and test for differential expression and regulation following their vignette.
327 Genes that met certain criteria (i.e. fold change of $> \pm 2.0$, q value (false discovery rate,
328 FDR of < 0.05) were accepted as significantly altered (40). Raw and processed data
329 were deposited in Gene Expression Omnibus (accession no. GSE167895 for *S.*
330 *agalactiae* 874391 Cu condition; GSE167894 (*S. agalactiae* 874391 control condition).

331 **Mammalian cell culture**

332 U937 monocytes were differentiated into human monocyte-derived macrophages
333 (MDMs) as follows. Briefly, monocytes were seeded (5×10^5 per well such that 1×10^5
334 adhere) into the wells of a 96-well tissue culture-treated plate (Falcon) essentially as
335 described elsewhere (41, 42), except that U937 monocytes were differentiated by
336 exposure to 30ng/mL phorbol 12-myristate 13-acetate (PMA) for 48h and cells
337 subsequently rested in media without PMA for 72h to enhance morphological and
338 phenotypic markers of MDMs (43). A multiplicity of infection (MOI) of 100 bacteria:
339 macrophage for 1h was used in RPMI without antibiotics. Non-adherent bacteria were
340 removed by five washes of 200 μ L PBS using a Well Wash Versa (Thermo Scientific).
341 RPMI containing 250U/mL penicillin, streptomycin (Gibco) and 50 μ g/mL gentamicin
342 (Sigma-Aldrich) were used for antibiotic protection assays to kill extracellular bacteria as
343 described previously by incubating for 1h at 37°C in 5% CO₂ (42). Samples were
344 processed after 1h (time zero), 24h or 48h after infection, monolayers were washed five
345 times with 200 μ L PBS and lysed by brief exposure to 50 μ L of 0.25% trypsin EDTA
346 (Gibco) and 0.1% Triton-X-100 (10 min) prior to dilution with 150 μ L PBS and estimation

347 of CFU/mL by serial dilution and plate counts on agar. Additional assays that ran in
348 parallel were identical except that 20 μ M Cu was added to RPMI culture media at all
349 stages post PMA-treatment of U937 cells.

350 **Animals and Ethics statement**

351 Virulence was tested using a mouse model of disseminated infection based on
352 intravenous challenge with 10^7 GBS (WT or Δ copA) as described elsewhere (26). This
353 study was carried out in accordance with the guidelines of the Australian National
354 Health and Medical Research Council. The Griffith University Animal Ethics Committee
355 reviewed and approved all experimental protocols for animal usage according to the
356 guidelines of the National Health and Medical Research Council (approval:
357 MSC/01/18/AEC).

358 **Statistical methods**

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

361

362 **Discussion**

363 Transcriptional and cellular responses of bacterial pathogens to metal ions including Cu
364 can influence host-pathogen interactions and thereby play a role in disease pathogenesis
365 (4). The role of Cu homeostasis and detoxification in the biology of GBS have not
366 hitherto been characterized. The principal finding of this study is the establishment of a
367 transcriptional and cellular connection between the response to Cu stress in GBS and
368 survival of the bacteria in conditions of Cu toxicity; this connection is mediated through
369 *copA* and controlled through *copY*, enabling the organism to resist Cu-mediated killing.
370 Additionally, this study establishes a connection between Cu stress resistance in GBS
371 and virulence in the host during systemic, disseminated infection. The new insights into
372 gene function in GBS viewed through the lens of the Cu stress transcriptome, combined
373 with the findings of enhanced virulence elucidate molecular mechanisms that underpin
374 GBS survival of intoxicating conditions, including those likely to be encountered in the
375 host.

376

377 The transcriptional remodelling in GBS that occurs in response to Cu stress, as defined
378 in this study at a global level, comprises an intriguingly constrained subset of genes
379 indicating a tightly controlled system of responses to Cu. Interestingly, equivalent low
380 numbers of genes were identified in previous transcriptional analyses of other
381 streptococci exposed to Cu stress (14-16). Our findings are consistent with these prior
382 reports, and support the notion that these transcriptional responses function in
383 housekeeping or homeostasis to set a low limit of Cu availability in the cytoplasm (15).
384 In GBS, *copA* is among the most strongly activated genes in the transcriptional

385 response to Cu stress, and the mutational analysis performed in this study shows that
386 *copA* is crucial for the bacteria to attain an essential Cu efflux response during Cu
387 stress. However, *copA* is only one of an assembly of genes engaged by GBS during Cu
388 stress and it is likely that other genes in the transcriptome contribute to Cu detoxification
389 via additional means. For example, we detected up-regulation of putative metal
390 transporters for Mn (*mtsABC*, *mntH2*) along with concurrent down-regulation of a
391 system that encodes Fe transport machinery (*fetAB*). These transcriptional insights are
392 interesting because they hint at additional stress response mechanisms that occur
393 during Cu stress in GBS, which extend beyond CopA and which need elucidation. Metal
394 content analysis by ICP-OES showed that high Cu stress disrupts additional pools of
395 intracellular trace metal, including for example causing modest elevations in levels of Fe
396 along with major increases in Cu content. The consequences of these alterations in
397 metal content in GBS for cell activity need further examination.

398

399 Transcription of metal import and export genes, including those mentioned above, is
400 typically controlled by metal-dependent regulatory proteins termed metalloregulators that
401 sense metal ion bioavailability and work to maintain cellular metal homeostasis (44). In
402 our study, we detected no major changes in the expression of genes for
403 metalloregulators (25, 44), such as for Mn (*mntR/mtsR*), Fe (*fur*), Zn (*adcR/sczA*)
404 transport and for the sensing of peroxide (*perR*) in GBS undergoing Cu stress. It would
405 be of interest to elucidate whether such regulators undergo mis-metalation in GBS
406 during Cu stress, since it is conceivable that Cu may displace Mn and Fe from proteins
407 as described in other studies (45).

408

409 In *S. pyogenes*, Mn uptake is facilitated by *mtsABC* and is protective against peroxide
410 induced stress (25) and controlled by the MtsR regulator (25, 46). Mn and Fe transport
411 are co-ordinated in order to control metalation of superoxide dismutase (25), which can
412 use Mn or Fe at its catalytic site in streptococci (36). The consequences of Cu
413 intoxication relating to oxidative stress was explored using killing assays with hydrogen
414 peroxide in this study. These revealed reduced viability in *copA*-deficient GBS, but only
415 in cells that had been pre-exposed to Cu. Taken with our observations that Cu
416 accumulates significantly in this strain following Cu intoxication (and to a lesser extent
417 Mn and Fe), these data hint at a role for oxidative damage as a killing mechanism. Fe
418 can produce hydroxyl radicals and Cu(II) can be oxidised to Cu(I) in reactions with
419 peroxide, both of which damage bacterial cells. A system for the dual import of Mn and
420 Fe in GBS is encoded by *mntH* that is regulated by pH (47). In some strains of GBS,
421 including the hypervirulent ST17 lineage used in this study, two homologues of MntH
422 exist, encoded by *mntH* and *mntH2*. This study demonstrates up-regulation of *mntH2*,
423 but not *mntH*, in response to Cu, however the role of *mntH2* or its regulation are
424 undefined in GBS. In *Bacillus subtilis* MntR co-ordinates Mn import (and efflux), by
425 control of genes homologous to GBS *mntH* and *mntH2* that import Mn (48). This may
426 provide context for elevated *mntH2* expression in the presence of modest Cu stress in
427 our study, since Cu-bound MntR has a much lower affinity for binding its target operator
428 sequence (49). We also note that *mtsABC* and *mntH2* are up-regulated in concert with
429 down-regulation of Fe-transporting *fetAB* in response to Zn stress in GBS (50).

430 Together, our data indicate that modulation of *mtsABC*, *mntH2* and *fetAB* expression
431 forms parts of a transcriptional signature of GBS exposed to Cu or Zn stress.
432

433 Our assays of bacterial growth *in vitro* in conditions of Cu stress revealed striking
434 differences in growth phenotypes of GBS between nutritionally rich (THB) and limited
435 (CDM) media, possibly reflecting relative quantities of compounds that confer a
436 protective advantage for survival during Cu stress, such as glutathione (15) or other
437 thiol-containing amino acids that may interact with free Cu ions in solution. Glutathione is
438 not included in CDM as a separate chemical constituent, but the quantities of
439 methionine, cystine and cysteine in our preparations of CDM are 30, 62.6 and 50 mg.L⁻¹
440 ¹, respectively. Importantly, Cu exposure assays *in vitro* are almost certainly influenced
441 by the compounds present in the medium that likely affect levels of Cu that become
442 inhibitory (15). For example, Cu buffering effects likely occur in THB, which would
443 influence toxicity of Cu towards the bacteria. Our rationale for using THB to analyse Cu
444 toxicity in GBS is that this medium is a standard rich growth medium for GBS and is
445 widely used for studies of this organism. The challenges inherent in such a rich media,
446 including for example, Cu buffering effects, led us to also examine the impact of Cu
447 toxicity on GBS in a defined, nutrient-limited medium (CDM), which revealed that lower
448 ranges of concentrations were required to initiate Cu intoxication. Some of the Cu
449 concentrations used in our study may be considered supra-physiological (1-1.5 mM) but
450 the magnitude of these concentrations is likely much greater than the magnitude of
451 'free' Cu ions (not bound by other molecules) that would be present and available to
452 react biologically. The concentration ranges used were informed by available literature

453 along with measures of expression of *copA*, since we and others have shown that
454 transcriptional activity of *copA* is influenced by cellular Cu. Thus, *copA* activity can be
455 considered a *bona fide* reporter of free cellular Cu; or a surrogate marker for exposure
456 to Cu stress. Consistent with this notion, we observed higher fold-change in expression
457 of *copA* (7.6 ± 2.3 fold up-regulated; not shown) in CDM with 100 μ M Cu than in THB
458 with 500 μ M Cu (4.9 ± 0.5 fold up; Figure 5). The Cu concentrations that are
459 encountered by GBS at infection sites or in different intracellular compartments in host
460 cells are unknown. It is also possible that the levels of Cu utilized in our assays in THB
461 are distinct from those in host niches. Cu is elevated in the host at sites infected with *S.*
462 *pyogenes* (15), including the blood and in infected skin lesions. For mycobacterial
463 phagosomes, investigators have reported Cu concentrations of 25 μ M, and in *M.*
464 *tuberculosis*-infected macrophages, intravacuolar concentrations of 0.4mM (51).
465 Notwithstanding considerations of possible physiological ranges of Cu, the Cu exposure
466 assays used here are beneficial to establish *bona fide* gene function and bacterial
467 responses to Cu intoxication.

468

469 Our finding that *copY* functions to repress *copA* in the absence of Cu is consistent with
470 previous reports in other bacteria (52, 53). Disrupting the genetic systems for Cu efflux
471 in GBS, via mutation of the CopA exporter, or the CopY regulator, reveals divergent
472 phenotypes that stem from loss of export or regulatory function, resulting in
473 accumulation ($\Delta copA$) or reduction ($\Delta copY$) of cellular Cu. These phenotypes will be of
474 interest to dissect in terms of the role of CopY in GBS biology in other models of
475 infection and disease in the future. The results of the current study indicate that GBS

476 might have a higher intrinsic level of resistance to Cu compared to other *Streptococcus*
477 spp., including *S. pyogenes*. However, we did not directly compare different species and
478 differences in experimental approaches or background solution, as our study
479 demonstrates, might affect these assays. Thus, further work characterising relative
480 resistance between streptococci more directly would be of interest to the field.

481

482 Studies have demonstrated increased Cu levels in the blood of humans during bacterial
483 infection (54, 55), however most of the insight into Cu-driven antimicrobial responses is
484 derived from studies of mammalian cells infected *in vitro*. In macrophages, bioavailability
485 of Cu correlates with antibacterial activities (56), and Cu 'hot spot' formation mediates
486 antimicrobial responses against intracellular bacteria (7). It would be of interest to
487 examine if Cu release within phagocytic cells co-localises with intracellular GBS. Our
488 findings based on *in vitro* macrophage infection showed no attenuation of GBS devoid
489 of CopA for survival in host cells, which was surprising given the important role of Cu
490 management in survival of other bacteria inside macrophages (6), and epithelial cells
491 (12). Notably, however, intracellular survival of *Salmonella* deficient in *cueO*, which
492 encodes an enzyme required for resistance to Cu ions, was not impaired in murine
493 macrophages in a previous study (57), leading the authors to suggest multiple host
494 factors are involved in clearance of the bacteria. In addition, *copA*-deficient *S. pyogenes*
495 were not impaired for survival in assays with human neutrophils [15]. Our study is
496 consistent with these findings. Other researchers have described the limitations of *in*
497 *vitro* tissue culture monolayer assays for determining intracellular survival of bacteria in
498 the context of Cu homeostasis (58).

499

500 Systemic infection of mice exposed a connection between the ability of GBS to generate
501 a Cu management response via CopA and bacterial virulence *in vivo*. Here, *copA* was
502 essential for GBS to fully colonize and survive in the blood, as well as in other tissues.

503 In demonstrating a significant attenuation of GBS deficient in CopA to be fully virulent in
504 mice, we suggest that Cu toxicity may represent a form of stress experienced by the

505 bacteria *in vivo* during systemic infection. In other bacteria, including *Pseudomonas*
506 *aeruginosa* and *Listeria monocytogenes*, compromised Cu transport leads to

507 attenuation for colonization in various infection models (58, 59). Attenuation of GBS for
508 colonization of the blood, liver and spleen indicates that Cu management in the bacterial

509 cell is essential not only for efficient survival of the bacteria in the bloodstream but also
510 for colonization of highly immunologically active tissues; *i.e.*, Kupffer cells and splenic

511 lymphocytes for innate and adaptive immune responses, respectively. It is plausible that
512 Cu stress that might be encountered by GBS *in vivo* could influence virulence factor

513 function, such as the hypervirulence associated adhesin, HvgA, which we have shown
514 is down-regulated under Cu stress. It would be of interest to analyse the effect of Cu

515 transport deficiency in GBS in other relevant models of infection, including in vaginal
516 colonization (60). Additional to defining the effects of metal homeostasis in GBS on the

517 nature of infection and disease caused by this organism, small-molecules probes might
518 hold promise for the identification of other molecular mechanisms of metal homeostasis

519 in GBS, as reported for Gram-positive bacteria (61).

520

521 In summary, this study shows that management of Cu export in GBS is essential for the
522 bacteria to survive in environments of Cu stress. Cu intoxication generates a
523 transcriptional signature that includes activation of the *cop* operon to confer bacterial
524 survival and virulence. The exact role for Cu ions as an antibacterial response against
525 GBS warrants further investigation.

526

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534

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728 mSphere 2.
729

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

Locus_ tag [†]	Genbank accession	Label	Annotation (NCBI)	FC	FDR
02565	ASZ00809.1	<i>copY</i>	CopY/TcrY family copper transport repressor	4.1	1.09E-12
02575	ASZ00811.1	<i>copZ</i>	carbonate dehydratase	4.0	1.02E-37
02570	ASZ00810.1	<i>copA</i>	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	<i>mtsA</i>	metal ABC transporter substrate-binding protein	2.9	2.15E-12
07960	ASZ01820.1	<i>mtsB</i>	metal ABC transporter ATP-binding protein	2.6	1.02E-15
10190	ASZ02237.1	<i>mntH2</i>	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	<i>mtsC</i>	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	<i>hvgA</i>	pathogenicity protein	-2.3	2.18E-07
04720	ASZ01199.1	<i>pcl1</i>	membrane protein	-4.3	1.38E-09
04185	ASZ01095.1	<i>fetB</i>	iron export ABC transporter permease subunit, FetB	-4.9	1.39E-08
04180	ASZ01094.1	<i>fetA</i>	ABC transporter ATP-binding protein FetA	-4.9	4.58E-11

731 [†] denotes locus tag of *S. agalactiae* 874391, preceded by CHF17_RS; FC, Fold-

732 Change; FDR, False Discovery Rate

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

Bacteria		
Strains	Characteristics*	Source
<i>E. coli</i> DH5α	<i>huA2 lac(Δ)U169 phoA glnV44 Φ80' lacZ(Δ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	Bethesda Research Labs
<i>S. agalactiae</i> 874391	Wild type, Sequence type-17, Serotype III strain, Vaginal isolate (Japan)	(62)
<i>S. agalactiae</i> GU2691	874391Δ <i>copA</i> (<i>copA</i> ⁻ mutant) Locus tag: CHF17_RS02570	This work
<i>S. agalactiae</i> GU2857	874391Δ <i>copY</i> (<i>copY</i> ⁻ mutant) Locus tag: CHF17_RS02565	This work
<i>S. agalactiae</i> GU3121	GU2691 (Δ <i>copA</i>) containing <i>copYAZ</i> complement construct pGU3112; Sp	This work
Plasmids		
pHY304aad9	<i>ori</i> (Ts); temperature-sensitive shuttle vector; Sp	(29)
pGU2650	pHY304aad9-derivative <i>copA</i> Δ construct; Sp	This work
pGU2847	pHY304aad9-derivative <i>copY</i> Δ construct; Sp	This work
pGU3112	pDL278 containing cloned <i>copYAZ</i> operon; Sp	This work

734 * Sp = Spectinomycin-resistant; Ts = temperature-sensitive

754 **FIG 1.** Global transcriptomic analysis of GBS in response to Cu stress. Volcano plot
755 showing data from RNASeq analysis of WT GBS cultures exposed to 0.5 mM Cu
756 compared to non-exposed controls. Transcripts detected as up- or down-regulated in
757 response to Cu ($n=4$, $>\pm 2$ -fold, FDR <0.05) are highlighted in red and blue,
758 respectively. Dotted lines show False discovery rate (FDR; q-value) and fold-change
759 cut-offs. Grey points indicate genes that were not significant changed in expression,
760 according to these analysis cut-offs. Selected genes are identified with black lines. B,
761 Validation of RNASeq data. Expression ratio (Fold-change) of *pcl1*, *hvgA* and *copY*
762 quantified by qRT-PCR in THB medium containing 0.5 mM Cu compared to THB without
763 Cu. Ratios for qRT-PCR were normalized using housekeeping *dnaN* and RNASeq ratios
764 were calculated using DESeq2. Bars show means and S.E.M from 4 independent
765 experiments.

766
767 **FIG 2.** Organisation of the *copY-copA-copZ* locus in GBS. A, *copY-copA-copZ* are
768 adjacent in the GBS genome and likely controlled by the promoter-proximal *copY*,
769 encoding a putative Cu-sensing repressor. Locus tags from the GBS 874391 genome
770 are indicated. B, Distribution of homologous *cop* genes in other Lactobacillales,
771 arranged by percentage identity of amino acid sequence to CopA of *S. agalactiae*. C,
772 Model of Cu efflux in GBS highlighting CopA as transmembrane Cu exporter,
773 transcriptionally repressed by CopY in the absence of Cu, which is likely inhibited by the
774 Cu-binding chaperone protein CopZ. Figure based on previous studies in other
775 lactobacillales (53, 63).

776 **FIG 3.** Growth curve analyses of GBS cultured in nutrient-rich THB medium (A), or in
777 THB supplemented with 0.5 mM Cu (B), 1.0 mM Cu (C) or 1.5 mM Cu (D), comparing
778 WT, $\Delta copA$ or $\Delta copY$ strains. Complementation of $copA$ mutation ($\Delta copA+C$) restored
779 growth of GBS to WT levels in conditions of Cu stress. Points and bars show mean and
780 S.E.M of several independent experiments (5 for WT, $\Delta copA$ and $\Delta copY$, 2 for
781 complemented strain) monitoring attenuation at 600nm.

782

783 **FIG 4.** Bactericidal effect of Cu on GBS viability. Time-kill assays comparing WT, $\Delta copA$
784 or $\Delta copY$ GBS (and complemented strain $\Delta copA+C$), incubated in CDM or in CDM
785 supplemented with 0.05, 0.1, 0.2, 0.5 or 1 mM Cu. Viable cells were quantified at 1h,
786 3h, 6h and 24h post incubation. [†] Viable Cell counts of 0 CFU/mL were assigned a value
787 of 1 to plot on log₁₀ y-axes. Points and bars show mean and S.E.M of 4 independent
788 experiments. Data were analysed by One-way ANOVA with Holm Sidak Multiple
789 comparisons. Significance markers represent comparisons of the number of surviving
790 bacteria in conditions of the defined Cu concentration [Cu] vs the number of bacteria in
791 the non-exposed control (0 mM Cu) for the same time point and same strain (* P < 0.05).

792

793 **FIG 5.** Expression analysis of $copA$ and intracellular metal content in GBS strains. A,
794 Expression ratio (Fold-change) of $copA$ quantified by qRT-PCR in THB medium
795 containing 0.25, 0.5, 1.0 and 1.5 mM Cu, compared to THB without Cu. B, Relative
796 $copA$ transcripts were quantified in WT and $\Delta copY$ strains with and without Cu
797 supplementation (0.5 mM) to demonstrate de-regulation of $copA$ expression in the
798 $\Delta copY$ background. Intracellular accumulation of Cu (C), Mn (D), Fe (E) and Zn (F) was

799 compared with and without Cu supplementation (0.5 mM) in WT, $\Delta copY$ and $\Delta copA$
800 strains. Ratios in A were calculated as described previously (35) using C_T values, primer
801 efficiencies and housekeeping *dnaN*. Bars show means and S.E.M from 3-4
802 independent experiments and compared by One-way ANOVA with Holm-Sidak multiple
803 comparisons (* $P < 0.05$; *** $P < 0.001$). Ctrl: Control condition (i.e., no supplemental Cu
804 in media). The ANOVA P value from comparisons of between strains was 0.06 for
805 Cellular Fe and 0.01 for Cellular Zn; subsequent pairwise Student's *t*-tests were used to
806 compare each strain in the Ctrl and Cu condition (* $P < 0.05$).

807

808 **FIG 6.** Interactions of GBS with human macrophage-like cells. Gentamicin protection
809 assays with WT and $\Delta copA$ strains in human (U937 monocyte-derived macrophage-like)
810 macrophages in media with and without 20 μ M supplemental Cu. Surviving bacteria are
811 expressed as CFU/mL, indicating the number of intracellular bacteria at 1h after
812 antibiotic treatment, and at 24h and 48h post infection (h.p.i). Data are means and
813 S.E.M of 3-4 independent experiments.

814

815 **FIG 7.** Virulence of WT (grey circles) or $\Delta copA$ (blue diamonds) GBS in a mouse model
816 of disseminated infection. C57BL/6 mice (6-8 weeks old) were intravenously injected
817 with 10^7 bacteria; bacteremia and disseminated spread to liver and spleen were
818 monitored at 24h post infection. CFU were enumerated and counts were normalized
819 using tissue mass in g. Viable Cell counts of 0 CFU/mL were assigned a value of 1 to
820 enable visualisation on $\log_{10}$ y-axes. Lines and bars show median and interquartile

821 ranges and data are pooled from 2 independent experiments each containing n=10

822 mice compared using Mann-Whitney U-tests (*P < 0.05, **P < 0.01).

823