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The role of CopA in *Streptococcus pyogenes* copper homeostasis and virulence

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ABSTRACT

Maintenance of intracellular metal homeostasis during interaction with host niches is critical to the success of bacterial pathogens. To prevent infection, the mammalian innate immune response employs metal-withholding and metal-intoxication mechanisms to limit bacterial propagation. The first-row transition metal ion copper serves critical roles at the host-pathogen interface and has been associated with antimicrobial activity since antiquity. Despite lacking any known copper-utilizing proteins, streptococci have been reported to accumulate significant levels of copper. Here, we report that loss of CopA, a copper-specific exporter, confers increased sensitivity to copper in *Streptococcus pyogenes* strain HSC5, with prolonged exposure to physiological levels of copper resulting in reduced viability during stationary phase cultivation. This defect in stationary phase survival was rescued by supplementation with exogeneous amino acids, indicating the pathogen had altered nutritional requirements during exposure to copper stress. Furthermore, *S. pyogenes* HSC5 Δ*copA* was substantially attenuated during murine soft-tissue infection, demonstrating the importance of copper efflux at the host-pathogen interface. Collectively, these data indicate that copper can severely reduce the viability of stationary phase *S. pyogenes* and that active efflux mechanisms are required to survive copper stress in vitro and during infection.

1. Introduction

Streptococcus pyogenes is a major human pathogen that can cause mild to severe group A streptococcal (GAS) infections, resulting in pharyngitis, tonsillitis, otitis, necrotizing fasciitis, meningitis, and sepsis. Asymptomatic carriage is common amongst children, with approximately 12% of colonized children having no obvious disease symptoms [1]. Globally, there are an estimated 616 million GAS pharyngitis cases, and 517,000 deaths yearly are due to severe GAS infections [2,3]. According to the Centers for Disease Control and Prevention, there are 9000 to 11,500 cases of invasive GAS disease each year in the United States, causing 1000 to 1800 deaths; 6% to 7% of which are necrotizing fasciitis. Although more rare, outbreaks of pneumonia caused by GAS can also occur [4]. The niches where *S. pyogenes* can exist as a commensal organism and a pathogen highlight this pathogen's versatility and underscore the bacterium's capacity to thrive in highly diverse environments.

Acquiring nutrients at the host-pathogen interface is essential to bacterial survival in the mammalian host. Nutritional immunity, whereby the host deploys specialized strategies to restrict microbial access to necessary nutrients, is a crucial component of how the host limits bacterial outgrowth during infection [5]. Limitation of metal ion bioavailability is a key example of nutrient restriction imposed by mammalian hosts during bacterial infection [5–7]. For example, individuals with underlying conditions resulting in elevated iron are at an increased risk of infection by several bacterial pathogens [8–10]. To

Abbreviations: CFU, colony forming unit; EDTA, ethylenediaminetetraacetic acid; GAS, group A streptococcus; ICP-MS, inductively coupled plasma-mass spectrometry; OD600, optical density at 600 nm; Rev, revertant; *S. pneumoniae*, *Streptococcus pneumoniae*; *S. pyogenes*, *Streptococcus pyogenes*; SDS, sodium dodecyl sulfate.

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circumvent this replication-limiting constraint imposed by the host, bacterial pathogens have evolved an array of metal ion scavenging mechanisms [11–13]. Not unsurprisingly, many of these acquisition mechanisms are required for virulence across a multitude of bacterial species in diverse host niches. Although metal ions are essential for bacterial replication, in excess they can be detrimental, as the chemistry of metal ions can be exploited by the host to mediate toxicity towards invading pathogens. This concept underscores that bacterial metal ion homeostasis, a process reliant upon efficacious import and efficient efflux of metal ions, is a critical aspect of virulence at the host-pathogen interface [14–16].

S. pyogenes can establish infection in a plethora of host tissues, all of which present distinct environments for metal ion bioavailability. Consequently, the relative importance of different pathways in metal ion homeostasis can vary depending on the niche of the infection site. Manganese (Mn) efflux is critical for *S. pyogenes'* response to oxidative stress and evasion from neutrophil-mediated killing [17]. Infiltration of neutrophils is a key feature of *S. pyogenes* tissue infection, noteworthy due to neutrophils' high concentration of the metal-chelating protein calprotectin, which is one of the main host strategies for manganese sequestration [18,19]. Perturbing zinc efflux via deletion of *czcD* imparts a similar phenotype, with isogenic mutants displaying heightened sensitivity to neutrophil-mediated killing and attenuation in murine models of infection [20]. Transposon mutagenesis screening of gene essentiality during soft tissue infection likewise identified metal-uptake systems, such as zinc uptake and manganese/iron transporters, that were required for survival of *S. pyogenes* in a necrotic lesion model of infection [21]. These data underscore the importance of metal homeostasis mechanisms for *S. pyogenes* disease progression, particularly those characterized by a high degree of inflammatory infiltrates, such as soft tissue infection.

Copper is an essential trace element found in all eukaryotes but also has potent antimicrobial properties that have been appreciated since antiquity. The mammalian host has exploited these antimicrobial properties to facilitate more-effective bacterial killing by innate immune cells, particularly in macrophages [22–25]. Macrophage-mediated clearance is a critical aspect of the initial clearance of *S. pyogenes* during infection, with animals depleted of macrophages displaying dramatically reduced survival to the pathogen [26]. However, there is evidence that GAS has mechanisms to evade these host defenses, such as the ability to survive and replicate intracellularly within macrophages during invasive disease in humans [27–29]. Reports suggest that copper intoxication is one of the mechanisms used by macrophages to mediate effective bacterial clearance [30]. Evidence for this inference arises from studies of the copper transporter ATP7a, which translocates copper into the phagosome and is associated with enhanced bacterial killing [22,23,31,32]. Additional evidence for the importance of copper efflux systems has been derived from by numerous studies in which these transporters are implicated in the virulence of both bacterial and fungal pathogens [33–35]. The copper resistance systems of Gram-positive bacteria, such as *Streptococcus pneumoniae* [36,37], *Enterococcus hirae* [38], *Streptococcus mutans* [39], and *Lactococcus lactis* [40], are typically comprised by a copper-binding metalloregulator (*copY/R*) that controls expression of a copper-binding metalloprotein (*cupA/copZ*) with chaperone functionality, and a copper-specific P_{1B}-type ATPase (*copA*), which exports cuprous ions in an ATP-dependent manner. Previous work has shown that *copA* is upregulated in the presence of copper stress and contributes to copper tolerance of an M1T1 clinical strain of *S. pyogenes* [41].

The broad distribution of copper efflux systems across kingdoms and bacterial species underscores the central importance of copper homeostasis across the Domains of life. We sought to investigate the role of the primary copper efflux system of *S. pyogenes* in the context of metal homeostasis and virulence. In agreement with previous studies, our data confirm that *S. pyogenes* encodes a prototypical P-type ATPase specific for copper resistance [41]. Underscoring a role for copper efflux during infection, *copA* mutant strains were significantly attenuated in a necrotic lesion model of infection. Sub-inhibitory copper stress reduced stationary phase survival, a defect that could be partially rescued via amino acid supplementation. These data highlight the importance of copper homeostasis in the pathogenesis of *S. pyogenes* and the transcriptional consequences of cellular copper accumulation.

2. Methods

2.1. Genetic manipulation

An in-frame truncation mutant of *copA* (*SPy_1715*), in which the bases from 216 to 595 that code for the consensus copper I binding domain were deleted, was cloned into the temperature-sensitive vector pJR233 and electroporated into *S. pyogenes* strain HSC5 (Tables S1 and S2) [42]. The truncated sequence was crossed into the chromosome via recombination when transformants were transferred to a non-permissive temperature. Following serial passage at permissive temperature (three overnight passages at 30 °C), strains were plated to recover individual colonies which were subsequently replica plates on ThyB with and without erythromycin selection (1 $\mu g.mL^{-1}$). Sensitive strains were then screened to either contain the full-length *copA* (referred to as the HSC5 revertant) or the truncated version (referred to as Δ*copA*). The revertant strain is included in assays as a passaged control as it has undergone the same passaging as the deletion strain, and hence serves as an important comparator when examining phenotypes of the generated mutant.

2.2. Phenotypic growth assays

S. pyogenes HSC5 (wild type), revertant, and Δ*copA* strains were grown overnight at 37 °C in ThyB (30 g Todd Hewitt Broth, 2 g Yeast Extract, 1 L dH_2O). Following overnight growth, 100 μL of the overnight culture was inoculated into triplicate samples of 10 mL of ThyB, pH 6.5, containing the following reagents: varying concentrations of $CuSO_4$; and/or 20 $\mu L.mL^{-1}$ Oxyrase; and/or 100 μM of $MnSO_4$, $FeSO_4$, or $ZnSO_4$. Bacteria were then incubated at 37 °C, and the optical density at 600 nm (OD600) was monitored to ascertain growth kinetics. To determine the impact of copper supplementation on prolonged viability, cultures were supplemented with copper at concentrations ranging from 25 to 100 μM immediately following a 1:100 back-dilution of overnight cultures. At 7–8 h (early stationary phase) and 24 h (prolonged stationary phase) post-inoculation, the three strains, wild type, revertant, and Δ*copA*, were serially diluted and plated onto ThyB plates and incubated overnight at 37 °C in a GasPack jar (BD, USA) to enable colony-forming units (CFU) enumeration. Supplementation of copper upon entry into stationary phase (7–8 h following back-dilution) was undertaken with the same copper concentrations and viability measured at 24 h (16 h following addition of copper).

2.3. Zone-of-inhibition assay

Overnight cultures of *S. pyogenes* HSC5, revertant, or Δ*copA* strain were plated (100 μL) on ThyB plates containing a centrally placed filter paper disk that had absorbed 10 μL of 1 M $CuSO_4$. Inoculated plates were incubated anaerobically at 37 °C for 12 h in a GasPack jar. The distance from the edge of the filter paper disc to the border of bacterial growth was measured as the zone of inhibition. Experiments were performed in biological triplicate.

2.4. Preparation of chromosomal DNA from S. pyogenes

S. pyogenes HSC5 was grown overnight in ThyB supplemented with 20 mM glycine. The bacterial pellet was collected by centrifugation at 7000 ×*g* for 10 min at 4 °C, washed in Tris-EDTA (TE) buffer before incubation with 50 mg lysozyme for 1 h at 37 °C. After centrifugation at 7000 ×*g* for 10 min at 4 °C, the pellet was frozen at −80 °C, freeze-

thawed thrice, resuspended in TE, and placed at 65 °C for 15 min in a mixture of 0.4 mL 10% (*w/v*) sodium dodecyl sulfate (SDS) and 0.5 mL Tween-20. Following incubation, 25 μL RNase (5 mg.mL^{-1}) was added to the sample, followed by a further incubation at 37 °C for 30 min. The lysate mixture was then subjected to routine phenol-chloroform extraction followed by DNA precipitation with 2.5 volumes of 95% (*v/v*) ethanol. DNA was pelleted by centrifugation at 14,000 ×*g* for 10 min, washed in 70% (v/v) ethanol, and resuspended in dH_2O.

2.5. Whole-cell metal ion accumulation

Total cell-associated metal ion content was determined essentially as described previously [43,44]. Succinctly, *S. pyogenes* HSC5, revertant, and the Δ*copA* strains were grown in metal-restricted, chemically defined media (CDM) (MP Biomedicals, #111009617) with or without 100 μM $CuSO_4$ to OD600 = 0.4 (mid-log phase) Bacterial pellets were harvested by centrifugation at 7000 ×*g* for 10 min at 4 °C. Bacterial pellets were washed three times with Phosphate Buffered Saline (PBS) supplemented with 5 mM ethylenediaminetetraacetic (EDTA), followed by three washes in PBS to remove any remaining EDTA. Bacterial pellets were collected by centrifugation at 10,000 ×*g* for 5 min and desiccated by overnight incubation at 95 °C. The mass of the dry cell material was measured, followed by resuspension in 35% (*v/v*) HNO_3. The digested samples were diluted 20-fold in ddH_2O to a final volume of 1 mL and analyzed in technical triplicate using on an Agilent 8900 triple quadrupole inductively coupled plasma-mass spectrometer (ICP-MS, Agilent Technologies). Samples were introduced directly from 1.5 mL polypropylene tubes via an integrated automation system autosampler (Agilent Technologies) using a peristaltic pump. Sample desolvation was performed via a MicroMist nebulizer (Glass Expansion, Australia). The instrument was calibrated for elements of interest (^{44}Ca, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, ^{66}Zn) using mixed 0, 5, 10, 25, 50, 100, 250 and 500 parts per billion standard calibration solutions in 1% (*v/v*) nitric acid (HNO_3) from commercially available certified reference standards (Multi-element Calibration Standard 2A, Agilent Technologies). A reference element solution containing 100 μg L^{-1} yttrium (Y) (Agilent Technologies) was introduced via T-piece positioned after the peristaltic pump and was used to normalize all measurements. The tuning solution for the instrument contained 1 μg.L^{-1} of cerium (Ce), cobalt (Co), lithium (Li), thallium (Tl) and Y in 2% (*v/v*) HNO_3 (ICP-MS Stock Tuning Solution, Agilent Technologies). Six independent biological samples were analyzed in technical triplicate, with the statistical differences between the sample means analyzed by one-way ANOVA with Šídák multiple comparison post-test (GraphPad Prism 9.3.1).

2.6. RNA extraction for microarray

For the microarray experiments, *S. pyogenes* HSC5, revertant, and Δ*copA* strains were grown in 30 mL of ThyB at 37 °C. At early exponential phase growth (OD600 = 0.3) the strains were exposed to a shock treatment of 100 μM $CuSO_4$ for 30 min. The treated cultures were then diluted into RNA Protect (Qiagen) at a ratio of 1:2 (culture:RNA Protect) and harvested by centrifugation at 5000 ×*g* for 10 min. Total RNA was extracted using a RiboPure RNA Purification kit (ThermoFisher) according to the manufacturer's protocols. The concentration and purity of extracted RNA for each sample were determined by the absorbance values ratio at 260/280 nm.

2.7. Microarray analysis

The *S. pyogenes* M1 *S. pyogenes* oligonucleotide 3x20K microarrays were purchased from MYcroarray (Ann-Arbor, USA). All hybridization, washing, and mapping steps were performed per the manufacturer's protocols. Microarray data are available as supplementary data files (Supplementary Files 1–6). Although a microarray signal for *copA* was observed, this was due to the oligonucleotide probe comprising the sequence retained after the in-frame deletion mutant was generated.

2.8. qRT-PCR

HSC5, Δ*copA* mutant, and the revertant were grown in 10 mL of ThyB media overnight at 37 °C. At 24 h, each strain was diluted 1:100 into 10 mL of fresh ThyB media and incubated at 37 °C. At an OD_{600} of 0.3, 4 replicates of HSC5, Δ*copA* mutant, and the revertant were exposed to 100 μM of $CuSO_4$ for 30 min at 37 °C. Control samples (4 replicates) were not exposed to $CuSO_4$ but allowed to incubate for an additional 30 min at 37 °C. Samples were then centrifuged at 5000 ×*g* for 10 min. ThyB media was removed, and the pellet was resuspended in 5 mL of RNAprotect Bacteria Reagent (Qiagen 76,506) and stored at −80 °C overnight. Samples were subsequently thawed and centrifuged at 5000 ×*g* for 10 min. RNA protect was removed and RNA was extracted using RNeasy Mini kit (Qiagen 74,104) with the following modifications to the manufacturer's protocol. Samples were resuspended with 800 μL RDE + β-mercaptoethanol (Sigma M3148-100ML) and placed into Fast Prep tubes (MP Biomedical 116,914,100) and cells were lysed for 45 s six times in Fast Prep – 24 (MP Biomedicals 116,004,500). Tubes were incubated at 70 °C for 10 min and cooled to room temperature. Samples was transferred to Qiashredder (Qiagen 79,656) and centrifuged at 10,000 ×*g* for 1 min. Flow through was then extracted with the RNeasy Mini kit (Qiagen 74,104) according to the manufacturer's protocol. RNA was quantified using a Nanodrop One (Thermo Scientific ND-ONE-W). A secondary DNA Removal kit (Invitrogen AM1906) was used according to manufacturer's protocol using 20 μg of RNA. Samples were quantified using the Nanodrop One and 1.5 μg of RNA was used for cDNA synthesis (Invitrogen 18,080–400) according to manufacturer's protocol. Each qRT reaction was run using 3 μL cDNA, 1 μL of each primer [10 μM], 10 μL 2× PowerUp SYBR Green (Applied Biosystems A25742) and 5 μL nuclease free water. All samples had two technical replicates. Data was analyzed by the ΔΔCt method and normalized to *gyrA*.

2.9. Amino acid supplementation in planktonic culture

Overnight ThyB cultures of the *S. pyogenes* HSC5, revertant, and Δ*copA* strains were diluted 1:20 into fresh ThyB. The cultures were then supplemented with 25 μM $CuSO_4$, a subinhibitory copper concentration. All amino acids were supplemented to media at a concentration of 100 μg/mL except L-cysteine and L-glutamine, which were supplemented at 50 μg/mL and 200 μg/mL, respectively. Unsupplemented (control) and supplemented cultures (25 μM $CuSO_4$; 25 μM $CuSO_4$ + 1 × amino acids) were incubated for 24 h at 37 °C. ThyB agar. For individual amino acid supplementations, *S. pyogenes* were grown overnight at 37 °C in ThyB and back diluted 1:100 into fresh ThyB supplemented with either 500 mg/L L-Cysteine or 100 mg/L L-Histidine, both with and without 25 μM $CuSO_4$ supplementation. Bacterial survival (CFU.mL^{-1}) was determined by serial dilution of the cultures, plating onto ThyB agar, and overnight incubation at 37 °C to enable CFU enumeration.

2.10. Subcutaneous SKH1 murine infection model

S. pyogenes HSC5, revertant, and Δ*copA* strains were grown in ThyB at 37 °C to an OD600 of 0.6. Bacteria were harvested by centrifugation at 7000 ×*g* for 10 min at 4 °C, sonicated (FS20, Fisher Scientific, maximal power) at room temperature for 60 s to disrupt streptococcal chains, and resuspended in phosphate buffered saline (PBS), pH 7.4. Anesthetized (2.5% isoflurane) 7-week-old SKH1 mice (Charles River Laboratories; strain code #686) were subcutaneously injected with 100 μL of the bacterial suspension, representing 2 × 10^7 CFUs, with groups of 5–10 mice infected for each strain. Infected mice were observed daily for signs of illness, and their lesions imaged for analysis by ImageJ version 1.52. Lesions were analyzed at 24 and 48 h for the area within the asymptomatic irregular border of each lesion. At 48 h, infected tissues were excised, weighed, and homogenized to determine the bacterial burden.

Enumeration of the bacterial load was performed by serial dilution of the tissue homogenate, followed by plating onto ThyB and overnight incubation at 37 °C. Note each mouse only had a single infection site/lesion.

2.11. Ethics statement

All experiments involving animals were performed with prior approval of and in accordance with guidelines of the St. Jude Institutional Animal Care and Use Committee. The St. Jude laboratory animal facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. Laboratory animals are maintained per the applicable portions of the Animal Welfare Act and the guidelines prescribed in the *Guide for the Care and Use of Laboratory Animals*.

3. Results

3.1. CopA is the primary copper efflux mechanism in S. pyogenes HSC5

In the *S. pyogenes* HSC5 strain (M14 serotype), *SPy_1715* represents the ortholog of *S. pyogenes* M1T1 *copA* and is predicted to contribute to bacterial copper homeostasis. The function of *SPy_1715*, henceforth *copA*, was investigated by generating an in-frame deletion of the gene in *S. pyogenes* HSC5. Copper tolerance of the *S. pyogenes* HSC5 wild type, Δ*copA*, and revertant strain was then examined in ThyB culture medium with or without 100 μM $CuSO_4$ supplementation. In the absence of copper supplementation all three strains had comparable growth kinetics in ThyB (Fig. 1A). Upon copper supplementation of the growth medium, the *S. pyogenes* HSC5 Δ*copA* strain demonstrated significantly reduced in vitro growth ($p \leq 0.05$ one-way, unpaired *t*-test) by comparison with the wild type and the revertant strains (Fig. 1A). This appeared to manifest at time points after 3 h growth, with the Δ*copA* strain not entering exponential phase growth in the presence of copper stress. Increasing copper supplementation from 0 to 1000 μM revealed that the *S. pyogenes* HSC5 Δ*copA* strain was hypersusceptible to copper stress (Fig. 1B). The cell density of the Δ*copA* strain in ThyB was significantly reduced ($p \leq 0.05$; two-way ANOVA with Tukey post-test), by comparison with the wild type and the revertant strains, at all copper concentrations. Notably, the viability of the Δ*copA* strain was essentially abrogated in concentrations of ≥500 μM $CuSO_4$ in contrast to the wild type and the revertant strains (Fig. 1B). The impact of copper exposure on *S. pyogenes* was further investigated using the sub-lethal concentration of 100 μM $CuSO_4$. Consistent with the growth analyses (Fig. 1B), survival of the Δ*copA* strain was significantly reduced relative to the wild type and HSC5 Rev strain ($p < 0.0001$; one-way ANOVA with Šídák post test) (Fig. 1C). Sensitivity of the Δ*copA* strain to copper was also observed on solid ThyB medium, with the mutant strain displaying a greater zone of inhibition ($p < 0.0001$; one-way ANOVA with Šídák post test) than the wild type or revertant strains (Fig. 1D).

CopA belongs to a subgroup of P_{1B}-type ATPases involved in the export Cu^+ ions and, in some instances, monovalent silver ions [45]. However, CopA is closely related to other soft metal-translocating P_{1B}-type ATPases, such as ZntA, that have more promiscuous metal ligand interactions and are associated with the export of divalent cations including zinc and cadmium [46]. Here, the role of CopA as a copper-specific efflux pathway in *S. pyogenes* HSC5 was further defined by examining the sensitivity of the Δ*copA* strain to divalent cation stress. Consistent with the predicted role of *S. pyogenes* CopA in Cu^+ efflux, the Δ*copA* strain did not show altered sensitivity to growth in the presence of divalent cation stress mediated by 100 μM $MgSO_4$, $MnSO_4$, $FeSO_4$, or $ZnSO_4$, in contrast to 100 μM $CuSO_4$ stress (Fig. S1). No growth rescue, nor enhanced toxicity, was observed in the 100 μM $CuSO_4$ treated cells when $MgSO_4$, $MnSO_4$, $FeSO_4$, or $ZnSO_4$ was supplemented in addition to

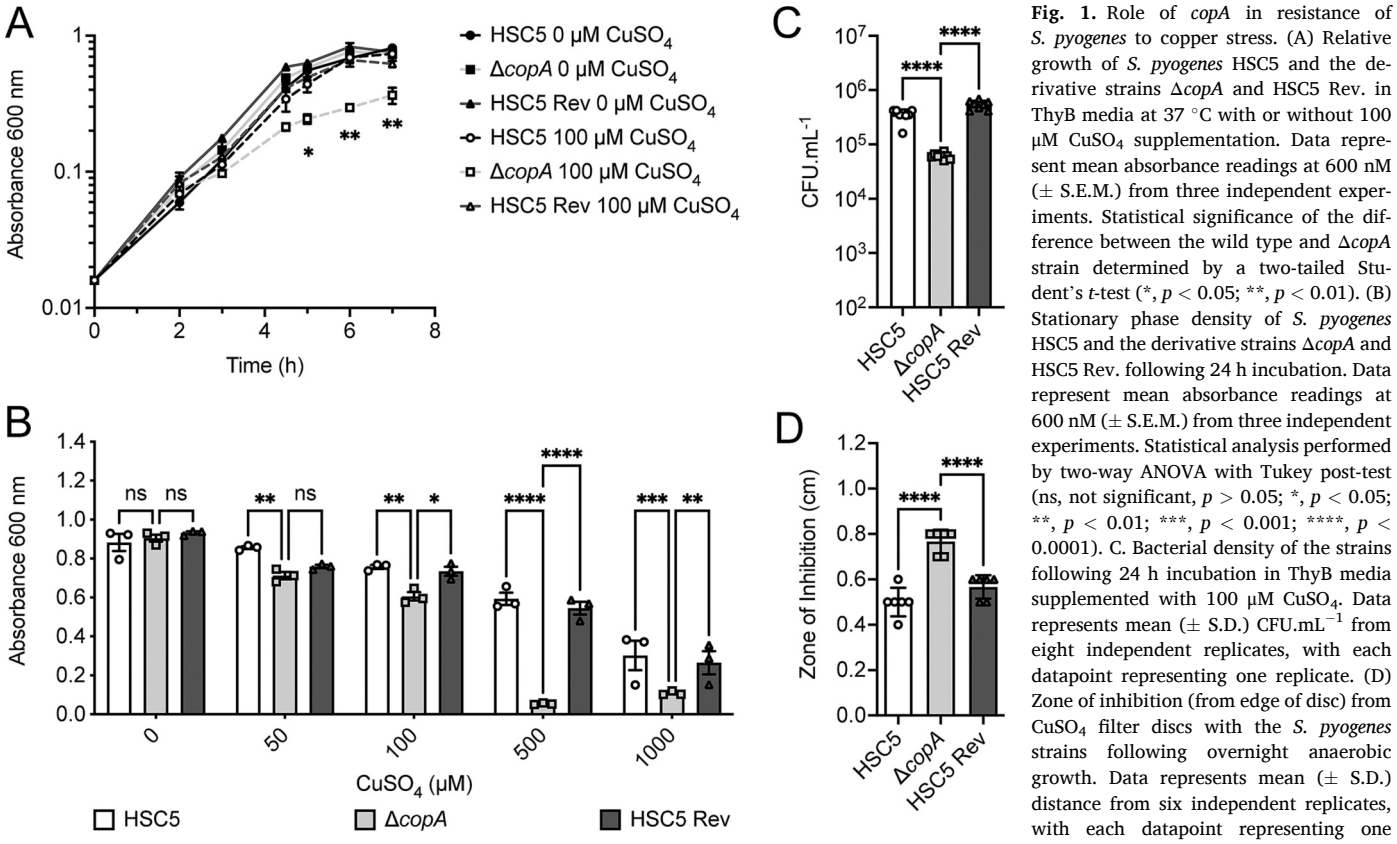

Fig. 1. Role of *copA* in resistance of *S. pyogenes* to copper stress. (A) Relative growth of *S. pyogenes* HSC5 and the derivative strains Δ*copA* and HSC5 Rev. in ThyB media at 37 °C with or without 100 μM $CuSO_4$ supplementation. Data represent mean absorbance readings at 600 nM (± S.E.M.) from three independent experiments. Statistical significance of the difference between the wild type and Δ*copA* strain determined by a two-tailed Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$). (B) Stationary phase density of *S. pyogenes* HSC5 and the derivative strains Δ*copA* and HSC5 Rev. following 24 h incubation. Data represent mean absorbance readings at 600 nM (± S.E.M.) from three independent experiments. Statistical analysis performed by two-way ANOVA with Tukey post-test (ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). C. Bacterial density of the strains following 24 h incubation in ThyB media supplemented with 100 μM $CuSO_4$. Data represents mean (± S.D.) CFU.mL^{-1} from eight independent replicates, with each datapoint representing one replicate. (D) Zone of inhibition (from edge of disc) from $CuSO_4$ filter discs with the *S. pyogenes* strains following overnight anaerobic growth. Data represents mean (± S.D.) distance from six independent replicates, with each datapoint representing one replicate. Statistical analysis in C and D performed using one-way ANOVA with Šídák post-test (****, $p < 0.0001$).

copper (Fig. S1). Taken together, these data support the role of CopA as a copper-specific efflux pathway involved in *S. pyogenes* copper homeostasis. The toxicity of copper towards the *S. pyogenes* HSC5 Δ*copA* strain appeared to be independent of oxygen availability. Bacterial growth in 100 μM $CuSO_4$ supplemented ThyB showed reductions in the cell density of the Δ*copA* strain relative to the wild type strain in both aerobic and anaerobic conditions (Fig. S2A,B). Unexpectedly, growth of the wild type was abrogated in 500 μM $CuSO_4$ supplemented ThyB under anaerobic conditions, whereas aerobic growth in 500 μM $CuSO_4$ supplemented ThyB was not substantially different to unsupplemented medium.

Collectively, these data support the role of CopA as the primary copper efflux pathway of *S. pyogenes* HSC5 consistent with the report from *S. pyogenes* GAS M1T1 strain 5448, supporting the inference of conserved functionality of this operon across *S. pyogenes* strains [47]. Further, these data show that copper-mediated killing occurs in an oxygen independent manner, suggesting that the molecular basis of toxicity may be attributable to mismetallation of enzymes in metabolic pathways or associated with other essential cellular processes.

3.2. The impact of copper stress on S. pyogenes HSC5 whole cell metal accumulation

Building on the above framework, the impact of the *copA* deletion on *S. pyogenes* HSC5 metal accumulation was investigated. The *S. pyogenes* HSC5 wild type, Δ*copA*, and revertant strain were grown in ThyB with or without 100 μM $CuSO_4$ supplementation and analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) (Fig. 2A-F). During growth in ThyB alone, no statistically significant differences were observed between the strains for any of the elements analyzed with the exception of a reduction in cellular ^{66}Zn for the revertant strain ($p = 0.0038$; one-way ANOVA with Šídák post-test) (Fig. 2F). Growth in 100 μM $CuSO_4$ supplemented ThyB resulted in a specific increase in ^{63}Cu accumulation in all strains, relative to untreated conditions, with the Δ*copA* strain

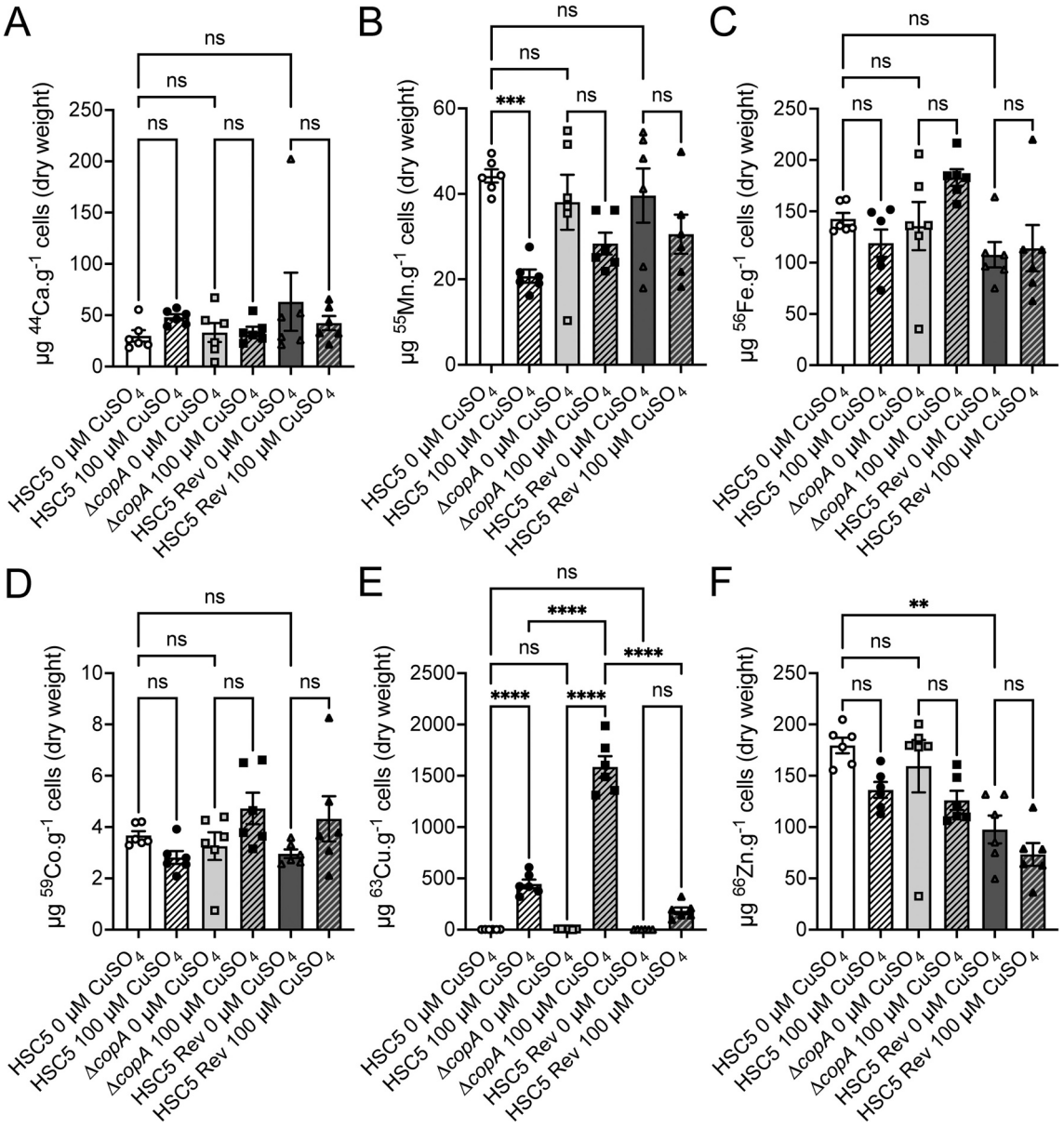

Fig. 2. Metal accumulation in *S. pyogenes* HSC5 wild type and derivative strains. Whole cell metal ion accumulation in *S. pyogenes* HSC5 and the derivative strains, Δ*copA* and HSC5 Rev., were analyzed by inductively coupled plasma mass spectrometry. Data represent the mean (± S.E.M.) concentrations (μg metal.g^{-1} of dry cell weight) of (A) ^{44}Ca, (B) ^{55}Mn, (C) ^{56}Fe, (D) ^{59}Co, (E) ^{63}Cu, and (F) ^{66}Zn, from six independent experiments. Statistical significance of the differences was determined by one-way ANOVA with Šídák post-test (ns, not significant, $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

showing a significant 3.5-fold increase in cellular accumulation relative to the wild type strain ($p < 0.0001$; one-way ANOVA with Šídák post-test) and an 8.4-fold increase relative to the revertant strain ($p < 0.0001$; one-way ANOVA with Šídák post-test) (Fig. 2E). The increased ^{63}Cu accumulation in the Δ*copA* strain was not associated with dysregulation of other cellular metals, by comparison with untreated conditions (Fig. 2A-D,F). In the wild type strain, a significant reduction in cellular ^{55}Mn accumulation was observed in the presence of copper stress ($p < 0.001$; one-way ANOVA with Šídák post-test) (Fig. 2B). Notably, reductions in cellular ^{55}Mn accumulation were also observed in the Δ*copA* and revertant strains (Fig. 2B), although these changes did not reach statistical significance. Nevertheless, these data suggest that manganese accumulation may be impacted by extracellular copper stress. This change could arise from competition between copper and manganese for binding to MtsA, the manganese-recruiting solute-binding protein ortholog of *S. pneumoniae* PsaA [48]. In vitro studies of *S. pneumoniae* PsaA showed that despite its role in manganese uptake, the protein was permissive for interaction with all first row transition metal ions including Cu^{2+} [49]. However, the binding of Cu^{2+} by PsaA renders the protein unable to release the metal on a physiological relevant timescale, abrogating its functionality in manganese import. Although *S. pyogenes* MtsA has not been directly shown to be susceptible to metal competition, the high similarity to *S. pneumoniae* PsaA (87% sequence identity over 308 amino acids) and strict conservation of the metal-binding site residues suggest that this may be a plausible inference.

Collectively, the cellular accumulation data are consistent with the role of CopA as a copper efflux pathway in *S. pyogenes* HSC5. The whole cell metal accumulation data also suggest that extracellular copper may exert an impact on manganese homeostasis, but further studies would be required to definitively elucidate the effect and the underlying molecular basis.

3.3. Transcriptional response of S. pyogenes HSC5 to copper exposure

We next investigated the transcriptional response of *S. pyogenes* HSC5 strains to copper stress via microarray. Here, wild type *S. pyogenes* HSC5 was subjected to a short (30 min) treatment with 100 μM $CuSO_4$ that revealed the primary pathways activated in response to copper exposure. Expression of 43 genes in *S. pyogenes* HSC5 (>2-fold; 22 upregulated, 21 downregulated) were altered by copper exposure (Table S3). Transcriptional analyses showed that genes associated with copper homeostasis, specifically: the copper-exporting ATPase, *copA*; the putative copper-binding metallochaperone *copZ*; and the putative transcriptional copper-binding metalloregulator *copY/R*, represented one of the most upregulated (>3-fold) gene clusters (Table S3). qRT-PCR analysis of *copZ*, a representative gene for this cluster, confirmed this response (Fig. S3). The next-highest upregulated genes were the cysteine protease *speB*, and several putative bacteriocins and their presumptive immunity factors. Notably, the ferrous iron exporting P_{1B}-ATPase *pmtA* (*Spy1434*) was downregulated (1.7-fold) in the presence of copper. Subsequent qRT-PCR analysis revealed that *Spy1434* was significantly downregulated in all strains following copper treatment, suggesting that even small increases in cellular copper abundance modulated *Spy1434* expression (Fig. S3). Previous studies have implicated *pmtA* orthologs in contributing to iron, cobalt, and zinc resistance [50–53], although recent studies have provided compelling data that *S. pyogenes* PmtA is primarily involved in Fe(II) export [52]. Expression of *pmtA* is dictated by PerR, a peroxide sensing Fur subfamily metalloregulator. PerR is a homodimeric protein and binds a zinc ion at a structural site located within the dimerization domain of each protomer. PerR also has a regulatory metal-binding site within the interdomain region that directly influences the activity of the protein in negatively regulating gene expression. The regulatory site can bind iron or manganese ions, although only iron is capable of catalyzing the oxidation of the metal-coordinating residues and thereby, enabling release of the metal from regulatory site in PerR [54]. Release of iron from PerR reduces its affinity for operator sites within the genome, enabling derepression of regulated genes such as *pmtA*. Here, the downregulation of *pmtA* suggests that exposure to copper decreases cytoplasmic peroxide stress which in turn is sensed by PerR and results in stronger repression of regulated genes in *S. pyogenes* HSC5.

Using this framework, we then analyzed the transcriptional impact of the *copA* gene deletion in the *S. pyogenes* HSC5 in ThyB without copper stress. This analysis provides the baseline differences between the parental and derivative strain to investigate more significant changes associated with the application of stress in subsequent experiments. Deletion of *copA* altered the microarray measured transcriptional abundance of 66 genes in standard ThyB growth conditions (>2-fold; 31 upregulated, 35 downregulated) (Table S4). Despite the low abundance of copper in ThyB, increased expression (~3–5-fold) of genes within the *cop* operon were observed, a response that was confirmed by qRT-PCR of *copZ* (Fig. S3). This can be attributed to the inability of the mutant to efflux copper as it enters the cell, resulting in persistent activation of this gene network. The transcriptional analyses also revealed the upregulation of other metal efflux systems including *cadC* (*Spy2163*; 3.3-fold) and *cadD* (*Spy2162*; 3.5-fold) (Table S4). The transcription of *cadD* was not significantly different between the wild type and Δ*copA* strain in the absence of copper by qRT-PCR (Fig. S3), but was substantially upregulated upon exposure to copper stress. This discrepancy can most likely be attributed to batch variation in ThyB copper levels, with *cadD* responding to increasing cellular copper. The activation of *cadD* and the *cop* operon indicate that there may be regulatory cross-talk between copper and other metal homeostasis transcriptional networks. The molecular basis for how this could occur remains speculative.

We next sought to model how intracellular copper accumulation differentially impacted transcriptional responses of *S. pyogenes* HSC5 Δ*copA*, again using a 30 min shock treatment of 100 μM $CuSO_4$. The microarray transcriptional profile of *S. pyogenes* HSC5 Δ*copA* during copper stress revealed 30 genes with differential expression between the Δ*copA* and wild type strains (>2-fold; 15 upregulated, 15 downregulated) (Table S5). The differentially regulated genes represent pathways primarily associated with carbon metabolism and energy generation. In addition to carbohydrate import, *lctO*, which encodes the pivotal metabolic enzyme lactate oxidase, was upregulated by ~1.5-fold. Lactate oxidase produces pyruvate by catalyzing the oxidation of lactate with molecular oxygen, with H_2O_2 generated as a reaction by-product. Therefore, the production of H_2O_2 would be predicted to increase peroxide stress within the cytoplasm. Collectively, the transcriptional analyses of *S. pyogenes* HSC5 reveal that copper stress activates genes associated with metal homeostasis and central carbon metabolism, suggesting that copper may impact bacterial metabolic pathways.

3.4. Copper efflux is required for stationary phase viability

The work *vide supra* establishes that copper stress can arrest exponential phase growth of the *S. pyogenes* HSC5 Δ*copA* strain. However, the copper concentration used to perturb growth was higher than that typically observed in host tissues [55–59]. Accordingly, we next investigated the impact of copper stress on bacteria within a prolonged stationary phase by performing growth assays with *S. pyogenes* HSC5 wild type, Δ*copA*, and the revertant strains in ThyB supplemented with increasing copper concentrations (0, 25, and 50 μM $CuSO_4$). Bacterial viability was determined upon entry into stationary phase (~7–8 h post-inoculation) and during prolonged stationary phase (24 h post-inoculation). Unexpectedly, we observed that copper supplementation at concentrations that had minimal impact on bacterial growth or viability upon entry into stationary phase exerted potent bactericidal activity during prolonged stationary phase (Fig. 3 A,B). The bactericidal activity of copper required its supplementation throughout all stages of growth, as ThyB supplementation upon entry into stationary phase was

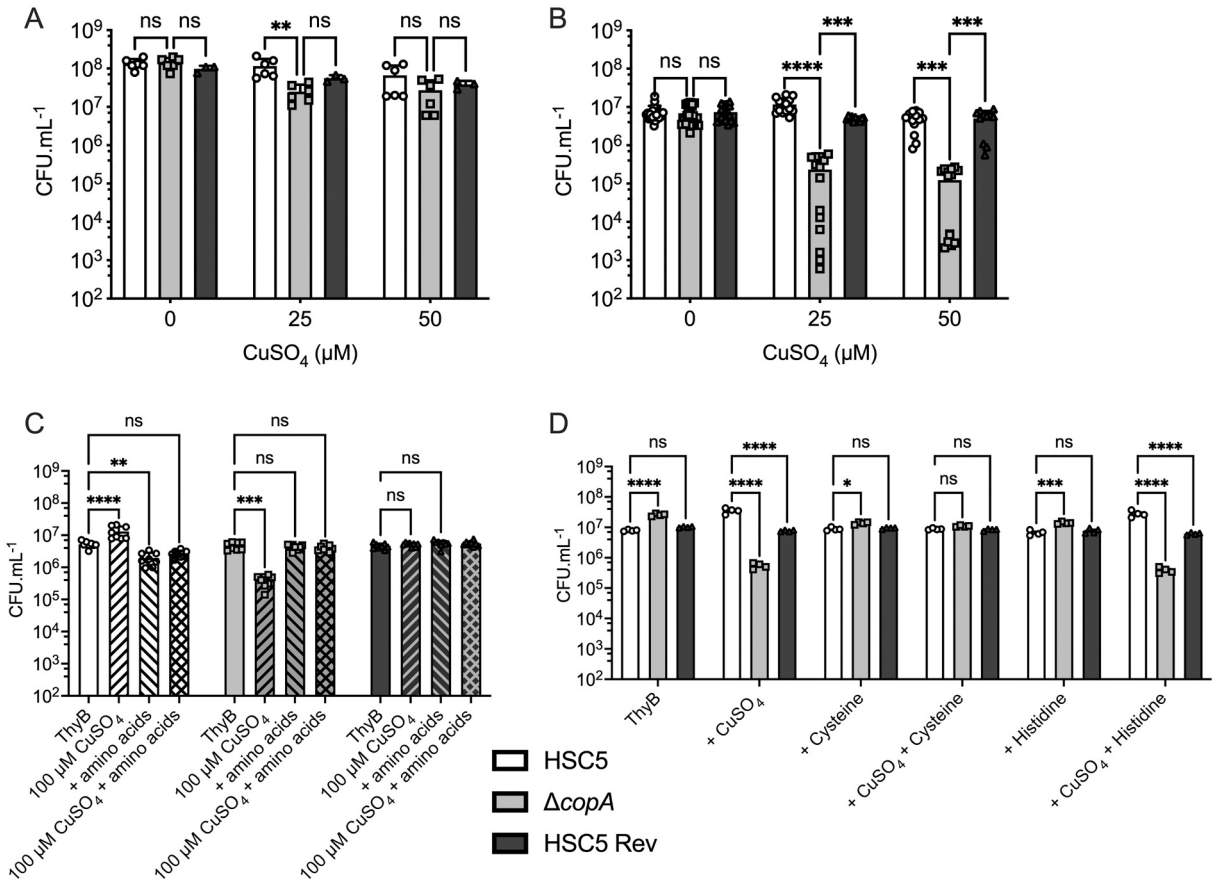

Fig. 3. Impact of prolonged copper exposure and exogenous amino acids on *S. pyogenes* survival. (A) Survival of *S. pyogenes* HSC5 and the derivative strains, Δ*copA* and HSC5 Rev., grown in ThyB media upon entry into stationary phase (8 h post inoculation) and supplemented with 25 μM or 50 μM copper. (B) Survival of *S. pyogenes* HSC5 and the derivative strains, Δ*copA* and HSC5 Rev., grown in ThyB media upon entry into late stationary phase (24 h post inoculation) and supplemented with 25 μM or 50 μM copper. (C) Survival of *S. pyogenes* HSC5 and the derivative strains, Δ*copA* and HSC5 Rev., grown in ThyB medium with or without amino acid supplementation and/or addition of 25 μM $CuSO_4$. (D) Survival of *S. pyogenes* HSC5 and the derivative strains, Δ*copA* and HSC5 Rev., grown in ThyB medium with or without cysteine or histidine supplementation and/or addition of 25 μM $CuSO_4$. Bacteria were incubated for 24 h at 37 °C and survival determined by colony-forming unit (CFU) enumeration. Data in all analyses represent the mean (± S.D.) from at least three independent replicates, with each replicate indicated by individual data points. Statistical analysis in A, B, and D performed by two-way ANOVA with Tukey post-test (ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$), and C using the Mann-Whitney *U* test (***, $p < 0.001$).

not observed to significantly reduce bacterial viability.

It is increasingly accepted that bacteria remain metabolically active during prolonged stationary phase and can maintain active protein production [60]. It logically follows that CopA can be expressed during prolonged stationary phase and under conditions such as amino acid limitation. Amino acid supplementation has been shown to confer protection against copper stress in some bacterial species [61–63]. Here, we hypothesized that amino acid starvation might contribute to the reduction in bacterial viability observed during prolonged stationary phase exposure to copper stress. To test this hypothesis, copper-treated stationary phase cultures were supplemented with exogenous amino acids. Supplementation of ThyB with all twenty amino acids significantly enhanced stationary phase survival of the *S. pyogenes* HSC5 Δ*copA* from copper exposure (Fig. 3C). These data suggest that copper intoxication of prolonged stationary phase cultures may be associated with an impaired ability to acquire or synthesize essential amino acids. However, an alternate inference could be that certain amino acids may provide a modest buffering capacity that indirectly protects the *S. pyogenes* HSC5 Δ*copA* strain. To test this, we assayed for the relative contribution of cysteine and histidine supplementation alone for their capacity to rescue viability during stationary phase. Histidine and cysteine are both capable of copper coordination by virtue of nitrogen and sulfur atoms respectively, and may therefore lower the bioavailability of copper for microbial cells [64]. Supplementation with cysteine, but not histidine, was found to significantly enhance survival of the Δ*copA* mutant in the presence of copper stress (Fig. 3D). Future studies would be warranted to elucidate the interplay between amino acid availability and enhanced survival of *S. pyogenes* during prolonged copper exposure.

3.5. The role of copper efflux in S. pyogenes HSC5 virulence

The impact of the *copA* deletion on the virulence potential of *S. pyogenes* HSC5 was investigated using a murine model of infection. Here, seven-week-old, hairless mice (SKH1) were subcutaneously infected with the *S. pyogenes* HSC5 wild type (both parental and revertant) or Δ*copA* strains and arising lesions areas measured daily (Fig. 4A). These data show that the Δ*copA* strain was associated with significantly smaller lesions ($p < 0.05$), by comparison with wild type and revertant strains, at 24 h post-infection (Fig. 4A). By 48 h post-infection, no significant differences in lesion size were observed. However, an ~1-$\log_{10}$ reduction in bacterial load for the Δ*copA* strain, relative to the wild type and revertant strains was observed at this time point (Fig. 4B). Taken together, these data indicate that that *copA* is required for full virulence in a subcutaneous lesion model of infection.

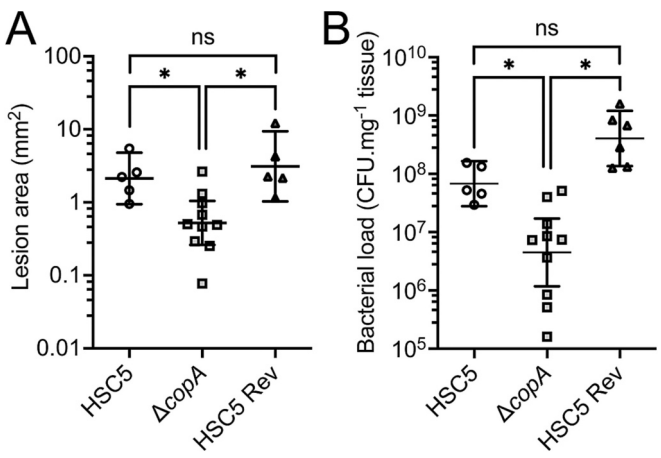

Fig. 4. Virulence of *S. pyogenes* Δ*copA* in a murine lesion model of infection. (A) Comparison of lesion areas caused by *S. pyogenes* HSC5 and the derivative strains, Δ*copA* and HSC5 Rev., at 24 h post infection. Lesion area of post subcutaneous infection were imaged and measured using ImageJ. (B) Bacterial load ($CFU.mL^{-1}$) recovered from lesions of *S. pyogenes* HSC5 and the derivative strains, Δ*copA* and HSC5 Rev., at 48 h post infection. Data are the geometric mean (with 95% confidence interval) with each data point representing a lesion from an individual mouse. Statistical analyses were performed using the Mann-Whitney U test (ns, not significant, $p > 0.05$; *, $p < 0.05$).

4. Discussion

Biological systems precisely maintain cellular ratios of essential metal ions to facilitate the cellular chemistry of life. However, the extracellular milieu's chemical complexity can disrupt this balance when cells encounter niches with high concentrations of metal ions. This exposure can result in cellular intoxication, with physiological impacts arising from the inappropriate interaction of the metals with non-cognate proteins, known as mismetallation, which perturbs or disrupts protein function; or the generation of reactive oxygen or nitrogen species leading to DNA, protein and lipid damage [65,66]. In mammalian systems, copper serves diverse roles, but has been implicated as contributing to the host-pathogen interaction as an antimicrobial metal ion. This inference is supported by the presence of copper tolerance and efflux systems present in many human bacterial pathogens [67–69]. Pathogenic streptococci, such as *S. pyogenes* and *S. pneumoniae*, respond to copper stress by upregulating copper-specific homeostasis genes encoded by the *cop* operon [36]. The work herein details the contribution of CopA to *S. pyogenes* HSC5 copper tolerance, the network of other genes associated with resistance to acute copper stress, the contribution of CopA to prolonged stationary phase survival, and its requirement for virulence in a murine model of soft tissue infection.

Our studies show that CopA is crucial to resist copper stress that can otherwise prevent exponential growth of the bacterium, consistent with analyses of CopA orthologs from other bacterial species [31]. Unexpectedly, this work also identified that during prolonged stationary phase, copper stress was acutely toxic to the Δ*copA* strain at concentrations that did not impact viability upon entry into stationary phase. This suggests that the inability of *S. pyogenes* to export copper during stationary phase perturbs one or more crucial cellular pathways. This phenotype could be ablated by amino acid supplementation. Still, it remains to be determined whether this protective effect was a direct effect due to one or more amino acids compensating for a nutritional deficiency or an indirect effect of the amino acids acting as a low affinity buffering pool for copper ions. The latter conclusion is supported, albeit to a limited extent, by the recent study of the *S. pyogenes* M1T1 5448 strain. In that work, deletion of the *copA* ortholog in *S. pyogenes* M1T1 5448 rendered late-stage exponential growth (~4 h post-inoculation) susceptible to copper intoxication, which could be rescued by supplementation with the tripeptide glutathione [47]. This was inferred to arise from glutathione serving as a low affinity buffering pool for copper.

That study of *S. pyogenes* M1T1 5448 copper homeostasis, using wild type and Δ*copA* derivative strains, also examined the transcriptional response to copper intoxication [47]. A comparison of the two transcriptional analyses showed that both *S. pyogenes* strains dramatically upregulated their respective copper homeostasis system, consistent with prior studies [41]. However, other than this single point of comparison, the two transcriptomes showed starkly different gene expression patterns. This may be partially attributed to the different nutritional profiles of the culture media used in each study, i.e., undefined and rich (ThyB; this work) vs. defined and minimal (glucose-based chemically defined media). In the *S. pyogenes* HSC5 strains, copper stress-induced gene expression changes centered on carbon source acquisition and central metabolism, notwithstanding the altered expression of the aforementioned *cop* operon and numerous genes with poorly defined/undefined cellular roles. Whereas analyses of the *S. pyogenes* M1T1 5448 strains showed a broader range of metal ion homeostasis with altered gene expression [47]. Nevertheless, metabolic arrest in late exponential phase of *S. pyogenes* M1T1 5448 was shown to arise from inappropriate interaction of copper with glyceraldehyde-3-phosphate dehydrogenase (GapA) and disruption of its function. The abrogated activity of GapA could be restored via glutathione supplementation, suggesting a functional collapse of the buffering pool. Many differentially regulated genes from the microarray data represent pathways primarily associated with carbon metabolism and energy generation. The genes with the highest level of upregulation, *SPy_1059* and *SPy_1060* (> 3-fold), belonged to the PTS family of transporters, while the maltose import pathway genes, *SPy_1294, SPy_1296,* and *SPy_1297*, were also upregulated (1–1.3-fold) (Table S5). The putative PTS genes are predicted to encode a carbohydrate-uptake system with specificity for mannose, maltose, fructose, or *N*-acetylgalactosamine. Taken together, these data indicate that the management of copper stress in *S. pyogenes* is associated with the upregulation of the *cop* operon, but may also involve alterations in central metabolic processes. However, these data also show key differences in how this latter resistance to copper can manifest. Notably, under the experimental conditions used in this work, copper-stress induced *S. pyogenes* HSC5 Δ*copA* to upregulate the expression of *lctO*, which encodes a flavin enzyme that oxidizes lactate to generate pyruvate. A by-product of this reaction is the generation of H_2O_2, which concomitantly leads to up-regulation of *pmtA* via PerR [70]. Collectively, these observations support the notion that resistance to copper intoxication in *S. pyogenes* HSC5 is associated with a switch to lactate utilization and/or other carbohydrates, although further study is required to confirm this inference.

Copper tolerance pathways have been implicated in the virulence of numerous bacterial pathogens, including *S. pneumoniae, Pseudomonas aeruginosa,* and *Neisseria gonorrhoeae.* However, it is important to note that considerable complexity is associated with copper's in vivo antimicrobial activity. This work shows that, in a soft tissue infection model, loss of CopA significantly reduced the virulence of *S. pyogenes* HSC5. By contrast, systemic infection with *S. pyogenes* M1T1 5448 Δ*copA* did not result in a virulence defect [47]. Although strain and murine mode differences cannot be excluded, these data further highlight that the application of host antimicrobial copper may be niche-specific and, potentially, spatially complex. In the context of this work, the reduced virulence of the *S. pyogenes* HSC5 Δ*copA* strain would be consistent with the expectation that tissue lesions can be highly enriched for copper [47]. During infection, the innate immune response can exploit the chemistry of copper to prosecute the killing of microbial pathogens. This has been established to occur within the phagolysosome of phagocytic cells, such as macrophages, in the context of some infection models. Thus, the reduction in *S. pyogenes* HSC5 Δ*copA* virulence may be attributable, at least in part, to this strain having a reduced capacity to survive prolonged periods of stationary phase in the presence of copper

stress that occurs within phagocytic cells or necrotic lesions. Collectively, this work shows that disruption of metal homeostasis in bacterial pathogens provides a route to further explore host-pathogen interaction and may provide a foundation for developing novel antimicrobial therapeutics [71]. This study highlights the importance of copper tolerance in *S. pyogenes*, a globally significant human pathogen, and provides new insights into the potential antimicrobial role of copper at sites infected by this pathogen.

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Author contributions

THD, MDLJ, SLN, AI, CAM, and JWR designed the study. THD, MDLJ, AI and JWR performed the microbiological and animal studies, with SLN and CAM performing the ICP-MS analyses. THD, MDLJ, and JWR wrote the first draft of the manuscript. CAM and JWR reviewed and revised the manuscript text. All authors read and approved the submitted version.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2023.112122.

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