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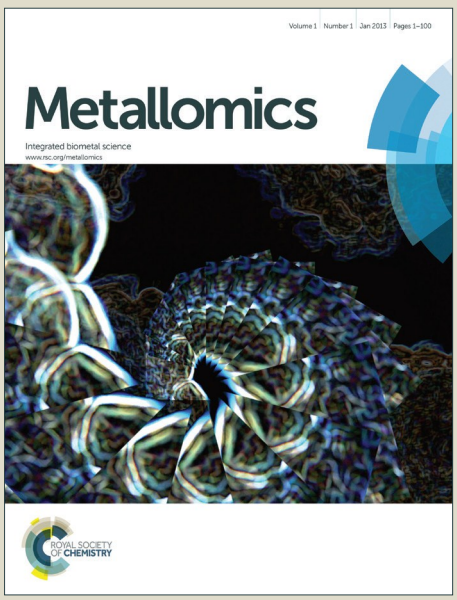

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Copper intoxication inhibits aerobic nucleotide synthesis in *Streptococcus pneumoniae*

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ABSTRACT

Copper is universally toxic in excess, a feature exploited by the human immune system to facilitate bacterial clearance. The mechanism of copper intoxication remains unknown for many bacterial species. Here, we demonstrate that copper toxicity in *Streptococcus pneumoniae* is independent from oxidative stress but, rather, is the result of copper inhibiting the aerobic dNTP biosynthetic pathway. Furthermore, we show that copper-intoxicated *S. pneumoniae* is rescued by manganese, which is an essential metal in the aerobic nucleotide synthesis pathway. These data provide insight into new targets to enhance copper-mediated toxicity during bacterial clearance.

1. INTRODUCTION

It is becoming increasingly apparent that manipulating the levels of transition metals during infection is a powerful weapon wielded by vertebrates to combat invading pathogens. These elements are essential for life but are toxic when present in excess. Highlighting the importance of maintaining appropriate intracellular metal levels is the observation that numerous acquisition and efflux systems contribute to bacterial pathogenesis.[1-3] Vertebrates take advantage of both the essential and toxic nature of these elements to combat infection.[4, 5] During infection, the host restricts the availability of iron, manganese, and zinc to pathogens in an attempt to starve them of these metals.[6-8] The host also uses the toxic properties of copper, which has antimicrobial and antiviral properties that have been appreciated since antiquity, to combat invading microbes.[9, 10 7, 11] Genes under the control of copper-responsive regulation contribute to bacterial pathogenesis in the mammalian host.[12] Understanding, how bacterial species adapt to fluctuations in metal availability is critical to understanding bacterial physiology and virulence.

S. pneumoniae, or pneumococcus, is a significant public health concern in pediatric and elderly populations and is a major cause of pneumonia, otitis media, and meningitis. Recent evidence indicates that *S. pneumoniae* experiences copper toxicity during infection.[2, 13] A widely used mechanism to counter copper toxicity is the expression of dedicated copper efflux systems.[2] These systems are functionally conserved amongst diverse bacterial species and typically mirror the pneumococcal system, which consists of a regulator to control expression (CopY), an ATPase to mediate efflux (CopA), and a copper chaperone (CupA).[14] Loss of CopA reduces the

virulence of pneumococcus in multiple infection models.[2] This observation underscores the importance of evading copper-mediated intoxication within the mammalian host. In addition to export, it seems likely that bacteria use other mechanisms to cope with metal toxicity because they can alter their metabolic profile in response to intoxicating concentrations of transition metals by altering their expression of amino acid biosynthesis and transport genes.[15] Alterations in multiple cellular pathways are not entirely unexpected given the involvement of metals in approximately 30% of cellular proteins.[16] This is especially true of copper because pneumococcus does not contain any known copper-utilizing proteins, yet cellular levels of copper are still approximately 10% of those of the widely utilized metals manganese and zinc, underscoring the potential for mismetallation.[17]

Although the antibacterial properties of copper are well established, the specific mechanism underlying copper toxicity remains unknown for many bacterial species, including pneumococcus. Copper can produce free radicals when oxidized via Fenton reactions. This process occurs when copper (I) is oxidized in the presence of hydrogen peroxide, producing copper (II) + $^{\cdot}OH$ + OH^{-}. The accumulation of free radicals can irreversibly damage bacterial cellular components, including proteins, lipids, and DNA.[18] This reaction is of particular concern in pneumococcus, which not only lacks catalase and other widely distributed antioxidant defenses but also produces millimolar amounts of hydrogen peroxide, a precursor to Fenton chemistry.[19] Oxidative damage can itself result in the mismetallation of proteins.[20, 21] However, copper-induced oxidative stress is not sufficient to kill *E. coli*, leading to the suggestion that additional mechanisms of copper-mediated toxicity may exist.[5, 22-24] Other proposed mechanisms of copper toxicity

include disrupting the photosystem oxidase HemN in *Rubrivivax gelatinosus*, inactivating solvent-exposed iron-sulfur clusters in dehydratases such as fumarase A and displacing manganese from the active site of superoxide dismutase.[25-27] Because of the universal nature of copper toxicity in bacteria, identification of both conserved and species-specific copper targets will enhance our understanding of how metals affect bacterial physiology and could provide potential targets for the development of novel therapeutics.

In this study, we elucidated a mechanism of copper toxicity in *S. pneumoniae* by examining the cellular consequences of copper stress. Copper toxicity in pneumococcus occurs independently of oxidative damage, as copper toxicity was readily observed under strict anaerobic conditions. Subsequent transcriptional profiling and mutagenesis studies revealed that the primary mechanism of copper toxicity in pneumococcus is inhibition of the essential manganese-dependent nucleotide synthesis pathway. This pathway has not been identified previously as a target of copper intoxication; thus, this work significantly expands our understanding of the underlying mechanisms of copper toxicity.

2. MATERIALS AND METHODS

2.1 Bacterial constructs

Mutations of SP_0202 (*nrdD*), SP_0727 (*copY*), SP_0728 (*cupA*), SP_0729 (*copA*), and SP_0766 (*sodA*) were created via the splicing by overhang extension method of PCR (SOE-PCR). Fragments approximately 1 kb upstream and downstream of the target gene were amplified and spliced to an erythromycin- (*copA*) or spectinomycin- (*nrdD* and *sodA*) resistance cassette. SOE-PCR products were subsequently used to transform pneumococcus (TIGR4), and the expected size of each knockout was verified by PCR to confirm insertion of the SOE-PCR product and deletion of the target gene.[28]

2.2 Growth curves and colony-forming units

Each *S. pneumoniae* strain was grown at 37°C in ThyB medium (30 g of Todd Hewitt Broth [Sigma], 2 g of yeast extract [Sigma], 1 L dH_2O, pH 6.5) under aerobic conditions, with varying amounts of $CuSO_4$ (hereafter, copper), other metals, and hydroxyurea (HU) as indicated. Bacteria was back-diluted 1:50 at optical density [O.D.] λ620 (unless otherwise noted) = 0.1 in experimental ThyB medium for overnight growth assessments. For colony-forming unit experiments, 10 µL of culture was serially diluted and plated on TSA (Sigma) sheep's blood (I-Tek) agar 8 hours after inoculation. All O.D. measurements used to construct growth curves were read at λ620 wavelength on a Turner Model 340 spectrophotometer. Because of HU's apparent instability at 37°C for extended time periods, 8-hour time points were reported for the HU studies.

2.3 Inductively coupled plasma mass spectrometry

Bacteria were grown in ThyB to an O.D. of 0.40, diluted by 37.5%, and grown for another hour. Then, 100 μM copper was added to the cultures, and cells were grown for an additional hour before being pelleted and washed with 10 mL PBS + 10 mM EDTA. Pelleted cells were resuspended in 1 mL PBS + 10 mM EDTA and transferred to an Eppendorf tube. Cells were then dried overnight at 70°C, collected in 10 mL of 10% nitric acid, vortexed vigorously for 1 minute, and incubated for 1 hour at 55°C. Samples were then vortexed and filtered for spectrophotometry (Varian 820 ICPMS System).

2.4 Macrophage intracellular growth assay

J774.1 murine macrophages (ATCC) were maintained in a 37°C, 5% CO_2 incubator with Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% (vol/vol) fetal bovine serum (FBS, Sigma), glutamine (2 mM, Sigma), penicillin (50 units/mL, Sigma), streptomycin (50 μg/mL, Sigma), and 0.015% sodium bicarbonate. Cells were grown to 80% confluence in 12-well tissue culture plates, washed twice with PBS, and resuspended in growth medium without antibiotics or FBS. Macrophages were then infected with 50 μL of *S. pneumoniae* at an O.D. of 0.1 for 90 minutes with or without 250 μM manganese supplementation. Wells were then washed twice: each wash was followed by a 1-minute incubation with DMEM containing gentamycin (50 μg/mL). Macrophages were lysed and serially diluted to determine intracellular bacterial content. Each value shown was normalized to the bacterial content of wild-type TIGR4 with no manganese supplementation.

2.5 Protein oxidation

S. pneumoniae were grown to an O.D. of 0.1 and stressed with 0 μM, 25 μM, 50 μM, or 100 μM copper for 1 hour. Each sample was serially diluted and plated to determine CFU titers used to ensure equivalent loading. Samples were centrifuged at 6000 x g for 5 minutes. Bacterial samples were lysed and processed by using the OxyBlot Protein Oxidization Detection Kit (Millipore), with 2% deoxycholate added to each sample to promote pneumococcal lysis. Samples added to the polyacrylamide gel were normalized by O.D. to the 0 μM copper inoculum of each individual sample after copper stress. Transfer, blocking, incubation with primary and secondary antibodies, and exposure were carried out according to manufacturer suggestions.

2.6 Hydrogen peroxide stress assay

Bacteria were grown to an O.D. of 0.1 in ThyB. Then, 500 μL of bacteria was combined with 0.1% (33 mM) hydrogen peroxide for 60 minutes. Samples were serially diluted in PBS and plated on TSA blood agar plates. Bar values represent percent survival compared to that of the respective strain grown without hydrogen peroxide added. Experiments were performed in triplicate.

2.7 RNA extraction

S. pneumoniae were grown to an O.D. of 0.1 in ThyB. Cultures were then diluted 50-fold in fresh ThyB and grown to an O.D. of 0.3. Indicated metals were added to each culture, and the mixtures were incubated for 15 minutes at 37°C. Culture samples (5 mL) were incubated with RNAprotect Bacterial Reagent (10 mL, Qiagen) for 20 minutes before the

bacteria were isolated via centrifugation. RNA was extracted from the cell pellets by using an RNeasy Mini Kit (Qiagen).

2.8 Quantitative real-time PCR assays

SuperScript III First-Strand Synthesis SuperMix (Invitrogen) was used to synthesize cDNA from the isolated RNA (50 ng/μL). SYBR Green (Invitrogen) was used to monitor dsDNA synthesis on an ABI-Prism 7300 Real-Time PCR machine (Applied Biosystems). Samples were normalized relative to *gyrA* expression. Fold-change was calculated by using the ΔΔCt method.

2.9 Zone-of-inhibition assays

Bacteria were grown in C+Y to an O.D. of 0.2 at 620 nm. Then, 100 μL of culture was spread onto a blood agar plate. A disc of filter paper with 10 μL of 1M copper was placed in the middle of the plate, and bacteria were grown aerobically in 5% CO_2 or anaerobically in a GasPak jar. The zone of inhibition was measured as the distance between the outer edge of the disc and that of bacterial growth.

2.10 Microarray analysis

The Qiagen RNeasy mini kit was used to harvest bacterial RNA from mid–log phase cultures (O.D. of 0.4 at 620 nm) grown in ThyB with or without 200 μM copper supplementation for 30 minutes. Microarray experiments were performed as described previously.[29] Briefly, whole-genome *S. pneumoniae* version 8.0 cDNA microarrays were obtained from the Pathogen Functional Genomics Resource Center (PFGRC) at the J. Craig Venter Institute. Microarray experiments were performed by the Functional

Genomics laboratory of the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital by using standard protocols provided by the PFGRC (http://pfgrc.tigr.org/protocols.shtml) as previously described.[1]

3. RESULTS

3.1 Copper stress does not affect accumulation of other metals

Although it is known that pneumococcus experience copper stress during infection,[5] the precise mechanism of copper-mediated toxicity has not been determined. Thus, we sought to elucidate the underlying mechanism of copper toxicity in pneumococcus. Previous studies in pneumococcus have revealed that elevated concentrations of zinc can interfere with the uptake of manganese by PsaA.[30] To evaluate whether copper can interfere with the uptake of other metals, the intracellular elemental composition was measured by performing inductively coupled plasma mass spectrometry on bacteria exposed to toxic levels of copper.[2] These experiments revealed that both wild-type and Δ*copA* mutant bacteria accumulate excess intracellular copper when exposed to high levels of this metal (Figure 1A). Consistent with the role of CopA in the copper efflux system, the mutant accumulated significantly more intracellular copper than did the wild type. While intracellular copper rapidly increased, the concentrations of the other metals examined (i.e., zinc, iron, manganese, and magnesium) were unaffected in wild-type and Δ*copA* mutant bacteria (Fig. 1A). This result indicates that intracellular concentrations of other metals are not perturbed under copper stress. Furthermore, this result emphasizes the specificity of the CopA transporter for copper.

3.2 Manganese Rescues Copper Toxicity

The interactions between copper and proteins are very stable. As such, elevated levels of copper could result in mismetallation of proteins that use metals with weaker affinities. Although there is no discernable intracellular perturbation of other metals

during short-term copper stress, we hypothesized that supplementation with different metals could overcome copper toxicity by preventing mismetallation. The Δ*copA* mutant was used in these studies because of its increased sensitivity to copper toxicity and heightened intracellular accumulation of copper. The addition of calcium or iron did not rescue the growth of the Δ*copA* mutant in the presence of toxic levels of copper. The addition of manganese partially alleviated the effects of copper toxicity in the Δ*copA* mutant, increasing both the overall optical density and colony forming units respective to the values observed after adding copper alone (Fig. 1B, C). Interestingly, supplementation with zinc, which also has a high affinity for proteins, exacerbated copper toxicity (Fig. 1B). These data indicate that excess manganese can partially rescue the inhibitory effects of copper. Because copper stress did not reduce intracellular manganese levels (Fig. 1A), manganese rescue is unlikely to be due to a competition for uptake by the manganese importer PsaA.[31] Manganese supplementation at concentrations used in this study have previously been shown to confer minimal alterations in the intracellular levels of other transition metals, indicating this effect is likely specific to manganese[1].

To determine whether the manganese rescue observed *in vitro* could be recapitulated in a more biologically relevant assay, we investigated whether the addition of manganese would enhance the survival of *S. pneumoniae* in J774 murine lung macrophages. During infection, macrophages are a primary source of copper toxicity encountered by pathogens.[7, 32, 33] Manganese supplementation enhanced the survival of TIGR4 and the Δ*copA* mutant by roughly 3-fold (Fig. 1D). Levels of manganese utilized were non-toxic and within range of those previously utilized to investigate the role of

manganese on macrophage function[34]. Thus, manganese supplementation partially rescues pneumococci under copper stress *in vitro* and during macrophage-mediated killing.

3.3 Oxidative Stress is not the Primary Cause of Copper Toxicity

Manganese is a vital aspect of the pneumococcal defense against oxidative stress.[35] Copper can generate free radicals via the Fenton reaction *in vivo;*[36] hence, we next investigated the effect of copper on oxidative stress in pneumococcus. First, we assessed the effect of copper toxicity on protein oxidation by using 2,4-dinitrophenylhydrazine to measure oxidized carbonyl groups in the individual *cop* operon mutants and the wild-type bacteria grown in standard media with or without copper stress. Increasing concentrations of copper led to progressively greater levels of protein oxidation in wild-type TIGR4 (Fig. 2A). The Δ*copA* mutant showed a similar trend with increasing copper concentrations but, overall, had more protein oxidation than did the wild-type. This result suggested that protein oxidation could partially explain some of the copper-mediated toxicity in the Δ*copA* mutant. However, Δ*copY*, which overexpresses *copA* and maintains wild-type virulence and sensitivity to copper, displayed even higher levels of protein oxidation. Additionally, the Δ*cupA* mutant experienced less protein oxidation than did the wild-type bacteria across all concentrations of copper yet is as sensitive to copper toxicity as Δ*copA* is (Fig. 2A).[2] These observations indicate that copper sensitivity does not correlate with protein oxidation in pneumococcus.

We also assessed pneumococcal susceptibility to exogenous hydrogen peroxide in the presence of toxic levels of copper. The Δ*copA* mutant had the greatest sensitivity to hydrogen peroxide stress, and the Δ*copY* and Δ*cupA* mutants had sensitivities similar to those of wild-type TIGR4 (Fig. 2B). Although the Δ*copA* mutant is sensitive to hydrogen peroxide, the Δ*cupA* mutant, which is equally sensitive to copper toxicity, is not.[2] These data indicate that copper sensitivity and hydrogen peroxide sensitivity do not correlate.

To further evaluate whether oxidative stress underlies copper toxicity, we investigated the contribution of superoxide dismutase (SodA) to resisting this stress. In other bacteria, SodA promotes resistance to transition metal stress.[37] Additionally, exposure to high levels of copper have been shown to interfere with the activity of manganese-dependent SodA.[38] We found that deletion of *sodA* in either the TIGR4 or the Δ*copA* background did not significantly alter sensitivity to copper stress. (Fig. 2C). Of note, manganese supplementation partially protected wild-type bacteria, the Δ*sodA* mutant, and Δ*sodA*/Δ*copA* double mutants equally from the toxic effects of copper (Fig. 2C). These results indicate that the manganese-mediated rescue of copper toxicity occurs independently of SodA activity. In total, these results suggest that oxidative damage is not the major factor in copper intoxication.

3.4 Intracellular Copper Alters Expression of Nucleotide Synthesis Pathways

To ascertain the potential targets of copper toxicity in pneumococci, we performed a microarray comparing the mRNA expression of wild-type TIGR4 and that of the Δ*copA*

mutant under copper stress. In addition to the expected changes in the *cop* operon, a rather limited transcriptional response to intracellular copper accumulation was observed (Table 1). The *czcD* zinc efflux system was upregulated, potentially indicating some degree of cross-talk between zinc and copper. The chaperones *clpP* and *clpE* were also upregulated, indicative of a cellular stress response. Additionally, genes encoding the anaerobic nucleotide (dNTP) synthesis pathway were upregulated. Pneumococci encode two functional dNTP biosynthesis pathways, one aerobic (SP_1458, SP_1178, SP_1179, SP_1180, SP_0158) and one anaerobic (SP_0202, SP_0205, SP_1297) (Fig. 3A). By using real-time PCR assays, upregulation of the anaerobic nucleotide synthesis pathway (SP_0202, NrdD) was observed in the Δ*copA* mutant during copper stress (Fig. 3B). Additionally, essential members of the aerobic nucleotide synthesis pathway (SP_1179 NrdH [electron transport] and SP_1713 NrdR [the Nrd regulator]) were more highly expressed by the Δ*copA* mutant during copper stress than during unstressed conditions (Fig. S1A). While it was surprising to observe both dNTP biosynthetic pathways being expressed concurrently, previous reports have shown detectable levels of both pathways under aerobic culture [39]. Taken together, these results indicate that pneumococci increase expression of the dNTP biosynthesis pathways in response to intracellular copper accumulation.

3.5 Copper Toxicity Reduces Aerobic Ribonucleotide Synthesis

The aerobic pathway is predicted to require a manganese cofactor and to be essential.[13, 40] The increased expression of the anaerobic dNTP synthesis pathway leads to the hypothesis that copper inhibits the aerobic pathway. This hypothesis

predicts that if the anaerobic pathway were disrupted, then the pneumococcus would display greater sensitivity to copper-mediated growth inhibition because it would lack a backup pathway. To test this hypothesis, we created a mutation in *nrdD* in both the wild-type and Δ*copA* backgrounds to disrupt the anaerobic pathway. Deletion of *nrdD* was chosen as this gene encodes the enzymatic catalytic subunit and NrdD alone has been previously shown to catalyze reduction of dNTPs [41]. The Δ*nrdD* mutants grew both aerobically and anaerobically, indicating that the aerobic nucleotide synthesis pathway retains some activity in anaerobic conditions (Fig. S1B). The zone of clearance around a copper-treated disk was assessed to test the sensitivity of the Δ*nrdD* and Δ*copA*/Δ*nrdD* mutants to copper toxicity. The sensitivity of the mutants to copper was assessed in both aerobic and anaerobic conditions. Consistent with previous data, the Δ*copA* mutant had larger zones of inhibition than did wild-type under both aerobic and anaerobic conditions (Fig. 3C).[2] The Δ*nrdD* single mutant had wild-type sensitivity under aerobic conditions. However, the mutant had a larger zone of clearance when grown anaerobically, indicating that copper toxicity prevented the aerobic pathway from supporting growth under these conditions. The Δ*copA*Δ*nrdD* double mutant had the largest zone of inhibition in both aerobic and anaerobic conditions (Fig. 3C). Similar experiments performed with other metals revealed no differences between wild-type TIGR4 and the mutants (Figure S1C). These data indicate that pneumococcus is more sensitive to copper intoxication when the metal-independent anaerobic pathway is deleted, suggesting that the aerobic pathway is not functional.

If intracellular copper toxicity inactivates the aerobic dNTP biosynthetic pathway, then further perturbation of this pathway should lead to an even greater sensitivity to

copper. Attempts to delete genes in the aerobic dNTP biosynthetic pathway were unsuccessful, likely because these genes are essential.[13, 42] Therefore, we used a chemical approach exploiting the ability of hydroxyurea (HU) to inhibit aerobic dNTP synthesis, which destroys a tyrosine free radical essential for enzymatic function in ribonucleotide reductase (NrdF).[43] To confirm that HU inhibited the aerobic pathway, the sensitivity of the Δ*nrdD* mutant to HU was assessed. The Δ*nrdD* mutant was significantly more sensitive to HU inhibition than was the parental TIGR4 (Fig. 3D, Fig. S1D). Consistent with copper targeting the aerobic nucleotide synthesis pathway, the growth of Δ*copA* was significantly more impaired by HU than was that of the wild-type TIGR4 (Fig. 3D), as might be expected if both copper and HU are targeting distinct steps of the same pathway. The addition of manganese partially reversed the growth defect of the Δ*nrdD* and Δ*copA*Δ*nrdD* mutants exposed to copper. However, the addition of manganese did not rescue the mutants when only HU was present (Figures S1D, S1E). These data suggest that copper toxicity and HU disrupt the aerobic dNTP synthesis via different mechanisms. [43] In total, these data indicate that intracellular copper may compete with manganese for a metal-dependent step in the aerobic dNTP biosynthetic pathway to facilitate bacterial killing.

4. Discussion

4.1 Oxidative stress is not the critical target of copper toxicity

In these investigations, we sought to clarify the mechanism by which copper is toxic to pneumococcus. We found that pneumococci do not alter the uptake of magnesium, iron, zinc, or manganese to combat the entrance of copper into the cell. In fact, manganese partially rescues the growth of wild-type TIGR4 and the Δ*copA* mutant under copper-stress conditions. This rescue by manganese may partially explain the enhanced sensitivity to zinc under copper-stress conditions, as zinc can effectively compete for manganese uptake when present at sufficiently high ratios. Theoretically, the manganese rescue of copper-stressed pneumococci could occur via reduction of oxidative damage, independent of the manganese-dependent SodA.[44] However, previous studies and data shown here indicate that copper stress does not cause significant oxidative stress in *E. coli* [22] or streptococci. Finally, the observations that culturing under strict anaerobic conditions did not alleviate copper sensitivity and that copper sensitivity did not correlate with oxidative damage further support a mechanism of copper toxicity that is independent of reactive oxygen species.

4.2 Copper inhibits the aerobic nucleotide synthesis pathway through potential mismetallation

Under copper stress conditions, the anaerobic nucleotide synthesis pathway is upregulated, implying that the anaerobic pathway may be partially complementing the aerobic pathway. Previous transcriptional data have also shown altered regulation of the anaerobic pathway in response to intracellular zinc stress in pneumococcus, indicating

that this potential mismetallation mechanism of intoxication may extend to other transition metals.[45] Because the anaerobic nucleotide synthesis pathway mutant (Δ*nrdD*) could grow in an anaerobic environment, it is likely that the aerobic nucleotide synthesis pathway can complement the anaerobic pathway in pneumococcus. Even so, in the Δ*nrdD* mutant, we observed heightened sensitivity to copper stress when only the aerobic pathway was functional, likely due to its still-intact copper export system. Copper sensitivity was further exacerbated in the absence of functional copper export. When the aerobic pathway was inhibited by hydroxyurea (as determined by no growth of the Δ*nrdD* mutant in the presence of 10 mM HU), the Δ*copA* mutant, which has increased intracellular copper, underwent less growth than did wild-type TIGR4. Therefore, we propose that copper disrupts aerobic nucleotide synthesis, which occurs through a manganese-dependent pathway.

Although the precise mechanism by which copper could be interfering with the aerobic dNTP pathway is unknown, proteomics data and structural predictions provide some insight. Interestingly, pneumococcal proteins in the aerobic dNTP pathway have been predicted to have copper-binding properties via proteomics approaches.[46] One hypothesis is that copper blocks aerobic nucleotide synthesis in *S. pneumoniae* by binding to and inhibiting the function of NrdF, a protein that is homologous (50% identity) to the manganese-dependent ribonucleotide reductase found in *E. coli* (Fig. S2A).[40, 47] This hypothesis is based on the promiscuous binding nature of metals toward ribonucleotide reductases such as NrdF, which has residues in its metallocenter that classically coordinate manganese or copper and typically have a higher affinity for the latter (Fig. S2B).[46, 48-50]

Our proposed model (Figure 4) is that when copper is added to the media, it enters the bacteria and triggers transcription of the *cop* operon. While the bacteria is attempting to alleviate the copper-induced stress by exporting copper, copper itself can inhibit aerobic nucleotide synthesis by binding to and inhibiting NrdF activity; this can lead to decreased replication and increased transcription of the anaerobic nucleotide synthesis pathway in an attempt to supplement the dNTP pool, which we observed transcriptionally. Addition of excess manganese, which binds to NrdF, can then potentially alleviate the copper stress by successfully competing for NrdF (Fig. S2A).[47] There is diversity in the strategies utilized by bacterial to synthesize nucleotides, with bacterial species encoding between one and three classes of ribonucleotide reductases [51]. Even amongst the streptococci there is inherent diversity, with *S. pyogenes* encoding a secondary functional *nrdEF* locus[52]. Hence, this mechanism of intoxication likely only extends to subset of bacterial species with similar classes of ribonucleotide reductases as the pneumococcus. Although these results indicate that copper toxicity can inhibit nucleotide biosynthesis, they do not preclude the possibility that other cellular pathways are inhibited by mismetallation with copper. Additional studies in pneumococcus and other bacteria are needed to elucidate the full effect of copper toxicity and will likely reveal other cellular pathways perturbed by accumulation of this metal.

4.3 Synergism between metal intoxication and starvation in host immunity

During infection, metal concentrations in various organs can be dramatically altered, underscoring the dynamic nature of the environments that bacteria encounter.[53] Furthermore, it is evident that both the absolute concentrations of various metals and the appropriate balance of these metals are vital for optimal cellular function. A prime

example of this is manganese import by PsaABC, which is thought to be inhibited by elevated zinc levels during infection.[5] The ability of this system to import manganese is influenced not by the absolute concentration of extracellular zinc but by the ratio of zinc to manganese.[53] The observation that manganese can mitigate the effects of copper toxicity further highlights the importance of metal homeostasis to infection. This result also suggests that the nutrient-withholding response and metal intoxication may function synergistically. The phagolysosome is a prime example of where this synergy may occur: In this organelle, copper is actively pumped into the lumen while manganese is removed. The reduced availability of manganese would enhance the ability of copper to inhibit nucleotide biosynthesis via the aerobic pathway, thus increasing the efficacy of macrophage-mediated killing. Manganese starvation induced by calprotectin, which restricts extracellular metal availability, may have a similar effect.[8] These findings and our data highlight the importance of understanding mechanisms of metal homeostasis under a dynamic range of metal bioavailability as well as understanding transporter-independent mechanisms by which bacteria circumvent metal toxicity. Although it is widely recognized that excess metals can be detrimental, the precise mechanisms by which metals intoxicate cells remain unknown for many bacterial pathogens whose sole environmental niche is the human host.

5. Conclusion

Although the antimicrobial properties of copper have been appreciated since antiquity, the mechanism underlying this activity in many bacterial species has remained poorly understood. The data presented here suggest that the main mechanism of copper

intoxication in pneumococcus is independent of oxidative damage and likely occurs via inhibition of dNTP biosynthesis. Knowing the precise cellular targets of metal intoxication may provide subjects for future investigations aimed at targeting bacterial pathogens with novel therapeutic strategies.

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Figure Legends

Figure 1. Manganese partially rescues *S. pneumoniae* from copper stress. (A) Inductively coupled plasma mass spectrometry of copper-stressed wild-type TIGR4 and the Δ*copA* mutant. Fold-increase is based on comparison to the strain without copper stress relative to bacterial CFU. *Student's *t*-test indicates $p < .01$ compared to copper-stressed, wild-type TIGR4. Error bars represent standard deviation (SD) n = 3. (B) Rescue of Δ*copA* under copper stress by manganese supplementation. Indicated metals were added (250 μM). Values represent optical density at 620 nm as a fraction of bacteria without copper. * Student's *t*-test indicates $p < .01$ compared to 50 μM copper alone for the Δ*copA* mutant. Error bars represent SD n = 5. (C) Replication under copper stress increases in the presence of manganese. The amount of copper used for each strain is relative to the estimated IC_{50} of copper stress respective to the strain's endpoint growth (500 μM for TIGR4 and 50 μM for the Δ*copA* mutant). Error bars represent SEM n = 3. * Student's *t*-test indicates $p < .01$. (D) Manganese rescues bacterial survival from macrophage-mediated killing. Survival is normalized to the amount of the individual strain (TIGR4 and Δ*copA*) without manganese added because of the heightened sensitivity of Δ*copA* to macrophage-mediated killing. * Student's *t*-test indicates $p < .01$. Error bars represent SEM n = 3.

Figure 2. Oxidative damage in *S. pneumoniae* does not correlate with copper stress. (A) Protein oxidation in response to copper. TIGR4 and the *cop* operon mutants at an O.D. of 0.1 were exposed to varying amounts of copper for 1 hour and probed for protein oxidation. (B) Sensitivity to hydrogen peroxide stress. TIGR4 and the *cop* operon mutants at an O.D. of 0.1 were exposed to 0.1% hydrogen peroxide for 60

minutes and plated to determine the number of colony-forming units (CFU) present. * Student's *t*-test indicates p < .01 compared to wild-type. Error bars represent SD n = 5. (C) Δ*sodA* mutants are equally susceptible to copper stress and equally rescued by manganese. End-point growth of wild-type, Δ*copA,* Δ*sodA*, and Δ*copA*/Δ*sodA* TIGR4 pneumococci. * Student's *t*-test indicates p < .01 compared to strains grown without manganese. Error bars represent SEM, n = 4.

Table 1. Microarray analysis comparing transcription in wild-type and Δ*copA* bacteria during intracellular copper accumulation. Data show the log(2) change in each transcript from 3 independent experiments comparing TIGR4 and Δ*copA* bacteria under copper-stress conditions. Experimental conditions are detailed in the Methods.

Figure 3. Copper downregulates aerobic nucleotide synthesis. (A) Pathways for aerobic and anaerobic nucleotide synthesis in *S. pneumoniae.* (B) The anaerobic nucleotide synthesis pathway gene *nrdD* (SP_0202) is upregulated in the Δ*copA* mutant after a sub-lethal dose of copper. Error bar represents SEM n = 3. * Student's *t*-test indicates p < .01 compared to real-time data of parental strain with no copper added. (C) The Δ*nrdD* mutant has increased copper toxicity in anaerobic conditions. Error bar represents SD n = 5. * Student's *t*-test indicates p<.01 compared to similarly treated TIGR4 strain in the copper zone-of-inhibition assay. (D) The Δ*copA* mutant is more susceptible to 10 mM HU than is wild-type TIGR4. Error bar represents SD, n = 4. * Student's *t*-test indicates p < .01 compared to parental strain with nothing added.

Figure 4. Graphical model of copper export and toxicity in *S. pneumoniae*

Table 1.

TIGR4 Gene Number	2^X change	SD	Gene Name	Predicted Function
SP0202	2.2	±0.21	*nrdD*	anaerobic ribonucleoside-triphosphate reductase
SP0203	1.8	±0.26		hypothetical protein
SP0204	2.2	±0.17		acetyltransferase, GNAT family
SP0205	2.2	±0.26	*nrdG*	anaerobic ribonucleoside-triphosphate reductase activating protein
SP0206	2.1	±0.18		hypothetical protein
SP0207	2.1	±0.25		conserved domain protein
SP0338	5.4	±0.47	*clpP*	ATP-dependent Clp protease, ATP-binding subunit, putative
SP0474	1.8	±0.18	*licC*	PTS system, cellobiose-specific IIC component
SP0478	1.6	±0.31	*lacE*	PTS system, lactose-specific IIBC components
SP0727	4	±0.11	*copY*	Copper repressor
SP0728	3.8	±0.24	*cupA*	Copper chaperone
SP0729	-6	±0.45	*copA*	Copper exporter
SP0820	1.7	±0.24	*clpE*	ATP-dependent Clp protease, ATP-binding subunit ClpE
SP1774	1.7	±0.15		transcriptional regulator, putative
SP1775	2.5	±0.31		hypothetical protein
SP1776	1.8	±0.08	*trxA*	Thioredoxin reductase
SP1857	3.4	±0.16	*czcD*	Zinc efflux protein
SP2026	1.5	±0.21		Iron containing alcohol dehydrogenase

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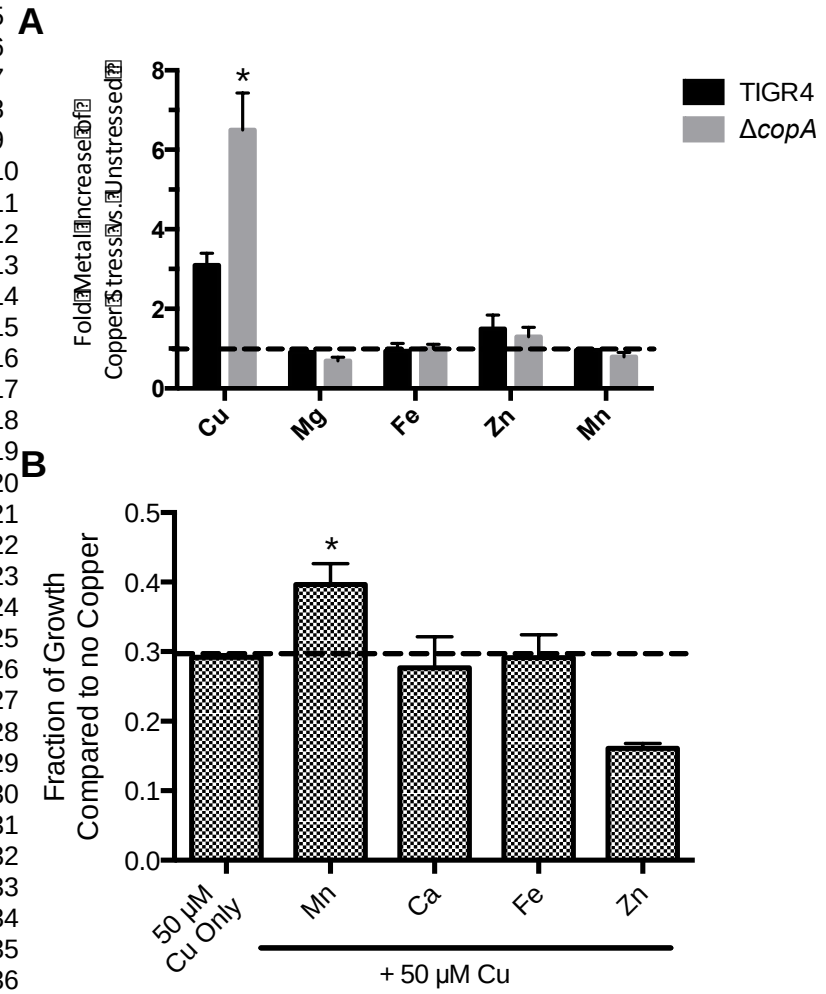


Figure 2

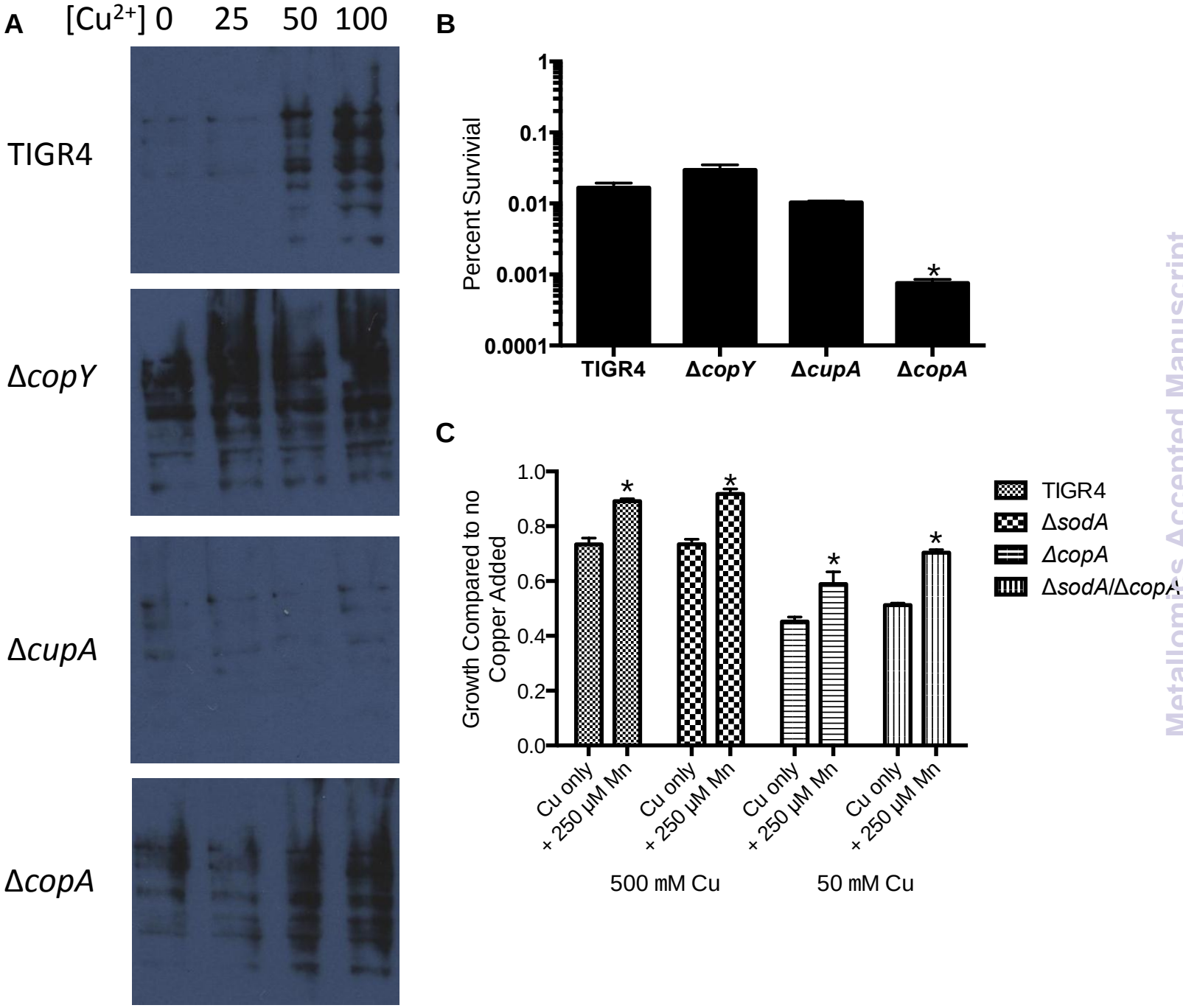
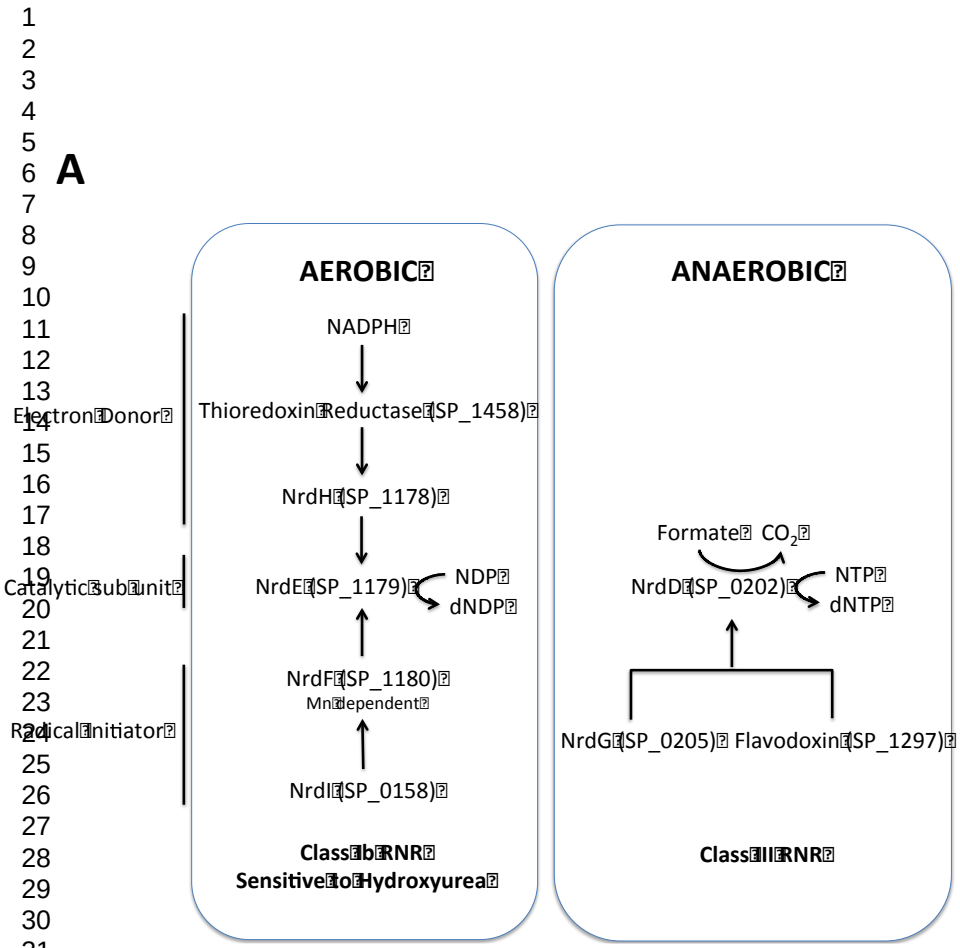
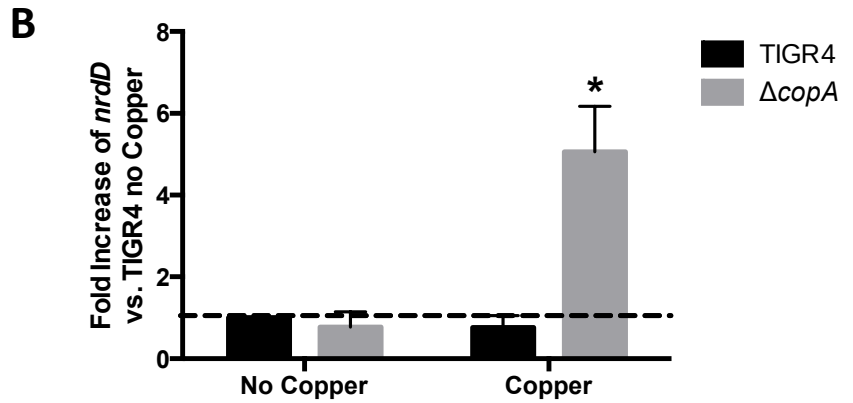
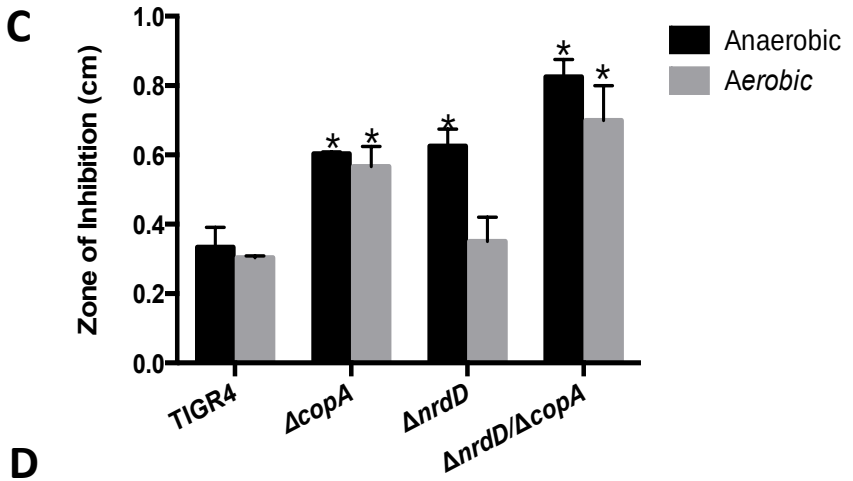


Figure 3







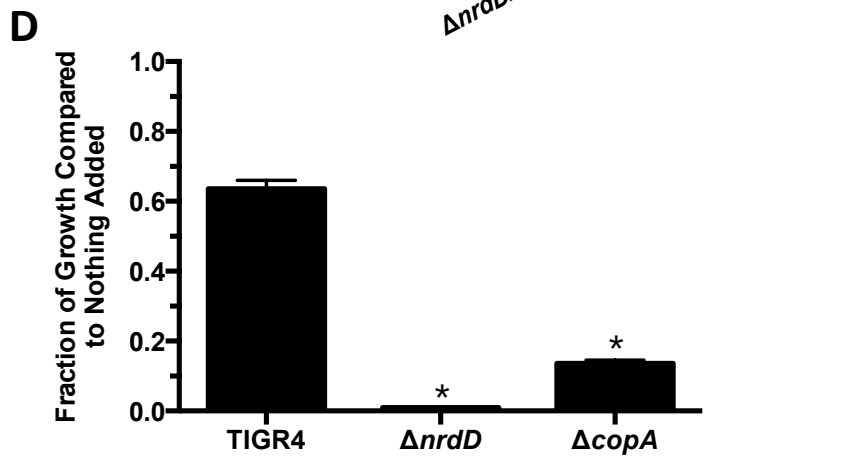


Figure 4