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Michael D L Johnson
St. Jude Medical

Thomas E. Kehl-Fie
University of Illinois at Urbana-Champaign

Roger Klein
St. Jude Medical

Jacqueline Kelly
Washington University School of Medicine in St. Louis

Corinna Burnham
St. Jude Medical

See next page for additional authors

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Role of Copper Efflux in Pneumococcal Pathogenesis and Resistance to Macrophage-Mediated Immune Clearance

Michael D. L. Johnson,[a] Thomas E. Kehl-Fie,[b] Roger Klein,[a]* Jacqueline Kelly,[a]* Corinna Burnham,[a] Beth Mann,[a] Jason W. Rosch[a]

Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee, USA[a]; University of Illinois Urbana-Champaign, Department of Microbiology, Urbana, Illinois, USA[b]

In bacteria, the intracellular levels of metals are mediated by tightly controlled acquisition and efflux systems. This is particularly true of copper, a trace element that is universally toxic in excess. During infection, the toxic properties of copper are exploited by the mammalian host to facilitate bacterial clearance. To better understand the role of copper during infection, we characterized the contribution of the *cop* operon to copper homeostasis and virulence in *Streptococcus pneumoniae*. Deletion of either the exporter, encoded by *copA*, or the chaperone, encoded by *cupA*, led to hypersensitivity to copper stress. We further demonstrated that loss of the copper exporter encoded by *copA* led to decreased virulence in pulmonary, intraperitoneal, and intravenous models of infection. Deletion of *copA* resulted in enhanced macrophage-mediated bacterial clearance *in vitro*. The attenuation phenotype of the *copA* mutant in the lung was found to be dependent on pulmonary macrophages, underscoring the importance of copper efflux in evading immune defenses. Overall, these data provide insight into the role of the *cop* operon in pneumococcal pathogenesis.

Streptococcus pneumoniae (pneumococcus) is a significant public health concern in pediatric and elderly populations and is a major cause of pneumonia, otitis media, and meningitis. The health risks are complicated by the fact that pneumococcus is common in the upper respiratory tract flora of most humans. In order to establish infection in these diverse host niches, *S. pneumoniae* must acquire necessary nutrients and avoid host-mediated clearance mechanisms in order to support survival and replication. One class of nutrients that impacts host-pathogen interactions are metals. These nutrients are predicted to be utilized by approximately 40% of all proteins and serve as important catalysts and structural components of many proteins (1). However, just as metals are essential nutritional requirements, they can also be detrimental when in excess. Accordingly, loss of bacterial metal import and export systems that facilitate metal homeostasis often corresponds with reduced virulence in murine models (2–5).

Bacteria express a myriad of systems dedicated to maintaining metal homeostasis, highlighting the importance of metals in bacterial physiology (6). Pneumococcus alone encodes systems dedicated to both influx and efflux of manganese and zinc, iron import, calcium efflux, and copper efflux, many of which play important roles during infection (2, 3, 6–11). These bacterial systems have likely evolved in response to the ability of vertebrates to harness both the essential and toxic nature of metals to combat infection. During infection, the host is known to sequester the essential metals iron, manganese, and zinc (12–14). Concurrent with the sequestration of essential metals, the host also exploits the toxic effects of metals, such as copper, to combat invading pathogens (15, 16). Copper has potent antimicrobial, antiviral, and antifungal properties and is utilized by mammalian hosts for these activities (17, 18). The ubiquitous distribution of systems for both metal acquisition and efflux across bacterial species underscores the importance of transition metal homeostasis in bacteria.

To counter copper toxicity, bacterial species have evolved dedicated copper efflux systems to eliminate excess cytosolic copper. One such elimination pathway is encoded by the *cop* operon. These systems can vary between species; however, essential elements include a copper repressor (CopY), which recognizes copper and allows for the operon's transcription, a copper chaperone protein (CupA) (19), and the copper exporter (CopA) (20). The fact that these copper export systems remain functionally conserved among diverse mammalian pathogens underscores the importance of copper homeostasis in the niches encountered within the host (21–23). This system represents a remarkably efficient system for eliminating intracellular copper, with the negative regulator having zeptomolar (10^{-21}) copper sensitivity in some systems, corresponding to less than one free atom per cell in *Escherichia coli* (24). The chaperone protein, CupA, facilitates efficient export of copper by CopA by binding and transferring the copper atom to CopA, which then facilitates export, as recently demonstrated in *S. pneumoniae* (25). There are a number of examples of the importance of copper in host-pathogen interactions (16, 17, 26). Pneumococci upregulate the *copA* copper export system during murine challenge in both the lungs and nasophaynx, as determined by relative transcript abundance, suggesting that copper toxicity is one of the mechanisms employed by the host to combat pneumococcal infection (4). This idea is further supported by studies that implicate *copA* and *cupA* in metal homeostasis and colonization of the nasopharynx in both the D39 and TIGR4 back-

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Address correspondence to Jason W. Rosch, jason.rosch@stjude.org.

* Present address: Roger Klein, Washington University School of Medicine, St. Louis, Missouri, USA; Jacqueline Kelly, University of Cincinnati School of Medicine, Cincinnati, Ohio, USA.

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grounds (4, 7). The role of these systems in invasive pneumococcal disease is less clear.

During infection, the pneumococcus migrates to the lower respiratory tract, where macrophages are one of the primary lines of host defense (27). Recent work suggests that copper intoxication is one of the mechanisms utilized by macrophages to kill bacteria (28). Evidence for this is derived from the copper transporter ATP7a, which pumps copper into the phagosome to mediate more effective bacterial clearance, as evidenced by *in vitro* models using both primary murine and RAW264.7 macrophages and Gram-negative bacterial intracellular killing assays (17, 28, 29). This strategy may extend to multiple pathogens, as copper detoxification is also a critical aspect of virulence in *Cryptococcus neoformans* (30). The notion of the importance of copper in pneumococcal virulence is supported by recent data showing that deletions in *copA* resulted in defects during murine nasopharyngeal colonization and transposon screens, thus implicating *copA* as an important virulence determinant in the murine lung, though the mechanisms underlying the attenuation remain unclear (4, 7, 31). Given the potential contribution of copper to controlling pneumococcal infection, studies were undertaken to further investigate the contribution of the *cop* operon to pneumococcal virulence. These investigations revealed that the Cop system is necessary during all stages of pneumococcal virulence and specifically protects the pneumococcus from macrophage-imposed copper toxicity.

MATERIALS AND METHODS

Bacterial constructs. Deletion disruption mutations of SP_0727 (*copY*), SP_0728 (*cupA*), SP_0729 (*copA*), and SP_0730 (*spxB*) were created by using splicing by overhang extension PCR (SOE PCR). Approximately 1-kb-long upstream and downstream fragments of the target gene were amplified and spliced to an erythromycin resistance gene (*ermB*). The SOE PCR products were subsequently transformed into pneumococci (strain TIGR4 or 19F), and all deletion disruption knockouts were verified by PCR to confirm insertion of the SOE PCR product and deletion of the target gene (32). Complementation for *copY* was done by performing SOE PCR to reintroduce the *copY* gene with a spectinomycin cassette immediately upstream from the gene, selecting for erythromycin-sensitive, spectinomycin-resistant colonies, and confirming by amplification of the *copY* gene. Complementation for *cupA* was done by cloning *cupA* into the shuttle vector pABG5 and selecting colonies for kanamycin resistance. Complementation for *copA* was adapted from the method in reference 2. Briefly, wild-type TIGR4 genomic DNA was transformed into the Δ*copA* mutant, and colonies growing on THYB (30 g Todd Hewitt broth [Sigma], 2 g yeast extract [Sigma], 1 liter distilled water, pH 6.5) plates supplemented with 100 μM copper under anaerobic conditions were selected. Colonies were replica plated onto tryptic soy agar (TSA) blood plates containing erythromycin. All 13 recovered colonies were erythromycin sensitive and contained the *copA* gene as confirmed by PCR.

Growth curves and CFU. Each *S. pneumoniae* strain was grown at 37°C in THYB medium either under aerobic conditions (5% CO_2) or with Oxyrase supplementation according to the manufacturer's guidelines for anaerobic conditions and with or without various amounts of $CuSO_4$. For all metal sensitivity growth curves, THYB medium was inoculated with 200 μl of bacteria (optical density [OD] of 0.1) to a final volume of 10 ml for overnight growth assessments. For CFU experiments, 20 μl of culture was serially diluted and plated on TSA (Sigma) supplemented with 3% sheep blood (I-Tek) at 8 h following inoculation. All OD measurements used to construct growth curves were read at 620 nm (OD_{620}) on a Turner model 340 spectrophotometer.

Hydrogen peroxide measurement. For measurement of H_2O_2 production, bacteria were cultured in THYB medium to an OD_{620} of 0.4, and then 1-ml aliquots were taken and bacteria were collected via centrifugation and resuspended in sterile phosphate-buffered saline (PBS). Bacteria were then incubated at room temperature for 20 min to allow H_2O_2 production. H_2O_2 production was measured with an H_2O_2 assay kit from National Diagnostics (Atlanta, GA). A PBS blank and a serial dilution of an H_2O_2 standard solution were used as a background measurement and a standard curve, respectively.

Mouse challenge. All experiments involving animals were performed with prior approval by and in accordance with the guidelines of the St. Jude Institutional Animal Care and Use Committee (IACUC). The St. Jude laboratory animal facilities have been fully accredited by the American Association for Accreditation of Laboratory Animal Care. Laboratory animals were maintained in accordance with the applicable portions of the Animal Welfare Act and the guidelines prescribed in the DHHS publication *Guide for the Care and Use of Laboratory Animals*. All mice were maintained in biosafety level 2 (BSL2) facilities, and all experiments were done while the mice were under inhaled isoflurane (2.5%) anesthesia. Mice were monitored daily for signs of infection. This work was approved under IACUC protocol number 538-100013-04/12 R1.

For survival studies, bacteria were introduced by intratracheal administration of 1×10^5 CFU of bacteria in PBS (100 μl), a model which effectively recapitulates the progression of disease from pneumonia to the development of sepsis and meningitis (33). Seven- to 8-week-old female BALB/cJ mice (Jackson Laboratory) were used for all murine experiments. Differences in time to death among the mice were compared via the Mantel-Cox log rank test. Tissues and blood were collected from animals 24 h following infection. For lung collection, mice were perfused with saline prior to organ collection to remove contaminating blood from the lung, which was then homogenized. For intraperitoneal and intravenous mouse infections, 7-week-old female BALB/cJ mice (Jackson Laboratory) were injected either intraperitoneally with 1×10^3 bacteria in 100 μl or intravenously with 3×10^5 bacteria in 100 μl. The parental TIGR4 bacteria and the respective mutants were quantified by serial dilution followed by enumeration of CFU on TSA blood plates. For the acute otitis media and sinusitis model, 1×10^5 BHN97x bacteria in a 100-μl volume were administered intranasally to 8-week-old female BALB/cJ mice (Jackson Laboratory). Mice were then monitored for luciferase levels by Xenogen imaging twice daily for the next 3 days, starting at 6 h postchallenge.

Depletion of macrophages in mouse lung. Anesthetized 7-week-old BALB/c mice (Jackson Laboratory) received intranasal administration of 100 μl of liposomal clodronate or Encapsome control (Clodrosome) (34). After 2 days, mice were infected with 1×10^5 bacteria in a 100-μl volume of PBS.

Macrophage killing 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 fetal bovine serum (FBS [10% vol/vol]; Sigma), glutamine (2 mM; Sigma), penicillin (50 units/ml; Sigma), streptomycin (50 mg/ml; Sigma), and sodium bicarbonate (0.015%). 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 OD of 0.1 for 90 min, corresponding to a multiplicity of infection of 10 bacteria per macrophage. Wells were then washed twice; each wash was followed by a 1-min incubation in DMEM containing gentamicin (50 μg/ml). Macrophages were lysed and serially diluted to determine the counts of viable intracellular bacteria. Data were normalized to the level of killing observed for the untreated TIGR4 bacteria for each assay.

Flow cytometry. *S. pneumoniae* cells (OD_{620} of 0.3) were added to 10 ml of PBS with 10 μM Vybrant carboxyfluorescein diacetate-succinimidyl ester (CFDA SE) stain (Invitrogen) and incubated for 30 min at 37°C. Bacteria were washed twice in PBS and used to inoculate J774.1 murine macrophages as described previously, except that macrophages were harvested after 15 min and fixed in 3% paraformaldehyde. Samples were examined on a BD FACSCanto II flow cytometer (BD Biosciences), and data were analyzed by using FlowJo version 7.6 (Treestar).

Adhesion and invasion assays. A549 adenocarcinomic human alveolar basal epithelial cells (ATCC) were maintained in a 37°C, 5% CO_2 incubator with F-12 medium containing 10% (vol/vol) FBS (Sigma). Cells were grown to 70% confluence in 12-well tissue culture plates, washed once in F-12 without FBS, and incubated with 1×10^7 bacteria at 37°C and 5% CO_2 for either 30 min (to allow bacterial adhesion) or 2 h (to allow bacterial invasion). For adhesion assays, cells were gently rinsed three times with PBS and incubated in 100 μl of trypsin for 5 min at 37°C to displace binding. Then, 900 μl of PBS was added, and serial dilutions of the resultant resuspensions were spread onto TSA blood plates for CFU enumeration.

For invasion assays, after 2 h of incubation, cells were washed three times with sterile PBS and then incubated in F-12 with 100 μg/ml gentamicin at 37°C and 5% CO_2 for 1 h. Cells were gently rinsed three times with PBS and incubated in 100 μl trypsin for 5 min at 37°C to displace cells. Then, 0.025% Triton X-100 in double-distilled water was added to lyse eukaryotic cells. The lysates were then diluted and spread onto TSA blood agar plates for CFU enumeration.

RNA extraction. *S. pneumoniae* cells were grown to an OD of 0.1 in THYB medium. Cultures were then diluted 50-fold in fresh THYB medium and grown to an OD of 0.3. Copper sulfate was added to each culture in the amounts indicated below, and the mixtures were incubated for 15 min at 37°C. Culture samples (5 ml) were incubated with RNAprotect bacterial reagent (10 ml; Qiagen) for 20 min before the bacteria were isolated via centrifugation. RNA was extracted from the cell pellets by using an RNeasy minikit (Qiagen).

Quantitative real-time PCR. SuperScript III first-strand synthesis supermix (Invitrogen) was used with the isolated RNA (50 ng/μl) to synthesize cDNA. SYBR green (Invitrogen) was used to monitor double-stranded DNA (dsDNA) synthesis on an ABI-Prism 7300 real-time PCR machine (Applied Biosystems). For each amplification curve, a cycle threshold (C_T) value was obtained for the specified gene transcripts, as well for *gyrA*, which served as the normalization control. The results for the samples were normalized to the levels of *gyrA* transcript, and the $\Delta\Delta C_T$ method was used to establish the relative fold changes in transcript abundance.

Growth curves. Bacteria were grown to an OD_{620} of 0.1 and subsequently diluted 1:100 into 200 μl of THYB medium in a 96-well format in triplicate. Samples were read at 620 nm every 30 min for 24 h.

RESULTS

Functional conservation of CopA. CopA from pneumococcal strain TIGR4 is a P-type ATPase containing a phosphatase domain (TGE, starting at residue 287), a phosphorylation motif (DKTGT, starting at residue 442), and the P-type ATP-binding motif (GDGXND, starting at residue 634) (Fig. 1A). These motifs are consistent with those of other reported CopA proteins in both *S. pneumoniae* and other bacteria (4, 25, 35, 36). Although many copper-specific ATPases contain the copper-binding motif GMX CXXC, TIGR4 CopA has a CXC copper-binding motif (starting at residue 398), with the sulfur in each respective cysteine putatively making the copper contacts (Fig. 1A). CopA also has a CupA-like fold in the first 99 residues, with the C. . .CXMXM motif beginning at residue 49 and ending at residue 99 (25). Thus, this protein is consistent with previously described copper export proteins in organisms like *Escherichia coli*, *Bacillus subtilis*, and *Rhodobacter capsulatus* (37–40).

Sensitivity profiles of the *cop* operon mutants. Transposon screens and targeted deletions have implicated *copA* and *cupA* in metal homeostasis and colonization of the nasopharynx in both the D39 and TIGR4 background; however, the role in invasive disease is less clear (4, 7). To clarify the contribution of the copper efflux system in resisting copper toxicity and invasive pneumococcal disease, stable deletion disruption mutations of *copY* (SP0727), *cupA* (SP0728), and *copA* (SP0729) in TIGR4 were generated via insertion of the *ermB* erythromycin resistance gene at the respective loci. Both Δ*copA* and the predicted cupredoxin Δ*cupA* mutants were highly sensitive to copper toxicity under aerobic conditions (Fig. 1B). Deletion of either of these genes rendered the pneumococcus approximately 10-fold more sensitive to copper stress, as evidenced by growth inhibition of the mutants at 50 μM copper, which was similar to the growth inhibition of the wild-type TIGR4 at 500 μM. Consistent with CopY acting as a repressor of the efflux system, the Δ*copY* mutant had a sensitivity profile similar to that of the wild type, with no heightened sensitivity being observed (Fig. 1B). We suspect the Δ*copY* mutant is not more resistant to copper stress because the pneumococcus rapidly upregulates this system, within 5 min, under conditions of copper stress *in vitro* (data not shown). All mutants grew at the same rate as TIGR4 under standard medium conditions, indicating that, overall, sensitivity to copper is not due to a general growth defect (Fig. 1C). No other metal tested resulted in significant changes in sensitivity between the wild-type TIGR4 and any of the respective mutants, including iron, manganese, cobalt, cadmium, nickel, and zinc (data not shown). Strains in which the respective deleted gene was reintroduced either by recombining the gene (Ω*copA* and Ω*cupY*) or on a plasmid (Ω*cupA*) resulted in restoration of copper sensitivity profiles to wild-type levels (Fig. 1D). In agreement with previous data about the role of this operon in copper homeostasis, *copA* is rapidly upregulated with increasing concentrations of copper (Fig. 1E). These data support the notion that CopA functions as part of a copper efflux system and is regulated by copper stress, as was recently demonstrated both transcriptionally in pneumococcal strain D39 and at the structural level when the CupA chaperone was shown to facilitate copper transfer to CopA (4, 25).

One concern with deletion disruption mutations is the potential for perturbation of downstream genes, which may confound subsequent phenotypic analysis. Of particular concern in this study is the gene immediately downstream from *copA*, *spxB*, encoding a pyruvate oxidase that is involved in hydrogen peroxide production and implicated in pneumococcal virulence (33, 41, 42). The deletion disruption of *copA* in the Δ*copA* strain resulted in no significant alteration of *spxB* transcript levels as measured by quantitative reverse transcription-PCR. The relative levels of transcript abundance were 1.0 ± 0.3 (mean ± standard deviation) for TIGR4, 1.32 ± 0.5 for the Δ*copA* mutant, 1.1 ± 0.4 for the Ω*copA* mutant, and undetectable for the Δ*spxB* deletion disruption mutant ($n = 3$ independent experiments). This finding was in agreement with the results of assays for hydrogen peroxide production, where the levels following a 30-min incubation were 137 ± 15 μM for TIGR4, 37 ± 3 μM for the Δ*spxB* mutant, 117 ± 6 μM for the Δ*copA* mutant, and 138 ± 18 μM for the Ω*copA* mutant ($n = 4$ experiments). These data indicate that the deletion disruption mutation in Δ*copA* did not have a significant effect on the transcriptional abundance or the functional activity of the downstream gene *spxB* and, thus, that the phenotypes observed in the Δ*copA* strain are not the result of polar effects on *spxB*.

CopA is required for pneumococcal virulence. Because copper exporters have been implicated in the virulence of pneumococci and other bacteria (4, 43), we determined the role of the individual *cop* genes in pneumococcal virulence during pulmonary infection. Mice infected with the Δ*copA* mutant had signifi-

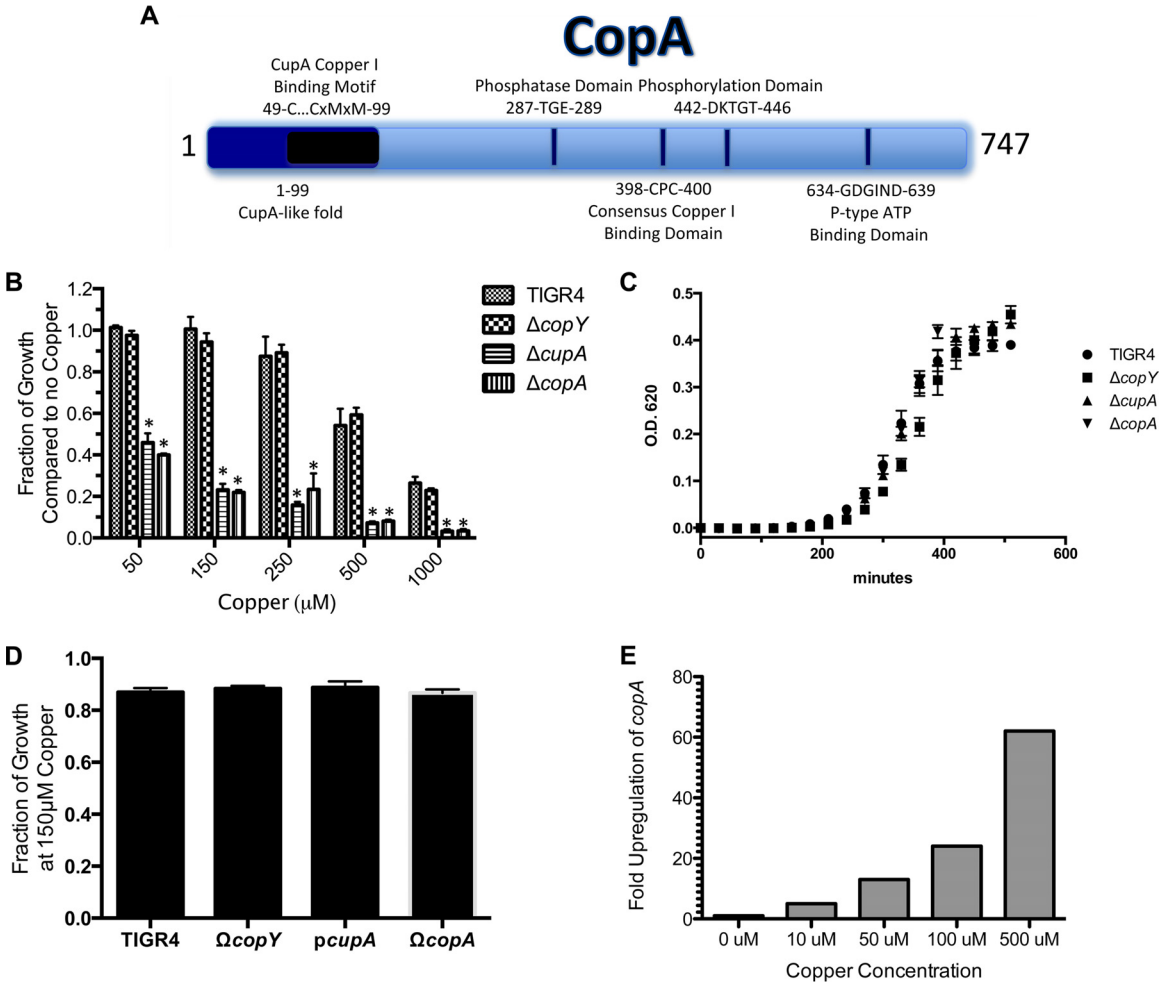

FIG 1 CopA is required to alleviate copper stress. (A) A bar representation of CopA from the TIGR4 strain of *S. pneumoniae*. The dark blue lines represent the motifs described above or below them. Motifs were predicted on the basis of 100% sequence homology by using InterProScan. (B) Endpoints of aerobic growth of wild-type TIGR4, Δ*copY*, Δ*cupA*, and Δ*copA* bacteria as determined by measuring OD values normalized to that of wild-type TIGR4 with no copper. *, $P <$ 0.01 for comparison by Student *t* test to the results for the wild-type TIGR4 counterpart unless otherwise noted. Error bars represent standard errors of the means from at least three independent experiments. (C) Growth curves of TIGR4 and the *cop* operon deletion disruption mutants. Data represent averages and standard deviations from two independent growth curves. (D) Growth of mutants with complementation mutations in THYB supplemented with 150 μM copper. Error bars represent standard deviations. (E) Real-time PCR data for *copA* expression with increasing amounts of copper; data are from three replicates and represent average fold changes in induction compared to the results with no copper.

cantly lower bacterial titers in the blood and lung and higher survival rates than mice infected with wild-type bacteria (Fig. 2C and D). Surprisingly, no significant differences in survival time or bacterial loads in the lungs were observed in mice infected with the Δ*cupA* mutant, indicating that the copper sensitivity phenotype was not necessarily predictive of virulence potential (Fig. 2A and D). The Δ*copY* mutant had a slightly but significantly ($P < 0.05$) heightened virulence phenotype in terms of overall mouse survival time and bacterial burden in the bloodstream (Fig. 2A and D). These data indicate that overexpression of *copA* and *copY* may lead to enhanced virulence in the context of the Δ*copY* mutant, though this slight difference is likely not biologically relevant. When *copA* (Ω *copA*) and *copY* (Ω *copY*) mutations were subsequently reintroduced into the respective knockout strains, no significant difference was observed in survival (Fig. 2F and G) or bacterial blood titers (data not shown) following infection compared to the results for infection with the wild type. These data further implicate *copA* in pneumococcal virulence during pulmonary infection.

To determine whether the attenuation was strain specific, the effect of *copA* deletion in a pneumococcal serotype 19F strain, BNH97, was assessed. This strain differs from TIGR4 in that it causes pneumonia and mucosal disease, such as acute otitis media, but does not progress to bacteremia like TIGR4 (44). In addition, while the TIGR4 strain does colonize the nasopharynx, progression to acute otitis media rarely occurs. Similar to the mice infected with the TIGR4 mutant, mice infected with 19F Δ*copA* had decreased bacterial burdens in the lungs 24 h following intratracheal instillation (Fig. 2E). The attenuation of the 19F Δ*copA* mutant extended to other mucosal sites, where this mutant was unable to establish acute otitis media within 24 h of challenge (Fig. 3A, C, and D), unlike the parental strain. The 19F Δ*copA* strain also displayed a defect in the capacity to cause sinusitis, as measured by Xenogen imaging (Fig. 3B, E, and F). Taken together, these data indicate that CopA is required for virulence during pneu-

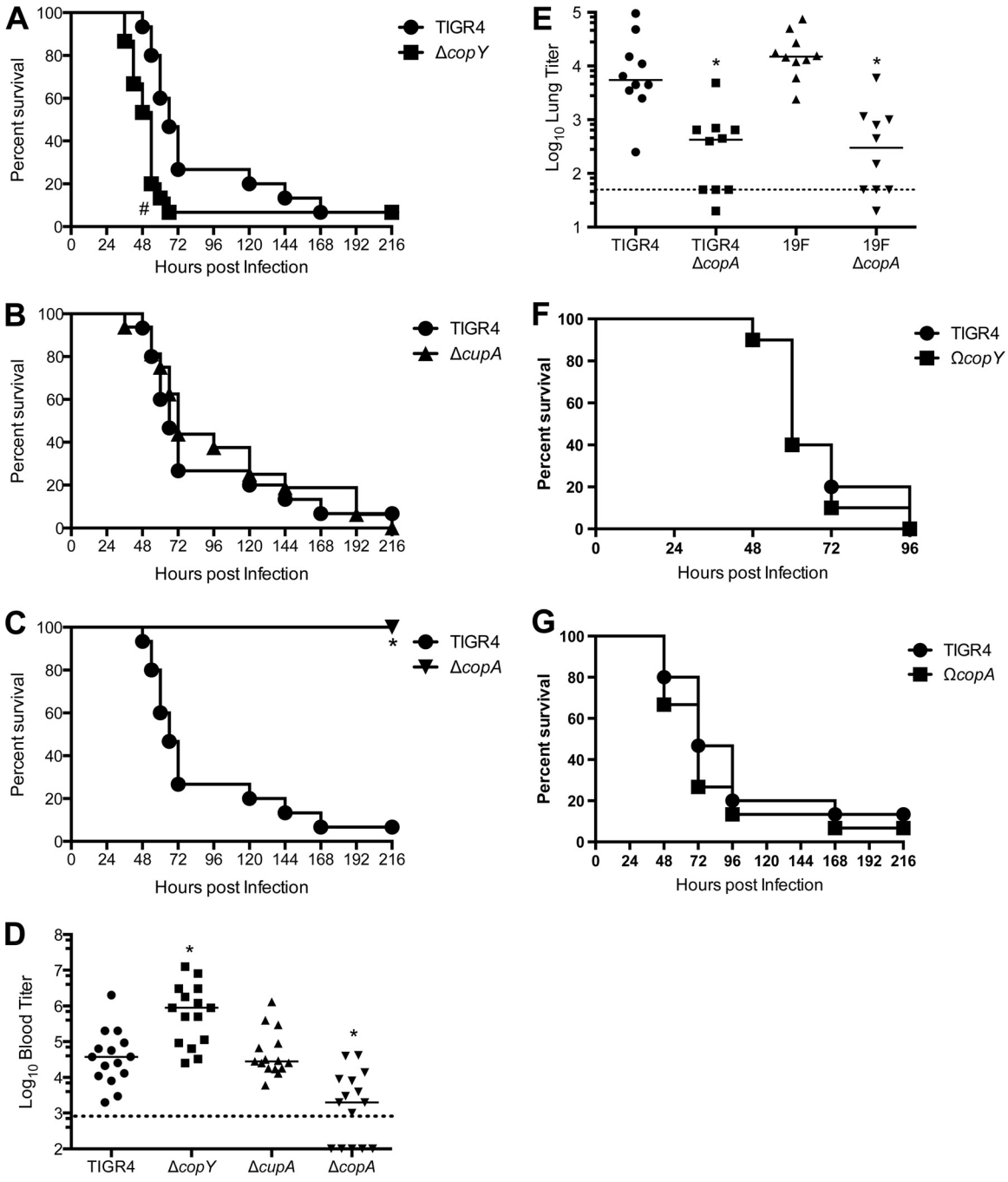

FIG 2 CopA is needed for virulence during intratracheal infections. (A to C) Survival curves of mice infected with wild-type TIGR4 or a deletion disruption mutant with the Δ*copY* (A), Δ*cupA* (B), or Δ*copA* (C) mutation after infection with the 100% lethal dose. *, $P < 0.01$, and #, $P < 0.05$, by Mantel-Cox test. (D) Bacterial burdens of wild type and mutants in the bloodstream of mice 24 h postchallenge. Horizontal rules represent median values. *, $P < 0.01$ for comparison by Mann-Whitney test to the results for mice infected with the parental wild type; $n = 15$ animals per group. (E) Mouse lung titers of the indicated strains 24 h following infection. Horizontal rules represent median values. *, $P < 0.01$ for comparison by Mann-Whitney test to the results for mice infected with the parental wild type; $n = 10$ animals per group. Dotted lines in panels D and E represent the lower limit of detection. (F) Survival curves of mice infected with TIGR4 wild type and the Ω*copY* revertant; $n = 15$ mice per group. (G) Survival curves of mice infected with TIGR4 wild type and the Ω*copA* revertant; $n = 15$ mice per group.

mococcal mucosal infection and that this role in pathogenesis is not strain specific.

CopA is required for bacteremia and dissemination through the epithelium. An important aspect of invasive pneumococcal disease is the capacity of this organism to adhere to and invade host cells. Perturbation of metal homeostasis can result in pleiotropic effects on the cell, including altered regulation of adhesins that mediate bacterial interactions with host cells. The contributions of members of the *cop* operon to host cell adhesion and invasion capacity in lung epithelial cells were assessed. Wild-type

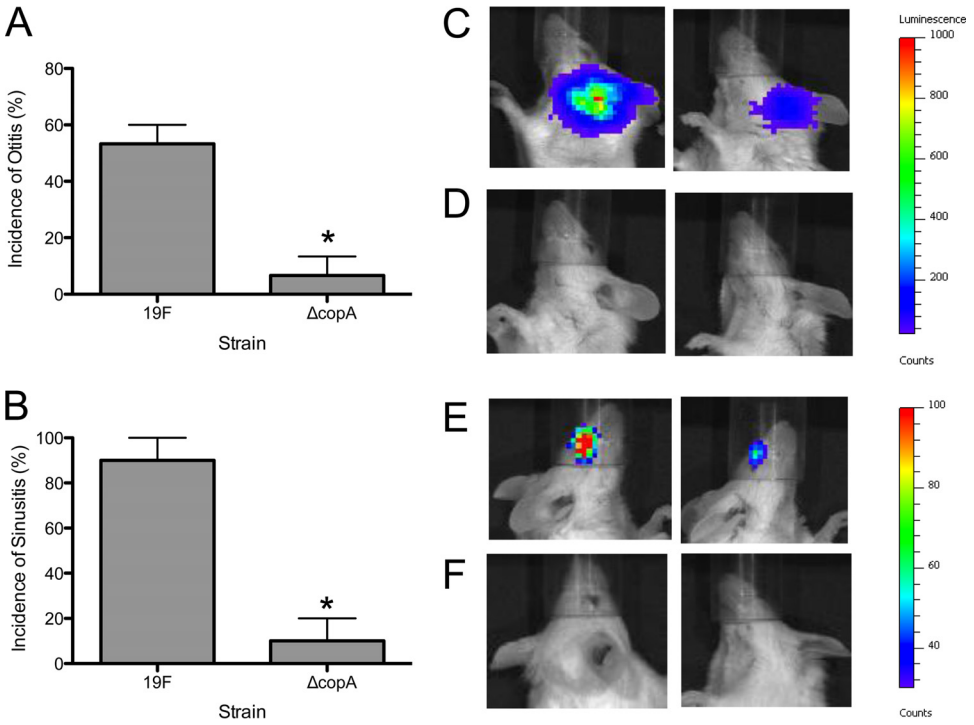

FIG 3 CopA is required for the development of acute otitis media and sinusitis. (A) Incidence of acute otitis media in mice infected with the 19F or Δ*copA* 19F strain. Data represent means and standard deviations from three independent experiments with five mice each. (B) Incidence of sinusitis 72 h postchallenge in mice infected with the 19F or 19F Δ*copA* strain. Data represent means and standard deviations from two experiments with five mice each. *, $P < 0.05$ by Fisher exact test for panels A and B. (C to F) Representative Xenogen images of mice infected with the 19F (C, E) and the 19F Δ*copA* (D, F) strains at 24 and 72 h postinfection, respectively. Luminescence scales are shown adjacent to the images.

bacteria invaded human lung epithelial cells better than the Δ*copY*, Δ*cupA*, or Δ*copA* mutants, with the Δ*copA* mutant having the lowest level of invasion ($P < 0.01$) (Fig. 4A). It should be noted that this decreased amount of intracellular bacteria could be the result of killing within the epithelial cells. Furthermore, only the Δ*copA* mutant had an adherence defect, indicating that it may also have deficiencies in initial epithelial adherence (Fig. 4B). These data may partially explain the heightened attenuation of the Δ*copA* mutant in relation to the results for the fully virulent Δ*cupA* mutant during the intratracheal challenges, as the Δ*copA* mutant has a significantly lower ability to adhere to and invade host cells than does the Δ*cupA* mutant.

To determine whether the Δ*copA* virulence defect was merely due to an inability to transverse the lung epithelium, intraperitoneal and intravenous models of infection were used. At 24 h following intraperitoneal challenge, the bacterial titers in the blood of mice infected with wild-type TIGR4 were 3 log higher than the titers in mice infected with the Δ*copA* mutant (Fig. 5A). This result corresponded with a significant increase in the survival of mice infected with the Δ*copA* mutant compared to the survival of mice infected with wild-type TIGR4 (Fig. 5B). Similar results were obtained from the intravenous infection model, where mice infected with the Δ*copA* mutant had significantly reduced blood bacterial titers and increased survival relative to the results for mice infected with the wild type (Fig. 5C and D). Taken together, these data indicate that the copper efflux system is an integral aspect of pneumococcal pathogenesis during both pulmonary and systemic infection.

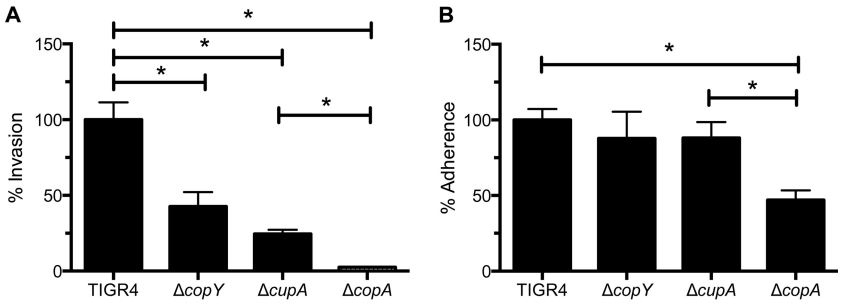

FIG 4 *S. pneumoniae* interactions with A549 lung epithelial cells. Relative levels of intracellular and adherent cells of TIGR4 and *cop* operon mutant strains on A549 lung epithelial cells during invasion (A) or adhesion (B) assays. Error bars represent standard errors of the means. *, $P < 0.01$ for comparison to the results for wild-type TIGR4 by Student *t* test; *n* = at least 3 independent experiments.

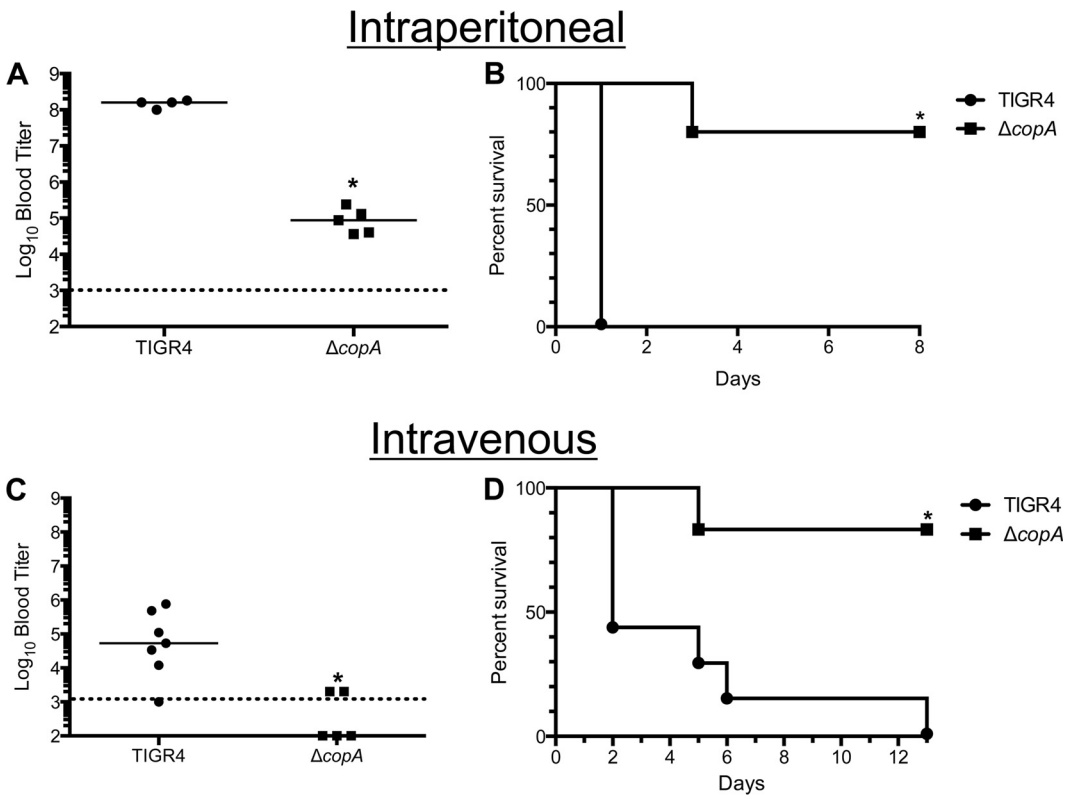

FIG 5 The Δ*copA* mutant has a bacteremia defect. Mice were infected with the indicated wild-type or mutant strain either intraperitoneally (A, B) or intravenously (C, D). (A, C) Blood titers of the bacteria in intraperitoneally (A) and intravenously (C) infected animals at 48 h postchallenge. Horizontal rules represent median values. *, $P < 0.01$ for comparison by Mann-Whitney test to the results for mice infected with wild-type TIGR4. Dotted lines in panels A and C represent the lower limit of detection. (B, D) Survival curves of mice infected with the TIGR4 or Δ*copA* mutant intraperitoneally (B) or intravenously (D); data are for the same mice whose blood titer results are shown in panels A and C. *, $P < 0.01$ for comparison by Mantel-Cox test to the results for mice infected with the parental strain; $n = 5$ animals per group.

The Δ*copA* mutant is more susceptible to macrophage-mediated killing. Macrophages are typically among the first host immune cells that bacteria encounter during lung infection. One mechanism employed by macrophages to kill bacteria is copper toxicity mediated by ATP7a, a copper transporter that actively imports Cu^{1+} into the phagolysosome and is upregulated in response to infectious challenge (28). We hypothesized that the attenuation of the Δ*copA* mutant was partially due to a heightened susceptibility to macrophage-mediated clearance, one of the first lines of host defense again pneumococcal infection. This was initially tested *in vitro*, where the Δ*copA* mutant was more sensitive to macrophage-mediated killing than the parental TIGR4 (Fig. 6A). This heightened sensitivity to macrophage-mediated killing was not due to differences in bacterial uptake, as equivalent rates of engulfment were observed when measured by flow cytometry (Fig. 6B). These data suggest that copper efflux is involved in the capacity of the pneumococcus to resist host innate clearance mechanisms.

To determine whether a heightened sensitivity to macrophage-mediated bacterial clearance was one of the underlying mechanisms of the Δ*copA* mutant's attenuation during pulmonary infection, murine lung macrophages were depleted prior to infection by using clodronate, which depletes macrophages and monocytes present in the lungs, but not circulating macrophages (34). Following 24 h of infection, the bacterial titers in the lungs of clodronate-treated mice infected with the Δ*copA* mutant were indistinguishable from those in the lungs of mice infected with TIGR4 (Fig. 6C). These data indicate that local depletion of macrophages rescues the attenuation observed for the Δ*copA* mutant. Consistent with the retention of circulating macrophages following clodronate treatment, fewer bacteria were recovered from the bloodstream of mice infected with the Δ*copA* mutant than from the blood of mice infected with the wild type (Fig. 6D). Additionally, both the clodronate- and mock-treated mice that were infected with the Δ*copA* mutant cleared the infection and were alive by day 7, while all of the mice infected with the wild type died or had to be euthanized due to sepsis by day 5 (Fig. 6E). This result is consistent with the results for both the intraperitoneal and intravenous challenges, where the Δ*copA* strain could not establish itself in the bloodstream during systemic infection. These data establish that the attenuation of the Δ*copA* mutant is partially due to heightened sensitivity to macrophage-mediated clearance and that macrophages are likely to be one source of copper toxicity in the lung during pneumococcal infection.

DISCUSSION

Despite not encoding any known copper utilization proteins, the pneumococcus contains a significant amount of this element—approximately 20 percent of the level of manganese, corresponding to approximately 100 nanograms of copper per milligram of

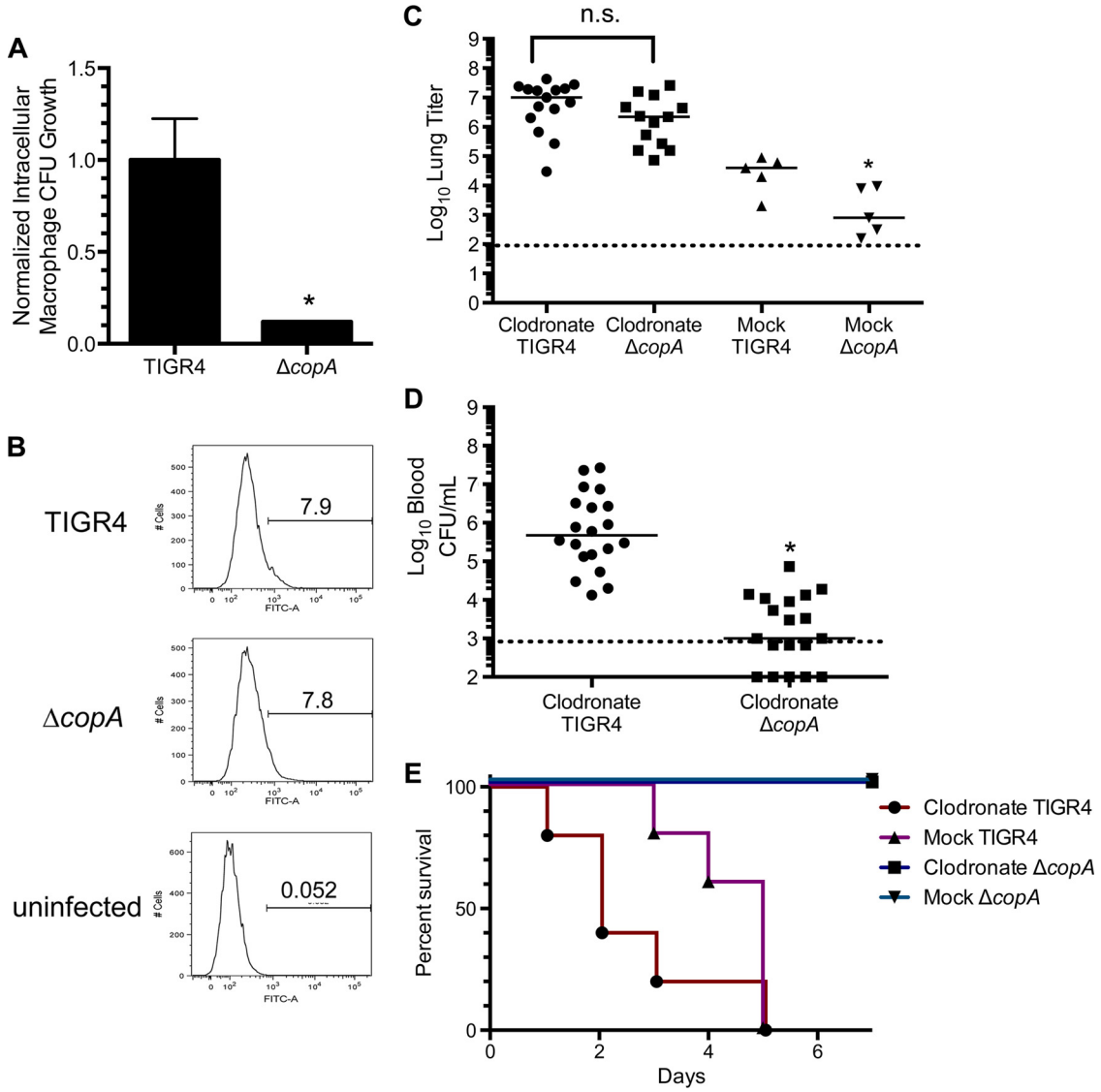

FIG 6 *S. pneumoniae* without CopA is more susceptible to macrophage-mediated killing. (A) The Δ*copA* mutant is more susceptible to J774 murine macrophage-mediated killing than wild-type TIGR4. Bacterial counts were normalized to the results for the wild type for data from triplicate samples. *, $P < 0.01$ by Student *t* test. Error bars represent standard deviations. (B) Measurements of intracellular bacteria in J744 macrophages. Populations were gated off at low percentages of uninfected macrophages. Plots are representative of the results of three experiments. (C) Bacterial titers in lungs of mice 24 h postchallenge. Macrophages were depleted from mouse lungs with clodronate liposomes; mock treatment with empty liposomes was used as a control. Horizontal rules represent median values. *, $P < 0.01$, and n.s., no significance, for comparison by Mann-Whitney test to the results for the TIGR4 strain in similarly treated mice. (D) Blood titers were measured 48 h after intratracheal infection of mice treated as described for panel C. Horizontal rules represent median values. *, $P < 0.01$ for comparison by Mann-Whitney test to the results for mice infected with the parental wild type. Dotted lines in panels C and D represent the lower limit of detection. (E) Survival curves of mock- and clodronate-treated mice infected with the TIGR4 and Δ*copA* mutants. *, $P < 0.01$ for comparison by Mantel-Cox test to the results for mice infected with the parental strain; $n = 5$ mice in each group tested.

cellular protein (45). While not having a known copper import mechanism, all sequenced pneumococci encode a highly efficient copper efflux system. This high degree of conservation among pneumococcal strains suggests an important role for this system in the human host, the only known reservoir for the pneumococci. In this study, we demonstrated that the copper exporter, CopA, was crucial for copper tolerance and virulence in *S. pneumoniae*. We also observed that eliminating the CopY repressor for the *cop* operon led to increased virulence. This indicates that increased expression of the copper export system prior to introduction into the mammalian host promotes more robust infection, potentially because the system is already expressed at high levels. Δ*copA* mutants were significantly less virulent during lung infection regardless of the strain background (TIGR4 and 19F) (Fig. 2D and E). Deletion of *copA* also severely attenuated the development of acute otitis media and sinusitis (strain 19F) (Fig. 3). Finally, the Δ*copA* mutant remained significantly attenuated during bloodstream infection (TIGR4) (Fig. 5). This is consistent with previous data demonstrating that the *cop* operon is required at the mucosal surface and with previously reported transposon-based screens (4, 7, 31). We observed more robust attenuation in the Δ*copA* mutant in TIGR4 and 19F strains than has been observed previously in the

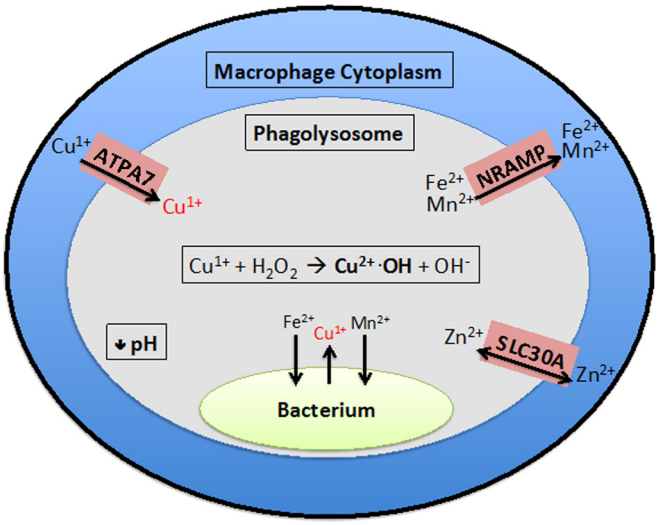

FIG 7 Model of macrophage-mediated bacterial killing. Model represents macrophage killing of bacteria via the phagolysosome using metal sequestration and intoxication.

D39 strain (4). These data implicate the CopA exporter as an integral aspect of pneumococcal pathogenesis in the mammalian host.

As the concentrations of copper in host tissues, which range from 6 to 11 μM in resting state and during infection (6), are not sufficient to explain the observed attenuation of the Δ*copA* mutant, we sought microenvironments that could have sufficiently high copper concentrations to facilitate clearance of the Δ*copA* mutant. As macrophages are known to utilize copper as one bactericidal mechanism, we hypothesized that part of the observed attenuation could be explained by increased susceptibility to macrophage-mediated clearance (28). This notion was supported by the results of our *in vitro* macrophage-mediated killing assays, where deletion of *copA* rendered the pneumococci more sensitive to clearance despite equivalent uptake by the immune cells. This was also modeled *in vivo*, where macrophage-depleted lungs restored the capacity of the *copA* mutant to replicate to wild-type levels of bacterial burden. These data implicate a role for copper efflux in resisting macrophage-induced copper toxicity and are summarized in Fig. 7. It should be noted that additional mechanisms of killing are likely playing accompanying roles in the observed phenotypes. For example, copper deficiency renders neutrophils less effective at microbial killing, though the precise mechanism has yet to be elucidated (46–48). Loss of copper efflux may also make the pneumococcus more sensitive to host sequestration of other essential transition metals, such as manganese or zinc, as the relative levels of transition metals are increasingly being recognized as an important aspect of metal homeostasis. Hence, the observed phenotypes very likely result from a combination of factors that perturb metal ion homeostasis.

Of note was the observation that *in vitro* copper sensitivity did not correlate with virulence potential, as the Δ*copA* and Δ*cupA* mutants displayed similar levels of sensitivity to copper stress *in vitro* but had differing degrees of virulence. The Δ*cupA* mutant had no discernible virulence defect based on both survival of infected mice and their bacterial burdens, while the Δ*copA* mutant was severely attenuated (Fig. 1 and 2). The CupA protein is annotated as a putative cupredoxin that chaperones copper to the CopA efflux pump, as has been elegantly described at the structural level (25). One potential explanation for this discrepancy is that deletion of *copA* results in overexpression of both the CopY repressor and the CupA chaperone in the absence of the cognate exporter (25). Thus, the increase in the levels of CupA could potentially be detrimental when the efflux pump is absent. Another possibility is that in the absence of CopA, the high overexpression of CupA facilitates the transfer of copper to inappropriate cellular targets.

Taken together, the observed attenuation of the Δ*copA* mutant is likely due to a combination of copper toxicity effects, consequences of incorrect expression of the remaining genes in this operon when *copA* is absent, inability to adhere to host cells, and inability to resist macrophage-mediated clearance. The significant increase in the capacity of the Δ*cupA* mutant to adhere to and invade lung epithelial cells compared to the adherence and invasion capacity of the Δ*copA* mutant is perhaps the most indicative reason why the Δ*cupA* mutant's overall virulence is greater than that of the Δ*copA* mutant, despite the two having equal susceptibilities to copper stress *in vitro* (Fig. 5). It should be noted that the decreased invasion could also represent impaired intracellular survival of the mutants. Based on both *in vivo* and *in vitro* models, the Δ*copA* mutant was seemingly unable to translocate through the lung epithelium and replicate in the bloodstream, despite high titers of bacteria in the lungs of clodronate-treated mice (Fig. 6). These data are likely the result of a combination of decreased translocation and enhanced bacterial clearance in the bloodstream, where monocytes continued to circulate. These data indicate that copper efflux is an essential aspect of pneumococcal biology during interactions at the mucosal surface and invasive infection. While these data indicate that evasion of copper-mediated toxicity is a critical aspect of pneumococcal pathogenesis, the precise mechanism of this toxicity remains unclear. In other bacterial species, intracellular accumulation of copper disrupts iron-sulfur clusters as one mechanism to mediate bactericidal activity (49). This mechanism of toxicity may not be universally conserved among bacterial pathogens, particularly in the pneumococcus, which does not contain a significant amount of iron-sulfur cluster-containing proteins (50). As maintaining appropriate intracellular ratios of transition metals is a vital aspect of bacterial physiology, one potential mechanism of copper-induced toxicity is incorrect metalation of an essential protein. Together, these data highlight the importance of copper homeostasis in multiple host niches and in response to innate immune clearance mechanisms.

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