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RESEARCH ARTICLE

Cytoplasmic Copper Detoxification in *Salmonella* Can Contribute to SodC Metalation but Is Dispensable during Systemic Infection

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ABSTRACT *Salmonella enterica* serovar Typhimurium is a leading cause of foodborne disease worldwide. Severe infections result from the ability of *S.* Typhimurium to survive within host immune cells, despite being exposed to various host antimicrobial factors. SodCI, a copper-zinc-cofactored superoxide dismutase, is required to defend against phagocytic superoxide. SodCII, an additional periplasmic superoxide dismutase, although produced during infection, does not function in the host. Previous studies suggested that CueP, a periplasmic copper binding protein, facilitates acquisition of copper by SodCII. CopA and GolT, both inner membrane ATPases that pump copper from the cytoplasm to the periplasm, are a source of copper for CueP. Using *in vitro* SOD assays, we found that SodCI can also utilize CueP to acquire copper. However, both SodCI and SodCII have a significant fraction of activity independent of CueP and cytoplasmic copper export. We utilized a series of mouse competition assays to address the *in vivo* role of CueP-mediated SodC activation. A *copA golT cueP* triple mutant was equally as competitive as the wild type, suggesting that sufficient SodCI is active to defend against phagocytic superoxide independent of CueP and cytoplasmic copper export. We also confirmed that a strain containing a modified SodCII, which is capable of complementing a *sodCI* deletion, was fully virulent in a *copA golT cueP* background competed against the wild type. These competitions also address the potential impact of cytoplasmic copper toxicity within the phagosome. Our data suggest that *Salmonella* does not encounter inhibitory concentrations of copper during systemic infection.

IMPORTANCE *Salmonella* is a leading cause of gastrointestinal disease worldwide. In severe cases, *Salmonella* can cause life-threatening systemic infections, particularly in very young children, the elderly, or people who are immunocompromised. To cause disease, *Salmonella* must survive the hostile environment inside host immune cells, a location in which most bacteria are killed. Our work examines how one particular metal, copper, is acquired by *Salmonella* to activate a protein important for survival within immune cells. At high levels, copper itself can inhibit *Salmonella*. Using a strain of *Salmonella* that cannot detoxify intracellular copper, we also addressed the *in vivo* role of copper as an antimicrobial agent.

KEYWORDS copper efflux, *Salmonella*, SodC

Salmonella enterica serovar Typhimurium is a leading cause of bacterial foodborne illness and is capable of causing life-threatening systemic disease (1). During extraintestinal infection, *Salmonella* resides inside host macrophages in a modified phagosome called the *Salmonella*-containing vacuole (SCV) (2–4). SodCI is important for *Salmonella* survival during systemic infection. Located in the periplasm, SodCI specifically defends against extracellular superoxide generated by the oxidative burst in macrophages (5). In the absence of SodCI, *S.* Typhimurium is approximately 10-fold

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attenuated (5–7). SodCI converts two molecules of superoxide into hydrogen peroxide and water, with the reaction being catalyzed by a copper atom in the active site; a zinc atom is also present for structural purposes (8). SodCI is exported to the periplasm via the Sec pathway, where the protein then folds and acquires metal cofactors (6).

Salmonella expresses a second Cu/Zn periplasmic superoxide dismutase (SOD), SodCII. Despite these superoxide dismutases being 60% identical at the amino acid level, our lab and others have previously shown that *Salmonella* strains lacking SodCII are fully virulent (6, 9–12). Two important properties that affect the contributions of SodCI and SodCII to virulence were identified. First, SodCI noncovalently binds peptidoglycan, whereas SodCII is released into the milieu upon partial disruption of the outer membrane, such as that caused by antimicrobial peptides (13). Second, SodCI is protease resistant, whereas SodCII is protease sensitive; proteases are an additional defense mechanism utilized by phagocytes. Modifying SodCII to remain tethered in the periplasm or protease resistant allows the enzyme to actively defend against superoxide during infection (13–15). SodCI has a higher affinity for its metal cofactors than SodCII (16). Importantly, this difference in metal affinity does not influence the differential function of SodCI and SodCII *in vivo* (6, 14, 16).

Copper is an essential transition metal used in a variety of biochemical reactions, including electron transfer, oxidation-reduction reactions, and free-radical scavenging (17). However, accumulation of intracellular copper is toxic. This creates a delicate balance between acquiring sufficient copper and avoiding deleterious side effects (18). ATP7A and ATP7B are human P_{1B}-type ATPases that transport copper across membranes throughout the body. Humans with mutations in ATP7A or ATP7B have Menkes' and Wilson's disease, respectively, both of which are debilitating genetic disorders caused by inability to appropriately traffic copper (19–21). Bacteria produce homologous P-type ATPases, which are necessary to prevent the accumulation of cytoplasmic copper (22). Based on *in vitro* experiments and studies in mammalian cells, it was originally hypothesized that cytoplasmic copper toxicity resulted from DNA damage mediated by Fenton-like reactions of Cu^{1+} with hydrogen peroxide, generating highly reactive hydroxyl radicals (23–25). Macomber et al. (26) demonstrated that copper does not catalyze formation of oxidative DNA damage in the cytoplasm but actually protects against iron-mediated Fenton reactions by competing with iron for binding. They went on to identify certain dehydratases as the primary cytoplasmic target of copper toxicity in *Escherichia coli* (26). The partially exposed nature of the iron-sulfur cluster in this enzyme family allows interaction with copper, which can displace iron, disrupting activity. The susceptible class of enzymes includes dihydroxy-acid dehydratase and isopropylmalate isomerase, which are involved in branched-chain amino acid synthesis, fumarase A, and 6-phosphogluconate dehydratase (27).

Copper toxicity necessitates that bacteria contain cytoplasmic copper detoxification systems. In *Salmonella*, two transcriptional regulators, CueR and GolS, detect intracellular copper. CueR regulates *copA*, *cueO*, and *cueP* (28–30). CopA is an inner membrane P-type ATPase capable of pumping copper from the cytoplasm into the periplasm. CueP is a periplasmic copper binding protein, and CueO is a periplasmic multi-copper-ion oxidase. GolS positively regulates *golT* and *golB* (31, 32). GolT is also a P-type ATPase, and GolB is a cytoplasmic copper binding protein (Fig. 1). CopA and GolT are functionally redundant in export of copper from the cytoplasm; single mutations in *copA* or *golT* do not confer significant growth defects in copper-supplemented medium *in vitro*, where a *copA golT* double mutant is growth inhibited (32). The periplasmic copper binding protein CueP binds copper exported by either CopA or GolT. Periplasmic copper can also be oxidized to Cu^{2+} by CueO, potentially hindering copper from crossing the inner membrane and/or preventing Cu^{+1} from reacting with hydrogen peroxide to generate hydroxyl radicals (33, 34). Previous reports suggest that a *Salmonella cueO* mutant is attenuated for survival in the spleen and liver after oral infection, based on reduced CFU recovery relative to that for the wild type (29). We have previously reported that a *cueO* mutant is not significantly attenuated in an intraperitoneal (i.p.) competition assay with the wild type (35).

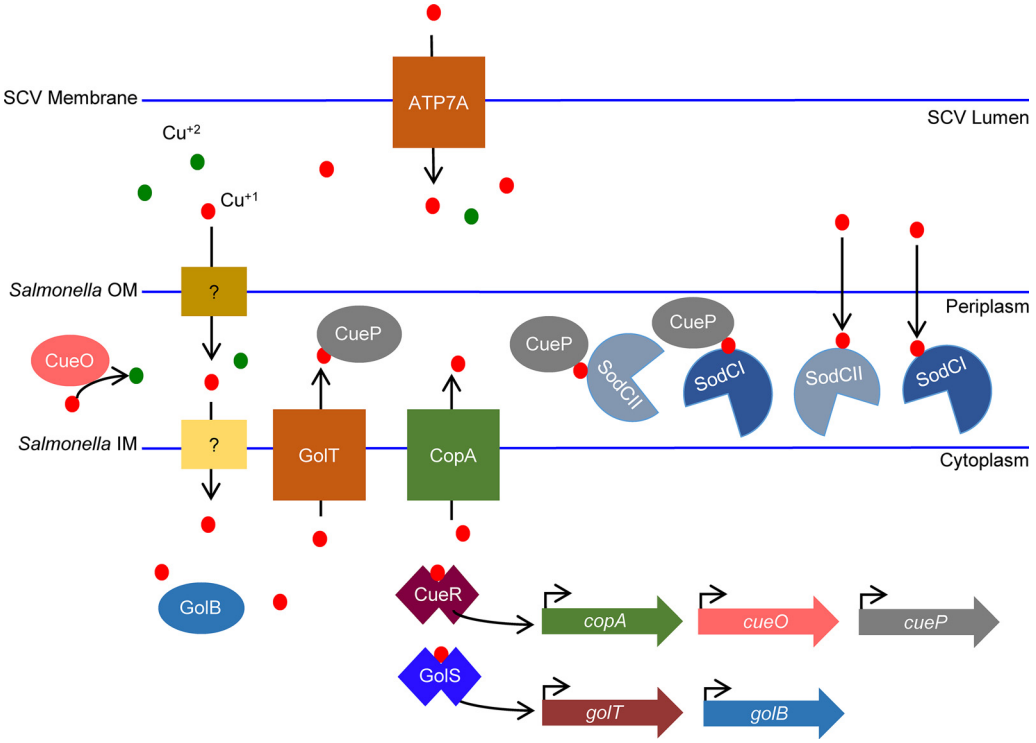

FIG 1 Schematic of copper trafficking in *S.* Typhimurium. Copper gains entry to the periplasm via nonspecific porins or transporters. In the periplasm, Cu^{1+} can be oxidized by CueO to Cu^{2+}. Cu^{1+} can cross the inner membrane via unspecified mechanisms. CueR and GolS are transcriptional regulators that bind Cu and activate target gene expression. CopA and GolT are inner membrane P-type ATPases that pump copper back to the periplasm, where it can be bound by CueP. CueP can deliver copper to SodCI and SodCII. SodCI and SodCII are also able to acquire copper via CueP-independent mechanisms. During infection, phagocytes upregulate expression of a copper-specific transporter, ATP7A, that potentially pumps copper into the phagosome.

The fate of periplasmic copper in *S.* Typhimurium, after being bound by CueP, is unclear. In addition to copper binding, it was suggested that under high copper concentrations, CueP confers additional protection to the cell by reducing Cu^{2+} to Cu^{0}, presumably blocking copper-mediated Fenton reactions in the periplasm (36). DsbC and DsbG are periplasmic disulfide bond isomerases (37). DsbC has been shown to be required to protect the periplasm from copper-mediated nonnative disulfide bond formation in *E. coli* (38). In addition, work in *Salmonella* suggests that DsbC contributes to maintaining CueP in a reduced state (39). Osman et al. have reported that, *in vitro*, CueP facilitates copper acquisition by SodCII in the periplasm (40). In the absence of CueP or both CopA and GolT, SodCII was produced but not completely metalated. The requirement of CopA or GolT implies that the copper source for CueP originates from the cytoplasm. Due to limitations in the experimental setup, SodCI was not tested.

During systemic infection, phagocytes alter normal copper trafficking and increase expression of transporters that import copper into the phagosome (41). Due to the antimicrobial properties of copper, it is appealing to presume that the host has evolved to use copper as an additional defense mechanism. One specific copper transporter, ATP7A, is actively recruited to phagosomes in response to bacterial infections. Macrophages with a mutation in this transporter have increased susceptibility to infection by *E. coli* (41). Recently, Ladomersky et al. (42) provided data supporting a role for ATP7A in resistance to *Salmonella* infection by increasing the concentration of copper in the *Salmonella*-containing vacuole (SCV). The overall impact of increased copper concentrations *in vivo* along with the role of cytoplasmic copper export in the ability of SodCI and SodCII to acquire copper in the SCV is unclear.

In this study, we first addressed the contribution of CueP to metalation of SodCI *in vitro*. Although more significant for SodCII, our data demonstrate that CueP and

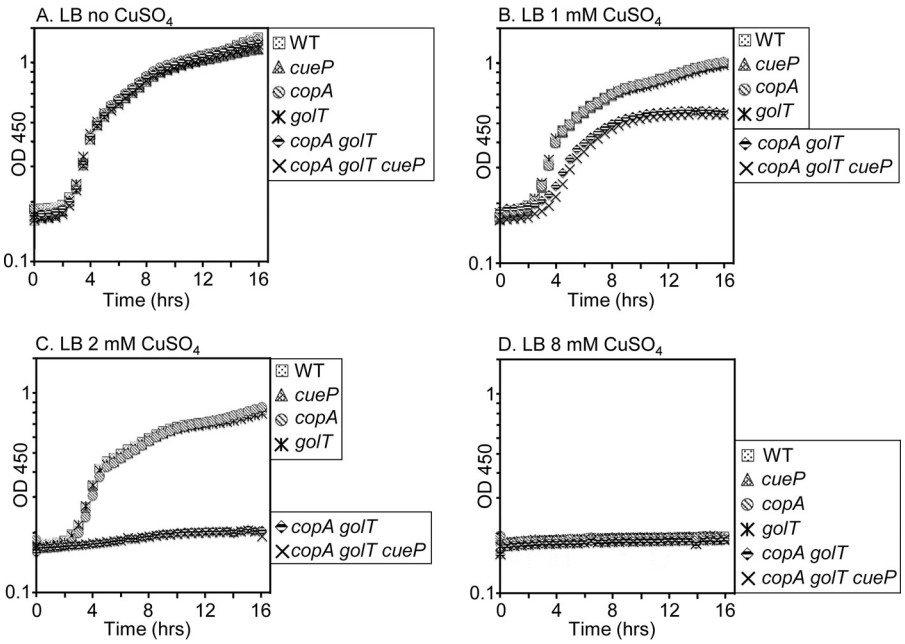

FIG 2 Growth curves of the wild type (WT) or mutants grown in increasing concentrations of copper sulfate ($CuSO_4$). Overnight LB cultures were diluted to an OD of 0.01 in LB with 0.2% glucose and grown highly aerated at 37°C until reaching an OD of 0.2. Each strain was then inoculated 1:50 into a 96-well plate containing a total of 250 μl LB with the indicated concentration of $CuSO_4$. The OD_{450} at 30-min intervals is plotted. The data are representative of three independent experiments. The strains used were 14028, JS2089, JS2087, JS2086, JS2091, and JS2092.

cytoplasmic copper export can also contribute to activation of SodCI. However, both enzymes can acquire copper independent of the copper efflux systems. We then moved to an *in vivo* infection model to determine if cytoplasmic copper detoxification was important for *Salmonella* survival during systemic infection and if SodCI or SodCII requires CueP-mediated activation to be fully virulent. SodCI and SodCII are sufficiently active independent of CueP or CopA and GolT. Moreover, our data suggest that *S.* Typhimurium does not experience significant cytoplasmic copper stress in the *Salmonella*-containing vacuole.

RESULTS

CopA or GolT is required to protect the cytoplasm from copper stress. To test the requirement for CopA, GolT, and CueP in copper metalation of SodCI and SodCII, we first generated gene deletions of *copA*, *golT*, and *cueP* (43) and then constructed a *copA golT* double mutant and a *copA golT cueP* triple mutant. To confirm that the *copA* and *golT* mutants were defective in copper export, growth assays were performed under increasing concentrations of copper sulfate ($CuSO_4$); accumulation of copper in the cytoplasm is expected to inhibit growth (27). Comparing the optical density at 450 nm during overnight growth in rich medium (lysogeny broth [LB]) without added $CuSO_4$, all mutants grew at similar rates and reached comparable endpoint optical densities (ODs) (Fig. 2A). With the addition of 1 mM $CuSO_4$, the *copA golT* double mutant and *copA golT cueP* triple mutant demonstrated a growth defect, reaching a final OD approximately 50% lower than that of any single mutant or the wild type (Fig. 2B). At 2 mM $CuSO_4$, the *copA golT* and *copA golT cueP* mutants did not grow (Fig. 2C); increasing the concentration to 8 mM $CuSO_4$ inhibited growth of all strains (Fig. 2D). This is in agreement with previous reports that *copA* or *golT* export systems are redundant in *Salmonella* (32). Metabolites in rich media are able to bind and sequester copper away from cells. We repeated the growth assay in M9 minimal medium and showed that even at 0.5 μM $CuSO_4$, a *copA golT* mutant exhibited a growth defect (Fig. 3B). Interestingly, at 1 μM $CuSO_4$, the *copA* and *golT* single mutants appear to have a

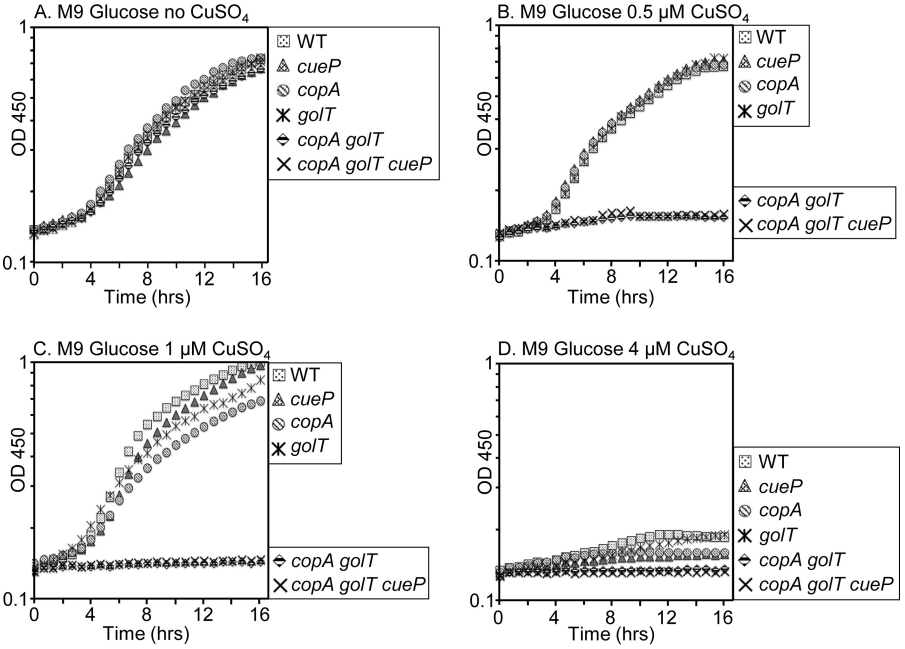

FIG 3 Growth curves of the wild type or mutants grown in increasing concentrations of copper sulfate. Strains were grown overnight in LB, washed with M9 glucose minimal medium, diluted to an OD of 0.01 in M9 glucose, and grown highly aerated at 37°C until reaching an OD of 0.2. Each strain was then inoculated 1:50 into a 96-well plate containing a total of 250 μl M9 glucose with the indicated concentration of $CuSO_4$. The OD_{450} at 30-min intervals is plotted. The data are representative of three independent experiments. The strains used were 14028, JS2089, JS2087, JS2086, JS2091, and JS2092.

slight growth defect compared to the *cueP* mutant and the wild type, whereas the double and triple mutants do not grow (Fig. 3C). At 4 μM $CuSO_4$ all six strains were severely inhibited for growth (Fig. 3D). A *cueP* mutant in these assays has no detectable phenotype, implying that the growth defect is due to cytoplasmic accumulation of copper. These experiments demonstrate that either CopA or GolT is sufficient to protect the cytoplasm from copper stress. At increasing concentrations of copper, 8 mM in rich medium and 4 μM in minimal medium, even wild-type *Salmonella* demonstrates a significant growth defect.

Cytoplasmic copper detoxification and CueP can contribute to metalation of SodCI and SodCII. Next we addressed the impact of the cytoplasmic copper detoxification system on the ability of SodCI and SodCII to acquire copper as a cofactor. Both enzymes are exported to the periplasm as unfolded polypeptides via the Sec pathway (11, 44). Presumably, both enzymes then acquire copper and zinc cofactors within the periplasm. Since copper is required for SodC activity, we can use enzyme activity as a proxy for metalation status. By adding exogenous copper, we can activate any apoenzyme to determine the total amount of enzyme present in each sample (6, 14). If CueP and CopA or GolT contribute to metalation of the SodC enzymes, we would expect that in the absence of these proteins, a reduced fraction of the total enzyme would be active before remetalation. We used the xanthine oxidase and cytochrome *c* method to assay superoxide dismutase activity (45).

To simplify the experiment, the SOD assays were done in a strain deleted for the two cytoplasmic superoxide dismutase genes, *sodA* and *sodB*, along with both *sodCI* and *sodCII*. The SOD gene of interest was then cloned into the expression vector pWSK29 (46) and transformed into the SOD-null strain, resulting in an ~10-fold increase in SodC activity (44). Overexpression of SOD should exacerbate the potential need for CueP and cytoplasmic copper export to metalate the enzyme, while also facilitating the detection of differences in SOD activity.

We first attempted to confirm previously published results suggesting that CueP contributes to SodCII activity by functioning as a copper chaperone (40). SodCII was

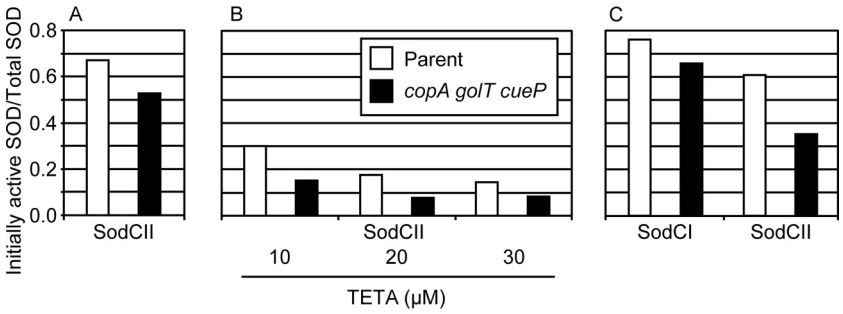

FIG 4 CueP can contribute to activation of SodCI and SodCII. SOD assays were performed using the xanthine oxidase-cytochrome *c* method. Cultures were grown overnight in 10 ml LB with TETA when indicated. Strains were devoid of all native SOD and expressed only the listed SodC from a pWSK29 vector. The osmotic shockates (A and B) or whole-cell extracts (C) were remetalated with 45 μM $CuSO_4$. Data shown are the initial SOD activity divided by the SOD activity after remetalation and are representative of three independent experiments. The strains used were JS2093 and JS2094 containing either pMR101 or pMR102.

isolated by osmotic shock in these experiments (6, 47). Osmotic shock partially disrupts the outer membrane, resulting in release of SodCII and other periplasmic proteins. Initially, we compared the activity of SodCII in a wild-type background to that of SodCII in a *copA golT cueP* triple mutant background. Data are presented as the fraction of active SOD in any given extract out of the total amount of SOD, as determined by remetalating any apoenzyme by addition of exogenous copper. Periplasmic extracts from the control strain, containing an empty vector, did not show any detectable SOD activity in a wild-type or triple mutant background (data not shown).

The data presented in Fig. 4A are representative of three independent experiments. Consistent with previous data (6, 14), only a fraction of SodCII is metalated. This fraction of active SodCII enzyme was consistently reduced in a triple mutant background compared to in the background with the export system intact. While it is clear from our data that cytoplasmic copper export and CueP can contribute to activation of SodCII, we did note variability in both the fraction of SodCII that was initially active and the relative contribution of the copper export pathway to activation of the enzyme. The SodCII activity was reduced 20 to 50% in the export mutant background compared to the parent strain background across all experiments. To try to further exacerbate the phenotype, we added triethylenetetramine (TETA), a copper-specific chelator, to the growth medium. We anticipated that the contribution of CueP-mediated copper delivery to SodCII might increase as the concentration of available copper became more limited. We compared the fractions of initial SodCII activity in medium containing increasing concentrations of TETA (Fig. 4B). As the concentration of TETA increased, the initial fractions of active SOD in the wild-type and triple mutant backgrounds both decreased. Surprisingly, the chelator did not enhance the importance of CueP, CopA, and GolT. In each case, the mutant background had approximately half the activity of the wild-type background.

Determining the metalation status of SodCI was more challenging because SodCI is not released by osmotic shock but remains tethered within the periplasm (10, 11). To assay SodCI, we used glass beads to mechanically disrupt cells to obtain whole-cell extracts. As a control to ensure that this method of isolation does not alter the metalation status, we also assayed SodCII using the same lysis protocol. In whole-cell extracts, we again saw that SodCII showed a reduced fraction of initial activity in a *copA golT cueP* mutant background compared to the wild-type background (Fig. 4C). Similar to our results for SodCII, SodCI was generally less active in a *copA golT cueP* background than in the wild-type background. A higher percentage of SodCI than of SodCII is initially active in both the wild-type and the triple mutant backgrounds, consistent with previous data demonstrating that SodCI has higher affinity for metal cofactors than SodCII (6, 14, 16). Overall, these data suggest that under certain conditions, the copper

TABLE 1 Competition assays of copper-trafficking mutants

Relevant genotype of *S.* Typhimurium strain[a]:		Mouse strain[b]	Median CI	No. of mice	*P* value[c]	Fold attenuation[d]
A	B					
Δ*cueP*	WT	BALB/c	1.16	4	NS	—
Δ*copA* Δ*golT*	WT	BALB/c	1.00	8	NS	—
Δ*cueP* Δ*copA* Δ*golT*	WT	BALB/c	3.14	4	NS	—
Δ*cueP* Δ*copA* Δ*golT*	WT	C3H	2.40	6	NS	—
Δ*cueP* Δ*copA* Δ*golT*	WT	C57	4.30	5	NS	—

[a]The strains used were 14028, JS2089, JS2090, and JS2092.
[b]Mouse genetic background: BALB/c, BALB/cAnNHsd; C3H, C3H/HeNHsd; C57, C57BL/6J.
[c]By Student's *t* test comparing CI versus inoculum. NS, not significant.
[d]Reciprocal of median CI, if significant. —, CI not significant.

detoxification pathway can also contribute to activation of SodCI. However, it is apparent that SodCI and SodCII do not absolutely require CueP and cytoplasmic copper export for activation; we can activate the enzyme simply by adding copper to an extract. In addition, strains lacking *copA*, *golT*, and *cueP* still have significant fractions of SodCI and SodCII that are active, although we did consistently observe a decrease in activity in the absence of the copper systems.

CopA, GolT, and CueP are not required for SodCI or SodCII activity *in vivo*. The results above suggest that, while not absolutely required, CueP can facilitate metalation of SodCI. We wanted to investigate if this supply pathway was important for SodCI activity during systemic infection. Previous work in our lab and others studying SodCI and SodCII found that only SodCI is important for *Salmonella* survival during systemic infection (6, 12). To determine the effect of *copA*, *golT*, or *cueP* mutations *in vivo*, we turned to the competition assay, a powerful tool to compare virulence of two strains. A *sodCI* mutant is 7- to 10-fold attenuated in a competition assay with the wild type (5). Mice were infected by the intraperitoneal route, bypassing intestinal invasion and directly testing the ability of each strain to survive systemically. Competition assays were done in 6- to 8-week-old BALB/c mice using an inoculum of ~500 bacteria mixed in a 1:1 ratio.

If CueP-mediated SodCI activation is important for defense against phagocytic superoxide, we would expect mutants with mutations in *cueP* or *copA* and *golT* to be attenuated relative to the wild type in a competition assay. This set of competition assays also allowed us to address whether an inhibitory amount of copper stress exists in the *Salmonella*-containing vacuole. Neither a *cueP* mutant nor a *copA golT* double mutant had any virulence defect compared to the wild type (Table 1). These results suggest that a sufficient amount of SodCI is able to obtain copper during systemic infection independent of the cytoplasmic copper detoxification pathway and CueP. To further probe the system, we tested the triple mutant against the wild type. Similarly to the single and double mutants, the triple mutant competed equally with the wild type.

Different mouse genotypes are known to have different susceptibilities to *Salmonella* infection (48). We repeated the competition with the *copA golT cueP* triple mutant versus the wild type in C3H (NRAMP1/Slc11a1 wild-type) and C57BL/6J mice. The results were similar to those of the BALB/c competition; the triple mutant was equally virulent as the wild type. From these data, we conclude that SodCI is able to acquire sufficient copper to defend against the oxidative burst independent of CueP, CopA, and GolT. The *in vitro* growth assays demonstrate that a *copA golT* mutant has a growth defect relative to the wild type in the presence of 1 mM copper in rich medium and 0.5 μM in minimal glucose medium. Based on these results, we can conclude that *Salmonella* does not encounter high enough concentrations of copper in the SCV for a *copA golT* mutant to be attenuated.

To more directly test if SodCI enzymatic activity is reduced in the absence of CueP and cytoplasmic copper export, we determined the phenotype of a *sodCI copA golT*

TABLE 2 Competition assays of Δ*sodCI* in copper-trafficking mutant backgrounds

Relevant genotype of *S.* Typhimurium strain[a]:		Median	No. of	*P*	Fold
A	B	CI	mice	value[b]	attenuation[c]
Δ*sodCI* Δ*cueP*	Δ*cueP*	0.17	7	0.001	6.0
Δ*sodCI* Δ*copA* Δ*golT*	Δ*copA* Δ*golT*	0.23	4	0.034	4.4
Δ*sodCI* Δ*copA* Δ*golT* Δ*cueP*	Δ*copA* Δ*golT* Δ*cueP*	0.13[d]	7	0.005	7.7
Δ*sodCI*	WT	0.15[d]	4	<0.005	6.7

[a]The strains used were 14028, JS2096, JS192, JS2095, JS2091, JS2097, and JS2092.
[b]By Student's *t* test comparing CI versus inoculum.
[c]Reciprocal of median CI, if significant.
[d]Not significantly different from one another; $P = 0.47$.

cueP strain competed against the *copA golT cueP* parent strain. The *sodCI copA golT cueP* strain was 8-fold attenuated, indistinguishable from the phenotype in a wild-type background (Table 2), showing that the enzyme has acquired sufficient copper.

The data above suggest that *Salmonella* has sufficient SodCI activity during systemic infection independent of cytoplasmic copper export. SodCI is reported to have higher affinity for metal cofactors than SodCII (Fig. 4C) (5, 10, 13). We have previously generated a hybrid SodCII protein that is protease resistant but still released by osmotic shock. Protease-resistant SodCII can complement SodCI *in vivo* yet has metal affinities similar to those of wild-type SodCII (14). Starting with a strain background that has deletions of *sodCI* and *sodCII*, the protease-resistant *sodCII* hybrid gene was integrated into the *sodCII* locus as described previously (14). The strain expressing the protease-resistant SodCII was approximately 5-fold more virulent than the *sodCI sodCII* double mutant strain, confirming the ability of this SodC to defend against phagocytic superoxide. A *cueP* mutant in the protease-resistant SodCII background was equally competitive in a competition assay against the SodCII protease-resistant strain (Table 3). This confirms that during systemic infection, CueP is not required to activate sufficient protease-resistant SodCII to functionally defend against phagocytic superoxide.

We can also use the competition assay to address the claim that CueP can directly scavenge superoxide (36). If CueP alone can protect against superoxide during infection, we would expect a *cueP sodC* strain to be attenuated relative to a *sodC* strain. A *cueP* mutant in a *sodCI sodCII* background was equally virulent as the parent strain (Table 3). This suggests that CueP does not have a direct role in protecting *Salmonella* against phagocytic superoxide. If copper is causing periplasmic stress due to inappropriate disulfide bond formation (38), we would expect a *dsbC dsbG* double mutant to be attenuated relative to the wild type. We find that the double mutant competes equally with the wild type (Table 3). Moreover, if *dsbC* were required to maintain CueP activity, which is required to activate SodC, we would expect a *sodCI* mutant in a *dsbC dsbG* background to compete equally with a *dsbC dsbG* strain. However, we see that a *sodCI dsbC dsbG* strain is 7-fold attenuated relative to the parent, the same phenotype we see when SodCI is fully functional (Table 3).

TABLE 3 Competition assays addressing the importance of CueP in defense against oxidative stress

Relevant genotype of *S.* Typhimurium strain[a]:		Median	No. of		Fold
A	B	CI	mice	*P* value[b]	attenuation[c]
Δ*sodCI* Δ*sodCII*	*sodCIIres*$^+$ Δ*sodCI* Δ*sodCII*	0.21	3	0.0085	4.8
Δ*cueP sodCIIres*$^+$	*sodCIIres*$^+$	0.86	3	NS	—
Δ*cueP* Δ*sodCI* Δ*sodCII*	Δ*sodCI* Δ*sodCII*	0.64	5	NS	—
Δ*dsbC* Δ*dsbG*	WT	1.60	5	NS	—
Δ*sodCI* Δ*dsbC* Δ*dsbG*	Δ*dsbC* Δ*dsbG*	0.14	5	<0.0005	7.1

[a]The strains used were 14028, JS2100, JS1176, JS2101, JS2102, JS2098, and JS2099.
[b]By Student's *t* test comparing CI versus inoculum; NS, not significant.
[c]Reciprocal of median CI, if significant. —, CI not significant.

DISCUSSION

We addressed the role of CueP and cytoplasmic copper export in *Salmonella* survival during systemic infection. First, we asked if cytoplasmic copper export and CueP-mediated copper delivery are required to activate sufficient SodC to defend *Salmonella* against the oxidative burst. Second, we determined if *Salmonella* experiences sufficient copper stress in the phagosome to cause growth inhibition and affect virulence.

Our *in vitro* data suggest that the copper detoxification pathway in *Salmonella* can contribute to metalation of both SodCI and SodCII. The phenotype is apparent only under conditions when copper is limited in the medium. If we grow the cultures with addition of exogenous copper, we no longer see any effect of CueP in SodC activation (data not shown). The ability to activate the enzyme in cell extracts simply by providing exogenous copper, even in strains lacking *cueP*, confirms that CueP-mediated delivery is not absolutely required for activation.

Our competition assay results show that sufficient SodC is active to defend *Salmonella* against phagocytic superoxide in strains lacking *copA*, *golT*, and *cueP*. This result is consistent across multiple mouse genotypes. Based on previous work, we can infer that far more SodC is produced than is necessary to defend *Salmonella* against superoxide generated within the phagosome (14). This implies that even a reduced fraction of active SodC in a *copA golT cueP* mutant background can be sufficient to protect *Salmonella* against phagocytic superoxide. Our assays are not sensitive enough to determine if the fraction of active SodC is also reduced *in vivo* in a *copA golT cueP* mutant. Our data also do not support CueP having any significant contribution to direct scavenging of reactive oxygen species (36). A *cueP* mutant competes equally with the wild type in a competition assay. In agreement, we do not see a phenotype for a *dsbC* mutant, and DsbC is reportedly necessary to keep CueP in a reduced state (39).

Copper has long been used as an antimicrobial agent (49), and many bacterial pathogens can be inhibited *in vitro* by addition of exogenous copper. The immune system has apparently evolved to take advantage of the toxic effects of copper. Notably, the ATP7A copper transporter is trafficked to the phagosome in *E. coli*-infected macrophages, increasing the concentration of copper in the vacuole (41, 42). Thus, bacterial copper efflux systems are often important for pathogenesis. For examples, in *Neisseria gonorrhoeae*, a *copA* mutation decreases intracellular survival in human cervical epithelial cells (50). *Streptococcus pneumoniae copA* mutants have decreased virulence in pulmonary, intravenous, and intraperitoneal infection models (51), while a copper efflux mutant of *Mycobacterium tuberculosis* shows decreased survival in guinea pigs (52).

Results on the role of copper in *Salmonella* Typhimurium virulence have been somewhat variable. Previous experiments in *Salmonella* Typhimurium have identified a phenotype for a *copA golT* double mutant in RAW264.7 cells (32). Similarly, Ladomersky et al. (42) reported that activated mouse peritoneal macrophages can kill *S.* Typhimurium and that this killing activity is partially dependent on ATP7A. Moreover, a *copA golT* mutant was more sensitive to macrophage killing, and this increased sensitivity was also dependent on ATP7A (42). In their study, a *copA golT* mutant of *S.* Typhimurium strain SL1344 was ~2-fold attenuated in an i.p. competition assay in C57BL/6J mice but equally virulent in mice lacking ATP7A in myeloid cells. In contrast, Osman et al. (32) reported no difference in bacterial CFU between SL1344 and the *copA golT* mutant in tissues of orally infected C57BL/6J mice. We also observed no phenotype conferred by deletion of *copA*, *golT*, and *cueP* in *S.* Typhimurium strain 14028 in i.p. competition assays in BALB/c, C57BL/6J, or C3H/HeN mice.

Our data suggest that the level of copper exposure in the SCV does not exceed amounts necessary to cause significant cytoplasmic stress. However, copper could still be playing an important role during *Salmonella* infection, potentially explaining the subtle differences observed in the various studies described above. There could be differences in the relative role of copper in different immune cells. For example, previous work found that neutrophils and inflammatory monocytes are able to kill

TABLE 4 Bacterial strains and plasmids

Strain or plasmid	Genotype[a]	Deletion endpoints (bp)[b]	Source or reference[c]
Strain			
14028	Wild type		ATCC
JS192	Δ*sodCI-1*::Aph		9
JS1176	Δ*sodCII*::*sodCII*$_{1-133}$ *sodCI*$_{136-157}$ (*sodCIIres*) Δ*sodCI-108*::Cm		14
JS2085	Δ*dsbC105*::Kn	3222825–3223540	
JS2086	Δ*golT1*::Kn	399253–401541	
JS2087	Δ*copA1*::Cm	558639–561140	
JS2088	Δ*dsbG106*::Cm	669962–670696	
JS2089	Δ*cueP2*::Cm	3850516–3851055	
JS2090	Δ*golT1*::Kn Δ*copA1*::Cm		
JS2091	Δ*golT1* Δ*copA1*		
JS2092	Δ*golT1* Δ*copA1* Δ*cueP2*::Cm		
JS2093	Δ*sodA101* Δ*sodB102* Δ*sodCI-105* Δ*sodCII-103*		
JS2094	Δ*sodA101* Δ*sodB102* Δ*sodCI-105* Δ*sodCII-103* Δ*cueP2* Δ*golT1* Δ*copA1*::Kn		
JS2095	Δ*golT1* Δ*copA1* Δ*sodCI-105*::Cm		
JS2096	Δ*cueP2*::Cm Δ*sodCI-1*::Aph		
JS2097	Δ*golT1* Δ*copA1* Δ*cueP2*::Cm Δ*sodCI-1*::Aph		
JS2098	Δ*dsbC105* Δ*dsbG106*::Cm		
JS2099	Δ*dsbC105* Δ*dsbG106*::Cm Δ*sodCI-1*::Aph		
JS2100	Δ*sodCI-105* Δ*sodCII-103*		
JS2101	Δ*cueP2*::Kn Δ*sodCII*::*sodCII*$_{1-133}$ *sodCI*$_{136-157}$ (*sodCIIres*) Δ*sodCI-108*::Cm		
JS2102	Δ*sodCI-105* Δ*sodCII-103* Δ*cueP2*::Cm		
Plasmids			
pMR101	pWSK29::*sodCI*$^+$		
pMR102	pWSK29::*sodCII*$^+$		

[a]All *Salmonella* strains are isogenic derivatives of *S. enterica* serovar Typhimurium strain 14028. Subscript numbers in hybrid genes indicate the amino acids of the mature protein encoded.
[b]Base pairs that are deleted (inclusive), defined by the *S.* Typhimurium 14028 genome sequence (NC_016856.1).
[c]The source is this study unless otherwise specified. ATCC, American Type Culture Collection.

Salmonella in vivo and that within different subsets of macrophages, survival can be variable (53). The *Salmonella* organisms that we recovered from infected mice are from those cells in which *Salmonella* survived. Our data suggest that in this case, copper toxicity does not significantly impact the results. However, in cells where *Salmonella* is effectively controlled, copper may have a more significant contribution. This could explain the phenotypes observed in *in vitro* activated mouse-derived macrophages, which are efficient at killing *Salmonella*. Also, the Atp7a^{lysMcre} mouse model created by Ladomersky et al. (42) is missing ATP7A in all macrophages and monocytes, impacting a simple interpretation of the results.

In those macrophages where *Salmonella* does survive, alterations in vesicular trafficking caused by the SPI2 type three secretion system (TTSS) effectors could be reducing delivery of the ATP7A copper transporter to the SCV, analogous to the role of the TTSS effectors in altering delivery of reactive oxygen or nitrogen species to the SCV (54, 55). Whereas ATP7A colocalizes with *E. coli*-containing phagosomes (41, 42), infection of bone marrow-derived macrophages with *Salmonella* resulted in formation of distinct copper-containing intracellular vesicles, which were dependent on copper uptake by the cell (56). However, these vesicles were distinct from the SCV or lysosomes. Perhaps a more detailed analysis of specific types of immune cells or infection models will further clarify the role of copper in *Salmonella* infection.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains and plasmids are listed in Table 4. All *Salmonella* strains used in this study are derived from *Salmonella enterica* serovar Typhimurium strain 14028 (American Type Culture Collection). Lambda Red-mediated recombination was used for allelic replacement of genes with antibiotic resistance cassettes (57). Primers for lambda Red and any PCRs were purchased from IDT Inc. Endpoints for each deletion are listed in Table 4. The resulting constructs were moved into an otherwise wild-type background via P22 HT105/1 *int*-201-mediated transduction (58) and confirmed via PCR. The temperature-sensitive plasmid pCP20 carrying FLP recombinase (59) was used to remove antibiotic resistance cassettes containing FLP recombination target (FRT) sites when necessary.

Growth conditions. Bacteria were grown in lysogeny broth (LB) broth at 37°C with shaking for 16 to 18 h, with the exception of strains containing pCP20 or pKD46, which were grown at 30°C. As necessary, antibiotics were used at the following concentration: chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; and ampicillin, 50 μg/ml. For growth curves, 2-ml overnight cultures were grown in LB, diluted to an OD of 0.01 in 3 ml LB with 0.2% glucose or M9 minimal medium with 0.4% glucose, and grown with shaking at 37°C in a 50-ml baffled flask until reaching an OD of 0.2. Each strain was inoculated 1:50 into a 96-well plate containing 250 μl LB (no glucose) or M9 glucose with various concentrations of $CuSO_4$. the OD at 450 nm (OD_{450}) was recorded every 15 min for 16 h using an ELx808IU absorbance reader (BioTek), with the temperature set to 37°C and a 15-s mixing prior to each OD_{450} reading.

SOD assays. Biochemical analysis of SOD enzymes was done using periplasmic extracts or whole-cell lysates. Periplasmic extracts were obtained by osmotic shock (14). To ensure that the measured SOD activity was specific to the SOD of interest, strains devoid of all four native SODs (SodA, SodB, SodCI, and SodCII) were transformed with pWKS29-derived plasmids encoding the specific SOD of interest or an empty vector. To study the impact of cytoplasmic copper export in this strain background, *copA*, *golT*, and *cueP* deletions were individually introduced via P22 transduction. Overnight 10-ml cultures were grown in LB with ampicillin. The copper-specific chelator triethylenetetramine (TETA) was added as indicated. Cultures were harvested via centrifugation at 4°C and then washed three times in an equal volume of cold 50 mM potassium phosphate buffer (pH 7.4). After the third wash, cells were resuspended in 5 ml of plasmolysis buffer (50 mM Tris, 2.5 mM EDTA, 20% [wt/vol] sucrose, pH 7.4) and allowed to sit at room temperature for 15 min before centrifugation for 5 min at 7,800 rpm. Cells were resuspended in 2.5 ml cold deionized water. After a 15-min incubation on ice, cells were recentrifuged, and the supernatant collected was considered the osmotic shockate. For experiments requiring whole-cell extracts, overnight cultures were washed twice in 50 mM potassium phosphate buffer (pH 7.4) and resuspended in 600 μl of the same buffer. Approximately 200 μl of glass disruptor beads (0.1 mm; USA Scientific) was added to the cells, and the mixture was placed in a Digital Disruptor Genie at 4°C. Cells were agitated at 3,000 rpm for 2 min, followed by a 2-min rest on ice. The cycle was repeated 3 additional times. The lysate was then centrifuged for 15 min at 4°C and 14,000 rpm. The supernatant was collected, transferred to a new 1.5-ml microcentrifuge, tube and spun a second time at 14,000 rpm. The resulting supernatant was considered the whole-cell extract.

Superoxide dismutase activity was determined using the xanthine oxidase-cytochrome *c* method (45). The protein concentration for osmotic shock or whole-cell lysates was determined using a Bradford assay (Bio-Rad). To determine the total amount of SOD present in a given extract, 45 μM copper sulfate was added to 100 μl of the initial extract and allowed to gently mix at room temperature for 30 min. The amount of SOD activity after remetalation was considered the total SOD activity. Dividing the initial activity by the total activity gives the initial fraction of active SOD. We previously determined that addition of copper sulfate to extracts does not affect the SOD assay, and thus we did not require dialysis to remove excess copper.

Competition assays. All animal work was reviewed and approved by the University of Illinois IACUC and performed under protocol 15214. Competition experiments were done in female 6- to 8-week-old mice. BALB/cAnNHsd and C3H/HeNHsd mice were purchased from Envigo (formerly Harlan), and C57BL/6J mice were purchased from The Jackson Laboratory. Strains for each competition were grown overnight in LB (16 h), mixed together in a 1:1 ratio, and then diluted to a target inoculum of ~500 CFU in 200 μl sterile 0.15 M NaCl. Infections were done via the intraperitoneal route. An aliquot of each inoculum was spread on LB agar to enumerate the total inoculum and replica plated to the appropriate selective medium to calculate the input ratio for each competition. At 4 to 5 days postinfection, animals were sacrificed, and their spleens were removed and homogenized. Dilutions of spleen homogenate were plated on LB and replica plated to appropriate selective medium to calculate the output ratio for each competition. The competitive index (CI) was calculated as (percent strain A recovered/percent strain B recovered)/(percent strain A inoculated/percent strain B inoculated). Statistical comparison of individual competitions was done using the Student *t* test.

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