Interaction of Copper Toxicity and Oxidative Stress in *Campylobacter jejuni*

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ABSTRACT Copper is both a required micronutrient and a source of toxicity in most organisms, including *Campylobacter jejuni*. Two proteins expressed in *C. jejuni* (termed CopA and CueO) have been shown to be a copper transporter and multicopper oxidase, respectively. We have isolated strains with mutations in these genes, and here we report that they were more susceptible to both the addition of copper in the growth media and to induced oxidative stress. Both mutant strains were defective in colonization of an avian host, and copper in the feed exacerbated the colonization deficiency. Overexpression of a cytoplasmic peptide derived from the normally periplasmic copper-binding region of CueO also caused copper intolerance compared to nonexpressing strains or strains expressing the non-copper-binding versions of the peptide. Taken together, the results indicate that copper toxicity in *C. jejuni* is due to a failure to effectively sequester cytoplasmic copper, resulting in an increase in copper-mediated oxidative damage.

IMPORTANCE Copper is a required micronutrient for most aerobic organisms, but it is universally toxic at elevated levels. These organisms use homeostatic mechanisms that allow for cells to acquire enough of the element to sustain metabolic requirements while ensuring that lethal levels cannot build up in the cell. *Campylobacter jejuni* is an important foodborne pathogen that typically makes its way into the food chain through contaminated poultry. *C. jejuni* has a metabolic requirement for copper and encodes a copper detoxification system. In the course of studying this system, we have learned that it is important for avian colonization. We have also gained insight into how copper exerts its toxic effects in *C. jejuni* by promoting oxidative stress.

KEYWORDS *Campylobacter*, copper

Copper is a required micronutrient for most aerobic organisms, in which it mainly participates in oxygen activation and electron transfer. In biological systems, copper typically exists in two oxidation states (Cu^+ and Cu^{2+}), and the facile cycling between the two makes it an excellent cofactor for enzymes that participate in oxidation and reduction of oxygen. Although copper has important physiological roles in the cell, copper in excess is toxic. Given the dual nature of copper requirements and toxicity, microorganisms have developed homeostasis mechanisms to provide the proper balance of this important micronutrient. These mechanisms have been best studied in Gram-negative bacteria, which use a coordinately regulated *cue* (*Cu* efflux) detoxification system (1–4). The main players of the *cue* detoxification system in *Escherichia coli* are *copA* and *cueO*, which encode a complementary system that keeps levels of copper low in the cytoplasm while oxidizing Cu^+ in the periplasm to the less toxic Cu^{2+} . CopA is a Cu^+ efflux P-type ATPase that transports cytosolic Cu^+ into the periplasm (5). CopA expression is turned on under micromolar copper concentrations (6). Therefore, basal cytosolic copper levels are kept quite low (4). CueO is a periplasmic multicopper oxidase, an enzyme that couples the oxidation of Cu^+ with the four-

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electron reduction of O_2 to water (7, 8). CueO itself is also a copper-containing protein, having 4 structural copper atoms identified as type 1 (T1), type 2 (T2), and 2 type 3 (T3) atoms. Up to 3 atoms are associated with a methionine-rich region that covers the active site (9). An amino acid substitution in one of the T1 copper-binding ligands in *E. coli* deactivates the enzyme (7). The synergistic effect of CopA and CueO together is capable of moderating the potential for copper to exert its toxic effects.

Campylobacter is responsible for the majority of foodborne gastroenteritis cases worldwide, and infection is often attributed to the consumption or handling of raw or undercooked poultry. Most *Campylobacter* infections are self-limiting; however, campylobacteriosis is an antecedent to Guillain-Barré syndrome and postinfectious irritable bowel syndrome (PI-IBS) (10–12). The genome of *Campylobacter jejuni* NCTC 11168 contains genes encoding 2 proteins annotated as copper enzymes, a *ccb*₃-type cytochrome *c* oxidoreductase (Cj1490c) and a copper-containing multicopper oxidase (Cj1516). Copper is also required for the full activity of the periplasmic iron transporter accessory protein P19 (Cj1658) (13). Two proteins in NCTC 11168 have been characterized as being involved in copper homeostasis: a multicopper oxidase termed CueO and a P_{1B-1} -ATPase transporter termed CopA. Mutations in either *cueO* or *copA* resulted in strains sensitive to added copper (14). Purified CueO was determined to be a cuprous oxidase, and growth was inhibited by added copper to both *cueO* and *copA* mutants (14). The *C. jejuni* multicopper oxidase crystal structure contains four copper atoms (15), however, isolated CueO is reported to contain 6.4 atoms per peptide (14), suggesting that the *Campylobacter* CueO may bind nonstructural copper atoms similarly to *E. coli*.

Despite the unequivocal toxicity of excess copper, the mechanism of copper-mediated cellular damage is still not entirely known. Relative to other physiologically occurring metals, copper binds especially well to amine, thiolate and carboxyl ligands present in proteins, effectively inactivating them (16–18). Copper also displaces iron atoms in iron-sulfur clusters of intracellular enzymes forming coordination compounds with sulfur or thiolate ligands (18, 19). This not only destroys the enzymatic function of the mismetallated protein, it also releases iron, which can cause oxidative damage through iron-based Fenton chemistry (20, 21). In a process comparable to the Fe^{2+} -catalyzed Fenton reaction, Cu^+ can react with H_2O_2 produced via oxygen metabolism to generate the hydroxyl radical ($HO^\bullet$) (22).

In this study, we show that two *C. jejuni* copper homeostasis genes, *copA* and *cueO*, are essential for copper resistance. Mutation in either gene results in a deficiency in avian host colonization. In our exploration into the mechanism of the toxic effect of copper in *C. jejuni*, we find that susceptibility to oxidative stress is a by-product of disruption of copper homeostasis. Mutations in *copA* and *cueO* result in cells that are more sensitive to methyl viologen challenge than the wild type parent, and copper preloading of cells exacerbates this sensitivity. Addition of the membrane permeable chelator 2,2'-dipyridyl, which protects cells from Fe^{2+} -catalyzed reactive oxygen species (ROS), renders copper-loaded *C. jejuni* cells more sensitive to challenge with H_2O_2 . Finally, by using expression plasmids, we are also able to demonstrate copper toxicity with a 78-aa peptide fragment containing the T1 copper-binding site derived from CueO. The results reported here suggest that the increased sensitivity to ROS is due to the interaction of ROS with copper, and that the mechanism *C. jejuni* uses to protect itself from copper toxicity is different than that in model systems such as *E. coli*.

RESULTS

Copper resistance in *C. jejuni* strains. Five wild-type strains of *C. jejuni* were initially tested for sensitivity to copper, and two strains of *C. jejuni* were chosen to further characterize the copper detoxification systems (Table 1). NCTC 11168 had the lowest level of resistance to copper (MIC = 1.67 mM $CuSO_4$) (Table 2) and was chosen because it was used in the initial characterization of CopA and CueO, while strain ATCC 35925 had the highest level of resistance to copper (MIC = 4.64 mM $CuSO_4$) (Table 2), and it proved to be readily transformable with *E. coli*-*C. jejuni* shuttle vectors (23). Insertion mutants were isolated for *cueO* and *copA* in both backgrounds (Table 1), as

TABLE 1 Strains used in this study

Species and strain	Description ^a	Source ^b
<i>C. jejuni</i>		
RM 1221	Type strain	D. Threadgill (41)
81-176	Type strain	Alain Stintzi (42)
ATCC 35918	Type strain	ATCC
NCTC 11168	Type strain	NCTC
SG1	<i>cat</i> inserted within <i>cueO</i> in NCTC 11168	This study
SG2	<i>cat</i> inserted within <i>copA</i> in NCTC 11168	This study
ATCC 35925	Type strain	ATCC
SG3	<i>cat</i> inserted within <i>cueO</i> in ATCC 35925	This study
SG9	<i>cat</i> inserted within Δ <i>cueO</i> in ATCC 35925	This study
SG4	<i>cat</i> inserted within <i>copA</i> in ATCC 35925	This study
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44</i> λ^- <i>thi-1 gyrA96 relA1</i>	Invitrogen
GC10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) F80 ϕ <i>lacZ</i> Δ M15 Δ <i>lacX74 endA1 recA1</i> Δ (<i>ara, leu</i>)7697 <i>araD139 galU galK nupG rpsL</i> λ^- T1R	Sigma

^aIncluding antibiotic cassette (if present).^bATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures.

well as for a 1,104-bp deletion in *cueO* in the ATCC 35925 background (SG9) (Table 1; see also Fig. S1A, C, and D in the supplemental material).

Disruption of *cueO* or *copA* resulted in greater sensitivity to copper (lower MIC) than wild-type parental strains. In addition to MIC tests, growth curves were performed to determine the effect of copper on the final density of cultures and calculate the effect of copper on growth rate. All strains exhibit sensitivity to copper, as shown by an increase in generation time in response to increasing copper concentrations (see Table S1 in the supplemental material). Additionally, even at levels below the MIC, the mutant strains, with the exception of SG3, had lower growth rates (Table S1).

Complementation and expression plasmids. Five expression plasmids were constructed for this study. p*OcopA* and p*OcueO* express full-length *copA* and *cueO* genes, respectively. The other plasmids express three versions of the 78 C-terminal amino acids of CueO, namely, the native sequence and two versions in which predicted T1 copper-binding ligands have been changed by site-directed mutagenesis (pO78_{C495A} and pO78_{C495H500A}) (Fig. 1; see also Table S2 in the supplemental material). These truncated versions were made to mimic the remaining portion of the *cueO* reading frame that is transcribed in the SG9 mutant (see Fig. S1C and D in the supplemental material). All expression plasmids use the *ompE* promoter for expression to ensure transcription of the cloned genes (24). A heterologous promoter was used because the native promoter regions are likely distant from the open reading frame. Quantitative

TABLE 2 Copper MICs of strains

<i>C. jejuni</i> strain	Copper MIC ^a of strain with:					
	Plasmid					
	No plasmid	p <i>OcopA</i>	p <i>OcueO</i>	pO78	pO78 _{C495A}	pO78 _{C495H500A}
RM1221	1.71 $\pm$ 0.37					
ATCC 35918	2.29 $\pm$ 0.10					
81-176	3.60 $\pm$ 0.22					
NCTC 11168	1.67 $\pm$ 0.25					
SG2	1.10 $\pm$ 0.30					
SG1	0.55 $\pm$ 0.34					
ATCC 35925	4.64 $\pm$ 0.47	5.00 $\pm$ 0.10	4.90 $\pm$ 0.20	2.69 $\pm$ 0.55	4.81 $\pm$ 0.37	4.83 $\pm$ 0.14
SG4	0.42 $\pm$ 0.14	4.93 $\pm$ 0.12				
SG3	1.70 $\pm$ 0.23		5.50 $\pm$ 0.0			
SG9	1.29 $\pm$ 0.18		3.46 $\pm$ 0.36			

^aMICs expressed as mM CuSO₄ $\pm$ the standard deviation.

FIG 1 Graphical representation (not to scale) of the C-terminal region of *cueO* that contains the T1 copper-binding residues. (A) The 78-aa version of *cueO*. (B) The single mutant 78_{C495A} was generated by substituting the codon corresponding to alanine for the codon corresponding to cysteine at position 495. (C) The double mutant 78_{C495H500A} was generated by substituting the codon corresponding to alanine for the codon corresponding to histidine at position 500.

reverse transcriptase PCR (qRT-PCR) confirmed that *copA* is overexpressed by ~50-fold over the that of the wild type in the strain harboring pO*copA*, and *cueO* is overexpressed by ~6.5-fold over that of the wild type in the strain harboring pO*cueO* (see Fig. S2A and B in the supplemental material). qRT-PCR of *cueO* downstream of the *cat* cassette suggests that the insertion does not disrupt downstream transcription (Fig. S2B), indicating that the remaining portion of *cueO* and the downstream genes are still transcribed.

CueO mutants are dominant negative in some assays due to expression of a C-terminal peptide fragment. pO*cueO* failed to fully complement the *cueO* mutants, including the SG9 mutant, in certain assays for both MIC of copper (Table 2) and for sensitivity to 2,2'-dipyridyl (Fig. 2). Quantitative reverse transcriptase-PCR determined that downstream transcription occurs downstream of the cassette (Fig. S2B), so we set out to determine if the defects are due to spurious translation of a downstream fragment of CueO. SG9 contains a 1,105-nucleotide (nt) deletion (from nt 160 to 1264) of *cueO*. Downstream (45 bp) of the *cat* cassette in SG9 is an in-frame methionine that, if used as a start codon, would produce a 78-amino acid (aa) C-terminal peptide of CueO that would remain cytosolic due the absence of a secretion sequence (Fig. 1A; see

FIG 2 Copper-loaded cultures of SG3, complemented SG3, and ATCC 35925 carrying plasmid pO78 are sensitive to 2,2'-dipyridyl, even in the absence of added ROS. Substituting the copper-binding ligands in pO78 relieves the 2,2'-dipyridyl sensitivity. Symbols represent log reduction values after a 1-h exposure to 0.1 mM 2,2'-dipyridyl. Bars indicate the mean values. ****, $P < 0.0001$.

also Fig. S1D). To test if this peptide could be responsible for the lack of complementation (a dominant negative phenotype), we created the plasmid pO78 to express this peptide from the naturally occurring methionine residue 437 (Fig. 1A). This truncated version includes the copper-binding ligands (H₄₃₉, C₄₉₅, and H₅₀₀) that make up the T1 copper site (15). pO78 carriage recapitulated the phenotype seen in SG9, lowering the MIC of copper to 2.59 mM versus 4.64 mM in ATCC 35925 that does not contain the plasmid (Table 2). Versions of the peptide in which the predicted copper-binding ligands are mutated abrogate the phenotype (Table 2; Fig. 2); pO78_{C495A} and pO78_{C495H500A} carriage had no effect on MIC (Table 2). The copper-binding ligands (Fig. 1) and by extension, copper binding, contribute to the sensitivity seen in these strains.

The combination of copper and 2,2'-dipyridyl is toxic to strains expressing cytoplasmic versions of CueO. Both SG3 and SG9 mutants experienced high levels of lethality during preincubation with 2,2'-dipyridyl, which interfered with our ability to test for H₂O₂ sensitivity. This phenotype was not seen in other strains, which revealed no loss of viability during preincubation. In order to investigate this further, we grew strains (with or without copper preincubation) that were then exposed to 2,2'-dipyridyl for 1 h and serially diluted to determine viability loss (Fig. 2). This phenotype is also dominant negative, as the complemented strain (SG3 carrying pOcueO) shows a similar log reduction (Fig. 2). Preloading the cells with copper is critical for 2,2'-dipyridyl toxicity, as cells that were not preexposed to copper had no loss of viability after the 2,2'-dipyridyl treatment (data not shown). ATCC 35925 cells carrying plasmid pO78 were significantly more sensitive to 2,2'-dipyridyl and copper than were wild-type cells alone (Fig. 2). Expression of the peptide containing the site-directed substitution of an alanine for the C₄₉₅ residue (or a combination of alanine-substituted C₄₉₅ and H₅₀₀) resulted in cells that were significantly less sensitive to 2,2'-dipyridyl and copper (Fig. 2).

The *copA* and *cueO* mutants are sensitive to oxidative stress. In order to investigate whether *copA* and *cueO* mutants are more sensitive to oxidative stress, we performed cell killing assays (expressed as log₁₀ CFU reduction) of cultures exposed to the ROS-producing compounds methyl viologen and H₂O₂. Methyl viologen is a cell-permeable redox-active dye that uses cellular reducing potential to catalyze a single-electron reduction of O₂ to produce superoxide (25). NCTC 11168 and its derived strains were grown in the presence of 0.125 mM CuSO₄ to mid-log phase and challenged with 0.5 mM methyl viologen for 1 h, and the remaining number of CFU were determined. The copper-treated SG2 mutant was significantly more sensitive to methyl viologen than was the wild type (see Fig. S3 in the supplemental material). ATCC 35925 strains are less sensitive to methyl viologen, so these strains were grown similarly but challenged with 5.0 mM methyl viologen. Both mutants were significantly more sensitive to methyl viologen in this background than was the wild type (Fig. S3). Cell killing by H₂O₂ was tested similarly. Cells were grown with or without 0.125 mM CuSO₄ to mid-log phase (optical density at 600 nm [OD₆₀₀], ~0.3) and treated with the chelator 2,2'-dipyridyl for 30 min, and all cells were then challenged with 0.1 mM H₂O₂ for 1 h. Pretreatment with 2,2'-dipyridyl was required to mitigate iron-mediated Fenton-based hydroxyl radical formation, as copper can potentially displace iron from iron-sulfur clusters (26). After 1 h, the cells were serially diluted and plated onto blood agar. CFU per milliliter calculated for all strains. The number of viable cells counted from mutant strains treated with both copper and 2,2'-dipyridyl before exposure to peroxide were reduced by 2 to 4 logs compared to that of the wild type; none of the strains were killed by H₂O₂ in the absence of added copper (Fig. 3A). SG4 was also 2 logs more sensitive to peroxide than either its parent or the complemented *copA* mutant, SG4 carrying pOcopA (Fig. 3B). Peroxide log₁₀ reduction values in SG4 ranged from 1.4 to 5, which likely stems from a Fenton chemistry catalytic cascade that would be set in motion when dying cells release Cu⁺ to interact with H₂O₂ to produce more hydroxyl radical. No data are presented for SG3, as the combination of 2,2'-dipyridyl and copper

FIG 3 *copA* and *cueO* mutants are sensitive to oxidative stress induced by the addition of H₂O₂. Log reduction values after 1 h treatment with 0.1 mM H₂O₂ for NCTC 11168 cells preloaded by growth overnight with 0.125 mM CuSO₄, where indicated (A), and ATCC 35925 cells (B). Overexpression of *copA* and complementation of SG4 results in wild-type resistance to H₂O₂. The bar represents the mean values. **, $P < 0.01$; ****, $P < 0.0001$.

alone for 1 h resulted in a severe reduction of viability, leaving too few cells to accurately measure H₂O₂-mediated killing (Fig. 2).

The *copA* and *cueO* mutants are deficient in host colonization. *Campylobacter* colonizes the epithelial mucosa of the gastrointestinal tract, particularly the cecum. Chickens that have been experimentally challenged have cecal contents that contain up to 10⁹ CFU/ml *Campylobacter* (27). In order to determine the ability of the mutants to colonize the gut based on the effect of copper present in the chicken gastrointestinal tract, colonization studies were carried out as previously described (28). Day-of-hatch chicks were separated into groups of 10 in Petersime brooder batteries and were fed either commercial chicken feed (containing 8 mg Cu/kg) or the same feed diet supplemented with 180 mg CuSO₄/kg. At 1 week, the chicks were inoculated with 10⁷ *C. jejuni* cells in 1 ml phosphate-buffered saline (PBS) by oral gavage. Three weeks postinoculation, the chickens were sacrificed by CO₂ asphyxiation, and the cecal contents were collected for enumeration of viable CFU per gram. The *copA* mutant colonized the cecum at significantly lower levels than did wild-type cells (Fig. 4). No *Campylobacter* was found in three of the chickens inoculated with the *cueO* mutant and fed the high-copper diet. The *cueO* mutant colonized at significantly lower levels than

FIG 4 Host colonization. In a 3-week trial, chickens experimentally gavaged with 10⁷ CFU/ml of SG1 were significantly less colonized than those with wild-type cells, especially when the feed was supplemented with CuSO₄. SG2 also colonized less than did the wild type, but the defect was less severe. Symbols represent CFU per gram recovered from the cecal samples of individual birds. Also shown are the median values and the detection limit for the assay. **, $P < 0.01$.

did wild-type cells in the presence of copper ($P < 0.01$) and without copper added to the feed (Fig. 4).

DISCUSSION

Failure to strictly regulate the concentration and oxidation state of cell-associated copper results in severe consequences (2, 29, 30), yet the cellular targets and mode of action of copper toxicity are still unresolved. Copper-mediated ROS production is implicated in damage to eukaryotic cells and tissues, as both DNA damage (29) and lipid oxidation is found within copper-induced liver tumors (31). In *E. coli* cells, however, the presence of excess copper is protective against ROS, and it was concluded that copper accumulation does not promote Fenton-like chemistry (22). In the course of characterizing copper tolerance in *C. jejuni*, we have discovered that this species is an ideal model for testing the direct effects of copper-mediated damage, as confounding protective effects are minimal in the presence of added copper.

Our survey of wild-type *C. jejuni* strains shows that most have MICs of copper that range from 1.67 to 4.64 mM CuSO_4 (Table 2). We chose to further examine the most resistant of the 5 tested strains (ATCC 35925), as well as the least resistant (NCTC 11168), which was the strain in which the copper detoxification genes *cueO* and *copA* were originally characterized. The genome of ATCC 35925 was sequenced and is reported elsewhere (23). The ATCC 35925 genome does not contain any obvious copper resistance genes in addition to those found in NCTC 11168, but it may be more resistant to copper-mediated ROS due to the presence of a gamma-glutamyl transpeptidase (B5D75_RS00190) and a thioredoxin (B5D75_RS00180) not found in NCTC 11168.

Both copper homeostasis genes *cueO* and *copA* were confirmed to be required for copper resistance. MIC values confirm a marked decline in resistance to copper in both strains for both mutations, with a modest increase in resistance when either is over-expressed (Table 2). In addition to MIC, we assayed the effect of sublethal concentrations of CuSO_4 on growth. Copper supplementation had a small, negative effect on generation time for all strains, especially for the mutants (see Table S1 in the supplemental material).

The importance of copper resistance in host colonization was confirmed. Chickens have a high tolerance for excess copper in feed and are often fed diets with high copper levels (100 to 200 mg/kg diet), both as a growth promoter and to prevent enteritis (32). Both the *copA* and *cueO* mutants displayed significant colonization defects compared to the wild-type strain. The defect was especially dramatic with the *cueO* mutant under the high-copper (188 mg CuSO_4 /kg diet) condition, as we were unable to recover any *C. jejuni* cells from 3 of the chickens in the group gavaged with the *cueO* mutant.

To determine if copper played a role in exacerbating oxidative stress, cells were exposed to either H_2O_2 or the superoxide-generating dye methyl viologen. Cell killing assays were performed in which the rate of cell death was monitored in response to a lethal concentration of compound. Mutant cells grown in copper-supplemented conditions were more sensitive to methyl viologen; those grown without copper did not show increased killing (Fig. S3). The sensitive phenotype of each mutant is rescued by complementation of both genes in the ATCC 35925 background. H_2O_2 is converted to the powerful oxidant hydroxyl radical, which can be catalyzed by either Fe^{2+} or Cu^+ (33, 34). In these assays, pretreating the cells with the iron chelator 2,2'-dipyridyl was used to differentiate Cu^+ - versus Fe^{2+} -catalyzed ROS. Although 2,2'-dipyridyl can bind both metals, 2,2'-dipyridyl prevents iron-mediated $\text{HO}^\bullet$ production (35), yet 2,2'-dipyridyl-copper complexes still allow for Cu^+ -catalyzed H_2O_2 reduction (22). Both mutants were significantly more sensitive to H_2O_2 than were the parents, but only under the copper-loaded condition.

We found that the combination of copper and 2,2'-dipyridyl is toxic to some *cueO* mutants, even in the absence of added ROS. The phenotype is dominant negative, as complementation with intact *cueO* does not restore the parent phenotype. In these mutants, the twin-arginine translocation (TAT) secretion signal is deleted and the

orientation of the insertion cassette allows for transcription of the 3' (downstream) portion of the gene (Fig. S2). Using expression plasmids, we were able to mimic the 2,2'-dipyridyl-copper toxicity with peptide fragments as small as 78 aa (Fig. 2), and this peptide contains all of the ligands for the T1 copper atom seen in the crystal structure of CueO (15) (Fig. 1B). Substitution of the copper-binding ligand C₄₉₅ residue with an alanine restored the wild-type phenotype, providing compelling evidence that a copper atom coordinated in the T1 copper-binding site is required for the toxicity (Fig. 2).

Overall, this work demonstrates that in *C. jejuni*, disruption of the cell's copper homeostatic mechanisms increases susceptibility to ROS. This is in contrast to work done in *E. coli*, in which copper treatment renders cells more resistant to ROS (18). Copper toxicity was suggested to result mainly from the free iron release via degradation of FeS-containing enzymes. In contrast, several lines of evidence suggest that in *C. jejuni*, copper participates directly in ROS production. First, addition of the iron chelator 2,2'-dipyridyl, which prevents iron-mediated Fenton chemistry, does not prevent copper-augmented cell killing by H₂O₂ (Fig. 3). Second, even in the absence of imposed ROS, 2,2'-dipyridyl and copper have a significant killing effect on the *cueO* mutants (Fig. 2). Lastly, we have shown that in the wild-type background, expression of a 78-aa peptide predicted to bind copper is lethal in the presence of the chelator (Fig. 2). The mechanism of toxicity is likely attributed to the disruption of cellular copper homeostatic mechanisms due to its copper-binding properties. Alterations of the predicted copper-binding ligands result in a form of the peptide that is no longer detrimental to the cell (Fig. 2), confirming that copper binding is crucial for the phenotype.

The cytoplasmic pathways for copper accumulation, sequestration, and ultimate mobilization into the copper-containing enzymes are not fully known. Disruption of these pathways has obvious negative consequences for the cell through the creation of ROS. Here, we show this can be achieved by either mutation of copper homeostasis proteins or by the expression of a cytoplasmic copper-binding peptide by introduction of the plasmid pO78. The exact mechanism of how this peptide exerts toxicity is yet to be determined, but the copper-binding ligands (and by extension copper) are critical. We believe these serendipitous findings will become an important tool for identifying and characterizing copper metabolic pathways and for studying the basis of copper-mediated lethality.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. jejuni* strains are listed in Table 1. Mueller-Hinton agar (MHA) (Difco, Sparks, MD) supplemented with 5% defibrinated sheep blood (Hemostat Laboratories, Dixon, CA) was used for growth of *C. jejuni*. *C. jejuni* strains were cultured microaerobically at 37°C in a trigas incubator (model 550D; Fisher Scientific, Hampton, NH), and maintained with 12% O₂, 5% CO₂, and 83% N₂. Liquid cultures were grown in Mueller-Hinton broth (MHB) (Difco, Sparks, MD). Growth medium was supplemented with chloramphenicol (12.5 µg/ml) and tetracycline (20 µg/ml) as indicated and incubated microaerobically at 37°C. Genetic manipulations were performed using the *E. coli* strain DH5α (Stratagene California, La Jolla, CA) or *E. coli* GC10 (Sigma-Aldrich, St. Louis, MO). Luria-Bertani broth and agar were supplemented with ampicillin (150 µg/ml), chloramphenicol (20 µg/ml), and tetracycline (20 µg/ml) as indicated.

Cloning and construction of *C. jejuni* mutants. Oligonucleotide primers (see Table S3 in the supplemental material) were designed by the DNA analysis software MacVector using the sequenced NCTC 11168 (36). PCR amplifications were performed with EconoTaq Plus 2X master mix (Lucigen Corporation, Middleton, WI), using genomic DNA as the template. The purified PCR products were cloned into pCR2.1-TOPOvector (Invitrogen, Waltham, MA), yielding pTOPO*copA* and pTOPO*cueO* (Table S2). The cloned fragments from both plasmids were then excised by digestion with EcoRI and ligated to EcoRI-digested pKSDH3 to yield pK*ScopA* and pK*ScueO* (Table S2). These plasmids were then digested with HindIII and treated with T4 DNA polymerase and deoxynucleoside triphosphates (dNTPs) to blunt the ends. They were then ligated to the chloramphenicol acetyltransferase gene (*cat*) (37), which was isolated from pJMA-001 by SmaI digestion (38). The resulting plasmids were termed pK*ScopA::cat* and pK*ScueO::cat* (Table S2). SG9 (*ΔcueO_{35925::cat}*) was similarly generated, using genomic DNA isolated from NCTC 11168. The purified PCR product was cloned into pCR2.1-TOPOvector (Invitrogen, Waltham, MA), yielding pTOPO*cueOC* (Table S2). The cloned fragment was excised by digestion with BamHI and XhoI and ligated into a BamHI- and XhoI-digested pKS to yield pK*ScueOC* (Table S2). The plasmid was then digested with HindIII and treated with T4 DNA polymerase and dNTPs to blunt the ends, then ligated to the *cat* cassette, resulting in pK*ScueOC::cat* (Table S2).

C. jejuni was transformed by electroporation, as previously described (39). After 16 h of nonselective incubation the cells were transferred by loop onto the appropriate selective antibiotic plate, and colonies appeared 2 days later (39, 40). Resistant colonies were screened by PCR-agarose gel electrophoresis, confirming that allelic replacement had occurred.

Construction of plasmids for overexpression and complementation of genes. Coding regions of *copA* and *cueO* were PCR amplified from NCTC 11168 genomic DNA using the primers CopA3 and CopA4 for *copA* and CueO3 and CueO4 for *cueO* (Table S3). In a separate reaction, the *ompE* promoter was amplified using pMEK91 (24) as a template and the primers OmpEF and OmpER (Table S3). OmpER was designed to have an 11-bp overlap with both the CopA3 and CueO3 primers, which allowed for an overlapping PCR. The purified *copA* or *cueO* amplicons were then combined separately with the purified *ompE* promoter PCR product and amplified in a second reaction, using the forward *ompE* primer and the CopA4 or CueO4 reverse primers to generate 2 gene fusion products, *ompEcopA* and *ompEcueO*. The gene fusion products were subsequently cloned into EcoRI-digested pMEK91, generating the shuttle vectors pOcopA and pOcueO (Table S2 and Fig. S1B and E). The plasmids were confirmed as correct by restriction analysis and were introduced into ATCC 35925, SG4 (*copA*_{35925::cat}), and SG3 (*cueO*_{35925::cat}) via electroporation, using the same conditions as for mutagenesis. Transformed cells were spotted onto blood agar plates containing 20 μ g/ml tetracycline. Tet^r colonies were passed and cultured under selection. The plasmids were isolated from the cultures by plasmid miniprep kit (Qiagen) and confirmed to contain *copA* or *cueO* via PCR amplification using the OmpEF and either CopA4 or CueO4 reverse primers.

Construction of plasmids for overexpression of *cueO78*. The coding region corresponding to the last 78 aa of *cueO* was PCR amplified from NCTC 11168 genomic DNA using the forward primer, CueO78, and the reverse primer, CueO4 (Table S3). The purified “78” amplicon was then combined separately with the purified *ompE* promoter PCR product and amplified in a second reaction, using the forward *ompE* primer and the CueO4 reverse primer and generating the gene fusion product *ompE78*. This gene fusion product was cloned into pCOTOPO2.1 vector (Invitrogen, Waltham, MA), generating pTOPO78 (Table S2). The *ompE78* gene fragment was excised with EcoRI and subsequently subcloned into EcoRI-digested pMEK91, generating the shuttle vector pO78 (Table S2 and Fig. S1F). pO78 was used to transform ATCC 35925 via electroporation. Tet^r colonies were passed and cultured under selection, and the plasmid was isolated via plasmid miniprep kit (Qiagen) and confirmed by PCR amplification using the OmpEF and CueO4 reverse primers.

Site-directed mutagenesis. Mutations in T1 copper-binding ligands of the 78-aa truncated version of *cueO* were generated through site-directed mutagenesis of the plasmid pTOPO78, using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the primers Cys495F and Cys495R (Table S3). pTOPO78_{C495A} was made by substituting the codon corresponding to alanine (GCC) for the codon corresponding to cysteine (TGC) at position 495 (Table S2). The double mutant (C495 and H500A) was generated similarly by substituting the codon corresponding to alanine (GCT) for the codon corresponding to histidine (CAT) at position 500, using the primers His500F and His500R (Table S3) and pTOPO78_{C495A}, resulting in the plasmid pTOPO78_{C495H500A} (Table S2). Mutations were confirmed by sequencing through Eton Bioscience, Inc. (Research Triangle Park, NC). The gene fusion sequences were subcloned by digestion with EcoRI and ligated with EcoRI-digested pMEK91 to make the shuttle vectors pO78_{C495A} and pO78_{C495H500A}.

Copper sensitivity. MIC for copper was determined using cultures grown to mid-log phase and adjusted to an OD₆₀₀ of 0.1 in Mueller-Hinton broth (MHB) supplemented with CuSO₄ (Fisher Scientific, Hampton, NH; supplier of all CuSO₄ in this report) at concentrations ranging from 0 to 6.0 mM. After 16 h of growth, the CFU per milliliter for each culture was determined by serial dilution and plating. The MIC was calculated as the lowest concentration of CuSO₄ in which the final CFU per milliliter was equal to or lower than the inoculated dose. Values are the averages from at least 3 biological replicates.

Methyl viologen sensitivity. Cultures of *C. jejuni* were grown to mid-log phase (OD₆₀₀ ~0.3) in MHB supplemented with 0.125 mM CuSO₄ and adjusted to an OD₆₀₀ of 0.2. An initial aliquot of cultures was removed and serially diluted to determine the initial CFU per milliliter. The remaining cultures were exposed to either 0.5 mM or 5.0 mM methyl viologen (Sigma-Aldrich, St. Louis, MO) for 1 h, then serially diluted into PBS, plated onto blood agar, and incubated microaerobically at 37°C. After 2 days of incubation, colonies were counted and log reductions were calculated. The data were analyzed by unpaired *t* test, using GraphPad Prism software version 7.

H₂O₂ sensitivity. *C. jejuni* was grown to mid-log phase (OD₆₀₀ ~0.3) in MHB supplemented with 0.125 mM CuSO₄ and adjusted to an OD₆₀₀ of 0.3. 2,2'-Dipyridyl was added to the cultures to a final concentration of 0.1 mM. After a 30-min incubation, an aliquot was removed and serially diluted to determine the initial CFU per milliliter. The cultures were then challenged with 1 mM H₂O₂ (Fisher Scientific, Hampton, NH) for 1 h and serially diluted into cold PBS. The first dilution in the series contained 4,000 U/ml bovine catalase (Sigma-Aldrich) to degrade any residual H₂O₂ present. The dilutions were then plated onto blood agar and incubated microaerobically at 37°C. After 2 days of incubation, colonies were counted and log reductions were calculated. The data were analyzed by unpaired *t* test, using GraphPad Prism software version 7.

2,2'-Dipyridyl sensitivity. Cultures were grown to mid-log phase (OD₆₀₀ ~0.3) in MHB supplemented with 0.125 mM CuSO₄ and then adjusted to an OD₆₀₀ of 0.3. An initial aliquot was taken to determine initial CFU per milliliter, and the remaining cultures were exposed to 0.1 mM 2,2'-dipyridyl for 1 h, then serially diluted into PBS, plated onto blood agar, and incubated microaerobically at 37°C. After 2 days of incubation, colonies were counted and log reductions were calculated. The data were analyzed by unpaired *t* test, using GraphPad Prism software version 7.

Quantitative reverse transcriptase PCR. The PCR primers designed for qRT-PCR are listed in Table S1 and were used to amplify gene fragments of the following sizes: 125 nucleotides for *copA*, 113 nucleotides for *cueO*, and 115 nucleotides for *gyrA*. Total RNA was isolated from the strains ATCC 35925, SG2 (*copA*_{35925::cat}), SG3 (*cueO*_{35925::cat}), ATCC 35925 carrying p*OCopA*, and ATCC 35925 carrying p*OCueO* using the MasterPure Complete DNA and RNA purification kit (Epicentre Technologies Corporation, Madison, WI). qRT-PCR was performed as previously described (40) by using the QuantiTect SYBR green RT-PCR kit (Qiagen, Valencia, CA). A standard curve was generated to determine the number of starting RNA molecules. Total RNA in each sample was normalized to that of the internal control *gyrA* (Cj1027c).

Chicken colonization. Day-of-hatch chicks were obtained from the NC State Lake Wheeler Chicken Education Unit and separated into groups of 10 in Petersime brooder batteries. Chicks were either fed commercial chicken feed containing 8 mg Cu/kg or the same feed diet supplemented with 180 mg CuSO₄/kg (32). On day 7, the chicks were inoculated by oral gavage with 0.1 ml PBS containing either 10⁷ CFU/ml of wild-type NCTC 11168, SG1, or SG2. The birds were kept on the same feeding regimen (with or without added copper) following inoculation. Two weeks after inoculation, the birds were humanely sacrificed by CO₂ asphyxiation and approximately 1 g of cecal contents was collected, serially diluted in PBS, and plated on selective blood agar prepared in-house, which contained 40 µg/ml cefoperazone, 40 µg/ml vancomycin, 10 µg/ml trimethoprim, and 100 µg/ml cycloheximide. The samples were incubated microaerobically at 37°C, and after 2 days, colonies were counted, and the number of CFU per gram of cecal contents was calculated. The data were analyzed by unpaired *t* test, using GraphPad Prism software version 7. All procedures were approved by the NC State Institutional Animal Care and Use Committee under protocol no. 12-156-A.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00208-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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