

ABSTRACT

Gardner, Susan Pandy. Interaction of Copper Toxicity and Oxidative Stress in *Campylobacter jejuni*. (Under the direction of Dr. Jonathan W. Olson).

Campylobacter jejuni is one of the leading causes of food-borne gastroenteritis in the world, yet its basic metabolism and mechanisms of host infections are still less well studied than other pathogens. This thesis is dedicated to advancing the knowledge about this pathogen with the hopes that it may be used to lessen the impact of this important pathogen on human health.

Chapters 1 and 2 are focused on the ability of *C. jejuni* to combat copper stress. Copper is both a required micronutrient and a source of toxicity in most organisms, including *C. 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. Overexpression of a cytoplasmic peptide derived from the copper-binding region of CueO also caused copper toxicity at normally sub-lethal concentrations. Expression of the peptide with the copper binding ligands mutated restored wild type sensitivities. Strains with mutations in either of the homeostatic genes did not colonize the avian host as well as wild type strains, and copper in the feed exacerbated the colonization deficiency. Taken together, the results indicated that copper toxicity in *C. jejuni* is due to an inability to sequester cytoplasmic copper, resulting in an increase in copper-mediated oxidative damage.

The appendix of this thesis deals current state of genetic transfer in *C. jejuni*. A major hurdle in investigating its physiology and pathogenesis comes from the

incompatibility of the molecular tools that have enabled advances in the characterization of other bacterial species. Most notably, the dearth of plasmid-based complementation, reporter assays, and plasmid-based unmarked mutagenesis procedures in many of the type strains has hindered research progress. The techniques themselves are not inadequate in *Campylobacter* species, but rather the barrier to genetic transfer of these genetic constructs from non-*Campylobacter* cloning stains. Also reviewed are two systems (CRISPR/Cas and Restriction modification) that are common to many strains of *C. jejuni* and should at least partly responsible for these barriers.

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

by
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Dedication

I would like to dedicate this manuscript to my husband John for encouraging my incredibly naïve, and crazy dream of going back to school.

Biography

Susan was born in Westfield, NY on May 7, 1959. In 1974 her family relocated from Amherst, NY, to Upper St. Clair, PA, where she met her husband John during their junior year of high school. Susan attended Duquesne University, in Pittsburgh, PA, where she received a B.S. in Music Education in 1981. She taught elementary school music for a few years, and gave private flute lessons for 15 years. She left teaching to raise her 4 children, David, Emily, Benjamin, and Nora, and through them, rediscovered her passion for science. After 20 years at home she returned to school by attending Meredith College in Raleigh, NC, where she fell in love with microbiology and molecular biology. She completed 2 senior undergraduate research projects while at Meredith. The first, under the direction of Dr. Reginald Shiflett, evaluated the development of a copper histidine model for amyloid plaque research. The second project identified bacterial species in the gut of *Apis mellifera*, under the guidance of Dr. Jason Andrus. During her time at Meredith, Dr. Andrus encouraged Susan to not only pursue undergraduate research in his lab, but to also become his teaching assistant, which she enjoyed very much. Dr. Andrus introduced her to her future thesis advisor, Dr. Jonathan Olson at North Carolina State University. Dr. Olson allowed Susan to volunteer in his lab for a year before she started in the microbiology graduate program. Susan remained in his lab throughout her graduate career. While at NC State, Susan had the opportunity to work for many semesters as a teaching assistant, and as an instructor for SCIBLS. Her love of research and teaching has made her want to pursue a career in academia.

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I would like to thank Dr. Jonathan Olson, for providing me the opportunity to enter the graduate program at NCSU. Working with you has taught me the importance of, and enabled me to become, a more independent researcher. It was a privilege working for you. Thank you for introducing me to the IBC Committee and facilitating my MBGSA involvement. I would like to thank Dr. Amy Grunden and Dr. Sid Thakur for encouraging and supporting me, and for giving me helpful feedback and advice about my projects. Dr. Hamilton, thank you, for taking the time to talk to me about science and teaching every single day, for thoughtfully answering my questions, for giving me meaningful career advice, and for being my friend. Thank you also for being so generous with supplies, and funding. I could not have done this without you. To Dr. Andrus, thank you for being such a wonderful mentor, and for being interested in, and supporting my career endeavors. You taught me how to connect to my students through trust, honesty, kindness, and respect. To my NCSU friends Jenn Stone, Christy Smith, Denise Aslett, and Jesse Noar: thank you all for your wonderful friendship. I don't think I have ever had friends who I've felt so deeply connected to, who love science as much as I do, or who made me laugh so hard. Finally, thank you to my family. John, thank you for encouraging me to go back to school, and for pushing me to finish when I had given up on having any kind of career in science. To my children, David, Emily, Ben, and Nora. Thank you for believing in me, studying with me, cooking for me, always cracking me up, and listening to me whenever I tried to explain my work to you. I am so very proud to be your mom.

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CHAPTER 1

Literature Review

Campylobacter

The genus *Campylobacter* consists of Gram negative microaerophilic bacteria that are pathogenic to humans and animals. *Campylobacters* are members of the class epsilon proteobacteria, and are of the order Campylobacterales. The order is comprised of two families: Helicobacteraceae and Campylobacteraceae. Helicobacteraceae includes *Helicobacter* and *Wolinella* species, and Campylobacteraceae includes the three genera: *Campylobacter*, *Arcobacter*, and *Sulfurospirillum*. Members of the family Campylobacteraceae are nonsaccharolytic, grow optimally between 37° - 42°C, have a low G +C content, and prefer an atmosphere of 85% N₂, 10% CO₂, and 5% O₂ (1, 2). The cells are slender, spiral-shaped rods that measure 0.2-0.8µm x 0.5-5µm. With the exception of *Campylobacter gracilis*, most *Campylobacter* species are motile, driven in a corkscrew motion by an unsheathed flagellum located at either one or both ends of the cell (2). *Campylobacter* predominantly inhabits the gastrointestinal tract of humans and animals, but can also be found in the oral cavity and reproductive organs (2). Today, the genus *Campylobacter* consists of 34 species and 14 subspecies (3).

***Campylobacter* History**

Perhaps the first identification of *Campylobacter* occurs in 1886 when Theodor Escherich identified a spiral shaped bacterium in stool samples isolated from children with diarrhea (4). In 1909, while investigating epizootic abortion in cattle and sheep, McFadyean and Stockman isolated a pure culture of a vibrio-shaped organism from ovine uterine mucus, and successfully infected previously healthy pregnant ewes with the bacteria (5). A similar looking “spirilla” shaped organism was isolated from cases of vibronic abortion in cattle by Smith and Taylor in 1919, and named *Vibrio fetus* (6).

Jones, et al. compared the morphology, motility, flagella, and nutrient requirements of pathogenic vibrios isolated from the bovine jejunum, and proposed the name *Vibrio jejuni* (7). *Vibrio coli* was the name assigned to the vibrio strain isolated from pigs (8). The first documented cases of human infection (most likely due to *V. jejuni*) occurred in 1938 in an outbreak amongst inmates who had consumed contaminated milk in two Illinois institutions (9).

In the late 1950's and early 1960's, Elizabeth King described differences between *Vibrio bulbulus*, *V. fetus*, *V. jejuni*, and what she called "related vibrios" through biochemical testing and varying growth conditions. She identified chickens as a major source of human infection (10, 11). In 1963, Sebald and Véron proposed the genus designation *Campylobacter* (derived from the Greek words for "curved" and "rod") for *V. fetus* and *V. bulbulus* after comparing the flagella, GC% content, sugar metabolism, and oxygen requirements of distinct *Vibrio* species (12). *V. jejuni* and *V. coli* were reclassified as *Campylobacter jejuni*, and *Campylobacter coli* by Véron and Chatelain in 1973 (13).

While it had been clear for some time that *Campylobacter* infection in humans caused inflammation of the small intestine, resulting in diarrhea, the ability to clinically isolate *Campylobacter* from stool samples was problematic because of overgrowth of other enteric microorganisms in the culturing process. Due to its small size in comparison to other gut microbes, filtration was the only means to isolate and identify *Campylobacter* from stool samples (14). Finally, in 1977, Martin Skirrow developed a selective media specifically for culturing *Campylobacter*, and his work identified *Campylobacter* as the "commonest identifiable cause of infectious diarrhea," (15). In 1982, the Centers for Disease Control and Prevention (CDC) initiated a national weekly *Campylobacter*

surveillance, and began collecting clinical data from state health departments (16). There were 4,027 laboratory-isolated cases reported that year (16).

***Campylobacter* and Public Health**

Today, an estimated 1.3 million people in the U.S. become infected with *Campylobacter* each year (17). According to the Foodborne Diseases Active Surveillance Network (FoodNet), the number of lab-confirmed cases of *Campylobacter* in 2014 accounted for approximately one third of all reported bacterial and parasitic illnesses transmitted by food, resulting in 11 deaths and 1,080 hospitalizations (17). Despite faster identification of food borne illnesses and changes in food industry practices to limit contamination, the number of confirmed *Campylobacter* infections in 2014 was 13% higher than the number reported in 2006 (17). There was, however, over the same time period, a 32% decrease in reported cases of Shiga-toxin producing *Escherichia coli* O157:H7, and a 27% decrease in confirmed *Salmonella enterica* Typhimurium infections (17).

In Europe, according to the European Food Safety Authority, (EFSA), there are 200,000 reported cases of campylobacteriosis annually, at a cost of € 2.4 billion (18). It is estimated that the actual number of cases per year is closer to 9 million (19). In Australia from 2000 to 2010, *Campylobacter* infections were the leading cause of hospitalizations for gastroenteritis, and over the same time period, the number of cases of foodborne campylobacteriosis increased from 139,000 to 179,000 (20).

A study funded by the World Health Organization (WHO), estimated worldwide that there are almost 170 million foodborne illnesses and 36,300 deaths due to *Campylobacter* every year (21). *Campylobacter* surveillance in low and middle income

countries is problematic due to the difficulty in culturing and identifying *Campylobacter*, the cost associated with the diagnostic platforms available, and variability in collecting and analyzing meaningful data to determine the disease burden (19). The Global Enteric Multicenter Study (GEMS) was developed to determine diarrheal pathogen burden of children living in developing countries. *Campylobacter* infection diagnosed between the ages of 0-56 months was associated with moderate to severe diarrhea in India, Bangladesh, and Pakistan (22). The burden of moderate to severe diarrhea in these children results in negatively impacted growth, and an 8.5% greater chance of dying (22).

Disease Manifestation

Although *Campylobacter* remains a significant health burden worldwide, most cases of campylobacteriosis are self-limiting, and patients usually recover within a week without having to undergo any specific treatment other than hydration therapy. *C. jejuni* and *C. coli* are the species most commonly associated with human gastroenteritis, and the infectious dose can be as low as 500 bacteria (23-25). Symptoms of campylobacteriosis include watery or bloody diarrhea, abdominal cramping, and fever, which usually occur within 1 to 7 days after exposure (15, 23). The severity of the illness depends upon the number of cells in the infectious dose, the strain, and the health of the patient. Patients who are immunocompromised or elderly tend to have a more severe, chronic infection associated with bacteremia (26, 27). In uncomplicated cases, diarrhetic episodes abate within 3 to 4 days, although *Campylobacter* has been detected in stool samples for as long as 69 days after the onset of infection (28). In instances of severe or prolonged infection, macrolides or fluoroquinolones are prescribed (23). In instances of systemic infections, gentamicin and tetracycline are also prescribed (29). *Campylobacter* however,

is becoming increasingly resistant to these antimicrobials that are critically important to human medicine. The public health issue of antibiotic resistance is further compounded by the fact that *Campylobacter* is a zoonotic pathogen.

***Campylobacter* sequelae**

Sequelae of campylobacteriosis include Guillain-Barré syndrome (GBS), reactive arthritis (ReA), and post-infectious irritable bowel syndrome (PI-IBS) (30-32). Guillain-Barré syndrome is a demyelinating polyneuropathy of the peripheral nervous system characterized by progressive motor weakness (33). An antecedent *Campylobacter* infection triggers an inappropriate autoimmune response due to molecular mimicry between *Campylobacter* lipooligosaccharide structures and human gangliosides (34). GBS associated with *Campylobacter* infection typically occurs 3-4 weeks after the onset of diarrhea (35). Globally, it is estimated that precedent infection with *Campylobacter* accounts for approximately one third of all GBS cases in high-income countries and over half in low-income countries (19).

An estimated 1-5% patients develop ReA following infection with *Campylobacter* (19). It's hypothesized that molecular mimicry leads to the synovitis associated with ReA (36). A US study from 2008 found that in people with laboratory confirmed cases of *Campylobacter*, *E. coli* O157:H7, *Salmonella*, *Shigella*, and *Yersinia*, the incidence of subsequent ReA was highest in those who had reported precedent campylobacteriosis (37).

Infection with *Campylobacter* has also been linked to chronic gastrointestinal diseases that include post-infectious irritable bowel syndrome (PI-IBS), inflammatory bowel disease (IBD), celiac disease, and functional dyspepsia. *Campylobacter* damages

intestinal epithelial cells through the production of cytolethal distending toxin (CDT), which in turn can lead to a state of chronic, low-level inflammation. There is evidence that infection may also upset the gut microbiota, resulting in competition between beneficial and pathogenic bacteria (38). Multiple studies that were initiated to follow patients who had suffered from campylobacteriosis found that the severity and duration of the precedent infection correlated with chronic abnormal bowel habits (32, 39, 40). A large cohort of over 13,000 patients in Denmark, who had laboratory confirmed infections with *Campylobacter* or *Salmonella*, were followed for 15 years to determine the risk of developing IBD after exposure to either pathogen. Patients in the study were compared to a control group matched in both age and gender. The researchers found that the risk of IBD was higher in the pathogen-exposed cohort, and that risk was higher in patients who had been hospitalized with acute enteritis preceding the diagnosis of IBD (41).

Modes of infection

Transmission of *Campylobacter* is usually foodborne, and attributed to the consumption of raw or undercooked poultry (42, 43). Large outbreaks are uncommon, but when they do occur, they are associated with the consumption of raw milk or contaminated water (9, 44). *Campylobacter* is a commensal in poultry, and can reach very high numbers in the cecal crypts of birds. *Campylobacter* is also found in high numbers on the skin and feathers of infected retail poultry. Consequently, contamination of the poultry carcass occurs throughout processing. In the US, previous studies estimated that up to 98% of commercial poultry products at retail were contaminated with *Campylobacter*, however data from 2013 indicated that the number of retail chicken

products testing positive for *C. jejuni* was 38% (44, 45). A cecal sample survey was also included in these data, and *Campylobacter* was isolated from 31% of market hogs, 43% of dairy cattle, 42% of beef cattle, 22% of chickens, and 9.5% of turkeys (45). The prevalence of *C. jejuni* was higher in the chickens and cattle surveyed, whereas *C. coli* was isolated most frequently from swine (45). Last year, a 12 month survey of samples taken by the Food Standards Agency in the UK from poultry products and packaging found that 73% of all chicken carcasses were contaminated with *Campylobacter*, 19% were highly contaminated (1×10^3 CFU/g), and 7% of the outer packaging tested positive for contamination (46).

***Campylobacter* control measures**

Current strategies for reducing *C. jejuni* contamination in retail chicken meat are ultimately ineffective. These intervention strategies include biosecurity measures, vaccine development, competitive exclusion, bacteriophage therapy, and bacteriocin-based treatment. While stringent hygienic and biosecurity measures have been found to be effective at reducing, or delaying *C. jejuni* colonization of industrial broiler flocks, they do not work in preventing the establishment of *C. jejuni* in the broiler population (47-50). There are too many sources of potential *Campylobacter* infection, from other animals, insects, and farm workers to make biosecurity measures cost effective.

Currently, there are no commercial poultry vaccines available. Vaccine development has been hampered by the fact that *Campylobacter* is a commensal in poultry, and there is a great deal of genetic and antigenic diversity among the many *Campylobacter* strains that could potentially infect broiler flocks. A vaccine would need to be developed with a broad spectrum of protection. In 2004, Wyszynska *et al.* were able

to demonstrate that oral immunization with avirulent *Salmonella* carrying the *Campylobacter cjaA* gene reduced the ability of *C. jejuni* 72Dz/92 to subsequently colonize the chicken cecum (51). Other vaccine research has included the development of genetically engineered live vectors expressing antigens specific to *Campylobacter*, flagellin-based subunit vaccines, and killed whole cells (52-54).

Competitive exclusion studies have explored the feasibility of inoculating chicks with microbiota from adult chickens to prevent infection with *Campylobacter*. While this may seem like a reasonable idea, commercially available competition exclusion products are ineffective in the industrial farm animal production setting (55). Attempts at treating broilers with pure cultures of *Lactobacillus acidophilus*, *Streptococcus faecium*, and *Saccaromyces boulardii* were unsuccessful at preventing *Campylobacter* colonization (56, 57).

Bacteriophages are ubiquitous in the environment. *Campylobacter*-specific phages have been found in broilers, abattoir effluents, sewage, and pig manure (58-61). Challenge studies utilizing bacteriophage therapy to eliminate *Campylobacter* colonization in broilers have looked promising, though results have been variable and influenced by the phage dosage and type (62, 63). Bacteriocin use has been studied as well. Bacteriocins are small peptides that display antimicrobial properties (64, 65). Stern *et al.* determined that bacteriocins purified from *Lactobacillus salivarius* and *Paenibacillus polymyxa* added as feed supplements were effective at reducing *C. jejuni* infection in chickens (66, 67). While there are many control measures that have been studied and developed to attempt to eliminate *Campylobacter* prevalence in poultry, none

of these measures to date have been effective on the scale needed by the US poultry industry.

Copper in Biology

Copper bioavailability occurred after oxygen became present in the earth's atmosphere, almost 2.5 billion years ago (68). Previously, the transition metal had existed in a water-insoluble Cu^+ form of inorganic sulfides, and was thus unavailable for biological systems (69). While copper became available to all forms of life as a cofactor for biochemical enzymes, as a free ion it was also toxic to cells, so copper resistance and acquisition mechanisms evolved. Copper has two oxidation states in nature, Cu^+ and Cu^{2+} , and is therefore a cofactor found in enzymes that catalyze redox reactions utilizing oxygen as a substrate. Copper-containing proteins can be found in aerobic organisms from bacteria to humans, and participate mainly in oxygen transport, activation, and electron transfer (69). In addition to cytochrome c oxidase, other cuproproteins include NADH-dehydrogenase 2 (ND2), copper, zinc-superoxide dismutase (SOD1), ascorbate oxidase, plantacyanin, polyphenol oxidase, nitrocyanin, plastocyanin, copper-containing nitrite reductase, copper amine oxidase, particulate methane monooxygenase (pMMO), CotA, and tyrosinase (70, 71).

The importance of copper to *Campylobacter* animal hosts

Copper is essential for the synthesis of hemoglobin in chickens. Copper deficiency can result in health issues that include anemia, aortic rupture due to decreases in the cross-linking of elastin, bone fragility, and decreased activities of the cuproenzymes SOD, cytochrome oxidase, ceruloplasmin (72, 73). A deficiency in copper can also cause reduced egg production, abnormal calcification of the eggshell, and

increased egg size (74). Chickens are oftentimes fed diets with high copper levels (100-200mg/kg diet) because it is thought to act as a growth promoter, much like certain antibiotics (75). A high copper diet results in the decrease of circulating lymphocytes in intestinal epithelial cells and connective tissues (76). Copper sulfate is also routinely added to water and feed in chicken production to prevent enteritis or candidiasis. Toxicity issues in poultry are not typical, although turkeys do not prefer to drink water to which copper sulfate has been added and will succumb to dehydration rather than drink the copper-treated water (77). Occasional copper poisonings occur after ingestion of copper sulfate crystals.

Copper is also essential in host control of infection via the innate immune system. During colonization of the host gastrointestinal tract, *C. jejuni* is targeted by macrophages for phagocytosis. Macrophages utilize copper in combination with reactive oxygen species (ROS) and nitric oxide species to kill off infectious microorganisms (78). In 2009, White *et al.* determined that the macrophage ATPase 1 copper transporter, ATP7A is essential for bacterial killing (79). Pretreating murine-leukemic monocyte-macrophage RAW 264.7 cells with copper increased the bactericidal activity of macrophages against *E. coli* (79). In the same study, an *E. coli* $\Delta copA$ mutant was found to be hypersensitive to killing by RAW 264.7 cells in comparison to wild type *E. coli* cells (79). Festa and Theile further elucidated the mechanism of copper uptake by host cells in order to fight invading pathogens. Ctr1, another well characterized and ubiquitous copper transporter, is induced by macrophage activation along with ATP7A, which localizes to the phagosomal membrane. Both transporters working concurrently result in an increase in the concentration of copper in the macrophage phagosome (80). It is interesting to point out

that at the same time macrophages are utilizing copper to kill pathogenic bacteria, they also retain the iron and manganese necessary for bacterial invasion, while the very same bacterial targets are trying to take up iron and manganese while keeping copper levels low (81).

One highly conserved and vital process that is dependent upon copper bioavailability is oxygen reduction by cytochrome c oxidase, a proton pump and terminal electron acceptor of many respiratory chains (82). Both mitochondria and *C. jejuni* express cytochrome oxidases (83). The *C. jejuni* NCTC 11168 *cbb₃*-type cytochrome c oxidoreductase (Cj1490c) is one of two respiratory terminal oxidases, and has been characterized in the Olson lab. The *cbb₃*-type oxidase operon of *C. jejuni* consists of four genes: *ccoN* (Cj1490c), *ccoO* (Cj1489c), *ccoQ* (Cj1488c), and *ccoP* (Cj1487c). The oxidase is a member of the superfamily of respiratory heme-copper oxidases, and catalyzes the reduction of dioxygen to water, translocating protons across the periplasmic membrane (82). This type of oxidase is often found in *Proteobacteria* to facilitate colonization of the anoxic environment found in the avian gut (82). Previous work determined that expression of the *cbb₃*-type oxidase is required for successful chicken colonization (84). The *C. jejuni* mutant in the *cbb₃*-type oxidase was demonstrated to be extremely sensitive to oxygen (84). *C. jejuni* also encodes a copper-containing oxidase (CueO), which will be discussed later in this review.

Copper toxicity

Although copper has important physiological roles in the cell, the ability of copper to undergo redox cycling also

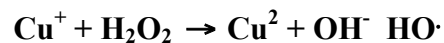


Figure 1. Copper reacts with endogenous H₂O₂ producing hydroxyl radicals

makes it toxic to cells. Copper reacts with hydrogen peroxide, much like iron, generating reactive oxygen species (ROS) in a process that's comparable to the Fenton reaction (Fig. 1). This process leads to production of the powerful hydroxyl radical that can then damage cellular components via lipid peroxidation, protein oxidation, DNA damage, and disruption of amino acid biosynthetic pathways (68, 85).

Copper toxicity also occurs through disruption of iron-sulfur clusters of intracellular enzymes or enzyme complexes via copper displacement of iron atoms by forming coordination compounds with sulfur or thiolate ligands (86, 87). This either causes damage to enzymes or disrupts enzyme activation or synthesis (85). Degradation of iron-sulfur clusters leads to the release of free iron, causing oxidative damage through iron-based Fenton chemistry (88). Copper toxicity is also independent of oxygen. Under the anaerobic, acidic conditions that enteric organisms encounter in the gastrointestinal tract, cells have been found to be significantly more sensitive to copper (69, 86, 89, 90). As a result, microorganisms have developed homeostasis mechanisms that allow for the uptake of copper ions necessary for cofactors in biochemical enzymes, while at the same time protecting the cell from the toxic effects of copper overload.

While copper oxidation by H_2O_2 leads to the generation of ROS *in vitro*, Macomber, Rensing, and Imlay demonstrated that *E. coli* challenged with copper were actually *less* sensitive to killing by H_2O_2 (91). This occurred even in cells lacking the two major copper detoxification pathways. Hydroxyl radical production could be observed in these cells, but H_2O_2 oxidizable copper was localized to the periplasm, thus sparing the DNA from damage. Moreover, they concluded that copper blocks iron-mediated H_2O_2 killing. They also found that cells grown in copper-enriched media had a ninefold

increase in catalase activity and generated the induction of superoxide dismutase. The work supports the idea that copper ions induce *E. coli*'s oxidative stress protection mechanisms (91).

Mechanisms of copper homeostasis

Copper homeostasis mechanisms have been well characterized in many Gram negative bacteria (69, 92, 93). In *E. coli*, enzymes that contain sensitive iron-sulfur clusters, such as dehydratases, are protected from copper toxicity in the cytoplasm where copper levels are kept low, while proteins that require copper as a cofactor are more commonly found in the periplasm (86). Intracellular copper toxicity and homeostasis in *E. coli* is controlled by three copper regulatory systems (69). The *cue* regulatory system (described below) is the primary copper homeostasis system in *E. coli*, however, when a higher threshold of copper toxicity is reached within the cell, the *cus* regulatory system is activated (90). The *cus* (Cu sensing) locus detoxifies the cell through activation of the large, four-component RND (resistance nodulation division)-type efflux pump, CusCFBA, which moves copper out of the cell and into the extracellular space (94, 95). The efflux pump is not synthesized until high levels of copper accumulate in the periplasm (86). CusS, a histidine kinase, senses the periplasmic copper, phosphorylates the response regulator, CusR, and activates transcription of CusCFBA (96). The CusCFBA complex senses the concentration of periplasmic copper through three conserved methionine residues in CusA, the inner membrane-bound transporter. CusF is a periplasmic metal chaperone, which delivers copper to the membrane fusion protein, CusB. CusC is the outer membrane component that copper then exits through (96). Additionally, some strains of *E. coli* and *Pseudomonas* carry the plasmid-encoded (*pco*)

copper defense system. This system includes a two-component regulatory system, a membrane transporter, a periplasmic copper-binding protein, and a multicopper oxidase (97, 98). Many bacteria that lack the *cus* system encode CueP, a periplasmic metal complex found in *Salmonellae*, *Yersinia*, *Erwinia carotovora*, *Citrobacter koseri*, *Vibrio shilonii*, and *Magnetospirillum magnetotacticum*, protects the cell, particularly under anaerobic conditions by binding copper, and is therefore thought to protect the cell from higher concentrations of copper as the CusCFBA complex does in *E. coli* (92).

The primary copper homeostatic system is in most Gram negative bacteria the *cue* (Cu efflux) system. Here the expression of *copA* and *cueO* is activated by the MerR homologue CueR upon binding to Cu⁺ (90, 96). CueR is a copper-responsive transcriptional activator that is characterized by a helix-turn-helix (HTH) DNA-binding motif and is the only regulator of *copA* in the presence of copper salts (99). In *E. coli*, CueR's affinity for DNA decreases in response to an increase in the concentration of Cu⁺ (99). Outten *et al.* proposed in their work that while CueR shares sequence similarities with ZntR and MerR, it has a different metal binding domain that may allow CueR to recognize differences between copper, zinc, and mercury (100).

CopA, a Cu⁺ translocating P-type ATPase, transports cytosolic Cu⁺ into the periplasm under both aerobic and anaerobic conditions (95). CopA is activated by low environmental copper concentrations (1.0 μM CuSO₄), keeping cytosolic copper levels quite low (100). P-type ATPases are ubiquitous transmembrane ATP-driven pumps, categorized by their substrate specificity. P-type ATPases are divided into five groups according to substrate specificity: P_I (K⁺, Cu⁺, Cu²⁺, Ag⁺, Cd²⁺, Zn²⁺, Pb²⁺, Co²⁺), P_{II} (Ca²⁺, Mn²⁺), P_{III} (H⁺/Mg²⁺ pumps), P_{IV} (phospholipid transport), P_V (no substrate

specificity) (101). The *E. coli* CopA contains two putative CXXC metal binding sites, however, it has been demonstrated that they are not metal-specific (102). Transcription of *copA* in *E. coli* is higher under anaerobic conditions, which suggests that cytosolic copper may be higher under anaerobic conditions than under aerobic conditions (90).

In *E. coli*, the multicopper oxidase, CueO, protects periplasmic enzymes by oxidizing Cu^+ to the less toxic Cu^{2+} (103). Grass *et al.* demonstrated that CueO safeguards periplasmic enzymes, such as alkaline phosphatase, from copper toxicity. That research also demonstrated that CueO along with cuprous oxidase activity, possesses ferroxidase activity and *p*-diphenol:dioxygen oxidoreductase activity (104). In addition to oxidizing Cu^+ , CueO can also oxidize other substrates, including Fe^{2+} , siderophores, and catechols (105).

Based on spectroscopic characteristics, multicopper oxidases contain an active center structure composed of four copper atoms per molecule at their oxygen binding site: a Type 1 (T1) blue copper ion, and a trinuclear cluster (TNC) composed of one Type 2 (T2) and two Type 3 (T3) copper ions (106). This type of multicopper oxidase, or laccase, combines the oxidation of a substrate near the T1 copper binding site with the four-electron reduction of dioxygen to water at the TNC (106). The T1 copper atom is buried in the interior of the protein by a methionine-rich region (107). The presence of this region blocking the T1 Cu binding site ensures binding specificity to small substrates such as copper. It was shown that deletion of the entire met-rich region in CueO resulted in a higher affinity for bulky substrate binding (108).

Methionine rich regions (MXXM) are common in CueO and CueO-like proteins, and also act as binding sites for labile, exogenous copper ions (109). A 5th copper atom,

C5, is bound to the methionine residue at the N-terminal end of the met-rich region of CueO near the T1 Cu binding site. This copper atom was designated the regulatory Cu (rCu) binding site by Roberts, *et al.* because mutations in the ligating residues of this binding site resulted in sensitivity to copper *in vivo*, and reduced or lost Cu²⁺ activity *in vitro* (109). Singh, *et al.* renamed the rCu site sCu, due to the Cu⁺ substrate binding capacity of the site (107). This work revealed 2 additional Cu⁺ atoms C6, and C7, ligated to methionine residues of the met-rich region. When the methionine residues of those binding sites were changed to serine residues through mutagenesis, there was a 4-fold decrease in the catalytic rates for the oxidation of Cu⁺ (107). Met-rich regions in multicopper oxidases also facilitate Cu⁺ transport, and protein-protein interactions (109-113). The copper transport protein Ctr1 (copper transport 1) in *Saccharomyces cerevisiae*, and the human copper transport and storage protein, ceruloplasmin, require at least one MXXM motif to move Cu⁺ through the cytoplasmic membrane (110).

Copper homeostasis in C. jejuni

C. jejuni NCTC 11168 encodes homologues for CueO (Cj1516), and The P_{1B}-type ATPase CopA (Cj1161c) (114, 115). Mutants were constructed in both enzymes by Hall *et al.*, established that a mutation in either gene rendered the cells more sensitive to copper than the wild-type strain, (114). The same work also used purified CueO protein to demonstrate that, as in *E. coli*, the *C. jejuni* multicopper oxidase possesses cuprous oxidase activity, ferroxidase activity, and phenoloxidase activity (114). The copper content of the multicopper oxidase was measured and estimated to be 6.4 atoms of Cu per polypeptide chain (114).

Silva *et al.* have subsequently solved the crystal structure of the *C. jejuni* multicopper oxidase, which they termed McoC. They determined that McoC is typical of other multicopper oxidases in structure and has a high efficiency (k_{cat}/K_m) for Cu^+ and Fe^{2+} atoms (116). The protein is comprised of three cupredoxin-like domains, and contains four copper atoms similarly arranged as in the *E. coli* CueO protein (116). There is a T1 blue copper atom located in domain 3, and a trinuclear cluster residing at the interface of domains 1 and 3. The T1 Cu atom is coordinated between His⁴³⁹, His⁵⁰⁰, Cys⁴⁹⁵ and Met⁵⁰⁵ (116). The T2 Cu atom is coordinated between His¹⁴¹, His⁴⁴², His¹³⁹, and His⁴⁴⁴ (116). One T3 Cu atom is coordinated between His⁴⁹⁶ and His¹⁸⁰, whereas the other T3 atom is coordinated between His⁴⁹⁴, and His¹⁸² (116). McoC also has a met-rich region that blocks the T1 Cu binding site, however, the additional Cu6 and Cu7 copper binding sites that exist along the met-rich region in CueO were not identified in this protein. This region resides between the residues Met³⁷⁹ and Gly³⁹¹. The rCu binding site is coordinated between Glu³⁸¹, His³⁸³, Ser⁴³⁵, and Met⁴³⁷ (116).

The research described here further characterizes the components of copper homeostasis in *C. jejuni*. By mutating the genes that encode CueO and CopA, this work demonstrates that the mutagenized strains are more sensitive to copper stress than their parental strains. This sensitivity was rescued by complementation of the genes back into the mutant strains. In order to elucidate the interplay between oxidative stress and copper toxicity, the strains were subjected to the superoxide generator, methyl viologen. The presence of copper exacerbated oxidative stress on the CueO and CopA mutants, which was relieved by complementation. When H_2O_2 and 2, 2'-dipyridyl were used to determine oxidative stress without the added effect of iron-mediated Fenton chemistry, it

was discovered that the CueO mutant was sensitive to the copper/2, 2'-dipyridyl combination without the addition of an oxidant other than O₂. Interestingly, complementation with *cueO* had no effect on the copper/2,2'-dipyridyl-sensitive phenotype observed. Our hypothesis was that this sensitive phenotype was due to the expression of a truncated, inactive cytoplasmic version of CueO that was acting as a copper sink, preventing copper from being sequestered or exported by components of copper homeostasis, and resulting in the copper-mediated production of ROS. We were able to generate the copper/2, 2'-dipyridyl sensitive phenotype in wild type cells by overexpressing a truncated version of CueO containing only the T1 copper binding site. Removal of one of the copper binding ligands (Cys₄₉₅) from the T1 binding site resulted in restoration of copper/2, 2'-dipyridyl sensitivity to wild type levels. Taken together, this research shows that unlike in the model organism *E. coli*, copper atoms in *Campylobacter jejuni* indeed cause oxidative stress by participating in Fenton chemistry. This work gives insight into how multiple resistance mechanisms work together in concert to bring about homeostasis within the cell, and demonstrates the importance of studying complex phenotypes in organisms other than *E. coli*.

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CHAPTER 2

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. Over expression of a cytoplasmic peptide derived from the copper-binding region of CueO also caused copper toxicity at normally sub-lethal concentrations. Expression of the peptide with the copper binding ligands mutated restored wild type sensitivities. Strains with mutations in either of the homeostatic genes did not colonize the avian host as well as wild-type strains, and copper in the feed exacerbated the colonization deficiency. Taken together, the results indicated that copper toxicity in *C. jejuni* is due to an inability to sequester cytoplasmic copper, resulting in an increase in copper-mediated oxidative damage.

Copper is a required micronutrient for most aerobic organisms where 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 can be toxic to living cells. Relative to other physiologically occurring metals, copper binds especially well to amine, thiolate and carboxyl ligands present in proteins, effectively inactivating them (1-3). Copper also displaces iron atoms in iron-sulfur clusters of intracellular enzymes forming coordination compounds with sulfur or thiolate ligands (3, 4). 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 (5, 6). In a process comparable to the Fe^{2+} -catalyzed Fenton reaction, Cu^+ can also react with H_2O_2 produced via oxygen metabolism to generate the hydroxyl radical ($\text{HO}\bullet$). 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 (7-10). The main players of the *cue* detoxification system in *E. coli* are *copA* and *cueO*, which encode the complimentary 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 (11). CopA expression is turned on under micromolar copper concentrations (12); therefore basal cytosolic copper levels are kept quite low (10). CueO is a periplasmic multicopper oxidase, an enzyme that

couples the oxidation of Cu^+ with the four electron reduction of O_2 to water (13, 14).

CueO itself is also a copper-containing protein, having 4 structural copper atoms and up to 3 atoms associated with a methionine-rich region that covers the active site (15). The synergistic effect of CopA and CueO together is capable of controlling 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, Miller Fisher syndrome, and post infectious irritable bowel syndrome (PI-IBS) (16-18). The genome sequence of *C. jejuni* NCTC 11168 contains 2 proteins annotated as copper enzymes: a *cbb3*-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) (19). Two proteins in 11168 have been characterized as being involved in copper homeostasis: a multicopper oxidase termed CueO and a $\text{P}_{1\text{B-1}}$ -ATPase transporter termed CopA (20). Purified CueO was determined to be a cuprous oxidase, and growth was inhibited by added copper to both *cueO* and *copA* mutants (20). CueO was shown to have a higher enzyme efficiency towards the metal ions Cu^+ and Fe^{2+} than aromatic substrates, which is probably due to an occlusion of the binding pocket by secondary structure elements that would prevent entry from larger substrates (21). The *C. jejuni* CueO structure contains 2 copper centers, a type 1 blue copper center, (T1), and a trinuclear cluster comprised of 1 type 2 (T2) copper, and two type 3 (T3) copper atoms (21). A Met-rich region (residues 355-399) that makes up part of the secondary structures

responsible for blocking the T1 binding site from larger substrate access has also been proposed for binding labile exogenous Cu^+ atoms (15, 22). The *C. jejuni* multicopper oxidase crystal structure contains 4 copper atoms (21), however, isolated CueO is reported to contain 6.4 atoms per peptide (20), suggesting that the *Campylobacter* may bind non-structural copper atoms similarly to *E. coli*.

In our exploration into the mechanism for the toxic effect of copper in *C. jejuni*, we have found that susceptibility to oxidative stress is a byproduct of disruption of copper homeostasis. Mutations in either *copA* or *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. This finding was surprising, as pre-treatment with copper in *E. coli* resulted in strains being less sensitive to killing by H_2O_2 (23), leading to the conclusion that copper blocks iron-mediated H_2O_2 killing. In *C. jejuni*, however, this is not the case. 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 . Taken together, we conclude 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 the model systems such as *E. coli*.

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 at 37°C microaerobically in a tri-gas incubator (model 550D; Fisher Scientific), and maintained with 12% O₂, 5% CO₂, and 83% N₂. Liquid cultures were grown in Mueller-Hinton broth (MHB) (Difco, Sparks, MD). Growth media was supplemented with chloramphenicol (12.5 µg/ml), and tetracycline (20 µg/ml) as indicated and incubated at 37°C microaerobically. Genetic manipulations were performed using the *E. coli* strain DH5α (Stragene) or *E. coli* GC10TM (Sigma-Aldrich). 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 (Table 2) were designed by the DNA analysis software MacVector using the sequenced strain 11168 (24). PCR amplifications were performed with EconoTaq® Plus 2X Master Mix (Lucigen Corporation, Middleton, WI) using genomic DNA isolated from either 11168 or 35925. The purified PCR products were cloned into pCRTM 2.1-TOPO®vector (Invitrogen), yielding pTOPO*copA*, and pTOPO*cueO* (Table 3). The cloned fragment from both plasmids was then excised by digestion with EcoRI and ligated to EcoRI-digested pKSAH3, to yield pK*ScopA*, and pK*ScueO* (Table 3). These plasmids were then digested with HindIII, and treated with T4 DNA polymerase and dNTPs to blunt the ends, and then ligated to the chloramphenicol acetyltransferase gene (*cat*) (25), which was isolated from pJMA-001 by SmaI digestion (26), and the resulting plasmids were termed pK*ScopA*::*cat*, and pK*ScueO*::*cat* (Table 3). SG9 (Δ *cueO*::CM35925) was generated similarly using genomic DNA isolated from 11168. The purified PCR product was cloned into pCRTM 2.1-TOPO®vector (Invitrogen), yielding pTOPO*cueOC* (Table 3).

The cloned fragment was excised by digestion with BamHI and XhoI and ligated to BamHI, XhoI digested pKS to yield pK*ScueOC* (Table 3). The plasmid was then digested with HindIII, and treated with T4 DNA polymerase and dNTPs to blunt the ends, and then ligated to the *cat* cassette, resulting in pK*ScueOC::cat* (Table 3).

In order to transform *C. jejuni*, electrocompetent cells (27) were incubated with 1 µg of plasmid DNA on ice for 10 minutes. The cells were added to a 2 mm electroporation cuvette and given a pulse of 2500 V in a ECM399 electroporator (BTX, San Diego, CA). 50 µl MHB was added to the cuvette, and the cells were incubated on ice for an additional 10 minutes, then plated onto cold, non-selective MHA (Difco, Sparks, MD), supplemented with 5% defibrinated sheep blood (Hemostat Laboratories, Dixon, CA). After incubating microaerobically at 37°C for 16 hours, the cells were transferred by loop onto the appropriate selective antibiotic plate (12.5 µg/ml chloramphenicol), and colonies appeared two days later (27, 28). Chloramphenicol-resistant colonies were screened by PCR for increase in amplicon size by agarose gel electrophoresis, which confirmed that the allelic replacement, or double crossover event, had occurred. This resulted in the disrupted targeted genes.

Construction of plasmids for overexpression and complementation of *cueO* and *copA*. Coding regions of *copA* and *cueO* were PCR amplified from 11168 genomic DNA using the primers CopA3 and CopA4 for *copA* and CueO3 and CueO4 for *cueO*. In a separate reaction, the *ompE* promoter was amplified using pMEK91 (29) as a template and the primers OmpEF and OmpER. OmpER was designed to have an 11 bp overlap with both the CopA3 and CueO3 primers that allowed for an overlapping PCR reaction. 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, generating 2 gene fusion products *ompEcopA*, and *ompEcueO*. The gene fusion products were subsequently cloned into the EcoRI digested pMEK91, generating the shuttle vectors pO*copA*, and pO*cueO*, (Table 3, Fig. 1B and 1E). The plasmids were confirmed as correct by restriction analysis and introduced into 35925, SG4 (*copA*₃₅₉₂₅::*cat*) and SG3 (*cueO*₃₅₉₂₅::*cat*) via electroporation using the same conditions as for mutagenesis. Transformed cells were spotted onto BA plates containing 20 µg/ml tetracycline. Tet^r colonies were passed and cultured under selection. The plasmids were isolated from the cultures via plasmid mini-prep (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 *cueO*78. The coding region corresponding to the last 78 aa of *cueO* was PCR amplified from 11168 genomic DNA using the forward primers CueO78, and the reverse primer, CueO4 (Table 2). 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, generating the gene fusion product *ompE*78. This gene fusion product was cloned into pCRTOP02.1 generating pTOPO78. The *ompE*78 gene fragment was excised with EcoRI, and subsequently subcloned into EcoRI-digested pMEK91, generating the shuttle vector pO78, (Table 3). pO78 was used to transform 35925 via electroporation. Tet^r colonies were passed and cultured under selection and the plasmid was isolated from the cultures via plasmid mini-prep (Qiagen), and confirmed via PCR amplification using the OmpEF and CueO4 reverse primers (Table 2).

Site directed mutagenesis. Mutations in the T1 copper binding site 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, LaJolla, CA) and the primers Cys495F and Cys495R. A single mutant was made by substituting the codon corresponding to alanine (GCC) for the codon corresponding to the cysteine (TGC) codon at position 495, resulting in the plasmid pTOPO78_{C495A}. The double mutant (C495, H500A) was generated similarly by substituting the codon corresponding to alanine (GCT) for the codon corresponding to a histidine codon (CAT) at position 500 using the primers His500F and His500R and pTOPO78_{C495A}, resulting in the plasmid pTOPO78_{C495H500A}. Mutations were confirmed by sequencing through Eton Bioscience, Inc. (Research Triangle Park, NC). Both plasmids were digested with EcoRI, and the gene fusion sequences were subcloned as above into EcoRI digested pMEK91, and used to transform 35925 via electroporation. The plasmids were also isolated from the cultures via plasmid mini-prep (Qiagen), and confirmed to contain *cueO78*, via PCR amplification using the OmpEF and CueO4 reverse primers (Table 2).

Copper sensitivity. In order to determine Minimum Inhibitory Concentration (MIC) for copper, cultures of *C. jejuni* were grown to midlog (OD₆₀₀ ~0.3) in Mueller Hinton broth then adjusted to OD₆₀₀ 0.1. Cultures were grown overnight in MHB supplemented with CuSO₄ at concentrations ranging from 0 to 6 mM. Serial dilutions of the cultures were spotted onto BA plates. Colonies were counted two days later and CFU/ml determined. The MIC was calculated as the lowest concentration of CuSO₄ in which the final CFU/ml was equal to or lower than the inoculated dose. Values are the

average of at least 3 biological replicates, standard deviations are not supplied as for 8 of the 10 conditions the MIC were the same for all assays.

Methyl viologen disk diffusion assay. Sterile filter paper disks 7.5 mm in diameter containing 0.5 mM methyl viologen were placed on MHA plates containing either 0 mM or 0.125 mM CuSO₄ that had been streaked for confluent growth. The cells were incubated overnight and the zone of inhibition measured. Each cleared zone was measured twice at perpendicular angles, and the two measurements were averaged. Values indicated are the average of at least 3 biological replicates for each strain/condition.

Methyl viologen culture sensitivities. Cultures of *C. jejuni* were grown to midlog (OD₆₀₀ ~0.3) in MHB supplemented with 0.125 mM CuSO₄, and adjusted to OD₆₀₀ 0.2. An initial aliquot of cultures was removed and serially diluted to determine the initial CFU/ml, the remaining cultures were exposed to either 0.5 mM or 5.0 mM methyl viologen for one hour, then serially diluted into PBS, plated onto BA, and incubated microaerobically at 37°C. After two days of incubation, colonies were counted and recorded. Log₁₀ reductions were calculated.

H₂O₂ sensitivity. *C. jejuni* was grown to midlog (OD₆₀₀ ~0.3) in MHB supplemented with 0.125 mM CuSO₄, and adjusted to OD₆₀₀ 0.3. 2,2' dipyridyl was added to the cultures to a final concentration of 0.1 mM. After a 30-minute incubation, an aliquot was removed and serially diluted to determine the initial CFU/ml. The cultures were then challenged with 1 mM H₂O₂ for one hour, and serially diluted into cold PBS. The first dilution in the series contained 4000 U/ml bovine catalase (Sigma-Adrich) to degrade any residual H₂O₂ present. The dilutions were then plated onto BA, and

incubated at 37°C microaerobically. After two days of incubation, colonies were counted and recorded. Log₁₀ reductions were calculated as for methyl viologen sensitivity.

2,2' dipyridyl sensitivity. Cultures were grown to midlog (OD₆₀₀ ~0.3) in MHB supplemented with 0.125 mM CuSO₄, then adjusted to OD₆₀₀ 0.3. An initial aliquot was taken to determine initial CFU/ml, then the remaining cultures were exposed to 0.1 mM 2,2' dipyridyl for one hour, then serially diluted into PBS, plated onto BA, and incubated microaerobically at 37°C. After two days of incubation, colonies were counted and recorded. Log₁₀ reductions were calculated as for methyl viologen sensitivity.

Quantitative reverse transcriptase polymerase chain reaction. The PCR primers designed for qRT-PCR are listed in Table 2 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 35925, SG2 (*copA*_{35925::cat}), SG3 (*cueO*_{35925::cat}), 35925[pO*copA*], and 35925[pO*cueO*] using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre Technologies Corporation, Madison, WI). qRT-PCR was performed as previously described (28) 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 separated into groups of 10 in Petersime brooder batteries. Chicks either fed commercial chicken feed (containing 8 mg Cu/kg), or the same feed diet supplemented with 188 mg CuSO₄/kg diet (30). On day 7 the chicks were inoculated by oral gavage with 0.1 ml PBS containing either 10⁷ CFU/ml of wild type

11168, SG1 (*cueO*₁₁₁₆₈::*cat*), or SG2 (*copA*₁₁₁₆₈::*cat*). The birds were kept on the same feeding regimen (+/- copper) following inoculation. Two weeks after inoculation, the birds were humanely sacrificed by CO₂ asphyxiation, and approximately 1 gram of cecal contents were collected, serially diluted (in PBS), and plated on selective BA containing 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/g of cecal contents was calculated. All procedures were approved by the NC State Institutional Animal Care and Use Committee under protocol #12-156-A.

Results

Strains and Plasmids. Five strains of *C. jejuni* were tested for sensitivity to copper, and MIC's ranged from 1.67 mM to 4.64 (Table 4). Two strains of *C. jejuni* were chosen further characterize the copper detoxification systems. 11168 had the lowest MIC, and was chosen because it was used in the initial characterization of CopA, and CueO, while 35925 showed the highest MIC, and is readily transformable with *E. coli*-*C. jejuni* shuttle vectors. Insertion mutants were isolated for both *copA* and *cueO* in both parental backgrounds (Table 1, Fig. 1A and 1C). A *cueO* deletion mutant was also generated in both parental backgrounds, in which 1104bp of the coding region was deleted and replaced with the *cat* cassette (Table 1, Fig. 1D).

Plasmids that express *copA* and *cueO* were constructed (Fig. 1B and 1E). The *ompE* promoter was used for expression to ensure transcription of the cloned genes. We chose to use this promoter because the native promoter regions are likely distant from the open reading frame, and *ompE* promoter has been shown to efficiently drive transcription

within the shuttle vector pMEK91, which was used as the backbone for these plasmids. Quantitative RT-PCR confirmed that *copA* is overexpressed ~50 fold over the wild type in the strain harboring pO*copA* and *cueO* is overexpressed ~ 6.5 fold over wild type in the strain harboring pO*cueO* (Fig. 2A and 2B). qRT-PCR of *cueO* downstream of the *cat* cassette indicates that the insertion does not disrupt downstream transcription (Fig. 2B), meaning the remaining portion of *cueO* and the downstream genes *moaD*, *moaE*, and *moaA2*, are still transcribed.

Copper inhibits growth of the both mutants. Strains with mutations in *copA* and *cueO* have previously been shown to be growth inhibited by copper, as measured by reduced optical density in a 16-hour end point assay (20). In order to learn more about the nature of the growth inhibition of copper, we conducted a series of experiments to determine the effect of copper on these strains. These included determining the maximum amount of copper the strains could withstand via Minimum Inhibitory Concentrations (MIC's), and the effect of sub-lethal levels of copper on growth rates. MICs were determined for 5 different *C. jejuni* strains as well as the strain/plasmid combinations that were generated (Table 4, Table 5). Copper was inhibitory to all strains tested, including the parents, but there were significant differences between strains. Strain 11168 exhibited a MIC of 1.67 mM CuSO₄, while SG1 and SG2 had MIC's of 0.55 mM CuSO₄ and 1.10 mM CuSO₄ respectively (Table 5). 35925 had a higher overall resistance to added copper, the parent strain having an MIC of 4.64 mM CuSO₄, with SG3, SG9, and SG4 having MICs of 1.75, 1.50, and 0.50 mM CuSO₄, respectively. Complementation with plasmids expressing either *cueO* or *copA* restored copper resistance in the SG3 and SG4 mutants to the level of the parent (Table 5).

Overexpression of *copA* increased the MIC over the wild-type strain from 4.64 mM to 5.00 mM CuSO₄, demonstrating over-expression of a copper efflux pump increased copper resistance. Overexpression of *cueO* also increased the MIC to 4.90 mM CuSO₄ (Table 5). Introducing the self-replicating plasmid containing the 78 aa truncated version of *cueO* into 35925 wild-type cells lowered the MIC to 2.69 mM CuSO₄. Introduction of the self-replicating plasmids pO78_{C495A}, and pO78_{C495His500A} into wild type cells, however, resulted in MICs of 4.81 and 4.83 mM CuSO₄ respectively.

In addition to MIC's we performed growth curves in such a way that we could determine the effect of copper on the final density of cultures and calculate the effect of copper on growth rate. Each strain was grown in triplicate under microaerobic conditions in MHB containing 0mM, 0.125mM, or 0.5mM added copper. These values were chosen as they are all below the MIC for each strain. All strains exhibit sensitivity to added copper, as shown by an increase in generation time in response to increasing copper concentrations (Table 6). Additionally, even at levels below the MIC, the mutant strains had slower growth rates, with the exception of SG 3, the *cueO* mutant in the 35925 background, which had doubling times similar to those of the wild-type strains in the 0.125mM and 0.5mM copper conditions (Table 6).

The *copA* and *cueO* mutants are deficient in host colonization. In birds *Campylobacter* colonizes the epithelial mucosa of the gastrointestinal tract, particularly the ceca, where they can reach very high numbers, as many as 10⁹ CFU/ml in birds that have been experimentally challenged (31). In order to determine the ability of the mutants to colonize in a chicken model, based on the effect of copper present in the chicken gastrointestinal tract, colonization studies were carried out as previously described (32).

Day of hatch chicks were separated into groups of 10 in Petersime brooder batteries, and were feed either fed commercial chicken feed (containing ~8mg Cu/kg), or the same feed diet supplemented with copper 188 mg CuSO₄/kg. At one week, the chicks were inoculated with ~10⁷ *C. jejuni* cells in 1 ml PBS by oral gavage. Three weeks post-inoculation the chickens were sacrificed by CO₂ asphyxiation and the cecal contents were collected for enumeration of viable CFU/g. The *copA* mutant colonized the cecum at significantly lower levels than wild type cells (Fig. 8). 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 wild-type cells in the presence of copper (p<0.01), and without added copper to the feed.

The *copA* and *cueO* mutants are sensitive to oxidative stress. We wanted to investigate the possibility that either increased cytoplasmic copper (as you would expect in the *copA* mutant) or an increase in Cu⁺ contributed to the lethality via an increase in oxidative stress. In order to do so, we performed a series of disk diffusion assays using the oxidative stress reagent methyl viologen, a cell permeable redox active dye that uses cellular reducing potential to catalyze a single electron reduction of O₂ to produce superoxide (33). Two types of assays were used to determine the methyl viologen sensitivity. In the first, disks saturated with 50 mM methyl viologen were placed onto the center of a MHA plate that had been inoculated with the strains. After overnight growth under microaerobic conditions, the inhibition zones were measured (Table 7). In both backgrounds, the *copA* and *cueO* mutants were more sensitive to methyl viologen, and the sensitivity increased when 0.125 mM CuSO₄ was included in the media. Copper only had an effect on the mutant strains; the parent strains displayed no difference in the zones

of inhibition in the copper and no copper conditions. The *cueO* mutants, SG1, and SG3 plated on the copper-containing medium had significantly larger zones of inhibition than wild type when plated on MHA alone (Table 7). Similarly, the *copA* mutants SG2, and SG4 had significantly larger zones of inhibition when plated on copper-containing MHA (Table 7).

Broth grown cultures were also tested for sensitivity to methyl viologen. The 11168 strains were grown in the presence of 0.125mM CuSO₄ to midlog phase, and challenged with 0.5 mM methyl viologen for one hour. The copper treated SG2 mutant was significantly more sensitive to methyl viologen than wild type (Fig. 3). 35925 strains are less sensitive to methyl viologen so these strains were grown similarly, in the presence of 0.125mM CuSO₄ to midlog phase, but challenged with 5.0mM methyl viologen. Both mutants were significantly more sensitive to methyl viologen than wild type in this background (Fig. 3).

Sensitivity to hydrogen peroxide was also tested using the log reduction assay. Cells were grown with or without 0.125 mM CuSO₄ to midlog (absorbance at 600nm of ~0.3 in MHB), treated with the chelator 2,2' dipyridyl for 30 minutes, and all cells were then challenged with 0.1 mM H₂O₂ for one hour. 2,2' dipyridyl pretreatment was required to mitigate iron-mediated Fenton-based hydroxyl radical formation, as copper can potentially displace iron from iron-sulfur clusters (34). After 1 hour, the cells were serially diluted and plated onto blood agar. CFU/ml were 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 as compared to wild type (Fig. 4A, B), or the complemented *copA* mutant, SG8 (Fig. 4B). No data is presented

for SG3 as the combination of 2,2' dipyridyl and copper alone for 1 hour resulted in a severe reduction of viability (Fig. 5A), leaving too few cells to accurately measure H₂O₂ mediated killing.

The combination of copper and 2,2' dipyridyl is toxic to SG3. In the course of performing H₂O₂ sensitivity experiments, a curious phenotype was discovered regarding SG3. This strain was showing high levels of lethality prior to being treated with H₂O₂, but only in the + copper conditions. Strains (+/- copper pre-incubation) were exposed to 2,2' dipyridyl for 1 hour and serially diluted to determine viability loss compared to the 0 time point. The results, (Fig. 5), show that even in the absence of any exogenously added ROS copper-loaded SG3 displays 1.5 log reduction, while the parent strain loses no viability in the same condition. The complemented strain, carrying the p*OcueO* plasmid, SG3[p*OcueO*] also showed a similar log reduction after exposure to copper and 2,2' dipyridyl (Fig. 5). We were surprised that complementation did not rescue the 2,2' dipyridyl-sensitive phenotype, as the plasmid clearly rescues both copper sensitivity (Tables 3 and 5) and methyl viologen sensitivity (Fig. 3). The mutation within *cueO* involves disrupting the coding sequence of the gene with a drug resistance cassette (*cat*) at nucleotide 160 of the 1,543-nucleotide gene (Fig. 1B). While this insertion certainly renders the enzyme inactive, qRT-PCR reveals that the transcription of the open reading frame downstream of the cassette occurs at wild-type levels (Fig. 2B). This 1,383 nt region includes 11 methionine residues (in-frame ATG's), and translation from any of these would result in a truncated version of CueO that is potentially constrained to the cytoplasm, as the TAT secretion leader sequence is 5' of the cassette. Additionally, these residues are possible copper-binding ligands (21), therefore, preloading the cells with

copper is critical for the 2,2' dipyridyl toxicity, as cells not pre-exposed to copper did not lose viability after the 2,2' dipyridyl treatment (Fig. 5).

We generated SG9 ($\Delta cueO_{35925::cat}$) to determine that if by deleting 1105 nt of the gene (including 10 of the 11 methionine residues), the sensitivity to the combination of 2,2' dipyridyl and copper would be alleviated. SG9 however, also displayed a 2,2' dipyridyl-copper sensitive phenotype (Fig. 6). p*OcueO* was transformed into SG9 to generate the complemented strain SG9[p*OcueO*], and similarly sensitive (Fig. 6), suggesting that the mutation with SG9 is dominant negative. In order to test this hypothesis, we created a plasmid (pO78) which would express a 78 amino acid peptide from the naturally occurring methionine residue 437 (Fig. 9). This truncated version includes the copper binding ligands (H₄₃₉, C₄₉₅, and H₅₀₀) that make up the T1 Cu site. 35925[pO78] was significantly more sensitive to 2,2' dipyridyl and copper than wild-type cells alone (Fig. 7). Substitution of an alanine for the C₄₉₅ residue through site-directed mutagenesis resulted in cells that were significantly less sensitive to 2,2' dipyridyl and copper (Fig. 7). Substitution of an alanine for both the C₄₉₅ and H₅₀₀ residues had a similar effect (Fig. 7).

Discussion

Failure to strictly regulate the concentration and oxidation state of cell-associated copper results in severe consequences (8, 35, 36), yet cellular targets and the mode of action of copper toxicity is still unsettled. Copper-mediated ROS production is implicated in eukaryotic cells and tissue damage, as both DNA damage (35) and lipid oxidation is found within copper-induced liver tumors (37). In *E. coli*, however, the presence of excess copper is protective against ROS, and it was concluded that copper

accumulation does not promote Fenton-like chemistry (23). In the course of characterizing the copper tolerance in *C. jejuni*, we have discovered that it is a ideal model for testing the direct effects of copper-mediated damage, as confounding protective effects are minimal in the presence of added copper. A survey of *C. jejuni* strains shows that most have MIC's for copper in range from 1.9-4.5 mM CuSO₄ (Table 4). We chose to further examine the most resistant of the 5 tested strains (35925) as well as the, least resistant (11168), which was the strain in which the copper detoxification genes *cueO* and *copA* were originally characterized.

Mutations for both of the copper homeostasis genes *cueO* and *copA* were made, with and confirmed to be required for copper resistance. The inclusion of strain 35925 (which accepts shuttle vectors) allowed us to investigate over-expression of the gene. 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 5). In addition to MIC, we assayed the effect of sub-lethal concentrations of CuSO₄ on growth. Copper supplementation had a small, negative effect on generation time for all strains, especially the mutants (Table 6). The 0.125 mM condition was subsequently chosen as our condition for “copper loaded” cells for all further tests.

The importance of copper resistance in host colonization was confirmed. *C. jejuni* colonizes the epithelial mucosa of the chicken gastrointestinal tract, particularly the ceca, where they can reach very high numbers, as many as 10⁹ CFU in birds that have been experimentally challenged (31). Chickens have a high tolerance for excess copper in feed, and are oftentimes fed diets with high copper levels (100-200mg/kg diet) both as a growth promoter and to prevent enteritis (30). 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 in the high copper (188 mg CuSO₄/kg diet) condition, as we were unable to recover any *C. jejuni* 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 H₂O₂ or the superoxide-generating dye methyl viologen. Two methods were used, a disk diffusion assay which is a proxy for the maximum concentration of drug used to inhibit growth, and a cell killing assay in which the initial rate of cell death is monitored in response to a lethal concentration of drug. In the disk diffusion assay, mutants were far more sensitive (larger zone of inhibition) when copper loaded, while the parent strains had the same sensitivities in both conditions. Over-expression of either *copA* or *cueO* was protective, having smaller zones of inhibition than the wild type strain (Table 7). Addition of lethal concentrations of methyl viologen to broth grown cultures yielded similar results (Fig. 3). Mutant cells grown in copper-supplemented conditions were more sensitive to methyl viologen, those grown without copper did not show increased killing. The sensitive phenotype is rescued by complementation of both genes in the 35925 background.

H₂O₂ killing was tested, as H₂O₂ is converted to the powerful oxidant hydroxyl radical, which can be catalyzed by either Fe²⁺ or Cu⁺ (38, 39). In these assays, pre-treatment with the cells with the iron chelator 2,2' dipyridyl, was used to differentiate Cu⁺ vs Fe²⁺ catalyzed ROS. Although 2,2' dipyridyl can bind both metals, 2,2' dipyridyl prevents iron-mediated HO• production (40), yet 2,2' dipyridyl-copper complexes still allow for Cu⁺ - catalyzed H₂O₂ reduction (23). Both mutants were significantly more

sensitive to H₂O₂ than the parents, but only in the copper-loaded condition. The addition of 2,2' dipyridyl made copper-loaded *C. jejuni* cells more sensitive to challenge with H₂O₂.

In the course of our H₂O₂ killing assay, we unexpectedly found that the combination of copper and 2,2' dipyridyl is toxic to some *cueO* mutants, even in the absence of added ROS. This phenotype is dominant negative, in that complementation with intact *cueO* does not restore the parental phenotype. We investigated the possibility that the phenotype is due to the presence of a truncated, cytoplasmic, version of CueO that still binds 1 (or more) of the 6 associated copper atoms found in full-length *cueO* (20). In both mutants, the TAT secretion signal is deleted, and the orientation of the insertion cassette allows for transcription of the 3' (downstream) portion of the gene (Fig. 2). Using expression plasmids, we were able to mimic the 2,2' dipyridyl-copper toxicity with peptide fragments as small as 78 aa (Fig. 7), and this peptide contains all of the ligands for the Cu1 atom seen in the crystal structure of CueO (21) (Fig. 9). Removal of copper-binding ligands through substitution of the C₄₉₅ residue of the substrate-binding site in CueO, restored the wild-type phenotype, providing compelling evidence that a copper atom coordinated in the T1 Cu binding site is required for the toxicity.

Overall, this work demonstrates that in *C. jejuni*, disruption of the cell's copper homeostatic mechanisms increases the susceptibility to ROS. This is in contrast to work done in *E. coli*, in which copper treatment makes the cells more resistant to ROS, and the authors concluded that copper does not directly participate in ROS production *in vivo* (3). These authors propose that instead, copper toxicity results from the inactivation of FeS-containing enzymes, and allowing free iron to participate in Fenton chemistry. 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 the copper augmented cell killing by H₂O₂ (Fig. 4). Second, even in the absence of imposed ROS, 2,2' dipyridyl and copper have a significant killing effect on the CueO mutants (Figs. 5 and 6). 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. 7). The mechanism of toxicity is likely due to disruption of cellular copper homeostatic mechanisms due to its copper-binding properties. Alterations of the predicted copper-binding ligands results in a form of the peptide that is no longer lethal to the cell (Fig. 7), confirming that copper binding is crucial to for the phenotype. Taken together, we conclude that any disruption of the normal cellular copper homeostatic mechanisms (by mutation or introduction of a novel copper binding peptide) allow for copper participation in the creation of ROS and cell killing.

The cytoplasmic pathways for copper accumulation, sequestration, and ultimate mobilization into the copper-containing enzymes are not fully known. These pathways are almost certainly comprised of here-to-for unidentified copper binding chaperones that perform these functions while keeping copper in a non-toxic form. The 78 aa peptide produced from pO78 obviously disrupts this pathway, perhaps through keeping copper from entering the system or being recognized as a substrate for the final “copper-insertase.” We believe this serendipitous finding may be an important tool for identifying and characterizing these copper metabolic pathways in the future.

Table 1. Strains used in this study

| Strain | Description of antibiotic cassette if any | Source |
|------------------|--|--------------------|
| <i>C. jejuni</i> | | |
| RM 1221 | Wild type | Deborah Threadgill |
| 81-176 | Wild type | Alain Stintzi |
| 35918 | Wild type | ATCC ^b |
| 11168 | Wild type | NCTC ^a |
| SG1 | <i>cat</i> inserted within <i>cueO</i> in 11168 | This study |
| SG10 | <i>cat</i> inserted within Δ <i>cueO</i> in 11168 | This study |
| SG2 | <i>cat</i> inserted within <i>copA</i> in 11168 | This study |
| SG12 | <i>aphA-3</i> inserted within <i>copA</i> in 11168 | This study |
| 35925 | Parent strain for <i>C. jejuni</i> strains (WT) | ATCC ^b |
| SG3 | <i>cat</i> inserted within <i>cueO</i> in 35925 | This study |
| SG9 | <i>cat</i> inserted within Δ <i>cueO</i> in 35925 | This study |
| SG4 | <i>cat</i> inserted within <i>copA</i> in 35925 | This study |
| SG13 | <i>aphA-3</i> inserted within <i>copA</i> in 35925 | This study |
| <i>E. coli</i> | | |
| DH5 α | F- Φ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (rK-, mK+) <i>phc1 gyrA96 relA1</i> | Invitrogen |
| GC10 | F- <i>mcrA D(mrr-hsdRMSmcrBC) F80dlacZDM15 DlacX74 endA1 recA1 D(ara, l galU galK nupG rpsL l- T1R</i> | Sigma |

^aNCTC – National Collection of Type Cultures

^bATCC – American Type Culture Collection

Table 2. Primers used in this study

| Primer | Description | Source |
|------------|--|---------------------|
| Cj1161cF | 5'- TGCATGAATTTGAAATCGGGC -3' | Eurofins MWG Operon |
| Cj1161cR | 5'- TCTGCAACAATGCTTTCTCCTTC -3' | Eurofins MWG Operon |
| Cj1516F | 5'- GCTTCCCAAAGTCCGCTACAAG -3' | Eurofins MWG Operon |
| Cj1516R | 5'- GAGGATGTGGATGATACCAGTAAGTTC -3' | Eurofins MWG Operon |
| CueOF | 5'- TTCAAACCTTTAGAAGAACCTAAGG -3' | IDT |
| CueOR | 5'- CAAACTGGCGGTAAAAGTGC -3' | IDT |
| OmpEF | 5'- GAATTCGGCTTTTTAGATCTCTTTAG-3' | Eurofins MWG Operon |
| OmpER | 5'- CATTAAAGCTTTCTCCTTGTCAAA-3' | Eurofins MWG Operon |
| CopA3 | 5'-GGAGAAAGCTTAAATGGAAGAATTGCGTATA -3' | Eurofins MWG Operon |
| CopA4 | 5'- TTAAATTCTTTAAGTCTTAAAG-3' | Eurofins MWG Operon |
| CueO3 | 5'- GGAGAAAGCTTAAATGAATAGAAGAAATTTTTTAAA-3' | Eurofins MWG Operon |
| CueO4 | 5'- TTATTCCTTACTTCTAAATTC-3' | Eurofins MWG Operon |
| CueO78 | 5'- GGAGAAAGCTTAAATGGATCATCCTTTC-3' | IDT |
| Cj1161cRTF | 5'- TCTAGCGGGGTTTTTTTACTTGAAG-3' | Eurofins MWG Operon |
| Cj1161cRTR | 5'- GTGTTTTTTTGCCTTGTATGCGTTC-3' | Eurofins MWG Operon |
| Cj1516RTF | 5'- TATGGGTTTAGCAGGGGCTTTTGTG-3' | Eurofins MWG Operon |
| Cj1516RTR | 5'- GGAATTTGAGCGTTTTTCATCAAGACG-3' | Eurofins MWG Operon |
| Cys495AF | 5'- GGACTTAGAATGTATCATGGCCATATTTTAGAACATGAAGATTTAGG-3' | IDT |
| Cys495AR | 5'- CCTAAATCTTCATGTTCTAAAATATGGGCATGATACATTCTAAGTCC-3' | IDT |
| His500AF | 5'- CCCATCATTCTAAATCTTCAGCTTCTAAAATATGGGCATGATAC-3' | IDT |
| His500AR | 5'- GTATCATGCCCATATTTTAGAAGCTGAAAGATTTAGGAATGATGGG-3' | IDT |

Eurofins MWG Operon, Huntsville, AL; IDT, Integrated DNA Technologies, Coralville, IA

Table 3. Plasmids used in this study

| Plasmid | Description | Source |
|-------------------------------|---|----------------|
| PCRTOP02.1 | Cloning vector | Invitrogen |
| pBluescript II KS (+) | Cloning vector | Agilent |
| pKSAH3 | Cloning vector | This study |
| pJMA-001 | PGEM-T containing <i>cat</i> insert | Jay Andrus |
| pHP1 | <i>aphA-3</i> containing construct | David McGee |
| pMEK91 | GFP containing <i>E. coli</i> - <i>C. jejuni</i> shuttle vector | Michael Konkel |
| pMEK80 | <i>E. coli</i> - <i>C. jejuni</i> shuttle vector | Michael Konkel |
| pTOPO <i>copA</i> | <i>copA</i> PCR product inserted into PCRTOP02.1 | This study |
| pTOPO <i>cueO</i> | <i>cueO</i> PCR product inserted into PCRTOP02.1 | This study |
| pK <i>ScopA</i> | <i>copA</i> cloned from pTOPO <i>copA</i> into pKSAHindIII | This study |
| pK <i>ScueO</i> | <i>cueO</i> cloned from pTOPO <i>cueO</i> inserted into the 51bp region between BamHI and XhoI of pKS | This study |
| pK <i>ScopA::cat</i> | <i>cat</i> inserted into HindIII site of pK <i>ScopA</i> | This study |
| pK <i>ScueO::cat</i> | <i>cat</i> inserted into HindIII site of pK <i>ScueO</i> | This study |
| pK <i>SompEcopA</i> | <i>ompEcopA</i> PCR product inserted into the EcoRI site of pGEM | This study |
| pK <i>SompEcueO</i> | <i>ompEcueO</i> PCR product inserted into the EcoRI site of pGEM | This study |
| pO <i>copA</i> | <i>ompEcopA</i> inserted into the EcoRI site of pMEK91 | This study |
| pO <i>cueO</i> | <i>ompEcueO</i> inserted into the EcoRI site of pMEK91 | This study |
| pTOPO <i>cueOC</i> | <i>cueOC</i> cloned from NCTC 11168 into PCRTOP02.1 | This study |
| pK <i>ScueOC</i> | <i>cueOC</i> cloned from pTOPO <i>cueOC</i> inserted into the 51bp region between BamHI and XhoI of pKS | This study |
| pK <i>ScueOC::cat</i> | <i>cat</i> inserted into HindIII site of pK <i>ScueOC</i> | This study |
| pTOPO78 | <i>ompE78</i> PCR product inserted into PCRTOP02.1 | This study |
| pTOPO78 _{C495A} | <i>ompE78</i> _{C495A} PCR product inserted into PCRTOP02.1 | This study |
| pTOPO78 _{C495AH500A} | <i>ompE78</i> _{C495AH500A} PCR product inserted into PCRTOP02.1 | This study |
| pO78 | <i>ompE78</i> inserted into the EcoRI site of pMEK91 | This study |
| pO78 _{C495A} | <i>ompE78</i> _{C495A} inserted into the EcoRI site of pMEK91 | This study |
| pO78 _{C495H500A} | <i>ompE78</i> _{C495H500A} inserted into the EcoRI site of pMEK91 | This study |

Table 4. Minimum Inhibitory Concentrations^a of strains to copper

| Strain | MIC |
|-----------------------------|------|
| <i>C. jejuni</i> NCTC 11168 | 1.67 |
| <i>C. jejuni</i> ATCC 35925 | 4.64 |
| <i>C. jejuni</i> 81-176 | 3.60 |
| <i>C. jejuni</i> RM1221 | 1.71 |
| <i>C. jejuni</i> ATCC 35918 | 2.29 |

^aMIC expressed as mM CuSO₄

Table 5. Minimum Inhibitory Concentrations^a of strains to copper

| Strain | Condition | | | | | |
|-----------------------------|------------|----------|----------|--------|--------------------------|------------------------------|
| | No plasmid | [pOcopA] | [pOcueO] | [pO78] | [pO78 _{C495A}] | [pO78 _{C495H500A}] |
| <i>C. jejuni</i> NCTC 11168 | 1.67 | - | - | - | - | - |
| SG2 | 1.10 | - | - | - | - | - |
| SG1 | 0.55 | - | - | - | - | - |
| <i>C. jejuni</i> ATCC 35925 | 4.64 | 5.00 | 4.90 | 2.69 | 4.81 | 4.83 |
| SG4 | 0.50 | 5.0 | - | - | - | - |
| SG3 | 1.75 | - | 5.50 | - | - | - |
| SG9 | 1.50 | - | 3.75 | - | - | - |

^aMIC expressed as mM CuSO₄

Table 6. Generation times^a

| Strain | Condition | | |
|----------------------|----------------|---------------------|-------------------|
| | MH (no copper) | MH + 0.125mM copper | MH + 0.5mM copper |
| 11168 | 1.10 ± 0.33 | 1.20 ± 0.32 | 1.30 ± 0.30 |
| SG2 | 1.20 ± 0.27 | 1.30 ± 0.33 | 1.70 ± 0.30 |
| SG1 | 1.20 ± 0.19 | 1.50 ± 0.18 | 1.90 ± 0.33 |
| 35925 | 0.97 ± 0.04 | 1.10 ± 0.12 | 1.24 ± 0.05 |
| SG4 | 1.14 ± 0.11 | 2.00 ± 0.38 | 3.39 ± 1.17 |
| SG4[p <i>OcopA</i>] | 1.25 ± 0.07 | 1.38 ± 0.03 | 1.48 ± 0.03 |
| SG3 | 0.97 ± 0.19 | 1.01 ± 0.18 | 1.25 ± 0.25 |
| SG3[p <i>OcueO</i>] | 0.77 ± 0.03 | 0.91 ± 0.08 | 1.10 ± 0.20 |

^aResults are expressed as generation time (hours) ± the standard deviation.

Table 7. Methyl Viologen Disk Diffusion Assay^a

| Strain | Condition | |
|------------------------|----------------|---------------------|
| | MH (no copper) | MH + 0.125mM copper |
| 11168 | 14.4 ± 3.6 | 14.8 ± 3.8 |
| SG2 | 19.0 ± 1.0 | 21.7 ± 1.8 |
| SG1 | 19.0 ± 4.5 | 27.5 ± 8.7 |
| 35925 | 16.8 ± 1.5 | 16.8 ± 1.9 |
| SG4 | 9.2 ± 1.9 | 26.0 ± 1.3 |
| 35925[p <i>OcopA</i>] | 9.7 ± 2.7 | 12.3 ± 1.8 |
| SG4[p <i>OcopA</i>] | 10.8 ± 3.6 | 12.7 ± 6.6 |
| SG3 | 14.5 ± 3.3 | 21.7 ± 2.6 |
| 35925[p <i>OcueO</i>] | 10.3 ± 5.8 | 15.9 ± 4.1 |
| SG3[p <i>OcueO</i>] | 12.2±2.3 | 8.5±3.9 |

^aResults are expressed as the zone of inhibition (mm) around the disk ± the standard deviation.

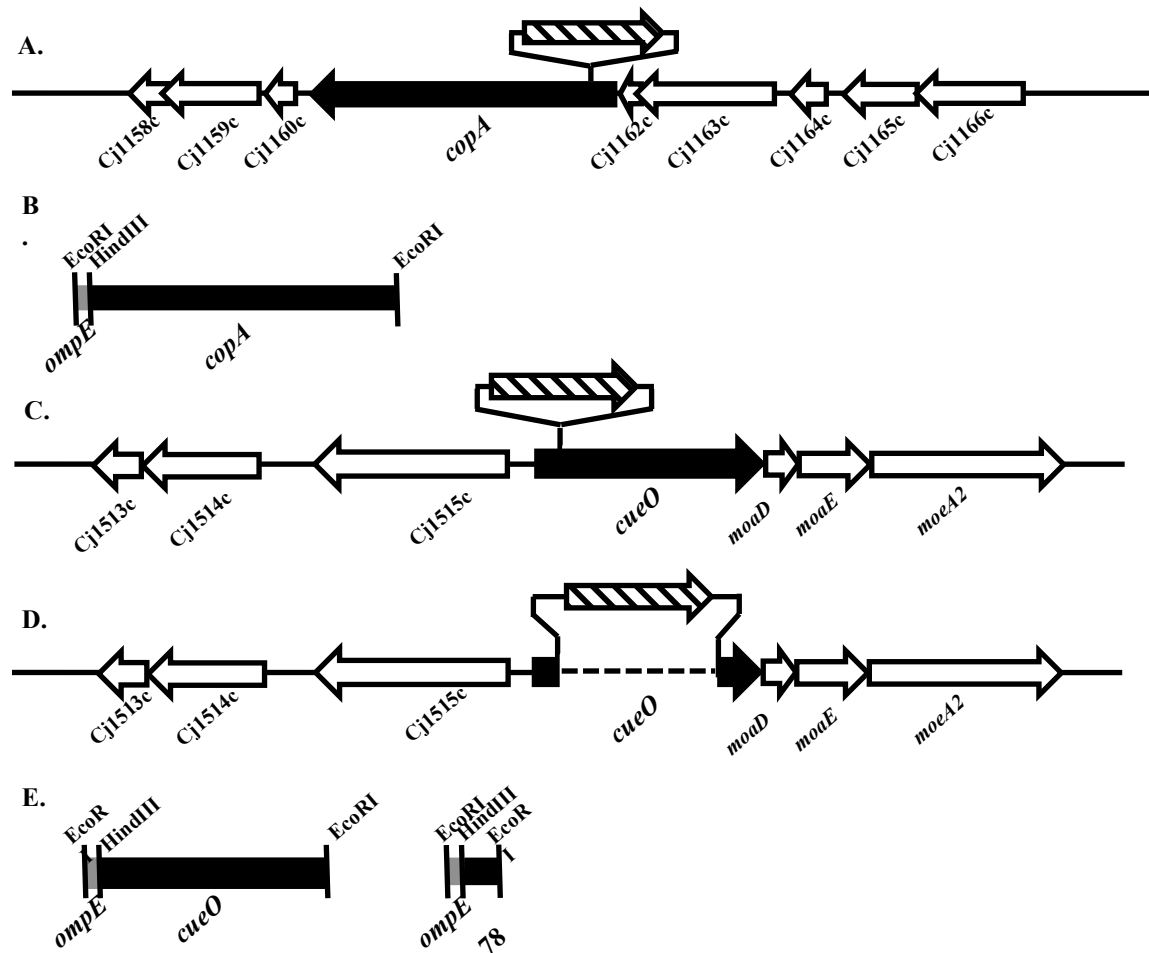


Figure 1. Graphical representation of the genomic context (for the sequenced strain NCTC 11168) of the genes studied in this work (black arrows) and surrounding genes (white arrows), in each case the direction of the arrow represents the orientation of the genes. (A) The *copA* (Cj1161c) locus. The striped arrow indicates the location of the *cat* cassette. (B) Representation of the promoter-gene fusion *ompEcopA* which was cloned into pMEK80 to form the expression plasmid pO*copA*. (C) The *cueO* (Cj1516) locus with the location of the inserted *cat* cassette (striped arrow). (D) Representation of the deleted region (dotted lines) in the mutant Δ *cueO*. (E) Representation of the promoter-gene fusions *ompEcueO*, and *ompE78*, which were cloned into pMEK80 to form the expression plasmids pO*cueO* and pO78 respectively.

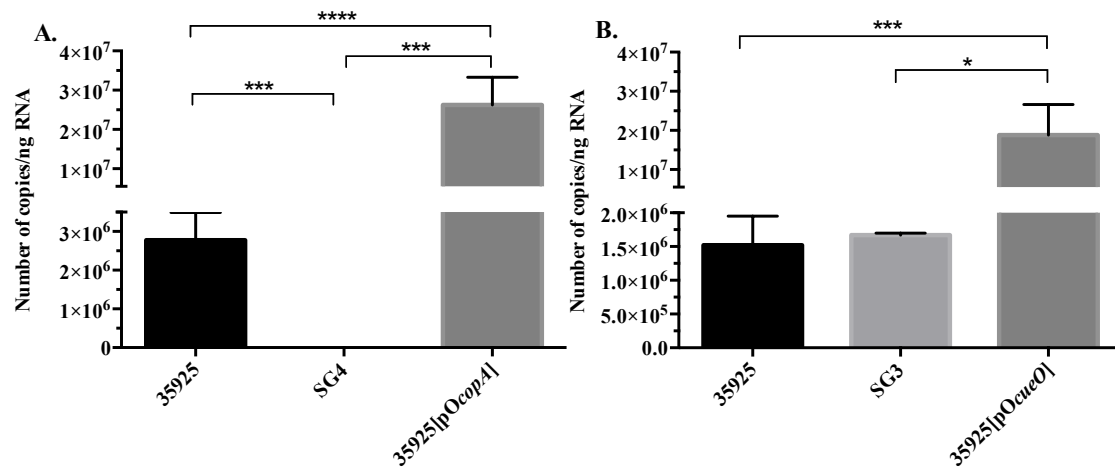


Figure 2. Expression level of *copA* in wild type 35925, SG4, and 35925[pO*copA*] (A), and *cueO* in wild type 35925, SG3, and 35925[pO*cueO*] (B). Expression levels are determined by qRT-PCR, and expressed as mRNA copies/ng total RNA. Error bars represent the standard deviation of three independent experiments.

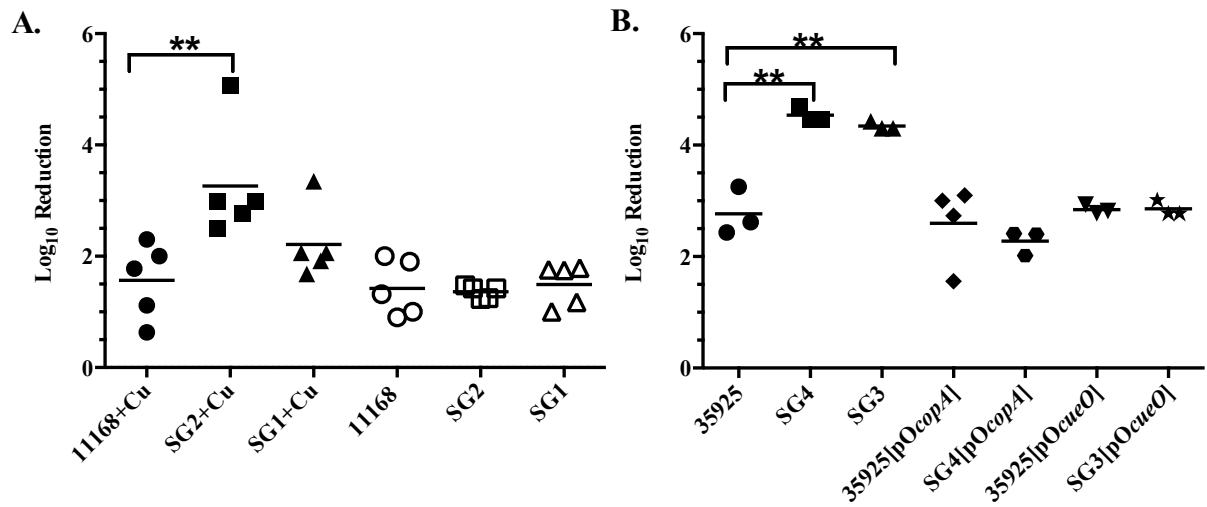


Figure 3. Sensitivities of strains to imposed oxidative stress by the addition of methyl viologen. The symbols represent Log₁₀ reduction values after 1 hour treatment with methyl viologen. (A) 11168 and derived strains exposed to 0.5mM methyl viologen and, (B) 35925 and derived strains exposed to 5.0mM methyl viologen. For each condition the bar represents the mean values, **p< 0.01.

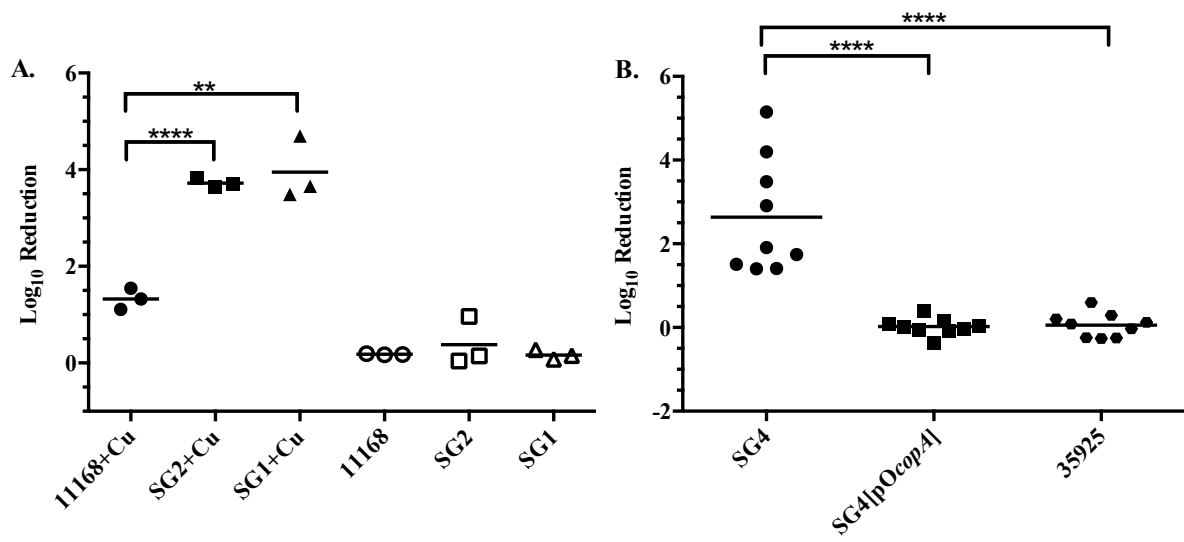


Figure 4. *copA* and *cueO* mutants are sensitive to oxidative stress induced by the addition of hydrogen peroxide. Log₁₀ reduction values after 1 hour treatment with 0.1mM H₂O₂ for (A) 11168, cells were pre-loaded by growth overnight with 0.125mM CuSO₄, where indicated, (B) and 35925. Overexpression of *copA* and complementation of SG4 result in wild type resistance to hydrogen peroxide. The bar represents the mean values. ** p<.01, **** p<0.0001.

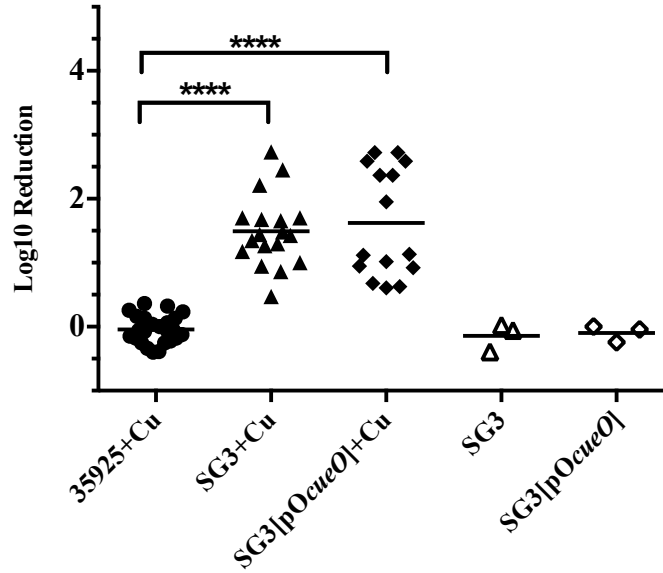


Figure 5. Copper-loaded cultures of SG3 and complemented SG3 are sensitive to 2, 2'-dipyridyl, even in the absence of added ROS. Symbols represent Log₁₀ reduction values after a 1 hour exposure to 0.1 mM 2, 2'-dipyridyl. **** p<0.0001.

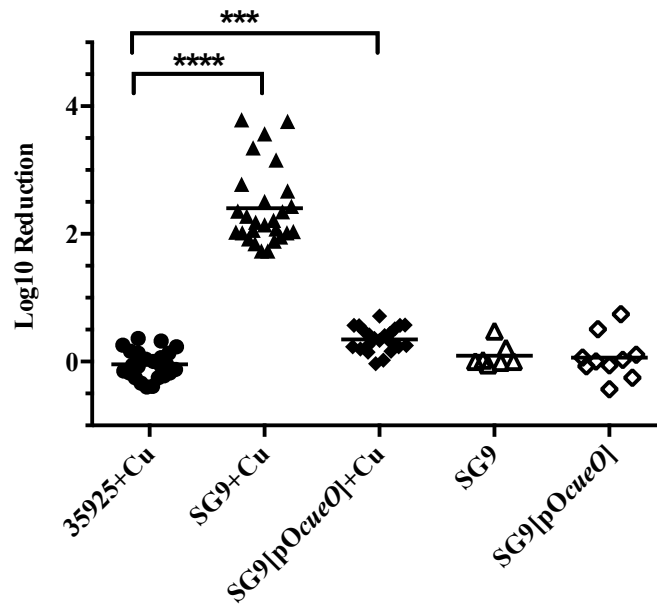


Figure 6. Copper-loaded cultures of SG9 and complemented SG9 are sensitive to 2, 2'-dipyridyl. Symbols represent Log₁₀ reduction values after a 1 hour exposure to 0.1 mM 2, 2'-dipyridyl. *** p<0.001, **** p<0.0001.

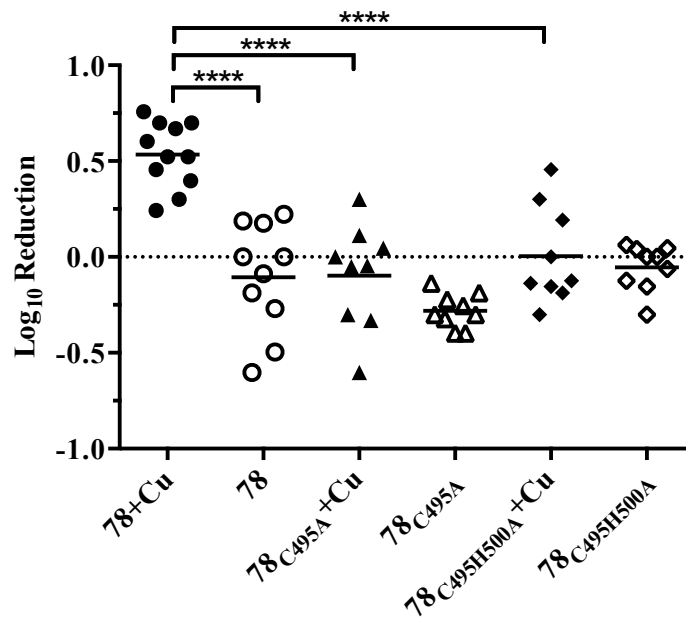


Figure 7. Copper-loaded cultures of 78 are sensitive to 2, 2'-dipyridyl. Symbols represent Log₁₀ reduction values after a 1 hour exposure to 0.1 mM 2, 2'-dipyridyl. The bar represents the mean values. **** p<0.0001

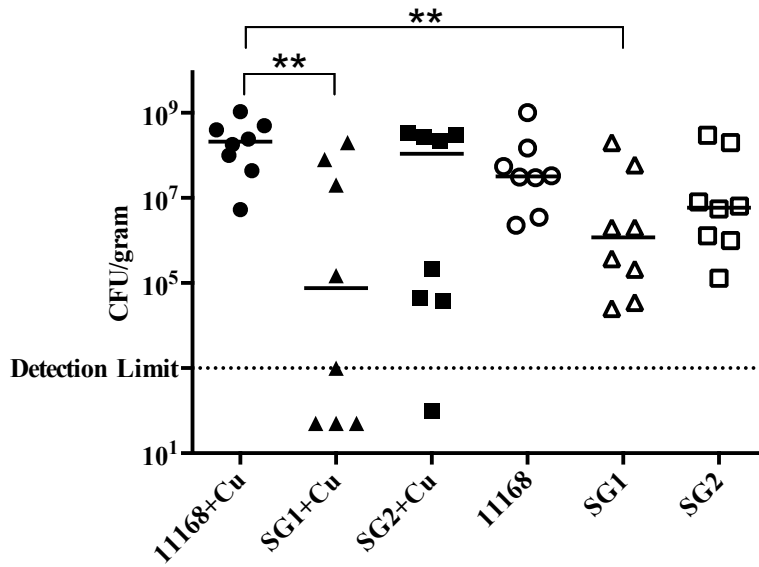


Figure 8. Host colonization. In a 3 week trial animals experimentally gavaged with 10⁶ CFU/ml of SG1 are significantly less colonized than those with wild type cells, especially when the feed was supplemented with CuSO₄. SG2 also colonized less than wild type, but the defect was less severe. Symbols represent CFU/gram recovered from the cecal samples of individual birds. Also shown are the median values and the detection limit for the assay, **, p<.01.

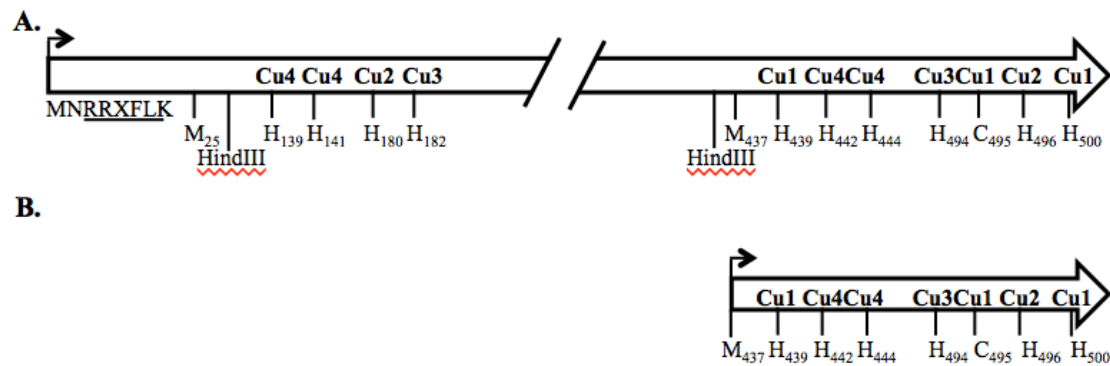


Figure 9. Graphical representation (not to scale) of *cueO*, including the Tat signal motif (underlined), the HindIII restriction sites used in mutagenesis, the copper binding sites, and the methionine residues in front of the copper binding sites. Only the N-terminal and C-terminal regions of the protein that contain copper binding residues are shown. (A) Full length protein. (B) The 78 aa version of *cueO*.

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Barriers to Horizontal Gene Transfer in *Campylobacter jejuni*

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Abstract

Campylobacter jejuni is among the most frequent agents of foodborne gastroenteritis in the world, but its physiology and pathogenesis is less well understood than other bacterial enteric pathogens. This is due in part to the incompatibility of the molecular tools that have enabled advances in the characterization of other bacterial species. Most notably, the dearth of plasmid-based complementation, reporter assays, and plasmid-based unmarked mutagenesis procedures in many of the type strains has hindered research progress. The techniques themselves are not inadequate in *Campylobacter* species, but rather the barrier to genetic transfer of these genetic constructs from non-*Campylobacter* cloning stains such as *Escherichia coli*. Here we review the modes of genetic transfer and the current state of each in *C. jejuni*. Also reviewed are two systems (CRISPR/Cas and Restriction modification) that are common to many strains of *C. jejuni* and should at least partly responsible for these barriers.

I. Introduction.

The evolution of organisms has traditionally been viewed as the vertical transmission (from parent to offspring) of naturally selected traits. Laterally acquired traits are also a major evolutionary force, this mechanism are termed horizontal gene transfer, or HGT. Recombination of genetic material has been shown to be an important evolutionary force in the human pathogen *C. jejuni*, generating twice the rate of evolution as mutation (1). It is also well documented that *C. jejuni* undergoes the best-characterized methods of HGT, transformation, conjugation, and transduction (2-4). Laboratory investigation of *C. jejuni* has not been able to use these powerful techniques to full effect, especially when compared to other gastric pathogens. Here we review the natural history of HGT in *C. jejuni*, and two of the systems that may provide the barriers to HGT. These include the recently characterized “bacterial immunity” modules named Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the CRISPR-associated (Cas) proteins (5), as well as the four types of restriction modification enzymes, all of which are represented in the genomes of sequenced *C. jejuni* strains (6).

II. Horizontal Gene Transmission

HGT involves the mobilization of genes from one organism to another laterally, and thus can greatly speed the scope of evolution. This is especially true among prokaryotic organisms, where all acquired traits will be passed on to subsequent generations. The exchange of genetic material has been shown to play an important role in bacterial evolution. Bacterial traits such as virulence factors, drug resistance, and metabolic properties, for example, are common outcomes of horizontal gene transfer (7).

Researchers have long taken advantage of a known natural mechanism of HGT to investigate biological processes. While HGT can lead to the acquisition of genes that provide improved fitness, unrestrained incorporation of foreign DNA into a genome comes with a fitness cost in that many mobile DNA elements are deleterious or pathogenic. Organisms must therefore erect barriers to HGT that select against DNA deemed dangerous while allowing some forms of HGT. This review will focus on the restriction barriers to HGT in the human enteric pathogen *Campylobacter jejuni*. The history of HGT in *C. jejuni* is a bit of enigma, as although it is quite apparent HGT has played a role in the evolution of *Campylobacter* species (spp.) (8), investigation into the physiology and pathogenesis of *C. jejuni* has been hampered by a HGT barrier that precludes many of the basic molecular techniques commonly used in other bacteria. A better understanding of the how HGT is blocked in *C. jejuni* will allow researchers to both get an understanding of the evolutionary history of *C. jejuni* and perhaps allow for the development of molecular tools that are unavailable at the present time.

By definition, all mechanisms of HGT involve the ability to physically transfer genetic information from a donor organism into a recipient organism. In bacteria, there are three generally recognized methods: bacteriophage mediated (transduction), the assimilation of naked DNA (transformation), and cell to cell mediated exchange (conjugation).

IIA. Methods of HGT: Transduction

Transduction is the transfer of DNA from one cell to another via a replicating virus. This exchange can occur either exclusively between prokaryotes (7), or between prokaryotes

and eukaryotes (9). The ability to transfer genes between species enables prokaryotes to adapt to new environments, greatly influencing prokaryotic diversification by not only transferring viral, but host genes (10). Phage mediated genetic exchange was described as early as 1951 in *Corynebacterium diphtheria*. It was demonstrated that virulence could be conferred to a nonvirulent strain of *C. diphtheria* after being infected with the phage strains that had previously infected the virulent *C. diphtheria* strain (11). Around the same time, Zinder and Lederberg documented phage-mediated genetic exchange between the *Salmonella enterica* serovars Typhi and Typhimurium and were the first to term this type of exchange “transduction” (12). Insight into the mechanism of transduction came from experiments with the *Escherichia coli* phage P1, demonstrating that small pieces of bacterial DNA are often incorporated into the phage capsid rather than phage DNA (13). Additionally, it has since been shown that phage-derived genes are found in most bacterial genomes (7). Phage genes that have been incorporated into bacterial genomes include virulence or fitness factors, such as exoenzymes, toxins and adhesins (14).

There are two main mechanisms by which genetic material is transferred between strains in bacteriophage-mediated transduction. Generalized transduction occurs during lytic replication when bacterial host DNA is picked up accidentally in place of viral genetic material, assembled into the capsid, and then integrated into a new host genome by recombination (15). The bacterial DNA incorporated into the transducing particle can be either chromosomal or plasmid DNA and this gets divided into segments similar in size to the phage genome (15). Because this is accidental, the likelihood of the production of

a transducing particle only rarely occurs (16). Perhaps the most well known examples of generalized transducing phages are P22 of *S. enterica* Typhimurium (12), and P1 of *E. coli* (17). Specialized transduction, on the other hand, occurs when bacterial genes located adjacent to a prophage integration site are excised along with the prophage during lysis. The phage and bacterial DNA are then incorporated through head-full packaging into the capsid and transferred into a new bacterial host via transduction (15). Because prophage integration sites occur more frequently on the chromosome, it is more likely that chromosomal DNA will be packaged into the transducing particles during specialized transduction (15).

IIB Methods of HGT: Transformation

Natural transformation occurs when competent recipient cells take up naked DNA from the environment (18). The idea of a non-heritable exchange of genetic information was first documented in 1928 with work on the pathogen *Streptococcus pneumoniae* (19). Griffith showed that virulence factors from a killed pathogenic strain could “transform” a nonvirulent strain to become virulent as well. Although it was unknown at the time of Griffiths experiment, DNA was determined to be the “transforming principle” responsible for the change from non-virulent to virulent that Griffith had documented in his classic experiments (20).

Species such as *S. pneumoniae* are naturally competent, competence is mediated by cell cycle, quorum sensing through secretion of a competence stimulating peptide, and specific competence proteins (21). Not all bacteria are naturally competent, however, but

can be forced to take up DNA using non-physiological techniques. *E. coli* was the first bacterium to be forced to become competent. Early transduction gave way to using helper phage-mediated transformation, to the development of a method using calcium ions to improve competency. This method allowed for successful transformation using recipient and donor DNA from *E. coli*, *Haemophilus influenzae*, *S. pneumoniae*, and *Bacillus subtilis*, (22, 23). Another breakthrough was the identification that multiple drug resistance in *Shigella* was found to be due to an episome, or plasmid-mediated transfer of antibiotic resistance, referred to as a resistance transfer factor, (RTF) (24). Due to their ease of manipulation and isolation of plasmids would become one of the most useful tools to molecular biologists. By 1989, transformation via electroporation of plasmid DNA into multiple Gram negative species had been well established, and enabled researchers to perform genetic manipulation of recipient cells with engineered plasmid DNA (25). Transformation quickly became the standard for introducing DNA in microbial organisms, and over 40 transformable bacterial species had been identified by 1994 (18).

II.C. Methods of HGT: Conjugation

Conjugation is the unidirectional transfer of genetic material between two cells that are in direct contact with each other. This mechanism was initially discovered by Tatum and Lederberg when they described a sexual mode of reproduction in *E. coli* K-12 mutants (26). This research showed that *E. coli* possesses the ability to be either a donor (male) or a recipient (female). Esther Lederberg coined the term “Fertility” factor F to describe the plasmid donated by the male or F⁺ cell to the female, or F⁻ cell (27). This plasmid

contains the transfer gene cluster operon (*tra*) (28) that encode the F-pilus which acts as a DNA conduit from the donor cell to the recipient cell (29). The F plasmid contains all the genes necessary for stabilization of the donor and recipient cells, surface exclusion proteins that keep donor cells from self mating, and genes that enable the transfer of DNA into the recipient cell during conjugation (30). It was initially believed that the F-pilus retracted and the cells engaged in membrane-to-membrane contact for conjugation to occur, however it has been shown that cells can be 12µm apart and still have the ability to transfer DNA via conjugation (29). The identification and characterization of self-mobilizing (conjugative) plasmids with broad host range (such as RP4 and RK2) were especially useful for molecular biologists, and RK2-based plasmids are still used in the transfer of plasmids from *E. coli* to *C. jejuni* by conjugation (31).

IID. Role of HGT in antibiotic resistance and pathogenicity

The importance of HGT in both macroevolution and microevolution of bacteria has long been established, and there have been many excellent articles about identification of genes transferred through HGT (32, 33), the mechanics of HGT in conferring antibiotic resistance (34), and bacterial pathogenesis (35). HGT enables bacteria to take advantage of the communal gene pool found in the surrounding environment. Often this gene pool is the source of the factors that confer antibiotic resistance and pathogenicity. If the environment itself contains bioactive molecules, it puts selective pressure on bacteria to develop resistance mechanisms. These antimicrobials can exist naturally or may be a consequence of human negligence, such as improper use and disposal of antibiotics (34). Once acquired, genes that have been altered in response to selective pressure are then

shared amongst the bacterial community. A frightening example of very rapid microevolution is that of *Mycobacterium tuberculosis*, in which totally drug resistant (TDR) strains have evolved in a relatively short timeframe (36).

III. HGT in *C. jejuni*

In addition to historical evidence for a role of HGT in *C. jejuni* evolution (1), *C. jejuni* can readily be made to exchange genetic information *in vivo*. In one experiment, chickens were co-colonized with two *C. jejuni* strains with resistance marker in different genes (*hipO* and *htrA*), and as early as 2 days after inoculation strains could be isolated with mutations in both genes (de Boer, *et al.*, 2002). While the mechanism for this genetic exchange cannot be known for certain, there are many known mechanisms which may have played a role.

IIIA. Known mechanisms for HGT in *C. jejuni*

Both *C. coli* and *C. jejuni* are naturally transformable by either chromosomal or plasmid DNA, without any special treatment of the cells (2). Competence in *C. jejuni* is due to environmental factors, as well as a number of well-characterized proteins and pathways. Proteins implicated include RecA, Gne (formerly GalE), VirB, as well as those of the N-linked glycosylation pathway and the type II and type IV secretion systems, (37-39).

Both environmental conditions and growth phase can dramatically affect transformation efficiencies in *C. jejuni*. Cells in later phases of growth are more readily transformable, early-log-phase grown cells were found to have transformation frequencies of 10^{-4} ,

whereas cells entering stationary phase had transformation frequencies of 10^{-6} (40). Growth of cells and transformation frequencies were also compared under different CO₂ concentrations. Although cells grown under an atmosphere of 10% CO₂ had the highest growth rate, they also had the lowest transformation frequencies. Conversely cells grown at a concentration of 0.7% CO₂ had the lowest growth rate but the highest transformation frequencies (40). Typically *C. jejuni* growth requires an atmosphere of between 1% and 10% CO₂ (41). Taken together, it appears that therefore, cells under stress (by CO₂-limitation or starvation) may be more amenable to transformation (42).

Proteins involved in the process of transformation have been identified all the way from the binding of extra-cellular DNA to the final incorporation into the genome. Many of these proteins were found through saturation mutagenesis (Wiesner, *et al.*, 2003), or simply by accident when looking at other cellular processes (38), but in each case mutants have significantly lower transformation efficiencies than the parent strains.

Quite possibly the first cellular structure encountered by free DNA are the surface carbohydrates. *C. jejuni* has an N-linked glycosylation pathway that affects surface and periplasmic proteins, which in turn influences invasion, adherence, colonization, and host immune response (43, 44). Cell surface carbohydrates of *Campylobacter* spp. include LOS, capsule, and the glycoprotein N-linked heptasaccharide (Pgl glycan) (45). These carbohydrates consist of galactose and N-acetylgalactosamine residues, and are important for cell-to-cell recognition. GalE, as it was originally annotated, is the only UDP-GlcNAc/Glc 4-epimerase found in *Campylobacter*, and epimerizes UDP-Glc into UDP-

Gal. This protein is important for not only adhesion and invasion, but competence as well. In experiments where *galE* was disrupted with a kanamycin resistance cassette, natural transformation with chromosomal DNA was reduced by over 20 fold, and adherence was significantly reduced (38). More recently, it was found that *galE* in *C. jejuni* NCTC 11168, encodes a bifunctional UDP-GlcNAc/Glc 4-epimerase, which is important for cell surface carbohydrate synthesis. This led to the updated annotation of *gne*, rather than *galE* (45).

Once the DNA has crossed the outer membrane, a number of proteins are involved in chaperoning the DNA into the cell. Cj0011c is a periplasmic DNA binding protein, that when inactivated, significantly decreased transformation frequency in *C. jejuni*.

Complementation of the mutant restored transformation frequencies to wild type levels (46). Cj1211, a putative membrane channel protein, and a *Helicobacter pylori* ComH3 homologue, was found to significantly reduce transformation frequencies when disrupted. Again, complementation of the mutant with a plasmid containing the gene restored transformation frequencies to wild type levels (47). Additionally, a number of putative type II secretion system and pilus genes were found to be important for transformation of *C. jejuni*. Weisner, et al determined through transposon mutagenesis, that there are 11 genes necessary for transformation, including the above mentioned type II and pilus genes. Deletion mutants in all these genes reduced the ability to undergo transformation by approximately 1000 fold (48). Furthermore, the authors determined that some of the proteins identified were homologous to proteins not normally considered necessary for

transformation in other bacterial species, but used instead for amino acid biosynthesis (48).

Once inside the cell, the genomic DNA needs to be incorporated into the recipient cell to be maintained. RecA repairs gaps and double stranded breaks in DNA through a recombinational repair mechanism (37), and is also important for nucleotide excision repair (NER) (49). While working on developing *recA* mutants for live attenuated vaccine strains of *C. jejuni*, Guerry, et al, found that the *recA* mutants from *C. jejuni* strains 81-176, 81-116, and VC83 exhibited, in addition to recombination defects, an inability to be naturally transformed with chromosomal DNA isolated from isogenic strains, thus indicating a role for RecA in transformation (50). This was later confirmed in *C. jejuni* strains NCTC 11168, and 2535, as inactivation of RecA (*Cj1673c*) resulted in phenotypes that could not be naturally transformed, (51).

Some strains of *Campylobacter* contain plasmids of different sizes that carry virulence factors along with antibiotic resistance genes (52). When the virulence plasmid pVir from *C. jejuni* 81-176 was sequenced, it was found to have proteins homologous to *H. pylori* proteins. These proteins include a *cag* pathogenicity island, and a type IV secretion system. Using insertional mutagenesis in the ComB3 homologue, the authors were able to determine that natural transformation efficiency was reduced by 80%, and adherence and invasion was reduced by 66% (39). Further investigation of pVir revealed that the type IV secretion system protein, VirB10 is also glycosylated by the protein glycosylation (*pgl*) pathway at 2 asparagine residues, N32, and N97, and glycosylation at N97 is

necessary for competence (53). The pVir type IV secretion system was most likely acquired through HGT from *Wollinella succinogenes*, which has genes with the closest homology to *C. jejuni* VirB10, because it has a putative N-linked glycosylation system quite similar to the *pgl* system (54).

While many *Campylobacter* spp. are naturally competent, there are DNases and periplasmic DNases that come from phage-like integrated elements that work to inhibit natural transformation. Transferring *dns*, the gene that encodes an extracellular DNase to a naturally transformable strain of *Campylobacter* resulted in reducing transformation efficiency, while inactivation of *dns* by insertion of a chloramphenicol cassette and then insertion into a non-naturally transformable strain enabled transformation (55). Further work with strains that possess DNases but not *dns*, demonstrated that nucleases encoded by the integrated elements CJIE2 and CJIE4 inhibit natural transformation of *C. jejuni* RM1221 (56).

Natural transformation of *E. coli*-derived plasmids are approximately 1000 fold lower than with small plasmids containing chromosomal markers (2). Electrotransformation of competent strains of *C. jejuni* with *E. coli*-derived plasmid DNA also occurs at much lower frequencies than that of chromosomal DNA (57). This implication that DNA isolated out of *C. jejuni* has been modified to allow transformation to occur was investigated previously, and it was found that there might possibly be multiple restriction modification systems, as methylating DNA at the EcoRI restriction site did not change the outcome of electrotransformation using *E. coli*-derived plasmids (58).

Conjugation

While *Campylobacter* is naturally competent and readily takes up chromosomal DNA from the environment, plasmid acquisition is more likely to occur through a conjugative process. Plasmids are particularly of interest in pathogens as antibiotic resistance determinates are often found on extra-chromosomal elements. *C. jejuni* isolated from pigs and chickens in recent years has been associated with resistance to enrofloxacin-ciprofloxacin, tetracycline, erythromycin, clindamycin, nalidixic acid, and ampicillin (59). Importantly for human health, azithromycin, erythromycin, ciprofloxacin, and nalidixic acid are first and second line drugs used to treat campylobacteriosis in patients where antibiotic intervention is indicated, (60).

Tetracycline resistance is associated with the *tet(O)* gene, which can be either carried on plasmids or found on the chromosome in different *Campylobacter* species. An Australian study of 46 *Campylobacter* isolates determined that all the identified strains had the *tet(O)* gene. Intraspecies conjugation was demonstrated between 8 strains and the *tet(O)* gene was then isolated from the transconjugants. The majority of strains that were found to have plasmids were able to transfer tetracycline resistance to another *Campylobacter* strain via conjugation (61). In another study, four tetracycline resistant *C. jejuni* isolates from diarrheal patients in Kuwait were also found to confer resistance to a tetracycline susceptible strain via conjugation (62).

C. jejuni strain 81-176 carries the plasmid pTet, that was sequenced and shown to be almost identical to the plasmid pCC31, from the *C. coli* strain CC31 (39). Interestingly, CC31 was isolated 20 years later from a patient in the UK (3). Both plasmids carry the tetracycline resistance gene and both carry 10 genes that are homologous to type IV secretion system genes, which are referred to as *Campylobacter* mating genes (cmg) due to their involvement in conjugation. The mating pair formation genes found in both plasmids are most similar to genes in pVT745 from *Actinobacillus actinomycetemcomitans*, but are similar to the type IV secretion system genes from *Brucella* species. This research shows that both plasmids are “self mobilizable” with conjugation frequencies of 10^{-4} and 10^{-6} , depending upon whether or not the host strain has restriction barriers. The authors conclude that tetracycline use in industrial poultry farming is probably a contributing factor to the wide dissemination of these plasmids (3).

Conjugation can occur between *Campylobacter* and the related species, *H. pylori* (63). Chromosomal DNA from a plasmid-free *H. pylori* strain carrying a streptomycin marker was transferred into *C. jejuni* by conjugation. Conjugation was unidirectional only. A membrane was used to show that cell-to-cell contact is necessary for conjugation to occur. The conclusion from this work was that the three known type IV secretion systems encoded by the *H. pylori* chromosome, the *cag* pathogenicity island, the *comB* system, and the type IV secretion system 3, aren't employed for conjugation, and transformation frequencies were higher when plated cultures were used rather than liquid cultures (63).

Transduction

C. jejuni is just as susceptible to phage predation as other prokaryotes, and *C. jejuni* phage can be isolated from human and avian intestine, as well as in the environment (4). Although transduction has not been explicitly demonstrated, phage do influence *C. jejuni* populations, at least in the chicken model. In broilers phage sensitive *C. jejuni* strains had a competitive advantage over strains insensitive to phage in the absence of phage pressure, while in the presence of phage, the opposite is true. During these same experiments a third strain was isolated that contained a large (112 kb) segment of DNA that was transferred between the original inoculated strains. Although the authors suggest that infection with phage facilitated the horizontal gene transfer, other methods of gene transfer cannot be ruled out (64).

IV. Known barriers

The key question for laboratory researchers is if HGT is prevalent in environmental settings, why is it so difficult to manipulate *C. jejuni* in the lab? One obvious answer is that the barriers that exist to keep exogenous DNA from being incorporated into the cell are stricter in *Campylobacter* species. These barriers include the recently characterized prokaryotic immune system, CRISPR, and the restriction modification system. Other possible answers that need to be considered are incompatibility with cloning strains of *E. coli*, which may include dramatically different C+G content, unrecognized promoter sequences, incompatible accessory genes, and finally an inability to synthesize *Campylobacter* peptides in *E. coli* (65).

IVA CRISPR-Cas

C. jejuni employs a prokaryotic immune system that identifies and interferes with incorporation of foreign DNA. DNA recognition is based on homology to short DNA sequences flanked by a series of clustered regularly interspaced short palindromic repeats (CRIPRs). CRISPRs have been found within ~40% of bacterial genomes and ~90% of archaeal genomes (66), and confer in these microorganisms an immunity-like resistance against plasmids and viruses. The CRISPR-Cas system consists of a genetic locus containing CRISPRs, non-repetitive, unique spacer sequences, and the adjacent 6 – 20 genes that encode the Cas (CRISPR associated) proteins (67).

The repeats are highly conserved within each CRISPR locus and vary between 23 to 47 base pairs, whereas the spacers are approximately 21 to 72 base pairs and are composed of extra-chromosomal elements (68). The majority of Cas loci contain less than 50 repeat/spacer units, however some *Chloroflexus* species contain up to 375 (68). There may also be more than one CRISPR-Cas locus located on a microbial genome. The genome of *Methanocaldococcus jannaschii* contains 18 CRISPR/Cas loci (67).

There are six core Cas proteins among the 13-45 Cas protein families that have been identified (5, 69). Cas1 and Cas2 are considered markers for CRISPR loci (69, 70), however the number of the other Cas proteins can vary between species (5). Cas1 is the most highly conserved of all the Cas proteins, occurs in all CRISPR loci (5), and is a DNA-specific, metal-dependent (Mn, Mg) endonuclease (71). Cas2 proteins are also

highly conserved, and act as metal-dependent endoribonucleases that cleave ssRNAs (72).

The Cas3 proteins contain an ATP-dependent helicase, and ssDNA nuclease that is also metal-dependent (Mg) (73). Cas4 appears to be a member of the RecB family of exonucleases, and contains 3 cysteine residues at the C terminus, which suggests DNA binding activity (5, 70). Cas5 proteins have been identified based on similar N-terminal domains and average 250 amino acids in length (5). Cas6 proteins are homologous at the C-terminus and are thought to act as endoribonucleases that cleave CRISPR RNA transcripts (74). The *cas6* gene is often determined to be the most distal to the CRISPR (5).

There are two stages or mechanisms that confer resistance to invasion by foreign genetic elements. The first is the immunization or adaptation stage. This occurs as exogenous DNA, usually phage or plasmid, is cleaved by a Cas protein complex into the spacers that are inserted into the leader end of the CRISPR array (71). Every time a new spacer is inserted into the CRISPR, a corresponding repeat is added to make an individual repeat/spacer unit (75). The spacers are derived from specific spacer precursors, or proto-spacers (76), and are what are thought to confer resistance against plasmid transformation or phage infection (68). Short (~4 nucleotides) sequences called proto-spacer-adjacent-motifs, or PAMs are thought to be what the Cas protein complex recognizes as the target for proto-spacer cleavage and transport into the CRISPR (77).

The immunity or interference stage begins with the transcription of the CRISPR array from the promoter located in the leader sequence upstream of the array. The resulting RNA is then cleaved by another Cas protein complex into short CRISPR RNAs, or crRNAs that match the spacer regions with their flanking repeats. The crRNAs correspond to invading extra chromosomal elements, and target these for cleavage by Cas-encoded endonucleases (78). Processing the RNA into crRNAs is either mediated by one enzyme, or as in the case of *E.coli*, by a larger complex, the CRISPR-associated complex for antiviral defense, or CASCADE (75).

Until recently, there was no consistent method of classification for the CRISPR-Cas system (5, 69). Previously there were 8 CRISPR subtypes that were named by their association with the species in which they were found: Ecoli, Ypest, Nmeni, Dvulg, Tneap, Hmari, Aperm, Mtube, and RAMP (repair-associated mysterious proteins) (5). Additionally, these subtypes were classified by length and periodicity of their repeats, spacer length, and core Cas proteins (5). Recently Markarova, et al, have proposed a new classification system with only three distinct CRISPR systems, all of which include the Cas1 and Cas2 proteins (75).

The type I CRISPR-Cas system includes the Cas3 family of proteins. Again, *cas3* encodes a protein complex that can act as a helicase and nuclease (73). Proteins from the RAMP superfamily are included in the type I CRISPR-Cas system, (75). These include the Cas5, Cas6, and Cas7 proteins. Exogenous DNA is targeted by the type I system for processing.

The type II CRISPR-Cas system contains the Cas1, Cas2, and Cas9 proteins, and is predominantly found in bacteria (Makarova, *et al.*, 2011). Cas9 has both a HNH nuclease domain, and a RuvC-like nuclease domain and is thought to cleave plasmid and phage DNA along with Cas1 and Cas2. In addition to cleaving exogenous DNA, type II systems are also thought to cleave pre-crRNA (75).

The type III CRISPR-Cas system differs from types I and II in that oftentimes Cas1 and Cas2 proteins are not found together. In these instances, the Cas1 protein is located in either a type I, or type II CRISPR-Cas system in a different location in the genome. The type III system contains the Cas2 and Cas6 proteins, ~2 RAMP proteins and a polymerase. The type III system has the ability to cleave both DNA and RNA, and is commonly found in Archaea (75).

In addition to the three major CRISPR-Cas systems, there are subtypes that correspond to the 8 CRISPR subtypes mentioned previously, along with each subtype-specific set of genes (5, 75). For example, genes associated with the Ecoli subtype are written as *cse1-cse4*; Nmeni subtype genes are written as *csn1-csn2*, and so on. This more recent classification system can be found on the NCBI CRISPR-Cas website (75). Cas5 family members in this typing system are written as *cas5*, followed by the letter according to subtype, so for example, *cas5* belonging to the Dvulg subtype would be written as *cas5d*.

IVB. *C. jejuni* CRISPR-Cas

The *C. jejuni* CRISPR-Cas system is considered a type II/Nmeni subtype, and typically consists of the Cas proteins, Cas1, Cas2, and Csn1 (Fig. 1). This subtype is lacking the Cas proteins, Cas3, Cas4, and Cas5, although Csn1 is thought to function perhaps as both a Cas3 and Cas4 type protein (5). *C. jejuni* NCTC 11168 exemplifies this subtype, and its CRISPR loci contains a series of 5 repeats, 4 spacers, and the Cas2, Cas1, and Csn1 proteins. The *C. jejuni* RM1221 CRISPR/Cas system locus differs from that of other *C. jejuni* CRISPR/Cas system loci in that the DNA sequence annotated as *CJE1697* immediately following *CJE1694* (Cas2) and *CJE1695* (Cas1) is listed as a pseudogene, due to the stop codon (TAA) that occurs at position 1597-1599 in the DNA sequence. A NCBI Protein BLAST search however, reveals that while this pseudogene is 99% identical to the CRISPR-associated protein Csn1 (encoded by *Cj1523c*) in *C. jejuni* NCTC 11168. This “psuedogene” is also shares close homology to the DNA sequence of two consecutive CRISPR-associated proteins (HNH endonucleases) encoded by *CSU_1791*, and *CSU_1790* found in *C. jejuni* 327. *CJE1697* is 97% identical to *CSU_1791* from amino acid 1 through amino acid 462. Following a gap composed of 12 amino acids, after the stop codon, *CJE1697* is then 98% identical to *CSU_1790* from amino acid 474 through 983. Because of this homology to the two proteins found in *C. jejuni* 327. In light of the fact that these polypeptides can act independently in *C. jejuni* 327, it is quite possible that *CJE1697* can function as an endonuclease even with the authentic stop codon.

V: Restriction modification.

The process we know of as restriction modification was first recognized as host-controlled variation of phage susceptibility in various bacterial species (79). This meant that the host range for any given phage preparation is dependent upon the host strain the phage was propagated in. It soon became clear that the variation was controlled by strain specific endonucleases that restricted foreign DNA, and in this case foreign DNA came from phage different host species (80). These endonucleases furthermore were shown to cleave specific DNA sequences that create discrete DNA fragments upon cleavage (81), by only cutting at unique DNA recognition sequences. Host variation (protection from self cutting) was imposed by blocking the recognition sequence via methylation of an adenine or cytosine base within (or near) the recognition sequence by a cognate methyltransferase. In this way, the methylase and endonuclease act as natural partners in the restriction modification system (82). The ability to cleave DNA at discrete sequences proved to be a boon to molecular biologists, spurring research into the mechanisms of the enzymes and broadening the search for enzymes with ever increasing DNA sequence specificity. This search has proved fruitful, with nearly 4,000 different restriction endonuclease enzymes currently known (83).

Restriction modification enzymes are grouped into three major classes and a fourth class type represented by a single representative type, McrBC. The enzymes systems are distinguished via their enzyme composition (6, 84). Each class also has unique cofactor requirements (such as ATP, S-adenosyl methionine (SAM), and Mg^{2+}), and cleavage patterns. Representatives of all four classes of RM systems have been found in several

different *C. jejuni* strains. Table 2 identifies the various RM enzymes found in three select *C. jejuni* strains.

IVA. Descriptions of the major RM types

Type I RM systems are perhaps the least characterized of the RM systems, and also the most complex. They typically consist of three polypeptides that work together in a complex, designated R (for restriction), M (modification), and S (specificity). The three proteins form a complex of stoichiometry $R_2M_2S_1$ that contains both the endonuclease and methyl transferase activities. The specificity subunit contains the “target recognition domain,” the region of the protein used to bind DNA (85). The recognition sequences of the characterized Type I RM systems are bipartite and asymmetrical, consisting of one 3-4 bp half and a second 4-5 bp half separated by 6-8 non-specific base pairs. The modification (M) subunit contains a well conserved, and contains the SAM binding motif and methylase activity. Methylation typically occurs on two adenine residues in both regions of the target sequence. The R subunit is less conserved than the M or S subunits and is the largest of the three subunits. A major characteristic of the type I RM systems is the site of DNA cleavage is distant from recognition sequence by a non-standard (and seemingly random) DNA sequence length. This is accomplished by a DNA translocation through the complex, with the R subunit acting as an ATP-dependent molecular motor (86). The two R subunits of the enzyme complex work independently, thus creating two loops of DNA on either side of the complex. Cleavage occurs on the DNA loop distant from the recognition sequence when the translocation is stopped, perhaps by interaction with an adjacent motor (87).

All three *C. jejuni* strains listed in table 2 contain Type I RM, and consistent with the class these are very large proteins, the R subunits typically being ~1,000 aa (1,031 in NCTC 11168 (88), with the M and S subunits being between 450-500 amino acids (469 and 500 in NCTC 11168, respectively) (6). Many of the sequenced strains encode at least one type I complex, the systems from NCTC 11168 and 81-176 share homology, and the Type I complex from RM1221 is unique (table 2). Although the sizes of the individual subunits is consistent with characterized Type I RM systems, the gene organization is slightly modified, as in all three instances in figure 1 intervening genes exist between the R, M, and S subunits. The typical arrangement is the M and S subunits are transcribed together in an operon, and the M subunit is transcribed separately but from an adjacent gene. The recognition sequence for any of the Type I RM systems from *C. jejuni* have not yet been characterized.

Type II RMs

The vast majority of characterized RM systems characterized to date belong to the type II class. They are also the simplest, the endonucleases typically require only Mg^{2+} and the methyltransferases only require SAM (84). The endonucleases are usually homodimers that act on symmetric sequences that comprise of 4-8 nt. Each strand of DNA is cleaved by one monomer, leaving a double strand break. In contrast, Type II methyltransferases act as monomers that methylate each strand of the recognition sequence independently (89). Because of the sheer number of Type II RM's known, there are several subdivision of Type II enzymes (designated by a following letter). The subdivisions are based upon

both sequence specificity, and enzyme composition. It is important to note the subdivisions are not mutually exclusive, meaning a Type II RM system can belong to more than one subdivision. An example of this would be the Type II enzymes found in *C. jejuni* (table 2), that are designated both Type IIs and Type IIg. Type IIs enzymes are a subset of the TypeII RM that exhibit a “shifted” cleavage pattern. This means that at least one strand of the DNA duplex is cut outside of the recognition sequence. Type IIg enzymes have the R and M subunits fused to form a single polypeptide, but otherwise resemble Type II RM systems, (84). Cj0031 (Table 2) is essentially a subgroup of the Type IIg/IIs Type RM systems based on homology to other enzymes of this type. Cj0031 is an extremely large protein of 1,243 aa, and there is still some ambiguity as to whether it is 1 or 2 authentic open reading frames. The original sequence annotation of NCTC 11168 (88) listed two proteins, Cj0031-Cj0032, separated by a homopolymeric tract of either 8, 9, or 10 guanine bases. When 8 G’s are present, translation continues into Cj0032, creating a 1,243 aa single polypeptide. Re-annotation of the genome in 2006 merged Cj0031 and Cj0032 to reflect the amino acid sequence of the gene regardless of phase (90).

Type III RMs

In Type III systems the R and M subunits are separate polypeptides, however the M subunit confers the sequence specificity for both. The M subunit can act alone as a methylase, the addition of the R subunit adds an endonuclease function. In this type of RM ATP is required for cleavage, SAM for methylation. The recognition sequence in Type III enzymes is asymmetric, and the uninterrupted recognition sequences can act as

hotspots for phase variation in certain host-adapted bacterial pathogens (91) (92). Strain RM 1221 has a Type III enzyme system (table 2), and interestingly also encodes poly G tracts which may be involved in phase generation (90). Type III RM systems exist, but appear to be more rare than other types of RM systems, but there are no Type III systems annotated in NCTC 11168 or 81-176. The M and R subunit in RM 1221 are adjacent on the genome, the methylase subunit annotated as Cje0731 and the endonuclease Cje0730. The recognition sequence has not been identified for these enzymes.

TYPE IV RMs

The Type IV RM systems (represented entirely by the McrBC family) are different from the Type I-III systems, in that with these enzymes only methylated DNA (fully or hemi) is cut, non-methylated DNA is not cut (93). In *E. coli* the two half sites of the recognitions sequence (G/A^mC) are located distant (up to 3,000 bp) to each other. Cleavage requires GTP, (93). Several *C. jejuni* strains carry the McrB (binding) subunit (Cj0139, Cje0134, CJJ8176_0174, table 2). The B subunit has been shown to be sufficient to bind to DNA, but it is unclear if one subunit is sufficient to also provide the enzymatic cleavage. This will have to be verified through experimentation.

Subunits with RM homology

There exist a number of “orphan” methylases (those without a cognate endonuclease associated) with defined sequence specificity that have been identified in several *C. jejuni* strains. CJ0208 (Cje0201 in RM1221, CJJ81-176_240 in 81-176) is an N6-adenine-specific DNA methyltransferase. It has been shown to methylate the GAATTC (the

EcoRI recognition sequence), however the cognate endonuclease, if it exists, has not been identified. It is known that DNA isolated from strains with an intact enzyme are resistant to cleavage by the EcoRI (data not shown). CjeI is an N-6 adenine specific DNA methylase that recognizes the sequence GAGNNNNGT. Site specific DNA methylase Cj1461 (and its homologs Cje1635 and CJJ81176-1454) has been purported to modify the sequence GATC (83) Purified Cj1461 has demonstrated methyltransferase activity, however it does not appear to affect enzymes typically inhibited by methylation of GATC (94). Curiously, *C. jejuni* strains with mutations in *cj1461* (the gene encoding Cj1461) have reduced motility and decreased host cell invasion (94), which might imply DNA methylation by this enzyme is involved in gene regulation rather than DNA protection. Also present are a host of orphan methylases with no known recognition sequences (Cj1300, 1325, 1419c, cj1420c, Cj1426c) conserved between at least two strains (Table 2). The significance of these enzymes are unknown, and the absence of a cognate endonuclease casts doubt on the role of these enzymes in restriction modification.

VI. Conclusions

The evolutionary history and the physiology of *C. jejuni* both support the idea that HGT is a major force in shaping the genetic makeup of this important human pathogen, (1). In spite of this fact, many strains of *C. jejuni* remain maddeningly resistant to many common laboratory techniques based on HGT. Here we discuss two systems that are suspected of providing much of the barrier to HGT between and within species. CRISPR-Cas systems have been shown to be important in limiting both phage and plasmid transmission (both of which contribute to HGT), and are a general feature of many *Campylobacter* genomes. In our laboratory, it appears that a knock out strain of either Cas1 or Cas5 (in the NCTC 11168 background) are more receptive to plasmid introduction, but transformation efficiency of plasmids isolated from *E. coli* is too low to calculate. It is likely then that CRISPR is more attuned to phage infection rather than plasmid transfer. Although NCTC 11168 is remarkably resistant to transformation by plasmids isolated from *E. coli*, we were able to isolate colonies harboring the green fluorescent protein expression vector pMEK91 (95). When pMEK91 is isolated from a CasI mutant, however, the parent strain NCTC 11168 was readily transformed, with transformation efficiency equal to plasmids isolated from CRISPR mutant can be transformed into parent (NCTC 11168) strain at high efficiency. These data indicate that for transformation, restriction modification system in *C. jejuni* is the major factor in limiting inter-species transfer. These plasmids are not susceptible to EcorI digestion (indication of methylation), however *in vitro* methylation of DNA with the EcorI methylase also does not increase the transformation efficiency of *E. coli* derived plasmids. These data are consistent with previously published results (58), indicating this

is not the major modification affecting transformation. It is important here to note that not all strains of *C. jejuni* employ the same HGT barriers. Indeed many strains of *C. jejuni* do not prove refractory to transformation from *E. coli* –derived DNA (95). However, research from our laboratory and others shows that barriers to transformation exist between different strains of *C. jejuni* as well. For instance, pMEK91 isolated from NCTC 11168 transforms naïve NCTC 11168 at high frequency (1×10^6 transformants per μg DNA), yet the transformation frequency of RM1221 is below quantitation level using the same plasmid preparation. The obverse is also true, RM1221-derived plasmid transforms NCTC 11168 poorly or not at all. This might not be too surprising due to the diversity in the RM systems of the sequenced *C. jejuni* strains (Table 2). This intra-species barrier, unfortunately, rules out the creation of a single “cloning” strain of *C. jejuni* with mutations in multiple RM systems and/or CRISPR through which plasmids isolated from *E. coli* could be cycled before introduction into the strain of interest. Further characterization of which systems are the most important to the HGT block would be of great value to labs interested in creating cloning stains, even if only for specific strains of *C. jejuni*.

Table 1. CRISPR/Cas regions from select *C. jejuni* strains

| <i>C. jejuni</i> Strain | Gene # | Repeats / Genomic Region |
|-------------------------|-----------------------------|----------------------------|
| NCTC11168 | CRISPR repeats ^a | 5 Repeats, 1455125:1455424 |
| Cj1521c | Cas2 | |
| Cj1522c | Cas1, NMENI subtype | |
| Cj1523c | Csn1 family | |
| RM1221 | CRISPR repeats ^a | 4 Repeats, 1594103:1594336 |
| Cje1694 | Cas2 | |
| Cje1695 | Cas1, SAG0894 family | |
| 81116 | CRISPR repeats ^a | 8 Repeats, 1440708:1441215 |
| C8J_1423 | Cas2 | |
| C8J_1424 | Cas1 | |
| C8J_1425 | Csn1 family | |

^aRepeats are identical between strains: GTTTTAGTCCCTTTTAAATTTCTTTATGGTAAAAT

Table 2. Restriction-Modification systems in select strains of *C. jejuni*

| RM Type | NCTC 11168 | RM1221 | 81-176 |
|--|-------------------|----------|---------------|
| Type I | | | |
| Type I RM protein hsdM | Cj1549c (1031 aa) | no match | CJJ81176_1534 |
| Type I restriction enzyme S | Cj1551c (469 aa) | no match | CJJ81176_1536 |
| Type I M protein hsdM | Cj1553c (500aa) | no match | CJJ81176_1539 |
| Type I RM, R subunit | no match | Cje1720 | no match |
| Type I RM, S subunit | no match | Cje1722 | no match |
| Type I RM,, R subunit | no match | Cje1724 | no match |
| Type II | | | |
| Type IIS RM enzyme | Cj0031 | Cje0031 | CJJ81176_0068 |
| N-6 adenine-specific methylase | Cj1051c | Cje1195 | no match |
| Type III | | | |
| Type III RM, methylase | no match | Cje0731 | no match |
| Type III RM enzyme | no match | Cje0732 | no match |
| Type IV | | | |
| McrB | Cj0139 | Cje0134 | CJJ81176_0174 |
| Orphan Methylases- known recognition sequence | | | |
| A-specific methyltransferase ^a | Cj0208 | Cje0201 | CJJ81176_0240 |
| <i>CjeI</i> DNA methylase ^b | Cj1051c | Cje1195 | no match |
| <i>CjeI</i> | Cj1461 | Cje1635 | CJJ81176_1454 |
| Orphan Methylases | | | |
| DNA adenine methylase | no match | Cje0220 | no match |
| | Cj0495 | Cje0603 | CJJ81176_0516 |
| | Cj0690c | Cje0789 | CJJ81176_0713 |
| | Cj0722c | Cje0822 | CJJ81176_0745 |
| | CJ0590 | Cje0693 | CJJ81176_0618 |
| | Cj0976 | Cje1058 | CJJ81176_0995 |
| | Cj0979c | Cje106 | CJJ81176_0998 |
| | Cj1300 | Cje1490 | CJJ81176_1314 |
| | Cj1325 | no match | CJJ81176_0207 |
| | Cj1419c | no match | CJJ81176_1418 |
| | Cj1420c | no match | CJJ81176_1419 |
| | Cj1426c | no match | no match |

^a*EcoRI* recognition sequence GAATTC

^bRecognition Sequence GAGNNNNNGT

^cRecognition sequence GATC

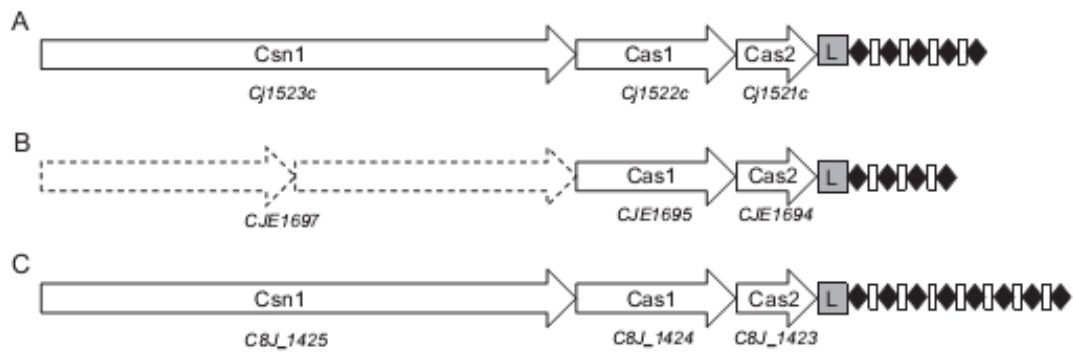


FIGURE 2.1 Graphical representation of the CRISPR-Cas locus from three *C. jejuni* strains: (A) NCTC 11168, (B) RM1221, and (C) 81116. Genes are represented as arrows, the pseudogene *CJE1697* is represented by two dashed arrows flanking the verified stop codon. The leader sequences are designated by a shaded box, the repeat elements are represented by black diamonds, and the phage or plasmid-derived spacers are indicated by white rectangles.

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