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Regulatory and mutational mechanisms for differential expression of the CmeABC multidrug efflux pump in *Campylobacter jejuni*

by

*Tara Leigh Grinnage-Pulley*

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

Program of Study Committee:
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Iowa State University
Ames, Iowa
2013

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DEDICATION

This dissertation is dedicated to:

The memory of my grandmothers: Mary Grinnage and Francis Watts

For feeding my love of books and learning.

The memory of David Morris DVM, PhD

Dr. Dave, without your encouragement I would not have considered

the alternatives to a clinical veterinary career.
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Campylobacter jejuni is a zoonotic, foodborne pathogen causing gastroenteritis in humans. The multidrug efflux pump CmeABC plays a key role in antimicrobial resistance by extruding structurally diverse compounds and is essential for intestinal colonization by mediating bile resistance. Expression of cmeABC is under the control of CmeR, a TetR family transcriptional regulator, and CosR, an oxidative stress response regulator. However, the molecular basis and functional consequences of differential CmeABC expression as well as the interactive role of CosR and CmeR in modulating cmeABC expression are still unknown. To address these questions, we performed two sets of studies. In the first study, we evaluated differential expression of cmeABC in naturally occurring C. jejuni isolates and identified the mutations associated with overexpression of cmeABC. It was found that 67% of examined C. jejuni isolates exhibited a CmeABC-overexpressed phenotype as determined by immunoblotting and real-time RT-PCR. This phenotype was further linked to mutations in the cmeABC promoter sequence that decreased the binding of CmeR to the promoter DNA or a reduced cmeR expression. Consequently, both types of mutation increased expression of cmeABC. Additionally, the CmeABC-overexpressed phenotype was associated with increased emergence of ciprofloxacin-resistant mutants in cultures treated with a fluoroquinolone antibiotic.

In the second study, we demonstrated that CmeR and CosR simultaneously bound to two separate sites in the cmeABC promoter, providing dual repression of cmeABC expression. The two regulators interact with the cmeABC promoter independently, but maximal repression by CmeR and CosR requires a 17 bp spacer between the binding sites.
as shortening the spacer interferes with CmeR binding of the promoter in the presence of CosR. Additionally, we demonstrated that CosR utilizes the single cysteine residue (C218) to sense oxidative stress as oxidation of C218 inhibited CosR binding to the promoter, providing a mechanistic explanation for oxidative-stress-induced, CosR-mediated overexpression of \textit{cmeABC}.

Together, these results reveal sophisticated mechanisms that modulate expression of \textit{cmeABC} and identify a new signal (oxidative stress) that interacts with this efflux system. Considering the important role of CmeABC in \textit{Campylobacter} pathobiology, the diverse mechanisms influencing \textit{cmeABC} expression may facilitate \textit{Campylobacter} adaptation to diverse environmental conditions.
CHAPTER 1: GENERAL INTRODUCTION

Introduction

*Campylobacter* is a gram-negative, microaerobic organism causing foodborne gastroenteritis in an estimated 845,024 people per year in the United States (1). *Campylobacter jejuni*, followed by *Campylobacter coli*, is the most common *Campylobacter* species associated with foodborne disease (2). *Campylobacter* is a commensal enteric organism of many animal species including birds (especially domestic poultry), swine, and ruminants (2). Undercooked poultry meat and unpasteurized (raw) milk are common vehicles for transmission of *Campylobacter* to humans (2, 3). Symptoms of campylobacteriosis include watery or bloody diarrhea, abdominal cramps, and fever. Illness may persist for 1 week and typically resolves with symptomatic treatment (4, 5). In immunocompromised individuals or persons with prolonged or severe disease, antimicrobial treatment is indicated (2). Fluoroquinolones and macrolides are drugs of choice for treatment of campylobacteriosis in humans, however increasing resistance, particularly to fluoroquinolones, complicates the treatment (4-6).

Antimicrobial extrusion mediated by multidrug efflux pumps is one mechanism bacteria utilize to provide intrinsic and acquired resistance to structurally diverse toxic compounds, including antimicrobials. Multidrug efflux pumps are classified into 5 major families, but in gram-negative bacteria, the resistance nodulation cell division (RND) family provides the majority of clinically relevant antimicrobial
resistance (7). CmeABC (Cme for \textit{Campylobacter} multidrug efflux) is a member of the RND family and is the predominant efflux pump in \textit{C. jejuni} (8). CmeABC is comprised of an inner membrane protein named CmeB, a periplasmic fusion protein named CmeA, and an outer membrane protein named CmeC, which are transcribed from a 3-gene operon (8). CmeABC extrudes structurally diverse compounds such as bile salts, detergents, ciprofloxacin, and erythromycin, and its extrusion of bile is essential for \textit{Campylobacter} to colonize the intestinal tract (8, 9). A TetR family regulator named CmeR functions as a repressor for \textit{cmeABC}, allowing a low, basal level of expression in wild-type organisms (10, 11). However, expression of \textit{cmeABC} can be induced by certain compounds. For example, bile induces expression of \textit{cmeABC}, which renders \textit{Campylobacter} more tolerant to bile (10, 12, 13). This induction is through CmeR. In the presence of bile, CmeR undergoes a conformational change, disassociating from the \textit{cmeABC} promoter and increasing \textit{cmeABC} expression (10, 12, 13).

By extruding antimicrobials, CmeABC functions synergistically with other acquired resistance mechanisms, such as target gene modifications, leading to high levels of resistance to macrolides, tetracyclines, and fluoroquinolones (14-17). Fluoroquinolone resistant \textit{Campylobacter} can outcompete sensitive bacteria even in the absence of antimicrobial treatment (18), leading to long-term persistence of fluoroquinolone-resistant \textit{Campylobacter}. Due to concerns over increasing fluoroquinolone resistance in \textit{Campylobacter}, this class of antimicrobials was withdrawn from U.S. poultry production in 2005. Unfortunately, the continued
persistence of fluoroquinolone resistant mutants has been documented on farms and in meat after the fluoroquinolone ban (19, 20).

Recently, a controversial mechanism of bactericidal antibiotic-mediated cell death was proposed involving reactive oxygen species (ROS) (21). ROS, such as hydrogen peroxide, superoxide, and hydroxyl radicals, induce oxidative damage to cells and can result in cell death (22, 23). Kohanski et al. reported the fluoroquinolone, β-lactam, and aminoglycoside classes of antibiotics induced formation of hydroxyl radicals and proposed that ROS production was a direct mechanism for bactericidal antibiotic-mediated killing of bacterial cells (21). It was further proposed that treatment with these antibiotics elevated intracellular iron levels and induced the Fenton reaction, increasing the level of hydroxyl radicals in treated bacterial cells (21). However, two recent studies by Keren et al. and Liu and Imlay were unable to confirm increased iron levels (24) or correlate ROS levels with cell death (25). In addition, contradictory results were shown at several levels (24, 25) in the pathway proposed by Kohanski et al., leading to the conclusion that antibiotic killing was not directly mediated by ROS. Regardless if ROS is the direct or indirect killing mechanism, oxidative stress is commonly encountered and bacterial organisms have developed multiple ways to deal with oxidative stress (22, 23, 26).

In bacteria, there are key regulatory proteins that modulate the response and resistance to oxidative stress (22, 26, 27). Interestingly, recent findings revealed that some regulatory proteins that modulate efflux pump expression are also involved in the oxidative stress response (22, 28-30). For example, MexR of Pseudomonas aeruginosa and MgrA of Staphylococcus aureus are two efflux pump regulators that
respond to oxidative stress (28, 30). Both regulators utilize cysteine residues to sense oxidative stress and modulate their DNA-binding function. Cysteine oxidation is a known mechanism for protein regulators to modulate gene expression (26, 31). The oxidized cysteine residues cause a conformational change in the regulator, altering their regulatory activities (26, 31).

CosR (*Campylobacter* oxidative stress regulator) is an orphan response regulator of *C. jejuni* that functions as an oxidative stress response regulator modulating the expression of several genes including *katA*, *sodB*, and *ahpC* (27, 32). These three genes encode enzymes to detoxify hydrogen peroxide, superoxide, and organic hydrogen peroxide, respectively (33-35). Recently, CosR was shown to bind to the promoter of *cmeABC* and repress its expression (32). How CosR senses oxidative stress and how it interacts with CmeR in modulating the expression of *cmeABC* have not been determined. CosR contains a single cysteine residue, C218, but it is unknown if this residue is involved in modulating CosR function in response to oxidative stress.

The genetic locus of *cmeABC* is highly conserved in *C. jejuni* and *C. coli* (36, 37), but its expression levels in naturally occurring *C. jejuni* isolates have not been measured. Considering the importance of CmeABC in *Campylobacter* pathobiology, the varied expression levels of CmeABC and its use of multiple regulatory pathways are expected to provide *Campylobacter* with flexibility to adapt to various environments and conditions. To address this hypothesis, we performed studies to examine differential expression of CmeABC in naturally occurring *C. jejuni* isolates, to analyze genetic mechanisms and functional consequences associated with
CmeABC differential expression, to evaluate how CosR senses oxidative stress, and how CosR interacts with CmeR in modulating the expression of *cmeABC*. Findings from these studies have significantly improved our understanding of the mechanisms governing the expression of *cmeABC* expression and provide new insights into the adaption of *Campylobacter* to various environmental conditions.

Dissertation Organization

This dissertation is organized in the alternative journal paper format. Chapter 1 is a general introduction. Chapter 2 is a review of the literature. Chapters 3 and 4 are manuscripts to be submitted to the *Journal of Bacteriology*. Tables and Figures immediately follow each manuscript. Tara Grinnage-Pulley (Ph.D. candidate) and Qijing Zhang (major professor) were the primary researchers responsible for experimental design, statistical analysis, and manuscript preparation. Tara Grinnage-Pulley performed the experiments with technical assistance from Dr. Orhan Sahin and Samantha Terhorst. Dr. Yang Mu constructed the pQECj0355c plasmid used in Chapter 4. Qijing Zhang will be the corresponding author upon manuscript submission. Chapter 5 is the general conclusions from this work. References are provided at the end of each chapter.
References


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CHAPTER 2: LITERATURE REVIEW

The Organism: *Campylobacter*

*Campylobacter*, currently known as a causative agent of foodborne gastroenteritis in humans, was first recognized as a cause of ovine abortion (1). The first published reports of the organism in 1913 resulted from an investigation into epizootic abortion of cattle and sheep initiated at the request of the British Board of Agriculture and Fisheries (1, 2). In carrying out those studies, John McFadyean and Stewart Stockholm documented isolation of a "comma shaped, actively motile" organism in 1906 from the uterine mucosa of a ewe (1). The same organism was also isolated from bovine abortions in the United States in 1919 and named *Vibrio fetus* (2). Identification of additional *Vibrio* species from veterinary cases continued in the following years, including *Vibrio jejuni* in 1931 from dysentery in calves and *Vibrio bubulus* from bovine semen and vaginal samples (2, 3). The first reports of disease in humans were from a 1938 outbreak of enteritis due to unpasteurized milk (4). Fecal cultures were negative, but fecal smears and blood cultures were observed to contain *V. jejuni*-like organisms (2, 4). *V. fetus* was successfully isolated in 1947 from the blood of pregnant women hospitalized for fever of unknown origin that ultimately aborted (2). *V. fetus* and a related *Vibrio* were reported in 1957 from human cases of enteritis (2). In 1968, the related *Vibrio* was finally cultured from human feces (2).

The genus *Campylobacter* was created with the organisms known as *V. fetus* and *V. bubulus* in 1963 (3). Separation from *Vibrio* was based on the low G+C content, non-
fermentative metabolism, and microaerophilic growth. In 1973, the taxonomy was revised to 4 species: *Campylobacter coli, Campylobacter jejuni, Campylobacter fetus,* and *Campylobacter sputorum.* *C. sputorum* was divided into two subspecies *C. sputorum* subspecies *sputorum* and *C. sputorum* subspecies *bubulus,* formerly *C. bulbulus* (3). Current taxonomic classification is *Epsilonproteobacteria* of the *Campylobacteraceae* family (5) with the genus *Campylobacter* including 27 species and 8 subspecies. The organism of study in this work *C. jejuni* is comprised of 2 subspecies *C. jejuni* subspecies *jejuni* and *C. jejuni* subspecies *doylei* (5). All references to *C. jejuni* in this work refer to *C. jejuni* subspecies *jejuni.*

*Campylobacter* is a gram negative, microaerobic, naturally transformable organism with one or two polar flagella (6-10). A microaerobic atmosphere with 5-7% oxygen, 10% carbon dioxide, and the balance in nitrogen or hydrogen can be created by commercially available gas generating packs or pumping the gas mixture from a gas tank into a sealed jar (11). A few species, *C. rectus, C. gracilis, C. showae,* are unique in their optimal growth under anaerobic conditions (3). Incubation is at 30-37° C for non-thermophillic species or 42° C thermophillic species and can range from 24 to 48 hours depending on species (11). Morphology of the rods varies with organisms being described as S-shaped, gull winged or comma, curved or spiral shaped (12). As cultures age they become more coccoid (5, 12).

Culture media for *Campylobacter* are continually undergoing development to optimize recovery from diverse habitats (13-17) and no single media has been reported as a 'gold standard' for isolation of all species (3). *Campylobacters* have been recovered from fecal samples, aborted fetal tissue, meat products, water, oral cavity, and milk (5, 7).
Basal media such as Columbia, Mueller Hinton, and Brucella, among numerous others, have been described for routine growth (11). Lysed horse blood or defibrinated blood from various species can be added at 5-15% to improve viability (12, 16). A variety of selective and enrichment medias for detection from environmental, food or fecal samples have been described (13, 15-17). Antimicrobials polymixin B, trimethoprim, rifampicin and various other combinations have been used to inhibit overgrowth of yeast and other bacteria (11, 16). Selective agents, temperature, and presence of blood can influence species recovery (11, 18, 19).

Differentiation between Campylobacter species by traditional biochemical methods and growth characteristics is possible, but is not always reliable, especially for the biochemical tests (3). C. jejuni and C. coli, the main human pathogens, are differentiated by the hippurate test. C. coli is always hippurate-negative, while most but not all C. jejuni strains are hippurate-positive. Based on their growth temperature, Campylobacter species have been characterized as non-thermophilic, such as C. fetus, and thermophilic such as C. coli and C. jejuni (16, 17). Molecular methods are commonly used to differentiate Campylobacter species and subspecies today. Some examples include pulse field gel electrophoresis (PFGE), restriction length fragment polymorphism (RFLP), multi-locus sequence typing (MLST), and amplification of 16SrRNA or species specific genes by PCR (20-24).
Epidemiology of *Campylobacter* as a Cause of Foodborne Disease

*Campylobacter* ranks fourth out of 31 known foodborne pathogens tracked by the Centers for Disease Control and Prevention (CDC) in pathogens causing disease in humans (25). Classification of foodborne pathogens by those causing illness, hospitalization, or death, places *Campylobacter* within the top 5 pathogens in each of these categories, a distinction shared only with Norovirus and non-typhoidal *Salmonella* (25). The CDC estimates campylobacteriosis causes illness in 845,000 people per year and 8,463 hospitalizations (25). *C. jejuni* infections account for 90% of foodborne cases associated with *Campylobacter*, while 5-10% of cases are caused by *C. coli* (6, 26). However, *C. fetus* subspecies *fetus*, *C. fetus* subspecies *veneralis*, *C. hyointestinalis*, *C. lari*, *C. upsalinesis*, *C. helveticus*, *C. concisus*, *C. curvus* *shanae*, *C. graciti*, *C. sputorum*, *C. rectus*, and *C. mucosalis*, have also been implicated in zoonotic disease.

Domestic poultry is an important reservoir for foodborne disease as avian species carry *Campylobacter* as a commensal organism (6, 27-29). Chickens are reported to harbor $10^9$ CFU/gram of *Campylobacter* in their cecum (6, 27-31) that can contaminate the carcass at slaughter during evisceration. Transmission to humans is primarily through undercooked poultry or poultry products, such as liver pâté (27, 32-34). Poultry meat can also serve as a source if raw foods (salads) or cooked foods are cross-contaminated with raw poultry (27, 32, 33). Unpasteurized milk and contaminated water are other sources of infection and usually involve larger outbreaks of disease (27, 32, 33). The infective dose for humans is 500-800 organisms with strain and host factors greatly influencing the dose (6, 8).
In animals, the majority of transmission is fecal-oral (28, 35) through ingestion of food or water contaminated by animals shedding *Campylobacter* in the feces. Pregnant animals infected with *Campylobacter* will also spread the organism from aborted fetal tissue and other uterine secretions to serve as a source of infection in the environment (36). *Campylobacter* is ubiquitous in the environment and can infect many animal species such as birds, cattle, sheep, and pet cats and dogs (6). Flies and wildlife species may serve as vectors for domestic species (37, 38). One exception to the oral transmission is bovine genital campylobacteriosis, which is transmitted sexually (39).

In poultry, *Campylobacter* is not usually found in chicks less than 2-3 weeks old (28, 40, 41). There is no consensus on the initial source of infection in poultry (28), but it is likely that multiple sources contribute to flock infection. Once a bird is colonized, the remainder of the flock quickly becomes positive (40, 41). Chickens require far fewer organisms compared to humans for colonization, only 35-40 organisms which may partially account for the rapid spread (42, 43).

**Clinical *Campylobacter* Infections**

**Disease in humans**

Campylobacteriosis occurs in all ages, with peaks in children less than 4 years old and in adults 15 to 39 years old in developed countries (44). In contrast, developing countries see a peak in clinical cases during childhood and then a sharp decline in cases with age (6). Immunocompetent individuals tend to have disease limited to the intestine and may clear the organism or become asymptomatic carriers (6). In developing
countries, the prevalence of asymptomatic infections (by fecal culture) are estimated at 15% (6). This appears to be mimicked in industrialized countries where occupationally-exposed individuals, such as poultry abattoir workers, experience clinical disease in the beginning of employment, but rarely develop clinical disease as employment continues (6, 44, 45).

Campylobacteriosis has an incubation period of 24-48 hours (46). Symptoms include watery or bloody diarrhea, abdominal pain, fever, and nausea with fever sometimes starting 1-2 days before the onset of diarrhea (46). Duration of clinical illness is typically 2-4 days, but symptoms can continue up to one week (6). Infections are usually self-limiting in immunocompetent individuals with home rehydration and symptomatic treatment used until symptoms resolve (7, 47, 48). Immunocompromised individuals particularly those with AIDS or humoral immune deficiencies tend to have more severe disease, often with bacteremia (6). Antimicrobial treatment is recommended for immunocompromised individuals with enteritis and in immunocompetent individuals when disease is severe or prolonged (7, 47).

Fluoroquinolones are the initial treatment choice for gastroenteritis of unknown bacterial origin as many enteric bacteria are sensitive to these drugs (47). However, with no pathognomonic symptoms, clinical campylobacteriosis is indistinguishable from many other causes of gastroenteritis. Increased resistance of *Campylobacter* to fluoroquinolones makes this drug class a poor choice for treatment in individuals infected with *Campylobacter*. Macrolides are drugs of choice in suspected or known *Campylobacter* infections (7, 47). Fluoroquinolones were withdrawn from poultry
production in 2005 by the United States Food and Drug Administration, in an effort to
decrease the level of fluoroquinolone resistant *Campylobacter* in foods of animal origin.

**Disease in animals**

In avian species, *Campylobacter* is regarded as a commensal organism, but clinical
disease can be seen in other species (35). Bovine genital campylobacteriosis in an
infection with *C. fetus* subspecies *veneralis* or *C. fetus* subspecies *fetus* causing infertility
in cattle (39). *C. coli* has been reported to cause diarrhea in weaning pigs (29). In sheep
and cattle, *C. jejuni* and *C. fetus* subspecies *fetus* are causes of abortion (29, 36, 49).

Recent emergence of *C. jejuni* as the primary cause of ovine abortion in the United
States is attributed to a highly virulent sheep abortion clone (22, 49). The clone is also
found causing abortion in cattle and goats. Additionally, this clone has been recovered
from bovine feces and healthy sheep (22). This clone is zoonotic as it has been associated
with a number of gastroenteritis cases in humans (22). The known zoonotic transmission
route for this clone is raw milk (22).

Numerous *Campylobacter* species have been recovered from domestic dogs and
cats including *C. upsaliensis*, *C. helveticus*, and *C. jejuni* (50-52). The role of
*Campylobacter* in disease of pets is unclear as *Campylobacter* can be found in healthy
animals and those with diarrhea (51, 53). Animals with enteritis can be treated, but
clearance of the organism, as denoted by negative culture, is not recommended as an
indicator of treatment efficacy (50, 51). Clearance may not be seen if the animal is re-
infected or if other pathogens were the source of infection and *Campylobacter* is carried
as a commensal (51).
Sequelae to *Campylobacter* Infection

Guillain Barré syndrome (GBS) and reactive arthritis (ReA) have been linked to *Campylobacter* infections (6, 26, 54). GBS is an autoimmune disease defined as “a progressive motor weakness of more than one limb with low or absent reflexes” by Asbury and Cornblath (54, 55). In severe cases the respiratory system can be affected with mortality rates of 2-3% (54). *Campylobacter* enteritis usually occurs 2-4 weeks prior to the onset of symptoms (6, 26, 54). Poropatich *et. al.* reported that 31% of GBS cases were related to previous *Campylobacter* infections (54). Individuals affected by GBS usually recover in 6 to 12 months (6). Reactive arthritis is a sterile inflammation of the joints occurring within 4 weeks of a bacterial intestinal or urinary infection (26). It occurs in 1-5% of *Campylobacter* infections (26). Affected individuals recover within 6 months (26).

Both GBS and ReA are believed to result from autoimmune disease secondary to immune recognition of *C. jejuni* like molecules, also known as molecular mimicry (26, 54). During infection, lipo-oligosaccharides of *Campylobacter* promote development of antibodies that cross-react with host antigens resulting in an inflammatory response (6). The host antigens in GBS are the sugar moieties of neuron gangliosides (6), but the exact antigen is unknown for ReA (26). Conflicting study results on the role for the human leukocyte antigen, HLA-B27 as the host target antigen in ReA have been reported and a definitive target for this disease is still under investigation (56, 57).

*Campylobacter* is also implicated in chronic gastrointestinal disorders such as inflammatory bowel disease (Crohn’s disease and ulcerative colitis) and irritable bowel
syndrome (58). Inflammatory bowel disease is characterized by activated T-lymphocytes in chronic, reoccurring intestinal inflammation (58). Irritable bowel disease is a non-inflammatory motility disorder of the colon (59). *Campylobacter* infections may serve to exacerbate disease or be a preceding event in both conditions (58).

**Pathogenesis of Infection**

After ingestion, *Campylobacter* must survive gastric acid, bile, and other host defense mechanisms to cause clinical disease in humans. Black *et. al.* observed an array of clinical symptoms during experimental infection of humans (60). Similar symptoms are observed in natural infections. It is unclear why diarrhea is watery or mucoid in some individuals while it is bloody in others, but host factors and strain virulence are suggested to play a role (44, 48). Host factors influencing disease outcome are immune status, the commensal and pathogenic organisms present in the intestines, and concurrent systemic infections (61). Pathogen factors include strain, dose, and challenge frequency (61). *C. jejuni* is naturally transformable, which has partially accounted for strain variability (9, 10, 46).

Two mechanisms are proposed for causing diarrhea in humans (6, 62). The first mechanism involves adhesion to intestinal epithelium and toxin production resulting in a secretory diarrhea (62). Alternatively, adhesion and intestinal epithelium invasion combined with the host inflammatory response result in bloody diarrhea (6).

Several virulence factors have been described for *Campylobacter* colonization and clinical disease. Motility is a requirement for colonization allowing *Campylobacter* to
reach the mucosal layer and intestinal epithelial cells (63-66). Motility requires the production of flagella (FlaA) and the flagellar apparatus is also involved in secretion of attachment and invasion proteins (63-68). CiaB, *Campylobacter* invasion antigen B, is one protein secreted by the flagellar apparatus that is also required for adhesion (68, 69). A fibronectin binding outer membrane protein, CadF, is also required for adhesion and invasion (70). Another essential factor for intestinal colonization is the CmeABC efflux pump as it is a key component of bile resistance (71, 72). Conflicting results have been reported on the necessity of a capsule in colonization (73, 74). In chickens, the capsule is not required for *C. jejuni* colonization (74), but is required to cause diarrhea in ferrets (73). *In vitro*, loss of the capsule increased *Campylobacter* sensitivity to serum and reduced adherence and invasion (73, 74). This reduced adherence and invasion may be due to production of hydrogen peroxide by the intestinal mucosa as a capsule is required in defense against this oxidative damage (74, 75).

Production of toxins is a common pathogenic mechanism for enteric bacterial pathogens, such as *Salmonella, E. coli, and Shigella* to induce diarrhea (76). *C. jejuni* encodes a single toxin, cytolethal distending toxin (CDT) (48, 77). CDT prevents eukaryotic cells from entering mitosis leading to cytoplasmic distention and cell death (48, 58, 62, 78). A decrease in the integrity of the intestinal barrier due to the death of intestinal epithelial cells may promote translocation of *Campylobacter* from the intestinal lumen into the mucosa (79). Additionally, CDT has pro-inflammatory effects in the intestines inducing production of CXCL-8 (IL-8) (80, 81). CXCL-8 can subsequently attract neutrophils to the intestines further inducing inflammation and cellular damage (44, 82). CDT influences the severity of enteritis in suckling mice (83) and NF-kappaB
deficient mice while in immunocompetent mice, CDT is required for persistent infection (84). CDT may be one of the strain related factors in clinical disease as CDT is detected in many, but not all C. jejuni strains (44).

**Antimicrobial Resistance Mechanisms in Campylobacter**

**Bile resistance**

Bile is a digestive secretion produced in the liver and excreted into the intestines to aid in digestions of dietary lipids (85). Bile consists of proteins, ions, cholesterol, pigments, and bile salts (85, 86). Bile salts are amphipathic molecules secreted into the intestines to emulsify lipids (85). This amphipathic nature allows bile to act as a detergent infiltrating fat particles and incorporating them into micelles for absorption (85). This infiltrative action is not restricted to dietary lipids, but also applies to the membranes of bacterial pathogens (85). Disruption of the bacterial phospholipid membrane results in a loss of selective permeability and cell death (85, 86). Bile is also thought to interact with bacterial DNA and proteins, disrupting signaling and growth processes, although the exact mechanisms are unknown (86). The killing action of bile is concentration dependent with the highest concentration in the duodenum where bile is secreted (85). These mechanisms allow bile to inhibit or kill most bacterial pathogens, unless adapted to the intestinal environment, as an innate defense against intestinal infection (85).

*C. jejuni* is recovered from the lower intestines and/or cecum of the hosts. As an enteric pathogen, *C. jejuni* has adapted to tolerate bile, which has been employed as a
component in selective media for isolation of *Campylobacter*. Bile modulates the expression of a number of genes in *C. jejuni*. For example, bile upregulates expression of flagellin, membrane or secreted proteins, and other virulence genes in *C. jejuni* such as *ciaB* and *cmeABC* (69, 87). Other genes differentially regulated by bile are involved in iron storage, energy utilization, and signal transduction (69, 87). Multiple factors contributing to bile resistance in *Campylobacter* have been identified. The multidrug efflux pump *CmeABC* is a key player for bile resistance by extruding bile from the interior of the cell (72). The *cmeABC* mutants lost the ability to grow in intestinal extracts and colonize chickens, indicating the essential function of *cmeABC* in bile resistance (71). Another contributing factor for bile resistance is *CbrR*, an orphan response regulator (88). Mutants of *CbrR* were unable to grow in bile-containing media and were severely attenuated in colonizing chickens (88). How *CbrR* contributes to bile resistance is unknown as the regulon of *CbrR* has not been identified. *CmeR*, a Tet-family regulator and pleiotropic regulator of *C. jejuni*, is also able to respond to bile (89, 90). *CmeR* interacts with bile and modulates the expression levels of *cmeABC* and other genes such as *Cj0561c* (89-91), a putative periplasmic protein.

**Multidrug efflux pumps**

Bacterial efflux pumps are cell membrane transporters extruding a variety of compounds and have physiologic roles in addition to providing antimicrobial resistance (92). Bacterial efflux pumps are single or multi-unit transporter proteins that extrude compounds from the cytoplasm and/or periplasma (93, 94). There are five families of efflux transporters: ATP binding cassettes (ABC), the resistance-nodulation cell division
RND), major facilitator superfamily (MFS), multidrug and toxin extrusion family (MATE), and small multidrug resistance family (SMR) (95). Based on energy utilization the transporters are categorized as primary or secondary transporters (96). Primary transporters, the members of the ABC family, utilize ATP-hydrolysis for energy (94, 96). The remaining transporter families are secondary transporters, utilizing ion gradients as an energy source (96).

ABC transporters are found in plants, archaea, prokaryotes, and eukaryotes (94). Although the transporters have evolved across the kingdoms, the basic structure has 2 transmembrane domains for transport and 2 nucleotide binding domains for ATP hydrolysis (94). MFS family transporters are H⁺-drug anti-porters (97). MFS transporters can function as monomers or form complexes with outer membrane and periplasmic fusion proteins (95). MATE family members can be either Na⁺ or H⁺ antiporters (98). In gram-negative bacteria, RND transporters are the predominant and most clinically relevant family in conferring antimicrobial resistance (95). RND transporters are typically a tripartite system consisting of an inner membrane protein, a periplasmic fusion protein, and an outer membrane protein that are encoded as an operon. Occasionally operons are found without the outer membrane protein such as the AcrAB-TolC pump of *E. coli* and the MexAB-OprM pump of *Pseudomonas aeruginosa* (95, 99, 100). Loss of a single component in a RND system will prevent function of the entire pump (93). The broad substrate range of RND transporters provides a major mechanism to mediate multidrug resistance (101). Thus, RND transporters often mask the function of MFS and MATE transporters, which share a similar, but slightly reduced substrate range than the RNDs (96, 98). SMR transporters are a subgroup of the drug metabolite superfamily and
are found only in prokaryotes. Cationic drugs are the substrate of this H\(^+\) antiporter system (102).

*C. jejuni* is predicted to contain 14 putative efflux pumps, (103, 104). Two RND family pumps, CmeABC (Cme for *Campylobacter* multidrug efflux) and CmeDEF, and a MFS family transporter CmeG have been characterized (105-108). CmeG has roles in both oxidative stress and antimicrobial resistance (105). Mutation of *cmeG* increases susceptibility to ciprofloxacin, erythromycin, tetracycline, gentamycin, rifampicin, ethidium bromide, cholic acid, and hydrogen peroxide (105).

CmeABC is comprised of an inner membrane protein (CmeB), a periplasmic fusion protein (CmeA), and an outer membrane protein (CmeC) transcribed from a 3-gene operon (*cmeABC*) (72). The sequence of *cmeABC* is variable, but the operon is conserved in both *C. jejuni* and *C. coli* (24, 109). CmeABC is required for colonization due to its role in bile resistance (71, 72). Bile, one of the substrates for CmeABC, induces *cmeABC* expression (71, 89). Other substrates for CmeABC include structurally diverse antimicrobials including fluoroquinolones, tetracyclines, and macrolides (72).

Expression of *cmeABC* is regulated by CmeR, a TetR family repressor (91). CmeR is located upstream of the *cmeABC* operon and transcribed in the same direction (91). Binding by CmeR to a 16-base inverted repeat within the *cmeABC* promoter region represses expression (91). CmeR also serves as pleiotropic regulator modulating the function of 27 other genes (110). One of these genes, *Cj0561c*, is tightly regulated by CmeR and strongly induced by bile (90, 110).

CmeR has an N-terminal DNA binding domain and a C-terminal ligand-binding domain, and functions as a dimer (111). Bile interacts with the ligand-binding domain of
CmeR and inhibits the binding of CmeR to its target promoters, resulting in increased expression of \textit{cmeABC} and \textit{Cj0561c} (89, 90, 110). This induction mechanism is due to the fact that binding of bile to the ligand pocket induces a conformational change in the DNA-binding domain of CmeR, releasing CmeR from the target promoter (89, 112). Different bile salts (conjugated and non-conjugated) had varied, yet substantial levels of induction of \textit{cmeABC} expression (89). Interestingly, taurocholate (a conjugated bile compound) induced \textit{cmeABC} expression in the absence of CmeR (89), suggesting that a CmeR-independent mechanism also exists in bile-mediated induction of \textit{cmeABC} (89).

CmeDEF is considered a secondary efflux pump and has synergistic roles with CmeABC (106). Although CmeDEF is also composed of an inner membrane protein (CmeF), a periplasmic fusion protein (CmeE), and an outer membrane protein (CmeD), it is not regulated by CmeR (107). CmeDEF contributes to resistance of various antimicrobials, detergents and bile salts at a modest level (106). Interestingly, mutation of both \textit{cmeF} and \textit{cmeB} was not possible in \textit{C. jejuni} strain 11168, but was successful in strains 81-176 and 21190. However, the double mutation reduced growth of strains 81-176 and 21190. These results suggest a role for CmeABC and CmeDEF in cell viability (106).

\textbf{Antimicrobial resistance}

Antimicrobial resistance of bacteria has been recognized as a major public health problem (113). Bacterial resistance to antimicrobials can be mediated by both intrinsic and adaptive mechanisms. Intrinsic resistance is inherent to the organism, such as lack of antimicrobial target, while development of adaptive resistance involves mutations or
acquisition of resistance genes (104, 113). Antibiotics are used for both human and veterinary medicine as well as animal production. The extensive usage of antibiotics serves as the selection force for the development and spread of antibiotic-resistant bacteria and is responsible for the antibiotic resistance problem seen today (113). In conventional food animal production systems, antimicrobials have been used for both growth promotion and therapeutic purpose (114). For growth promotion, all animals of a herd or flock are treated with a subtherapeutic level of drug, in feed or water, in the absence of disease. The mechanisms underlying growth promotion by antimicrobials are unknown (115). This subtherapeutic level of treatment provides a constant, low level of selection pressure, allowing proliferation of organisms resistant to the drug. In Europe, restriction on the use of antimicrobials for growth promotion in food animals began in 1986 (114). Restrictions varied by country and drug until 2006 when all antimicrobials for growth promotion were banned in the European Union (114, 116). In the United States, fluoroquinolones were used for poultry production before 2005, but have been banned since then due to concerns of antimicrobial resistance to these drugs (117).

Antimicrobial resistance mechanisms in *Campylobacter* are well documented for aminoglycosides, tetracyclines, macrolides, and fluoroquinolones (104). Aminoglycosides bind to the 16S rRNA within the small ribosomal subunit of bacteria and blocks assembly of the 30S and 50S subunits, preventing protein synthesis (118). Resistance to aminoglycosides in *Campylobacter* is mediated by *aphA* genes encoding 3’-O-aminoglycoside phosphotransferase (104, 113). This enzyme adds phosphate groups to the 3’ hydroxyl group in aminoglycosides, causing inactivation (104, 113). Three genes have been described in *Campylobacter* mediating aminoglycoside resistance
including \textit{aphA-1, aphA-3, and aphA-7}, which can be found on the chromosome or plasmids (119-122). Resistance to aminoglycosides, represented by gentamycin, in \textit{C. jejuni} was $< 1\%$ in U.S. (123), but the resistance rate is much higher in some other countries (124-126).

Tetracyclines bind to 16S rRNA within the small ribosomal subunit, inhibiting the elongation phase of protein synthesis (118). There are multiple tetracycline resistance mechanisms in bacteria, but tetracycline resistance in \textit{Campylobacter} is mediated by Tet(O), a ribosomal protection protein (104, 113). After tetracycline has bound to the ribosome inhibiting elongation, Tet(O) binds to the ribosomal A site and induces a confirmational change that disrupts tetracycline binding and allows protein synthesis to continue (104, 127). Tet(O) can be found on the chromosome or on a plasmid (128). The tetracycline class is widely used in animal production in the U.S. and tetracycline resistance is highly prevalent in \textit{Campylobacter}. For example, the National Antimicrobial Resistance Monitoring System (NARMS) Executive Report indicated that tetracycline resistance in \textit{C. jejuni} was 43\% for all isolates in 2010 with resistance generally higher in isolates from turkeys and chickens (123). Tetracyclines are the only approved drug to prevent infectious causes of abortion in sheep in the U.S. (36). However, a tetracycline-resistant \textit{C. jejuni} clone [carrying the tet(O) gene] responsible for the majority of sheep abortion in the U.S. was recently described, limiting the usefulness of this class of antibiotics for preventing abortion (22, 49).

Macrolides target the 23S rRNA in the large ribosomal subunit, blocking translocation of the peptide chain during protein synthesis (118, 129). Macrolide resistance in \textit{C. jejuni} is mediated by mutations in the 23S rRNA genes and the L4 and
L22 ribosomal proteins (130-134). *C. jejuni* carries 3 copies of the 23S rRNA genes (135, 136). For stable macrolide resistance, at least 2 of the copies must carry the mutation (134). These resistance-conferring mutations occur at nucleotide positions 2074, 2075, or rarely both (131, 134, 137). Resistance mediated by mutation at nucleotide 2075 is more common than the 2074 mutation, but mutations at both positions can confer high-level resistance (129, 131, 134). Mutations of the ribosomal proteins L4 and L22 by amino acid substitution and amino acid insertion result in a moderate-level macrolide resistance (130). According to NARMS’ data, *C. jejuni* resistance to macrolides was <2% in 2010 in the U.S. (123), but the resistance rate is much higher in some other countries (124-126, 138-142).

Fluoroquinolones target DNA gyrase, a type II topoisomerase necessary for DNA supercoiling and required for DNA replication (118). Fluoroquinolones form a complex with DNA gyrase on the DNA strand being replicated, causing double stranded DNA breakage (143, 144). DNA gyrase is encoded by *gyrA* and *gyrB*. In *Campylobacter* several mutations in GyrA have been described influencing the level of fluoroquinolone resistance (145, 146). Among the reported GyrA mutation, the Thr-86-Ile change confers high-level resistance to fluoroquinolones (103). This mutation affects the DNA supercoiling activity of GyrA and contributes to the increased fitness in fluoroquinolone-resistant *C. jejuni* (103, 147).

In addition to the mechanisms discussed above, efflux pumps also play important roles in antibiotic resistance in *Campylobacter*. As the major multidrug efflux pump in *Campylobacter*, CmeABC extrudes diverse antibiotics including tetracyclines, macrolides, aminoglycosides, and fluoroquinolones (72). Thus CmeABC plays a
synergistic role with other resistance mechanisms in conferring antibiotic resistance (103, 148, 149). Additionally, CmeABC facilitates the emergence of spontaneous mutants resistant to ciprofloxacin under selection pressure (150), contributing to the development of fluoroquinolone resistance. NARMS data indicated that fluoroquinolone resistance is 20-22% for *Campylobacter* cultured from animal, retail meat, and humans samples from the U. S. (123), but the resistance rate is higher in some other countries (124-126, 139, 141).

**Oxidative stress response and defense**

An enteric microaerobic organism, *C. jejuni* is susceptible to oxidative stress created by internal and environmental sources (151). Superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (‘OH), are some of the reactive oxygen species (ROS) that induce oxidative stress (151, 152). *C. jejuni* requires low levels of oxygen for optimal growth and can utilize oxygen as a terminal electron acceptor during aerobic respiration (153). Aerobic respiratory enzymes contain redox cofactors necessary for electron transfer and these co-factors can be autoxidized to produce ROS (152). Superoxide is generated as a byproduct of auto-oxidation of respiratory chain co-factors (151, 152). In addition, the presence of unbound ferrous iron (Fe$^{2+}$) in the cytoplasm can trigger the Fenton reaction producing hydroxyl radicals (151, 152). Intracellular hydrogen peroxide is produced as a byproduct of NADH reductase reactions and during the breakdown of superoxide (154).

ROS are also encountered upon attachment to epithelial cells and invasion of the intestinal epithelium and lamina propia (29, 60, 75). Production of hydrogen peroxide by
intestinal cells was demonstrated after adhesion and invasion of *C. jejuni* (75). Invasion of the intestinal tissue brings *Campylobacter* into contact with macrophages and neutrophils, which produce nitric oxide, hydrogen peroxide, and superoxide (82). Secondary reactions can produce hydroxyl radicals (82).

ROS damage cells by oxidizing proteins, lipids and DNA, causing disruption of bacterial metabolism, membrane integrity, signaling and replication (155). Superoxide and hydrogen peroxide can oxidize iron sulfur proteins releasing ferric (Fe$^{3+}$) iron from the protein into the cytoplasm and inactivating the protein (155). The cytoplasm of bacteria is a reduced environment (152, 155). This causes reduction of free ferric iron to ferrous iron via the Haber-Weiss reaction (Equation 1) (152, 155). Ferrous iron then enters the Fenton reaction (Equation 2) and perpetuates the production of ROS (152, 155). As ROS continue to oxidize cellular components, organic hydroperoxides are generated, causing further oxidative damage (155).

(Eq. 1) Haber-Weiss reaction: Fe$^{3+}$ + O$_2^·$ → Fe$^{2+}$ + O$_2$

(Eq. 2) Fenton reaction: Fe$^{2+}$ + H$_2$O$_2$ → Fe$^{3+}$ + ‘OH + OH$^·$

To minimize the effects of ROS, bacteria encode oxidative stress regulators to coordinate the response of oxidative stress genes. In *E. coli*, OxyR and SoxRS, mediate this response with additional contributions by RpoS and Fur (151). OxyR and SoxRS regulate the response to hydrogen peroxide and superoxide, respectively (151). RpoS is a general stress response regulator and Fur is an iron responsive regulator (151). These regulators modulate the expression of genes encoding superoxide dismutase, catalase and alkyl hydroperoxide reductase that detoxify superoxides and peroxides (151). There are no enzymes to detoxify hydroxyl radicals (152).
In *E. coli* there are multiple catalase, alkyl hydroperoxide reductase, and superoxide dismutase genes, but *C. jejuni* contains only one of each (151, 156-158). Superoxide dismutase (*sodB*) provides resistance to superoxide and peroxides (Equation 3) (158). Catalase (*katA*) and alkyl hydroperoxide reductase (*ahpC*) provide resistance to peroxides (Equation 4) (156, 157). Catalase is specific for hydrogen peroxide while *ahpC* breaks down organic hydroperoxides (156, 157).

(Eq. 3) Superoxide detoxification: \(2O_2^- + 2H^+ \rightarrow H_2O_2^+ + O_2\)

(Eq. 4) Peroxide detoxification: \(2H_2O_2 \rightarrow O_2 + H_2O^-\)

*C. jejuni* does not contain OxyR, SoxRS, or RpoS (159). PerR, Fur, Cj1556, and CosR contribute to regulation of oxidative stress response genes (160-165). PerR is a Fur homolog regulating the response to iron and peroxide stress (163, 164). PerR represses *ahpC* and *katA* (163, 164). Fur is an iron responsive regulator that also negatively regulates *katA* (163, 165, 166). Cj1556, a regulator of oxidative and aerobic stress, positively regulates *katA*, *ahpC* and *perR* while negatively regulating *sodB* (162). CosR, an orphan response regulator, negatively regulates *ahpC* while positively regulating *sodB* and *katA* (160, 161).

**CosR as an oxidative stress response regulator**

Garénaux *et. al.* first recognized the role of CosR (*Cj0355c*) in oxidative stress response in a proteomic study examining the response of *C. jejuni* to paraquat, a generator of superoxide radicals (167). In that study, genes involved in oxidative stress response, redox, and virulence were overexpressed while CosR was downregulated (167). Attempts to knockout CosR were unsuccessful and it was classified as an essential gene.
(88, 167). The lethal effects of cosR mutagenesis were circumvented by gene inhibition using peptide nucleic acid (PNA) by Hwang et. al. 2011(160).

PNA is a synthetic DNA mimic able to bind complementary DNA or RNA following Watson-Crick binding principles (168, 169). Nielsen et. al. designed PNA to be homomorphous to DNA with the exception of the deoxyribose backbone (169). 2-Aminoethylglycine units form the non-charged polyamine backbone of PNA, replacing the deoxyribose sugars (169). PNA binds optimally to DNA or RNA in an anti-parallel manner with the amino terminal of PNA directed to the 3’ end of the DNA or RNA (168, 170). PNA has been used for inhibiting gene expression in both eukaryotic and prokaryotic systems (170). Target inhibition by PNA occurs at the level of translation in bacteria (170, 171).

In C. jejuni PNA has been used to successfully inhibit protein expression from cmeA and cosR (160, 161). cmeA-specific PNA inhibited CmeA expression, impairing the function of the CmeABC efflux pump (172). The decreased pump function was reflected as an increase in antimicrobial susceptibility (72, 172). cosR-specific PNA reduced the expression of CosR while still maintaining the viability of C. jejuni (160). Using PNA inhibition, it was found that CosR regulates 93 genes in C. jejuni (161). Notably, CosR regulates sodB, ahpC, and katA by binding to a 21-base pair binding site within the promoter region of these genes (160, 161). SodB activity was increased while KatA activity decreased with inhibition of CosR, indicating the negative and positive regulation of the respective genes (160, 161). Thus, inhibiting CosR increases resistance to oxidants in accordance with its role as a negative regulator of many oxidative stress defense genes (160). However, resistance is dependent on the type of oxidant. When
exposed for 1 hour to equal amounts of paraquat and hydrogen peroxide, paraquat was more effective than hydrogen peroxide in killing *C. jejuni* independent of CosR levels (160). This is likely explained by the fact that paraquat treated cells reduced gene and protein expression of CosR, but hydrogen peroxide did not (160). Hydrogen peroxide-mediated cell death was suggested to be independent of CosR (160).

**Oxidative stress and efflux pumps**

Many bacterial regulators sense oxidative stress through redox reactions of residues. The thiol group of cysteine is readily susceptible to redox reactions and can be oxidized to form disulfide bonds or sulfonic acid or undergo S-nitrosylation or S-alkylation (173). Regulatory proteins containing metal ion binding sites utilize metals, such as iron (Fe\(^{2+}\)), zinc (Zn\(^{2+}\)), or manganese (Mn\(^{2+}\)), to oxidize histidine residues (155). The oxidative stress regulator families OxyR and OhrR contain cysteine residues that can be modified by redox reactions while PerR is a metal responsive regulator containing critical histidine residues (155, 173).

OxyR redox modulation is still under study but it is believed to occur through two mechanisms: general or selective (173). In the general mechanism two cysteine residues are oxidized to form an intramolecular disulfide bond inducing a conformational change (173). The selective mechanism involves a single cysteine modification that can vary. The variation in the modification presumably allows selection of certain genes, resulting in differential gene expression (155, 173). Interestingly, OxyR is functional in both the oxidized and reduced states acting as a repressor and activator (155).
The OhrR family is a subgroup of the MarR family that includes MgrA and SarZ of *Staphylococcus aureus* (155). Proteins may contain one or two cysteines that function in redox reactions. For proteins with a single cysteine, oxidation produces a mixed disulfide bond with a free thiol group or forms a cyclic amide that interferes with DNA binding activity (155). The proteins with two cysteines undergo intermolecular disulfide binding (155).

PerR is a member of the metal responsive Fur family (155, 163). PerR contains two metal binding sites, one for Zn$^{2+}$ and the second for Fe$^{2+}$ or Mn$^{2+}$ (155). The preference for Fe$^{2+}$ or Mn$^{2+}$ is not understood (155). Three histidine residues and two aspartate residues align the metal ions within the binding site resulting in a protein confirmation that stabilizes the DNA binding domains (155). In this confirmation PerR binds to DNA, repressing gene expression (155). Disassociation is mediated by oxidation of a histidine residue (155). Hydrogen peroxide is reduced by Fe$^{2+}$ generating a hydroxyl radical (155). The hydroxyl radical oxidizes a histidine, usually H37, disrupting the metal binding site confirmation, and PerR dissociates from the DNA (155).

Kohanski *et. al.* hypothesized that bactericidal antibiotics induce bacterial cell death through oxidative stress (174). An increase in hydroxyl radical production was observed after treatment with bactericidal, but not bacteriostatic antibiotics. Addition of a radical quencher increased the viability of cells treated with bactericidal drugs. This killing by bactericidal drugs involved intracellular iron from iron-sulfur cluster proteins. In addition, NADPH levels decreased after bactericidal drug treatment. Blockage of the TCA cycle also reduced death in cells treated with bactericidal antibiotics (174).
Based on these findings, Kohanski et al. proposed the following mechanism for bactericidal antibiotics, including fluoroquinolones, aminoglycosides, and β-lactams, to mediate cell death (174). Treatment with bactericidal antibiotics leads to an increase in NADPH consumption as NADPH is converted to NAD+, producing superoxide (174, 175). Superoxide oxidizes iron sulfur proteins, releasing ferric iron that also reacts with superoxide via the Haber-Weiss reaction to produce ferrous iron. Excess ferrous iron production drives the Fenton reaction increasing hydroxyl radicals (174). Hydroxyl radical damage results in cellular death.

However, two recent studies independently disputed the mechanism proposed by Kohanski et al. (176, 177). Liu and Imlay were unable to demonstrate increased iron or hydrogen peroxides levels that were postulated to drive the Fenton reaction (176). In addition, respiratory chain function was observed to decrease or remain the same after bactericidal drug treatment (176). Also catalase and peroxidase genes were not activated during antimicrobial treatment (176). In another study, Keren et al. were not able to correlate hydroxyl radical formation with cell death caused by antibiotics (177). They also found similar levels of killing under aerobic and anaerobic conditions (177). Both studies concluded that ROS were not involved in antibiotic-mediated cell death.

In spite of the controversy over ROS as a mechanism of antibiotic-mediated killing of bacteria, oxidative stress appears to affect many cellular processes including antibiotic efflux systems. The pleiotropic regulators MexR of Pseudomonas aeruginosa, MgrA and SarZ of Staphylococcus aureus, and AsrR of Enterococcus faecium sense oxidative stress (178-181). MexR and MgrA are regulators of efflux pumps (182-185) and oxidation has been shown to affect their binding to promoter DNA (178, 180). In addition, the efflux
pumps CmeG of *C. jejuni*, AcrAB of *Salmonella enterica* serovar Typhimurium, and P55 of *Mycobacterium tuberculosis* were found to be responsive to oxidative stress (105, 186, 187). AcrAB appears to have multiple pathways to respond to oxidants (186). CmeG contributes to oxidative stress resistance as inactivation of cmeG increased the susceptibility of *C. jejuni* to hydrogen peroxide (105). In addition, CmeABC of *C. jejuni* was recently described to be repressed by CosR and that the cmeABC promoter contains a CosR binding site upstream of the CmeR binding site (161). These findings suggest that oxidative stress response and antibiotic efflux systems are intertwined or linked, but the detailed mechanisms for the interaction and how efflux systems contribute to defense against oxidative stress are largely unknown.

Control of *Campylobacter*

Control of *Campylobacter* has focused primarily on the broiler industry as chicken meat or meat products are the most common source of infection for humans (42). *Campylobacter* is highly prevalent in live birds and colonizes in high numbers. Thus, intervention on farm or at pre-slaughter will have a great impact on control of *Campylobacter*. In general, biosecurity, immunologic approaches, and antimicrobial alternatives have been used for intervention (42). Biosecurity focuses on restriction, barriers, and sanitation and is widely used on farms (37, 42). Access to birds is restricted by limiting the number of persons onto the farm or into the house (52, 188), limiting the number of visits into house (189, 190), and pest control (189, 191-193). Pest control applies not only to poultry house, but also to feed to prevent contamination with feces
from other animals or wild birds (194). Barriers methods include footbaths, clothing changes, or showering for entry/exit of house (37, 188). Basic sanitation of feed and watering devices as well as decontamination and disinfection of houses and equipment between groups of birds are also used (188). Biosecurity measures are limited by practicality, cost, and consistency of implementation (37, 42).

Immunological interventions rely on host defenses to exclude or reduce *Campylobacter* from the avian intestinal tract (42). Attenuated, killed, and subunit vaccines have been studied, but no commercial product is currently available (42). The ideal *Campylobacter* vaccine in poultry would be delivered orally, stimulate immunity to kill or reduce *Campylobacter* in the intestinal tract, and have no effect on humans consuming the food product (42, 195). In addition, the 6-week growth cycle of broilers requires a vaccine that induces immunity quickly (42). Live attenuated vaccines are a concern for potential reversion to pathogenicity. Killed vaccines tested so far have not achieved the expected level of reduction (195). Several subunit vaccines have appeared promising, but need additional work (195-197).

Other intervention methods include competitive exclusion and improving poultry genetics. Competitive exclusion involves treatment of flocks with defined cultures or crude intestinal extracts (198). Several competitive exclusion products are commercially available, but efficacy is variable (42, 194). The bacteria comprising these mixtures may or may not be identified (194, 198). Concerns exist for the potential transmission of pathogens and/or antimicrobial resistance in undefined cultures (42, 198). Some poultry lines show differences in resistance to colonization by *Campylobacter* (199-201). Studies
into the chicken immune response are ongoing for selective breeding to improve resistance to *Campylobacter* (200, 202-204).

Bacteriocins and bacteriophages are potential alternatives to antimicrobials (42). Bacteriophages, viruses of bacteria, kill susceptible bacteria. Current concerns for using bacteriophages are the potential for bacteria to develop resistance to the bacteriophage and the transmission of antimicrobial resistance or virulence genes by phages (42). Bacteriocins are antimicrobial peptides produced by bacteria, such as members of *Lactobacillus*, *Paenibacillus*, *Enterococcus*, *Lactococcus*, *Pediococcus*, *Carnobacterium*, *Escherichia*, *Bacillus*, *Staphylococcus*, *Pseudomonas* and *Clostridium*, to kill other bacteria (42). The exact mechanism of killing is unknown, but disruption of bacterial membranes through pore formation is one suggested mechanism (42, 205). Bacteriocins have shown promise in producing high level reductions of *Campylobacter* levels ranging from 2-6 logs in poultry (42, 206). Additionally, bacteriocins do not induce cross-resistance to other bacteriocins or antimicrobials and antimicrobial resistance does not confer bacteriocin resistance (205, 207). Also, since bacteriocins are likely produced in humans and animals by their own microflora and they are susceptible to proteases, bacteriocins are not expected to cause toxicity in humans (205). Several bacteriocins have been described to reduce *Campylobacter* levels in chickens and turkeys (42, 205, 208-210), but commercialization of the product has not happened.

For postharvest control of *Campylobacter*, the focus is on limiting cross contamination. To promote emptying of the gastrointestinal tract, feed is withdrawn for 8-12 hours prior to processing (194). This limits fecal contamination of the crates and birds during transportation, preventing cross contamination from farm to processing plant
Once in the plant, scalding loosens feathers and reduces surface pathogens (211). Inside–out bird washers rinse carcasses after evisceration to remove fecal material that may have been released from the intestinal tract (211). Carcass chilling is usually through immersion in water with sanitizers, and the type of sanitizer used is dependent on current regulations for chemicals in food (194). However, air chilling is common in Europe (194). Temperature of water, length of time for chilling, pressure, water flow, cleanliness of water source and sanitizer concentration all impact the effectiveness of these methods (194, 211). Freezing is another control method, but is precluded in production of fresh poultry products (194). Irradiation, although effective in reduction of pathogens (212), is not commonly used due to public perception of irradiation safety (194).

Summary

*Campylobacter jejuni* is an important zoonotic pathogen and a main cause of foodborne disease. Its ability to survive bile, ROS, antimicrobials, host defenses and varying environmental conditions highlights the adaptability and resilience of the organism. However, resistance to fluoroquinolone and macrolide drugs is problematic as these are drugs of choice for treatment of campylobacteriosis. Drug specific antimicrobial resistance mechanisms, particularly those for fluoroquinolones and macrolides, are synergized by the multidrug efflux pump CmeABC. CmeABC is essential for colonization and also appears to have a role in oxidative stress, being repressed by CosR, an oxidative stress regulator. Improved understanding of the
pathobiology of C. jejuni may facilitate development of better Campylobacter control mechanisms. The following chapters examine the mechanisms influencing expression of the CmeABC efflux pumps in Campylobacter jejuni and its interaction with an oxidative stress regulator named CosR.

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CHAPTER 3: GENETIC BASIS AND FUNCTIONAL CONSEQUENCES OF DIFFERENTIAL EXPRESSION OF THE CMEABC EFFLUX PUMP IN CAMPYLOBACTER JEJUNI ISOLATES

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Abstract

The CmeABC multidrug efflux transporter of Campylobacter jejuni plays a key role in antimicrobial resistance. CmeR, a transcriptional repressor, maintains transporter expression at a basal level. Overexpression of CmeABC has been observed in laboratory-generated mutants, but it is unknown if this phenotype occurs naturally in C. jejuni and if it has any functional consequences. Expression of cmeABC was examined in natural isolates obtained from broiler chickens, turkeys and humans, to determine the genetic mechanisms and role of cmeABC differential expression in antimicrobial resistance. Phenotypic classification of 64 C. jejuni isolates as overexpression (OEL) or wild-type expression level (WEL) of cmeABC, determined by immunoblotting and real time RT-PCR comparisons with strain NCTC 11168, identified 43 isolates as OEL. Representative mutations of the cmeABC promoter and/or CmeR-coding sequence were analyzed using electrophoretic mobility shift assays and transcriptional fusion assays. Reduced CmeR binding to the mutated cmeABC promoter sequences or decreased CmeR levels increased
cmeABC expression. Amino acid substitutions in CmeR did not affect cmeABC promoter binding and some OEL isolates harbored no mutations in known regulatory elements, suggesting that cmeABC is also regulated by unidentified mechanisms. Overexpression of cmeABC did not affect the susceptibility of C. jejuni to most tested antimicrobials except for chloramphenicol, but promoted the emergence of ciprofloxacin-resistant mutants under antibiotic selection. These results link CmeABC overexpression in natural C. jejuni isolates to various mutations and this phenotypic change promotes the emergence of antibiotic-resistant mutants under selection pressure. Thus, differential expression of CmeABC may facilitate Campylobacter adaptation to antibiotic treatments.

Introduction

Multidrug efflux pumps play key roles in bacterial physiology, conferring intrinsic and acquired resistance to diverse toxic compounds. There are multiple types of drug efflux systems in bacteria, but in gram negative bacteria, the resistance-nodulation-cell division (RND) family of efflux pumps is of primary importance for antimicrobial resistance (1). ArcAB-TolC, MexAB-OprM, and MexXY-Z in Escherichia coli and Pseudomonas aeruginosa are examples of well-characterized RND efflux pumps that extrude bile salts, organic cations, detergents, and various classes of antimicrobials (2-5). These efflux pumps have been associated with intrinsic and acquired resistance to antimicrobial compounds. CmeABC, also a member of the RND family, is the predominant efflux pump in Campylobacter jejuni (6) and plays a key role in the resistance to structurally diverse compounds, such as bile salts, ciprofloxacin,
erythromycin, ethidium bromide, and various detergents (6, 7). Synergistic effects of CmeABC with other resistance mechanisms (such as target gene mutations) contribute to high-level resistance to macrolides, tetracyclines, and fluoroquinolones in *Campylobacter* (8-11). Due to its significant role in bile resistance, CmeABC is essential for *Campylobacter* colonization and adaptation in the intestinal tract of animals (7).

CmeABC is comprised of an inner membrane transporter (CmeB), a periplasmic fusion protein (CmeA), and an outer membrane protein (CmeC). The three components are encoded by a three-gene operon (6). Transcription of this operon is repressed by CmeR, a TetR family transcriptional regulator (12). The *cmeR* gene is located immediately upstream of the *cmeABC* operon. The CmeR protein contains a C-terminal ligand-binding domain and a N-terminal DNA-binding domain (7). The DNA-binding domain interacts specifically with a 16-base inverted repeat within the promoter region of the *cmeABC* operon (13). This binding inhibits the transcription of the *cmeABC* operon. However, mutation of CmeR or alteration of the promoter sequences affects the binding of CmeR, resulting in increased expression of CmeABC (12, 14). Additionally, *cmeABC* expression is inducible by bile and this induction is through the interaction of bile with the ligand-binding pocket of CmeR, which triggers a conformational change in the DNA-binding domain, releasing CmeR from the *cmeABC* promoter (15, 16).

Gastroenteritis caused by *Campylobacter* is estimated to affect 845,024 people and cause 8,463 hospitalizations per year in the United States (17). *C. jejuni* and *C. coli* are the most common *Campylobacter* species associated with foodborne disease and are commensals in avian species (particularly poultry), swine, and ruminants (18).

*Campylobacter* contamination frequently occurs with poultry meat and unprocessed milk.
Thus, undercooked poultry and unpasteurized milk are common vehicles for foodborne transmission of *Campylobacter* to humans (18, 19). Clinically, campylobacteriosis is manifested as diarrhea, abdominal cramps, and fever, which typically resolves in 1 week without medical intervention. However, when antimicrobial treatment is indicated with severe or prolonged cases, or in immunocompromised patients, fluoroquinolones and macrolides are the drugs of choice (18, 20). Increasing resistance to these antibiotics in *Campylobacter* is problematic, especially to fluoroquinolones, as *Campylobacter* is highly adaptable to fluoroquinolone treatment and acquisition of mutations associated with fluoroquinolone resistance does not impose a fitness cost on this organism (21-23).

In all clinically relevant antibiotic resistance, CmeABC plays an important role as inactivation of *cmeABC* rendered *Campylobacter* much more susceptible to various antimicrobials (6).

Considering the significance of CmeABC in *Campylobacter* pathobiology, its varied expression levels are expected to affect antimicrobial resistance. Under toxic conditions or in the adaptation to harsh environments, enhanced expression of *cmeABC* may confer a survival advantage on *Campylobacter*. The advantage may occur directly through increased extrusion of toxic substrates, which reduces their harmful effect and increased frequency of emergence of antimicrobial resistant mutants. Although inactivation of *cmeABC* or overexpression of this efflux pump has been examined under experimental conditions by using insertional mutagenesis or stepwise selection of mutants on antibiotic containing plates (12, 14), it is unknown if differential expression of *cmeABC* occurs in naturally occurring isolates and if the differential expression has any functional consequences. In this study, we investigated the expression of *cmeABC* in
C. jejuni isolates from turkeys, chickens, and humans, examined the mechanisms associated with the differential expression, and measured the functional consequences associated with the differential expression.

Materials and Methods

Bacterial Strains and Growth Conditions

Sixty four naturally occurring Campylobacter isolates were randomly selected from conventionally raised broiler chickens (24), conventionally raised turkeys (24), and clinical diarrheal cases of humans. Eight of the human isolates including E46972, H30769, H49024, S13530, T37957A, X77136, M402 and W11805 were used in a previous study (25). Other human isolates were F6501, W14861, H52022, X60179, T59822, M63885, H2958, X39768, W64861, W28752, M76927, M37523, M33323, M32506, F15871, M36292, S47645, X7199, W52546. The 20 broiler isolates, 17 turkey isolates, and 27 human isolates were confirmed to be C. jejuni using the reported mapA and 16S rRNA primers (26, 27). Key PCR primers used in this study are listed in Table 1. In addition to these isolates, several laboratory strains were also used, including NCTC 11168 (28), 81-176 (29), 81-176ΔcmeR (12), 11168ΔcmeR (12) and the quality control C. jejuni strain ATCC 33560 (30, 31), which are listed in Table 2. All strains were routinely cultured in Mueller Hinton (MH) agar or MH broth (Difco, Detroit, MI) at 42°C under microaerobic conditions (5%O2, 10%CO2, 85%N2). Media were supplemented with kanamycin at 30 µg/mL or chloramphenicol at 4 µg/mL when needed.
E. coli strains (Table 2) DH5α (Invitrogen), DH5αpMW10 (32), DH5αpRK2013 (33), XL1-Blue (Agilent), and JM109 (Agilent) were cultured at 37°C. Luria-Bertani (LB) broth or agar (Difco) was supplemented with 30 µg/mL of kanamycin or 100 µg/mL of ampicillin when needed.

**Immunoblotting**

Isolates were initially screened for CmeABC expression by immunoblotting with polyclonal antibodies against CmeABC. All clinical C. jejuni isolates, NCTC 11168 and 11168ΔcmeR were cultured in MH broth. Samples were pelleted, and re-suspended in SDS loading buffer for a final concentration of 5 x 10⁹ CFU/mL. The protein samples were analyzed by SDS-PAGE and immunoblotting as described previously using antibodies against CmeA, CmeB, CmeC (6) and MOMP (9). Bands for CmeA, CmeB, and CmeC from the clinical isolates were compared to those of NCTC 11168 and 11168ΔcmeR by visual inspection and densitometric analysis using the AlphaEaseFC Software (Version 3.2.3 Rev C; Innotech).

Primary classification as wild type-level (WEL) or overexpression level (OEL) of CmeABC was based on analysis of CmeB by densitometry and CmeA was utilized as a secondary factor for the classification. The 64 clinical C. jejuni isolates were analyzed on 8 immunoblots with NCTC 11168 and 11168ΔcmeR used as controls for WEL and OEL, respectively. The threshold for CmeABC overexpression based on densitometric analysis was a 2-fold increase for the CmeB band in relation to the expression level in NCTC 11168. Each immunoblot was examined individually to ensure the threshold for overexpression was met. This phenotypic classification was further confirmed by
measuring the *cmeB* transcript using real time RT-PCR. OEL isolates showed overexpression of CmeB on immunoblotting and/or real time RT-PCR expression of *cmeB*. CmeA expression based on a threshold of 2-fold for overexpression was used for isolates that remained between the two phenotypic groups after real time *cmeB* expression levels were analyzed.

The expression level of CmeR was also evaluated by immunoblotting in selected clinical isolates that harbored mutations in the *cmeR* gene. The whole cell samples were prepared from isolates M63885, CT9:7, CB2:6, CB2:8, CB2:11, S13530, T37957A, X7199, CT2:2, NCTC 11168, and 11168ΔcmeR. Samples were loaded onto a 12% SDS PAGE gel for electrophoresis in Lameilli buffer at 80 V for 30 minutes followed by 200V for 60 minutes. The gel and PVDF membrane were equilibrated in Towbin transfer buffer for 30 minutes, assembled onto the transfer apparatus, and transferred at 60 V for 40 minutes in Towbin transfer buffer. The membrane was blocked in blocking buffer (5% skim milk with 0.01% Tween-20 in PBS) at 4°C on a rocker. Then it was incubated with rabbit anti-CmeR diluted 1:100 in blocking buffer for 90 minutes at room temperature on a rocker. The membrane was washed 3 times for 10 minutes in washing buffer (0.01% Tween-20 in PBS) and further incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (KPL) (1:1000 in blocking buffer) for 1 hour at room temperature. After three washings, the membrane was developed with the 4CN Horseradish Peroxidase Substrate system (KPL). Densitometric analysis for CmeR was performed in the same manner for CmeABC.

SDS-PAGE and immunoblotting were also performed for the recombinant mutant CmeR named rCmeR-tr to determine if the protein was recognizable by CmeR.
antibodies. This recombinant protein was derived from mutations in strain CT2:2 (Table 3). The recombinant CmeR named rCmeRSS was used as a control. Both rCmeRSS and rCmeR-tr were loaded at 400 ng onto a 12% SDS-PAGE gel. Electrophoresis and immunoblotting were performed using the same methods used for immunoblotting of CmeR in clinical isolates.

Real time RT-PCR

Transcription of cmeB and cmeR was detected by real time RT-PCR to confirm the results of immunoblotting. RNA was isolated from 24-hour cultures of clinical isolates, NCTC 11168, and 11168ΔcmeR as described previously (13). Real time RT-PCR was performed for all isolates for cmeB and for selected isolates for cmeR as described previously (12, 13, 16, 34). The primers used for real time RT-PCR are shown in Table 1. Relative expression based on NCTC 11168 was calculated with the Pfaffl Method (35). Overexpression of cmeB was defined as greater or equal to 3 fold of NCTC 11168 expression. cmeB expression measured by RT-PCR was compared with the result of immunoblotting for final determination of a CmeABC phenotype. For those isolates where RT-PCR and immunoblotting data did not align, less weight was given to real time RT-PCR data due to reported variability in cmeB coding sequence (36).

DNA Sequencing

DNA sequencing was performed to determine if isolates were carrying mutations in cmeR, the Cj0369c-cmeR promoter or the cmeABC promoter. All clinical isolates classified as having a phenotype of CmeABC overexpression, some selected clinical
isolates with the wild-type expression levels, and 81-176 were amplified with RIGA-F and RIGA-R primers (Table 1) for sequence analysis of the region between cmeR to the 5’end of cmeA, which covers the whole ORF of cmeR and the entire promoter of cmeABC. Additionally, the predicted promoter region of Cj0369c-cmeR (the two genes share a single promoter located upstream of Cj0369c) was examined to determine if mutations in the promoter region were involved in differential CmeR expression. The Cj0369c-cmeR promoter was amplified with Cj0370-F and Cj0368c-R (Table 1).

**Electrophoretic mobility shift assays (EMSA)**

EMSA was used to assess the binding of CmeR to the cmeABC promoter sequences from various clinical isolates. These clinical isolates were observed to contain sequence polymorphisms within the cmeABC promoter region. A recombinant CmeR named rCmeRSS (37) with cysteines 69 and 166 replaced with serine was used for EMSA as described previously (12, 38). The Cys-Ser substitution does not affect the binding activity, but significantly improves the stability of recombinant CmeR as the Cys residues are sensitive to oxidation during *in vitro* binding assay. The 170-bp cmeABC promoter sequences were amplified from genomic DNA of NCTC 11168, 81-176, CT3:7, CT1:1, CT1:9, CT9:20, and X7199 with primers GSF and GSR1(12) (Table 1). The amplified products were purified (QIAgene PCR Purification kit, Qiagen) and then labeled with DIG-11-dd-dUTP using the DIG Oligonucleotide 3’ End Labeling kit (Roche). The labeled promoter DNA were used as probes in EMSA.

The cmeABC promoter probes were named for their strain of origin: 11168, 81-176, CT3:7, CT1:1, CT1:9, CT9:20 and X7199. Promoter probes from clinical isolates
(CT3:7, CT1:1, CT1:9, CT9:20 and X7199) were compared with the promoter probes of 11168 or 81-176 (laboratory strains), depending on their similarity to the CmeR binding site in the two laboratory strains. The CmeR-binding site contains an A to T substitution in the 81-176 strain compared to 11168, which is considered a naturally occurring variation (12).

The promoter probes (0.05 pmol each) were incubated with 0, 60, 120, and 180 ng of rCmeRSS in 22 µL of binding buffer according to the method of Alekshun et. al. and Lin et. al. 2005 (12, 38). The reaction mixtures were incubated for 30 minutes at room temperature and Promega DNA loading buffer was added to each reaction. Samples were separated by electrophoresis at 200V for 45 minutes on a 6% polyacrylamide gel in 0.25X TBE Buffer and transferred to a positively charged nylon membrane by vacuum (12). Chemiluminescent detection using alkaline phosphatase-conjugated anti-DIG antibody and CDP-Star (Roche) was performed as previously described (12).

**Construction of promoter fusions and β-galactosidase assays**

The observed sequence polymorphisms in the promoter sequences of cmeABC were assessed for their impact on cmeABC transcription by constructing transcriptional fusions with a promoter-less lacZ gene. Genomic DNA templates (NCTC 11168, 81-176, CT1:1, CT 1:9, CT9:20, CT3:7, M32506 and X7199) were used for amplification of a 578-bp sequence containing the cmeABC promoter with primers PF (12) and PX (Table 1). These PCR products were purified (QIAquick PCR Purification kit), digested with XbaI and BamHI (Promega), and re-purified using the QIAquick kit. Vector pMW10 (32) was purified (QIAprep Spin Miniprep kit, Qiagen) from DH5αpMW10 (Table 2),
digested with the same enzymes, and re-purified. Vector and PCR product inserts were ligated with T4 ligase (Roche) and transformed into DH5α. Transformants were selected on LB agar plates supplemented with kanamycin (30 µg/mL). Plasmid constructs (Table 2) were purified from DH5α and sequenced with pMW10-F and pMW10-R (Table 1) to confirm the appropriate sequences and fusion.

To transfer the plasmids into C. jejuni, tri-parental mating using C. jejuni 81-176, E. coli DH5αpRK2013 (33), and the various DH5α pMW10 transcriptional fusion constructs was performed as described previously (33). After transfer into C. jejuni 81-176, plasmids pMW11168, pMW81-176, pMW1:1, pMW1:9, pMW3:7, pMW9:20, pMWM32506, and pMWX7199 (Table 2) were purified and electroporated into 81-176ΔcmeR. The empty vector, pMW10, was also transferred to 81-176 and 81-176ΔcmeR by the same methods and used as a background control. Cultures were grown for 20 hours in MH broth supplemented with kanamycin (30 µg/mL), then β-galactosidase assays were performed as described previously (39). Three independent experiments were conducted. Student’s t-test with Welch’s correction was used to compare the expression data from various promoters and was done using GraphPad InStat® (Version 3.06) with the significance level set at 0.05.

Sequence polymorphisms were also observed in the Cj0369c-cmeR promoter. To determine if these mutations affected CmeR expression, a second set of promoter fusions was created using the same method. Briefly, primers 370BamH-F and 369XbaI-R1 (Table 1) were used to amplify a 238-base pair segment containing the Cj0369c-cmeR promoter from genomic DNA templates of NCTC 11168, 81-176 and X7199. Construction of plasmids, transformation into DH5α and sequencing was performed as
described above. Plasmid constructs pMW11168-R, pMW81-176-R and pMWX7199-R were electroporated into C. jejuni 81-176 (Table 2). The empty vector, 81-176pMW10, from the prior assay was used as a background control. β-galactosidase assays for 3 independent experiments and statistical analysis were performed as described in the assays with the cmeABC promoters.

**Construction, purification, and functional analysis of various CmeR variants**

Sequence polymorphisms were detected in CmeR among the analyzed isolates. To determine if the sequence variations affected the DNA binding activity of CmeR, we generated various forms of recombinant CmeR using site-directed mutagenesis. Plasmid pQECmeRSS (37) was used as a template for site directed mutagenesis, which was done using the Stratagene QuikChange II kit. Site-specific primers (Table 1) were used to produce the amino acid changes in CmeR from isolates CT2:2, M63885, CT9:7, and T37597A. All amino acid substitutions, corresponding nucleotide sequences and protein names are listed in Table 3.

Isolate M63885 contains 2 amino acid substitutions in CmeR, which were introduced simultaneously into the pQECmeRSS template. The M63R17-F and M63R17-R primers were used to mutate the threonine to isoleucine at residue 3 and the M63475-F and M63475-R primers were used to mutate the glutamate to lysine at residue 159. This mutated plasmid was named pQECmeR-IK. Isolates CT9:7 and T37957A both contain a single amino acid substitution in CmeR. Primers T97547G-F and T97547G-R were used to change the proline to arginine at residue 159 as observed in strain CT9:7, creating the plasmid named pQECmeR-R. Primers T3X250A-F and T3X250A-R introduced the
glutamate to lysine substitution at residue 84 as observed in strain T37957A. This plasmid was named pQECmeR-K. Isolate CT2:2 contains 2 amino acid substitutions in CmeR and a nucleotide insertion. This mutated CmeR was created using 2 rounds of mutagenesis. The T insertion after nucleotide 583 was introduced into template pQECmeRSS with primers T22583-F1 and T22583-R1 along with the first amino acid substitution at residue 144, a glycine to alanine substitution, with primers r431GA-F and r431GA-R to create an intermediate plasmid. The mutations were confirmed in the intermediate plasmid prior to introduction of the final mutations. In the second round of mutagenesis, this intermediate was used as a template to introduce the final amino acid change, a serine to glycine substitution at residue 207 with primers r619621-F and r619621-R to create plasmid pQECmeR-tr.

All mutations were introduced into the respective templates by one cycle of 95°C for 30 seconds followed by 16 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 68°C for 4 minutes. The amplified product was cooled on ice for 2 minutes before Dpn-I digestion of parental DNA at 37°C for 1 hour. Each product was transformed into JM109 or XL1-Blue (Agilent) and the transformants were selected on LB agar supplemented with ampicillin (100 µg/mL). The specific mutations were confirmed by sequencing with primers pQETYPEIII/IV-F and pQEREverse-R (Table 1).

The CmeR variants including rCmeR-tr, rCmeR-R, rCmeR-K, and rCmeR-IK were induced and purified from their respective E. coli strains (Table 2) under native conditions (40). After purification, proteins were desalted using PD-10 desalting columns (GE Healthcare). Proteins of rCmeR-tr and rCmeR-K were concentrated in PBS using Amicon Centricon YM-10 Columns (Millipore).
For functional analysis, EMSA was used to assess the ability of the mutated versions of CmeR to bind to the *cmeABC* promoter. Binding by the purified mutant proteins rCmeR-tr, rCmeR-R, rCmeR-K or rCmeR-IK was compared to binding by the rCmeRSS protein. The 11168 *cmeABC* promoter probe (0.05 pmol) was incubated with 0, 60, 120, and 180 ng of rCmeRSS or one of the mutant rCmeR proteins in 22µL of reaction buffer according to the method of Alekshun *et. al.* and Lin *et. al.* 2005 (12, 38). The reaction mixtures were incubated for 30 minutes at room temperature and Promega DNA loading buffer was added to each reaction. Electrophoresis, transfer, and detection were performed using the same methods as described for the EMSA assay.

**Antimicrobial susceptibility testing**

Agar dilution test was performed in MH agar and MH agar supplemented with 12,500 µg/mL of sodium choleate according to the CLSI-recommended method (31). Ciprofloxacin, kanamycin, erythromycin, tetracycline, ampicillin, clindamycin, chloramphenicol, ethidium bromide, sodium choleate, cholic acid and taurocholic acid were tested. In addition to the 21 WEL and 43 OEL clinical isolates, 3 laboratory strains classified as WEL isolates, NCTC 11168, ATCC33560, and 81-176, were also tested. At least 2 experiments were performed for each isolate. Distribution of the minimum inhibitory concentration (MIC) around the median was tested with the Brown Forsythe test (SAS version 9.2) comparing the 43 OEL and 24 WEL isolates. The significance level set at 0.05.
Fluctuation Assays

Fluctuation assays were performed to assess if CmeABC expression levels affected the spontaneous mutation rate to ciprofloxacin in *C. jejuni*. The assays were conducted using the methods described by Luria and Delbrück (41) with some modifications (42-46). Selected WEL isolates (NCTC 11168, 81-176, CB8:14, CT10:18, H2958, CB6:8) and OEL isolates (CT9:7, CB4:22, M76297, CT9:14, CB3:1, T37957A, 11168ΔcmeR, 81-176ΔcmeR) were cultured on antimicrobial-free MH plates, adjusted to $10^8$ CFU/mL in MH broth, and serially diluted to $10^4$. Thirty-six parallel cultures of 200 µL were inoculated on a 96-well plate and incubated for 24 hours. Total cell counts were determined by plating on antimicrobial free MH agar while spontaneous mutant counts were plated on MH agar supplemented with 4 µg/mL of ciprofloxacin.

Total cell counts for each set of cultures (36 parallel cultures) were estimated from 5 random wells. The 5 wells were selected using the Random Integer Set Generator (Random.org). From each of these wells, a 10 µL sample of culture was removed and serially diluted to $10^{-7}$. A 90 µL sample from the $10^5$, $10^6$, and $10^7$ dilutions was spread to MH agar. Plates were incubated for 2 days for total CFU counts. Counts from the 5 random wells were averaged to determine the total count for each set of 36 parallel cultures.

To determine the number of spontaneous mutants, all 36 wells were plated to MH agar containing 4 µg/mL of ciprofloxacin. Plates were checked after 2 days for colony size and incubated for an additional day to ensure colonies were large enough for counting. Mutation rate was calculated by the Ma-Sandri-Sarkar Maximum Likelihood Estimator method using the Fluctuation Analysis Calculator (47). Average mutation rates
for WEL and OEL were log transformed and compared with the Student’s t-test using GraphPad InStat® (Version 3.06) with the significance level set at 0.05.

**In vitro ciprofloxacin treatment**

Inactivation of CmeABC reduced the emergence of ciprofloxacin-resistant mutants in *C. jejuni* under antibiotic selection, while inactivation of *cmeR* (resulting in overexpression of *cmeABC*) increased mutant emergence, suggesting that expression levels of CmeABC influences the emergence of antibiotic resistant mutants. To assess if differential expression of *cmeABC* in naturally occurring isolates affects their adaptation to antibiotic treatment, we examined the emergence frequencies of ciprofloxacin-resistant mutants in isolates of different CmeABC phenotypes. Three WEL (CB6:8, F15871, CT10:18) and three OEL isolates (T37957A, CT7:20, CB8:14) were used for this experiment, which were cultured on non-selective media (MH agar) and then treated with 4 µg/mL of ciprofloxacin using the method of Han *et. al.* 2008 (48) with some modifications. Briefly, MH broth supplemented with 4 µg/mL of ciprofloxacin was inoculated to an initial concentration of $10^7$ CFU/mL in a 20 mL of culture with 3 replicates per isolate. Cultures were incubated for 3 days. Samples (0.5 mL) were taken on days 0, 1, 2, and 3 - post inoculation for enumeration of ciprofloxacin-resistant mutant and total cell counts. Total counts were cultured on MH agar according to the plate drop method (49) for days 0, 1, 2, and 3. For days 0 to 2 mutants were counted by direct plating 100 µL of culture onto MH-ciprofloxacin (4 µg/mL) agar in duplicate and according to the plate drop method (49) onto MH-ciprofloxacin (4 µg/mL) agar. For day 3, total plate counts and mutants were all determined by the plate drop method. The serial
dilutions used in the plate drop method were dilutions $10^{-2}$ to $10^{-7}$ for day 0 and $10^{-3}$ to $10^{-8}$ for days 1 to 3. Two independent experiments were performed for each isolate. The cell counts were calculated for each culture and log transformed. Data analysis was performed with Graph Pad Prism® (Version 6.0c) with multiple unpaired t-tests and Holm-Šidák method for multiple comparisons. The significance level was set at 0.05.

In the second experiment, cultures were inoculated to an initial density of $10^6$ CFU/mL. Samples were collected in the same manner as the first experiment. For each day total counts were cultured on MH agar according to the plate drop method (49). Mutants were counted by direct of plating 100 µL of culture to MH-ciprofloxacin (4 µg/mL) agar in duplicate and serial dilutions for plate drop method (49) onto MH-ciprofloxacin (4 µg/mL) agar. The serial dilutions used in the plate drop method were dilutions $10^{-2}$ to $10^{-7}$ for day 0 and $10^{-3}$ to $10^{-8}$ for days 1 to 3. Two independent experiments were performed for each isolate. Data analysis was performed as described in the prior experiment.

Results

Phenotypic classification of isolates

Initial screening for phenotypic classification of CmeABC expression was done through immunoblotting (Fig.1) and real time RT-PCR for expression of cmeB. Analysis of the 64 C. jejuni isolates for cmeB expression identified 43 isolates with overexpression levels (OEL) of CmeABC and 21 isolates with wild-type expression levels (WEL) of CmeABC. The region spanning from cmeR to cmeA was sequenced for all OEL isolates,
4 of the WEL isolates, and C. jejuni strain 81-176 to identify genetic mutations that were potentially involved in mediating the differential cmeABC expression. All isolates except 1 OEL isolate harbored mutations in the sequenced region compared to the same region in strain NCTC 11168.

To refine the identification of mutations mediating differential cmeABC expression, the cmeABC promoter was analyzed for mutations unique to the OEL isolates and cmeR was analyzed for DNA polymorphisms resulting in amino acid changes. The CmeR binding site of the cmeABC promoter contains an A to T substitution at base 10 of the 16 base inverted repeat in strain 81-176 (Fig. 2A) that is considered a natural variation (81-176 variation) (6). Among the clinical isolates, 34 carried this mutation. Isolates that contained only the 81-176 variation in the CmeR binding site were excluded from further analysis. There were 14 OEL isolates carrying mutations within the CmeR binding site of the cmeABC promoter other than the 81-176 A to T substitution (Figure 2A). These isolates also carried amino acid mutations in CmeR. All of the observed amino acid mutations were also seen in isolates with no mutations in the cmeABC promoter. These 14 isolates were categorized as cmeABC promoter mutants.

Analysis of cmeR for DNA polymorphisms found numerous mutations leading to amino acid changes in CmeR. DNA polymorphisms that did not result in amino acid changes in CmeR were excluded. Three isolates harbored unique mutations in CmeR (Table 3). Other OEL isolates carried amino acid changes in CmeR that were also seen in WEL isolates. Five OEL isolates and one WEL isolate with a unique combination of substitutions at residues 144, 183 and 207 were also selected for analysis (Table 3). None
of these isolates, except X7199, had mutations in the *cmeABC* promoter. Together these 9 clinical isolates were categorized as *cmeR* mutants.

**Mutation of the CmeR binding site affects cmeABC expression in clinical isolates**

Figure 2A illustrates the region of the *cmeABC* promoter containing the CmeR binding site. Two categories of substitutions were observed in the CmeR binding site: 5 isolates with a G to A substitution at base 2 of the inverted repeat and 7 isolates with an A to G substitution at base 14. The 81-176 variation is found in 7 isolates carrying the substitution at base 14 and one isolate carrying the substitution at base 2. Two isolates carrying the 81-176 variation and a 5 base deletion in the *cmeABC* promoter were detected. The last 3 bases of the CmeR binding site and the following 2 bases are absent in isolate CT9:20 (Fig 2A). Isolate X7199 has a 5 base deletion 5' to the CmeR binding site in addition to an A to G substitution at base 14 of the CmeR binding site (Fig 2A). These 14 isolates are considered *cmeABC* promoter mutants.

From the *cmeABC* promoter mutants, 6 representative sequences were selected for analysis by EMSA and transcriptional fusion (Fig. 2A). The promoters from the isolates X7199 and CT9:20 were selected for their unique deletions. The M32506 and CT3:7 promoters both contain the 14 A to G mutations, while the CT3:7 promoter also contains the 81-176 variation. The CT1:1 and CT1:9 promoters carry the 2 G to A mutation.

The ability of CmeR to bind to the mutant *cmeABC* promoter sequences was assessed by EMSA. The selected mutant *cmeABC* promoter sequences from clinical isolates were paired with either the 11168 promoter (promoter from strain NCTC 11168) (Fig. 2B, panels I to III, lanes 1 to 4) or 81-176 promoter (Fig. 2B, panels IV to VI, lanes
1 to 4) for use in EMSA based on the presence or absence of the A to T 81-176 variation in the CmeR binding site. Five of the 6 mutant promoters, CT1:1, CT1:9, M23506, CT3:7, and CT9:20 (Fig 2B, panels I, II, III, V, and VI respectively, lanes 5 to 8) showed decreased binding to rCmeRSS as manifested by the increased amounts of unbound probe and/or decreased amounts of probe-rCmeRSS complexes. The CT1:1 (Fig. 2B, panel I, lanes 5 to 8), CT1:9 (Fig. 2B, panel II, lane 5 to 8) promoters showed increased amounts of free probe. The M32506 (Fig. 2B, panel III, lane 5 to 8), CT3:7 (Fig 2B, panel V, lane 5 to 8), and CT9:20 (Fig. 2B, panel VI, lane 5 to 8) probes display decreased intensity in the CmeR-DNA complexes and increased amounts of free probe. The CT9:20 cmeABC promoter (Fig. 2B, panel VI, lanes 5-8) showed the largest reduction in CmeR binding as indicated by the reduced intensity of rCmeRSS-DNA complexes (Fig. 2B, panel VI lanes 7-8) and increased free probe amount compared to the 81-176 probe. The X7199 promoter displayed no difference in binding to rCmeRSS compared to the 81-176 promoter (Fig. 2B, panel IV). These results suggest that most of the examined mutations in the cmeABC promoter sequence affected binding by CmeR.

To further quantify the effect of the promoter mutations on cmeABC expression, transcriptional fusion of the mutant cmeABC promoters to the promoterless lacZ gene was performed in the presence and absence of CmeR. In the 81-176 wild-type background, CmeR is expressed and binds to the cmeABC promoter, repressing its transcription. The 81-176ΔcmeR strain is an isogenic mutant that does not express CmeR, resulting in a loss of repression for the cmeABC promoter. Without this repressor, cmeABC is overexpressed. Transcription from the 11168 promoter in the 81-176 wild-type background (CmeR is present) was defined as the basal level of cmeABC.
transcription and used as a control. In the presence of CmeR (81-176 wild-type background) (Fig. 3A), all examined cmeABC promoters including 81-176 have significantly increased ($p<0.05$) transcription compared to basal levels. Expression from the 81-176 promoter increased 2.4-fold ($p = 0.0096$), the CT1:1 promoter increased 4.6-fold ($p = 0.0232$), the CT1:9 promoter increased 5.5-fold ($p = 0.0073$), the CT3:7 promoter increased 5.6-fold ($p = 0.0425$), the M32506 promoter increased 6.3-fold ($p = 0.0036$), and the X7199 promoter increased 5.4-fold ($p = 0.0150$) over basal transcription levels. These increases are indicative of decreased repression by CmeR due to the mutations in the cmeABC promoters. Notably, the CT9:20 promoter with the deletion of the last 3 bases of the CmeR binding site, had the highest increase in transcription at 8-fold ($p = 0.0208$) over the basal levels, consistent with its most obvious reduction in binding by CmeR on EMSA.

Transcription from the CT1:1 and CT1:9 promoters, with the same sequence but carried in different isolates, was not significantly different. This indicates that choosing a single representative isolate is sufficient to evaluate the other isolates in the group. The transcription from the X7199 and CT3:7 promoters is not significantly different, although there is 5-base pair deletion upstream of the CmeR binding site in the X7199 promoter. This result suggests that the substitutions in the CmeR binding site alone (represented by CT3:7) is sufficient to alter expression of cmeABC.

In the absence of CmeR, transcription for all promoters except X7199, was not significantly different ($p >0.05$) compared to the 11168 promoter (Fig. 3B). Interestingly, transcription from the X7199 promoter was significantly increased 2.2-fold ($p = 0.0472$) over 11168 in the absence of CmeR and represents a 14.1-fold ($p = 0.0154$)
increase over the basal transcription level. Comparatively, the 11168 promoter increased 6.3-fold ($p = 0.014$) over basal levels while the 81-176 promoter increased 6.0-fold ($p < 0.0001$). This suggests that the additional increase for the X7199 promoter does not involve CmeR. Comparisons for each promoter showed the deletion of CmeR significantly increased expression ($p < 0.05$) of the cmeABC promoters form 11186, 81-176 and M32506 (Fig 3C). Transcription in the absence of CmeR (81-176 ΔcmeR background) showed a 6.3-fold increase for the 11168 promoter ($p = 0.014$) and a 2.5-fold increase for the 81-176 promoter ($p = 0.0039$) and a 2.6-fold increase for the X7199 promoter ($p = 0.0367$) (Fig 3C). The smaller change for the 81-176 and X7199 promoters compared to the 11168 promoter is likely due to already elevated expression in the wild-type background mediated by the CmeR binding site mutations. Transcription from the M32506 promoter, surprisingly, was statistically significant ($p = 0.0410$) in the absence of CmeR compared to the wild-type. However this 0.8-fold difference is not biologically significant and is the same for the CT9:20 promoter (Fig. 3C). Examination of M32506 transcription revealed this was likely due to a single, low level measurement in the wild-type background. Compared to their transcription in the wild-type background, the expression of CT1:1, CT1:9, CT3:7, and CT9:20 promoters were not significantly different ($p > 0.05$), suggesting that inactivation of cmeR did not further increase transcription from these promoters or M32506 (Fig. 3C).

**Varied expression levels of cmeR in clinical isolates**

Several DNA polymorphisms were detected in the cmeR gene of clinical isolates, resulting in amino acid changes in this regulatory protein (Table 3). Immunoblotting of
whole cell proteins was performed to determine the CmeR expression level from 8 OEL isolates and 1 WEL isolate harboring representative mutations. The anti-CmeR antibody detected the CmeR protein from 8 of the 9 cmeR mutants (Fig. 4A). The remaining cmeR mutant isolate (CT2:2) did not produce a band reactive with the antibody (Fig. 4B, lane 2), suggesting that the CmeR protein was not translated in this isolate. CT2:2 contained a T insertion after base 583 in cmeR, resulting in a frame shift and premature truncation (Table 3). Additional immunoblotting failed to detect any portion of the truncated CmeR from CT 2:2.

To confirm the results of immunoblotting, cmeR expression was evaluated by real time RT-PCR. Real time expression levels of cmeR varied dramatically among the 9 isolates. Expression ranged from 0.006 to 33 fold of that in NCTC 11168 (Table 3), however this was not correlated with CmeR expression levels on immunoblotting (densitometric data not shown). Expression levels of cmeR from CT2:2 were negligible at 0.006 fold of that in NCTC 11168, consistent with the lack of protein expression as detected by immunoblotting.

cmeR and Cj0369c form a two-gene operon and share a single promoter located in front of Cj0369c (13). The predicted promoter for the Cj0369c-cmeR operon contains an inverted repeat with two half sites separated by a 12-base spacer that may represent an unknown regulatory mechanism (13). Sequence analysis of this region was performed on several isolates to determine if mutations occurred and if they could be correlated with the varying cmeR expression levels identified by real time RT-PCR. Mutations of the Cj0369c-cmeR promoter were found in some isolates after comparison to the sequence of NCTC 11168 and were divided into 2 groups (data not shown). The first group carried a
single base deletion one base 5’ to the second half site of the inverted repeat in strains 81-176, T37957A, and E46972. The second group, consisting of isolates X7199, W52546, and S13530, contained a T insertion in the second half site of the inverted repeat after the eighth base and a G to A substitution at base 5 of the spacer. The Cj0369c-cmeR promoter from CT2:2 had no mutations, suggesting that transcription from this promoter is unlikely the source of the decreased production as observed by immunoblotting and real-time PCR.

Transcriptional fusion of representative Cj0369c-cmeR promoters from strains 81-176, X7199, and NCTC 11168 was performed to determine if the observed polymorphisms affected transcription. The 81-176, NCTC 11168, and X7199 promoters produced low levels of transcription (Miller units in the range 2-16), which were not significantly different (p > 0.05) (data not shown), suggesting that these mutations were not associated with the expression levels of cmeR. However, this was not consistent with the real time cmeR expression data (Table 3). The mutations observed in the Cj0369c-cmeR promoter for X7199 and the S13530 were identical but, cmeR expression was 10.9 and 0.2 fold of NCTC 11168 respectively. The reason for this discrepancy is unclear.

Truncation, but not amino acid substitution affected CmeR binding to the cmeABC promoter

The amino acid changes in CmeR observed in clinical isolates were categorized into 5 groups represented by the 9 cmeR mutant isolates (Table 3). Site-directed mutagenesis and recombinant CmeR production were performed for 3 representative isolates with amino acid substitutions and a single isolate with substitution and truncation
of CmeR (Table 3). The 4 proteins produced were named rCmeR-tr, rCmeR-IK, rCmeR-K and rCmeR-R. The rCmeR-IK from M63885 contains 2 amino acid substitutions at residues 6 and 159 replacing threonine with isoleucine and glutamate with lysine, respectively. The rCmeR-K from T37957A and rCmeR-R from CT9:7 contain single amino acid substitutions of the glutamate at residue 84 for lysine in rCmeR-K and the proline 183 residue for arginine in rCmeR-R. The rCmeR-tr from isolate CT2:2 contains a glycine to alanine substitution at residue 144 and a nucleotide insertion after base 583 resulting in pre-mature truncation of CmeR to 193 amino acids (the full-length CmeR is 210 amino acids). The S207G substitution observed in CT2:2 occurs downstream of the truncation and was not expected to affect CmeR function. rCmeR-tr was detected by immunoblotting with the anti-CmeR antibody and presented as a band of 23 kD (Fig. 5A, lane 3), slightly smaller than the full-length rCmeRSS at 24kD (Fig. 5A, lane 2), consistent with the predicted truncation.

EMSA was performed with all 4 mutant proteins to assess the binding to the NCTC 11168 cmeABC promoter (11168 promoter). The rCmeR-R, rCmeR-IK, or rCmeR-K proteins bound to the cmeABC promoter in a manner similar to the rCmeRSS control (data not shown). However, rCmeR-tr failed to bind to the cmeABC promoter at all tested concentrations (Fig. 5B, lanes 5-8) suggesting that the truncation abolished the ability to bind promoter DNA.

**Differences in antimicrobial susceptibility**

*In vitro* antimicrobial susceptibilities did not differ between the OEL and WEL isolates in the presence or absence of bile for most of the tested antimicrobials except for
chloramphenicol. Without bile, the median MIC for chloramphenicol was 4 µg/mL for both the OEL and WEL isolates (Table 4). However, the distribution of MICs around the median was significantly different between the two phenotypic groups ($p < 0.05$). The WEL isolates had chloramphenicol MICs of 2 to 4 µg/mL with 67% of the isolates at 4 µg/ml, while the OEL isolates had MICs ranging from 2 to 16 µg/mL with 21% of the isolates at 8 and 16 µg/mL (Table 4). With bile in the testing media, the MIC distribution between the two phenotypes was also significantly different ($p < 0.05$) (Table 4).

Although the median remains at 4 µg/mL for both groups, 81% of the OEL isolates were at this MIC, while 58% of the WEL isolate were at this MIC. Both OEL and WEL isolates have MIC ranges of 2 to 8 µg/mL after addition of bile. Compared to the non-bile media, addition of bile shifted the MIC to the upper end for WEL isolates and shifted the MIC to the median the for OEL isolates.

**Emergence of ciprofloxacin-resistant mutants**

The spontaneous mutation rate to ciprofloxacin was examined for selected isolates using the fluctuation assay, which was not significantly different between OEL and WEL isolates (data not shown). However, OEL isolates showed increased emergence of ciprofloxacin-resistant (Cip$^R$) mutants during *in vitro* treatment (Fig. 6). Two experiments were performed with the initial inoculum levels of $10^7$ and $10^6$ CFU/mL, respectively. For the inoculums at $10^7$ CFU/mL, there were no significant difference in the mean numbers of pre-existing Cip$^R$ mutants between the WEL and OEL cultures on day 0 (Fig. 6A). Cip$^R$ populations from both WEL and OEL cultures expanded over days 1 to 3. The mean Cip$^R$ mutant populations were 0.9 logs higher for OEL on day 1, 1.5
logs higher on day 2, and 2 logs higher than WEL on day 3. However, the means were not significantly different between the OEL and WEL groups ($p > 0.05$).

For the inoculum of $10^6$ CFU/mL, the means for pre-existing Cip$^R$ mutants on day 0 were not significantly different for the WEL and OEL cultures. The mean Cip$^R$ mutant numbers on day 1 were 2 logs ($p = 0.0175$) higher than the mutants in the WEL cultures. This trend continued on days 2 and 3 with OEL means being 3.7 logs ($p = 0.0053$) and 4.2 logs ($p = 0.0016$) higher than the WEL means, respectively. These results indicate OEL cultures produced significantly higher numbers of Cip$^R$ mutants than the WEL cultures during ciprofloxacin treatment.

Discussion

This study demonstrates that differential expression of CmeABC naturally occurs in *Campylobacter* isolates derived from different host species. The differential expression was linked to multiple mechanisms including mutations in the *cmeABC* promoter region and coding sequences of CmeR as well as decreased expression of CmeR, the repressor for the *cmeABC* operon. Additionally, there are unidentified mechanisms that also modulate *cmeABC* expression as some OEL isolates did not have any mutations in the known regulatory elements for *cmeABC*. Differential expression of *cmeABC* was linked to altered antimicrobial susceptibility and enhanced the emergence of Cip$^R$ mutants under antibiotic selection. These findings suggest that differential expression of CmeABC is selected under natural conditions and may facilitate *Campylobacter* adaptation to various environments.
Of the 64 isolates examined in this study, 43 (67%) were phenotypically classified as OEL isolates. CmeABC is normally repressed by CmeR, which binds to the promoter region of \textit{cmeABC} (12). Thus, mutations in CmeR and/or the \textit{cmeABC} promoter sequence were investigated to determine the genetic basis associated with overexpression of \textit{cmeABC} in these clinical isolates. Those mutations that occurred in the CmeR binding site or resulted in amino acid changes in CmeR and were absent from the majority of the WEL isolates were selected for detailed analysis. Additionally, those isolates harboring the same mutation as the one in the CmeR binding site of strain 81-176 were excluded from analysis as this mutation has been characterized previously (12). Using these selection criteria, we selected 14 isolates harboring promoter mutations and 9 isolates harboring CmeR mutations for detailed analysis.

Most of the detected amino acid substitutions in CmeR did not affect its function as determined by EMSA. However, a single nucleotide insertion at the 3’ end of the \textit{cmeR} gene resulted in a frame-shift and presumably led to truncation of the CmeR protein in CT2:2. CmeR was not detected by immunoblotting in CT2:2 (Fig. 4B) and the \textit{cmeR} transcript level was also significantly reduced. Additionally, CT2:2 did not contain any mutations in the predicted \textit{Cj0369c-cmeR} promoter region, excluding the possibility that lack of \textit{cmeR} expression was due to altered transcription initiation. These findings suggest that the single nucleotide insertion could have destabilized the \textit{cmeR} transcript or the frame shift rendered the CmeR protein unstable in \textit{C. jejuni}, leading to the lack of CmeR in this isolate. However, a recombinant version of the truncated CmeR (rCmeR-tr) was successfully generated (Fig. 5A), suggesting that this truncated CmeR is stable in the \textit{E. coli} host. Interestingly, rCmeR-tr failed to bind to the promoter DNA of \textit{cmeABC} as
determined by EMSA (Fig. 5B), suggesting that even if this truncated version is made in *C. jejuni*, it would not be able to control the expression of *cmeABC*. The lack of CmeR production and the inability of the truncated CmeR to bind to promoter DNA fully explain the overexpression of *cmeABC* in isolate CT2:2.

It is interesting that rCmeR-tr lost the ability to bind DNA despite the fact that the truncation occurred in the C-terminal end of CmeR and the DNA-binding domain remained intact. The truncation occurred in the α10 helix of CmeR. CmeR functions as dimer *in vivo* and the crystal structure of CmeR identified that α helices 6, 8, 9, and 10 are involved in dimer formation (12, 15, 50). Thus, the truncation in rCmeR-tr likely affects dimer formation and ultimately the function of CmeR. This result suggests that the C-terminal sequence of CmeR is also important for its interaction with target DNA.

Recently, *C. jejuni* ATCC 33560, a quality control strain used for antimicrobial susceptibility testing in *C. jejuni*, was found to contain a frame shift mutation in *cmeR*, which led to truncation of the CmeR protein (51). The truncation occurs in α helix 8 of CmeR and presumably results in non-functional CmeR (51). Together, these findings indicate that frame-shift mutations in CmeR occur under natural conditions. As CmeR is a pleiotropic regulator (regulating other genes in addition to *cmeABC*) (13), truncation of CmeR likely affects multiple functions in *C. jejuni*.

The majority of the mutations in the *cmeABC* promoter were found to affect *cmeABC* expression. The *cmeABC* promoter contains a 16 base inverted repeat that serves as the specific binding site for CmeR (12). Mutations in the CmeR binding site within the *cmeABC* promoter have been described previously after *in vitro* stepwise selection with erythromycin (14) and ciprofloxacin (12). This study is the first to describe
the occurrence of this type of mutation in *C. jejuni* isolates from natural sources and various hosts including humans, turkeys, and chickens. These mutations inhibited binding by CmeR, resulting in increased transcription from the *cmeABC* promoter. This was demonstrated by reduced binding of the mutant promoters by CmeR on EMSA (Fig. 2B, panels I, II, III, V, and VI) and increased transcription of *cmeABC* as determined by transcriptional fusion assays (Fig. 3). In addition to the substitution, deletions within the CmeR binding site were found in one isolate, CT9:20. This promoter showed the greatest inhibition of CmeR binding on EMSA and the largest increase in transcription in the presence of CmeR (Fig. 3A). These findings indicate that mutations in the *cmeABC* promoter commonly occur and these mutations influence the expression of this multidrug efflux pump.

Multiple mutations were also identified in a single isolate. For example, isolate X7199 harbored a substitution in the CmeR binding site and a 5 base deletion upstream of the CmeR binding site (Fig. 2A). This isolate also contained mutations in *cmeR* (Table 3). The *cmeABC* promoter in this isolate was identical to the promoter in CT3:7 except for a 5-base pair deletion upstream of the CmeR binding site (Fig. 2A). While the CT3:7 promoter demonstrated reduced binding to CmeR on EMSA (Fig. 2B, panel V), the X7199 promoter had similar binding as the 81-176 *cmeABC* promoter (Fig. 2B, panel IV). However, both the X7199 and CT3:7 *cmeABC* promoters demonstrated similar, elevated expression by transcriptional fusion assay compared with the 11168 promoter in the presence of CmeR (Fig. 3A). This discrepancy between the results of EMSA and transcriptional fusion suggests that the EMSA assay has a lower sensitivity than the transcriptional fusion assay, or alternatively, there is another regulatory mechanism that
also modulates *cmeABC* expression. Indeed, when the X7199 promoter was examined in the absence of CmeR using transcriptional fusion (Fig. 3B), its expression was significantly increased compared to its own expression level in the presence of CmeR (Fig. 3C). This result is consistent with the EMSA result and suggests that the X7199 promoter is still under the repression by CmeR.

In some isolates, the OEL phenotype was not linked to the known mechanisms modulating *cmeABC* expression as there were no mutations detected in CmeR or the promoter region. Additionally, even though some isolates harbored mutations in CmeR, these mutations did not affect the function of CmeR. Furthermore, the regulation of the X7199 promoter cannot be fully explained by a CmeR-dependent mechanism. These findings suggest that there may be additional regulatory mechanisms modulating *cmeABC* expression. Previously, Lin *et. al.* 2005 also described a CmeR-independent mechanism modulating *cmeABC* expression (16). Bile is a known inducer of CmeABC and mediates increased expression by altering the confirmation of CmeR, resulting in disassociation of CmeR from the *cmeABC* promoter (15, 16). It was noticed that in the absence of CmeR, the expression of *cmeABC* was further induced by taurocholate, suggesting this bile compound induced expression of *cmeABC* through a CmeR-independent mechanism (16). Taken together, observations from this study and previous reports suggest that multiple mechanisms modulate the expression of *cmeABC*.

The functional consequence of *cmeABC* overexpression was evaluated in relation to antimicrobial treatments. Antimicrobial susceptibility was unaffected by *cmeABC* overexpression for most of the tested antibiotics, with the exception of chloramphenicol. This was not surprising as a previous study using genetic manipulation revealed that
overexpressing *cmeABC* by inactivating *cmeR* had a modest effect on MICs, but inactivation of *cmeABC* significantly increased the susceptibility of *C. jejuni* to antibiotics (6, 12). For chloramphenicol, the OEL isolates displayed a larger range of MIC values than WEL isolates, with more MICs distributed above the median value (Table 4). This suggests that overexpression of CmeABC had an effect on the MIC distribution of chloramphenicol. Interestingly, when chloramphenicol MIC was measured in the presence of bile, the MICs of the WEL isolates shifted above the median value, while the MICs of the OEL isolates shifted toward the median value. This difference is probably due to the fact that bile is an inducer for *cmeABC* and the possibility that induction was more prominent in the WEL isolates than in the OEL isolates. For the OEL isolates, *cmeABC* was already overexpressed due to less inhibition by CmeR or other unidentified mechanisms. Thus, the bile-mediated induction through CmeR is expected to be less effective in the OEL isolates than in the WEL isolates.

Fluoroquinolone resistance in *Campylobacter* is mediated by DNA gyrase mutations and the function of CmeABC (6, 8, 9). These two mechanisms function synergistically in mediating resistance to fluoroquinolones (9). Additionally, CmeABC promotes the emergence of fluoroquinolone-resistant mutants under antibiotic selection (8, 23). In this study, we examined the correlation between the OEL phenotype and ciprofloxacin resistance. It was found that the basal spontaneous mutation rate was not affected by overexpression of *cmeABC* as measured by the fluctuation assay. However, the OEL isolates showed higher level of emergence of CipR mutants under antibiotic selection (Fig. 6). This was consistently shown by using two inoculation doses (10^6 and 10^7 CFU/mL). For the 10^7 CFU/mL inoculum, the difference between the OEL and WEL
groups were obvious, but were not statistically significant. For the $10^6$ CFU/mL inoculum, the numbers of Cip$^R$ mutants from the OEL isolates were significantly higher than those from the WEL mutants. The lack of statistical significance with the $10^7$ CFU/mL inoculum was probably due to the presence of pre-existing Cip$^R$ mutants in the inoculum (Fig. 6) that somewhat reduced the difference between the OEL and WEL groups. Thus, reducing the inoculum to $10^6$ CFU/mL allowed clear detection of differences between the two groups. These results suggest that the OEL phenotype may facilitate Campylobacter to adapt to fluoroquinolone treatment by promoting the emergence of resistant mutants. This finding has practical implication as fluoroquinolone antibiotics are used for both human medicine and animal production. Thus, the detection of a large number of C. jejuni with an OEL phenotype from different host species might be the result of antibiotic usage that has served as a selection force for the OEL phenotype.

In summary, this study reveals that overexpression of CmeABC commonly occurs in C. jejuni isolates derived from various host species. The overexpression is mediated by multiple mechanisms including mutations in the cmeABC promoter sequence and in the CmeR coding sequence. Additionally, results from this study suggest that there are other unidentified mechanisms that modulate the expression of CmeABC. Overexpression of cmeABC promotes the development of resistant mutants upon treatment with fluoroquinolone antibiotics and may contribute to the survival and persistence of C. jejuni in animal reservoirs where antibiotics are commonly used. These findings provide new insights into the adaptive mechanisms of C. jejuni and further
highlight the potential to control antibiotic resistant *Campylobacter* by targeting CmeABC.

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<td>16srRNA-F</td>
<td>AATCTAATGCTTAAACCATTA</td>
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<tr>
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<tr>
<td>GSF</td>
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</tr>
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<td>GSR1</td>
<td>GCAACACACCTAAAGCTAAA</td>
<td>(12)</td>
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<tr>
<td>PF</td>
<td>AAAAGGATCTCTAAATGGGAAATCAATGCTCC (BamHI)</td>
<td>(12)</td>
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<tr>
<td>PX</td>
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</tr>
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<td>This study</td>
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<tr>
<td>pQETlplII/IV-F</td>
<td>CG GATAACAATT TCACACA G</td>
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<td>370BamH-F</td>
<td>CAGTCCGATCCACCTTTC (BamHI)</td>
<td>This study</td>
</tr>
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<td>369Xbal-R1</td>
<td>AAATACTGTTTTTTTCTAGAGTTTGTAAAT (XbaI)</td>
<td>This study</td>
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<td><strong>Real time RT-PCR</strong></td>
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<tr>
<td>16S-F</td>
<td>TACCTGGCGTTGATATCC</td>
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<tr>
<td>16S-R</td>
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<td>(34)</td>
</tr>
<tr>
<td>cme-B-F</td>
<td>ACGATTCACCTTTTTCCAGC</td>
<td>(34)</td>
</tr>
<tr>
<td>cmeB-R</td>
<td>TTGGCTACTTGGGAAATCGCTTC</td>
<td>(34)</td>
</tr>
<tr>
<td>F3</td>
<td>ATTTTCAATCAACAGGATCTG</td>
<td>(16)</td>
</tr>
<tr>
<td>R1</td>
<td>TCCATGGAATGGATGTCTATC</td>
<td>(16)</td>
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1 Restriction sites are indicated by underlined sequence
### TABLE 2: Bacterial strains or plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid or Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMW10</td>
<td><em>E. coli – Campylobacter</em> shuttle vector carrying promoter-less lacZ, Kan^R</td>
<td>(32)</td>
</tr>
<tr>
<td>pMW11168</td>
<td>pMW10 carrying the <em>cmeABC</em> promoter from NCTC11168 fused to lacZ, Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pMW81-176</td>
<td>pMW10 carrying the <em>cmeABC</em> promoter from 81-176 fused to lacZ, Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pMX7199</td>
<td>pMW10 carrying the <em>cmeABC</em> promoter from X7199 fused to lacZ, Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pMWM32506</td>
<td>pMW10 carrying the <em>cmeABC</em> promoter from isolate M32506 fused to lacZ, Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pMW1:1</td>
<td>pMW10 carrying the <em>cmeABC</em> promoter from isolate CT1:1 fused to lacZ, Kan^R</td>
<td>This study</td>
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<tr>
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<td>pMW10 carrying the <em>cmeABC</em> promoter from isolate CT1:9 fused to lacZ, Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pMW3:7</td>
<td>pMW10 carrying the <em>cmeABC</em> promoter from isolate CT3:7 fused to lacZ, Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pMW9:20</td>
<td>pMW10 carrying the <em>cmeABC</em> promoter from isolate CT9:20 fused to lacZ, Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pMW11168-R</td>
<td>pMW10 carrying the <em>Cj0369c-cmeR</em> promoter from NCTC 11168 fused to lacZ, Kan^R</td>
<td>This study</td>
</tr>
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<td>pMW81-176-R</td>
<td>pMW10 carrying the <em>Cj0369c-cmeR</em> promoter from 81-176 fused to lacZ, Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pMX7199-R</td>
<td>pMW10 carrying the <em>Cj0369c-cmeR</em> promoter from isolate X7199 fused to lacZ, Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pQE30</td>
<td>Expression vector for N-terminal 6-His tagged proteins, Amp^R</td>
<td>Qiagen</td>
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<tr>
<td>pQECmeRSS</td>
<td>pQE30 carrying CmeR with the C69S and C166S mutations</td>
<td>(37)</td>
</tr>
<tr>
<td>pQECmeR-K</td>
<td>pQE30 carrying CmeRSS with the E84K mutation, Amp^R</td>
<td>This study</td>
</tr>
<tr>
<td>pQECmeR-R</td>
<td>pQE30 carrying CmeRSS with the P183R mutation, Amp^R</td>
<td>This study’</td>
</tr>
<tr>
<td>pQECmeR-IK</td>
<td>pQE30 carrying CmeRSS with the T6I and E159K mutations, Amp^R</td>
<td>This study’</td>
</tr>
<tr>
<td>pQECmeR-tr</td>
<td>pQE30 carrying CmeRSS with the G144A and S207G amino acid mutations. Also carries T insertion at nucleotide 583 causing frame shift after amino acid 193, Amp^R</td>
<td>This study’</td>
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<td>Table 2: continued</td>
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<tr>
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<tr>
<td><strong>Plasmid or Strain</strong></td>
<td><strong>Description</strong></td>
<td><strong>Source</strong></td>
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<td><strong>Campylobacter jejuni</strong> strains</td>
<td></td>
<td></td>
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<tr>
<td>NCTC 11168</td>
<td>Wild type; genome sequence known</td>
<td>(1)</td>
</tr>
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<td>11168ΔcmeR</td>
<td>Derivative of NCTC 11168, <em>cmeR::cat</em></td>
<td>(2)</td>
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<tr>
<td>ATCC 33560</td>
<td><em>C. jejuni</em> quality control strain</td>
<td>(3, 4)</td>
</tr>
<tr>
<td>81-176</td>
<td>Wild type; isolated from a human</td>
<td>(5)</td>
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<tr>
<td>81-176pMW10</td>
<td>Derivative of 81-176 carrying pMW10</td>
<td>This Study</td>
</tr>
<tr>
<td>81-176pMW11168</td>
<td>Derivative of 81-176 carrying pMW11168</td>
<td>This Study</td>
</tr>
<tr>
<td>81-176pMW81-176</td>
<td>Derivative of 81-176 carrying pMW81-176</td>
<td>This Study</td>
</tr>
<tr>
<td>81-176pMWX7199</td>
<td>Derivative of 81-176 carrying pMWX7199</td>
<td>This Study</td>
</tr>
<tr>
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<td>Derivative of 81-176 carrying pMWM32506</td>
<td>This Study</td>
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<td>This Study</td>
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<td>Derivative of 81-176 carrying pMW1:9</td>
<td>This Study</td>
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<td>Derivative of 81-176 carrying pMW3:7</td>
<td>This Study</td>
</tr>
<tr>
<td>81-176 pMW9:20</td>
<td>Derivative of 81-176 carrying pMW9:20</td>
<td>This Study</td>
</tr>
<tr>
<td>81-176ΔcmeR</td>
<td>Derivative of 81-176, <em>cmeR::cat</em></td>
<td>(2)</td>
</tr>
<tr>
<td>81-176ΔcmeR pMW10</td>
<td>Derivative of 81-176ΔcmeR carrying pMW10</td>
<td>This Study</td>
</tr>
<tr>
<td>81-176ΔcmeR pMW11168</td>
<td>Derivative of 81-176ΔcmeR carrying pMW11168</td>
<td>This Study</td>
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<td>81-176ΔcmeR pMW81-176</td>
<td>Derivative of 81-176, <em>cmeR::cat</em> carrying pMW81-176</td>
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<td>This Study</td>
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<td>81-176ΔcmeR pMW1:9</td>
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<td>This Study</td>
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<td>This Study</td>
</tr>
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<td>81-176ΔcmeR pMW9:20</td>
<td>Derivative of 81-176ΔcmeR carrying pMW9:20</td>
<td>This Study</td>
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<td>Derivative of 81-176 carrying pMW11168-R</td>
<td>This Study</td>
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<td>Derivative of 81-176 carrying pMW81-176-R</td>
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<td>81-176pMWX7199-R</td>
<td>Derivative of 81-176 carrying pMWX7199-R</td>
<td>This Study</td>
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</tbody>
</table>

**Escherichia coli** strains

<p>| DH5α | F <em>Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (tλ m-) phoA supE44 thi1 gyrA96 relA1 | Invitrogen |
| DH5cRK2013 | IncP Km</em> Tra RK2 ΔrepRK2 repE1* | (6) |
| DH5cPMW10 | DH5α derivative carrying pMW10 | (7) |</p>
<table>
<thead>
<tr>
<th>Plasmid or Strain</th>
<th>Description</th>
<th>Source</th>
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<td>E. coli strains, continued</td>
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<tr>
<td>JM109</td>
<td>e14('McrA') recA1 endA1 gyrA96 thi-1 hsdR17(t, m&lt;sup&gt;-&lt;/sup&gt;) supE44 relA1 Δ(lac-proAB) [F' traD36 proAB lac&lt;sup&gt;5&lt;/sup&gt;ZΔM15]</td>
<td>Agilent</td>
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<tr>
<td>XL-1 Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tet&lt;sup&gt;+&lt;/sup&gt;)]</td>
<td>Agilent</td>
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<tr>
<td>JM109pQECmeRSS</td>
<td>Derivative of JM109 carrying pQECmeRSS Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>JM109pQECmeR-K</td>
<td>Derivative of JM109 carrying pQECmeR-K, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>JM109pQECmeR-R</td>
<td>Derivative of JM109 carrying pQECmeR-R, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<td>JM109pQECmeR-IK</td>
<td>Derivative of JM109 carrying pQECmeR-IK, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>XL1-Blue pQECmeR-tr</td>
<td>Derivative of XL1-Blue carrying pQECmeR-tr, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>Clinical Isolate</td>
<td>cmeR Expression Fold Change*</td>
<td>Nucleotide Mutation</td>
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<td>0.006</td>
<td>G431A 583 T insertion</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>C621A</td>
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<td>M63885</td>
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<td>T37957A</td>
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<td>G250A</td>
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<td>CT9:7</td>
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<td>CB2:8</td>
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<td>CB2:11</td>
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<td>CB2:6**</td>
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<td>S13530</td>
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<td>X7199</td>
<td>10.861</td>
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*In relative to the expression level in NCTC 11168

**Isolate is phenotypically classified as WEL
TABLE 4: Chloramphenicol MIC distribution (% for each MIC) among the tested isolates

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<th>MIC</th>
<th>2 µg/mL</th>
<th>4 µg/mL</th>
<th>8 µg/mL</th>
<th>16 µg/mL</th>
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<tbody>
<tr>
<td>MH agar</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>WEL</td>
<td>33</td>
<td>67</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OEL</td>
<td>42</td>
<td>37</td>
<td>19</td>
<td>2</td>
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<tr>
<td>MH agar with ox-bile $^1$</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WEL</td>
<td>33</td>
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<td>0</td>
</tr>
<tr>
<td>OEL</td>
<td>16</td>
<td>81</td>
<td>2</td>
<td>0</td>
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</tbody>
</table>

$^1$Ox bile 12,500 µg/mL
FIGURE 1: Differential expression of CmeABC in clinical *Campylobacter jejuni* isolates. Expression was determined by immunoblotting of whole cell proteins from NCTC 11168 (lane 1), clinical isolates (lanes 2-11), and 11168ΔcmeR (lane 12) with anti-CmeB, anti-CmeC, anti-CmeA, and anti-major outer membrane protein (MOMP) antibodies. These broiler isolates in lanes 2 to 11 are CB1:6, CB1:14, CB1:18, CB2:6, CB2:8, CB2:11, CB3:1, CB3:5, CB3:14, and CB3:21. Isolates CB2:8, CB2:11, CB3:1, CB3:5, CB3:14 and CB3:21 (lanes 6-11) were designated as having overexpression levels of CmeABC and isolates CB1:6, CB1:14, CB1:18, and CB2:6 (lanes 2-5) as having wild-type levels of CmeABC. The major outer membrane protein (MOMP) was used as an internal control.
FIGURE 2: Binding of CmeR to variants of the *cmeABC* promoter in different isolates.

(A) Sequence alignment of the *cmeABC* promoter region illustrating the 16-base inverted repeat of the CmeR binding site shown in lowercase letters. The strain names are listed on the left of each sequence. All mutations differing from the 11168 promoter are highlighted in bold. (-) indicates a deleted base. (B) EMSA showing the binding of rCmeRSS to different promoter variants. The control probes include the NCTC 11168 probe (lanes 1-4) in panels I-III and the 81-176 probe (lanes 1-4) in panels IV to VI. The variant promoter probes include CT1:1 (panel I, lanes 5-8), CT1:9 (panel II, lanes 5-8),
FIGURE 2 continued: M32506 (panel III, lanes 5-8), X7199 (panel IV, lanes 5-8), CT3:7 (panel V, lanes 5-8), and CT9:20 (panel VI, lanes 5-8). For each probe, the amount of rCmeRSS used for each reaction was 0 (lanes 1 and 5), 60 (lanes 2 and 6), 120 (lanes 3 and 7), and 180 ng (lanes 4 and 8), respectively. The rCmeRSS-DNA complexes are indicated with a “C” and the unbound promoter probe is indicated with a “P”.
FIGURE 3: Effect of various mutations in CmeR and the promoter region on transcription of *cmeABC* as measured by transcriptional fusions and β-galactosidase assays. The names of the promoters are indicated under each panel. Each promoter was assayed in the wild-type 81-176 background (A) and the 81-176ΔcmeR background (B). The data represent means with standard deviation from three independent experiments.
FIGURE 3 continued: The relative difference in transcription (fold change) due to repression by CmeR for each promoter is shown in (C) and was determined by comparison of transcription in the absence of CmeR (B) to the presence of CmeR (A). The unpaired Student’s t-test with Welch’s correction was used for comparison of the means with significance set at 0.05.
FIGURE 4: Expression of CmeR in various isolates and its correlation with CmeABC expression. (A) Immunoblotting of whole cell proteins from NCTC 11168 (lane 1), clinical isolates (lanes 2-9), and 11168ΔcmeR (lane 10) with the anti-CmeR antibody. The clinical isolates in lanes 2 to 9 are M63885, CT9:7, CB2:6, CB2:8, CB2:11, S13530, T37957A, and X7199, respectively. (B) Immunoblotting of whole cell proteins from 11168ΔcmeR (lane 1), CT2:2 (lane 2), and NCTC 11168 (lanes 3) with anti-CmeR, anti-CmeB, and anti-CmeA antibodies.
FIGURE 5: The inability of the recombinant CmeR from isolate CT2:2 to bind to the promoter DNA of *cmeABC*. (A) Immunoblotting of purified rCmeRSS (lane 2; wild-type CmeR with C69 and C166 replaced with serine) and rCmeR-tr (lane 3; truncated CmeR after residue 193 from isolate CT2:2) with the anti-CmeR antibody. Lane 1 is the protein standard ladder. (B) EMSA showing binding of the 11168 *cmeABC* promoter by rCmeRSS (lanes 1-4) and rCmeR-tr (lanes 5-8). Proteins were added at 0, 60 (lanes 2 and 6), 120 (lanes 3 and 7), 180 ng (lanes 4 and 8). The locations of protein-DNA complexes and the probe are indicated.
FIGURE 6: Emergence of ciprofloxacin-resistant mutants from WEL (circle) and OEL (triangle) isolates during treatment with ciprofloxacin. In panel A, the experiment was performed with an initial inoculum of $10^7$ CFU/ml of each isolate, while in panel B, the inoculum was $10^6$ CFU/ml for each isolate. The culture medium was MH broth containing 4 µg/mL of ciprofloxacin. Three WEL and OEL isolates were used in each experiment with cultures prepared in triplicate. Each point represents the number of ciprofloxacin-resistant mutants from a single culture. Bars represent mean $\log_{10}$CFU/mL for each group. Means for each phenotypic group were compared for each day with
FIGURE 6 continued: multiple unpaired Student’ t-tests and Holm-Šidák method for multiple comparisons. The significance level was set at 0.05.
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CHAPTER 4: INTERACTION OF COSR AND CMER IN MODULATING THE
EXPRESSION OF CMEABC AND ROLE OF CYSTEINE OXIDATION IN COSR
SENSING OF OXIDATIVE STRESS

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Abstract

During transmission and intestinal colonization, Campylobacter jejuni, a major foodborne human pathogen, experiences oxidative stress. CosR, a response regulator in C. jejuni, modulates oxidative stress response and defense and represses expression of the CmeABC multidrug efflux pump. CmeABC, a key component in the resistance to toxic compounds including antimicrobials and bile salts, is also under negative regulation by CmeR, a TetR family transcriptional regulator. How CosR interacts with CmeR in controlling cmeABC expression and how CosR senses oxidative stress are still unknown. To answer these questions, we conducted various experiments utilizing electrophoretic mobility shift assays, transcriptional fusion assays, and immunoblotting. CosR and CmeR bound independently to two separate sites of the cmeABC promoter, simultaneously repressing cmeABC expression. This dual binding of CosR and CmeR required 17 bp between the two binding sites as mutations that shortened the distance enhanced cmeABC expression. Additionally, the single cysteine residue (C218) of CosR
was sensitive to oxidation, which altered the DNA-binding activity of CosR, dissociating CosR from the cmeABC promoter. Replacement of C218 with serine rendered CosR insensitive to oxidation, confirming the role of C218 in sensing oxidative stress and providing a mechanism for CosR-mediated response to oxidative stress. These findings reveal an interactive role of CosR and CmeR in modulating cmeABC expression and identify a previously unrecognized mechanism that explains overexpression of cmeABC in response to oxidative stress. CmeR and CosR mediated differential expression of cmeABC in response to different signals may facilitate adaptation of Campylobacter to various environmental conditions.

Introduction

*Campylobacter jejuni* is a microaerophilic, gram-negative pathogen causing foodborne gastroenteritis in humans. In some animal species, such as birds, *C. jejuni* is a commensal organism well adapted to the enteric environment. Colonization in the intestines requires *C. jejuni* to be resistant to the antimicrobial action of bile. The CmeABC multidrug efflux pump is one of the known mechanisms required for intestinal colonization (1). CmeABC is a tripartite efflux system composed of the inner membrane protein CmeB, the periplasmic fusion protein CmeA, and the outer membrane protein CmeC (2). This three-gene operon is regulated by a TetR family regulator named CmeR (3), which binds to a 16-base inverted repeat within the cmeABC promoter and inhibits the expression of cmeABC (3). CmeABC is an important player for antibiotic resistance and is the predominant mechanism for bile resistance in *C. jejuni*, making it essential for
intestinal colonization (1, 2, 4). The expression of cmeABC is inducible by bile, and the induction is mediated by the binding of bile to CmeR, which triggers a conformational change in the DNA-binding domain of CmeR, thereby releasing CmeR from the promoter and increasing the pump expression (4, 5).

As a microaerobic organism, Campylobacter is sensitive to atmospheric oxygen and to oxidative stresses from host immune systems including hydrogen peroxide produced by intestinal epithelium (6). Hydrogen peroxide is one of the reactive oxygen species (ROS) that induce oxidative damage to cells (7, 8). Other ROS include superoxide and hydroxyl radicals. Oxidative stress is one of the mechanisms the immune system employs to defend against pathogens (8). Recent studies have identified the orphan response regulator CosR as an oxidative stress response regulator in C. jejuni, modulating the expression of oxidative stress response and resistance genes including katA, sodB, and ahpC (9, 10). Interestingly, CosR also represses the expression of CmeABC by binding to a region in the cmeABC promoter containing a CosR binding site (10). This finding suggests a link between the oxidative stress response and antibiotic efflux system in Campylobacter. Regulation of antibiotic efflux pumps has been previously linked to oxidative stress in other bacteria. For example, MexR of Pseudomonas aeruginosa senses oxidative stress through two cysteine residues (11). The reduced form of MexR serves as a repressor for the MexAB-OprM efflux pump, but once oxidized, MexR is dissociated from the promoter, leading to overexpression of MexAB-OprM (11, 12). Collectively, these observations suggest that oxidative stress response and antibiotic efflux systems are intertwined in bacteria.
HP1043 is a homologue of CosR found in *Helicobacter pylori* (13). CosR can functionally substitute for HP1043 when expressed in *H. pylori* from the HP1043 promoter (13). HP1043 forms a dimer and contains 2 cysteine residues that modulate its regulatory function (13-15). CosR contains a single cysteine residue (C218) that corresponds to C215 of HP1043. Based on the HP1043 sequence and its crystal structure, the single cysteine residue of CosR likely resides in the dimer interface (15). It has been known that oxidation of cysteine residues at the dimer interface affects the conformation and function of regulatory proteins (16, 17), but it is unknown if modification of C218 in CosR modulates its binding activities to promoter DNA.

Previous work suggested that CmeABC is also likely regulated by a CmeR-independent mechanism, because *cmeABC* was further induced by bile in the absence of CmeR (4). The excess induction in the absence of CmeR was attributed to an unknown mechanism regulating *cmeABC* expression (4). Additionally, our recent work studying various *cmeABC* promoter mutations further indicated that multiple regulators may bind to the promoter sequence of *cmeABC* (18). These observations and the recent publication on CosR binding to the promoter of CmeABC (10) suggest that the regulation of *cmeABC* is complex and likely involves interaction of multiple regulators. Therefore, we hypothesize that CosR and CmeR function as a dual mechanism in modulating the expression of CmeABC and that C218 in CosR serves as a sensor for oxidative stress. To test this hypothesis, we examined the interactive role of CosR and CmeR in the regulation of *cmeABC* and the effect of cysteine oxidation on the function of CosR.
Material and Methods

**Bacterial strains and growth conditions**

*C. jejuni* strains X7199 (19), NCTC 11168 (20), 81-176 (21), 81-176ΔcmeR (3), and 11168ΔcmeR (3) were used in this study (Table 1) and they were routinely cultured on Mueller Hinton (MH) agar or in MH broth (Difco, Detroit, MI) at 42°C under microaerobic conditions (5% O$_2$, 10% CO$_2$, 85% N$_2$). Media was supplemented with kanamycin at 30 µg/mL or chloramphenicol at 4 µg/mL as needed.

*Escherichia coli* strains DH5α (Invitrogen) and JM109 (Agilent Technologies) were routinely cultured at 37°C with Luria-Bertani (LB) broth or LB agar (Difco), which was supplemented with 30 µg/mL kanamycin or 100 µg/mL ampicillin when needed.

**Recombinant CosR construction and purification**

Recombinant CosR was produced using the pQE30 (Qiagen) expression system. Amplification of the *cosR* (*Cj0355c*) sequence from NCTC 11168 was performed with primers *Cj0355c*-F1 and *Cj0355c*-R1 (Table 2). This PCR product and the pQE30 plasmid were digested with *BamH*I and *KpnI* (Promega). The digested PCR product and pQE30 were purified using the QIAquick PCR purification and QIAprep Spin Miniprep kits (Qiagen), respectively. The vector and insert were then ligated with T4 ligase (Roche) and transformed into *E. coli* JM109. The transformants were selected on LB agar supplemented with ampicillin (100 µg/mL). The plasmid was purified from transformant JM109pQECj0355c (Table 1) and was sequenced to confirm there were no mutations in the cloned *Cj0355c* gene. The recombinant CosR, named rCosRWT, was
induced and purified from JM109pQECj0355c under native conditions as described in the QIAExpressionist (22). Following purification, the protein was desalted with a PD-10 desalting column (GE Healthcare).

To mutate the single cysteine residue (C218) in CosR, pQECj0355c was used as a template for site directed mutagenesis of *cosR*. Primers CosR652-F and CosR652-R (Table 2) were designed to introduce a T to A substitution at nucleotide 652, resulting in the replacement of cysteine residue 218 by serine. The QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies) was used to introduce the mutation by one cycle of 95°C for 30 seconds followed by 16 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 68°C for 4 minutes. The amplified product was cooled on ice for 2 minutes before *Dpn*-I digestion of parental DNA at 37°C for 1 hour. The product, pQECj0355c652 (Table 1), was then transformed into *E. coli* strain JM109 and the transformants were selected on LB agar supplemented with ampicillin (100µg/mL). The specific mutation was confirmed by sequencing. This mutated version of CosR was named rCosRC218S and was purified using the same method as with rCosRWT.

**Electrophoretic mobility shift assays (EMSA)**

EMSA was used to assess binding of CosR to the *cmeABC* promoter or its derivatives. Primers GSF and GSR1 (3) (Table 2) were used to amplify a 170-bp region of the *cmeABC* promoter from strains 81-176, NCTC 11168 (named 11168 promoter) and X7199 as described previously (18). An internal fragment of *cmeA* was amplified with primers AF and AR (Table 2) and was used as a negative control probe (3). A 14-base pair deletion between the CosR and CmeR binding sites of *cmeABC* was designed based
on the sequence of 81-176 and this probe was named P14D. A pUC57 vector carrying the P14D sequence was synthesized (Genscript) and then amplified using the GSF and GSR1 primers (3) (Table 2). All probes were purified with QIAquick PCR purification kit and labeled with DIG-11-dd-UTP as described previously (3).

To assess the binding specificity of CosR to the cmeABC promoter, the 11168 promoter probe or the cmeA probe (0.05 pmol) were mixed with 250 ng of rCosRWT or rCosRC218S in reaction buffer (14.4 µL total) according to the method of Alekshun et. al. (23) and Lin et. al. 2005 (3). A control reaction was prepared with the 11168 promoter probe without addition of protein. Reactions were incubated for 30 minutes at room temperature. Promega DNA loading buffer was added to each reaction and then the reaction was run at 200V for 45 minutes on a 6% polyacrylamide gel in 0.25X TBE buffer. Transfer to a positively charged membrane by vacuum and detection of DIG with CDP Star (Roche) were performed as previously described (3).

To assess the effect of oxidation on binding of CosR to the cmeABC promoter, the 11168 promoter probe (0.05 pmol) was mixed with 250 ng of rCosRWT or rCosRC218S, incubated for 30 minutes at room temperature, and then hydrogen peroxide was added to the reactions at final concentrations of 0, 5, 10, or 20 nM (final volume 14.4 µL). Reactions were incubated for an additional 30 minutes at room temperature. Electrophoresis, transfer and detection were performed as described above.

To determine if CosR and CmeR interfere with each other in binding to the cmeABC promoter, dual binding EMSA assays were performed using promoter DNA that had varied lengths of spacing between the CmeR-binding site and the CosR-binding site. Dual binding of CosR and CmeR utilized 81-176 cmeABC promoter probe which has 17-
base pairs between the CmeR and CosR binding site as a full length promoter control. The second probe containing a reduced distance between the CmeR and CosR binding sites was either the promoter probe of X7199, which has a 5 base-pair deletion, or probe P14D with a 14 base pair deletion. Each probe (0.05 pmol) was incubated with 200 ng of rCmeRSS alone, 400 ng of rCosRC218S alone, or both rCmeRSS and rCosRC218S at 200 ng and 400 ng, respectively, in the reaction mixture (14.4 µL). A control reaction was prepared for each probe without the protein. Reactions were incubated at room temperature for 30 minutes. Promega DNA loading buffer was added to each reaction, which was then run at 200V for 55 minutes on a 6% polyacrylamide gel in 0.25X TBE buffer. Transfer and detection were performed as described above.

**Immunoblotting**

To determine if inhibition of CosR affects expression of CmeABC, immunoblotting was performed using cultures that were incubated with the anti-CosR peptide nucleic acid (PNA). The PNA (KFKFKFFKFKK-O-CATTGTCTATCTCTT) (9) was obtained from PNA Bio, Inc. *C. jejuni* NCTC 11168 and 11168ΔcmeR were grown on MH agar and then adjusted in MH broth to OD~0.07. Cultures were incubated with and without the anti-CosR PNA at a final concentration of 1.5 µM with shaking at 180 rpm for 8 hours at 42 °C under microaerobic conditions (9). After incubation, cultures were adjusted to 1.5 x 10^8 cells, centrifuged, and re-suspended in 1X SDS loading buffer to 1.5 x 10^6 cells/µL. SDS-PAGE and immunoblotting for CmeA and CmeB were performed as described by Lin et. al. 2002 (2). Densitometric analysis was performed on the immunoblots using AlphaEaseFC Software (Version 3.2.3 Rev C;
Transcriptional fusion and β-galactosidase assay

Various \textit{cmeABC} promoters were fused to the promoter-less \textit{lacZ} gene in pMW10 (24). Construction of strains 81-176 and 81-176Δ\textit{cmeR} containing the plasmids pMW10, pMW11168, pMW81-176, and pMWX7199 was described previously (Table 1) (18). Plasmids pMW11168, pMW81-176, and pMWX7199 contained the \textit{cmeABC} promoter from NCTC 11168, 81-176, and X7199, respectively, fused to the \textit{lacZ} reporter gene, while pMW10 contains the reporter gene, \textit{lacZ}, without a fused promoter. Plasmid pMW561 carries the \textit{Cj0561c} promoter from strain NCTC 11168. \textit{Cj0561c} is repressed by CmeR, not CosR, serving as a negative control for the effect of anti-CosR PNA.

pMW561 (25) was extracted from 11168W7pMW561 (25) (Table 1) using the QIAprep Spin Miniprep kit and transformed into DH5α. Tri-parental mating was used to transfer the plasmid into \textit{C. jejuni} strains 81-176 and 81-176Δ\textit{cmeR} (26).

Overnight cultures of 81-176 or 81-176Δ\textit{cmeR} with pMW10 (24), pMW11168, pMW81-176, pMWX7199, or pMW561 were grown on MH agar supplemented with kanamycin (30 µg/mL) and then harvested in MH broth with kanamycin. Cultures were adjusted in MH-kanamycin broth to OD$_{600}$~0.07 and aliquoted to two tubes. The first tube was incubated with 1.5 µM anti-CosR PNA and the second was incubated without the anti- CosR-PNA. All tubes were incubated by shaking at 180 rpm for 8 hours at 42 ºC under microaerobic conditions (9). β-galactosidase assays were performed in triplicate samples for three independent experiments (27). Means were calculated and statistical analysis was performed using Student’s t-test with Welch’s corrections.
(GraphPad InStat® Version 3.06). The repressive effects (fold changes) for each regulator were statistically analyzed between promoters by log2 transformation of the transcriptional data and one-way analysis of variance (ANOVA) in SAS (version 9.3).

Results

**CosR binds specifically to the cmeABC promoter**

EMSA was used to demonstrate the specific binding of CosR to the cmeABC promoter. As shown in Figure 1, the control reaction containing the 11168 promoter probe (Fig. 1, lane 1) and no CosR demonstrated the migration of unbound 11168 promoter probe at the lower end of the gel. In the presence of rCosRWT or rCosRC218S, DNA-protein complexes were formed and migrated above the unbound probe (Fig. 1, lanes 2 and 3). No complexes were seen with the control probe (cmeA internal fragment) (Fig. 1, lanes 4 and 5), indicating that CosR did not bind to the internal cmeA fragment. The result is consistent with the finding described by Hwang et. al. 2012 (10) and indicates that CosR binds specifically to the cmeABC promoter.

Notably, the rCosRC218S-DNA band (Fig. 1, lane 3) was darker than the rCosRWT-DNA band (Fig. 1, lane 2), indicating stronger binding of the DNA probe by rCosRC218S. The two rCosR proteins differ in one amino acid, with cysteine 218 replaced by serine in rCosRC218S. Cysteine residues are sensitive to oxidation, while serine is not susceptible to oxidation (16). The EMSA reactions were carried out under aerobic conditions and the reduced binding of rCosRWT compared to rCosRC218S suggested that this protein was subject to oxidation under aerobic conditions. Repeated
EMSAs showed rCosRWT was always less active than rCosRC218S in DNA binding assayed under the in vitro condition (data not shown), suggesting a potential role of cysteine oxidation in modulating the function of CosR.

**Oxidation of C218 in CosR reduced DNA binding**

To further demonstrate the effect of C218 oxidation on DNA binding, hydrogen peroxide was used to treat the reactions in the EMSA assay. As shown in Figure 2, the binding of the cmeABC promoter by rCosRWT decreased as hydrogen peroxide concentration increased (lanes 2-5). At 20 nM of hydrogen peroxide (Fig. 2, lane 5), the binding of rCosRWT to the DNA probe was totally inhibited and the unbound probe was at the level of the free probe control (Fig. 2, lane 1). In contrast, the promoter binding by rCosRC218S was not affected by treatment with hydrogen peroxide (Fig. 2, lanes 6-9). These results indicated that the C218 in CosR was sensitive to hydrogen peroxide and oxidation of this residue interfered with CosR binding to promoter DNA.

**Dual binding of the cmeABC promoter by CosR and CmeR**

The cmeABC promoter contains binding sites for both CmeR and CosR (9, 10) (also see Fig. 3A). To determine if binding of one protein interferes with binding of the other, promoter sequences with various lengths between the two binding sites were used as probes in EMSA. Both the 11168 and 81-176 cmeABC promoter probes had 17 bp between the CosR and CmeR binding sites (Fig. 3A). This distance was reduced to 12 bp in the promoter from strain X7199 (Fig. 3A), a human isolate, while the P14D probe contained only 3 bp between the binding sites (Fig. 3A).
Each promoter probe was incubated with rCmeRSS or rCosRC218S individually or in combination. Evaluation of individual protein binding showed that rCosRC218S bound equally well to the 81-176 (Fig. 3B and C, lane 2), X7199 (Fig. 3B, lane 7), and P14D (Fig. 3C, lane 7) promoter probes. However, binding of rCmeRSS to the promoter probes varied (Fig. 3A and B, lanes 2 and 6). rCmeRSS binding to the 81-176 cmeABC promoter produced 2 banding patterns: in Figure 3C (lane 2), there are 3 distinct bands representing the rCmeRSS-DNA complexes, while in Figure 3B (lane 2) the upper band of the rCmeRSS-DNA complexes produced a wide streak, blurring the edges of the band with a shadow effect. Binding of rCmeRSS to the X7199 promoter produced 3 distinct dark bands (Fig. 3B, lane 6), while rCmeRSS binding to the P14D probe yielded 3 light bands (Fig. 3C, lane 6), suggesting reduced interaction between the protein and the P14D. This indicates that the cmeABC promoter binding by CmeR, but not CosR, is affected by spatial arrangement of the CosR and CmeR binding sites.

Evaluation of the cmeABC promoter for binding by the rCosRC218S and rCmeRSS proteins when added in combination demonstrated simultaneous binding of rCosRC218S and rCmeRSS to the promoter probe. Dual binding to the 81-176 promoter probe produced 3 dark bands that shifted higher than those from a single protein binding (Fig. 3B and C, lane 4), while binding to probe X7199 produced 3 lighter bands (Fig. 3B, lane 8). Dual binding of rCosRC218S and rCmeRSS to P14D also created 3 bands, but the bands were thinner in width (Fig. 3C, lane 8) compared to the 81-176 promoter (Fig. 3C, lane 4). The decrease in band size or intensity is indicative of reduced binding. Binding was not affected by the order of protein addition to the probes (data not shown).
These results suggest that reducing the distance between the binding sites of CosR and CmeR interferes with dual binding by CosR and CmeR.

**CmeR and CosR independently modulate CmeABC expression in vivo**

Based on the results of EMSA, CosR and CmeR can bind to the *cmeABC* promoter simultaneously, constituting a dual mechanism for regulating the expression of *cmeABC*. To further understand the interaction of these two regulators in modulating *cmeABC* expression, immunoblotting was used to assess the expression of this efflux pump under different conditions. Since *cosR* appears to be an essential gene and cannot be inactivated in *Campylobacter*, we used anti-CosR PNA to assess its impact on *cmeABC* expression in *C. jejuni*. The wild-type NCTC 11168 and its isogenic *cmeR* mutant (11168Δ*cmeR*) were grown with or without the PNA, and the whole cell proteins were analyzed by immunoblotting using anti-CmeA, anti-CmeB, and anti-major outer membrane protein (MOMP) antibodies (Fig. 4). MOMP was used as a control as its expression is not affected by CosR (9, 10) and CmeR (3, 25). Indeed, MOMP showed similar levels of expression under all culture conditions (Fig. 4). In contrast, the expression of CmeA and CmeB increased in the absence of CmeR or presence of the anti-CosR PNA (Fig. 4), and the effects were further quantified by densitometric analysis.

In the 11168Δ*cmeR* strain (Fig. 4, lane 3), *cmeR* deletion resulted in a 2.1-fold increase in CmeB and a 2.7-fold increase in expression of CmeA compared to the expression level in wild-type 11168 (Fig. 4, lanes 1). This represents the effect of CmeR on *cmeABC* expression in the presence of CosR. Treating the cultures with the anti-CosR PNA also increased the expression of *cmeABC* (Fig. 4, lanes 2 and 4). When treated
with the PNA (Fig. 4, lanes 2 and 4), expression of CmeB increased 1.2-fold in both the wild-type 11168 and the 11168ΔcmeR cultures relative to the untreated cultures (Fig. 4, lanes 1 and 3). For CmeA, its expression increased 1.3-fold over the untreated culture (Fig. 4, lane 1) in wild-type 11168. This represents the effect of CosR in the presence of CmeR. A similar effect was observed in the 11168ΔcmeR culture treated with the anti-CosR-PNA (Fig. 4, lane 4) as expression of CmeA increased 1.5-fold (densitometric data not shown) over the untreated 11168ΔcmeR culture (Fig. 4, lane 3). The increased expression of CmeB and CmeA in the wild-type background in the presence of the PNA indicates that CosR is able to repress the efflux pump expression in the presence of CmeR, consistent with the EMSA finding that CosR and CmeR bound to the cmeABC promoter simultaneously. In addition, the similar effects of CosR in both the wild-type and cmeR mutant backgrounds indicate that CosR functions independently and is not affected by CmeR.

**CosR functions as a secondary transcriptional regulator of cmeABC**

Additionally, *in vivo* transcriptional fusion assays were performed to assess cmeABC expression under various conditions. Plasmids containing the cmeABC promoter from strains NCTC 11168 (11168 promoter), 81-176, or X7199 (Fig. 3A) were fused to a promoterless lacZ gene. These plasmids were transferred into wild-type 81-176 (Fig. 5A) and 81-176ΔcmeR (Fig. 5B), which were then cultured and treated with the anti-CosR PNA (Fig. 5). The effect of CmeR and CosR on transcription was determined by comparison of the same promoter under various conditions. Inactivation of CmeR resulted significant increases (*p* < 0.05) in cmeABC transcription: 4.6-fold for the 11168
promoter, 3.7-fold for the 81-176 promoter, and 2.6-fold for the X7199 promoter as determined by comparing 81-176ΔcmeR with the wild-type 81-176 in the absence of the anti-CosR PNA (Fig. 5). In cultures treated with the anti-CosR PNA (81-176ΔcmeR and 81-176), the loss of CmeR also had a significant \((p < 0.05)\) effect on cmeABC transcription with 3.5-, 2.3-, and 2.1-fold increases for the 11168, 81-176, and X7199 promoters, respectively. These two sets of fold changes represent the levels of CmeR inhibition on cmeABC expression in the presence of CosR (Table 3) and under CosR suppression (Table 4), respectively for each promoter. These effects further confirm that CmeR functions independently of CosR.

The independent repression of CosR was confirmed by comparison of cultures treated with anti-CosR PNA and without anti-CosR PNA for each promoter. Inhibiting cosR in wild-type 81-176 (with a functional CmeR) caused a significant \((p < 0.05)\) increase in transcription of all tested promoters (Fig. 5A). For the 11168, 81-176, and X7199 promoters, the increases were 1.8-, 2.1-, and 1.7-fold, respectively which indicates that CosR functions as a repressor for cmeABC in the presence of CmeR, consistent with the immunoblotting result (Fig. 4). The transcriptional fusions were also evaluated in the 81-176ΔcmeR background (Fig. 5B). In the absence of CmeR, inhibition of CosR further increased the transcription of the cmeABC promoters compared to the non-treated controls, but this was not statistically significant. Inhibition of cosR resulted in a 1.4-fold increase for the 11168 and 81-176 promoters and 1.3-fold for the X7199 promoter. These two sets of fold changes represent the levels of CosR inhibition on cmeABC expression in the presence of CmeR (Table 3) and in the absence of CmeR (Table 4), respectively for each promoter. The lower magnitude of inhibition on cmeABC by CosR
compared with CmeR suggests that CmeR functions as a primary regulator and CosR as a secondary regulator for \textit{cmeABC}.

**Reduced spacer length affects dual binding of the \textit{cmeABC} promoter by CmeR and CosR**

EMSA demonstrated that dual binding of CmeR and CosR to the \textit{cmeABC} promoter was influenced by the length of the spacer between the two promoter binding sites on EMSA (Figure 3). Transcriptional fusion assays confirmed that transcription of \textit{cmeABC} was significantly ($p < 0.05$) increased for each promoter when CosR was inhibited and CmeR was inactivated (81-176ΔcmeR, with anti-CosR PNA). Transcriptional increases of 6.3-, 4.9-, and 3.5- fold for the 11168, 81-176, and X7199 promoters, respectively, were observed and these increases represent the collective effects of CosR and CmeR (Table 4). Notably, the collective effect on transcription was significantly lower ($p = 0.0102$) for the X7199 promoter than the 11168 promoter. Mutations in the CmeR binding site and/or the spacer between the CosR and CmeR binding sites, but not the CosR binding sites are seen in the promoters in Figure 3A. The transcriptional effects of CosR, 1.8-, 2.1, and 1.7- fold in the presence of CmeR (Table 3) and 1.4 and 1.3-fold in the absence of CmeR (Table 4), were not significantly different among the promoters, suggesting that repression of these promoters by CosR is not influenced by the spacer region, consistent with the EMSA results (Fig. 3B and C).

Examination of individual effect of CmeR for each promoter showed that the effect is similar ($p > 0.05$) for the 81-176 and X7199 promoter at 2.3- and 2.1-fold, but both are significantly ($p < 0.05$) reduced from the 3.5-fold effect for the 11168 promoter
(Table 4). This decrease is consistent with the mutations in the CmeR binding site of the X7199 and 81-176 promoters. Interestingly, in the presence of CosR, the effect of CmeR for the X7199 promoter was only 2.6-fold compared to 4.6- and 3.7-fold for the 11168 and 81-176 promoter (Table 3). These effects are similar ($p > 0.05$) for the full length promoters 81-176 and 11168, but significantly lower ($p < 0.05$) for the X7199 compared to either of the full length promoters. This suggests repression of $cmeABC$ by CmeR is reduced by the shortened space between the binding sites in the presence of CosR for the X7199 promoter.

To confirm this effect of CmeR was related to the decreased distance between the binding sites and not mutations in the CmeR binding site, transcription levels in the presence of dual regulators (wild-type 81-176 without CosR-PNA) were compared between the full length and shortened promoters (Fig. 5A). Transcription from the X7199 promoter showed a 2.9-fold ($p < 0.01$) increase over the 11168 promoter compared to the 1.4-fold ($p > 0.05$) increase of the 81-176 promoter over the 11168 promoter. To confirm the larger increase for the X7199 promoter was not solely due to the mutations in the CmeR binding site, transcription from the X7199 $cmeABC$ promoter was compared to the 81-176 promoter and showed a 2.1-fold increase over the 81-176 promoter in the presence of both repressors (Fig. 5A). Thus even with mutations in the CmeR binding site in both promoters, repression by CmeR is further reduced for the X7199 promoter. Taken together, these results indicate that reduction of the spacer length between the binding sites decreases the binding by CmeR during dual repression of $cmeABC$ by CmeR and CosR and leads to increased $cmeABC$ levels.
The effects of each regulator individually and collectively was also analyzed for the possibility of an additive or synergistic effect during dual binding. Addition of the calculated individual effects for CmeR and CosR, determined that the sum of the individual effects for each promoter was 4.9-fold for the 11168 promoter, 3.7-fold for the 81-176 promoter, and 3.4-fold for the X7199 promoter (Table 3). The difference between the collective effect (6.3-, 4.9-, and 3.5-fold) and sum the individual effects (4.9-, 3.7-, and 3.4-fold) was not significantly different at 1.4- 1.2-, or 0.1-fold for the 11168, 81-176, and X7199 promoters respectively (Table 3). Notably the magnitude of this effect was smallest for the shortened X7199 promoter, although this was not statistically significant compared to the full-length promoters (Table 3). The inability to detect a statistical difference between the promoters for this value may be due to the indirect measurement of this value. However, the presence of a difference between the sum and collective effects suggests there might be a synergy between CmeR and CosR in repressing cmeABC in the full-length promoters.

**CosR specifically inhibits CosR in vivo**

To confirm that CosR is specific for inhibiting expression of cmeABC, the Cj0561c promoter was also examined in the presence of the anti-CosR PNA. Cj0561c is known to be repressed by CmeR (25, 28), but there is no evidence that it is regulated by CosR. As shown in Figure 5A, expression from the Cj0561c promoter did not differ significantly with or without the CosR PNA in the wild-type 81-176 background. A similar result was obtained in the 81-176ΔcmeR background (Fig. 5B). This indicates that inhibiting CosR did not affect the expression of Cj0561c, confirming the specificity
of CosR to cmeABC. In contrast, when transcription of Cj0561c was compared between wild-type 81-176 and 81-176ΔcmeR, a significant ($p < 0.01$) increase in transcription was observed in the 81-176ΔcmeR backgrounds regardless of the CosR levels, confirming that deletion of cmeR increases the transcription of Cj0561c. The ability of CosR to inhibit cmeABC transcription, but not Cj0561c transcription demonstrates that CosR specifically regulates cmeABC.

Discussion

The multidrug efflux pump CmeABC is well known for its roles in antimicrobial resistance and bile resistance (1, 2, 4, 29-33). This efflux system is under negative regulation by CmeR (3-5, 25, 34-36). Recently, CosR, an oxidative stress response regulator, was also found to modulate the expression of cmeABC (10). In this study we demonstrate that CosR and CmeR bind simultaneously to the promoter sequence of cmeABC and function as a dual regulatory mechanism for this efflux pump. The interaction of CosR and CmeR with the promoter DNA does not depend on each other, but the spacing between the two binding sites for the two regulators influences CmeR repression during dual binding of the cmeABC promoter. In addition, we found that the sole cysteine (C218) of CosR is sensitive to cysteine oxidation and it likely serves as a mechanism for CosR to sense oxidative stress. These findings provide new information on the complex regulatory mechanisms of CmeABC and the diverse signals that may modulate the expression and function of this predominant efflux system in C. jejuni.
Both CmeR and CosR have a specific binding site in the \textit{cmeABC} promoter (3, 9). CosR was described to recognized to a 21 base binding site, ttaAanAaAAaTtAtagaTTt, which occurs in multiple promoters (9), and this CosR binding site is found within the \textit{cmeABC} promoter region (10) upstream of the Cme binding site (3). The inability of CosR to bind to the \textit{cmeA} internal fragment on EMSA (Fig. 1) or to influence Cj0561c expression as measured by transcriptional fusion (Fig. 5) was correlated with the lack of this specific binding site within \textit{cmeA} and the promoter of Cj0561c. The specific interaction of CosR with the binding site was recently demonstrated by the DNA footprinting assay using the \textit{katA} promoter (10). CosR positively regulates \textit{katA}, but \textit{katA} is also negatively regulated by PerR and Fur, peroxide and iron responsive regulators (37-40). The \textit{katA} promoter contains multiple binding sites for PerR and holo-Fur regulators in addition to the CosR binding site (10, 41). CosR binds to the CosR-specific binding site, which does not overlap with the sites for other regulators (10). A similar situation occurs in the \textit{cmeABC} promoter, where CosR and CmeR bind to two separate sites (Fig. 3A).

The CosR binding site resides 17 bases upstream of the CmeR binding site within the \textit{cmeABC} promoter. Dual regulation of the \textit{cmeABC} promoter by CosR and CmeR was demonstrated \textit{in vitro} by EMSA (Fig. 3) and was confirmed by \textit{in vivo} expression using immunoblotting (Fig. 4) and transcriptional assays (Fig. 5). An interesting finding of this study is that the dual binding of the \textit{cmeABC} promoter by CmeR and CosR is influenced by the distance between the two binding sites. The \textit{cmeABC} promoter from NCTC 11168 was used as a reference and the wild-type control for expression. The other promoters, including 81-176, X7199, and P14D, contained various mutations in the CmeR binding
site or in the spacer between the two binding sites (Fig. 3A). Specifically, the 81-176 promoter contained an A to T substitution in the CmeR binding site, but had the full-length (17 bp) spacer between the CmeR and CosR binding sites. The X7199 promoter and the constructed P14D probe had the same A to T substitution, but the spacer length in the two promoter sequences were reduced to 12 and 3 bp, respectively. In addition, the X7199 promoter had an A to G substitution at base 14 of the CmeR binding site. This additional substitution in the CmeR binding site of the X7199 promoter did not affect CmeR binding on EMSA (Fig. 3B, lane 6) or transcription of cmeABC compared to the 81-176 promoter when CmeR was present and cosR was inhibited by the PNA (individual effects of CmeR, Table 4). Both the X7199 and P14D promoters displayed reduced dual binding by CmeR and CosR, with the greatest reduction in binding observed when the spacer was reduced to only 3 bp (Fig. 3B and C). The collective effect of CmeR and CosR on cmeABC transcription was also the lowest for the X7199 promoter (Table 5). These results suggest a steric interference between CosR and CmeR in their interaction with the promoter DNA. The reduction in spacer was confirmed to increase transcription of cmeABC from the X7199 promoter (Fig. 5). This was shown by comparing the transcriptional rates between the 81-176 promoter and the X7199 promoter in the presence of both CmeR and CosR (Fig. 5A and Table 3). Both promoters had substitutions in the CmeR binding site, but transcription from the X7199 promoter was significantly higher than that of the 81-176 promoter, attributing the difference to the deletion in the spacer region. In addition, the effect of CmeR repression was similar between the 81-176 and X7199 promoters when CmeR was evaluated alone (Table 4), but reduced for the X7199 promoter when the effect of CmeR was measured in the
presence of CosR (Table 3). This result suggests that maximal repression of \textit{cmeABC} by CmeR and CosR requires at least 17 bp between the regulator binding sites.

The effect of the spacer length on the binding of CosR and CmeR was further demonstrated by using an artificial DNA probe, P14D, in which the majority of the spacer sequence was deleted (Fig. 3A). This significant shortening of the spacer even reduced the binding of P14D by CmeR alone (Fig. 3C), while binding of the X7199 promoter by CmeR was not affected by the 5 bp deletion (Fig. 3B). Additionally, shortening the spacer sequence did not affect the binding of CosR to the X7199 and P14D promoter probes (Fig. 3B and C; lane 7). However, both probes showed decreased complexes during dual binding by CosR and CmeR (Fig. 3A and 3B; lane 8). Based on these findings, it can be speculated that the spacer sequence facilitates CmeR binding to its binding site, but is not required for CosR to interact with its binding site. Thus when both CosR and CmeR bind to the X7199 promoter, CosR may create a steric hindrance for CmeR binding, resulting in reduced binding by CmeR and consequently enhanced transcription of \textit{cmeABC} (Fig. 5). This observation further supports the notion that a sufficient length of the spacer is required for maximum inhibition of CosR and CmeR.

In addition to the dual binding, we also demonstrated that CosR functions independently of CmeR as a secondary repressor for \textit{cmeABC}. The transcriptional fusion assay indicated that the level of CosR repression in the presence of CmeR was similar for the 11168, 81-176, and X7199 promoters (Fig. 5 and Table 3), demonstrating the ability of CosR to provide additional repression when CmeR is present. The effect of CosR repression in the absence of CmeR was also similar for all tested promoters, confirming that the spacer length did not affect the function of CosR (Fig. 5 and Table 4).
The observed magnitude of CosR repression on \textit{cmeABC} was lower than that of CmeR, indicating CosR functioned as a secondary regulator for \textit{cmeABC}. However, this finding is based on PNA inhibition of \textit{cosR}, which did not completely abolish CosR. This also limited our ability to evaluate the additive or synergistic effects of CosR and CmeR on \textit{cmeABC} expression. The small difference between the collective and sum of individual effects of 1.2- and 1.4-fold for the 81-176 and 11168 promoters in comparison with the 0.1-fold of the X7199 promoter is suggestive of a synergistic effect. But this possibility requires further investigation. The inhibition of CosR represents a limitation of this study due to the essential nature of CosR to \textit{C. jejuni} (9, 42), which prevents deletion of this gene from \textit{C. jejuni} as we did with \textit{cmeR}. Without a way to delete the gene, the magnitude of inhibition of CosR on \textit{cmeABC} expression and the synergistic effect of dual CmeR and CosR repression must be interpreted cautiously.

Many genes in the CosR regulon are involved in the oxidative stress response (9, 10). Under oxidative stress, reactive oxygen species (ROS) cause oxidative damage to cellular components, reducing growth, and at high levels, can cause cell death. Thus, CosR-mediated response and defense against oxidative stress is important for \textit{Campylobacter} physiology. Although the role of CosR has been defined, how it senses oxidative stress is not known. Examination of the CosR sequence identified a single cysteine residue, C218, which is predicted to be localized at the dimer face. Cysteine residues are known sites subject to modification by ROS, reactive nitrogen species, and reactive electrophilic species and are involved in redox sensing by regulatory proteins (11, 16, 17, 43). In this study, we demonstrated that C218 in CosR is sensitive to oxidative stress and oxidation of this cysteine disassociated CosR from the \textit{cmeABC}
promoter (Fig. 2, lanes 1 to 5). This result suggests that cysteine modification affects the function of CosR. This conclusion is further supported by findings in other studies, in which expression of cmeABC was induced under oxidative stress (10). As CosR modulates the expression of multiple genes in C. jejuni (10), altered function of CosR by cysteine modification would conceivably affect the expression of multiple genes involved in oxidative stress response and defense. Thus, cysteine oxidation likely serves as a mechanism for sensing oxidative stress by CosR, which may help Campylobacter to deal with oxidative stress.

The nature of C218 modification and how it affects the function of CosR is still unknown. Cysteine oxidation can result in disulfide bond formation, which often alters protein conformation and modulates DNA binding activity (16). OxyR, MgrA, AsrR, and MexR are regulators that utilize cysteine oxidation as a mechanism to regulate DNA binding (11, 16, 44, 45). Considering the similarity in regulatory mechanisms between CosR and other regulators, here we propose that oxidation of C218 in CosR results in disulfide bond formation and conformational changes in the protein, inhibiting its ability to bind to the cmeABC promoter. This model will be examined in future studies.

CosR positively regulates katA and ahpC, but negatively regulates others genes such as sodB, dps, and cmeABC (9, 10). OxyR, a well characterized oxidative stress regulator present in other bacteria but absent in C. jejuni, has been shown to acts as an activator and repressor for katA in Pseudomonas aeruginosa (46). OxyR can be modified to multiple oxidation and activation states, which mediate the opposing effects (46). Results from two-dimensional gel analysis suggest CosR undergoes protein modifications in C. jejuni as CosR was identified as three parallel spots (9). The nature of the
modification is unknown, but evidence suggests that CosR may not be modified by phosphorylation as both CosR and HP1043 (an ortholog of CosR in *H. pylori*) lack the conserved aspartate residue that is required for phosphorylation (9, 15, 47). Regardless of the nature of the modification, we speculate that the three modified forms of CosR may have different functions providing both repression and activation of genes in the regulon (9, 10). This possibility remains to be examined in future work.

Although CosR and CmeR co-repress *cmeABC*, the two regulators respond to different signals. CosR senses and responds to oxidative stress, while CmeR responds to bile (4, 5), salicylate (36), and possibly other unidentified compounds. As a microaerophilic zoonotic pathogen prevalent in food producing animals, *Campylobacter* frequently encounters environmental stresses such as antimicrobials, bile, and oxidative challenges (6, 48). *Campylobacter* utilizes multiple mechanisms for environmental adaptation, but CmeABC is a key player for antibiotic resistance and intestinal colonization by mediating resistance to antimicrobials and bile (1, 3, 4, 25). Findings from this study suggest that CmeABC is also involved in oxidative stress response via a CosR-mediated mechanism. How CmeABC contributes to oxidative stress defense is unknown and remains to be examined in future studies. Nevertheless, the sophisticated mechanisms of regulation and the newly discovered role in oxidative stress response further signify the importance of this efflux system in *Campylobacter* pathobiology and indicate that its functions are more diverse than previously expected. All together, these observations further justify CmeABC as a potential target for the development of anti-*Campylobacter* interventions.
Acknowledgements

The authors would like to thank Yang Mu for construction of the pQECj0355c plasmid. TGP was supported by Fort Dodge Animal Health Fellowship, Pfizer Animal Health Scholarship for Combined DVM-PhD, and a Career Development Grant from the National Institutes of Allergy and Infectious Diseases (Award Number U54AI057160). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Disease, the National Institutes of Health, Pfizer or Fort Dodge.
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<tr>
<th>Plasmid or Strain</th>
<th>Description</th>
<th>Source</th>
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<td>pMW10</td>
<td><em>E. coli</em> – <em>Campylobacter</em> shuttle vector carrying promoterless <em>lacZ</em>, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pMW11168</td>
<td>pMW10 carrying the <em>cmeABC</em> promoter from NCTC11168 fused to <em>lacZ</em>, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(18)</td>
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<td>pMW81-176</td>
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<td>pMWX7199</td>
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<td>pMW10 carrying the <em>Cj0561c</em> promoter fused to <em>lacZ</em>, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pQE30</td>
<td>Expression vector for N-terminal 6-His tagged proteins, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>pQECmeRSS</td>
<td>pQE30 carrying <em>CmeR</em> with the C69S and C166S substitutions</td>
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<tr>
<td>pQECj0355c</td>
<td>pQE30 carrying <em>Cj0355c</em> with the T to A mutation at nt 652</td>
<td>This study</td>
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<td>pQECj0355c652</td>
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<td>Cloning vector, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Genscript</td>
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**Campylobacter jejuni strains**

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<td>NCTC11168Δ<em>cmeR</em></td>
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<td>Human clinical isolate</td>
<td>(18)</td>
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<td>Derivative of 81-176 carrying pMW11168</td>
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<td>81-176pMW561</td>
<td>Derivative of 81-176 carrying pMW561</td>
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<td>Derivative of 81-176, <em>cmeR::cat</em></td>
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**Escherichia coli strains**

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<td>JM109</td>
<td>e14('McrA') recA1 endA1 gyrA96 thi-1 hsdR17(t&lt;sub&gt;R&lt;/sub&gt;m&lt;sub&gt;R&lt;/sub&gt;) supE44 relA1 Δ(lac-proAB) [F' traD36 proAB lac&lt;sub&gt;2&lt;/sub&gt;ΔZ1M15]</td>
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<td>AR</td>
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1 Restriction sites are indicated by underlined sequences
TABLE 3: Effects of CmeR and CosR on repression of \textit{cmeABC} during dual regulation$^1$

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<tr>
<th>Promoter</th>
<th>Effect of CosR in presence of CmeR$^2$</th>
<th>Effect of CmeR in presence of CosR$^3$</th>
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<tr>
<td>11168</td>
<td>1.8$^a$</td>
<td>4.6$^c$</td>
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<tr>
<td>81-176</td>
<td>2.1$^a$</td>
<td>3.7$^b$</td>
</tr>
<tr>
<td>X7199</td>
<td>1.7$^a$</td>
<td>2.6$^c$</td>
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$^1$ One-way ANOVA was performed on log2 transformed transcriptional data to determine difference between promoter effects. Effects with different letters indicate a significant difference ($p < 0.05$).

$^2$ The effect of CosR in presence of CmeR was calculated as the relative fold change in transcription levels in the 81-176 wild-type background with and without the anti-CosR PNA.

$^3$ The effect of CmeR in presence of CosR was calculated as the relative fold change in transcription between the 81-176Δ\textit{cmeR} background and the 81-176 wild-type background without the anti-CosR-PNA.
TABLE 4: Collective and individual effects of CmeR and CosR on repression of cmeABC

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Collective Effect</th>
<th>Individual Effect of CosR</th>
<th>Individual Effect of CmeR</th>
<th>Sum of individual effects</th>
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<td>1.4a</td>
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<td>4.9a</td>
<td>1.4a</td>
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<td>1.3a</td>
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<td>0.1a</td>
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1 One-way ANOVA was performed on log2 transformed transcriptional data to determine difference between promoter effects. Effects with different letters indicate a significant difference (p <0.05).

2 The collective effect was calculated as the relative fold change in transcription levels between the 81-176ΔcmeR background with anti-CosR PNA and the 81-176 wild-type background without anti-CosR-PNA.

3 Individual effect of CosR was calculated as the relative fold change in transcription levels in the 81-176ΔcmeR background with and without the anti-CosR-PNA.

4 Individual effect of CmeR was calculated as the relative fold change in transcription levels between the 81-176ΔcmeR and the 81-176 wild-type background, in the presence of the anti-CosR-PNA.
FIGURE 1: Specific binding of CosR to the *cmeABC* promoter as shown by EMSA.

rCosRWT (lanes 2 and 4) and rCosRC218S (lanes 3 and 5) were incubated with the DIG-labeled 11168 *cmeABC* promoter (lanes 2 and 3) or the internal *cmeA* fragment (lanes 4 and 5). Lane 1 contains the 11168 *cmeABC* promoter without added protein (probe-only control). All probes are 0.05 pmol per reaction and the protein (rCosRWT or rCosRC218S) concentration is 250 ng per reaction.
FIGURE 2: Sensitivity of CosR to oxidation by hydrogen peroxide. Binding of 250 ng of rCosRWT (lanes 2-5) or rCosRC218S (lanes 6-8) to DIG-labeled 11168 cmeABC promoter DNA (0.05 pmol). No protein was added to lane 1 as a probe-only control. The promoter probe and protein were incubated for 30 minutes prior to addition of hydrogen peroxide. Hydrogen peroxide was added for final concentrations of 0 nM (lanes 2 and 6), 5 nM (lanes 3 and 7), 10 nM (lanes 4 and 8), or 20 nM (lanes 5 and 9). After addition of hydrogen peroxide, all reactions were incubated for an additional 30 minutes before electrophoresis.
FIGURE 3: Dual binding of CosR and CmeR to various variants of the cmeABC promoter sequence. (A) Alignment of cmeABC promoter sequences from strains NCTC 11168 (11168), 81-176, X7199, and the artificially designed P14D probe. The known CosR binding site is in bold and the CmeR binding site is in lowercase, underlined italics. Mutations in the CmeR binding site are indicated in bold, lowercase, underlined italics. (-) indicates a deleted base. (B) EMSA results of the cmeABC promoter probes from 81-176 (lanes 1-4) and X7199 (lanes 5-8) incubated with 200 ng of rCmeRSS (lanes 2 and 6),
FIGURE 3 continued: 400 ng of rCosRC218S (lanes 3 and 7), or both (lanes 4 and 8). No protein was added to lanes 1 and 5 as probe-only controls. (C) EMSA results of the cmeABC promoter probes from 81-176 (lanes 1-4) and P14D (lanes 5-8) incubated with 200 ng of rCmeRSS (lanes 2 and 6), 400 ng of rCosRC218S (lanes 3 and 7), or both (lanes 4 and 8). No protein was added to lanes 1 and 5 as probe-only controls. All promoter probes were 0.05 pmol per reaction.
FIGURE 4: Effect of cosR inhibition on expression of CmeB and CmeA. Immunoblotting of wild-type 11168 (lanes 1 and 2) and 11168ΔcmeR (lanes 3 and 4) whole cell proteins with anti-CmeB, anti-CmeA, and anti-major outer membrane protein (MOMP) antibodies, respectively. The anti-CosR PNA was added to samples loaded in lanes 2 and 4 at 1.5 µM to inhibit cosR expression.
FIGURE 5: Effect of inhibiting CosR by the anti-CosR-PNA on transcription from the cmeABC promoter in the presence (A) or absence (B) of CmeR. Transcriptional fusions and β-galactosidase assays were used to measure the expression from 11168, 81-176, or X7199 cmeABC promoters or the Cj0561c promoter (561). Presence or absence of CmeR was determined by using 81-176 wild-type (A) and 81-176ΔcmeR (B) for the transcriptional fusions. Cultures were incubated with (+ CosR PNA; 1.5 μM) or without
FIGURE 5 continued: (-CosR PNA) the PNA. The Cj0561c promoter was used as a control to demonstrate its regulation only by CmeR, not by CosR. Data are means with standard deviation from three independent experiments. The unpaired Student’s t-test with Welch’s correction was used for comparison of the means with significance set at 0.05.
References


CHAPTER 5: GENERAL CONCLUSIONS

Using various molecular and biochemical methods, we showed that CmeABC was differentially expressed in natural *C. jejuni* isolates derived from different host species with the majority of the isolates exhibiting overexpression of *cmeABC* compared to the reference strain, *C. jejuni* NCTC 11168. Overexpression of *cmeABC* in some isolates was linked to mutations in the *cmeABC* promoter that affected binding of CmeR, a transcriptional repressor for CmeABC. CmeR levels were undetectable in one isolate, CT2:2, resulting in increased *cmeABC* expression. Interestingly, this isolate also harbored a frame shift mutation that caused truncation of the CmeR protein and abolished the DNA binding activity in the recombinant CmeR harboring this mutation. Thus even if CmeR was produced, this isolate would continue to overexpress *cmeABC*.

Additionally, isolates with mutations (amino acid substitutions) in CmeR that overexpressed *cmeABC* were identified, but the examined mutations did not affect the DNA-binding activity of CmeR. Other isolates did not harbor mutations in the known *cmeABC* regulatory regions (*CmeR* coding sequence or *cmeABC* promoter) suggesting that the increased *cmeABC* expression in these isolates was due to unidentified regulatory mechanisms. This work is the first to evaluate differential CmeABC expression in natural isolates and identifies multiple mutations that influence *cmeABC* expression.

*cmeR* is the second of gene of the *Cj0369c-cmeR* operon and these genes are transcribed as a single transcriptional unit (1). No regulatory mechanisms for this operon have been described and post-translational investigation into the regulation of CmeR has not been evaluated. Investigation into the mechanisms of *cmeR* regulation may provide
an explanation why CT2:2 did not produce any CmeR. In addition, if \textit{cmeR} regulation has other mechanisms than transcriptional control, this may also explain the varied \textit{cmeR} transcript levels detected by real time RT-PCR in selected isolates with a phenotype of CmeABC overexpression.

Differential CmeABC overexpression had functional consequences. In the cultures treated with ciprofloxacin, \textit{cmeABC} overexpression promoted the emergence of ciprofloxacin-resistant mutants, suggesting that the isolates with this phenotype may be better prepared to deal with antibiotic treatment. Thus, the high prevalence of isolates with \textit{cmeABC} overexpression (67%) might have been due to selection by antibiotic usage.

In addition to examining differential CmeABC expression and natural mechanisms altering expression, we also evaluated an additional regulator of \textit{cmeABC} named CosR. We determined how CosR interacts with CmeR in binding to the \textit{cmeABC} promoter and how CosR senses oxidative stress. It was found that the two regulators provide a dual and possibly synergistic repression of \textit{cmeABC}. Maximal repression by the two regulators requires sufficient spacing between the CosR and CmeR binding sites. Shortening of the spacer region causes steric hindrance and reduces dual binding by the two regulators, increasing expression of \textit{cmeABC}. Additionally, we found that CosR utilizes the single cysteine to detect oxidative stress and modulates \textit{cmeABC} expression. These findings indicate that \textit{cmeABC} is co-repressed by dual regulators that are inducible by different signals (bile, oxidative stress, antimicrobials, etc).

Based on the results from our study we propose the following mechanisms of \textit{cmeABC} regulation (Figure 1). In the absence of environmental stimuli, such as bile or
oxidative stress, \textit{cmeABC} is expressed at a low, basal level (Fig. 1A) (2), but can be induced by modifying the functions of CmeR (Fig. 1B), CosR (Fig. 1C), or both (Fig. 1D). The presence of bile in the intestinal lumen would induce \textit{cmeABC} expression through CmeR (Fig. 1B) (3, 4), which would undergo a conformational change upon binding by bile and disassociate from the \textit{cmeABC} promoter, increasing the expression of the efflux pump (3, 5). This inducible expression of \textit{cmeABC} likely contributes to \textit{C. jejuni} adaptation in the intestinal tract. Additionally, association of \textit{C. jejuni} with the epithelial cells triggers the release of hydrogen peroxide (6). Hydrogen peroxide oxidizes CosR, resulting in its disassociation from the \textit{cmeABC} promoter and overexpression of the transporter system (Fig. 1D). Furthermore, CosR-mediated induction of \textit{cmeABC} may occur when \textit{C. jejuni} encounters phagocytes or after contact with enterocytes, where ROS are produced. The up-regulation of \textit{cmeABC} under oxidative stress may provide a defense mechanism against oxidation, but how CmeABC contributes to this defense is unknown and awaits further investigation.

Given the importance of CmeABC in \textit{C. jejuni}'s adaptation to various environments, it is plausible that this efflux machinery can be targeted to control \textit{Campylobacter}. One possible way is to inhibit expression of \textit{cmeABC} by using peptide nucleic acids, which can be designed for specific inhibition of CmeABC (7). Another approach could be use of efflux pump inhibitors, which may block the function of CmeABC (8). Either reduced expression or blocked function of CmeABC would sensitize \textit{Campylobacter} to bile and other antimicrobials. Alternatively, a compound that binds to CmeR and make it un-inducible by environmental cues could be developed and used to prevent CmeR from disassociating from the \textit{cmeABC} promoter, thus repressing
the expression of CmeABC. Furthermore, CosR could be targeted for the control of *C. jejuni*. Since CosR is essential for the viability of *C. jejuni* and is important for oxidative defense, a compound that inhibits the expression or function of CosR should produce anti-*Campylobacter* effects. These potential approaches should be examined using both *in vitro* systems and *in vivo* models in future studies.

In summary, this work demonstrates the versatile mechanisms and signals that modulate the expression of CmeABC. Given that CmeABC is an important player in antimicrobial resistance and intestinal colonization, differential expression of this efflux system will provide *Campylobacter* with flexibility in the adaptation to diverse environments. The findings from this work further support the notion that CmeABC is a potential target for the development of intervention strategies as inhibiting the expression or function of CmeABC will sensitize *Campylobacter* to antibiotics and bile, making the organism unable to survive antibiotic treatment or colonize in the intestinal tract. CosR also provides an attractive target that would eliminate the concern over CmeABC expression levels. Future efforts are warranted to explore these possibilities.
FIGURE 1: Regulatory and induction mechanism of \textit{cmeABC} expression. A partial promoter sequence for \textit{cmeABC} is shown. The ribosome binding site is underlined and the ATG start codon of \textit{cmeA} is indicated in bold italics. The CosR binding site is indicated in
FIGURE 1 continued: bold lowercase letters and the CmeR binding site is shown in bold uppercase letters. A) Basal level of \textit{cmeABC} transcription in the absence of environmental stimuli with dual repression by CmeR and CosR. B) Induction of \textit{cmeABC} by bile through CmeR. Binding of bile to CmeR alters its confirmation, resulting in disassociation of CmeR from the promoter and an increase in \textit{cmeABC} transcription. C) Induction of \textit{cmeABC} by oxidation through CosR. ROS oxidize CosR, resulting in its disassociation from the \textit{cmeABC} promoter and increased transcription of \textit{cmeABC}. D) Induction of \textit{cmeABC} by ROS and bile, results in disassociation of both CosR and CmeR from the promoter and high levels of \textit{cmeABC} transcription.
References


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