Molecular mechanisms involved in the emergence and fitness of fluoroquinolone-resistant Campylobacter jejuni

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Molecular mechanisms involved in the emergence and fitness of fluoroquinolone-resistant Campylobacter jejuni

by

Jing Han

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

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Iowa State University
Ames, Iowa
2009

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DEDICATION

This dissertation is dedicated to:

My beloved one-Husband, Hailin Tang

My son Eric (Jiale) H. Tang, who was born in February 2008

   My Mother, Xingzhi Zhao

   My Father, Futing Han:

Thanks for your love and support.
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CHAPTER 1. GENERAL INTRODUCTION

1. Introduction

Since the 1970s, *Campylobacter*, a Gram-negative microaerobic bacterium, has emerged as a major foodborne pathogen and a common causative agent of human enterocolitis. *Campylobacter jejuni* is responsible for approximately 90% cases of human campylobacteriosis, followed by *Campylobacter coli*, which accounts for approximately 10% cases of human campylobacteriosis. Signs and symptoms of campylobacteriosis often start as a cramping pain in the abdomen, followed by watery or inflammatory diarrhea. Some individuals may develop fever, headache, dizziness and myalgia. *Campylobacter* infection causes more than 2 million cases of diarrhea each year in the U.S. alone. *C. jejuni* infections in humans may also trigger a degenerative neurological disorder called Guillain-Barré syndrome.

Majority of *Campylobacter* infections are mild, self-limiting and usually resolve within a few days, no specific treatment is required for most patients with *Campylobacter enteritis*. However, in specific clinical circumstances such as in severe infections, prolonged illness or where the patient is pregnant, young, elderly, or has compromised immunity, antimicrobial treatment is usually warranted. Fluoroquinolone (FQ) antimicrobials are one of the antibiotics that are often prescribed for clinical treatment of diarrhea caused by enteric bacterial pathogens including *Campylobacter*. However, *Campylobacter* is increasingly resistant to FQ antimicrobials and FQ-resistant (FQ\textsuperscript{R}) *Campylobacter*, developed in food-producing animals, can be transmitted to humans via the food chain, which has become a major concern for public health. In *Campylobacter*, the main targets of FQs are DNA gyrase, and resistance to FQ antimicrobials is mediated by point mutations in the quinolone resistance-determining region (QRDR) of \(gyrA\) in conjunction with the function of the multidrug efflux pump CmeABC. Acquisition of high-level FQ resistance in *Campylobacter* does not require stepwise accumulation of point mutations in \(gyrA\). Instead, a single point mutation in \(gyrA\) can lead to clinically relevant levels of resistance to FQ antimicrobials. Specific mutations at positions Thr-86, Asp-90 and Ala-70 in GyrA have been linked to FQ
resistance in \textit{C. jejuni}. The Thr-86-Ile mutation in GyrA confers a high-level resistance to fluoroquinolone. One unique feature of FQ resistance development in \textit{Campylobacter} is the rapid emergence of FQR mutants from a FQ-susceptible (FQS) population when treated with FQ antimicrobials and \textit{C. jejuni} possesses a high mutation rate to FQ resistance. The rapidness and magnitude of FQ resistance development in \textit{Campylobacter} in response to FQ treatment suggest that both selective enrichment of pre-existing spontaneous mutants and adaptive gene expression may contribute to the emergence of FQR \textit{Campylobacter}, but how \textit{Campylobacter} responds to FQ treatment is unknown. Once developed, FQR \textit{Campylobacter} carrying the Thr-86-Ile change in the GyrA subunit of DNA gyrase can persist in the absence of antibiotic selection pressure. Notably, FQR \textit{Campylobacter} carrying the Thr-86-Ile substitution in the GyrA subunit of DNA gyrase outcompeted the FQS strains in chickens, suggesting that acquisition of FQ resistance enhances the \textit{in vivo} fitness of FQR \textit{Campylobacter}. How the resistance-conferring mutation affects \textit{Campylobacter} fitness remains to be determined.

To close these important gaps in our understanding of the mechanisms underlying the rapid emergence of FQR mutants and fitness of the FQR mutants, we conducted a series of \textit{in vitro} and \textit{in vivo} studies to determine how \textit{Campylobacter} responds to FQ treatment, what facilitates the emergence of FQR mutants in \textit{Campylobacter}, and what are the molecular mechanisms contributing to the enhanced fitness in FQR \textit{Campylobacter}. The findings from this project will significantly improve our understanding of the molecular mechanisms underlying the development of FQR \textit{Campylobacter} and the fitness of the FQR \textit{Campylobacter} and will facilitate the design of strategies to reduce the emergence and spread of FQR \textit{Campylobacter}.

\textbf{2. Dissertation Organization}

This dissertation is organized in the alternative format with five chapters. Chapter 1 contains a general introduction. Chapter 2 contains a literature review. Chapter 3 through 5 each contains a manuscript published or to be submitted to the ASM journals. Chapter 6 is
the general conclusion. At the end of each chapter are appended references cited followed by tables and figures.
CHAPTER 2. LITERATURE REVIEW

1. *Campylobacter*: The Organism

1.1 Taxonomy

The pathogenic bacteria *Campylobacter* spp. were first observed and described as non-culturable spiral-shaped or *Vibrio*-like bacteria in 1886 by Theodor Escherich (130) and first isolated from an aborted bovine fetus in 1913 by McFadyean and Stockman (36). At that time, these bacteria were classified in the genus *Vibrio*. In 1963, based on their low G+C composition, their microaerophilic growth requirements, and their nonfermentative metabolism, Sebald and Veron transferred two of these *Vibrio* species, *Vibrio fetus* and *Vibrio bubulus*, into a new genus *Campylobacter* as *Campylobacter fetus* and *Campylobacter bubulus*, respectively. With the development of new isolation technique, additional *Campylobacter*-like organisms were isolated from a variety of human, animal, and environmental sources and some of those species were described and added to *Campylobacter* genus. In 1991, Vandamme and De ley accommodated the genera *Campylobacter* and *Arcobacter* into a new bacterial family, the *Campylobacteraceae*. Currently, the genus *Campylobacter* includes 18 species: *C. jejuni*, *C. coli*, *C. concisus*, *C. curvus*, *C. fetus*, *C. gracilis*, *C. helveticus*, *C. hominis*, *C. hyointestinalis*, *C. insulaenigrae*, *C. lanienae*, *C. lari*, *C. mucosalis*, *C. rectus*, *C. showae*, *C. sputorum*, *C. upsaliensis* and *C. canadensis*. Although most of the *Campylobacter* spp. have been associated with veterinary and human infections, *C. jejuni* and *C. coli* are the most important and major cause of food-borne *Campylobacter* infection in humans.

1.2 General characteristics of *Campylobacter*

*Campylobacter* spp. are Gram-negative, nonspore-forming, S- or spiral-shaped rod bacteria that are approximately 0.2 to 0.8 µm wide and about 0.5 to 5 µm long (58). Although normally a curved-rod shape, other forms of *Campylobacter* such as spherical or coccoid occur in response to stress or deleterious conditions (47, 58). Most *Campylobacter* are motile with distinctive corkscrew-like darting motions, using a single polar, unsheathed
flagellum at one or both ends (58). Some *Campylobacter* species (*C. gracilis*) are nonmotile while some (*C. showae*) have multiple flagella (58).

The members of *Campylobacter* spp. are slowly-growing, fastidious organisms and require a microaerophilic atmosphere for optimal growth (47, 201). Typical *Campylobacter* spp. requires an atmosphere containing approximately 3 to 15% O₂ and 2 to 10% CO₂ for optimal growth. Although most *Campylobacter* can grow at 37 °C, thermophilic *Campylobacter* spp. such as *C. jejuni*, *C. coli*, and *C. lari* show optimal growth at 42 °C on artificial media (47, 221). The ideal environment for recovery of *Campylobacter* is at 37 °C or 42 °C under the atmosphere containing approximately 5% O₂, 10% CO₂, and 85% N₂. *Campylobacter* spp. are unable to utilize glucose and other hexoses as carbon sources. The main carbon and energy sources used by *Campylobacter* for growth are derived from the degradation of amino acid or tricarboxylic acid cycle intermediates (123, 124). Therefore, nutrient-rich media, such as Mueller-Hinton broth, are often used for culturing *Campylobacter*.

*Campylobacter* spp. have a small genome of approximately 1.6-1.7 Mb with AT-rich DNA and low G+C content ranging from 29-34 mol% (183, 191). *C. jejuni* NCTC 11168 has 1,643 open reading frames (ORFs) and it lacks plasmids (191). Other features found in the *C. jejuni* NTCT 11168 are the lack of insertional sequences or phage-associated sequences, and few repeat sequences (183). One of the most striking themes in the *C. jejuni* genome is the presence of hypervariable sequences encoding the biosynthesis or modification of surface structures such as flagellum, capsular polysaccharides, and lipooligosaccharides (LOS).

2. Epidemiologic Aspects of *Campylobacter* Infection

*Campylobacter jejuni* is one of the most prevalent bacterial foodborne pathogens in the United States and many other developed countries, causing more than 2 million cases of diarrhea each year in the U.S. alone (7, 75, 184, 214, 236). Person-to-person transmission of *Campylobacter* is rare, and the main source of human *Campylobacter* infection is via food, water, or milk contaminated by *Campylobacter* (11, 56, 75). In general, people can become
infected with *Campylobacter* spp. by four different ways: (i) by handling raw meats and meat products, (ii) by consumption of raw or undercooked meats, unpasteurized milk and dairy products, (iii) by consumption of foods that are usually eaten raw or without further cooking such as salads and breads, which may become cross contaminated with raw meats in the kitchen, or (iv) by direct contact with infected animals/pets (25, 224, 225). Domestic poultry are considered to be a major risk factor for human *Campylobacter* infection (7, 229). The correlation between the consumption of chicken meat and *Campylobacter* infection in humans has been demonstrated in several studies (74, 209, 226). *C. jejuni* is highly prevalent in the intestinal tract of market-age broiler chickens. Several survey studies revealed that at least 70% of retail chickens sold in the United States are contaminated with *Campylobacter* (270). The sources of *C. jejuni* infections in poultry have not yet been elucidated. Potential sources for horizontal transmission on poultry farms include old litter, untreated drinking water, other farm animals, domestic pets, wildlife species, house flies, insects, equipment and transport vehicles (113, 174, 196, 229, 245). Besides poultry meats, other major cause of human *Campylobacter* infection includes consumption of raw or unpasteurized milk, improperly/untreated water, and contacts with pets (7, 25, 75, 126, 225).

Unlike *Salmonella* infection, the majority of *Campylobacter* infections in humans occur as sporadic cases; outbreaks of *Campylobacter* infection are rare (181, 184). Sporadic cases of *Campylobacter* infection have different epidemiological characteristics from outbreaks. Most outbreak cases of *Campylobacter* infection are associated with raw/unpasterized milk and untreated/contaminated water (1, 33, 70, 184), while the majority of sporadic cases of *Campylobacter* enteritis result from the handling or consumption of undercooked poultry and/or other foods that are cross-contaminated with raw poultry meat during food preparation (7, 12, 31, 54, 108). The milk- and water- associated outbreaks usually occur in the spring and autumn and are uncommon in the summer, while sporadic *Campylobacter* infections often occur during summer (184).

Although *Campylobacter* enteritis is the most common enteritis worldwide, the epidemiology of *Campylobacter* infections in developing countries is markedly different
from that in the developed countries (181). First, the incidence of campylobacteriosis in developing countries is much higher than in developed countries. Second, in developing countries, *Campylobacter* infection occurs mostly in the first few years after birth and declines with age. Most infections become asymptomatic among adults, presumably because of the acquisition of immunity due to multiple exposures to several serotypes of *Campylobacter* in the early stages of the life. In developed countries, infections can occur at any age and the infections are more severe. Inflammatory (bloody) diarrhea is more common among patients in developed countries. Third, the route of transmission in developing countries differs from that in developed countries. In developed countries, infections are often caused by uncooked food, while in developing countries, the presence of livestock in close proximity to living areas severely increases the risk of infection with *Campylobacter*. Fourth, in developed countries, *Campylobacter* infections typically increase during summer and fall. This seasonal variation is not significant in developing countries.

### 3. Clinical Significance of *Campylobacter* Infections

#### 3.1 *Campylobacter* infection in human

*Campylobacter* species, particularly *C. jejuni* and *C. coli*, have been recognized as important causes of bacterial enteric infections in humans since the late 1970’s (37). *C. jejuni* and *C. coli* account for 85-95% and 5-15% of human campylobacteriosis, respectively (170). There is no difference between the characteristics of infections caused by *C. jejuni* and *C. coli*. The infectious dose for humans can be as low as 500 bacterial cells (32). The mean incubation period of the infections is 3.2 days which is longer than that of most other intestinal infections and the range can extend from 1-8 days (31, 32, 37). Infections with *Campylobacter* spp. are of variable severity ranging from asymptomatic to severe. The main clinical symptom of campylobacteriosis in humans is acute enteritis, which is characterized by acute diarrhea with abdominal cramps, fever, headache, and nausea. Some patients may also vomit.

The symptoms of *Campylobacter* enteritis are different between developed countries and developing countries. Patients in developed countries usually have more severe
symptoms than those in developing countries (181). In developed countries, the common clinical features of *Campylobacter* spp. infection are acute gastroenteritis which is characterized by diarrhea, fever, and abdominal cramps (7). The diarrhea may range in severity from loose stools to profuse, watery stools with 10 or more bowel movements within one day (31). The initially watery diarrhea may develop into severe bloody diarrhea with fecal leucocytes as the disease progress. The acute diarrhea usually lasts 2-3 days, but it may persist for 1 week or longer particularly in patients with an acute colitis. Usually the gastroenteritis is self-limiting without the need for treatment (32). In developing countries, the typical clinical symptom is milder form of gastroenteritis which is usually characterized by wetery, non-bloody, non-inflammatory diarrhea and symptomatic infections are extremely common in children younger than 2 years old (45, 127, 188). In contrast, in developed countries, infections occur in both adults and children. The highly endemic nature of infections in developing countries result in the early exposure to *Campylobacter* in life and increased *Campylobacter*-specific antibody levels with age which may account for the less severe clinical symptoms in adults.

In some patients, the diarrhea is not significant, while acute abdominal pain is the predominant feature of *Campylobacter* infections. Since the abdominal pain is usually cramping in nature and most often occurs at the right lower quadrant of the abdomen, severe abdominal pain may mimic acute appendicitis which can lead to a mistaken diagnosis (7, 30-32, 37).

The symptoms of *Campylobacter* infection usually last for 3-4 days, and the disease gradually resolves over a week (32). A longer relapse may occur in 15-25% of affected patients who did not receive antibiotic treatment and last for several weeks (7, 31, 32). Patients may shed *Campylobacter* in their feces for several weeks after the clinical symptoms have disappeared unless treated with antibiotics (32).

### 3.2 *Campylobacter* infection in animals

In animals, *C. jejuni* and *C. coli* usually do not cause clinical diseases. They are
commensal inhabitants of the intestinal tracts of a wide range of domestic animals and pets, as well as wild animals and birds (12, 110, 234). Commercial poultry such as broilers, layers, turkeys, ducks and free-living birds are the natural reservoirs of *C. jejuni* and *C. coli* (220). Recent studies indicated that *C. jejuni* is increasingly associated with ovine abortions and it has replaced *C. fetus* as the predominant *Campylobacter* species causing sheep abortion in the United States (59, 213).

### 3.3 Complications of *Campylobacter* infection in human

In general, gastrointestinal complications due to *Campylobacter* infections rarely occur. However, *Campylobacter* spp. may spread from the gastrointestinal tract and lead to cholecystitis, pancreatitis, or massive gastrointestinal hemorrhage (7, 37). In addition to the gastrointestinal tract, *Campylobacter* also can cause some extraintestinal diseases, such as bacteremia, meningitis, endocarditis, septic arthritis, osteomyelitis and abortion (7, 32, 60).

The most important post-infectious complication of *C. jejuni* infection is Guillain-Barré Syndrome (GBS), an acute immune-mediated neuromuscular paralysis that may lead to respiratory muscle compromise and death (8, 131, 172). About 20 to 50% of patients with GBS symptoms have a preceding *Campylobacter* infection and symptoms of GBS typically start 1 to 3 weeks after a *Campylobacter* infection (26, 37). Although *C. jejuni* infection is a common trigger of GBS, the risk of developing GBS after *C. jejuni* infection is actually quite low (172). Among the different *C. jejuni* strains involved with GBS, Penner serotype O:19 is most commonly associated with GBS in the United States (9, 172). It has been hypothesized that the molecular mimicry between the peripheral nerve gangliosides and sialylated *Campylobacter* lipooligosaccharide (LOS) may play a role in the pathogenesis of *Campylobacter*-induced GBS (131, 267). The antibodies induced by *Campylobacter* LPS cross-react to gangliosides, causing inflammation and tissue damage and eliciting the autoimmune disease GBS (7, 26, 37, 132).

### 4. Virulence and Pathogenesis of *Campylobacter* Infection
Although *Campylobacter* has emerged as a leading cause of bacterial food-borne illness during the past decade, relatively little is known of the molecular pathogenesis and virulence of *Campylobacter* infections compared to other enteric pathogens (127, 199, 247). This is partly due to the lack of an ideal animal model to evaluate the pathogenesis and virulence of *Campylobacter* (266). As a food-borne bacterial pathogen, *Campylobacter* enters the human body with food or water, survives the stomach acid and highly alkaline secretion from the bile duct, then colonizes the distal ileum and colon (109). Following colonization of the mucus blanket and adhesion to the intestinal cell surfaces, *Campylobacter* perturbs the normal absorptive capacity of the intestine by damaging epithelial cell function through direct cell invasion and/or toxin production, or indirectly by inducing host inflammatory reactions (260). Thus, factors involved in motility, intestinal adhesion, colonization, invasion, protein secretion, intracellular survival and toxin production are thought to be the major contributors to the pathogenicity of *Campylobacter* spp. Numerous studies using *in vitro* cell cultures and *in vivo* model systems have been performed to identify and characterize potential bacterial cell components involved in the pathogenesis of *Campylobacter*. In *C. jejuni*, the bacterial factors involved in disease pathogenesis include adhesins, flagella, capsular polysaccharides, cytolethal distending toxin (CDT) and many others.

### 4.1 Adhesins

The first step involved in *Campylobacter* colonization and pathogenesis is adherence to intestinal epithelial cells. The bacterial cell components involved in adhesion have been studied extensively using *in vitro* cell cultures and *in vivo* model systems. The adhesins identified in *C. jejuni* includes CadF, PEB1 (CBF 1), JlpA, and CapA.

CadF (*Campylobacter* adhesion to fibronectin) binds specifically to fibronectin, which is located at the basolateral epithelial cells, and is required for maximal binding and invasion by *C. jejuni* (134, 167, 168). The binding of *C. jejuni* to immobilized fibronectin can be blocked by specific anti-CadF antibody (167). *cadF* mutants show a great reduction in
binding and invasion of INT407 cells and are unable to colonize chickens compared with wild-type isolates (167, 168, 273).

PEB1 (CBF 1) is periplasmic and shares homology with the periplasmic-binding proteins of amino acid ATP-binding cassette (ABC) transporters involved in nutrient acquisition (78, 194). *C. jejuni* mutants that lack *peb*1 exhibit a reduction in the adherence to HeLa cells, as well as reduced colonization of mice when compared with wild-type isolates (194, 195).

JlpA (*C. jejuni* lipoprotein A) is a surface-exposed lipoprotein that binds to Hsp90α on the surface of HEp-2 cells (115). Binding of JlpA to Hsp90α activates NF-κB and p38 mitogen-activated protein kinase, which contribute to proinflammatory responses (194). Ablation of *jlpA* reduces *C. jejuni* adherence to HEp-2 cells by 18-19.4% compared to the wild-type strain (115).

CapA is an autotransporter, and *capA* mutants show reduced adherence to Caco-2 cells and decreased colonization and persistence in chickens (17).

### 4.2 Flagella

Flagella are the most extensively studied virulence determinant of *Campylobacter*. They are involved in several aspects of pathogenesis. *Campylobacter* have one or two unsheathed polar flagella. The unique characteristics of the flagellum, combined with the spiral shape of the bacterial cell, enable the organism to move using a darting motility. *C. jejuni* flagella and flagellar motility are vital to many aspects of *C. jejuni* pathogenesis, including host colonization, virulence in ferret models, protein secretion and host invasion. Numerous studies have shown that *Campylobacter* must be flagellated to colonize a host and cause disease (10, 92, 102, 133, 235, 254, 264). During the initial stages of infection, the flagella help *Campylobacter* overcome the clearing action of peristalsis and allow it to enter and cross the viscous mucosal layer covering the intestinal epithelia, where it adheres to the host cell surface.
*Campylobacter* flagellum is composed of two flagellin monomers, FlaA and FlaB, encoded by *flaA* and *flaB* respectively (95). The *flaA* and *flaB* genes are tandemly arranged in the *Campylobacter* genome and transcribed from σ28- and σ54-dependent promoters with its own promoter respectively (10, 95, 180, 235). The major subunit FlaA and the minor subunit FlaB share about 90% amino acid identity and are capable of assembling independently into functional filaments (95, 180, 235). Mutagenesis studies showed that a flagellar filament composed exclusively of the FlaA is indistinguishable in length from that of the wild type, but shows a slight reduction in motility, while the flagellar filament composed exclusively of the FlaB is severely truncated in length and greatly reduced in motility (95, 235). Thus, both FlaA and FlaB are required for full-length flagella expression and motility. Study by Wassenaar *et al.* (254) demonstrated that mutants with reduced motility but intact FlaA can colonize chickens at levels similar to the parent strain; by contrast, mutants with full motility but inactivated flaA gene were significantly reduced in their ability to colonize chickens, which suggests the presence of FlaA, rather than motility, is necessary for optimal bacterial colonization of chicken intestine.

Flagella also play an active role in the process of epithelia cell invasion. Mutagenesis and invasion studies showed the presence of a flagellum consisting of the FlaA protein is essential for invasion of INT-407 cells, whereas FlaB protein is not required (118, 235, 253). However, immotile mutants still penetrate at low levels, indicating that factors other than the flagella are also involved in invasion (265).

In addition to a role in motility, *C. jejuni* flagella also serve as a type III secretion system (T3SS) to secrete both flagellar and non-flagellar proteins that contribute to cell adhesion and invasion (137, 208, 228). The protein CiaB (*Campylobacter* invasion-associated antigen B) secreted by the flagellar secretion system is required for the invasion of INT-107 cells (135-137, 228). Disruption of *ciaB* reduces the colonization of *C. jejuni* in chicken (272). The mutation of FlaC which is also secreted by the flagellar secretion system affects Caco-2 cell invasion (228).
4.3 Lipooligosaccharide (LOS)/Capsular polysaccharides (CPS)

LOS and CPS (high molecular weight lipopolysaccharides) are common features on bacterial surfaces. Both LOS and CPS are the major surface antigens of Campylobacter and are highly variable among different Campylobacter strains (72, 122, 190). As mentioned earlier, the molecular mimicry between the Campylobacter LOS and human neuronal gangliosides is thought to lead to autoimmune disorders, including GBS (131, 267).

Studies have shown that these surface polysaccharide molecules play important roles in the interaction of the bacteria with animal/human hosts and are required for cell invasion in vitro, chicken colonization, and full virulence in the ferret animal model (22, 23, 76, 118, 120, 122, 161). Mutations in the genes involved in LPS synthesis and transport, such as galE, waaF and kpsE, significantly affect mutant cell adhesion to and invasion of INT-407 cells in vitro and increase sensitivity to some antibiotics compared that of the wild-type strain (76, 120).

4.4 Cytolethal distending toxin (CDT)

One of the mechanisms involved in the virulence of Campylobacter is the production of toxins, which are important in diarrheal diseases. C. jejuni produces cytolethal distending toxin (CDT) (116), which is also produced by several other Gram-negative bacteria, including E. coli, Shigella, and Helicobacter (52, 117, 182). The action mechanism of CDT produced by C. jejuni is to cause sensitive eukaryotic cells to arrest at either the G1/S or G2/M transition of the cell cycle, depending on the cell type (100, 101, 141, 142, 256).

The active holotoxin CDT consists of three subunits (CdtA, CdtB and CdtC) encoded by three adjacent or slightly overlapping genes cdtABC (41, 142). CdtB is the enzymatically active subunit of CDT and it shares homology with mammalian DNase I, however, CdtB has weak DNase activity in vitro (141). Whether DNA damage in vivo is a direct or indirect result of CdtB activity is still debatable (101, 159, 219, 248, 256). The exact function of CdtA and CdtC are unclear. Studies have shown both CdtA and CdtC can bind Hela cells...
It is suggested that CdtA and CdtC may mediate the binding and subsequent internalization of CdtB into the host cell (55, 142).

The role of CDT in *C. jejuni* pathogenesis remains unclear. Studies showed that CdtA, CdtB and CdtC are all required for the induction of pro-inflammatory cytokine IL-8, a hallmark of *C. jejuni* pathogenesis (103) and CDT may have a role in eliciting inflammatory response *in vivo* (73). However, it seems that CDT is not required for colonization since mutants that lack CDT colonize chickens as well as wild-type strains (29).

### 4.5 Glycosylation

*Campylobacter* expresses two glycosylation systems: a general N-linked protein glycosylation pathway and an O-linked protein glycosylation pathway, which makes it unique in bacteria (23). Both of these pathways are important in intestinal colonization.

The N-linked protein glycosylation pathway, which is encoded by a single gene cluster named *pgl*, is responsible for post-translational modification of asparagine residues on many surface proteins (23, 235). The N-linked glycan that is assembled by the Pgl system is conserved in all *C. jejuni* and *C. coli* strains that have been examined (23, 235, 248). Numerous studies have shown that *C. jejuni pgl* mutants have significantly reduced ability to adhere to and invade host cells and colonize animal models; these studies have linked N-linked protein glycosylation to bacterial virulence (23, 102, 119, 121, 125, 143). The *pglH* mutant of strain 81116 results in deficient glycosylation of a number of proteins and showed reduced adherence and invasion of human Caco-2 cells and colonization of chickens (121). An 81176 strain lacked glycoprotein Cj1469c was defective for both adherence to INT-407 human epithelium cells *in vitro* and colonization of chicken gut (119).

The O-linked protein glycosylation pathway is responsible for glycosylation of serine or threonine residues on flagellar proteins (23). O-linked glycosylation of flagellin is essential for the successful assembly of the flagellar filament (90). Defects in O-linked protein glycosylation result in the loss of motility, reduced adhesion and invasion of host
cells, and decreased virulence in ferret (96, 239).

5. Clinical Treatment of *Campylobacter* Infection in Human

Since the majority of *Campylobacter* infections are mild, self-limiting and usually resolve within a few days, no specific treatment is required for most patients with *Campylobacter* enteritis other than the oral replacement of fluid and electrolytes that are lost through diarrhea and vomiting (7, 32, 37).

However, in specific clinical circumstances such as in severe infections (persistent high fever, bloody diarrhea, or more than eight bowel movements within one day), prolonged illness (clinical symptoms last longer than 1 week), or where the patient is pregnant, young, elderly, or has compromised immunity, antimicrobial treatment is usually warranted (7, 32, 37).

For clinical antibiotic treatment of confirmed *Campylobacter* infection, erythromycin (a macrolide) is the drug of choice because of its efficacy, safety, low cost and relatively narrow spectrum of activity (less inhibitory effect on fecal flora) (7, 31, 37, 67). In addition to erythromycin, fluoroquinolone (FQ) antimicrobials (e.g., ciprofloxacin) have been commonly used for the treatment of enteritis caused by *Campylobacter* because of their broad spectrum activity against enteric pathogens, especially when bacterial gastroenteritis is suspected and the cause of diarrheal illness has not yet been identified (7, 32, 37). However, the increasing prevalence of FQ-resistant (FQ<sup>R</sup>) *Campylobacter* and macrolide-resistant *Campylobacter* has compromised the effectiveness of these treatments (67, 97, 110, 255). For serious systemic infection, tetracyclines and aminoglycosides, such as gentamicin, are the drugs of choice (32). Since up to 60% of strains may be resistant to tetracycline, care should be taken before prescribing this antibiotic (32).

6. Antimicrobial Resistance of *Campylobacter*

As mentioned in the previous section, FQ and macrolide antibiotics are the
antimicrobial agents considered for treatment of human *Campylobacter* enteritis when therapeutic intervention is needed (7, 45). In addition to use in human, these antibiotics have been used in animal husbandry for therapeutic purposes or as growth promoters (4). However, *Campylobacter* resistant to these clinically important antibiotics is increasingly prevalent, which is a major public concern.

**6.1 Action mode of antibiotics**

FQs, such as ciprofloxacin, enrofloxacin, and levofloxacin, are a class of structurally-related synthetic antimicrobials that are effective against a wide range of Gram-positive and Gram-negative bacterial and are used in both treatment and prophylaxis of bacterial infections (16, 105, 107). FQs are bactericidal by inhibiting DNA synthesis. The main targets of FQs in bacteria are the type II topoisomerase DNA gyrase and/or topoisomerase IV (64, 106). DNA gyrase catalyzes ATP-dependent negative supercoiling of DNA, which is an essential step in DNA replication, recombination, and transcription (42). Once inside bacterial cells, FQ antimicrobials form a stable complex with the target enzymes and trap the enzymes on DNA, resulting in double-stranded breaks in DNA and bacterial death (63, 223, 242, 259). In Gram-negative bacteria, DNA gyrase is the primary target of FQ antibiotics, while topoisomerase IV is the main target of FQs in Gram-positive bacteria (106).

Macrolides include erythromycin, tylosin, clarithromycin, azithromycin, and telithromycin, which have similar structures and are bacteriostatic agents that are active against most Gram-positive and some Gram-negative bacteria. They are important drugs for the treatment of upper and lower respiratory tract infections and gastric diseases caused by *Helicobacter pylori* and *Campylobacter* in humans (206, 268). Macrolides are protein synthesis inhibitors that reversibly bind to the 50S ribosome subunit and physically block tunnel entrance of the ribosome, causing the dissociation of peptidyl-tRNAs from the ribosome in the elongation cycle (86, 165, 186, 206, 242).

Tetracyclines are an important class of antibiotics with a broad spectrum of activity, being active against many Gram-negative and Gram-positive bacteria and have been widely
used in both human and animal medicine (268). The tetracyclines can be divided into two groups, the atypical tetracyclines (e.g. anhydrotetracycline and 6-thiatetacycline) and typical tetracyclines (e.g. tetracycline and minocycline) (6, 48). The atypical tetracyclines function by disrupting bacterial membranes, whereas the typical tetracyclines are bacteriostatic and act on the ribosome as protein synthesis inhibitors. Once inside the bacteria cell, the tetracycline binds reversibly to the 30S subunit of the ribosome, which prevents the binding of the incoming aminoaacyl-tRNA to the A site of the ribosome, resulting in elongation cycle halt and thus inhibition of nascent polypeptide synthesis (48, 69, 268).

Aminoglycosides, such as streptomycin or gentamicin, are potent bactericidal antibiotics that have been widely used in human and animal medicine. The bactericidal activity of aminoglycosides is primarily exerted through irreversible binding of the 30S ribosome subunit, which interferes with the normal proofreading activity of the ribosome and prevents the translocation of the growing peptide from the A site to the P site of the ribosome (114, 156, 268). As a result, the elongation of nascent proteins is disrupted.

6.2 Mechanisms of antibiotic resistance in *Campylobacter*

6.2.1 FQ resistance

The dominant mechanisms involved in FQ resistance are 1) target (DNA gyrase and/or topoisomerase IV) modification resulting in reduced affinity of these enzymes for fluoroquinolones, 2) reduced antibiotic intracellular accumulation by increasing efflux activity or decreasing outer membrane permeability, 3) reduced target enzyme expression, and 4) target protection mediated by the Qnr protein which can protect DNA gyrase from inhibition by the FQs (106, 192, 241). Of these resistance mechanisms, the first three are encoded by chromosomal elements, while the Qnr is encoded by a plasmid-carried quinolone-resistance gene and only confers a low level of resistance to FQs (192). In *Campylobacter*, the resistance to FQs is mediated by a point mutation in the quinolone resistance determining region (QRDR) of DNA gyrase subunit A (GyrA) (21, 81, 155, 192, 268). No mutations in DNA gyrase subunit B (GyrB) have been involved in FQ resistance in
**Campylobacter** (21, 193, 198). Unlike the FQ resistance in other enteric organisms (e.g. *Escherichia coli* and *Salmonella*), acquisition of high-level FQ resistance in *Campylobacter* does not require stepwise accumulation of point mutations in *gyrA*. Instead, a single point mutation in the QRDR of *gyrA* is sufficient to lead to clinically relevant levels of resistance to FQ antimicrobials (72, 155, 210). The most commonly observed mutation in FQ-resistant isolates of *Campylobacter* is the C257T change in the *gyrA* gene which leads to the Thr-86-Ile substitution in the gyrase A subunit and confers high-level resistance to ciprofloxacin (155, 268). Other mutations reported include Thr-86 Lys, Ala-70-Thr, and Asp-90-Asn, which are less common and only confer intermediate-level FQ resistance (67, 91, 155, 210, 252). Mutations in *parC*, which encodes topoisomerase IV, were reported to confer high-level FQ resistance (84); however, subsequent studies conducted by multiple independent investigators failed to identify the *parC-parE* gene in *Campylobacter*, which excluded the role of *parC-parE* mutations in *Campylobacter* resistance to FQ antimicrobials (21, 155, 193, 198). In addition to the mutations in the GyrA, the multidrug efflux pump also contributes to FQ resistance in *Campylobacter*. The main efflux pump involved in FQ resistance in *Campylobacter* is CmeABC, which is encoded by a three-gene operon (*cmeA*, *cmeB*, and *cmeC*) located on the *Campylobacter* chromosome. CmeABC functions as an energy-dependent efflux system and contributes to both intrinsic and acquired resistance of *C. jejuni* to FQ antimicrobials (81, 147, 155, 204). CmeABC plays a major role in ciprofloxacin resistance in *C. jejuni* by reducing the accumulation of FQs in *Campylobacter* cells (81, 147, 155). Thus, CmeABC functions synergistically with the *gyrA* mutations in mediating FQ-resistance. All of the known FQ-resistance determinants in *Campylobacter* are chromosomally encoded, and plasmid-mediated quinolone resistance determinants, such as *qnr, aac(6')-Ib-cr* and *qepA*, have not been reported in *Campylobacter* (268).

### 6.2.2 Macrolide resistance

There are three mechanisms involved in macrolide resistance in bacteria: 1) modification of the antibiotic, 2) modification of the antibiotic target, and 3) drug efflux (144, 268). Target modification and active efflux are most commonly associated with macrolide resistance in *Campylobacter* (40, 83, 148, 157, 268). Enzyme-mediated target
methylation and inactivation of antibiotics have not been identified in *C. jejuni* or *C. coli* (86, 268).

The most common mechanisms for macrolide resistance in *C. jejuni* and *C. coli* are point mutations in domain V of the 23S rRNA target gene at positions 2074 and 2075, which correspond to positions 2058 and 2059, respectively, in *E. coli* (53, 148, 192, 268). So far A2074C, A2074G, A2074T, A2075G, A2075T, and A2075C mutations have been reported in erythromycin-resistant *Campylobacter* (268). Among these reported mutations, the A2074C, A2074G, and A2075G mutations all confer high-level resistance to macrolide antibiotics with erythromycin MIC > 128 µg/ml in *C. jejuni* and *C. coli* (38, 53, 83, 148, 157, 268). But the A2075G mutation is the predominant mutation detected among the clinical- and field- isolated *C. jejuni* and *C. coli* (86, 138, 192). There are three copies of the 23S rRNA gene in the chromosome of *Campylobacter* and the resistance-associated mutations are usually identified in all three copies of the 23S rRNA gene (71, 191, 268). However, the wild-type and mutated alleles can coexist in some isolates and evidence suggests that at least two mutated copies are required to confer macrolide resistance (83, 148).

In addition to the mutations in 23S rRNA, mutations affecting macrolide binding have also been identified in the ribosome protein L4 and L22 (40, 53). The G221A change in the L4 protein which leads to the Gly-74-Asp substitution and an insertion at position 86 or 98 of the L22 protein can result in low levels of macrolide resistance in *C. jejuni* (40).

As with FQ resistance, active efflux also contributes to macrolide resistance in *Campylobacter* (38, 40, 138, 148, 157, 268). Studies have shown that the CmeABC efflux pump contributes to both intrinsic and acquired resistance to erythromycin in *C. jejuni* and *C. coli*. Inactivation of CmeABC significantly reduced the MICs of macrolides in both the isolates with intermediate- or low-level resistance and the isolates with high-level resistance, which indicates that CmeABC functions synergistically with target mutations to confer resistance to macrolides in *Campylobacter* (40, 53, 87, 157).
6.2.3 Tetracycline resistance

There are four mechanisms of tetracycline resistance: 1) efflux pumps that reduce drug accumulation, 2) protection of the ribosomal binding site of tetracycline by ribosomal protection proteins (RPPs), 3) drug modification, and 4) ribosomal modifications that alter the ability of the antibiotic to bind. In *C. jejuni* and *C. coli*, tetracycline resistance is mainly conferred through the ribosomal protection protein Tet(O) and the efflux pump CmeABC (268). The tet(O) gene is widespread in *Campylobacter*, and it is mainly encoded in transferable plasmids, but the gene located on chromosome has also been reported in tetracycline-resistant *Campylobacter* (56, 158, 169, 200, 238). Based on sequence homology, G+C content (40%), codon usage and Southern blot studies, it appears that *Campylobacter* acquired the tet(O) gene from Gram-positive bacteria through horizontal gene transfer (HGF) (27, 181, 237, 268). Studies have shown that Tet(O) recognizes an open A site on tetracycline-blocked ribosomal complexes and binds to the A site, which induces a conformational change in the region of the decoding site and results in the release of the bound tetracycline molecule. The conformational changes induced by the binding of the Tet(O) can persist after the release of the Tet(O), thus allowing the aa-tRNA to bind to the ribosomal A site (48, 49). Tet(O) functions synergistically with CmeABC to confer high-level resistance to tetracycline (87, 147).

6.2.4 Aminoglycoside resistance

Bacterial resistance to aminoglycosides is generally mediated by four mechanisms: 1) reduced accumulation of the drug in the intracellular environment, usually mediated by either increased efflux activity or by decreased outer membrane permeability, 2) 16S rRNA methylation that abolishes the intermolecular contacts between 16S rRNA and the drug, 3) ribosomal mutations that result in decreased affinity for the drug, and 4) enzymatic modification of the drug, one of the most important mechanisms of aminoglycoside resistance (114, 156, 268). Based on the type of reaction they catalyze, the intracellular aminoglycoside-modifying enzymes are classified into three different groups: aminoglycoside phosphotransferases, aminoglycoside adenylyltransferases, and
aminoglycoside acetyltransferases (2, 6). The modifications mediated by these enzymes result in the loss of antibacterial activity of the drug due to abolished affinity for the bacterial ribosomal aminoacyl-tRNA site (150). So far only 3’ aminoglycoside phosphotransferases and aminoglycoside adenyltransferase have been described in *Campylobacter*; 3’ aminoglycoside phosphotransferases account for the majority of aminoglycoside-modifying enzymes in *Campylobacter* spp (85, 145, 187, 268).

### 6.3 Emergence of antibiotic resistance in *Campylobacter*

As mentioned in the previous section, target mutations are the major mechanisms by which *Campylobacter* develop resistance to FQs and macrolide antibiotics. Studies have shown FQ<sup>R</sup> mutants emerge rapidly from a FQ<sup>S</sup> population when treated with FQ antimicrobials (94, 155, 160, 217, 228, 244). Since there are different types of spontaneous point mutations that may occur in the QRDR region of the gyrA gene and different point mutations confer different levels of resistance to FQ antibiotics, the frequencies of emergence of FQ<sup>R</sup> GyrA mutants range from approximately 10<sup>-6</sup> to 10<sup>-8</sup> in culture media, depending on the concentrations of ciprofloxacin used on the enumerating plates (263). The expression level of cmeABC also influences the frequencies of emergence of spontaneous FQ<sup>R</sup> mutants and the same type of gyrA mutation confers different levels of resistance to FQs in different genetic background with varying levels of CmeABC expression (264). In chickens infected with FQ<sup>S</sup> *Campylobacter*, treatment with enrofloxacin resulted in the emergence of FQ<sup>R</sup> *Campylobacter* mutants, detected in feces within 24-48 hours after the initiation of treatment, and the FQ<sup>R</sup> population continued to expand during the treatment and eventually occupied the intestinal tract at densities as high as 10<sup>7</sup> CFU/g feces (155, 160, 244). Considering that FQs use in poultry promotes the development of FQ<sup>R</sup> *Campylobacter* species in an originally FQ<sup>S</sup> population and FQ<sup>R</sup> *Campylobacter* can be transmitted to humans via the food chain, the Food and Drug Adminidtration (FDA) suspended all FQs use in poultry production in 2005 in the USA. Also FQ<sup>R</sup> *C. jejuni* could develop in *Campylobacter*-infected patients treated with ciprofloxacin (66, 217, 261). These findings suggest that *Campylobacter* is highly mutable to FQs treatment.
In contrast to FQ resistance, there are several distinct features associated with erythromycin resistance development in Campylobacter. First, the spontaneous mutation rates for macrolide resistance are very low in Campylobacter (approximately $10^{-10}$) (148). Second, the development of macrolide resistance in Campylobacter is very slow. Treatment of chickens with therapeutic doses of tylosin in drinking water for a short time did not select for erythromycin-resistant mutants (139, 148) This finding is in clear contrast with the emergence of FQR mutant Campylobacter in poultry, which occurred in enrofloxacin-treated chickens rapidly after the initiation of treatment (155). However, erythromycin resistant mutants emerged in chickens after prolonged exposure to tylosin, which suggested that development of erythromycin-resistant mutants in Campylobacter need a prolonged exposure to macrolide (38, 148). Third, the mutants with low to intermediate levels (MIC = 8 to 64 µg/ml) of resistance to erythromycin harbor mutations different from those found in high-level erythromycin-resistant mutants. Low-level erythromycin-resistant Campylobacter have mutations in the L4 and L22 proteins or no detectible mutations at all, which are not stable in the absence of macrolides. The high-level erythromycin resistant mutants (MIC ≥ 512 µg/ml) usually harbor a specific point mutation in the 23S rRNA gene, and most of these 23S rRNA mutations can be stably maintained in the absence of macrolide antibiotics (38, 129, 148).

Fourth, although macrolide resistance is generally more prevalent in C. coli isolates than in C. jejuni isolates, studies have shown there is no significant difference in the frequencies of emergence of erythromycin-resistant mutants between these two strains, suggesting that C. coli is not intrinsically more mutable then C. jejuni with regard to developing macrolide resistance (148).

6.4 Prevalence of antibiotic resistant Campylobacter

Owing to the widespread use of these antibiotics in both animals and humans, a rapid increase in the proportion of Campylobacter strains resistant to antimicrobial agents has been reported all over the world, which has become a major public health concern in recent years (6, 67, 97, 111, 169, 173, 255).
The emergence of FQ$^R$ Campylobacter strains was first noticed in the early 1990s after the approval of the use of FQs in animal production (169). In countries where FQs have been licensed for animal use, there were increased rates of isolation of FQ$^R$ Campylobacter strains in both humans and animals following the licensure (169). Many publications have shown that the use of FQs in poultry and livestock is associated with increased FQ$^R$ strains in humans (173, 205, 211, 227, 246).

In the United States, there were no reports of FQ$^R$ Campylobacter strains isolated from humans before 1992. However, several recent studies showed that about 19%-40% of Campylobacter strains isolated from humans were resistant to ciprofloxacin (97, 173). In Canada, no FQ$^R$ Campylobacter strains were isolated during 1980-1986, but recent reports indicated that ciprofloxacin resistance among C. jejuni has dramatically increased since 1992 and reached 47% in 2001 (79, 80). In Europe, there is also a clear tendency of emerging FQs resistance in Campylobacter and the rate of FQ resistance has rapidly increased in the last decade (6, 32, 77, 98, 152, 153, 169, 189, 197). In Asia, studies showed that FQ$^R$ Campylobacter strains isolated from humans increased rapidly since 1993 and the FQ resistance rates have reached more than 80% in Thailand, Taiwan, and Hong Kong (44, 111, 240). In contrast, although FQ resistance in Campylobacter isolates was also been observed in Australia and New Zealand, the rate of FQ$^R$ Campylobacter isolates in these regions was much lower than that from the other regions (6, 61, 88, 222). The lack of FQs use in veterinary medicine probably accounts for the low frequency of FQ resistance in these regions.

Since the 1990s, an increase in the prevalence of macrolide resistance among C. jejuni and C. coli has also been reported in both developed and developing countries (20, 24, 28, 67, 86, 211), but the rate of macrolide resistance among Campylobacter is highly variable among different countries (13, 67, 68, 99, 104, 112, 166, 178, 212, 218). In Spain, resistance to macrolide has been reported to be as high as 90%, but trends over time for macrolide resistance show stable and low rates in countries such as Japan, Sweden, and Finland (99,
Several studies showed that resistance to macrolides is more prevalent in *C. coli* than *C. jejuni* in both human and animal isolates (67, 188).

### 6.5 Fitness of antibiotic resistant *Campylobacter*

Several studies have shown that FQ\textsuperscript{R} *Campylobacter* mutants carrying gyrA mutations can be stably maintained in the absence of antibiotic selection pressure and FQ\textsuperscript{R} *Campylobacter* continue to persist on poultry farms even after FQs withdrawal (15, 202, 203). The persistence of FQ\textsuperscript{R} *Campylobacter* in the absence of antibiotic selection pressure may be due to the enhanced fitness of the FQ\textsuperscript{R} *Campylobacter*. Recently Luo et al. examined the persistence and biological fitness of FQ\textsuperscript{R} *Campylobacter* harboring the Thr-86-Ile mutation in GyrA in chickens in the absence of antibiotic selection pressure by using in vivo-derived, clonally-related isolates and defined isogenic mutants (154). Results from this study showed that when mono-inoculated into chickens, both FQ\textsuperscript{R} and FQ\textsuperscript{S} strains were able to colonize and persist in chickens with equal efficiency in the absence of antibiotic usage. When co-inoculated into chickens as a pairwise competition, the FQ\textsuperscript{R} *Campylobacter* outcompeted the FQ\textsuperscript{S} *Campylobacter* and quickly became the dominant population in the chicken host in the majority of pairs, which indicated that acquisition of FQ\textsuperscript{R} mutants carrying the Thr-86-Ile mutation in GyrA enhances the in vivo fitness of FQ\textsuperscript{R} *Campylobacter* instead of causing a fitness burden. And the prolonged colonization (upto 3 months) in vivo did not result in the loss of the resistance phenotype and the reversion/loss of the specific resistance associated mutation in GyrA (154, 269). Since there are no compensatory mutations in GyrA and GyrB, which are the targets of FQ antimicrobials in *Campylobacter*, the enhanced fitness likely is likely due to the effect of the C257T mutation in gyrA.

For erythromycin-resistant *Campylobacter*, there is no published information about the ecological fitness of the resistant mutants. One study in Denmark showed the reduction in tylosin usage as a growth promoter in pigs has led to a significant reduction in the occurrence of erythromycin-resistant *C. coli* isolated from pigs (3).
7. Prevention and Control

7.1 Control of Campylobacter on poultry farms

Since poultry is the major reservoir of Campylobacter and contaminated poultry meat is considered as an important source for human campylobacteriosis, reduction of Campylobacter contamination in poultry would have an important impact on human public health. It is not effective to control Campylobacter during processing because of the high prevalence of Campylobacter in poultry flocks. Therefore, control of Campylobacter infection in poultry farm should start from the preharvest stage (249). Currently, two major strategies have been used for control and prevention of Campylobacter infection at the farm level: 1) biosecurity to eliminate Campylobacter from the farm; and 2) reduction/prevention of Campylobacter colonization by competitive exclusion, phage treatment, and vaccination (57, 151, 175, 250, 251).

7.1.1 Biosecurity

Since external environmental contamination during the life of the flocks is likely to be the primary source of Campylobacter infection, strict hygiene and biosecurity measures have been used in an attempt to control Campylobacter infections in chicken flocks (12, 82, 245). Empirical biosecurity measures to prevent Campylobacter colonization of poultry flocks include use of an all-in all-out policy, cleaning and disinfection of buildings between flocks, maintaining buildings in good condition, controlling and keeping buildings free of vermin and wild birds, restricting other domestic animals on the farm, proper disposal of dead birds, along with change and disinfection of boots and outer protective clothing for workers, restriction on staff and equipment entering broiler house, and chlorination of drinking water (175). However, intensifying biosecurity can only reduce the colonization level or delay the onset time of colonization of birds by Campylobacter, but can not eliminate the introduction of Campylobacter into broiler flocks (175, 220, 249).

Because Campylobacter are commonly present in the poultry farm environment and
poultry flocks can be infected by multiple sources, it is unlikely that flock exposure to 
*Campylobacter* can be prevented by use of strict biosecurity measures alone (175, 176). In 
addition, stringent biosecurity measures are expensive and difficult to maintain (151). 
Therefore, other supportive measures, such as competitive exclusion (CE), phage treatment, 
antibacterial agents and vaccination, have also been explored.

### 7.1.2 Competitive exclusion (CE)

With CE, defined or undefined gut flora or probiotic anaerobic bacteria is used to 
prevent target bacteria colonization. It is thought that CE works through the generation of 
bactericidal metabolites, such as organic acids, or though nutritional competition. Although 
CE has been developed as a successful approach for the control of organisms like *Salmonella*, 
its efficacy for *Campylobacter* is variable and unpredictable (163, 177, 215, 216, 249). Some 
studies showed that CE could help reduce the colonization level and prevalence of 
*Campylobacter* in broilers, but in other experiments, these CE materials had no effect (5, 164, 
177, 215, 216, 249). Since CE materials are derived from live adult birds, it is not surprising 
that they are inconsistent and are influenced by the status of the donor birds and the site from 
which the materials are collected (140, 164, 175, 215, 216). Recently, studies showed that 
when given as feed supplements, bacteriocins extracted from *Bacillus circulans*, 
*Paneibacillus pylomyxa* and *Lactobacillus salivarius* were highly effective in eliminating 
*Campylobacter* colonization from broilers and young commercial turkeys (46, 232, 233). 
Several probiotics from *Saccharomyces boulardii*, *Lactobacillus acidophilus* and 
*Streprococcus faecium* have been found to be able to partially reduce *Campylobacter* 
colonization in broiler chickens under laboratory conditions (43, 149, 171). Even though 
these CE materials seem to work under experimental conditions, their abilities to control and 
prevent *Campylobacter* colonization in chickens under production conditions remain to be 
investigated. Currently none of the commercially available CE materials appear to be 
effective in excluding *Campylobacter* from broiler flocks under production conditions (162).

### 7.1.3 Vaccination
Since *Campylobacter*-specific antibodies can effectively reduce *Campylobacter* colonization of chickens (57, 243), vaccination seems to be a feasible approach for control and prevention of *Campylobacter*. Several vaccine development strategies are currently being investigated. These reported immunization studies have used killed whole-cell vaccines, flagellin-based vaccines, or genetically engineered live vectors expressing *Campylobacter*-specific antigens. The protective effect of these vaccines against *Campylobacter* was variable. In general, the studies suggest that the *Campylobacter*-specific antibodies generated provide some, but not biologically significant protective effects in chickens (39, 57, 128, 179, 207, 249, 250, 257, 258, 271). However, a recent study reported that oral immunization of chickens with an avirulent *Salmonella* strain expressing the *C. jejuni* CjaA antigen (the substrate-binding component of an ABC transport system) drastically reduced the colonization of *Campylobacter* in the majority of birds upon homologous challenge (262). The encouraging results from this study suggest the possibility of using CjaA protein in combination with the attenuated *Salmonella* vector as a protective vaccine against *Campylobacter* colonization in poultry. Passive immunization by oral administration of anti-*Campylobacter* antibodies has also been reported to have both therapeutic and prophylactic properties in chickens (231, 243, 249). Therefore, it may be possible to immunize parent flocks to produce passively protected offspring. Overall, the limited results from the experimental vaccines suggest that vaccination may be a feasible strategy to control *Campylobacter* infection on poultry farms.

### 7.1.4 Phage therapy

Bacteriophages are viruses that can infect, multiply, and kill susceptible bacteria. Like most bacterial species, *Campylobacters* also have their own specific bacteriophages. *Campylobacter*-specific bacteriophages can be readily isolated from broiler chickens and the farm environment (19, 50, 51, 65, 151, 251). Bacteriophages have shown to be a promising approach to reduce *Campylobacter* colonization in broilers, but currently no commercial product is available. Recent studies showed that bacteriophages significantly reduce the *Campylobacter* number in both therapeutic and preventive treatment of chickens with *Campylobacter*-specific bacteriophage. However, the level of reduction was variable and was
affected by many factors, such as bacteriophage type, dosage, bacteriophage dynamics and bacteriophage ecology in chickens. More work will be needed to determine if phage therapy is an effective and/or practical biological control measure for the reduction of *Campylobacter* in poultry.

### 7.1.5 Other strategies

Other intervention strategies that may be used on poultry farm to control and prevent *Campylobacter* colonization in broiler flocks include use of inbred chicken lines, which are resistant to cecal colonization by *Campylobacter* (35, 230) or supplementation of feed with prebiotics (e.g. fructooligosaccharide, lactose, mannose-oligosaccharide), immune response stimulators (e.g. selenium, beta-glucan), antimicrobials (e.g. salinomycin, flavophopholipol), or active charcoal (14, 34, 62). Similar to the strategies mentioned previously, these strategies have shown limited success in reducing the incidence of *Campylobacter* colonization in chickens.

### 7.2 Control of *Campylobacter* at the slaughterhouse during processing

Multiple measures in the slaughterhouse have been explored to reduce carcass contamination by *Campylobacter* during slaughter. Currently, the most promising control strategy is the separation of *Campylobacter*-positive and *Campylobacter*-negative flocks during slaughter (“scheduled processing”) and decontamination of the meat from positive flocks (250). However, one issue of the scheduled processing is the sensitivity of the on-farm test. False-negative tests would result in misidentification of positive flocks which would be treated as safe meat. The feasibility of scheduled processing remains an issue to the poultry industry (249).

In addition being used to reduce *Campylobacter* colonization in live poultry, bacteriophages have also been used as a disinfection agent for poultry meat in processing facilities. Several studies investigating the direct application of bacteriophages onto raw chicken skin showed a significant decrease in *Campylobacter* numbers on the surface of
experimentally-contaminated chicken skin (18, 89). However, these studies only involved experimentally-infected chickens and only showed efficacy in the laboratory. Additional studies are needed to assess the possibility of using bacteriophage to control natural contaminants and improve food safety in a real-life setting (93).

8. Reference


CHAPTER 3. KEY ROLE OF MFD IN THE DEVELOPMENT OF FLUOROQUINOLONONE RESISTANCE IN CAMPYLOBACTER JEJUNI

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1. Abstract

*Campylobacter jejuni* is a major foodborne pathogen and a common causative agent of human enterocolitis. Fluoroquinolones are a key class of antibiotics prescribed for clinical treatment of enteric infections including campylobacteriosis, but fluoroquinolone-resistant *Campylobacter* readily emerges under the antibiotic selection pressure. To understand the mechanisms involved in the development of fluoroquinolone-resistant *Campylobacter*, we compared the gene expression profiles of *C. jejuni* in the presence and absence of ciprofloxacin using DNA microarray. Our analysis revealed that multiple genes showed significant changes in expression in the presence of a suprainhibitory concentration of ciprofloxacin. Most importantly, ciprofloxacin induced the expression of *mfd*, which encodes a transcription-repair coupling factor involved in strand-specific DNA repair. Mutation of the *mfd* gene resulted in approximately 100-fold reduction in the rate of spontaneous mutation to ciprofloxacin resistance, while overexpression of *mfd* elevated the mutation frequency. In addition, loss of *mfd* in *C. jejuni* significantly reduced the development of fluoroquinolone-resistant *Campylobacter* in culture media or chickens treated with fluoroquinolones. These findings indicate that Mfd is important for the development of fluoroquinolone resistance in *Campylobacter*, reveal a previously unrecognized function of Mfd in promoting mutation frequencies, and identify a potential molecular target for reducing the emergence of fluoroquinolone–resistant *Campylobacter*. 
2. Author Summary

As a foodborne bacterial pathogen, Campylobacter jejuni is a common causative agent of gastrointestinal illnesses in humans. Development of antibiotic resistance in Campylobacter, especially to fluoroquinolone (a broad-spectrum antimicrobial), compromises clinical treatments and presents a major public health threat. It is poorly understood why Campylobacter is highly adaptable to fluoroquinolone treatment and how it acquires mutations associated with fluoroquinolone resistance. Understanding the molecular mechanisms involved in the resistance development will help us to reduce the emergence of FQ-resistant Campylobacter. Using DNA microarray and other molecular methods as well as animal studies, we uncovered the key role of Mfd in promoting spontaneous mutations and development of fluoroquinolone resistance in Campylobacter. Mfd is a transcription-repair coupling factor involved in DNA repair and was not previously known for its role in promoting mutations conferring antibiotic resistance. Our findings not only reveal a novel function of Mfd, but also provide a potential molecular target for reducing the emergence of fluoroquinolone-resistant Campylobacter.

3. Introduction

Campylobacter jejuni, a Gram-negative microaerobic bacterium, is one of the most prevalent bacterial foodborne pathogens in humans, causing more than 2 million cases of diarrhea each year in the U.S. alone [1, 2, 3]. As an enteric pathogen, this organism causes watery diarrhea and/or hemorrhagic colitis. Campylobacter infection is also the most common antecedent to Guillain-Barre syndrome, an acute flaccid paralysis that may lead to respiratory muscle compromise and death [4, 5]. In developed countries, person-to-person transmission of Campylobacter is rare, and the main source of human Campylobacter infections is via food, water, or milk contaminated by Campylobacter [6].

Fluoroquinolone (FQ) antimicrobials are often prescribed for clinical treatment of diarrhea caused by enteric bacterial pathogens including Campylobacter [7, 8]. However,
Campylobacter is increasingly resistant to FQ antimicrobials, which has become a major concern for public health [9, 10, 11]. FQ-resistant (FQ\textsuperscript{R}) Campylobacter developed in food producing animals can be transmitted to humans via the food chain. Poultry are considered the major reservoir for C. jejuni and a significant source for FQ\textsuperscript{R} Campylobacter infections in humans, because the majority of domestically acquired cases of human campylobacteriosis result from consumption of undercooked chicken or food contaminated by raw chicken [2, 12, 13]. Although FQ antimicrobials have been banned since 2005 in poultry production in the U.S., FQ\textsuperscript{R} Campylobacter continue to persist on poultry farms [14, 15, 16].

The main targets of FQs in bacteria are DNA gyrases and/or topoisomerase IV [17, 18]. In Campylobacter, the resistance to FQ antimicrobials is mediated by point mutation in the quinolone resistance-determining region (QRDR) of gyr\textit{A} in conjunction with the function of the multidrug efflux pump CmeABC [10, 19, 20, 21]. Acquisition of high-level FQ resistance in Campylobacter does not require stepwise accumulation of point mutations in gyr\textit{A}. Instead, a single point mutation in gyr\textit{A} can lead to clinically relevant levels of resistance to FQ antimicrobials [19, 20, 22]. Specific mutations at positions Thr-86, Asp-90 and Ala-70 in Gyr\textit{A} have been linked to FQ resistance in C. jejuni [10, 19]. When enumerated by ciprofloxacin (CIPRO)-containing plates, spontaneous FQ\textsuperscript{R} Campylobacter mutants occur at a frequency as high as 10\textsuperscript{-6} [23], suggesting that C. jejuni possess a high mutation rate to FQ resistance. CmeABC, an energy-dependent efflux system, contributes significantly to the intrinsic and acquired resistance to FQs in C. jejuni by reducing the accumulation of the antibiotics within Campylobacter cells [19, 20, 24, 25]. The expression level of cmeABC also influences the frequencies of emergence of spontaneous FQ\textsuperscript{R} mutants [23].

One unique feature of FQ resistance development in Campylobacter is the rapid emergence of FQ\textsuperscript{R} mutants from a FQ-susceptible (FQ\textsuperscript{S}) population when treated with FQ antimicrobials. This has been observed in Campylobacter-infected animals or patients treated with FQs [19, 26, 27, 28, 29, 30]. In chickens infected with FQ\textsuperscript{S} Campylobacter, treatment with enrofloxacin resulted in the emergence of FQ\textsuperscript{R} Campylobacter mutants that were
detected in feces within 24-48 hours after the initiation of treatment, and the FQ\(^R\) population continued to expand during the treatment and eventually occupied the intestinal tract at a density as high as \(10^7\) CFU/g feces [19, 29, 30]. As shown in a comparison study, the same FQ treatment did not result in the development and enrichment of FQ\(^R\) *E. coli* in chickens [29], suggesting that *C. jejuni* has a unique ability to adapt to FQ treatment. This high frequency of emergence of FQ\(^R\) *Campylobacter* mutants in response to the selection pressure may have directly contributed to the global prevalence of FQ\(^R\) *Campylobacter*. For example, multiple studies have shown the temporal link between the approval of FQ antimicrobials for use in animal production and the rapid increase of FQ\(^R\) *Campylobacter* isolates from both animals and humans [9, 31, 32, 33, 34, 35, 36, 37, 38, 39]. In some regions of the world, the vast majority of *Campylobacter* isolates have become resistant to FQ antimicrobials [22, 40, 41].

The rapidness and magnitude of FQ resistance development in *Campylobacter* in response to FQ treatment suggest that both selective enrichment of pre-existing spontaneous mutants and adaptive gene expression may contribute to the emergence of FQ\(^R\) *Campylobacter*, but how *Campylobacter* responds to FQ treatment is unknown. Within bacterial cells, FQ antimicrobials form a stable complex with gyrase and DNA, which generates double-stranded breaks in DNA and leads to bacterial death [18]. In other bacteria, antibiotic treatments (including FQs) induce the SOS response, which upregulates multiple genes involved in DNA repair, recombination, and mutation as well as other functions [42,43,44,45]. The SOS response is controlled by LexA, a transcriptional repressor. DNA damage triggers LexA autocleavage, which derepresses the SOS genes controlled by LexA. Once activated, SOS response promotes the development of drug resistance, horizontal transfer of genetic materials, and production of virulence factors [45, 46, 47]. Unlike many other bacterial organisms, *epsilonproteobacteria* including *Campylobacter* and *Helicobacter* do not have a LexA ortholog [46] and also lack many genes involved in DNA repair, recombination, and mutagenesis, such as the *mutHL* genes (methyl-directed mismatch repair), the *umuCD* genes (UV-induced mutagenesis), and SOS-controlled error-prone DNA polymerases [48, 49, 50]. These observations suggest that *Campylobacter* may not have the
typical SOS response system. In light of this possibility, it is intriguing to determine how Campylobacter copes with FQ treatment and what facilitates the emergence of FQ\textsuperscript{R} mutants in Campylobacter.

In this study, we examined the gene expression profiles of \textit{C. jejuni} NCTC 11168 in response to treatment with CIPRO. Consistent with the prediction, a typical SOS response was not observed in \textit{Campylobacter} treated with CIPRO. However, 45 genes showed ≥1.5-fold (p < 0.05) changes in expression when \textit{Campylobacter} was exposed to a suprainhibitory dose of CIPRO for 30 min. One of the up-regulated genes was \textit{mfd} (mutation frequency decline), which encodes a transcription-repair coupling factor involved in DNA repair. The \textit{mfd} gene in \textit{E. coli} was originally linked to the phenotype of mutation frequency decline [51, 52]. Subsequently it was found that Mfd functions as a transcription-repair coupling factor and promotes strand-specific DNA repair [53, 54]. DNA lesions stall RNA polymerase during transcription. Mfd displaces the stalled RNA polymerase from the DNA lesions in an ATP-dependent manner, recruits the UvrABC excinuclease complex, and enhances the repair of the DNA lesions on the transcribed strand [54, 55]. Thus, Mfd couples transcription with DNA repair and contributes to mutation frequency decline. Recently it was reported that depending on the nature of DNA damage and the availability of NTPs, Mfd can also promote the forward translocation of arrested RNA polymerase in the absence of repair, leading to transcriptional bypass of non-repaired lesions [55]. In contrast to its previously known function in the decline of mutation frequency in other bacterial organisms [51, 52], Mfd in \textit{Campylobacter} was found to promote the emergence of spontaneous FQ\textsuperscript{R} mutants and the development of FQ\textsuperscript{R} mutants under FQ treatments in this study. These findings define a novel function of Mfd and significantly improve our understanding of the molecular mechanisms underlying the development of FQ\textsuperscript{R} \textit{Campylobacter}.

4. Results

\textit{Transcriptional analysis of \textit{C. jejuni} response to FQ treatment}

To understand the adaptive response of \textit{Campylobacter} to FQ treatment, DNA microarray was used to analyze the transcriptional changes in \textit{C. jejuni} NCTC 11168 after
exposure to CIPRO. When the Campylobacter cells were treated with a subinhibitory concentration (0.06 µg/ml; 0.5 x the MIC) of CIPRO for 1.5 hours, no genes showed ≥1.5-fold changes in expression, suggesting that the transcriptional response to the low dose of CIPRO was very limited. When the Campylobacter cells were treated with a suprainhibitory concentration (1.25 µg/ml; 10 x the MIC) of CIPRO for 30 min, 45 genes showed ≥1.5-fold (p < 0.05) changes in expression (Table 1), among which 13 were up-regulated and 32 were down-regulated. The up-regulated genes are involved in cell membrane biosynthesis, cellular processes, and transcription-coupled DNA repair or have unknown functions, while the majority of the down-regulated genes are involved in energy metabolisms (Table 1). Consistent with the lack of LexA, the core genes involved in SOS responses in other bacteria, such as recA, uvrA, ruvC, ruvA, and ruvB, did not show significant changes in expression. The expression of other genes involved in DNA repair and recombination also did not change significantly. These findings indicate that C. jejuni does not mount a typical SOS response or upregulate the general DNA repair system in the early response to CIPRO treatment. Notably, cj1085c, a homolog of mfd, was upregulated in the presence of CIPRO. Two up-regulated genes, uppP and uppS, encode products involved in cell wall production [56,57], while pldA encodes an outer membrane phospholipase that has been implicated in hemolysis, capsular production, and virulence [58, 59]. According to the Q values, the identified genes would have an estimated false discovery rate (FDR) of 20%. However, quantitative real-time RT-PCR (qRT-PCR) confirmed all of the 11 genes selected from the microarray list (Table 1), suggesting that the actual FDR is lower than the estimation.

**Characteristics of Mfd**

Cj1085c (978aa) was annotated as Mfd [48] and shows 31.5% amino acid identity to the E. coli Mfd protein (1148 aa). In addition, it contains the characteristic domains conserved in Mfd proteins, such as the ATP/GTP-binding site motif and the superfamily II helicase motif. Mfd in other bacteria has been shown to be involved in strand-specific DNA repair by displacing lesion-stalled RNA polymerase and recruiting enzymes involved in recombination events [54,60]. The mfd locus is highly conserved in Campylobacter and is present in all Campylobacter species and C. jejuni strains that have been sequenced to date.
The Mfd proteins in different *Campylobacter* species share 57-79% identity to the Mfd in *C. jejuni* NCTC 11168. Within *C. jejuni*, the Mfd proteins are 98-100% homologous among different strains. The *mfd* gene is located in the middle of a gene cluster, whose transcription is in the same direction (partially shown in Fig. 1A). The downstream gene *Cj1084c* encodes a putative ATP/GTP binding protein, while the upstream gene *Cj1086c* encode a hypothetical protein [48]. It is unknown if *mfd* and its flanking genes form an operon, but it appeared that *Cj1086c* and *mfd* were co-transcribed because a RT-PCR product spanning both ORFs was amplified (data not shown).

**Expression levels of mfd influence the frequency of emergence of spontaneous FQ-resistant mutants.**

Since *mfd* was the only DNA repair related gene that showed a significant change in expression in the early response of *C. jejuni* to CIPRO treatment (Table 1), we examined its role in the emergence of spontaneous FQ\(^R\) mutants in *Campylobacter*. Firstly, the *mfd* gene was inactivated by insertional mutagenesis (Fig. 1B). As shown in Fig. 2, the *mfd* mutant (JH01) showed a approximately 100-fold reduction in the frequencies of emergence of spontaneous FQ\(^R\) mutants detected using plates containing three different concentrations (1, 2, and 4 \(\mu\)g/ml, respectively) of CIPRO. Complementation of the *mfd* mutant *in trans* by a plasmid-carried *mfd* restored the frequencies of mutant emergence to the wild-type level (JH02 in Fig. 2). As determined by qRT-PCR, the expression level of *mfd* in the complemented construct (JH02) was fully restored (1.7 x the wild-type level). pRY112 alone (without the cloned *mfd* gene) did not complement the *mfd* mutant in the mutation frequency (data not shown). These results indicate that Mfd contributes significantly to the rate of spontaneous mutations to FQ resistance.

Secondly, we determined if the enhanced expression of *mfd* increases the mutation frequency. For this purpose, we constructed strain JH03, which was a wild-type 11168 strain containing an extra copy of *mfd* carried on a shuttle plasmid. In JH03, the mRNA of *mfd* increased 3.8 times compared with that in 11168 as determined by qRT-PCR. When compared with the wild-type 11168, the frequency of emergence of FQ\(^R\) mutants from JH03
increased about 10-fold (Fig. 2). The increase was reproducible in multiple experiments and was statistically significant (P < 0.05). These results indicated that overexpression of mfd increases the frequency of emergence of spontaneous FQR mutants.

Given that there is only one nucleotide between the mfd gene and its downstream gene cj1084c, it was prudent to determine if the mfd mutation resulted in a polar effect on the expression of cj1084c. RT–PCR showed that cj1084c was transcribed at a comparable level in both the mfd mutant and the wild-type NCTC 11168 (Fig. 1C). RT-PCR was also performed using 10-fold serial dilutions of the RNA template, which yielded comparable results between the two strains (data not shown). PCR without the reverse transcriptase did not yield a product (Fig. 1C), indicating that the mRNA templates had no DNA contamination. These results suggested that the insertional mutation in the mfd gene did not cause an apparent polar effect on expression of the downstream gene. This finding plus the complementation data (Fig. 2) strongly indicate that loss of Mfd is responsible for the observed reduction in the mutation frequency in JH01.

**Loss of mfd does not affect the susceptibility of C. jejuni to antibiotics.**

To examine if the reduction in the emergence of spontaneous FQR mutants is caused by the increased susceptibility of the mfd mutant to CIPRO, we compared the MICs of several antibiotics in the mfd mutant with those in the wild type. Our results did not reveal any differences between the mutant and the wild type in their susceptibility to the tested antibiotics including erythromycin, ampicillin, streptomycin, and CIPRO (data not shown). In addition, there was no apparent difference in growth kinetics between the wild-type and the mfd mutant either in MH broth (without antibiotics) or in MH broth supplemented with a subinhibitory concentration (0.06 µg/ml) of CIPRO (Fig. 3). The growth rates of the mfd over-expressing strain (JH03) and the complemented mutant (JH02) were also similar to that of the wild type (Fig. 3). Thus, the reduced spontaneous mutation rate to FQ resistance in the mfd mutant was not attributable to decreased growth rate or increased susceptibility to antibiotics. In addition, the CIPRO-resistant colonies examined for gyrA mutations all carried
the C257T mutation in gyrA and had a CIPRO MIC of > 32 µg/ml regardless of the backgrounds (11168 or JH01) from which the mutants were selected.

**Mfd contributes to the emergence of FQR Campylobacter under in vitro treatment**

FQR Campylobacter mutants emerge rapidly from a FQ-susceptible population once treated with FQ antimicrobials [19,26,27,28,29,30]. To determine if Mfd influences the development of FQR Campylobacter under selection pressure, we conducted in vitro growth experiments, in which C. jejuni was treated with a suprainhibitory concentrations of CIPRO (4 µg/ml). In the first treatment experiment, 10⁹ CFU of bacterial cells were inoculated into each flask containing 100 ml MH broth with 4 µg/ml of CIPRO, yielding an initial cell density of 10⁷ CFU/ml. At the beginning of the treatment, 1-3 CFU/ml of FQR mutants were detected in the flasks inoculated with 11168, while no FQR mutants were detected in the cultures inoculated with JH01 (Fig. 4A). One day after the initiation of the treatment, the numbers of FQR mutants in the 11168 cultures grew to a level ranging from a few hundreds to a few thousands CFU/ml, while no mutants or about 1 CFU/ml of FQR mutants were detected in the cultures of JH01 (Fig. 4A). The FQR populations expanded on day 2 in both strains, but the FQR population of JH01 was still about 1,000-fold less than that of 11168. Due to the continued enrichment of the FQR mutants by CIPRO and the fact that the mutants of 11168 was entering the stationary phase, the average difference between 11168 and JH01 on day 3 decreased, but was still more than one order of magnitude (Fig. 4A). In the second experiment, 2 x 10⁷ CFU bacterial cells of 11168 or JH01 were inoculated into each flask containing 20 ml of MH broth with 4 µg/ml of CIPRO, yielding an initial cell density of 10⁶ CFU/ml. At the beginning of the treatment, no FQR mutants were detected from either 11168 or JH01 (Fig. 4B). On day 1 after the initiation of the treatment, FQR Campylobacter emerged from some of the cultures of 11168 and continued to expand in numbers on day 2 and day 3. In contrary to 11168, no FQR mutants emerged from any of the JH01 cultures during the three-day incubation (Fig. 4B). In the third experiment, the inoculum was decreased to 2 x 10⁴ CFU per flask (initial cell density = 10³ CFU/ml), and no FQR mutants were detected from either 11168 or JH01 after three day’s incubation (data not shown).
These results indicated that emergence of FQR mutants under treatment with CIPRO was influenced by the initial bacterial cell density and facilitated by the function of Mfd.

*Mfd affects the emergence of FQR mutants in vivo*

To determine if Mfd influences the emergence of FQR *Campylobacter* during *in vivo* therapeutic treatment, broiler chickens were infected with 11168 or JH01 and then treated with enrofloxacin administered in drinking water (50 ppm). The birds in both groups were quickly colonized by *C. jejuni* after inoculation (Fig. 5). Before the treatment with enrofloxacin, all birds were colonized by *Campylobacter* and the colonization levels (CFU/g feces) were similar in both groups (*p* > 0.05). One day after initiation of the treatment, the number of colonized chickens and the levels of colonization decreased drastically in both groups, with *Campylobacter* detectable only in three chickens that were inoculated with the wild-type strain (Fig. 5A). After that, the numbers of *Campylobacter* in both groups rebounded. On day 3 after the initiation of the treatment, all of the birds in the 11168 group were re-colonized by *Campylobacter* and remained colonized until the end of the experiment. For the group inoculated with JH01, 6 of the 11 birds became positive with *Campylobacter* on day 3 after initiation of the treatment (Fig. 5A) and 3 birds remained negative until the end of the experiment. On days 3, 5 and 7 after initiation of the treatment, the average colonization level of the JH01-inoculated group was approximately 3 log units lower than that of the 11168-inoculated group (Fig. 5A) and the differences were statistically significant (*p* < 0.05). The number of FQR *Campylobacter* in each chicken was also monitored. Prior to the treatment, no FQR *C. jejuni* was detected in any of the chickens (Fig. 5B). On day 1 after initiation of the treatment, the three *Campylobacter*-positive birds of the 11168-inoculated group still carried FQ-susceptible *Campylobacter*. However, FQR *C. jejuni* appeared on day 3 in all birds of the 11168-inoculated group and in some birds of the JH01-inoculated group (Fig. 5B). Comparison of the total *Campylobacter* counts (Fig. 5A) with the numbers of FQR *Campylobacter* (Fig. 5B) revealed that the birds were re-colonized by FQR mutants after initiation of the treatment. The average numbers of FQR *Campylobacter* in the JH01-inoculated group were approximately 3 log units lower than those of the 11168-inoculated group (Fig. 5B) and the differences were statistically significant (*p* < 0.05). These results
indicate that loss of Mfd significantly reduced the rates of emergence of FQ\textsuperscript{R} \textit{Campylobacter} in enrofloxacin-treated chickens.

Representative \textit{Campylobacter} isolates obtained at different sampling times from both groups were tested for CIPRO MICs using E-test strips. The result showed that before treatment all the tested isolates from both groups were susceptible to CIPRO (MICs = 0.094 - 0.125 µg/ml). The majority of the tested isolates from day 1 after initiation of the treatment were still susceptible to CIPRO (MICs = 0.094 - 0.5 µg/ml). On day 3 after the initiation of treatment, 21 of the 22 tested isolates (from both groups) had a CIPRO MIC of > 32 µg/ml and the other one had an MIC of 8 µg/ml. Similarly, the majority (44 out of 49) of the tested isolates from days 5 and 7 had a CIPRO MIC of >32 µg/ml and the rest had MICs from 1-24 µg/ml. The MIC results further confirmed the differential plating results that the chickens were re-colonized by FQ\textsuperscript{R} \textit{Campylobacter}.

5. Discussion

When \textit{Campylobacter} cells were treated with a subinhibitory concentration (0.06 µg/ml, 0.5 x the MIC) of CIPRO for 1.5 hours, no significant changes in gene expression were detected using the cut-off criteria defined in this study. This result was somewhat similar to the study with \textit{Haemophilus influenzae} [61] in that the treatment with a low concentration of CIPRO induced few changes in gene expression, but was different from that study because several genes involved in SOS response were upregulated in \textit{Haemophilus influenzae}. Prolonged treatment of \textit{Campylobacter} with the subinhibitory concentration of CIPRO may reveal noticeable changes in gene expression, but culturing \textit{Campylobacter} with 0.06 µg/ml of CIPRO reduces its growth rate (Fig. 3), which will make the comparison with the non-treated control unfeasible and complicate the interpretation of the microarray results. To mimic clinical treatment, \textit{C. jejuni} cells were exposed to a suprainhibitory dose (1.25µg/ml, 10 x the MIC) of CIPRO. This dose is within the concentration range of CIPRO in gut contents during FQ treatment in chickens [62]. The reason that we treated the samples for 0.5 hour instead of a longer time was to detect the primary response triggered by CIPRO, instead of the secondary response caused by cell death. When \textit{Campylobacter} cells were
treated with this suprainhibitory dose for 0.5 hour, the expression of multiple genes was significantly altered (Table 1). Notably, the majority of the affected genes were downregulated and many of them are involved in cellular processes and energy metabolism (Table 1). This result is similar to the findings obtained with other bacteria [43, 44, 61] and supports the notion that reducing cellular metabolism is a common strategy utilized by bacteria to cope with antibiotic treatment.

Within bacterial cells CIPRO interacts with gyrase and DNA, blocking DNA replication and transcription [18]. When exposed to CIPRO, the expression of gyrA and gyrB in various bacteria was either altered or unchanged [43, 61, 63]. In this study, we found that the expression of gyrA, gyrB, and topA was not significantly affected in Campylobacter by CIPRO. In addition, the expression of the genes encoding enzymes involved in DNA repair, recombination, or mutagenesis, such as recA, ruvABC, uvrABC, and mutS, did not change significantly. Only two genes involved in DNA metabolism (mfd and dnaE) were affected by CIPRO under the conditions used in this study (Table 1). These observations indicate that C. jejuni does not mount a typical SOS response under the treatment with FQ. These findings are also consistent with the fact that C. jejuni lacks LexA, the key regulator of bacterial SOS responses [46].

In addition to transcription-coupled DNA repair, Mfd has been associated with other functions in bacteria [64]. For example, Mfd of Bacillus subtilis is involved in homologous DNA recombination and stationary-phase mutagenesis [65, 66]. Inactivation of the mfd gene of B. subtilis resulted in a great reduction in the number of prototrophic revertants to Met+, His+, and Leu+ during starvation [66], indicating that Mfd promotes adaptive mutagenesis. This finding is in contrast to the known function of Mfd in mediating mutation frequency decline and could be explained by the role of Mfd in promoting transcriptional bypass and consequently increasing the adaptive mutagenesis rates [66].

In this study we found that Mfd increases the frequency of emergence of spontaneous FQR mutants in Campylobacter (Fig. 2). Furthermore, the mfd mutation also decreased the
frequency of emergence of spontaneous streptomycin-resistant mutants in \textit{Campylobacter} (data not shown). Together, the results convincingly showed that Mfd is an important player in modulating the mutation rates in \textit{Campylobacter}. To our knowledge, this is the first report documenting the key role of Mfd in promoting spontaneous mutation rates in a bacterial organism. How Mfd contributes to the increased mutation rates in \textit{Campylobacter} is unknown, but it can be speculated that transcriptional bypass mediated by Mfd may actively occur in replicating non-stressed \textit{Campylobacter} populations, resulting in an elevated level of retromutagenesis (fixed changes in DNA sequence due to transcriptional mutation [67]) that contributes to the size of the mutant pools. This possibility remains to be examined in future studies. Although \textit{mfd} contributes significantly to the mutation rate (Fig. 2), its expression level was not precisely proportional to the mutation frequencies. For example, expression of \textit{mfd} was upregulated 3.8-fold in JH03, but its mutation frequency increased 10-fold. This difference is probably due to the fact that emergence of spontaneous mutants is a multi-step process and Mfd only contributes to one of the steps in the process. It is also possible that Mfd interacts with other proteins in modulating the mutation frequency. Thus, the changes in \textit{mfd} expression level and the mutation frequency are not exactly at the same scale.

Another interesting observation of this study is the upregulation of \textit{mfd} by CIPRO. The enhanced expression may be needed for transcription repair because CIPRO treatment causes DNA damage, which stalls RNA polymerase. Alternatively, the increased production of Mfd may enhance transcriptional bypass of the non-repaired DNA lesions in order to maintain cell viability and/or promote mutations for resistance. This possibility is high given the facts that massive DNA damage incurred by a suprainhibitory dose of CIPRO may overwhelm the DNA repair system and \textit{Campylobacter} must maintain certain levels of transcription to survive the treatment, that Mfd contributes significantly to the mutation rates to FQ resistance (Fig. 2), and that \textit{Campylobacter} does not have the error-prone DNA polymerases, such as Pol II, Pol IV, and Pol V [48]. \textit{E. coli} and other bacteria have these error-prone DNA polymerases [68, 69], which are repressed by LexA, but upregulated by the SOS response triggered by DNA damage. Once produced, the enzymes perform translesion DNA synthesis, allowing replication to continue without DNA repair. This special functional
feature results in reduced genetic fidelity, but allows for bacterial survival under stress. The outcome of the enhanced expression of the error-prone enzymes is the increased mutation rates, which contribute to the emergence of drug resistance [70]. In the absence of a SOS response and the error-prone DNA polymerases, *Campylobacter* may use Mfd as an alternative pathway to increase mutation rates. Thus, enhanced expression of mfd may represent an adaptive response of *Campylobacter* to the stresses imposed by CIPRO treatment. How CIPRO upregulates *Campylobacter* Mfd is unknown and further work in this direction is warranted.

FQ\textsuperscript{R} *Campylobacter* readily emerges from a FQ-susceptible population when treated with FQ antimicrobials (Figs. 4 and 5). As shown in the *in vitro* experiment, the development of FQ\textsuperscript{R} population under CIPRO treatment is influenced by the initial cell density (Fig. 4 and the corresponding text) as well as the functional state of Mfd. Considering the differences in spontaneous mutation rate between 11168 and JH01 (Fig. 2), it was likely that the 11168 and JH01 inocula had different numbers of pre-existing FQ\textsuperscript{R} mutants, which were selected by CIPRO and contributed to the differences in the FQ\textsuperscript{R} population detected in the cultures of the two strains. The inoculum-dependent emergence of FQ\textsuperscript{R} mutants in both 11168 and JH01 suggests that development of FQ\textsuperscript{R} *Campylobacter* under FQ treatment involves selection of preexisting mutants. However, the magnitude and dynamics of FQ\textsuperscript{R} development can not be totally explained by selection. For example, in some cultures FQ\textsuperscript{R} mutants were not detectible until the 2\textsuperscript{nd} day of the incubation (Fig. 4). A single mutant at time zero in a culture flask would grow to a population of more than 2,000 cells in one day (the generation time of *C. jejuni* in MH broth is about 2 hours), which would be readily detected by the plating method on day 1. Thus, if selection was the only factor in the development of FQ\textsuperscript{R} *Campylobacter*, the latest time for detecting the pre-existing mutants in the mutant-positive flasks would be day 1 after initiation of the treatment. Obviously, this was not the case for all of the cultures because some of them did not show FQ\textsuperscript{R} mutants until day 2 (Fig. 4). In addition, some cultures were negative with FQ\textsuperscript{R} mutants at time zero, but showed a large number of mutants at day 1, which could not be easily explained by sole selection of a few preexisting mutants from the inocula. Considering these unexplainable observations and the
fact that a small fraction of the FQ-susceptible inoculum survived the killing effect as long as one day after the initiation of the treatment (data not shown), it was possible that new FQ\textsuperscript{R} mutants were developed during the treatment. If this occurs, Mfd may enhance the emergence of new mutants by promoting transcriptional bypass or other mechanisms, which may partly explain the differences between 11168 and JH01 in the dynamics of emergence of FQ\textsuperscript{R} mutants. Thus, there is a possibility that both selection of pre-existing mutants and de nova formation of mutants are involved in the development of FQ\textsuperscript{R} *Campylobacter* during treatment with FQ antimicrobials.

The role of Mfd in the development of FQ\textsuperscript{R} mutants was further shown by the *in vivo* experiment, in which *Campylobacter*-infected chickens were treated with enrofloxacin (Fig. 5). Previous studies have shown that therapeutic use of FQ antimicrobials in chickens promotes the emergence of FQ\textsuperscript{R} *Campylobacter* \cite{19, 27, 28, 29, 30}, which can be potentially transmitted to humans via the food chain. In this study, we showed that inactivation of *mfd* significantly reduced the development of FQ\textsuperscript{R} *Campylobacter* in chickens (Fig. 5). In fact, several birds in the JH01-inoculated group became negative with *Campylobacter* once the treatment was initiated. Since the *mfd* mutant did not show a growth defect *in vitro* (Fig. 3) and colonized chickens as efficiently as the wild-type strain (see the colonization level before treatment in Fig. 5), the observed differences in the development of FQ\textsuperscript{R} mutants were not due to changes in growth characteristics. These *in vivo* results (Fig. 5) plus the *in vitro* findings (Fig. 4) clearly showed that Mfd plays an important role in the development of FQ\textsuperscript{R} *Campylobacter* mutants under the selection pressure. To our knowledge, this is the first report that documents the role of Mfd in the development of FQ resistance in a bacterial pathogen. Since Mfd is highly conserved in bacterial organisms \cite{64}, it would be interesting to know if this finding applies to other bacterial pathogens. In addition, inhibition of Mfd functions may represent a feasible approach to reducing the emergence of FQ\textsuperscript{R} *Campylobacter*.

### 6. Materials and Methods
**Bacterial strains and growth conditions**

*C. jejuni* strain NCTC 11168 (ATCC 700819) was used in this study. The strain was routinely grown in Mueller-Hinton (MH) broth (Difco) or on MH agar at 42 °C under microaerobic conditions (5% O₂, 10% CO₂, and 85% N₂). The media were supplemented with kanamycin (50 µg/ml) or chloramphenicol (4 µg/ml) as needed. *Escherichia coli* cells were grown at 37 °C with shaking at 200 r.p.m. in Luria Bertani (LB) medium which was supplemented with ampicillin (100 µg/ml) or kanamycin (30 µg/ml) when needed.

**DNA microarray and qRT-PCR**

DNA microarray was used to identify genes that were differentially expressed in *C. jejuni* 11168 treated with CIPRO. For RNA isolation, *Campylobacter* cells were grown for 24 hours to the mid exponential phase (OD₆₀₀ ≈ 0.1 ~ 0.15) and split into two equal portions, one of which was treated with CIPRO and the other served as a non-treated control. A subinhibitory concentration (0.06 µg/ml, 0.5 x the MIC) and a suprainhibitory dose (1.25 µg/ml, 10 x the MIC) of CIPRO were used in the treatments. For the treatment with 0.06 µg/ml of CIPRO, the treated and non-treated samples were incubated at 42 °C for 1.5 hours under microaerobic conditions, while for the treatment with 1.25 µg/ml of CIPRO, the samples were incubated at 42 °C for 30 min under microaerobic conditions. Immediately after the incubation, RNAProtect™ Bacteria Reagent (Qiagen, Valencia, CA) was added to the cultures to stabilize mRNA. The total RNA from each sample was extracted using the RNeasy Mini Kit (Qiagen). The purified RNA samples were treated with On-Column DNase Digestion Kit (Qiagen) followed by further treatments with DNase to remove residual DNA contamination. RNA samples were extracted from 6 independent treatments with each concentration of CIPRO. Absence of contaminating DNA in the RNA samples was confirmed by RT-PCR. The concentration of total RNA was estimated with the NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA), and the integrity and size distribution of the purified RNA was determined by denaturing agarose gel electrophoresis and ethidium bromide staining. The quality of total RNA was further analyzed using the
Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), which showed the good quality and integrity of the RNA samples (Data not shown).

cDNA synthesis and labeling, microarray slide (Ocimum Biosolutions) hybridization, Data collection and normalization, and statistical analysis were performed as described in a previous publication [71]. For each type of treatment (0.06 µg/ml for 1.5 hours or 1.25 µg/ml for 30min), six microarray slides were hybridized with RNA samples prepared from 6 independent experiments. For this study, we chose $p$-value < 0.05 and the change $\geq$ 1.5-fold as the cutoff for significant differential expression between the treated and non-treated samples. Representative genes identified by the DNA microarray were further confirmed by qRT-PCR as described in a previous work [72]. The primers used for qRT-PCR are listed in Table 2.

Insertional mutation of mfd

An isogenic mfd (cj1085c) mutant of strain NCTC 11168 was constructed by insertional mutagenesis. Primers mfd-F2 (5’-TGTTGATGGAGAGTTAAGTGGTAT-3’) and mfd-R2 (5’-AATAGCATTCAATAGCGACTTCTGTT -3’) were designed from the published genomic sequence of this strain [48] and used to amplify a 1.8-kb fragment spanning the 5’ region of mfd. Amplification was performed with Pfu Turbo® DNA Polymerase (Stratagene, La Jolla, CA, USA). The blunt-ended PCR product was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), ligated to Smal-digested suicide vector pUC19, resulting in the construction of pUC-mfd, which was then transformed into E. coli DH5α. Since a unique EcoRV site (which generates blunt ends) occurs in the cloned mfd fragment, pUC-mfd was digested with EcoRV to interrupt the mfd gene. Primers KanNco-F (5’-CTT ATC AAT ATA TCC ATG GAA TGG GCA AAG CAT 3’) and KanNco-R (5’-GAT AGA ACC ATG GAT AAT GCT AAG ACA ATC ACT AAA 3’) were used to amplify the aphA3 gene (encoding kanamycin resistance) from the pMW10 vector [73] by using Pfu Turbo® DNA polymerase (Stratagene). The aphA3 PCR product was directly ligated to EcoRV-digested pUC-mfd to obtain construct pUC-mfd-aphA3, in which the aphA3 gene was inserted within mfd (the same direction as the transcription of mfd)
and the insertion was confirmed by PCR using primers mfd-F2 and Kana-intra (5’ GAA GAA CAG TAT GTC GAG CTA TTT TTT GAC TTA 3’). The pUC-mfd-aphA3 construct, which served as a suicide vector, was electroporated into *C. jejuni* NCTC 11168. Transformants were selected on MH agar containing 10 µg/ml of kanamycin. Inactivation of the *mfd* gene in the transformants by insertion of the *ahpA3* gene was confirmed by PCR using primers mfd-F2 and mfd-R2 (Fig. 1B). The *mfd* mutant of NCTC 11168 was named JH01.

**Complementation of the *mfd* mutant in trans**

The entire *mfd* gene including its putative ribosome binding site was amplified from strain 11168 by PCR using primers mfd-F5 (5’-CGCTTCCGCGAAGTAAATGGAAGATATATC-3’) and mfd-R3 (5’-GGCTTTAAATAATCTTTTCGACCTCTATAAATT-3’). The underlined sequences in the primers indicate the restriction sites for SacI and SacII, respectively. The PCR product was digested with SacI and SacII, and was then cloned into the plasmid construct pRY112-pABC to generate pRY112-mfd, in which the *mfd* gene was fused to the promoter of *cmeABC*. pRY112-pABC was made by cloning the promoter sequence of *cmeABC* [74] to shuttle plasmid pRY112 [75]. The promoter DNA of *cmeABC* was amplified by primers BSF (5’ AAAAGGATCTCTAATGGAATGCAATAG 3’) and AR2 (5’ TGATCTAGATCATAAGGAG 3’), digested with BamHI and XbaI, and cloned into pRY112. There were two reasons that we used the promoter of *cmeABC* in the expression of *mfd*. First, the 5’ end of *mfd* overlaps with its upstream gene and the native promoter for *mfd* was unknown. Second, the promoter of *cmeABC* is moderately active in *Campylobacter* [74], preventing over- or under-expression of *mfd*. The constructed plasmid pRY112-mfd was sequenced and confirmed that no mutations in the cloned sequence occurred. For complementation, the shuttle plasmid pRY112-mfd was transferred into JH01 by conjugation. The complemented strain was named JH02. Limited passage of JH02 in MH broth without antibiotics indicated that the complementing plasmid was stable in the construct (data not shown). The shuttle plasmid carrying the *mfd* gene was also transferred to wild-type 11168 to generate strain JH03 for overexpression of the *mfd* gene.
**Growth rates in MH broth with or without CIPRO**

To compare the growth kinetics of the mfd mutant with that of the wild-type, a fresh culture of each strain was inoculated into MH broth (initial cell density of OD600 = 0.05) and the cultures were incubated at 42 °C under microaerobic conditions. To determine if the mutation affects *C. jejuni* growth with a subinhibitory concentration of CIPRO, the various strains were grown in MH broth with 0.06 µg/ml of CIPRO (0.5 x the MIC). Culture samples were collected and measured for OD$_{600}$ at 0, 3, 6, 12, 24 and 48 hours post-inoculation.

**Antibiotic susceptibility test**

The minimum inhibitory concentration (MIC) of CIPRO was determined by using E-test strips (AB Biodisk, Solna, Sweden) as described in the manufacturer's instructions. The detection limit of the E-test for CIPRO was 32 µg/ml. The MICs of erythromycin, ampicillin and streptomycin for *C. jejuni* NCTC 11168, JH01, JH02, and JH03 were determined using a standard microtiter broth dilution method described previously [24]. Each MIC test was repeated at least three times to confirm the reproducibility of the MIC patterns. The antibiotics used in this study were purchased from Sigma Chemical Co. (erythromycin, ampicillin, streptomycin) or ICN Biomedicals Inc. (CIPRO).

**Frequencies of emergence of spontaneous FQR mutants in vitro**

Wild-type 11168, JH01, JH02 and JH03 were compared for the spontaneous mutation rates to CIPRO resistance. In each experiment, each of the 4 strains was inoculated into three flasks, each of which contained 30 ml of antibiotic-free MH broth. The cultures were incubated to the mid logarithmic phase (OD$_{600}$ ≈ 0.15) under microaerobic conditions. The culture in each flask was collected by centrifugation and resuspended in 1 ml of MH broth. The total CFU in each culture was measured by serial dilutions and plating on MH agar plates, while the number of FQR mutants was detected using MH agar plates containing 1, 2 or 4 µg/ml CIPRO. The frequency of emergence of FQR mutants was calculated as the ratio of the CFU on CIPRO-containing MH agar plates to the CFU on antibiotic-free MH agar.
plates after 2 days of incubation at 42°C under microaerobic conditions. This experiment was repeated five times. The mutation frequency data were log-transformed for statistical analysis. One-Way ANOVA followed by Tukey test was used to determine the significance of differences in the levels of spontaneous mutation rates among the strains. The data were also analyzed by the Wilcoxon rank-sum test to allow for non-normality. For the comparisons discussed in Results, the conclusion of the two tests was the same at significance level 0.05.

**Sequence analysis of the QRDR of gyrA**

Representative FQR colonies were selected for determination of the point mutations in gyrA. The QRDR of gyrA was amplified by PCR using primer pair GyrAF1 (5'-CAACTGGTTCTAGCCTTTTG-3') and GyrAR1 (5'-AATTTCACTCAGCCTACG-3') [76]. The amplified PCR products were purified with the QIAquick PCR purification kit (Qiagen) prior to sequence determination. DNA sequence analysis was carried out using an automated ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA, USA) and analyzed by the Omiga 2.0 (Oxford Molecular Group) sequencing analysis software.

**In vitro treatment with CIPRO**

To determine if Mfd affects the development of FQR mutants under treatment with CIPRO, wild-type 11168 and JH01 were treated in MH broth with 4 µg/ml (32 x the MIC) of CIPRO. Wild-type 11168 and JH01 were grown on antibiotic-free MH agar plates under microaerobic conditions. After 20 hours of incubation, the cells were collected and resuspended in MH broth for inoculation. Three treatment experiments were conducted using three different initial cell densities. In experiment 1, each strain was inoculated into 3 100-ml flasks with MH broth containing 4 µg/ml of CIPRO and the initial cell density was 10^7 CFU/ml. The cultures were incubated microaerobically at 42 °C. Aliquots of the cultures were collected at different time points (0, 1, 2, 3 days post-inoculation) and plated onto regular MH plates for enumeration of the total bacterial number and onto MH plates containing 4 µg/ml of CIPRO for counting FQR colonies. In experiments 2 and 3, the cultures were treated in the same way, but the initial cell densities were 10^6 and 10^3 CFU/ml,
respectively. Experiment 1 was repeated three times, while experiments 2 and 3 were each repeated twice.

**The transcription level of Cj1084c**

To determine if the insertional mutation in *mfd* affected the expression of the downstream gene *Cj1084c* (encoding a possible ATP/GTP-binding protein), RT-PCR was performed to assess the expression of *Cj1084c*. Total RNA was isolated from *C. jejuni* 11168 and JH01 using the RNeasy Kit (Qiagen). The purified RNA samples were treated with On-Column DNase Digestion Kit (Qiagen) followed by further treatments with DNase to remove DNA contamination. The *Cj1084c*-specific primers Cj1084cF (5' TTG CCT TAG CAG ATA TCA T 3') and Cj1084cR (5' ACC ACT TCT ACT TGC TCT TA 3') were used to amplify a 430 bp region of the gene in a conventional one-step RT-PCR by using the SuperScript™ III One-Step RT-PCR kit (Invitrogen®). An RT-PCR mixture lacking the RT was included as a negative control.

**Emergence of FQ<sup>R</sup> mutants in enrofloxacin-treated chickens**

To examine if Mfd plays a role in the emergence of FQ<sup>R</sup> *Campylobacter* during *in vivo* FQ treatment, a chicken experiment was performed using 11168 and JH01. Day-old broiler chickens (Ross X Cobb) were obtained from a commercial hatchery and randomly assigned to 2 groups (11 birds per group). Each group of chickens was maintained in a sanitized wire-floored cage. Feed and water were provided *ad libitum*. Prior to inoculation with *Campylobacter*, the birds were tested negative for *Campylobacter* by culturing cloacal swabs. At day 3 of age, the two groups of chickens were inoculated with 11168 and JH01, respectively, at a dose of 10<sup>6</sup> CFU/chick via oral gavage. Six days after the inoculation, the birds were treated with 50 ppm enrofloxacin. The treatment was administered in drinking water and lasted for five consecutive days. During the treatment, only medicated water was given to the birds to ensure enough consumption. Cloacal swabs were collected periodically before and after enrofloxacin treatment until the end of the experiment. Each swab was serially diluted in MH broth and plated onto two different types of MH plates: one containing
Campylobacter-specific growth supplements (SR 084E and SR117 E; Oxoid Ltd., Basingstoke, England) for the enumeration of total Campylobacter cells and the other containing 4 µg/ml of CIPRO in addition to the same selective agents and supplements to recover FQ$^R$ Campylobacter in each chicken. At each sampling time, at least one Campylobacter colony from each chicken were selected from the regular MH agar plates (no CIPRO) for the determination of CIPRO MICs using the E-test (AB Biodisk). The colonization data (CFU/g feces) were log-transformed and used for statistical analysis. The significance of differences in the level of colonization between the two groups was determined using Student’s t-test, Welch’s t-test to allow for non-constant variation across treatment groups, and the Wilcoxon rank-sum test to allow for non-normality. The conclusion of all three tests was the same at significance level 0.05.

Microarray data accession number

The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) database and the accession number is GSE10471.

7. References


### TABLE 1. Genes differentially expressed in the presence of ciprofloxacin

<table>
<thead>
<tr>
<th>Gene ID and Functional Category</th>
<th>P-Value</th>
<th>Q-Value</th>
<th>n-Fold change</th>
<th>Microarray</th>
<th>qRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell membrane</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cj0205 appP, undecaprenyl-diphosphatase</td>
<td>0.0135</td>
<td>0.130143</td>
<td>1.59</td>
<td>6.1</td>
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</tr>
<tr>
<td>Cj0735 putative periplasmic protein</td>
<td>0.0186</td>
<td>0.14811</td>
<td>1.70</td>
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<td>Cj0824 appS, undecaprenyl diphosphate synthase</td>
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<td>0.120356</td>
<td>1.52</td>
<td>2.1</td>
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<tr>
<td>Cj1351 pldA, phospholipase A</td>
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<td>0.094812</td>
<td>2.02</td>
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<td>Cj0633 putative integral membrane protein</td>
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<td>-1.52</td>
<td>NT</td>
<td></td>
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<tr>
<td>Cj0179 exbB1, biopolymer transport protein</td>
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<td>NT</td>
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<td>Cj0486 putative sugar transporter</td>
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<td>Cj0834c ankyrin repeat-containing possible periplasmic protein</td>
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<td>Cj1013c putative cytochrome C biogenesis protein</td>
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<td>NT</td>
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<td>Cj1662 putative integral membrane protein</td>
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<td>Cj1663 putative ABC transport system ATP-binding protein</td>
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<td>0.067622</td>
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<td></td>
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<td><strong>DNA replication, recombination and repair</strong></td>
<td></td>
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<td>Cj1085c mfd, transcription-repair coupling factor</td>
<td>0.0029</td>
<td>0.082832</td>
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<td>Cj0718 dnaE, DNA polymerase III, alpha chain</td>
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<td><strong>Cellular process and energy metabolism</strong></td>
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<td>Cj0041 putative flagellar hook-length control protein</td>
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<td>Cj0103c lepA, GTP-binding protein homolog</td>
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<td>0.210454</td>
<td>1.54</td>
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<td>Cj1280c putative ribosomal pseudouridine synthase</td>
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<td>0.170564</td>
<td>1.50</td>
<td>NT</td>
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<td>Cj0099 gltD, glutamate synthase (NADPH) small subunit</td>
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<td>0.067622</td>
<td>-1.74</td>
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<td>Cj0123c putative tRNA-dihydrouridine synthase</td>
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<td>0.076881</td>
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<td>-2.1</td>
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<tr>
<td>Cj0227 argD, acetylornithine aminotransferase</td>
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<td>0.151677</td>
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<tr>
<td>Cj0283c cheW, chemotaxis protein</td>
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<td>Cj0415 putative GMP oxidoreductase subunit</td>
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<td>Cj0490 ald putative aldehyde dehydrogenase C-terminus</td>
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<tr>
<td>Cj0537 oorh, OORb subunit of 2-oxoglutarate:acceptor oxidoreductase</td>
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<tr>
<td>Cj0734c hisJ, histidine-binding protein precursor</td>
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<td>0.214389</td>
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<td>Cj0764c speA, biosynthetic arginine decarboxylase</td>
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<td>Cj0767c kdbB,3-deoxy-D-manno-octulosonic-acid transferase</td>
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<td>Cj1264c hydD, Putative hydrogenase maturation protease</td>
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<td>0.067622</td>
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<td>Cj1265c hydC, NiFe-hydrogenase B-type cytochrome subunit</td>
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<td>Cj1266c hydB, NiFe-hydrogenase large subunit</td>
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<td>Cj1364c fumC, fumarate hydratase</td>
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<td>NT</td>
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<td>Cj1476c pyruvate-flavodoxin oxidoreductase</td>
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<td>Cj1566c muoN, NADH dehydrogenase I chain N</td>
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<td>Cj1682c gdiA, citrate synthase</td>
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<td>0.142007</td>
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<tr>
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<td>Cj0163c hypothetical protein</td>
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* NT: Not tested
TABLE 2. Oligonucleotide primers used in qRT-PCR

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<td>16s RNA F</td>
<td>5’-TAC CTG GGC TTG ATA TCC TA-3’</td>
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<td>16s RNA R</td>
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<td>Cj0123cF</td>
<td>5’ CGC CTT GAT CTT TGT AGT GTT TT 3’</td>
<td>cj0123c</td>
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<td>Cj0123cR</td>
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Fig. 1. Insertional mutation of *mfd* and its impact on the transcription of *cj1084c*. (A) Diagram depicting the genomic organization of *mfd* and its flanking regions. ORFs and their directions of transcription are indicated by boxed arrows. The location of the inserted kanamycin resistance gene (*aphA3*) in *mfd* is indicated. (B) PCR confirmation of the *aphA3* insertion into the *mfd* gene in JH01. Lane 1 shows the PCR product from 11168, while Lane 2 shows the PCR product of JH01. The primers used in the PCR were *mfd*-F2 and *mfd*-R2. Lane M contains 1 kb DNA size markers (Promega). (C) RT-PCR analysis of *cj1084c* expression in strains 11168 and JH01. The same amount of total RNA from 11168 (Lane 1) and JH01 (Lane 2 and 3) were used as template in the RT-PCR. Lanes 1 and 2 are normal RT-PCR reactions. Lane 3 is a RT-PCR reaction without reverse transcriptase (DNA-free control for the RNA preparation). In Lane 4, genomic DNA of 11168 was used as template (positive control for PCR).
Fig. 2. Frequencies of emergence of spontaneous FQ$^R$ mutants in different *C. jejuni* strains including the wild-type 11168, the *mfd* mutant (JH01), the complemented *mfd* mutant (JH02), and the *mfd*-overexpressing construct (JH03). Three different concentrations of CIPRO (1, 2, and 4 µg/ml, respectively) were used in the detection plates to count FQ$^R$ colonies. Each bar represents the mean ± standard deviation of frequencies from three independent cultures. The bars labeled with different letters indicate that they are significantly different ($P < 0.05$).
Fig. 3. Growth kinetics of various *C. jejuni* constructs in culture media. The strains were grown in MH broth (A) or MH broth supplemented with 0.06 µg/ml of CIPRO (B).
Fig. 4. Development of FQ\textsuperscript{R} mutants from 11168 (solid circle) and JH01 (triangle) grown in MH broth supplemented with 4 µg/ml of CIPRO. In panel A, the initial cell density (at time 0) of each culture was 10\textsuperscript{7} CFU/ml, while in panel B the initial cell density was 10\textsuperscript{6} CFU/ml. Each symbol represents the number of FQ\textsuperscript{R} mutants in a single culture. Each horizontal bar represents the mean log\textsubscript{10} CFU/ml from each strain at a given time. Panel A displays the results of 3 independent experiments, while panel B represents the results of two independents experiments. The detection limit of the plating method is 1 CFU/ml.
Fig. 5. Development of FQR *Campylobacter* mutants in chickens initially infected with FQ-susceptible *Campylobacter*, but treated with enrofloxacin. (A) The level of total *Campylobacter* in each chicken inoculated with the wild-type 11168 (open circle) or the mfd mutant strain (JH01; solid circle). The treatment with enrofloxacin started on day 0 and lasted for five consecutive days (indicated by a bracket on top of the panel). (B) The level of FQR *Campylobacter* in each chicken inoculated with the wild-type (open circle) or the mfd mutant (solid circle). In both panels, each symbol represents the number of *Campylobacter* in a single bird. Each group includes eleven chickens and the mean of each group at a given time is indicated by a horizontal bar. A chicken is considered negative if the level of colonization was below the detection limit (100 CFU/ g of feces).
CHAPTER 4. FLUOROQUINOLONONE RESISTANCE ASSOCIATED MUTATION IN GYRA AFFECTS DNA SUPERCOILING IN CAMPYLOBACTER JEJUNI

A paper to be submitted to Journal of Bacteriology

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Ames, Iowa 50011

1. Abstract

Our previous work showed that acquisition of fluoroquinolone (FQ) resistance via a specific GyrA mutation (Thr-86-Ile) enhances the in vivo fitness of FQ-resistant mutants in the absence of antibiotic selection pressure. In this study, we further confirmed the role of the Thr-86-Ile mutation in modulating Campylobacter fitness by reverting the mutation. Reversion of this mutant allele to a wild-type allele resulted in the loss of the fitness advantage, consistent with our previous findings. To understand how the Thr-86-Ile mutation affects Campylobacter fitness, we initiated work to determine if this mutation alters the physiological function of DNA gyrase. Recombinant wild-type gyrase and mutant gyrases with three different types of mutations (Thr-86-Ile; Thr-86-Lys; and Asp-90-Asn) were generated in E. coli and compared for their supercoiling activities using an in vitro assay. The mutant gyrase with the Thr-86-Ile change showed a greatly reduced supercoiling activity compared with the wild-type gyrase and other mutant gyrases. In addition, we measured DNA supercoiling within Campylobacter cells using a reporter plasmid. Consistent with the results from the in vitro supercoiling assay, the FQ-resistant mutant Campylobacter carrying the Thr-86-Ile change in GyrA showed much less DNA supercoiling than the wild-type strain
and the mutant strains carrying other mutations. Together, these results clearly indicate that
the Thr-86-Ile change in GyrA confers a high-level resistance to FQs and significantly
reduces the supercoiling activity of the enzyme in *Campylobacter*.

2. Introduction

*Campylobacter jejuni*, a Gram-negative microaerobic bacterium, has emerged as the
leading bacterial cause of foodborne diseases in the United States and other developed
countries (45). The estimated number of human cases of campylobacteriosis in the U.S. is
more than 2 million per year (30, 42). The medical and productivity costs resulting from *C.
jejuni* infection are estimated at 0.6 to 1.0 billion dollars each year in the U.S. (8). Antibiotic
treatment using fluoroquinolone (FQ) or erythromycin is recommended when the infection
by *Campylobacter* is severe or occurs in immunocompromised patients (12, 35). However,
*Campylobacter* is increasingly resistant to FQ antimicrobials, which has become a major
concern for public health (12, 16, 48). Although FQ antimicrobials have been banned since
2005 in poultry production in the U.S., FQ-resistant (FQR) *Campylobacter* continues to
persist on poultry farms (25, 39, 40).

The main targets of FQs in bacteria are DNA gyrase and/or topoisomerase IV (11,
18). In Gram-negative bacteria, DNA gyrase, a type II topoisomerase, is the primary target of
FQ antibiotics, while topoisomerase IV is the main target of FQs in Gram-positive bacteria
(18). Once inside bacterial cells, FQ antimicrobials form stable a complex with the target
enzymes and trap the enzymes on DNA, resulting in double-stranded breaks in DNA and
bacterial death (10, 44, 49). Bacterial DNA gyrase is essential for bacterial viability. It
catalyzes ATP-dependent negative supercoiling of DNA and is involved in DNA replication,
recombination, and transcription (9). The enzyme consists of two subunits (subunits A and B) that combine into the $A_2B_2$ complex to form a functional enzyme. The two subunits are encoded by the genes gyrA and gyrB, respectively. In *Campylobacter*, resistance to FQ antimicrobials is mediated by point mutation in the quinolone resistance-determining region (QRDR) of *gyrA* in conjunction with the function of the multidrug efflux pump CmeABC (3, 12, 13, 27). No mutations in *gyrB* have been implicated in FQ resistance in *Campylobacter* (3, 37, 38). Acquisition of high-level FQ resistance in *Campylobacter* does not require stepwise accumulation of point mutations in *gyrA*. Instead, a single point mutation in *gyrA* can lead to clinically relevant levels of resistance to FQ antimicrobials (13, 27, 41). Specific mutations at positions Thr-86, Asp-90 and Ala-70 in GyrA have been linked to FQ resistance in *C. jejuni* (12, 27, 47). Specifically, the Thr-86-Ile change (mediated by the C257T mutation) is the most commonly observed mutation in FQR *Campylobacter* isolates and confers high-level (ciprofloxacin MIC ≥16 µg/ml) resistance to FQ, whereas the Thr-86-Lys and Asp-90-Asn mutations are less common and are associated with intermediate-level FQ resistance (15, 27, 41, 47).

Genes targeted by antibiotics are usually essential for bacterial physiology. Thus resistance-conferring mutations in target genes are often associated with changes in physiological processes, resulting in reduced growth rate and fitness in the absence of antibiotic selection (1, 2, 20, 21). However, bacteria can develop compensatory mutations to ameliorate the fitness defect associated with antimicrobial resistance (1, 6, 7, 33, 34). In some situations, antibiotic-resistant mutants show little or no fitness cost even without compensatory mutations (43). A previous study by Luo *et. al.* showed FQR *Campylobacter* carrying the C257T mutation (leading to a Thr-86-Ile substitution in the GyrA subunit) was
able to colonize and persist in chickens as efficiently as the FQ-susceptible (FQ\textsuperscript{S}) strains in the absence of FQ. Pairwise competitions using clonally or isogenic strains further demonstrated that the FQ\textsuperscript{R} isolates outcompeted the FQ\textsuperscript{S} strains in the majority of the competing pairs in chickens (26), suggesting that acquisition of FQ resistance enhances the \textit{in vivo} fitness of FQ\textsuperscript{R} \textit{Campylobacter}. How the resistance-conferring mutation affects \textit{Campylobacter} fitness is unknown. GryA plays an essential role in maintaining the topological configuration of DNA by introducing negative superhelical turns in DNA. Work performed with \textit{Escherichia coli} and \textit{Pseudomonas} revealed that antibiotic resistance-conferring mutations in GyrA reduced the supercoiling activity of the enzyme in (5, 20). It is also known that changes in DNA supercoiling affect bacterial adaptive responses to various environmental signals including heat shock, cold shock, osmolar stress, and oxidative stress (24, 46). Based on the available evidence, we hypothesize that the resistance-conferring mutations in FQ\textsuperscript{R} \textit{Campylobacter} may affect the enzymatic function of GyrA and modulate the DNA supercoiling status within the bacterial cells, which leads to fitness changes in FQ\textsuperscript{R} \textit{Campylobacter}.

To begin to examine our hypothesis, we conducted a series of experiments in this study to determine the impact of various point mutations on the supecoiling activities of GyrA. We first confirmed the specific role of Thr-86-Ile mutation of GyrA in influencing \textit{Campylobacter} fitness in chickens by reverting the mutation to a wild-type allele. We then prepared recombinant gyrases with three different types of mutations [C257T (Thr-86-Ile), C257A (Thr-86-Lys), and G268A (Asp-90-Asn)] and compared their supercoiling activity with that of the wild-type gyrase in the presence or absence of ciprofloxacin using an \textit{in vitro}
supercoiling assay. Also we determined the impact of the mutations on in vivo supercoiling (within Campylobacter cells) using a reporter plasmid. Our results consistently showed that the Thr-86-Ile change in GyrA was directly linked to the fitness change in Campylobacter and greatly reduced the supercoiling activity of GyrA, while other GyrA mutations, although reduced the susceptibility to ciprofloxacin, did not affect the supercoiling activity of the gyrase.

3. Materials and Methods

**Bacterial strains and growth conditions**

The FQ^S^ strains and FQ^R^ mutant strains (carrying different point mutations in gyrA) used in this study are listed in Table 1. The strain was routinely grown in Mueller-Hinton (MH) broth (Difco) or agar at 42 °C under microaerobic conditions (10% CO2, 5% O2, and 85% N2). Campylobacter-specific growth supplements and selective agents (Oxoid) were added to media when needed to recover Campylobacter from chicken feces. MH media were supplemented with kanamycin (50 µg/ml) or chloramphenicol (4 µg/ml) as needed.

*Escherichia coli* (E. coli) cells were grown at 37 °C with shaking at 200 r.p.m. in LB medium which was supplemented with ampicillin (100 µg/ml) or kanamycin (30 µg/ml).

**Reversion of the gyrA mutation (Thr-86-Ile)**

To formally define the role of the C257T mutation in influencing Campylobacter fitness, the specific mutation (C257T) in gyrA of isolates 62301R33 was reverted to wild-type sequence by using a method reported by Ge et. al. (13). A chloramphenicol resistance
(Cm') cassette, which was used as a selective marker, was inserted in the *cj1028c* gene immediately upstream of *gyrA*. *Cj1028c* and *gyrA* are tandemly positioned on the chromosome of *C. jejuni*. An 850-bp fragment containing the 3' region of *Cj1028c*, the intergenic region, and the *gyrA* sequence up to the mutation site was amplified by PCR using *C. jejuni* 62301R33 as a template and *gyrA*1028F and *gyrA*257WR (corresponding to the wild-type *gyrA* sequence) as primers (Table 2) and then cloned into a pGEMT-Easy (Promega). The construct was linearized using EcoRV, which cuts once within the cloned *cj1028c* sequence, ligated with a blunt-ended Cm' cassette, and electroporated into *E. coli* JM109. Recombinant plasmids were purified from *E. coli* JM109 and used to transform the parent strains *C. jejuni* 62301R33 by electroporation. Transformants were selected on MH agar containing chloramphenicol 10 mg/L and analyzed by PCR and DNA sequencing, confirming the insertion of the *cat* cassette into *Cj1028c* and the simultaneous replacement of the mutant *gyrA* with the wild-type allele. This revertant was named 62301R33S. To make an isogenic pair for the revertant for *in vivo* competition, the *cat* gene was also inserted into *Cj1028c* of isolate 62301R33 without changing the C257T mutation in *gyrA*. Primers *gyrA*1028F and *gyrA*257m(T) were used for this purpose (Table 2). The obtained construct was named 62301R33R. The ciprofloxacin MIC in the revertant 62301R33S was restored to the wild-type level (0.125µg/ml), while 62301R33R with the *cat* gene inserted into *Cj1028c* retained the ciprofloxacin MIC at 32 µg/ml. S3B R33S and 62301R33R were equally motile as determined by motility assay. Using the same strategy, a point mutation (C257T) in *gyrA* was introduced into FQ5 NCTC 11168. The generated FQ5 mutant strain and its isogenic FQ5 strain, both of which contained a Cm' insertion in *cj1028c*, were named 11168 (R) and 11168 (S), respectively (Table 1).


*In vivo colonization and pairwise competition*

Chicken experiments were performed using pairwise competition as described previously (26). Three groups (10-11 birds/group) of *Campylobacter*-free chickens were inoculated with about $10^7$ CFU of 62301R33S, 62301R33R, or a mixture (approximately 1:1) of the two strains via oral gavage. During the entire experiment, antibiotic-free feed and water were given to the chickens. Thus, antibiotic selection pressure was not involved in the competition. After inoculation, cloacal swabs were collected from the chickens at days 3, 6, and 9 for culturing *Campylobacter*. Each fecal suspension was serially diluted in MH broth and plated simultaneously onto two different types of culture media: conventional *Campylobacter* selective plates for recovering the total *Campylobacter* colonies and the selective MH plates supplemented with 4 µg/ml ciprofloxacin for recovering FQR*Campylobacter* colonies. Competition index (CI) was calculated as described in reference (26).

To confirm the results from the differential plating, 10–15 *Campylobacter* colonies were selected randomly for each group from the conventional selective plates (no ciprofloxacin) at each sampling time and tested for ciprofloxacin MICs using Etest strips (AB Biodisk, Solna, Sweden). In the second chicken experiment, the same pairwise experiment was performed using the isogenic pair of 11168(S) and 11168(R).

In both experiments, the detection limit of the plating methods was 100 CFU/g of feces. The statistic analyses that were used to determined the significance of differences in
the level of colonization between the two groups were performed as described in a previous publication (17).

**Production and purification of GyrA and GyrB**

Full-length histidine (His)-tagged recombinant gyrases including wild-type (WT) GyrA, three mutant GyrA, and GyrB were produced in *E. coli* by using the pQE-30 vector of the QIAexpress expression system (Qiagen). The complete coding sequences of *gyrA* in strains 62301S2 (no mutation in GyrA), 62301R33 (with the Thr-86-Ile change in GyrA), 52901-II2 (with the Thr-86-Lys change in GyrA) and 62301R37 (with the Asp-90-Asn change in GyrA) were amplified using primers gyrA-F-2 and gyrA-R. The complete coding sequence of *gyrB* in strain 62301S2 was amplified using primers gyrB-F-J2 and gyrB-R-J. A restriction site (underlined in the primer sequences) was attached to the 5’ end of each primer to facilitate the directional cloning of the amplified PCR product into the pQE-30 vector. The amplified PCR product was digested with SphI and Sall, and then ligated into the pQE30 vector, which was previously digested with the same enzymes. Each plasmid in the *E. coli* clone expressing a recombinant peptide was sequenced, revealing no undesired mutations in the coding sequence. These His-tagged recombinant GyrA and GyrB proteins were expressed and purified to near-homogeneity under native conditions by following the procedure supplied with the pQE-30 vector. Then these proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). To remove imidazole, the purified proteins were washed extensively with 10 mM Tris-HCl using Centricon YM-50 (Millipore).
**In vitro supercoiling assay**

DNA supercoiling activity was assayed by monitoring the conversion of relaxed pBR322 to its supercoiled form. WT or mutant GyrA was mixed with WT GyrB in a ratio of 1:1. Each supercoiling reaction contained 500 ng of relaxed pBR322 DNA (TopoGen, Inc) and 300 μM gyrase in 1X assay buffer (TopoGen, Inc). The reaction mixtures were incubated at 37 °C for 1h. Assays were terminated by the addition of 0.2 volume of the stop buffer (TopoGEN) and 1 volume of chloroform-isooamyl alcohol (24:1). The reactions were analyzed on 1.0% agarose gels. The gels were stained with ethidium bromide for 30 min and then destained in 1X TAE buffer for 1h.

**MEC (minimum effective concentration) determinations**

To determine the susceptibility of various gyrases to the inhibitory effect of ciprofloxacin, the *in vitro* supercoiling assay using recombinant gyrases was also performed with added ciprofloxacin as described previously (28). Ciprofloxacin was added into each reaction prior to the addition of DNA gyrase. The minimum effective concentration (MEC) was defined as the lowest concentration of ciprofloxacin that shows an observable inhibition on supercoiling of the plasmid DNA.

**Examination of in vivo supercoiling**

To determine if the mutations in GyrA changed the DNA supercoiling in *Campylobacter* cells, the shuttle plasmid pRY107 was used as a reporter to monitor the relative difference in the levels of DNA supercoiling between the FQ\(^\text{S}\) and FQ\(^\text{R}\) strains.
pRY107, which carries a kanamycin resistance marker (50), was transferred into 62301S2, 62301R33, 52901-II2, 62301R37, 11168, and 1168CT by conjugation. The conjugates were grown in MH broth (Difco) for one day at 42 °C under microaerobic conditions (10% CO2, 5% O2, and 85% N2), then plasmid DNA was isolated using QIAGEN Plasmid Midi kit (Qiagen Inc.) and analyzed by agarose gel electrophoresis in the absence or presence of chloroquine diphosphate salt, as described previously (20, 31). Agarose gels (1%) were run for 20 h at 2 V/cm in 1 x TAE buffer containing 20 µg/ml of chloroquine and were washed for 4 h in distilled water before staining with ethidium bromide. The topoisomers of the plasmid DNA from each strain were visualized using a digital imaging system (Alpha innotech). The relative amounts of supercoiled vs. relaxed DNA in each sample reflecting the level of DNA supercoiling in each strain were determined by densitometry scanning and were used to indicate the difference in DNA supercoiling between the FQ<sup>S</sup> and FQ<sup>R</sup> strains.

**Cloning and sequence of topA genes**

The entire topA gene from *C. jejuni* 62301S2 and 62301R33 were amplified by PCR with the forward primer topA-F and reverse primer topA-R. The forward primer is 286 bp upstream of the AUG start codon, and the reverse primer extends 239 bp beyond the UAG stop codon of topA. The PCR was performed in a volume of 50 µl containing 100 µM each deoxynucleoside triphosphate (dNTP), 200 nM primers, 2.5 mM MgSO4, 100 ng of *Campylobacter* genomic DNA, and 5 U of Taq DNA polymerase (Promega). The cycling conditions consisted of an initial polymerase activation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 3 min, with a final
extension at 72 °C for 10 min. The amplified PCR products were purified with the QIAquick PCR purification kit (Qiagen) and subsequently sequenced.

**Real-time quantitative RT-PCR (qRT-PCR) analysis of topA transcription in FQ^S and FQ^R Campylobacter**

The transcription level of topA in *C. jejuni* 62301S2 and 62301R33 was compared by qRT-PCR as described in a previous work (22). Briefly, total RNA of 62301S2 and 62301R33 was isolated from overnight cultures by using the RNasy mini kit (QIAGEN) according to the manufacturer's instructions. Isolated RNA was further treated with TURBO DNA-free (Ambion) to remove DNA contamination. The qRT-PCR reactions were conducted using the iScript one-step RT-PCR kit with SYBR green (Bio-Rad). topA-specific primers (cj1686c-F1 and cj1686c-R) and primers specific for the *Campylobacter* 16S RNA gene (16SF and 16SR; for normalization) were used for qRT-PCR. Triplicate reactions, each in a volume of 20 µl, were performed for each dilution of the RNA template. Thermal cycling conditions were as follows: 10 min at 50 °C, 5 min at 60°C followed by 5 min at 95 °C, and then 40 cycles of 10 s at 95 °C and 30 s at 55 °C (for 16S RNA) or 56 °C (for topA). Cycle threshold values were determined with the MyiQ software (Bio-Rad). The relative changes (n-fold) in topA transcription between the S3B 62301S2 and 62301R33 were calculated using the \(2^{-\Delta \Delta CT}\) method as described by Livak and Schmittgen (23).

**Antibiotic susceptibility test**
The minimum inhibitory concentration (MIC) of CIPRO was determined by using E-test strips (AB Biodisk, Solna, Sweden) following the manufacturer's instructions. The maximum detection limit of the E-test for CIPRO was 32 µg/ml.

**Campylobacter motility assay**

*Campylobacters* were grown overnight on fresh MH plates and then were collected using MH broth. The optical density at 600 nm (OD$_{600}$) was adjusted to 0.3. Approximately 1 µl of this suspension was then stabbed into a MH motility plate (MH broth+0.4%Bacto Agar). The low density of the agar allows the bacteria to move within the agar, forming a halo of growth around the point of inoculation. Following microaerobic growth at 42 °C for approximately 30 h, the radius of the ring was measured relative to that of the ring produced by the control strain.

**GenBank Accession No.**

The assigned GenBank accession no. for *topA* in strain 62301S2 and 62301R33 is FJ795047.

4. **Results**

**Role of the Thr-86-Ile change in enhanced fitness**

Our previous study showed that FQ$^{R}$ *Campylobacter* carrying the C257T mutation (leading to a Thr-86-Ile substitution in the GyrA subunit) outcompeted its isogenic FQ$^{S}$
strains in chickens, suggesting that acquisition of FQ resistance enhances the in vivo fitness of FQ\textsuperscript{R} \textit{Campylobacter}. To further confirm the link of this specific mutation with the enhanced fitness, we reverted the mutation back to the wild-type allele and conducted pairwise competition in chickens using 62301R33S and 62301R33R. After inoculation with either of the two isolates or mixed populations, all of the groups of birds were colonized by \textit{Campylobacter} at similar levels (Fig. 1A). When separately inoculated into chickens, the two strains showed no significant differences ($P > 0.05$) in the level of colonization (the number of \textit{Campylobacter} shed in feces) (Fig. 1A). Differential plating by using ciprofloxacin-containing plates showed that the group inoculated with the FQ\textsuperscript{S} revertant alone shed homogeneous FQ\textsuperscript{S} \textit{Campylobacter}, whereas the group inoculated with FQ\textsuperscript{R} strain shed homogeneous FQ\textsuperscript{R} \textit{Campylobacter} during the entire course of the experiment. However, in the group inoculated with mixtures of the two strains, the FQ\textsuperscript{S} revertant strain was outcompeted by its parent FQ\textsuperscript{R} \textit{Campylobacter} ($P < 0.05$) (Fig. 1B). Testing of randomly selected \textit{Campylobacter} colonies (10–15 colonies per group per time point) by using Etest confirmed the results of differential plating. This result clearly indicates that once the C257T mutation in gyr\textit{A} is reverted, \textit{Campylobacter} loses its fitness advantage in chickens, confirming our previous results using clonally related and isogenic strains (26). To further show the specific effect of the C257T mutation on fitness, we introduced this mutation into a different strain background (NCTC 11168) and conducted chicken competition using the isogenic pair of constructs. As shown in Fig 1C, 11168(S) was outcompeted by 11168(R) ($P < 0.05$), indicating the mutation had the same impact on fitness in a \textit{C. jejuni} strain different from the previously tested ones. These new findings plus our published studies (26)
convincingly showed that the C257T mutation in *gyrA* is directly responsible for the enhanced fitness of *Campylobacter* in chickens.

**No compensatory mutations in topA.**

*C. jejuni* has only two types of topoisomerases, type I (TopA) and type II (gyrase). The gyrase introduces negative supercoiling to DNA, while TopA relaxes DNA to prevent excessive supercoiling. Thus, the two enzymes are the key proteins modulating the level of DNA supercoiling in *Campylobacter*. Previously we demonstrated that isolate 62301R33 (FQ<sup>R</sup>), which carried the Thr-86-Ile mutation and outcompeted FQ<sup>S</sup> *Campylobacter* (62301S2) in chickens, did not harbor any compensatory mutations in GyrA and GyrB (26). In this study, we sought to determine if there were any mutations in TopA that might potentially offset the impact of the Thr-86-Ile mutation in GyrA. The whole ORF of *topA* was PCR amplified from 62301R33 and sequenced, which showed that the *topA* sequence was identical to that in 62301S2. In addition, the expression level of *topA* in 62301R33 and 62301S2 were compared by qRT-PCR, which did not reveal a significant difference. These results suggest that the Thr-86-Ile mutation in GyrA was not accompanied by changes in *topA* sequence or expression.

**Effect of GyrA mutations on in vitro supercoiling.**

To determine the impact of the resistance-conferring mutations on the enzymatic activities of GyrA, we produced the recombinant forms of various gyrases. As estimated by SDS-PAGE, the recombinant GyrA and GyrB were 97 kDa and 87 kDa, respectively,
comparable with the calculated molecular masses of the two proteins (Fig. 2A). The mutant GyrA carrying Thr-86-Ile, Thr-86-Lys, or Asp-90-Asn mutation migrated at the same position as the wild-type GyrA on the SDS-PAGE (data was not shown), indicating that the point mutations in GyrA did not change its migration on SDS-PAGE. The supercoiling activity of the WT and mutant DNA gyrase was analyzed in vitro by using the recombinant proteins. Both GyrA and GyrB are required for supercoiling the plasmid DNA and no detectable activity was observed when only GyrA or GyrB was used in the assay (data were not shown). The mutant GyrA carrying the Thr-86-Lys or Asp-90-Asn mutation exhibited supercoiling activities comparable to that of the WT GyrA, whereas the supercoiling activity of the mutant GyrA carrying the Thr-86-Ile change was substantially reduced comparing to that of the wild-type GyrA. In fact, the most supercoiled band was absent with this mutant GyrA (Fig. 2B). The results were consistently shown in multiple experiments with different concentrations of gyrases (up to 1200 nM; data were not shown).

Effect of the GyrA mutations on in vivo supercoiling

The recombinant mutant GyrA with the thr-86-Ile mutation showed reduced supercoiling activity in vitro, but it is important to know if the same mutation also affects DNA supercoiling in vivo (in Campylobacter cells). For this purpose, we used the shuttle plasmid pRY107 as a reporter plasmid to monitor the relative levels of DNA supercoiling in the FQ and FQR strains. The plasmid pRY107 was transferred into 62301S2 (wild-type strain), 62301R33 (carrying the Thr-86-Ile mutation in GyrA), 52901-II2 (carrying the Thr-86-Lys mutation in GyrA), and 62301R37 (carrying the Asp-90-Asn mutation), which were
clonally related and all derived from strain S3B (26). Plasmid DNA was re-isolated from these constructs and analyzed by agarose gel electrophoresis in the presence of chloroquine. Under the condition utilized in this study (20 µg/ml chloroquine), negatively supercoiled forms migrated slower than relaxed topoisomers, which was confirmed by negatively supercoiled plasmid pBR322 and relaxed plasmid pBR322 (results not shown). Compared to the pRY107 from 62301S2, the plasmid topoisomers from 62301R33 harboring the Thr-86-Ile mutation in GyrA shifted to lower positions, indicating less supercoiling (Fig. 3A). As determined by densitometrical analysis, the percentage of the most supercoiled DNA in the total population of the plasmid topoisomers extracted from 62301S2 and 62301R33 was 87.4% and 20.2%, respectively. The plasmids from 52901-II2 and 62301R37 showed topoisomer distribution patterns similar to that of 62301S2 (Fig 3A). These results indicate that the GyrA mutant with the Thr-86-Ile mutation reduced DNA supercoiling in Campylobacter cells, while the mutants with the Thr-86-Lys or Asp-90-Asn changes in GyrA did not alter DNA supercoiling. To further confirm the impact of the Thr-86-Ile change on DNA supercoiling, we introduced pRY107 to a different strain background, NCTC 11168 and 11168CT. The only known difference between this pair of isolates is the C257T mutation in gyrA. Similar to the result from 62301R33, the plasmid topoisomers from 11168CT shifted to lower positions compared with NCTC 11168 (Fig 3B). Together, the in vivo supercoiling results were consistent with the findings from the in vitro supercoiling assay and indicated that the Thr-86-Ile mutation reduced DNA supercoiling in Campylobacter.

GyrA mutations reduce the susceptibility to ciprofloxacin
The effects of ciprofloxacin on the supercoiling activity of Campylobacter DNA gyrases were determined using the in vitro supercoiling assay. As shown in Fig. 4, the MECs of CIPRO to wild-type GyrA and the two mutants GyrA carrying the Thr-86-Lys or Asp-90-Asn change were 32 and 1024, respectively, indicating the two mutations significantly reduced the susceptibility of GyrA to the inhibition by CIPRO. With the mutant GyrA carrying the Thr-86-Ile change, the inhibitory effect of CIPRO was not measurable because the mutation itself abolished the ability of the enzyme to form the supercoiled band in the in vitro assay (Fig. 4). These findings provide a molecular basis for the reduced susceptibility of FQ<sup>R</sup> mutants to CIPRO.

5. Discussion

Antibiotic resistance-associated mutations often affect the physiological functions in bacteria and may impose a biological cost in the absence of antibiotics. The fitness cost associated with topoisomerase mutations has been reported in several bacteria (4, 14, 20, 51). In Salmonella typhimurium, the FQ<sup>R</sup> mutants selected in vitro or in vivo (chicken) showed different growth characteristics in culture medium and in the chicken host in the absence of FQs (14). The S. typhimurium mutants selected by in vitro plating were highly resistant to FQs, but grew significantly slower in culture medium and on solid media, and failed to colonize chickens. On the contrary, the in vivo selected resistant isolates exhibited intermediate susceptibility to FQs, had normal growth in liquid medium, and were able to colonize chickens as efficiently as or lower than that of the wild-type strains (14, 51). In the case with FQ<sup>R</sup> E. coli, single mutations in DNA gyrase or topoisomerase IV confer low-level resistance to FQs, while accumulation of multiple mutations in the enzymes result in high-
level resistance (4, 32). Interestingly, the single mutations were not associated with fitness reduction, but the mutants carrying multiple mutations showed a significant fitness disadvantage when assayed in competition experiments *in vitro* and in a mouse model (19). These findings suggest that resistance-conferring GyrA mutations generally impose a fitness cost in bacterial pathogens.

In contrast to the findings in other bacteria, our previous study using clonally related isolates and isogenic mutants generated by natural transformation showed that FQ$^R$ *Campylobacter* isolates outcompeted the FQ$^S$ strains in chickens in the absence of FQ antimicrobials and the enhanced fitness was linked to the single point mutation (C257T) in *gyrA*, which confers on *Campylobacter* a high-level resistance to FQ antimicrobials (26). In the previous study, the isogenic mutants were generated by natural transformation and ciprofloxacin was used to select for the isogenic transformants. There was a concern that ciprofloxacin might also have selected for spontaneous FQ$^R$ mutants with unknown compensatory mutations that could affect the fitness of *Campylobacter*. To address this concern, we further defined the role of the Thr-86-Ile mutation in GyrA in enhanced fitness by reverting the mutation back to the wild-type allele. In the creation of the revertant, a Cm$^R$ cassette, which was inserted into *cj1028c* upstream of *gyrA*, was used as selective marker to revert the mutant *gyrA* allele by homologous recombination. This strategy allowed us to test if a compensatory mutation was involved in the enhanced fitness of the FQ$^R$ mutants. The *in vivo* fitness study showed that once the Thr-86-Ile mutation in GyrA of 62301R33 was reverted, *Campylobacter* lost its fitness advantage in chickens, confirming the specific effect of the point mutation on fitness. In addition, introducing the Thr-86-Ile mutation into the
GyrA of the wild-type strain NCTC11168, which is divergent from S3B derivatives used in our previous work (26), also enhanced its fitness in chickens. Together, these findings conclusively establish that the C257T mutation in gyrA enhances fitness of C. jejuni in chickens.

How the Thr-86-Ile mutation affects Campylobacter fitness remains a question and was the main reason for performing in this study. Gyrase introduces and maintains negative supercoils in DNA, which is important for DNA replication and transcription. Thus, resistance-conferring mutation in GyrA may affect its enzymatic function. Indeed, it has been shown in other bacteria that antibiotic resistance-conferring mutations in GyrA affected the supercoiling activity of the enzyme (5, 20). A previous study conducted by Barnard et. al. showed that a FQ\textsuperscript{R} E. coli mutant carrying single (Ala\textsuperscript{87}) or double mutations (Ala\textsuperscript{83} Ala\textsuperscript{87}) in GyrA exhibited reduced supercoiling compared to that of the wild type (5). In Pseudomonas aeruginosa, it was also shown that a FQ\textsuperscript{R} GyrA Ile\textsuperscript{83} mutant and a Tyr\textsuperscript{87} mutant had decreased supercoiling, which was associated with reduced growth rate (20). In this study, we demonstrated that in Campylobacter the Thr-86-Ile change in GyrA resulted in a greatly reduced supercoiling activity compared to that of the wild-type enzyme (Fig. 2). The change in the enzymatic activity was further confirmed by the reduced supercoiling status in Campylobacter cells (Fig. 3). These findings suggest that development of FQ resistance impacts DNA supercoiling homeostasis within Campylobacter. Despite this effect, FQ\textsuperscript{R} Campylobacter grow well \textit{in vitro}, can colonize and persist in chickens as efficiently as the FQ\textsuperscript{S} strains in the absence of antibiotic usage when monoinoculated into the chickens, and can outcompete the FQ\textsuperscript{S} parent strains when coinoculated into chickens.
In contrast to the Thr-86-Ile mutation, the supercoiling activity of the mutant gyrases harboring the Thr-86-Lys or Asp-90-Asn mutation was comparable to that of the wild-type gyrase (Fig. 2), indicating that these two types of mutations did not affect the function of the enzyme. Interestingly, the Thr-86-Ile and Thr-86-Lys mutations occurred at the same place, but had a different impact on the enzyme function. This is probably due to the fact that Ile and Lys have different chemical properties. Ile is aliphatic, hydrophobic and neutral, while Lys is polar, hydrophilic, and positively charged. In Campylobacter GyrA, Thr-86 is equivalent to Ser-83 in Escherichia coli GyrA(47). It has been known that Ser-83 is located in the QRDR region, which are situated close to the catalytic cleavage residues Tyr122, and Ser-83 has an important role in the interaction of gyrase-quinolone-DNA complex (5). Thus changes at Ser-83 (Thr-86 in Campylobacter) can conceivably prevent FQ binding, but is also accompanied by altered supercoiling activities of the gyrase.

The results presented in this study strongly indicate that the Thr-86-Ile mutation impacts DNA supercoiling in Campylobacter. It would be interesting to know if FQ_R mutants acquire compensatory mutations to offset the impact of the Thr-86-Ile mutation. In Campylobacter, the genes encoding topoisomerase IV (parC/parE) are absent (36), and supercoiling homeostasis is controlled by topoisomerase I (TopA) and topoisomerase II (GyrA and GyrB). Our previous study has shown that the FQ_R isolates had no compensatory mutations in GyrA and GyrB (26). Since topoisomerase I, encoded by topA gene, is involved in the relaxation of DNA supercoiling and also has an important role in the maintaining of the topological state of DNA, it is necessary to see if there is any compensatory mutation in
topA and whether the expression of the topA is changed that contributes to the enhanced fitness. Our studies showed that the primary sequences of topA gene were identical for 62301S2 and 62301R33 and there was no difference in the expression of topA between these two strains, which excludes the possibility that mutations or altered expression of topoisomerase I is involved in the fitness change associated with FQ\textsuperscript{R} Campylobacter.

The MIC is the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism after incubation, while the MEC is the lowest concentration of a drug that shows any inhibition of supercoiling activity (15). In this study, we demonstrated that the mutant gyrases carrying the Thr-86-Lys or Asp-90-Asn mutation had significantly higher MECs of CIPRO than the wild-type GyrA, indicating the mutant gyrases are more resistant to CIPRO. Interestingly, the MECs of CIPRO with the enzymes were significantly (at least 250 times) higher than the MICs of CIPRO in these strains. This finding was consistent with the results from other studies (15, 29) that MECs and MICs differ by one or two orders of magnitude. The \textit{in vitro} results (Fig. 4) provide a molecular explanation for the resistance of FQ\textsuperscript{R} Campylobacter mutants to FQ antimicrobials.

To our knowledge, this is the first report that a FQ\textsuperscript{R}-conferring mutation in gyrase reduces its supercoiling activity, but enhances the fitness of the mutant strain. This result is in contrast to the results from a recently published paper (20), in which reduced fitness of FQ\textsuperscript{R} mutants of \textit{Pseudomonas aeruginosa} was associated with decreased DNA supercoiling. Usually decreased supercoiling causes an increased accumulation of positive supercoils in front of a replication/transcription, which in turn reduce the growth rate (20). This apparently
did not happen with FQ\textsuperscript{R} \textit{Campylobacter} because it grows as efficiently as FQ\textsuperscript{S} \textit{Campylobacter} in both culture media and animal hosts. The intriguing question is whether the reduced supercoiling activity of the gyrase affects \textit{Campylobacter} physiology and directly contributes to the enhanced fitness in chickens. DNA supercoiling is important for gene expression, and it is possible that the change in DNA supercoiling homeostasis affects the expression of certain genes in \textit{Campylobacter}. This possibility remains to be investigated in future studies.

6. References


**Table 1. Bacterial strains used in this study**

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### Table 2. Key PCR primers used in this study

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Fig. 1 Pairwise competition between FQ$^S$ and FQ$^R$ strains in chickens
Fig. 1. Pairwise competition between FQ\textsuperscript{S} and FQ\textsuperscript{R} strains in chickens. (A) The level of total *Campylobacter* in each chicken inoculated with 62301R33S (solid circle), 62301R33R (open circle), or 1:1 mixture of the two stains (half solid half open circle). (B) Differential enumeration of FQ\textsuperscript{S} (solid circle) and FQ\textsuperscript{R} (open circle) *Campylobacter* in the group inoculated with a mixture of 62301R33S and 62301R33R (left panel) and competition index of the competitive fitness (right panel). (C) The level of FQ\textsuperscript{S} (solid triangle) and FQ\textsuperscript{R} (open triangle) *Campylobacter* in the second chicken experiment in which the chickens were inoculated with a 1:1 mixture of 11168(S) and 11168(R) (left panel) and competition index of the competitive fitness (right panel). The mean colonization level of each group at a given time is indicated by a horizontal bar. The detection limit of the plating method is about 100 CFU/g of feces. DAI: days after inoculation.
Fig. 2. (A) SDS-PAGE analysis of purified recombinant GyrA and GyrB. Lane 1, Purified GyrB; Lane 2, Purified WT GyrA; Lane 3, Purified mutant GyrA (T86I); and Lane M, protein size markers. (B) Supercoiling activities of the WT gyrase and the three mutant gyrases measured by an *in vitro* assay. Lane 1, DNA ladder; Lane 2, relaxed pBR322 (control); Lane 3, WT GyrA+GyrB; Lane 4, mutant GyrA(T86I)+GryB; Lane 5, mutant GyrA(T86K)+GryB; Lane 6, mutant GyrA(D90N)+GryB; and Lane 7, supercoiled pBR322 (control). SC indicates supercoiled DNA and R represents relaxed DNA.
Fig. 3 Agarose gel electrophoresis analysis of plasmid topoisomers extracted from different strain background (A) 1 2 3 4 SC 87.4% 20.2% 88.8% (B) 1 2 SC 87.2% 25.8%
Fig. 3. Agarose gel electrophoresis analysis of plasmid topoisomers extracted from different strain backgrounds. The plasmid DNA was run on a 1.0% agarose gel containing 20 µg /ml chloroquine. (A) Plasmid pRY107 isolated from strain S3B derivatives. Lane 1, pRY107 from 62301S; Lane 2, pRY107 from 62301R (Thr-86-Ile mutation in GyrA); Lane 3, pRY107 from 52901-II2 (T86K mutation in GyrA); and Lane 4, pRY107 from 62301R37 (D90N). (B) Plasmid pRY107 isolated from strain 11168 backgrounds. Lane 1, pRY107 from NCTC 11168 and Lane 2, pRY107 from 11168CT (T86I mutation in GyrA). The results of densitometric scanning are shown to the right of each gel image. The numbers on the left of the densitometric scanning correspond to the lane numbers of the gel image. The number below each lane indicates the percentage of the most supercoiled DNA in the total population of plasmid topoisomers as measured by densitometry.
Fig. 4. Inhibition of DNA gyrase supercoiling activity by ciprofloxacin as measured by an *in vitro* supercoiling assay. A, WT GyrA+GyrB; B, mutant GyrA (Thr-86-Lys)+GyrB; C, mutant GyrA (Asp-90-Asn)+GyrB; and D, mutant GyrA (Thr-86-Ile)+GyrB. The numbers on the top of each panel are the concentration of ciprofloxacin (µg/ml) used in the reaction. SC indicates supercoiled DNA and R represents relaxed DNA.
CHAPTER 5. TRANSCRIPTOMIC AND PROTEOMIC COMPARISON OF CAMPYLOBACTER JEJUNI STRAINS THAT ARE RESISTANT OR SUSCEPTIBLE TO FLUOROQUINOLONE

A paper to be submitted to the Journal of Bacteriology

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1. Abstract

Our previous work showed that acquisition of fluoroquinolone (FQ) resistance via a specific GyrA mutation (Thr-86-Ile) enhances the in vivo fitness of FQ-resistant mutants in the absence of antibiotic selection pressure and the Thr-86-Ile change in GyrA both confers a high-level resistance to FQs and significantly reduces the supercoiling activity of the enzyme in Campylobacter. To understand how the reduced supercoiling activity of mutant DNA gyrase affects Campylobacter fitness, we initiated work to determine if this altered supercoiling activity affects the gene transcription and protein expression in the bacterial organisms. Using both DNA-microarray and 2-D DIGE (two dimensional differential in-gel electrophoresis) methods, we compared the transcription patterns and protein profiles between the FQ<sup>R</sup> and FQ<sup>S</sup> Campylobacter grown in laboratory media. Results revealed that the expression of multiple genes was changed in FQ<sup>R</sup> strains compared to FQ<sup>S</sup> stains. Notably, the iron-response system and proteins involved in energy metabolism were upregulated in FQ<sup>R</sup> Campylobacter, which may contribute to its enhanced fitness in the chicken host.

2. Introduction

Campylobacter jejuni is a major foodborne pathogen worldwide and causes diarrhea, abdominal pain, colitis and other clinical diseases in humans (40). Clinically, Campylobacter
infections are mild, self-limiting and usually resolve within a few days. No specific treatment is required for most patients with Campylobacter enteritis, but antibiotic treatment is needed in severe cases or in immunocompromised patients (2, 8, 9, 11, 14, 35). Fluoroquinolones (FQs) or erythromycin are the antibiotics of choice for treating Campylobacter infection (14, 35). However, Campylobacter has become increasingly resistant to FQs worldwide over the past 15 years, which is a major concern for public health (14, 21, 34, 45). Although FQ antimicrobials have been banned since 2005 in poultry production in the U.S., FQ-resistant (FQR) Campylobacter continues to persist in poultry farms (29, 37, 38).

In Campylobacter, resistance to FQs is mediated by a point mutation in the quinolone resistance-determining region (QRDR) of gyrA in conjunction with the function of the multidrug efflux pump CmeABC (6, 14, 17, 31). Usually antibiotic resistance-conferring mutations in target genes are associated with changes in physiological processes, resulting in reduced growth rate and fitness in the absence of antibiotic selection pressure (5). However, in Campylobacter FQR mutants carrying the Thr-86-Ile change in the GyrA subunit of DNA gyrase were able to colonize and persist in chickens as efficiently as the FQ-susceptible (FQS) strains in the absence of FQ and showed enhanced fitness in chickens. This altered phenotype was directly linked to the resistance conferring mutation and was confirmed by using isogenic mutants as well as revertants (30).

DNA gyrase plays an essential role in maintaining the topological configuration of DNA by introducing negative superhelical turns in DNA. Our previous study has shown that the Thr-86-Ile mutation in GyrA greatly reduced the supercoiling activity of DNA gyrase both in vitro and in vivo (within Campylobacter cells). Whether the changed supercoiling function of the mutant gyrase is responsible for the enhanced fitness is unknown. It has been well established that DNA supercoiling affects gene transcription in bacterial organisms (1). DNA supercoiling is a key player in regulating bacterial adaptive responses to environmental challenges such as stress. A number of studies have shown that changes in environmental conditions such as temperature, osmolarity, and oxidative stress affect DNA supercoiling and consequently alter the expression of a number of genes involved in the adaptation to the
changes (12, 42). DNA topological changes are also linked to intracellular adaptation, expression of virulence factors such as surface proteins, and invasins of pathogenic organisms (3, 15, 32). In *E. coli*, alteration of DNA supercoiling by mutations in TopA and GyrB affects the relative abundance of more than 80 proteins (41). Based on the available evidence, we hypothesize that the reduced supercoiling activity of mutant DNA gyrase (Thr-86-Ile mutation in GyrA) alters gene expression in the *Campylobacter* cells, which contributes to the enhanced in-host fitness.

To examine our hypothesis, we used whole-genome microarray to compare the transcription patterns in FQ<sup>S</sup> and FQ<sup>R</sup> strains. Microarray results identified 23 genes with ≥1.5-fold (*p* < 0.05) changes in expression, among which 17 were up-regulated and 5 were down-regulated in FQ<sup>R</sup> mutant (62301R33) compared with FQ<sup>S</sup> (62301S2). In addition, we conducted 2-D DIGE (two dimensional differential in-gel electrophoresis) to compare the protein profiles of FQ<sup>R</sup> (62301R33R) and FQ<sup>S</sup> (62301R33S) *Campylobacter*. The 2-D DIGE results revealed 42 protein spots that were significantly differentially expressed in 62301R33R compared with 62301R33S, among which 39 were up-regulated and 3 were down-regulated. Ten spots of interest were identified by MALDI-TOF/TOF.

3. Materials and Methods

*Bacterial strains and growth conditions*

The various *Campylobacter* strains used in this study are listed in Table 1. The *Campylobacter* strains were routinely grown in Mueller-Hinton (MH) broth (Difco) or on MH agar plates at 42°C under microaerobic conditions (10% CO<sub>2</sub>, 5% O<sub>2</sub>, and 85% N<sub>2</sub>).

*Bacterial RNA isolation*

*Campylobacter* isolates 62301S2 and 62301R33 were grown in MH broth for 48 hours (OD<sub>600</sub> ≈ 0.45) and were immediately treated with 2 volumes of RNAprotect Bacterial Reagent (Qiagen, Valencia, CA) to stabilize the total bacterial RNA. After incubation for 10
min at room temperature, the culture was centrifuged at 10,000 × g for 10 min. Bacterial RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Isolated RNA was treated with an on-column DNase Digestion Kit (Qiagen), which was followed by removal of the DNase using an RNeasy Mini Kit (Qiagen). The absence of DNA contamination in the RNA samples was confirmed by reverse transcription-PCR (RT-PCR). The concentration of total RNA was estimated with the NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA), and the integrity and size distribution of the purified RNA was determined by denaturing agarose gel electrophoresis and ethidium bromide staining. The quality of total RNA was further analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), which showed the good quality and integrity of the RNA samples (Data not shown). The purified RNA was kept at −80 °C until it was used. RNA samples extracted from three independent experiments were used for the microarray hybridization experiments.

**cDNA synthesis and labeling**

cDNA synthesis and fluorescence labeling were performed using ChipShot™ Indirect cDNA Labeling and Clean-Up Protocols (Promega) according to the manufacturer's instructions. Briefly, 5µg of total RNA was reverse transcribed using ChipShot™ reverse transcriptase, random primers and Oligo(dT) primers in the presence of an aminoallyl-dNTP mix together with 5X reaction. After incubation at 42°C for 2 hours, RNase H was added to the reaction mix to degrade RNA. The synthesized aminoallyl-cDNA was purified using ChipShot™ Membrane columns (Promega) to remove unincorporated nucleotides and eluted with 65µl of 100mM sodium bicarbonate (pH 9.0). Sixty µl purified aminoallyl cDNA were then labeled by coupling with one dried aliquot of post-labeling CyDye™ NHS ester, Cy3 or Cy5 (GE Healthcare Bio-Sciences). A final purification step using ChipShot™ Membrane Columns (Promega) removed unreacted dyes, and the amount of labeled cDNA was estimated with the NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). The pmol of dye incorporated and the frequency of incorporation (FOI) were calculated accordingly. The eluted cDNA with good quality was stored in a lightproof container at 4 °C until it was used to hybridize microarray slides.
Microarray hybridization

*C. jejuni* NCTC 11168 microarray slides were purchased from Ocimum Biosolutions. The array contained 1,632 oligonucleotide (50-mer) probes covering the entire transcriptome of NCTC 11168. To compare the gene expression of FQ\(^R\) strain (C257T mutation in *gyrA*) with that of FQ\(^S\) strain, equal volumes of Cy3- or Cy5-labeled cDNAs from the 62301S2 strain and 62301R33 strain were combined and dried with a SpeedVac and then resuspended in 120 µl of hybridization buffer (Ocimum Biosolutions). The probes were denatured at 95°C for 3 min, briefly cooled on ice, and then hybridized to the microarray slide using microarray gene frames (Ocimum Biosolutions). The slide were placed in a wet hybridization chamber and incubated on a shaker for 20 h at 42°C. After hybridization, the slide was washed sequentially with wash buffer 1 (2x SSC, 0.1 % SDS) for 10 min, wash buffer 2 (1 x SSC) for 10 min, and wash buffer 3 (0.1 x SSC) for 10min. Each wash step was repeated three times. The washing buffers were prewarmed at 32°C, and all of the washing steps were performed at 32°C. Finally, the slides were dried by centrifugation at 500 × g for 3 min. The microarray slides were hybridized with the swaped-dye labeled cDNA samples to ensure dye balance in the experimental design. The hybridization experiments were performed using RNA extracted from three biological replicates and each of the experiments consisted of two technical replicates (slides).

Data collection and analysis

Hybridized slides were scanned at a wavelength of 650 nm for Cy5 and at a wavelength of 550 nm for Cy3 using a General Scanning ScanArray 5000 (PerkinElmer, Boston, MA) at 10 µm resolution. The fluorescence intensities were collected with the ImaGene software (BioDiscovery, El Segundo, CA). Genes with fluorescence signals within less than 2 standard deviations of the background were considered insignificant. Background subtraction, Lowess normalization, scale normalization, and median centering were performed using the R statistical package (version 2.0.1; The R Foundation for Statistical Computing) and the normalized data were subjected to a mixed-linear model analysis using
the SAS statistical package. For this study, we chose a P value of < 0.05 and a change equal to or greater than 1.5-fold as the cutoff for significant differential expression in comparisons of the FQ<sup>R</sup> and FQ<sup>S</sup> strains.

**Real-time quantitative PCR (qRT-PCR)**

Representative genes identified by the DNA microarray were further confirmed by Real-time quantitative PCR (qRT-PCR) as described in a previous work (27). Before being used for quantitative reverse transcriptase PCR (RT-PCR), all RNA templates and primer sets were tested with a conventional One-step RT-PCR Kit and a regular PCR kit (Invitrogen) to ensure specific amplification from the target mRNA and no detectable DNA contamination in the RNA preparation. The RT-PCRs were conducted using the iScript one-step RT-PCR kit with SYBR green (Bio-Rad). Thermal cycling conditions were as follows: 10 min at 50°C, 5 min at 60°C followed by 5 min at 95°C, and then 40 cycles of 10 s at 95°C and 30 s at 58°C. Samples were normalized using 16S RNA as an internal standard. Cycle threshold values were determined with the MyiQ software (Bio-Rad). The relative changes (n-fold) of specific genes between the FQ<sup>R</sup> and FQ<sup>S</sup> samples were calculated using the 2<sup>−ΔΔCT</sup> method as described by Livak and Schmittgen (28). Three independent PCR experiments were performed to get the mean of the fold change.

**Proteome analysis by 2-D DIGE**

2-D DIGE was performed by Applied Biomics (Hayward, CA). Briefly, overnight fresh bacterial cells 62301R33S and 62301R33R were harvested form MH plates and centrifuged. The cell pellets were washed with PBS 4 times and then were dissolved in 2-D lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS and 30 mM Tris-HCl, pH 8.5). The samples were stored at -80 °C until they were shipped on dry ice to Applied Biomics (Hayward, CA) for proteomic analysis. Total protein was extracted from lysed cells and equal amount of protein extract from 62301R33S and 62301R33R were labeled with the Cy3 or Cy5 dyes, (GE Healthcare, Piscataway, NJ) respectively. The labeled protein extracts were mixed and subjected to a single 2-D gel electrophoresis, using isoelectric focusing (IEF) in the first
dimension and SDS polyacrylamide gel electrophoresis (SDS-PAGE) in the second
dimension. After electrophoresis, the gel was scanned using a Typhoon Image Scanner (GE
Healthcare, Piscataway, NJ). Each scan revealed one of the CyDye signals (Cy3 and Cy5).
Scanned images were analyzed by Image Quant Software (version 5.0, GE Healthcare) and
then subjected to in-gel analysis using the DeCyder software (version 6.5, GE Healthcare).
The ratio change for differentially expressed protein spots was obtained from the in-gel
DeCyder software analysis. Protein spots of interest were picked up by Ettan Spot Picker
(GE Healthcare) following the DeCyder software analysis and spot picking design. The
selected protein spots were subjected to the in-gel trypsin digestion, peptides extraction,
desalting and followed by mass spectrometry (MALDI-TOF/TOF) (ABI 4700, Applied
Biosystems, CA). Peptide sequence information was used for protein identification in the
NCBIInr databases.

**SDS-PAGE and Immunoblotting**

To prepare whole-cell lysates, overnight culture of 62301S2 and 62301R33 were
grown in MH broth to late logarithmic phase (about 3 x 10^9 cells/ml), harvested by
centrifugation, and solubilized by boiling for 10 min in sodium dodecyl sulfate-
polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Approximately 10^8 whole
cells were loaded in each lane and separated by SDS-PAGE with a 12% (wt/vol)
polyacrylamide separating gel. After SDS-PAGE, the gels were equilibrated for 30 min in the
transfer buffer (0.025 M Tris base and 0.192 M glycine with 20% methanol; pH 8.3). Proteins
in the gels were then electrophoretically transferred to nitrocellulose membranes (Bio-Rad) at
60 V for 1 h on ice. The membranes were incubated with blocking buffer (5% Nestle skim
milk powder in PBS) for 4 h at 4°C prior to incubation with anti-FlaA. After incubation at
room temperature for 1 h, the blots were washed three times with PBS containing 0.05%
Tween 20 and subsequently incubated with secondary antibodies (goat anti-rabbit
immunoglobulin G-horseradish peroxidase; Kirkegaard & Perry) at room temperature for 1 h.
After washing, the blots were developed with the 4-CN Membrane Peroxidase Substrate
System (Kirkegaard & Perry). Prestained molecular mass markers (Bio-Rad) were
coelectrophoresed and blotted to allow estimation of the sizes of the proteins.
4. Results

**DNA microarray identified multiple genes differently expressed in the FQ\textsuperscript{R} mutant**

To compare the gene transcription patterns in FQ\textsuperscript{S} and FQ\textsuperscript{R} (Thr-86-Ile) strains, DNA microarray was used to analyze the differences in transcriptoms between 62301S2 and 62301R33. The two strains are clonally related and were well characterized in terms of genotypic and phenotypic characteristics (30). Microarray results showed 23 genes with \( \geq 1.5 \)-fold \((p < 0.05)\) changes in expression, among which 17 were up-regulated (Table 2) and 5 were down-regulated in FQ\textsuperscript{R} mutant (62301R33) compared with FQ\textsuperscript{S} (62301S2).

Notably, 11 (indicated by an asterisk in Table 2) of the 17 upregulated genes are known *Campylobacter* genes whose expression is increased in iron-limited conditions (24, 36). ChuA (Cj1614) which functions as a haemin receptor involved in iron uptake (43). Cj1613c encodes an orthology (39% aa identity) of HugZ, which is involved in heme iron utilization (20, 23, 43). ExbB1 (Cj0179) is a biopolymer transport protein. Cj1661 and cj0176c encode the ferric-binding protein of a putative ABC transport system. Hypothetical protein Cj0427 co-transcribed with Cj0426, which is a putative ABC transporter ATP-binding protein. Ahpc (Cj0334) is an alkyl hydroperoxide reductase. Cj1664 encodes putative periplasmic thioredoxin. Both Cj0334 and Cj1664 are involved in the oxidative stress response of *Campylobacter*. Cj1383c and cj1384c are tandemly positioned on the *Campylobacter* chromosome and encode hypothetical proteins of unknown function. Cj0089 encodes a putative lipoprotein. The other upregulated genes include flaA, which encodes the major subunit of flagellum and has an important role in the motility of *Campylobacter* (18, 44). Cj1002c encodes a putative phosphoglycerate/ bisphosphoglycerate mutase, which catalyzes reactions involving the transfer of phosho groups between the 3 carbon atoms of phosphoglycerate. Cj0843c encodes a putative secreted transglycosylase. Cj1626c and cj0200c encode putative periplasmic protein. The transcription of 7 genes selected from the microarray list was further confirmed by three independent qRT-PCR experiments (Table 2).
The down-regulated genes include \( cj0262c, cj0246c, cj1127c, cj1139c, \) and \( cj1180c \). pgIJ (Cj1127c) is a GalNAc transferase involved in the N-glycan biosynthesis in \( C.\) jejuni (26). \( Cj0262c \) encodes a putative methyl-accepting chemotaxis signal transduction protein. \( Cj0246c \) encodes a putative MCP (methyl-accepting chemotaxis protein)-domain signal transduction protein. \( Cj1139c \) encodes a putative galactosyltransferase. \( Cj1180c \) encodes a putative ABC transporter ATP-binding protein. None of the five down-regulated genes is among the ones known to be regulated by iron concentration.

**Proteomic analysis of 62301R33S and 62301R33R**

2-D DIGE was used to investigate the difference in the protein profiles between 62301R33S and 62301R33R. Using fold change \( \geq 1.5 \)-fold as cutoff, the Decyder analysis of 2D-DIGE image revealed 42 protein spots that were significantly differentially expressed in 62301R33R compared with 62301R33S (Figure 1 and 2), among which 39 were up-regulated and 3 were down-regulated (Table 3). Ten spots of interest were identified by MALDI-TOF/TOF using MASCOT search engines on NCBInr sequence database (Table 4). These proteins include flavodoxin (FldA), flagellar hook protein (FlgE), translation elongation factor Tu (Tuf), HAD-superfamily hydrolase subfamily IA variant 1 family protein, ribosomal proteins L23 and L7/L12, DNA-binding protein HU, F0F1 ATP synthase subunit epsilon, and a hypothetical protein. Flavodoxins are electron-transfer proteins that function in various electron transport systems. FlgE is a flagellar hook subunit protein. The function of the flagellar hook is to connect the filament to the basal body and acts as a joint to transmit the rotation of the rod of the basal body to the filament. Elongation factor Tu promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis. HAD-superfamily hydrolase, subfamily IA, variant 1 represents part of one structural subfamily of the HAD-superfamily of aspartate-nucleophile hydrolases. The function of F0F1-ATP synthase, which is composed of membrane-embedded F_{0}-portion and hydrophilic catalytic F_{1}-portion, is to couple ATP synthesis/hydrolysis with transmembrane proton transport. HU is a histone-like DNA-binding protein that plays an important role in bacterial nucleoid organization and is involved in numerous processes including transposition, recombination and DNA repair (10, 25). Ribosomal proteins L23 and L7/L12 are 50S...
ribosomal proteins. L23 is one of the proteins that surrounds the polypeptide exit tunnel on the outside of the ribosome and forms the main docking site for trigger factor binding to the ribosome. L7/L12 is a stalk proteins that implicated in the ribosomal interaction with the translocase (33).

5. Discussion

DNA gyrase plays an essential role in maintaining the topological configuration of DNA, which affects gene transcription in bacterial organisms and plays an important role in regulating bacterial adaptive responses to environmental challenges such as stress (1). Our previous study showed that the FQR-associated Thr-86-Ile mutation in GyrA greatly reduced the supercoiling activity of DNA gyrase both in vitro and in vivo (within Campylobacter cells). Whether the change in DNA supercoiling homeostasis alters the expression of certain genes in Campylobacter cells is unknown. To examine our hypothesis, we conducted transcriptome analysis to compare the mRNA level in FQS and FQR strains. One of the unregulated genes identified by DNA microarray is flaA, which encodes the major subunit of flagellum and is essential for the motility and colonization of Campylobacter (18, 44). Although 62301R33 and 62301S2 have similar motility, and both have intact flagellar structures when observed by electron microscopy, it is still possible that the increased expression of flaA may have functional consequences in vivo by facilitating the adaptation of 62301R33 in the intestine, contributing to the enhanced fitness. However, the difference in the expression level of FlaA was be detected using immunoblotting (data not shown). This suggests that the transcriptional change is not reflected at the translational level.

One interesting finding from the microarray results is that among the 17 upregulated genes in 62301R33, 11 are known to be controlled by the Fur regulator and their expression is increased in iron-limited conditions (24, 36). One of the upregulated genes is ChuA (Cj1614) which functions as a haemin receptor involved in iron uptake. Inactivation of the chuA gene rendered Campylobacter unable to grow in media in which haemin was the sole iron source (43). Cj1613c, which is immediately upstream of chuA and divergently transcribed, encodes an orthology (39% aa identity) of HugZ, which is involved in haeme...
utilization (20, 23, 43). Recently, Pfam domain pyridoxamine 5'-phosphate oxidase, which is a flavin mononucleotide (FMN) flavoprotein that catalyses the oxidation of pyridoxamine-5-P (PMP) and pyridoxine-5-P (PNP) to pyridoxal-5-P (PLP), was identified within CDS of Cj1613 (19). ExbB1 (Cj0179) is a biopolymer transport protein and it also involved in transport of iron. Cj1661 encodes the ferric-binding protein of a putative ABC transport system. The protein encoded by Cj0426 and Cj0427, which overlap 10 nucleotides and are co-transcribed, are likely to form an ABC transport system. Although cj0426 is not on the list of known genes regulated by iron stress, expression of cj0427 was increased under iron-limited conditions (24), suggesting that cj0426 might also be subject to iron regulation. Cj0334 and Cj1664 encode proteins involved in oxidative stress response, which is known to be regulated by iron concentration (7, 43). AhpC (Cj0334) is an important determinant of the ability of Campylobacter to survive oxidative and aerobic stress (7). Insertional mutagenesis of the ahpC gene resulted in an increased sensitivity to oxidative stresses. The iron-regulated expression of AhpC might also be significant in survival of Campylobacter in the intestinal epithelium of infected chickens (7). These results strongly suggest that the iron-response system is enhanced in FQR Campylobacter. Since iron is essential for microbial physiology and free iron is limited to the bacterial organism in animal hosts (24, 36), it is possible that the changed DNA supercoiling homeostasis in FQR Campylobacter increases the expression of the genes involved in iron stress response, which facilitate the iron acquisition and subsequently contributes to the enhanced in-host fitness.

We also used 2-D DIGE method to compare the protein profiles between the FQR and FQS Campylobacter grown in laboratory media. 2-D DIGE is an advanced version of classical two-dimensional gel electrophoresis (2-D PAGE). Compared to classical 2-D PAGE, 2-D DIGE has many advantages. First, in 2-D DIGE, the protein samples are pre-labeled with fluorescent dyes (CyDye) and then separated by 2D-PAGE. Different protein samples are labeled with different fluorescent dyes, mixed together and separated by the identical gel. Analyzing multiple samples on one 2-D DIGE rather than running single samples on individual 2D-PAGE gels reduces experimental gel-to-gel variations, which are the most severe problem in the gel-based proteomics. Second, fluorescent dyes (CyDye technique)
used in 2-D DIGE have much more sensitivity than Coomassie blue and silver staining used in traditionally 2D-PAGE gels. Third, with silver and Coomassie staining, the gels need to be fixed and stained / destained using time-consuming techniques. Gels are then compared using computer software for changes in protein expression. However with DIGE, immediately after completion of electrophoresis each gel is scanned multiple times by laser scanners with different wavelengths for different CyDye used. The multiple images obtained by 2-D DIGE are analyzed by the image software such as DeCyder software. The normalization of Cy5 intensity with Cy3 intensity is automatically achieved for all protein spots by the software and subtle changes in protein expression detected very accurately. Using proteomics, we identified 42 protein spots that were differentially present between 62301R33R and 62301R33S. Among the 10 proteins that were upregulated in 62301R33R, 3 are involved in energy metabolism and 5 are involved in macromolecule metabolism. Two of the most upregulated proteins were both identified as flavodoxin (FldA), which is a small FMN-containing electron transferases (16). It functions as the electron acceptor of the pyruvate-oxidoreductase complex (POR) that catalyzes pyruvate-oxidative decarboxylation to produce NADPH (39). Several studies have shown that flavodoxin is an essential protein for the survival of bacteria. Inactivation of FldA precludes the survival of Helicobacter pylori, which is a microaerophbic pathogen that is a close relative to C.jejuni (13). fldA is a vital gene in E. coli and a FldA mutation is lethal for E. coli under both aerobic and anaerobic stress conditions (16). In Campylobacter, the expression of fldA is also upregulated in iron-limited conditions (24). This result plus the results from DNA microarray strongly suggest that the iron-response system is enhanced in FQ Campylobacter.

No significant correlation between the microarray data and proteomic data was found in this study. Since posttranscriptional mechanisms control the turnover of proteins and the posttranscriptional modifications of proteins, it is not surprising to see the inconsistency between the mRNA and protein data. Similar findings have been reported in other studies (4, 22). Since the whole genome sequence of C.jejuni S3B (from which all of the isolates used in this study were derived) is not available, we used the DNA microarray designed from strain NCTC 11168. Thus there is a possibility that the genes that are uniquely present in the S3B
series may not be detected by the microarray even though their expression is altered in 62103R33. On the other hand, proteomics is capable of identifying all protein spots that show differential production between the paired strains. Due to budget constraints, we were not able to identify all of the 42 protein spots. Further work is needed to identify the remaining spots, which might reveal genes that were detected by the DNA microarray. In addition, whole cell proteins were used in the proteomics work, which might reduce the sensitivity of detecting expression changes of low-abundance proteins. Thus, fractioning cell proteins prior to 2-D DIGE may be needed to further enhance the detection sensitivity of the proteomics method. Nevertheless, our DNA microarray and proteomics studies revealed multiple changes in gene expression and protein production in FQ R Campylobacter carrying the Thr-86-Ile mutation in GyrA. Future research will be needed to assess the contribution of the altered gene expression to the enhanced fitness of FQ R Campylobacter in chickens.

6. References


Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>S3B</th>
<th>nt mutation in gyrA</th>
<th>aa mutation in GyrA</th>
<th>Genotype</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FQ&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62301S2</td>
<td>None</td>
<td>None</td>
<td>gyrA (Thr-86)</td>
<td>0.125</td>
</tr>
<tr>
<td>strain</td>
<td>62301R33S</td>
<td>None</td>
<td>None</td>
<td>Cj1028c::Cm&lt;sup&gt;r&lt;/sup&gt;; gyrA (Thr-86)</td>
<td>0.125</td>
</tr>
<tr>
<td>FQ&lt;sup&gt;r&lt;/sup&gt; strains</td>
<td>62301R33</td>
<td>C257T</td>
<td>Thr-86-Ile</td>
<td>gyrA (Ile-86)</td>
<td>&gt;32</td>
</tr>
<tr>
<td></td>
<td>62301R33R</td>
<td>C257A</td>
<td>Thr-86-Ile</td>
<td>Cj1028c::Cm&lt;sup&gt;r&lt;/sup&gt; gyrA (Ile-86)</td>
<td>&gt;32</td>
</tr>
</tbody>
</table>
Table 2. Genes upregulated in the FQ<sup>R</sup> strain identified by DNA microarray

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Description</th>
<th>Bayesian P</th>
<th>Fold change (Microarray)</th>
<th>Fold Change (RT-PCR)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Cj1339c</td>
<td><em>flaA</em>; encoding the major flagellin subunit</td>
<td>3.4</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>*Cj1613c</td>
<td>Ortholog of <em>hugZ</em> involved in haemin utilization</td>
<td>2.9</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>Cj0426</td>
<td>Probable ABC transporter ATP-binding protein</td>
<td>2.0</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>*Cj0427</td>
<td>Hypothetical protein; co-transcribed with Cj0426</td>
<td>1.7</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>*Cj1614</td>
<td>chuA, Haemin uptake system outer membrane receptor</td>
<td>2.1</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>*Cj1384c</td>
<td>Hypothetical protein</td>
<td>2.2</td>
<td>2.0</td>
<td></td>
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<tr>
<td>Cj1002c</td>
<td>Probable ABC transporter ATP-binding protein</td>
<td>2.4</td>
<td>2.5</td>
<td></td>
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<tr>
<td>*Cj1383C</td>
<td>Hypothetical protein</td>
<td>2.1</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>*Cj0176C</td>
<td>Ferric-binding protein</td>
<td>2.0</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>*Cj0334</td>
<td>AhpC (involved in oxidative stress)</td>
<td>1.6</td>
<td>NT</td>
<td></td>
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<tr>
<td>*Cj0179</td>
<td>ExbB1; transport protein</td>
<td>1.9</td>
<td>NT</td>
<td></td>
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<tr>
<td>*Cj1661</td>
<td>Putative ABC transporter protein; ferric-binding protein</td>
<td>1.8</td>
<td>NT</td>
<td></td>
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<td>*Cj1664</td>
<td>Thioredoxin</td>
<td>1.9</td>
<td>NT</td>
<td></td>
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<tr>
<td>*Cj0089</td>
<td>Probable lipoprotein</td>
<td>1.6</td>
<td>NT</td>
<td></td>
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<td>Cj1626c</td>
<td>Probable periplasmic protein</td>
<td>1.8</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>Cj0200c</td>
<td>Probable periplasmic protein</td>
<td>1.7</td>
<td>NT</td>
<td></td>
</tr>
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<td>Cj0843c</td>
<td>Probable secreted transglycosylase</td>
<td>1.6</td>
<td>NT</td>
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</table>

* Genes whose expression is known to be upregulated under iron-limited conditions.
# Means of three independent PCR experiments. NT, not tested.
### Table 3. Differentially expressed protein spots between 62301R33R and 62301R33S identified by 2-D DIGE

<table>
<thead>
<tr>
<th>Assigned spot No.</th>
<th>Volume Ratio (62301R33R/62301R33S)</th>
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<tr>
<td>1</td>
<td>1.66</td>
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<tr>
<td>2</td>
<td>1.68</td>
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<tr>
<td>3</td>
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<td>6</td>
<td>1.84</td>
</tr>
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<td>7</td>
<td>1.8</td>
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<tr>
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<td>1.84</td>
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<td>9</td>
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<td>19</td>
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<td>38</td>
<td>2.69</td>
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Table 3. Differentially expressed protein spots between 62301R33R and 62301R33S identified by 2-D DIGE (Continued)

<table>
<thead>
<tr>
<th>Assigned spot No.</th>
<th>Volume Ratio $(62301R33R/62301R33S)$</th>
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<tbody>
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<td>39</td>
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<tr>
<td>40</td>
<td>1.72</td>
</tr>
<tr>
<td>41</td>
<td>1.6</td>
</tr>
<tr>
<td>42</td>
<td>1.82</td>
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<tr>
<td>43</td>
<td>1.48</td>
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</table>
### Table 4. Differentially expressed proteins between 62301R33R and 62301R33S identified by 2-D DIGE

<table>
<thead>
<tr>
<th>Assigned Spot No.</th>
<th>Top Ranked Protein Name(Species)</th>
<th>NBCInr accession No.</th>
<th>Protein MW</th>
<th>Protein PI</th>
<th>No. of matched peptides</th>
<th>Protein Score</th>
<th>Protein Score C.I.%</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>flagellar hook protein FlgE [C. jejuni RM1221]</td>
<td>gi</td>
<td>57236913</td>
<td>89851</td>
<td>4.9</td>
<td>25</td>
<td>352</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>translation elongation factor Tu [C. coli RM2228]</td>
<td>gi</td>
<td>57504721</td>
<td>43580.2</td>
<td>5.11</td>
<td>22</td>
<td>505</td>
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<tr>
<td>20</td>
<td>hypothetical protein CJE0800 [C. jejuni RM1221]</td>
<td>gi</td>
<td>57237003</td>
<td>25979.7</td>
<td>4.44</td>
<td>12</td>
<td>498</td>
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<tr>
<td>23</td>
<td>HAD-superfamily hydrolase, subfamily IA, variant 1 family protein [C. jejuni RM1221]</td>
<td>gi</td>
<td>57238105</td>
<td>23722.1</td>
<td>6.08</td>
<td>11</td>
<td>229</td>
<td>100</td>
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<tr>
<td>28</td>
<td>flavodoxin FldA [C. jejuni RM1221]</td>
<td>gi</td>
<td>57238425</td>
<td>17124</td>
<td>3.89</td>
<td>7</td>
<td>486</td>
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<tr>
<td>31</td>
<td>flavodoxin FldA [C. jejuni RM1221]</td>
<td>gi</td>
<td>57238425</td>
<td>17124</td>
<td>3.89</td>
<td>4</td>
<td>168</td>
<td>100</td>
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<tr>
<td>41</td>
<td>F0F1 ATP synthase subunit epsilon [C. jejuni RM1221]</td>
<td>gi</td>
<td>57237115</td>
<td>13683</td>
<td>4.85</td>
<td>6</td>
<td>311</td>
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<tr>
<td>42</td>
<td>DNA-binding protein HU [C. jejuni subsp. jejuni 84-25]</td>
<td>gi</td>
<td>88596497</td>
<td>6393.3</td>
<td>6.07</td>
<td>3</td>
<td>220</td>
<td>100</td>
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<tr>
<td>43</td>
<td>50S ribosomal protein L7/L12 [C. jejuni RM1221]</td>
<td>gi</td>
<td>57237531</td>
<td>13022.9</td>
<td>4.7</td>
<td>7</td>
<td>255</td>
<td>100</td>
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</tbody>
</table>
Fig. 1. Proteomic analysis of protein expression in *Campylobacter*. The image shows the overlay of 2-D DIGE patterns of 62301R33R versus 62301R33S. 62301R33R is in red and 62301R33S is in green. The 42 differentially expressed protein spots were circled. Red indicates increased expression in 62301R33 and green indicates decreased expression in 62301R33 in comparison to 62301R33S. Yellow indicates similar expression levels of between the two strains.
Fig. 2. Representative DeCyder display of relative abundance of selected protein spots in 62301R33R versus 62301R33S (fold change in parentheses). A: FldA (17.52); B: translation elongation factor Tu (3.65); C: F0F1 ATP synthase subunit epsilon (1.60); and D: DNA-binding protein HU (1.82).
CHAPTER 6. GENERAL CONCLUSIONS

The increasing FQ resistance rate of *Campylobacter* isolated from food-producing animals is a continuing concern for food safety and public health. How to reduce the occurrence and spread of FQ<sup>R</sup> *Campylobacter* has become more and more important in the control of foodborne campylobacteriosis. Understanding the mechanisms underlying the rapid emergence of FQ<sup>R</sup> and the enhanced fitness of FQ<sup>R</sup> mutants in *Campylobacter* will help to reduce the occurrence and spread of FQ<sup>R</sup> *Campylobacter*.

In this project, we conducted a series of studies to determine how *Campylobacter* responds to FQ treatment, what facilitates the emergence of FQ<sup>R</sup> mutants in *Campylobacter*, and what are the molecular mechanisms contributing to the enhanced fitness in FQ<sup>R</sup> *Campylobacter*. In the first study, we examined the gene expression profiles of *C. jejuni* NCTC 11168 in response to treatment with ciprofloxacin (CIPRO) using microarray and found that 45 genes showed ≥1.5-fold (p <0.05) changes in expression when exposed to a suprainhibitory dose of CIPRO for 30 min. Most of the differentially-expressed genes involved in cell membrane biosynthesis and DNA repair are up-regulated, while the genes associated with cellular processes and energy metabolism are down-regulated following exposure to CIPRO. These findings suggest that *C. jejuni* modulates membrane biosynthesis, increases spontaneous mutation rates, and decreases metabolism in response to FQ treatment. One of the up-regulated genes was *mfd* (mutation frequency decline), which encodes a transcription-repair coupling factor involved in DNA repair. We found that in this study Mfd promotes the emergence of spontaneous FQ<sup>R</sup> mutants and the development of FQ<sup>R</sup> mutants under FQ treatments. These findings define a novel function of Mfd and significantly improve our understanding of the molecular mechanisms underlying the development of FQ<sup>R</sup> *Campylobacter*. In the second study, we formally defined the role of the Thr-86-Ile mutation of GyrA in the enhanced fitness FQ<sup>R</sup> *Campylobacter* in chickens by reverting the mutant allele. Then we conducted *in vitro* supercoiling assay using recombinant gyrase to assess the impact of various resistance-associated mutations on the enzymatic activities of DNA gyrase. We found that compared with that of the wild-type gyrase, the mutant gyrase with the Thr-
86-Ile change showed greatly reduced supercoiling activity, while other GyrA mutations, although reduced the susceptibility of Campylobacter to ciprofloxacin, did not affect the supercoiling activity of the gyrase. Subsequently we determined the impact of the GyrA mutations on in vivo supercoiling (within Campylobacter cells) using a reporter plasmid. The in vivo supercoiling result was consistent with the in vitro supercoiling findings and revealed that the Thr-86-Ile mutation altered the DNA supercoiling state in FQR Campylobacter. In the third study, we examined if the altered function of mutant gyrase affected gene and protein expression in Campylobacter. We compared the differences in transcriptomes and protein profiles between FQR and FQS strains using DNA microarray and 2-D DIGE. The microarray data and 2-D DIGE data showed that the expression of multiple genes was altered between the FQR strains and FQS strains. Especially, the iron-response system and proteins involved in energy metabolism were upregulated in FQR Campylobacter, which may contribute to its enhanced fitness in the chicken host. Together, these findings from this project have significantly improved our understanding of the molecular mechanisms underlying the development of FQR Campylobacter and the fitness of the FQR Campylobacter. Findings from these studies will facilitate the design of strategies to reduce the emergence and spread of FQR Campylobacter.
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