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Development, Stability, and Molecular Mechanisms of Macrolide Resistance in *Campylobacter jejuni*

Dave Bryson Caldwell
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To the Graduate Council:

I am submitting herewith a thesis written by Dave Bryson Caldwell entitled "Development, Stability, and Molecular Mechanisms of Macrolide Resistance in *Campylobacter jejuni*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Jun Lin, Major Professor

We have read this thesis and recommend its acceptance:

Kelly R. Robbins, Barton W. Rohrbach, Stephen P. Oliver

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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Development, Stability, and Molecular Mechanisms of Macrolide Resistance in *Campylobacter jejuni*

A Thesis Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Dave Bryson Caldwell
December 2007

DEDICATION

I would like to dedicate this thesis to my family, Dwight, Melisa, and Ashley Caldwell. Thank you for your unending support.

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ABSTRACT

Previous studies on macrolide resistance in *Campylobacter* were primarily focused on the isolates from various origins using *in vitro* systems. In this study, both *in vitro* and *in vivo* experiments were conducted to examine the development, stability, and genetic basis of macrolide resistance in *C. jejuni*. All *in vitro* and *in vivo* selected Ery^R mutants were derived from the same parent strain *C. jejuni* NCTC 11168. To determine if long-term exposure of low-level Ery^R *C. jejuni* to low-dose tylosin selects for high-level Ery^R mutants (MIC > 512 μg/ml), two low-level Ery^R mutants (MIC = 32 or 64 μg/ml) were used to inoculate chickens at 15 days of age in two independent experiments. Total and Ery^R *C. jejuni* populations in swabs collected at different time points were determined by differential plating and MIC test. The *in vitro* stability was tested by repeated subculturing of Ery^R mutants in Ery-free broth medium. The *in vivo* stability was tested by inoculating 3-day-old chickens (12-13 birds/group) receiving non-medicated feed with Ery^R mutants and collecting cloacal swabs from each chickens at 12, 22, 38, and 47 days of age. Total and Ery^R *C. jejuni* populations in culture (after 10, 20 and 33 passages) or swab were determined by differential plating and MIC test. Genomic DNA from each of 63 selected Ery^R mutants was used for PCR amplification and sequence analysis of 23S rRNA gene and ribosomal proteins L4 and L22. Mutation in CmeABC multidrug efflux pump was transferred to Ery^R strains to determine the role of CmeABC efflux pump in Ery resistance. Chicken studies showed that the length of exposure time to subtherapeutic level of tylosin is not a sole factor contributing to the emergence of highly Ery^R *Campylobacter*. Prolonged exposure of low-level Ery^R *C. jejuni* (MIC = 32 or 64 μg/ml) to tylosin did not select for highly Ery^R mutants. The low-

level Ery resistance (MIC = 32μg/ml) was stable after 10 passages *in vitro* but majority of *C. jejuni* were sensitive to Ery after 20 passages. The instability of low-level Ery resistance was also observed in chickens as early as 9 days postinoculation and Ery^R mutants were rarely isolated 35 days postinoculation. However, high-level Ery resistance (MIC > 512μg/ml) displayed remarkably stability *in vitro* and *in vivo*. All high-level Ery^R mutants selected *in vivo* displayed the A2074G mutation in 23S rRNA gene, distinct from the specific mutation (A2074C) observed in all highly Ery^R mutants selected *in vitro*. No mutations were observed in ribosomal proteins L4 for all *in vitro* selected Ery^R mutants but specific mutations in L4 (G74D or G57D) were widely found in low level Ery^R mutants selected *in vivo* (Ery MIC = 8-64 μg/ml). Insertion of three amino acids TSH at position 98 in L22 was only observed in mutants selected *in vitro* with Ery MIC ranging from 32-512 μg/ml. The CmeABC efflux pump worked synergistically with other mechanisms to confer Ery resistance in *C. jejuni*. Together, these findings indicated that *C. jejuni* utilize complex and different mechanisms to develop Ery resistance *in vitro* and *in vivo*.

Key Words: *Campylobacter*, macrolide resistance, development, stability, mechanisms

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1. REVIEW OF LITERATURE

Animal antibiotic use and antibiotic resistance in foodborne pathogens

The intensity of food animal production systems presents opportunities for the introduction and spread of disease. Therefore, antibiotics are often administered to food animals to improve health and growth performance. According to a recent estimate, 24.6 million pounds, over half of the antibiotics produced each year in the US, are used in agriculture (Mellon *et al*. 2001). In animal agriculture, antibiotics can be administered whenever symptoms appear in a few animals (therapeutic treatment), or in anticipation of symptoms (prophylactic treatment). In addition, some antibiotics are widely administered as feed additives at sub-therapeutic levels in food animals to enhance growth rates and improve feed efficiency (growth promotion) (Dibner & Richards 2005; Frost & Woolcock 1991). The antibiotics used for growth promotion are also called 'antibiotic growth promoter' (AGP) and have been used worldwide in food animals more than 50 years (Dibner & Richards 2005; Frost & Woolcock 1991). To date, many antimicrobials with different mechanisms of action have been used as AGPs in the food animal industry (Chapman & Johnson 2002; Frost & Woolcock 1991; Gaskins *et al.* 2002). In poultry, antibiotics including bacitracin, virginiamycin, tylosin, bambermycins, and lincomycin have been used as AGPs with no withdrawal period required (Animal Health Institute 2005; Chapman & Johnson 2002; Gaskins *et al.* 2002). Usually a combination of different AGPs instead of single AGP is used in feeds during a complete production cycle to maximize the efficacy of AGPs. In poultry, bacitracin was used more frequently than other antibiotics in the starter and grower feed while virginiamycin was used more

frequently in finisher feed (Chapman & Johnson 2002).

Foodborne human pathogens are increasingly resistant to antimicrobial agents, compromising the effectiveness of currently available drugs for treating human illness (White *et al*. 2002). Development of antimicrobial resistance in bacteria may also directly affect human disease development through various mechanisms such as increased virulence (Barza 2002). One of the driving forces behind the progression of antibiotic resistance is the selective pressure exerted by the widespread use of antimicrobial agents in food animal production (Teuber 1999; Wegener 2003a). Inappropriate use of antibiotics in agriculture can result in increased antibiotic-resistant organisms, not only among pathogens but also among commensal microflora of animals that can be subsequently transferred to human via the food chain (McEwen & Fedorka-Cray 2002; Wegener *et al.* 1997). This possibility is particularly strong with foodborne human pathogens, such as *Campylobacter* and *Salmonella*, which are primarily transmitted from animals to humans via contaminated food (Slutsker *et al*. 1998). Since person-to-person transmission rarely occurs with these foodborne human pathogens in developed countries, the primary sources of human infections with resistant bacteria are likely from food producing animals (White *et al*. 2002). Therefore, food animals can serve as a reservoir for resistant bacteria (Witte 2000) and/or resistant gene clusters which can then enter the environment and be transmitted to human pathogens (Heuer *et al.* 2006; Lipsitch *et al.* 2002).

Therapeutic use of antibiotics has been demonstrated to select drug resistant human pathogens in food animals (Aarestrup 2005; Angulo *et al.* 2004). Recent studies have shown an increase in *Campylobacter* resistance to fluoroquinolones (FQs), a drug of

choice for treating human infections, following the approval of FQs for treatment of food animal infections in 1995 (Gaudreau & Gilbert 2003; Hein *et al.* 2003; Nachamkin *et al.* 2002). Laboratories studies have demonstrated the emergence of FQ-resistant *Campylobacter* in experimental chickens (Luo *et al*. 2003; McDermott *et al*. 2002) and pigs (Delsol *et al*. 2004) treated with FQ antibiotics. Strikingly, FQ treatment of chickens infected with FQ-sensitive *Campylobacter* promoted the emergence of FQ-resistant *Campylobacter* mutants; almost all *Campylobacter* shed by chickens were FQ-resistant after just 3 days of enrofloxacin treatment (Luo *et al*. 2003). These findings highlight the need for the prudent use of FQ antibiotics. Consequently, to prevent and control *Campylobacter* resistance to FQs, the FDA issued a ban on the use of Baytril (a FQ used in animal) in poultry in 2005 (www.fda.gov/bbs/topics/news/2005/new01212.html). Evidence of an association between the use of gentamicin in food animals and gentamicin resistant *Enterococci* isolated from food of animal origin and humans was also recently reported. Resistant rates of more than 30% were reported on swine and poultry farms in Michigan, Wisconsin, and Indiana (Hershberger *et al.* 2005). The same gentamicin resistance genes present in *Enterococci* isolated from food animals were also found in isolates from food products of the same animal species (Donabedian *et al.* 2003).

Although it has been widely believed that the low dosages of AGP used for growth promotion are an unquantified hazard, long-term use of AGP in feed could exert great selection pressure for developing antibiotic-resistant bacteria (WHO, 2004) Epidemiological studies have strongly linked AGP application to the emergence of antibiotic-resistant bacteria, consequently posing a serious threat to public health (Wegener 2003a). For example, vancomycin resistant *Enterococci* have long been

associated with the use of avoparcin (a vancomycin analogue) as growth promoters in food animals (Bager *et al.* 1997; Wegener *et al.* 1999) Thus, Denmark banned all AGPs in 1998 and European Union member nations banned all AGPs in January 2006 (Dibner & Richards 2005; Wegener 2003b). However, in Europe, it has been observed that the levels of antibiotic resistance in animals and food, and consequently in humans, have been markedly reduced after the termination of AGP use (Wegener 2003b). In Switzerland, Boerlin *et al.* (2001) reported a clear decrease in *Enterococci* resistance to macrolides, lincosamides, and tetracycline after the ban of AGPs. In Tawain, the occurrence of vancomycin resistant *Enterococci* decreased in association with a ban on avoparcin as a feed additive on chicken farms (Lauderdale *et al*. 2007). Other studies also noted a decrease in vancomycin- resistant enterococci following the ban on avoparcin as a growth promoter, although the resistant strains did not disappear completely (Heuer *et al.* 2002; Borgen *et al.* 2001). Furthermore, several studies have also noted a reduction of vancomycin resistant *Enterococci* in food-animal products and humans following AGP ban in food animals (Klare *et al.* 1999; van den Bogaard *et al.* 2000; Witte 2000; Bager *et al.* 1999; Pantosti *et al.* 1999). Together, these findings provide compelling evidence for the role of AGPs in selecting resistant bacteria. Using experimental chicken model system, Lin *et al*. (2007) demonstrated that long-term use of a macrolide as a growth promoter selects for the emergence of erythromycin-resistant *Campylobacter* in animal reservoirs. However, chickens subjected to single or multiple treatments with therapeutic dose of macrolide did not select for erythromycin-resistant *Campylobacter* (Lin *et al*. 2007).

There is a worldwide trend of limiting AGP use in food animals (Dibner &

Richards 2005). Although there has been little regulatory activity regarding AGP use in the United States, consumer pressure, market limitations, and export restrictions are pressuring commerce to withdraw AGPs from market (Dibner & Richards 2005). For example, KFC and McDonalds have claimed that they do not accept chicken meat grown using AGP with similar structure to the antibiotics used in humans in 2002 and 2005, respectively (Dibner & Richards 2005b).

Campylobacter colonization in poultry

Campylobacter species are the most common cause of human gastrointestinal enteritis worldwide, accounting for 2.5 million cases each year in the United States (Friedman *et al*. 2000). Each year in the United States, 1.5 to 8 billion dollars in medical and production costs are attributed to *Campylobacter* infections (Buzby *et al.* 1997). Human *Campylobacter* infections are characterized by abdominal pain, watery or bloody diarrhea lasting a few days, and also fever, headache, nausea, and vomiting (Skirrow & Blaser 2000). The diarrhea typically lasts a few days. Although complications are rare, infection with *Campylobacter* can lead to Guillain- Barré syndrome an acute autoimmune disease affecting the peripheral nervous system that can result in respiratory compromise and death (Nachamkin *et al*. 1998). Human *Campylobacter* illness is caused primarily by *C. jejuni* and secondarily by *C. coli*. Both *Campylobacter* species are closely related and require microaerophilic conditions (e.g. 5% O2, 10%CO2, and 85% N2) and elevated temperature (42 ^{o}C) for optimal growth.

Although *Campylobacter* widely colonizes wild and domestic animals, poultry are considered the major reservoir of *Campylobacter* (Nesbit *et al*. 2001). *Campylobacter* is

highly prevalent in poultry and most human infections have been linked to consumption of undercooked poultry products or other food contaminated by these products (Allos 2001). Other sources of human contamination include raw milk, undercooked beef or pork, and house pets (Corry & Atabay 2001). Interestingly, chickens mainly harbor *C. jejuni*, while turkeys tend to have more *C. coli*. Despite wide prevalence of *Campylobacter* in poultry, this organisms does not cause clinical signs of infection in poultry host under natural conditions (Newell & Fearnley 2003; Sahin *et al.* 2002).

Although prevalence estimates of *Campylobacter* vary, recent studies showed that 80- 100% of broiler flocks in the US were contaminated with *C. jejuni* (Croft *et al.* 2007; Jacobs- Reitsma 1997; Stern *et al.* 2001b). *Campylobacter* is highly prevalent in organic, free-range, and conventional poultry production systems (Avrain *et al.* 2003; Heuer *et al.* 2001; Luangtongkum *et al.* 2006; Luangtongkum *et al.* 2006), indicating that different production systems are equally vulnerable to invasion by this organism. The seasonality of *Campylobacter* infections, with a peak in the summer months, is also well documented (Evans & Sayers 2000; Sahin *et al.* 2002; Wedderkopp *et al.* 2000; Wedderkopp *et al.* 2001; Newell & Wagenaar 2000).

The prevalence of *Campylobacter* in broiler flocks depends on the age of the birds (Evans & Sayers 2000; Newell & Fearnley 2003). In commercial conditions, *Campylobacter* is rarely detected in broilers less than 2- 3 weeks of age (Stern *et al.* 2001a; Evans & Sayers 2000), although experimental inoculation of newly hatched chicks with *Campylobacter* can establish colonization successfully (Sahin *et al.* 2003b; Young *et al.* 1999). The reasons for this lag phase are unknown but might be attributed to multiple factors, such as presence of maternal antibodies, antibiotic feed additives,

intestinal development, and intestinal microbial flora (Newell & Wagenaar 2000; Sahin *et al.* 2003b).

Although *Campylobacter* can be isolated from most intestinal sites of broiler chickens, it is mainly found in the cecal and cloacal crypts where it does not adhere to epithelial cells but is found in the mucous layer (Achen *et al.* 1998; Beery *et al.* 1988). While more than one strain of *Campylobacter* has been isolated from the same flock (Hiett *et al*. 2002b), individual chickens are rarely infected with more than one strain (Korolik *et al*. 1998). Experimental inoculation of chickens has shown that colonization rate can be influenced by dose of inoculum and route of challenge (Sahin *et al*. 2002).

Once the first bird in a flock becomes colonized, infection spreads to the entire flock in just a few days. This rapid spread of *Campylobacter* throughout the flock is likely a result of fecal-oral transmission, compounded by communal water and feed (Lee & Newell 2006). In broiler chickens, colonization persists for the lifetime of the animal that is usually less than 47 days, consequently leading to carcass contamination at the slaughter facility.

Although the sources of infection of poultry flocks are still unclear, horizontal transmission (transmission within a population) is considered the most likely mechanism (Sahin *et al*. 2002). Some potential sources include feed, water, and carryover from previous flocks (Newell & Fearnley 2003). *Campylobacter* also exists in the environment surrounding the broiler house and could be transferred into the house by wildlife species, houseflies, insects, equipment, and farm workers (Sahin *et al*. 2002). However, molecular typing of *Campylobacter* isolates from various sources including broilers, houses, humans, and the environment surrounding the broiler house did not clearly identify the

sources of *Campylobacter* (Nesbit *et al.* 2001; Petersen *et al.* 2001a). *Campylobacter* is generally unable to persist in feed or water due to the stringent growing conditions (Jacobs- Reitsma 2000; Van De Giessen *et al.* 1998). Litter is also a potential but unlikely source of transmission (Stern *et al*. 2001b). Vertical transmission, although unlikely, has been suggested as a source of *Campylobacter* infection in broiler flocks. The 2-3 week lag phase presents evidence against vertical transmission. Also, broiler flocks are often infected with different strains than the corresponding breeder flocks (Petersen *et al*. 2001b). *Campylobacter* has not been found in hatcheries or young hatchlings, and isolation from eggs has also proven difficult (Hiett *et al.* 2002a; Sahin *et al.* 2003a).

Antibiotic resistance in *Campylobacter*

Most human *Campylobacter* infections are self-limited and treated with fluid replacement (Allos 2001). However, antimicrobial treatment is often necessary for severe, prolonged, or systemic infections, or infections in immunocompromised patients (Engberg *et al*. 2001). In the US and other developed countries, *Campylobacter* isolates resistant to multiple drugs have been cultured from clinical and food samples (Boonmar *et al.* 2007; Gallay *et al.* 2007; Moore *et al.* 2005). *Campylobacter* isolates are increasingly resistant to FQs and macrolides, the major drugs of choice for treating human campylobacteriosis (Engberg 2006; Friedman *et al.* 2000; Moore *et al.* 2005; Smith *et al.* 2002; van den Bogaard *et al.* 2000). *Campylobacter* could also acquire resistance to other antibiotics such as tetracycline, β lactam, chloramphenicol, and aminoglycosides (Trieber & Taylor 2000). *Campylobacter* species display intrinsic resistance to rifampin and trimethoprim (Trieber & Taylor 2000). Thus, these two

antibiotics are included as selective agents in *Campylobacter*- specific growth supplement (SR0117E; Oxoid). *Campylobacter* develops three general mechanisms of antibiotic resistance: 1) inactivation of antibiotic itself (e.g. acetylation of chloramphenicol) 2) target site alteration (e.g. mutations in ribosome affecting macrolide binding) 3) active drug efflux (e.g. *CmeABC* multidrug efflux pump) (Trieber & Taylor 2000).

Fluoroquinolones inhibit the activity of DNA gyrase and/ or DNA topoisomerase IV in bacteria (Drlica & Zhao 1997). There are two general mechanisms for *Campylobacter* resistance to FQs; which include modification of DNA gyrase and active efflux (Luo *et al.* 2003; Zhang *et al.* 2003). Specific mutations such as Asp-90-Asn and Thr-86-Lys in gyrase A were linked to FQ resistance in *C. jejuni* (Luo *et al.* 2003; Zhang *et al.* 2003). Unlike other bacteria, acquisition of high-level FQ resistance in *Campylobacter* does not require stepwise accumulation of point mutations in *gyrA*. Instead, a single step mutation in gyrA can create clinically relevant levels of resistance to FQs (Luo *et al.* 2003; Zhang *et al.* 2003). The simplicity of the *gyrA*-mediated resistance mechanism may facilitate the rapid emergence of FQ-resistant *Campylobacter* in response to FQ treatment (Zhang *et al.* 2003). It is still not clear why *C. jejuni* displays a hypermutable phenotype and FQ- resistant mutants emerge rapidly in infected chickens under the selective pressure of FQ antibiotics. A recent study indicated that the adaptive gene expression in *Campylobacter* may contribute to the rapid emergence of FQ-resistant mutants (Han & Zhang 2007). A multidrug efflux pump, named CmeABC, in *Campylobacter* contributes to both intrinsic and acquired FQ resistance in clinical isolates (Lin *et al.* 2002; Luo *et al.* 2003; Zhang *et al.* 2003). CmeABC is constitutively

expressed in *Campylobacter* and is essential for maintaining high-level FQ resistance. However, over-expression of CmeABC is not required for conferring the resistance, which distinguishes *Campylobacter* from other Gram-negative bacteria. Increasing resistance of *Campylobacter* isolates to FQs is associated with FQ usage in animals (Aarestrup & Wegener 1999; Nielsen *et al.* 2006; Smith *et al.* 2002). Regarding *in vivo* development of FQ resistance in *Campylobacter*, recent studies using chicken and pig models showed that FQ treatment has resulted in rapid emergence of FQ-resistant *Campylobacter* isolates (Delsol *et al.* 2004; McDermott *et al.* 2002; Luo *et al.* 2003). Particularly, Luo *et al.* (2003) noticed that after just 3 days of treatment with enrofloxacin (a quinolone antibiotic), 100% of *Campylobacter* shed by chickens were FQ-resistant. FQ resistance in *Campylobacter* has been shown to be stable even after treatment has stopped (McDermott *et al*. 2002; Luo *et al*. 2005).

Increased *Campylobacter* resistance to FQs has resulted in a decrease of FQ usage in clinics. Erythromycin (a macrolide antibiotic) has become the best and a major choice for treating human *Campylobacter* infections recently (Nachamkin *et al*. 2000). However, an increase in *Campylobacter* resistance to macrolides in human and animal isolates has been documented recently, arising more rapidly in developed countries (Gibreel & Taylor 2006). More detailed information regarding macrolide resistance in *Campylobacter* is described below.

Macrolide resistance in *Campylobacter*

Macrolide antibiotics are the metabolic products of *Steptomyces* spp., which inhibit bacterial protein synthesis by binding to the 50S subunit of ribosome (Walsh

2003). This class of antibiotics including erythromycin, tylosin, spiramycin, tilmicosin, roxithromycin are approved for both growth promotion and therapeutic purpose in animal agriculture in the US (Mcewen & Fedorka-Cray 2002).

The use of macrolides in food animals has been associated with resistance in *Campylobacter* and presents opportunity for resistant isolates to reach humans via direct or indirect contact with contaminated food products (Aarestrup 2000; Gibreel & Taylor 2006). The reported macrolide resistance rates vary with *Campylobacter* species and the animal hosts. *Campylobacter coli* usually has higher macrolide resistance rates than *C. jejuni*, regardless of the source of isolation (Aarestrup & Engberg 2001; Engberg *et al.* 2001; Kim *et al.* 2006). Likewise, pigs and turkeys tend to harbor higher numbers of macrolide resistant *Campylobacter* than other animal species. For example, in a study involving human, chicken, and pig isolates, *C. coli* was more resistant to Ery and ciprofloxacin than *C. jejuni* (Gallay *et al.* 2007). *C. coli* isolated from retail raw meats also showed higher rates of resistance to Ery and ciprofloxacin (Ge *et al.* 2003). In Denmark, withdrawal of tylosin from swine feed additives significantly reduced Ery^R *Campylobacter* in pigs (Boerlin *et al*. 2001). A study examining Ery^R *Campylobacter* from broilers in France, before and after the growth promoter ban (1998), found no difference in resistance rates in *C. jejuni*, while a higher number of resistant *C. coli* were isolated after the ban. It is possible that the sample period was too close to the ban to detect true differences (Desmonts *et al*. 2004). A high prevalence of *Campylobacter* has been shown in poultry from both organic and conventional production operations, while *Campylobacter* isolated from conventional poultry operations had significantly higher resistance rates to FQs, Ery, clindamycin, kanamycin, tetracycline, and ampicillin than

Campylobacter isolated from organically raised poultry (Luangtongkum *et al*. 2006). While Ery^R *Campylobacter* have been isolated from organically and intensively reared retail poultry, the isolates with the highest Ery MIC values were from intensively reared retail poultry (Soonthornchaikul *et al*. 2006). These observations suggest that different production practices influence the frequency of macrolide-resistant *Campylobacter* in animal reservoirs.

Direct experimental information supporting the relevance of macrolide use in food animals with emergence of macrolide resistant *Campylobacter* is very limited. A recent study found that long-term exposure of chickens to tylosin as a growth promoter selected for Ery^R *Campylobacter* mutants, while short-term (therapeutic) exposure did not (Lin *et al.* 2007). In this study, chickens experimentally infected with a macrolide-sensitive *Campylobacter* strain were exposed to single or multiple therapeutic treatments of tylosin in water or exposed to tylosin in feed as a growth promoter. However, Ery^R mutants were not isolated from chickens receiving therapeutic tylosin treatment. In contrast, Ery^R mutants were isolated from chickens after 17 days of exposure to tylosin supplemented in feed as a growth promoter. In another recent study, Berrang et al (2007) isolated *Campylobacter* from carcasses of chickens that received feed supplemented with tylosin or unmedicated feed. Although the feed treatment did not affect actual numbers of *Campylobacter* on carcasses after washing, carcasses from chickens fed tylosin in feed as a growth promoter were found to harbor Ery^R *Campylobacter*, while carcasses from chickens fed unmedicated feed were not. The information from these studies suggests that use of tylosin as a growth promoter in feed results in Ery^R *Campylobacter*.

Mechanisms of macrolide resistance in *Campylobacter*

Macrolide antibiotics inhibit bacterial growth by binding to the ribosome, inhibiting protein synthesis by causing premature dissociation of the growing peptidyl tRNA (Corcoran *et al.* 2006; Franceschi *et al.* 2004). Macrolide resistance in bacteria is attributed to many mechanisms including target modifications (e.g. methylation of 23S rDNA, point mutations in 23S rDNA, and mutations in ribosomal proteins), and drug efflux (Gibreel & Taylor 2006). In *Campylobacter*, methylation of 23S rDNA is not reported to confer macrolide resistance. Modifications of the ribosomal target site (e.g. 23S rDNA and ribosomal proteins L4 and L22) and active efflux (e.g. CmeABC efflux pump) are major mechanisms conferring macrolide resistance in *Campylobacter*. (Gibreel & Taylor 2006; Lin *et al.* 2002; Payot *et al.* 2006; Franceschi *et al.* 2004).

Resistance in *C. coli* and *C. jejuni* has been associated with mutations in domain V of the 23S rRNA gene (Engberg *et al.* 2001; Gibreel & Taylor 2006; Mamelli *et al.* 2005). These point mutations within the 23S rRNA gene occur at base position 2074 (A2074C, A2074G, or A2074T) or 2075 (A2075G or A2075C), or both (which correspond to positions 2058 and 2059, respectively, in *E. coli*) (Gibreel *et al.* 2005; Jensen & Aarestrup 2001). There are three copies of the 23S rRNA gene in *C. jejuni* and *C. coli* (Parkhill *et al*. 2000) and wild-type and mutant copies can both be present in a single macrolide-resistant mutant (Gibreel *et al*. 2005). The most common mutation in macrolide-resistant *Campylobacter* is A2075G, which is associated with high-level erythromycin resistance (Gibreel & Taylor 2006). The A2074G mutation also confers high-level resistance but was thought to negatively affect the fitness of the organism, and was relatively unstable (Gibreel *et al*. 2005). Recently, all high- level erythromycin-

resistant *Campylobacter* mutants selected *in vivo* displayed the A2074G mutation in the 23S rRNA gene (Lin *et al*. 2007).

Mutations in ribosomal proteins L4 and L22 have been reported to be associated with Ery resistance in *Campylobacter* (Corcoran *et al*. 2006). Gibreel et al (2005) did not find significant macrolide resistance-associated alterations in either L4 or L22 protein from five erythromycin-resistant clinical isolates. However, the involvement of some modifications in ribosomal proteins L4 and L22 in conferring macrolide resistance in *Campylobacter* has recently been demonstrated. A G74D mutation in protein L4 and insertions in protein L22 (ins_{86}ARAR and ins_{98}TSH) contributed to macrolide resistance (Cagliero *et al*. 2006b). Further investigations of modifications in these proteins are necessary to determine the exact effects of specific mutations on resistance.

The CmeABC efflux pump has been well documented as the major drug efflux pump in *Campylobacter* (Lin *et al.* 2002; Luo *et al.* 2003). This pump has action against a variety of compounds (Gibreel & Taylor 2006). In the presence of efflux pump inhibitor, *Campylobacter* isolates displayed increased susceptibility to bile salts and reduced colonization in chickens (Lin *et al.* 2003; Lin & Martinez 2006). CmeABC efflux pump could play an important role in conferring macrolide resistance in *Campylobacter* (Cagliero *et al.* 2006b; Lin *et al.* 2002; Payot *et al.* 2004). Inhibition of the efflux pump has resulted in increased susceptibility to macrolides (Cagliero *et al.* 2006b; Cagliero *et al.* 2006a; Corcoran *et al.* 2006; Lin *et al.* 2007; Payot *et al.* 2004), reducing both intrinsic and acquired resistance. Mamelli *et al.* noticed that low-level macrolide resistant isolates were susceptible to efflux pump inhibitor. The antibiotic-susceptible wild-type strain also showed increased susceptibility upon inhibition of the

efflux pump (Mamelli *et al*. 2005). The CmeABC efflux pump works in synergy with mutations in 23S rRNA and inactivation of the pump has resulted in increased susceptibility of high-level and low-level resistant human, pig, and poultry *Campylobacter* isolates to erythomycin (Cagliero *et al*. 2005; Lin *et al*. 2002, 2007).

Fitness and stability of antibiotic resistant *Campylobacter*

The ecological fitness and stability of the antimicrobial resistance is a key parameter influencing the incidence, transmission, and persistence of antibiotic resistant bacteria in humans and in animal reservoirs (Andersson 2006). Acquiring drug resistance usually incurs a fitness cost in bacteria (Andersson & Levin 1999; Andersson 2003; Bjorkman & Andersson 2000; Gillespie 2001; Kanai *et al.* 2004; Levin *et al.* 2000; Maisnier-Patin & Andersson 2004; Normark & Normark 2002; Sander *et al.* 2002). Antibiotic-resistant bacteria frequently acquire compensatory mutations to avoid a fitness cost and maintain competitiveness with parent sensitive strains (Andersson 2006). However, some resistance-conferring mutations in bacteria incur very little fitness cost, none at all, or even enhanced fitness (Luo *et al.* 2005; Sander *et al.* 2002; Spratt 1996).

Using delicately designed experiments, Luo *et al.* (2005) recently demonstrated that the FQ-resistant *Campylobacter* do not show a fitness cost *in vivo* and are ecologically competitive in the colonization of chickens even in the absence of antibiotic selection pressure. When separately inoculated into chickens, sensitive and resistant strains, derived from the same parent strain, showed similar levels of colonization. The resistance was persistent throughout this colonization. However, when both strains were co-inoculated into the same chicken host, the resistant strain was able to outcompete the

sensitive strain. Specifically, when co-inoculated into chickens, the FQ-resistant strain outcompeted the FQ-sensitive strain, completely replacing the FQ-sensitive strain in just 3 days, and the specific resistance-conferring mutation did not disappear after prolonged colonization *in vivo* (Luo *et al.* 2005). This enhanced fitness was the result of a single point mutation in *gyrA*, instead of compensatory mutations, as evidenced by similar findings using isogenic gyrA mutants together with parent FQ-sensitive strain (Luo *et al*. 2005). Fluoroquinolone-resistant *Campylobacter* have been shown to be competitive with FQ-sensitive strains *in vivo*, displaying similar levels of colonization. Fluoroquinolone-resistant *Campylobacter* have been cultured from farms where FQ usage had been discontinued (Luangtongkum *et al.* 2006; Pedersen & Wedderkopp 2003; Price *et al.* 2005). Fluoroquinolone and macrolide- resistant *Campylobacters* were found on organically and intensively-reared chickens purchased from retail outlets (Soonthornchaikul *et al*. 2006). These results indicate that FQ resistance-conferring mutations in *Campylobacter* are stable, and the resistant strains can persist even in the absence of antibiotic selection pressure, presenting an unusual problem. Humans infected with FQ-resistant *Campylobacter* have had prolonged diarrhea and hospitalization, and increased risk of complications in comparison to humans infected with FQ-sensitive *Campylobacter* (Nelson *et al*. 2004), and this might be attributed to increased virulence resulting from enhanced fitness of the FQ-resistant *Campylobacter*. Other studies also showed that in comparison with FQ-sensitive *Campylobacter*, infections with FQ-resistant *Campylobacter* have resulted in prolonged illness (Engberg *et al*. 2004) and increased risk of adverse health events (invasive disease or death within 90 days) (Helms *et al*. 2005).

Little information exists concerning fitness cost and stability of macrolide resistant *Campylobacter*. A recent study on the stability of erythromycin-resistant *Campylobacter* with mutations in 23S rRNA gene (corresponding to *E. coli* numbers A2059G, A2058C, and A2058G), revealed that the A2059G and A2058C mutations were stable after 15, 35, and 55 passages on MH agar plates (Gibreel *et al*. 2005). Because *Campylobacter* resistance to macrolides is on the rise, stability of macrolide resistance in *Campylobacter* should be thoroughly investigated to develop effective farm-based strategies to prevent and control emergence and transmission of Ery^R *Campylobacter*.

2. INTRODUCTION

Campylobacter jejuni is recognized as a leading cause of human gastrointestinal enteritis worldwide (Gibreel *et al.* 2005; Nachamkin *et al.* 2000). The majority of *Campylobacter* infections involve diarrhea and abdominal pain, although *Campylobacter* is also associated with Giullain- Barré syndrome, an acute demyelinating disease that can result in respiratory compromise and death (Moore *et al.* 2005; Nachamkin *et al.* 1998). In the United States, the estimated number of cases of campylobacteriosis exceed 2 million per year (Friedman *et al.* 2000; Mead *et al.* 1999). The medical and production costs associated with *Campylobacter* infections are estimated at 1.5 to 8 billion dollars each year in the United States (Buzby *et al.* 1997). Most cases of campylobacteriosis are self- limiting, but antimicrobial treatment is often necessary for severe, prolonged, or systemic cases, or cases in immunocompromised patients.

In parallel to its increased prevalence, *Campylobacter* is increasingly resistant to antibiotics including Fluoroquinolone (FQ) and macrolide antibiotics, the drugs of choice for treating human *Campylobacter* infections (Altekruse *et al.* 1999; Engberg *et al.* 2001). FQ antibiotics are losing their effects on *Campylobacter* because this pathogen has become highly resistant to FQ's. Therefore, erythromycin (Ery), a macrolide antibiotic, has been considered the best choice for treating human *Campylobacter* infections (Allos 2001). Unfortunately, *Campylobacter* resistance to macrolides is also on the rise and this class of antibiotics could eventually lose effectiveness against *Campylobacter* (Engberg *et al.* 2001; Smith *et al.* 2007). To develop effective measures to control and prevent the spread of macrolide-resistant *Campylobacters*, the mechanisms of macrolide resistance in

Campylobacter are critical to understand, but still are not clear.

Campylobacter species colonize the intestinal tract of wild and domestic animals (Nesbit *et al.* 2001). Most human *Campylobacter* infections are associated with consumption of undercooked poultry meat, as well as unpasteurized milk and untreated water (Friedman *et al.* 2000). Epidemiologic studies have revealed poultry as the major reservoir of *Campylobacter* (Corry & Atabay 2001; Jacobs- Reitsma 1997). Macrolide usage in food producing animals is considered to be a major factor influencing the emergence of erythromycin-resistant (Ery^R) *Campylobacter* (Gibreel & Taylor 2006). However, the direct impact of macrolide usage on erythromycin resistance development has not been formally determined in experimental systems until recent examination on the dynamics of *Campylobacter* populations in chickens treated with a tylosin, a macrolide antibiotic (Lin *et al.* 2007). Exposure of chickens to therapeutic doses of tylosin did not select for Ery^R mutants in the treated birds. However, when tylosin was given to the chickens in feed at a growth-promoting dose, Ery^R mutants emerged in the birds after prolonged exposure to the antibiotic (Lin *et al* 2007). Specifically, the chickens inoculated with a sensitive *C. jejuni* strain at 17 days of age started to shed Ery^R mutants on day after inoculation (DAI) 17 in response to long-term exposure to growth promoter tylosin; but all Ery^R mutants only showed low-level Ery resistance with MIC ranging from 8 to 16 μg/ml. Interestingly, when chickens were inoculated with the same Ery sensitive *C. jejuni* strain at 3 days of age, Ery^R mutants were only detected in the chickens on DAI 31 and 38 but not in chickens at DAI 17, and 24; majority of Ery^R mutants displayed high-level Ery resistance (MIC > 512 μg/ml). Although these findings provide compelling evidence that long-term use of macrolide as a growth promoter

selects for the emergence of Ery^R *Campylobacter* in chickens, the length of exposure time to macrolide seems not the only factor contributing to the emergence of Ery resistance in *Campylobacter*.

The stability of the resistant phenotype is a key parameter influencing the development and transmission of antibiotic resistance (Andersson 2006). In many bacterial species, antibiotic resistance confers a reduction in bacterial fitness and thus antibiotic resistance phenotype is not stable in the absence of antibiotic selection pressure (Nachamkin *et al.* 2000). However, distinct from other bacteria, FQ resistance in *Campylobacter* is very stable and FQ-resistant mutants do not show a fitness cost *in vivo* and are ecologically competitive in the colonization of chickens even in the absence of antibiotic selection pressure (Luo *et al.* 2005). The stability of Ery resistance in *Campylobacter* is still largely unknown. To develop effective farm-based strategies to prevent and control emergence and spread of Ery resistant *Campylobacter*, *in vitro* and *in vivo* stability of Ery^R *C. jejuni* mutants with different levels of Ery resistance should be thoroughly investigated.

Significant progress has been made in elucidating molecular mechanisms of macrolide resistance in *Campylobacter*. Modifications of the ribosomal target site and active efflux are the major mechanisms that confer *Campylobacter* resistance to macrolides (Engberg *et al.* 2001; Trieber & Taylor 2000; Lin & Martinez 2006). However, previous studies on the mechanisms of macrolide resistance in *Campylobacter* either focused on the comparison of isolates from various origins or examined macrolide resistant mutants selected *in vitro*, greatly limiting the interpretation of association of specific molecular mechanisms with acquired Ery resistance and limiting elucidation of

macrolide resistance development *in vivo* in *Campylobacter* (Cagliero *et al.* 2006b; Mamelli *et al.* 2005). Examination of *in vivo*- as well as *in vitro*-selected macrolide resistant mutants that are all derived from the same parent sensitive strain would greatly improve our understanding on the molecular basis and development of macrolide resistance in *Campylobacter*.

Based on this published information and our previous work on antibiotic resistance in *Campylobacter*, we speculate that length of exposure to growth promoter tylosin and other *in vivo* factors affect the dynamics of emergence of Ery^R *Campylobacter* mutants. Different molecular mechanisms together contribute to the stability and level of Ery resistance in *C. jejuni*. To test these hypotheses and move towards the goal of controlling macrolide resistance in *Campylobacter*, we pursued the following three specific objectives:

1. Determine if long-term exposure of low-level Ery^R *C. jejuni* to growth promoter tylosin selects for high-level Ery^R mutants using chicken model system.
2. Determine *in vitro* and *in vivo* stability of the macrolide-resistant phenotype in *Campylobacter*.
3. Determine the molecular mechanisms of macrolide resistance in *C. jejuni* mutants selected *in vitro* and *in vivo*.

3. MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The key *C. jejuni* strains used in this study are listed in Table 1. *Campylobacter jejuni* NCTC 11168 is an Ery-sensitive strain whose whole genome sequence has been completed and published (Parkhill *et al.* 2000). NCTC 11168 was purchased from ATCC (Cat. No 700819). The NCTC 11168 was used as a parent strain for *in vitro* selection of macrolide resistant mutants in this study. The NCTC 11168-derived Ery^R mutants isolated from chickens (Lin *et al.* 2007) were also used in this study. All *Campylobacter* strains used were cultured in Mueller-Hinton (MH) broth or agar, or media containing *Campylobacter* growth supplement and Preston *Campylobacter* selective supplement (Oxoid) when necessary. MH media were supplemented with various concentrations of Ery or tylosin when necessary. Strains were grown at 42˚C under microaerophilic conditions using CampyGen gas pack (Oxoid) in enclosed jars.

Antibiotic susceptibility test

Minimum Inhibitory Concentrations (MICs) were determined using the standard agar dilution method as recommended by the CLSI (Formerly NCCLS) (CLSI, 2006). *C. jejuni* ATCC 33560 was used as a quality control strain and the quality control range of Ery MIC is 1-4 μg/ml when incubated at 42˚C for 24 hours. Erythromycin stock solutions of concentration 25 mg/ml in 100% ethanol were stored at -20˚C. To prepare plates for MIC test, Ery stock solution was vortexed vigorously and diluted to 5.12 mg/ml (highest concentration) and subsequently diluted two-fold with sterile dd H_20 until a final

concentration of 0.005 mg/ml was obtained. Two-ml of desired concentration of Ery and 1 ml of defibrinated sheep blood (Cleveland Scientific, Cleveland, OH) were added in 17 ml agar deep for preparing agar dilution plates with 11 concentrations ranging from 512 µg/ml to 0.5 µg/ml. Plates with Ery concentration of 0 µg/ml were made with dd H_20 in place of Ery and were used as positive control. To prepare inoculum, well-isolated *C. jejuni* colonies from a fresh agar plate (grown at 42°C for 24 hrs) were suspended in MH broth to a turbidity equivalent to that of a 0.5 McFarland standard using VITEK Colorimeter (bioMerieux, Inc., Durham, NC). Agar dilution test was performed using Cathra Replicator System (Oxoid). The growth and end points were monitored following 24 hr of microaerophilic incubation at 42°C. According to the new breakpoints recommended by CLSI (CLSI, 2006), Ery MIC ≤8 µg/ml and ≥ 32 µg/ml are considered susceptible and resistant, respectively. Ery was purchased from Sigma chemical Co., St. Louis, MO.

***In vitro* selection of macrolide-resistant mutants**

Erythromycin (SIGMA) or tylosin tartrate (MP Biomedicals) was used as selective agent for selecting spontaneous macrolide-resistant mutants *in vitro.* Briefly, 100 µl of wild-type Ery-sensitive NCTC 11168 cultures were plated on MH plates and grown overnight at 42˚C under microaerophilic conditions. The fresh cells were then harvested from plates using sterile MH broth. Cell suspension were then plated on increasing concentrations of erythromycin and tylosin (4- to 16-fold the initial MIC of NCTC 11168). Following 3-5 days incubation under microaerophilic conditions at 42˚C, single macrolide resistant colony on selective plates were selected, cultured in MH broth

and stored at -80 ˚C. The Ery-resistant and tylosin-resistant mutants obtained from the first round of selection were grown in MH broth containing sublethal concentrations of Ery and tylosin, respectively, and then plated on selective plates again for selecting mutants with higher level of resistance. If needed, the procedure was repeated to obtain high level macrolide resistant mutants. All *in vitro*-selected mutants were subjected to MIC test using standard agar dilution method as described above. In addition, genomic DNA was prepared from each mutant and used for PCR amplification and sequence analysis of 23S rDNA and ribosomal proteins L4 and L22 genes as detailed below.

Polymerase Chain Reaction (PCR)

PCR was performed to analyze the sequences of domain V of the 23S rRNA genes, ribosomal proteins L4 and L22 genes, and major outer membrane protein gene *cmp* in *C. jejuni*. All primers used for PCR are described in Table 2. PCR was performed with a total volume of 50µl containing 1µl of 10 mM concentration of each of the deoxynecleoside triphosphates, 5µl of Mg^{2+}-free thermophilic DNA polymerase 10X buffer, 5µl of 25mM $MgCl_2$, 2µl of forward primer (5 pmol/µl), 2µl of reverse primer (5 pmol/µl), 2.5µl of DNA template, 32µl of dd H_20, and 0.5µl of Taq DNA polymerase (2.5 U). Cycle for all reactions was 95˚C for 5 minutes followed by 33 cycles of: 95˚C for 30 seconds for denaturation, 50˚C for 30 seconds for annealing, followed by 72˚C for 1 minute for extension. In all PCR reactions, boiling samples were used as DNA templates. Briefly, cultures were grown in MH broth at 42˚C for 48 hours under microaerophilic conditions. One ml of *C. jejuni* cells were centrifuged for 3 min at 10,000 x g and pellet was suspended in 100µl sterile ddH_20. The tube containing cell

suspensions was placed in boiling water for 10 minutes, followed by centrifugation for 3 minutes at 10,000 x g. Seventy µl of the supernatant was carefully transferred to a clean tube and used as a DNA template for PCR. The PCR products were run together with 1 kb DNA ladder (Promega) in 1% agarose gel at 100 constant volts for 30 minutes. The gel was stained by ethidium bromide and digital photographs of gels were taken using FluoChem 5500 digital imaging system (Alpha Innotech).

Sequence Analysis

PCR products were purified using QIAquick PCR Purification Kit (Qiagen). Purified products were sequenced using ABI 3730 DNA Sequencer at UTK Molecular Core Facility. Sequences were aligned suing NCBI BLAST software at NCBI website (http://www.ncbi.nih.gov/blast/bl2seq/wblast2.cgi) and mutations identified.

Insertional mutagenesis of *cmeB* gene

Isogenic *cmeB* mutants of various *Campylobacter jejuni* isolates were constructed using natural transformation. Wild-type strain NCTC 11168 or its Ery-resistant derivatives selected *in vitro* or *in vivo* was spread on MH agar plates, followed by incubation at 42° C for 48 hours. Cells were harvested with MH broth and adjusted to the approximate concentration of 3 x 10^9 CFU/ml. 500µl of *C. jejuni* cell culture was added to 15ml tubes containing 1ml MH agar. The tubes were incubated 3 hours. Following uncubation, 5µl of *C. jejuni* 9B6 genomic DNA was mixed with 500µl of *C. jejuni* cells by pipetting. Tubes were again incubated for 3 hours. To select transformants, cultures were spread on MH plates containing kanamycin 30µg/ml and incubated 48 hours. All

incubation was performed under microaerophilic conditions at 42° C.

Exposure of low-level Ery resistant *Campylobacter* mutants to subtherapeutic dose of tylosin in chickens

Two independent experiments were conducted in chickens using tylosin at the dose used for growth promotion. In each experiment, day-old broiler chicks (gift from commercial company Hubbard Hatchery, Pikeville, TN) were randomly assigned to two groups (9-10 per group). All birds were placed in sanitized wire cages with unlimited access to feed and water. In the first experiment, chickens in the control group received nonmedicated feed. Chickens in the treatment group received the complete feed supplemented with tylosin (Elanco Animal Health). All feed was prepared by the feed mill at Johnson Animal Research and Teaching Unit. Medicated feed was prepared in accordance with the label for preparation of medicated feed used for growth promotion of chickens to obtain a final concentration of 50 mg/kg. To confirm that the birds were *Campylobacter*-free, on day 12 (prior to inoculation with *C. jejuni*), sterile swabs were used to take cloacal swab samples from each bird. Swabs were immediately placed in small sterile tubes containing 1 ml MH broth. Tubes were briefly vortexed and 100 µl of each sample spread on MH plates containing growth and selective supplements, and plates were incubated at 42˚C under microaerophilic conditions for 48 hours. All birds were *Campylobacter*-free prior to inoculation. At 15 days of age, all birds were inoculated with approximately 10^7 CFU of fresh *C. jejuni* DC2 (Ery MIC= 32µg/ml; Table 1) culture via oral gavage. Cloacal swabs were then collected on 17, 22, 29, 36, 43, and 50 days of age. Samples from each bird were were diluted serially, and each dilution

was spread onto two different types of MH plates: one containing *Campylobacter*-specific growth and selective supplements (SR084E and SR117E; Oxoid) to recover the total *Campylobacter* populations, and the other containing 8 µg/ml Ery (for control group) or 128µg/ml Ery (for treatment group) in addition to the same growth and selective supplements to recover the *Campylobacter* populations that were considered not susceptible to Ery. *Campylobacter* colonies were counted following 48 h of incubation at 42^{o}C under microaerophilic conditions. Individual colonies were also collected from selective plates for each chicken and was used for MIC testing. Representative isolates were also selected for PCR amplification and sequence analysis of 23S rDNA and *rplD* and *rplV* genes encoding ribosomal proteins L4 and L22. Poultry is usually *Campylobacter*-free for the first 2-3 weeks of age in commercial conditions. Therefore, inoculating birds with *C. jejuni* at day 15 in this study mimics commercial broiler production conditions.

In the second experiment, chickens were randomly assigned to two groups and chickens in both group received the feed supplemented with tylosin with a final concentration of 50 mg/kg feed. At 15 days of age, each chicken within each group was either inoculated with approximately 10^{7} CFU of fresh *C. jejuni* DC2 (Ery MIC= 32µg/ml) or DC26 (Ery MIC= 64µg/ml; obtained from the first chicken experiment) via oral gavage. Cloacal swabs were collected on 20, 34, and 48 days of age. Representative isolates from each bird on each sample day were subjected to differential plating, MIC testing, and PCR sequencing analysis as described above.

In vitro stability of Ery resistance

One low-level Ery resistant mutant (DC2; Ery MIC= 32μg/ml) and two high-level Ery resistant mutants (DC3 and DC6; Ery MICs> 512μg/ml) (Table 1) were used for *in vitro* stability test and all these mutants are chicken isolates derived from the same parent strain NCTC 11168 (Lin *et al,* 2007). Experimental design is outlined in Figure 1. To perform *in vitro* stability test, all mutants were inoculated in antibiotic-free MH broth and grown in microaerophilic conditions at 42˚C. Every 48-72 hours, 10 μl of each culture was subcultured in 4 ml fresh MH broth (1:400 dilution) for a total of 33 passages. Following passages 10, 20, and 33, the cultures were serially diluted (10-fold dilution) in MH broth and plated on both MH agar plates and MH agar plates supplemented with Ery at final concentration of 8 μg/ml (DC2) or 128 μg/ml (DC3, DC6). Plates were incubated at 42˚C under microaerophilic conditions for 48 hours. Total numbers of colonies on each type of plate were counted and compared. In addition, following passage 33 differential plating, 10 colonies for each mutant were randomly chosen from MH agar plates and were subjected to Ery MIC test using standard agar dilution method as described above.

In vivo stability of Ery resistance using chicken model system

Day-old broiler chicks (gift from commercial company Hubbard Hatchery, Pikeville, TN) were randomly assigned to two groups (12-13 birds per group). All birds were placed in sanitized wire cages with unlimited access to feed and water. Feed was nonmedicated feed without any antibiotic additives and was prepared by the feed mill at Johnson Animal Research and Teaching Unit. At 3 days of age, all birds were inoculated with approximately 10^7 CFU fresh *C. jejuni* culture via oral gavage. Chickens in group A

were inoculated with *C. jejuni* DC2, a low-level Ery resistant *C. jejuni* mutant (MIC= 32μg/ml) that was obtained from previous chicken study (Lin *et al* 2007). In group B, all chickens were inoculated with a highly Ery^R mutant DC6 (MIC> 512μg/ml) that was also obtained from previous chicken study (Lin *et al* 2007). Cloacal swabs were then collected on day 12, 22, 38, and 47. Isolation of *Campylobacter* and differential plating for enumerating the proportion of the mutant colonies were conducted as described above. Samples from group A were subjected to differential plating on MH plates supplemented with growth and selective supplements, and with or without Ery 8μg/ml. Samples from group B were subjected to differential plating on MH plates supplemented with growth and selective supplements, and with or without Ery 128μg/ml. Representative isolates from each chicken were selected for Ery MIC test. Selected isolates were also subjected to PCR amplification and sequence analysis of 23S rDNA and *rplD* and *rplV* genes encoding ribosomal proteins L4 and L22. To confirm that *C. jejuni* strain used for inoculation was the same strain collected from cloacal swabs, major outer membrane protein gene *cmp* (MOMP), a good target for molecular typing in *Campylobacter* (Huang *et al*. 2005), was PCR amplified in the input strain and a representative output isolates and subjected to sequencing analysis. MOMP gene sequencing revealed no difference between input strain and output isolates.

Detection limit and statistical analysis

In all chicken experiments, the detection limit of the plating methods is approximately 100 CFU/g of feces. Student's *t* test was used to examine the significance of differences in *Campylobacter* colonization levels (log transformed CFU/g feces) at

each sampling point between two treatment groups. A P value of <0.01 was considered significant.

4. RESULTS

Colonization of Ery^R mutants in response to tylosin treatment.

In the first chicken experiment in which *C. jejuni* DC2 was used to inoculate chickens either receiving medicated or nonmedicated feed, all chickens, including the ones with the medicated feed, were successfully colonized by *C. jejuni* DC2 on d 17 (two days after inoculation) (Figure 2). The shedding level of *Campylobacter* in feces was consistently higher (up to 1.8 $\log_{10}$ units) in the chickens given nonmedicated feed than in those given tylosin-containing feed, except that on d 50 the shedding levels of *C. jejuni* for the nonmedicated group was slightly lower (but the difference was not statistically significant: $P > 0.05$) than those from medicated group (Figure 2).

In the second chicken experiment, chickens were assigned to two groups that were inoculated with either low-level Ery^R *C. jejuni* DC2 (Ery MIC= 32μg/ml) or DC26 (Ery MIC= 64μg/ml) but all chickens were treated with medicated feed throughout the study. All chickens were successfully colonized by DC2 or DC26 at 20 days of age (5 days after inoculation) (Figure 3). Both groups of chickens displayed similar shedding levels of *Campylobacter* throughout the study although DC2 seemed colonize slightly better than DC 26 on day 20 (Figure 3).

Representative *C. jejuni* isolates derived from chickens were analyzed by PCR (for the MOMP gene) (Figure 4) and subsequent sequence analysis, which revealed that the MOMP gene sequences of the mutants (output) were identical to strain used as inoculum (input).

Effect of prolonged low dose tylosin treatment on the emergence of high-level Ery^R *C. jejuni* in chickens

In the first experiment, day-old broiler chicks were placed in two groups, receiving feed supplemented with tylosin at a concentration of 50mg/kg (treatment group), or nonmedicated feed (control group). After inoculation of chickens with DC2 at 15 days of age, cloacal swabs collected every 2-7 days and used to determine the percentage Ery^R populations of *C. jejuni*, and levels of Ery resistance for both groups. In control group, all isolates obtained from chickens at 29 days of age became susceptible to Ery as determined by MIC test (Figure 5, Table 3), which was also confirmed by differential plating method. Each chicken in the treatment group consistently shed Ery^R *C. jejuni* throughout the study (Figure 5, Table 3). By day 29, and every sample day thereafter, mutants with Ery MIC of 64 μg/ml were isolated in the treatment group (Table 3). However, no high-level Ery^R organisms were selected throughout the study. These results suggest that low-level Ery resistance in *C. jejuni* is not stable in the absence of antibiotic selection pressure and long-term exposure of *C. jejuni* to low-dose tylosin may not be enough for developing high-level Ery resistance in *C. jejuni*.

To further confirm the finding from above chicken experiment, DC2 together with its new derivative DC 26 (Ery MIC = 64 μg/ml; from above chicken study) were used to challenge chickens receiving tylosin-containing feed (50 mg/ml). As shown in Table 4, regardless of the mutants used for inoculation, none of chickens shed high-level Ery^R *C. jejuni* (MIC > 512 μg/ml) with up to 33 days continuous tylosin treatment, which is also consistent with differential plating result. However, in the group inoculated with *C. jejuni*

DC2, a mutant with Ery MIC of 256μg/ml was obtained on day 48 (Table 4) while in the group inoculated with DC26 mutants with MIC of 128μg/ml were selected on each sampling day (Table 4). Together, these findings confirmed the results from the first chicken experiment and indicated that long-term antibiotic selection pressure should work together with other factors to select high-level Ery resistance in *Campylobacter*.

In vitro stability of Ery resistance in *C. jejuni*

C. jejuni strains DC2 (Ery MIC= 32μg/ml) and two high-level Ery^R mutants DC3 and DC6 (Ery MIC> 512μg/ml) were used for *in vitro* stability test. All these three Ery^R mutants are chicken isolates derived from NCTC 11168 in a previous chicken experiment (Lin *et al*. 2007). As shown in Figure 6, more than 60% of DC2 populations could still be selected on MH plates containing 8 μg/ml Ery after 10 passages while all DC3 as well as DC6 populations grew well on MH plates containing 128 μg/ml of Ery. By passage number 20 and 33, Ery-resistant populations in DC2 culture were dramatically dropped to 13% and 7%, respectively (Figure 6). However, both DC3 and DC6 displayed high stability after 20 and 33 passage; near100% populations could still be recovered on MH plates containing 128 μg/ml of Ery (Figure 6). The differential plating results (Figure 6) were also confirmed by Ery MIC tests of representative isolates selected on MH plates for passage 33. Majority of DC2 isolates showed Ery MIC of 2μg/ml that is comparable with the MIC of wild-type sensitive level. However, all tested DC3 and DC6 had Ery MIC> 512μg/ml. Together, these results indicated that high-level Ery resistance (Ery MIC > 512μg/ml) in *C. jejuni* is stable *in vitro* while low-level Ery resistance is not.

In vivo stability of Ery resistance in *C. jejuni*

Both DC2 and DC6 strains were chosen for *in vivo* stability test of Ery resistance in *C. jejuni* using chicken model system. At 12 days of age (9 days after *C. jejuni* inoculation), each chicken in both groups was colonized by DC2 or DC6 with average colonization level from 3.7 to 4.9 Log_{10} units (Figure 7). Differential plating showed no Ery^R *C. jejuni* mutant was detected in DC2-inoculated chickens by day 38 using MH selective plates containing 8 μg/ml Ery (Figure 8). However, high-level Ery resistance was maintained throughout the study; approximate 100% of *C. jejuni* populations from chicken inoculated with DC6 grew on MH selective plates containing 128 μg/ml of Ery for the entire study, suggesting high-level Ery resistance is very stable *in vivo* (Figure 8). MIC test of all randomly selected isolates from each individual chickens further confirmed these findings (Table 5). For low-level Ery^R mutant DC2, the instability of low-level Ery resistance was also observed in chickens as early as 9 days after inoculation (day 12); two of 12 isolates from 12-day-old chicken displayed reduced Ery MIC (16 μg/ml) (Table 5). With further growth of DC2 in chickens without tylosin selection pressure, majority of isolates from chickens inoculated with DC2 displayed Ery MIC similar to that of wild-type sensitive strain on day 38 and 47f (Table 5). In contrast, all isolates from chicken inoculated with DC6 consistently displayed high-level Ery resistance (MIC> 512μg/ml), regardless sampling date (Table 5).

Molecular mechanisms of Ery resistance in mutants selected *in vitro* and *in vivo*

Thirty *in vitro*-selected and thirty three *in vivo*-selected Ery^R *C. jejuni* mutants (all NCTC 11168 derivatives) were subjected to PCR amplification of 23S rRNA gene and

rplD and *rplV* genes encoding ribosomal proteins L4 and L22, respectively (Figure 9). The sequences of these PCR products were aligned to corresponding sequence of parent strain NCTC 11168 to identify specific mutations occurred in ribosome.

With respect to 23S rRNA gene, all high-level Ery^R isolates selected *in vitro* (JL287-289) displayed an A→C point mutation at position 2074 (Table 6) while all high-level Ery^R isolates selected *in vivo* (DC3, 6, 9, 31, 32, 33) displayed a different point mutation at the same position (A2074G) (Table 7). According to sequence results, it appeared that A2074C mutation was present in two of the three copies of the 23S rRNA gene in mutants selected *in vitro*, because the sequence chromatogram showed double peaks in the same position where the C peak was two times higher than the A peak (Figure 10). Regardless of selection environment, none of Ery^R mutants with Ery MIC ≤ 512μg/ml displayed point mutation in the domain V of the 23S rRNA genes (Table 6 and 7).

Changes in ribosomal protein L4 were not detected in mutants selected *in vitro* (Table 6) and were only observed in majority of low-level Ery^R isolates selected *in vivo* (22 of 25 isolates, MIC range 8- 64μg/ml) (Table 7). Specifically, these mutants showed a G→D change either at positions 74 or 57 (Table 7, Figure 12). The G74D modification in ribosomal protein was stable in the presence of continuous tylosin selection pressure (DC21-27 in Table 7). However, in the absence of tylosin selection pressure in feed, the Ery MIC of randomly selected isolates decreased (DC28-30), consistent with the loss of specific G74D point mutation in L4 protein (Table 7).

Modification in ribosomal protein L22 was only observed in Ery^R mutants selected *in vitro* but not in the mutants selected *in vivo* (Table 7). Specifically, a 3-

amino acid insertion (TSH) at position 98 in protein L22 was observed in isolates selected *in vitro* with wide Ery MIC range from 32 to 512 μg/ml (Table 6, Figure 11). It seemed that use of specific selective agent (Ery or tylosin) did not affect development of Ins_{98}TSH modification in L22 protein (Table 6).

The different mutations in Ery^R mutants were summarized in Table 8. Interestingly, some of Ery^R mutants, either selected *in vivo* or selected *in vitro*, did not display any mutations in the sequenced three gene targets (Table 6-8). Such mutants include four *in vivo*-selected mutants (DC1, DC4, DC11, and DC29) with Ery MIC ranging from 4 to 64 μg/ml (Table 7 and 8) and 50% *in vitro*-selected mutants with Ery MIC ranging from 8-128 μg/ml (Table 6 and 8).

Contribution of CmeABC efflux pump to the acquired Ery resistance

To determine the contribution of the CmeABC efflux pump to the acquired Ery resistance, CmeABC mutation was transferred to selected Ery^R mutants and Ery MIC of each isogenic CmeB mutant was measured by standard agar dilution method. As shown in Table 9, regardless the presence of specific mutation in 23S rRNA gene (DC32, JL287, JL288), in L4 protein (DC22, DC27), in L22 protein (Jl290, JL301, JL303), or absence of any mutation (JL283, JL284), inactivation of *cmeB* dramatically reduced Ery MIC (8 – 1024 folds) when compared to its parent strain (Table 9), indicating that CmeABC works synergistically with other mechanisms to maintain high-level and low-level Ery resistance in *C. jejuni* selected *in vivo* and *in vitro*.

5. DISCUSSION

The results of this study reveal several unique features with respect to macrolide resistance development, stability, and associated molecular mechanisms in *Campylobacter jejuni*. First, exposure time to subtherapeutic level of tylosin is not a sole factor contributing to emergence of highly Ery^R *Campylobacter*. Prolonged exposure of low-level Ery^R *C. jejuni* (MIC = 32 or 64 μg/ml) to growth promoter tylosin did not select for high-level Ery^R *C. jejuni* (Table 3, 4). Second, high-level Ery resistance (MIC >512 μg/ml) displayed remarkably stability *in vitro* and *in vivo*. (Figures 6, 8, Table 5). However, the low-level Ery resistance (MIC = 32 μg/ml) was not stable *in vitro* and majority of *C. jejuni* were sensitive to Ery after 20 passages (Figure 6). The instability of low-level Ery resistance was also observed in chickens as early as 9 days post inoculation and Ery^R mutants were rarely isolated 35 days post inoculation (Table 5, Figure 8). Third, sequencing analysis of 23S rDNA, L4 and L22 genes revealed that molecular mechanisms contributing to Ery resistance in *C. jejuni* differ between high-level and low-level resistant isolates and between isolates selected *in vivo* and *in vitro*. (Figures 10,11,12, Tables 6,7,8, 9). Fourth, mutation in CmeABC efflux pump drastically reduced MIC of Ery for Ery^R mutants selected *in vitro* and *in vivo*, indicating CmeABC worked synergistically with other mechanisms to confer Ery resistance in *C. jejuni* (Table 9). Together, these findings indicated that *C. jejuni* utilize complex and different mechanisms to develop Ery resistance *in vitro* and *in vivo*

Recently, Lin *et al.*, (2007) conducted a study to examine the development of Ery resistance in *C. jejuni* in chickens upon exposure to growth promoter tylosin. When

tylosin was given to the chickens in feed at a growth-promoting dose, Ery^R mutants emerged in the birds after prolonged exposure to the antibiotic. In experiment 1, when chickens were inoculated with Ery sensitive *C. jejuni* strain at 3 days of age, Ery^R mutants were finally detected in the chickens on 31 days after inoculation (DAI 31) and DAI 38 but not in chickens at DAI 17, and 24; majority of Ery^R mutants from DAI 31 and 38 displayed high-level Ery resistance (MIC > 512 μg/ml). In experiment 2, the chickens inoculated with the same sensitive *C. jejuni* strain at 17 days of age started to shed Ery^R mutants at DAI 17; but all Ery^R mutants only showed low-level Ery resistance with MIC ranging from 8 to 16 μg/ml. Further 1-week exposure did not promote emergence of any mutants with higher level Ery resistance at DAI 24. Although these findings indicate that long-term use of tylosin as a growth promoter selects for Ery^R *Campylobacter* in chickens, it is intriguing why highly Ery-resistant *Campylobacter* emerged without pre-emergence of low-level Ery^R mutants at DAI 24 in experiment 1. Is the length of exposure time a major factor contributing to emergence of high-level Ery^R *C. jejuni*? Does emergence of highly Ery^R *Campylobacter* mutants require stepwise accumulation of various identified mutations in ribosome? To address these issues, in this study, we first conducted two chicken experiments to determine if long-term exposure of two low-level Ery^R *Campylobacter* mutants results in emergence of high-level Ery resistant mutants. The findings from this study (Table 3 and 4) strongly suggest that exposure time is not a sole factor selecting for high-level Ery resistant *C. jejuni*. Some unknown factors in conjunction with continuous antibiotic selection pressure may determine the rate of occurrence of highly Ery^R *Campylobacter*. For example, gut microflora may play an important role. *Campylobacter* species have an exceptional

ability for taking up heterologous DNA. Thus, interspecies exchange of genetic material between *Campylobacter* and other bacterial flora (e.g. macrolide resistant populations) in intestine may lead to the emergence of Ery resistant *Campylobacter*. Other factors, such as anatomy, physiology, and specific tissue concentration of tylosin, may also affect the rate of emergence of high-level Ery^R *Campylobacter*.

Sequence analysis revealed that all high-level Ery^R mutants (MIC >512 μg/ml) selected *in vivo* displayed the specific A2074G mutation in 23S rRNA gene while specific mutations in L4 (G74D or G57D) were widely found in low level Ery^R mutants (Ery MIC = 8-64 μg/ml) selected *in vivo* (Table 7). No single Ery^R mutant displayed mutations in both 23S rRNA gene and L4 protein, which suggest that specific mutations in L4 contribute to low-level Ery resistant but are not essential for further development of high-level resistance in *C. jejuni* in chicken. Similarly, no single *in vitro*-selected Ery^R mutant displayed mutations in both 23S rRNA gene and L22 protein either (Table 6). This finding is also consistent with recent *in vitro* study (Cagliero *et al.* 2006b) and also suggests that 23S rRNA gene mutation and change in L4 protein or L22 protein may not co-exist in Ery^R *Campylobacter*. However, it does not mean that low-level Ery resistance is not required for developing high-level Ery resistance. We have obtained some Ery^R mutants that do not have any mutations in domain V of the 23S rDNA, L4 or L22 genes, such as DC1, DC4, and DC11 selected *in vivo*, and JL283-289 selected *in vitro* (Tables 6,7,8), The Ery resistance mechanisms in these mutants needs to be determined in the future and the novel mechanisms required for low-level resistance in these mutants may make *C. jejuni* survive better under selection pressure and facilitate the development of high-level Ery resistance in *Campylobacter*.

Campylobacter jejuni has displayed unique feature with respect to the fitness and stability of antibiotic resistance (Luo *et al.* 2005; Zhang *et al.* 2003). Chromosomal mutation- or plasmid acquisition-mediated antibiotic resistance generally incur a fitness cost in bacteria (Andersson & Levin 1999; Levin *et al.* 2000). However, Luo *et al* (2005) demonstrated that FQ resistance in *Campylobacter* is very stable and FQ-resistant mutants do not show a fitness cost *in vivo*. Interestingly, FQ resistant *Campylobacter* mutants are ecologically more competitive in the colonization of chickens than their FQ-sensitive parent strain even in the absence of antibiotic selection pressure (Luo *et al.* 2005). Does Ery resistance in *Campylobacter* have the same distinct feature? It has been observed that high-level Ery resistance that is associated with A2075G and A2074C mutations in 23S rRNA in *Campylobacter* was stable *in vitro* in the absence of macrolide selection pressure (Gibreel *et al.* 2005; Kim *et al.* 2006). In this study, both *in vitro* and *in vivo* experiments provided compelling evidence that low-level Ery resistance is not stable in *C. jejuni* while high-level Ery-resistant mutants displayed remarkably stability. The phenotype of Ery resistance stability in *C. jejuni* could be contributed by two factors: reverse mutation rate of specific Ery resistance associated mutation (e.g. A2074G in 23S rRNA gene) and fitness cost of Ery^R *Campylobacter* mutant due to acquisition of specific mutation(s). It is technically difficult to assess reverse mutation rate of specific Ery resistance associated mutation. However, given the fact that the emergence of spontaneous Ery^R mutants *in vitro* is at a low frequency in *Campylobacter* (Lin *et al.* 2007), it is likely that *in vitro* reverse mutation rate is also low. With respect to point mutation in 23S rRNA that is associated with high-level Ery resistance, acquisition of such mutation either alone or with unknown compensatory mutation(s) may result in little

fitness cost. Consequently, the highly Ery^R *C. jejuni* mutants may not be outcompeted by sensitive strain after successive multiplication either *in vitro* or *in vivo* with removal of antibiotic selection pressure, leading to high stability or persistence phenotype as we observed in this study. In contrast, changes in L4 or L22 may result in increased fitness cost in low-level Ery^R *C. jejuni* mutants according to our findings in this study. Thus, as soon as sensitive *C. jejuni* emerges in total populations due to the loss of specific characterized mutation (e.g. G74D in L4) or other unknown mutation(s), the sensitive populations, which display higher fitness than low-level Ery^R *C. jejuni* mutants, will eventually become dominant. The randomly selected isolates (DC28-30) from the 47-day old chickens that have been inoculated strain DC2 (Ery MIC = 32 ug/ml) and received non-medicated feed (Table 7) showed reduced MIC (1-8 ug/ml) with or without specific G74D mutation in L4, strongly suggesting that G74D mutation together with other uncharacterized mutation(s) contribute to low-level resistance and none of these mutations is stable *in vivo*.

Previous investigation on the mechanisms of macrolide resistance in *Campylobacter* either focused on the comparison of isolates from various origins or examined macrolide resistant mutants selected *in vitro*, greatly limiting the interpretation of association of specific molecular mechanisms with acquired resistance and limiting elucidation of macrolide resistance development *in vivo* (Cagliero *et al.* 2006b; Corcoran *et al.* 2006; Gibreel *et al.* 2005; Kim *et al.* 2006; Mamelli *et al.* 2005). Distinct from these studies, we examined a panel of *in vitro*- and *in vivo*-selected Ery^R *C. jejuni* mutants that are all derived from the same parent strain *C. jejuni* 11168. Sequence analysis and Ery MIC test revealed several unique findings with respect to the molecular mechanisms of

Ery resistance development in *Campylobacter*. First, with respect to high-level Ery resistance (MIC > 512 μg/ml), all mutants selected *in vivo* displayed A2074G mutation while all mutants selected *in vitro* displayed A2074C mutation in 23S rDNA. Different environment (*in vitro* vs. *in vivo*) may be a major factor causing such difference. However, we cannot rule out the possibility that different selective agents may select for different point mutation. In this study, tylosin was supplemented in feed for selecting Ery^R mutants developed in chicken and erythromycin was used *in vitro* to select high-level Ery^R mutants (JL287-289). We have made extensive efforts to select high-level Ery^R mutants using tylosin as selective agent *in vitro*. However, we failed to obtain high-level Ery^R mutants with point mutation in 23S rDNA using tylosin for mutant selection *in vitro*, further suggesting selection pressure resulting from tylosin alone may not be enough to promote emergence of high-level Ery^R *C. jejuni* mutants. Second, with respect to low- and intermediate-level Ery resistance development, growth environment seems to determine specific type of modification in ribosomal protein that confers Ery resistance. Although one study (Cagliero *et al*, 2006b) showed one *in vitro*-selected Ery^R *C. jejuni* mutant acquired G74D mutation in ribosomal protein L4, we only identified such mutation and another novel mutation G57D in L4 in mutants selected *in vivo* but not *in vitro*. Modification in ribosomal protein L22 (TSH_{98} insertion) was only found in Ery^R mutants selected *in vitro* with Ery MIC from 32 μg/ml to 512 μg/ml, regardless specific selective agent used (tylosin or Ery). Finally, some of *in vivo*-selected mutants (DC1, DC4, and DC11) and *in vitro*-selected mutants (JL276-JL279, Jl283-JL286, and JL292-JL298) do not have any mutations or modifications in domain V of 23S rRNA gene and in ribosomal proteins L4 and L22. Some of these mutants have Ery MIC as high as 128

μg/ml (e.g. JL284-286). Molecular mechanisms of Ery resistance in these isolates need to be examined in the future. Taken together, these findings indicated that *C. jejuni* utilize complex and different mechanisms to develop Ery resistance *in vitro* and *in vivo*.

CmeABC efflux pump is widely noted as being linked to macrolide resistance in *C. jejuni* (Lin et al., 2007; Cagliero et al., 2006; Gibreel et al., 2005; Mamelli et al., 2005; Martinez and Lin, 2006). In these studies, inactivation of *CmeABC* by site-directed mutagenesis or inhibition of *CmeABC* by efflux pump inhibitor dramatically reduced Ery MIC in wild-type sensitive strains or in Ery-resistant mutants, indicating *CmeABC* contributes to both intrinsic and acquired Ery resistance. In this study, regardless of the presence of specific target mutations in the ribosome, inactivation of *CmeB* greatly reduced the Ery resistance of all Ery^R isolates (Table 9), indicating that *CmeABC* multidrug efflux pump is a significant player in maintaining the acquired resistance to Ery. In all low-level resistant isolates from *in vivo* and *in vitro* studies, inactivation of *CmeB* led to a drastic reduction in Ery MIC to a level that was even below that of the wild-type strain. This can be explained by the known role of *CmeABC* in the intrinsic resistance to various antibiotics (Lin *et al.* 2002). With respect to three high-level resistant isolates (MIC>512μg/ml), inactivation of *CmeB* led to the decrease of Ery MIC to 64 μg/ml in DC32 and JL288 and to 0.5 μg/ml in JL287. It is still unknown why inactivation of *CmeB* in two similar *in vitro*-selected highly Ery-resistant mutants (JL287 and JL288) led to different MIC reductions. The mechanism of how *CmeABC* synergistically functions together with other mechanism(s) to confer Ery resistance in these mutants needs be examined.

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APPENDIX

Table 1. Key *Campylobacter jejuni* strains used in this study.

Strain	Description	Source
NCTC 11168	wild-type, Ery[a] sensitive	ATCC (cat No. 700819)
JL11	ATCC 33560, quality control strain for MIC testing	ATCC
9B6	*cmeB*::kan, *C. jejuni* 81-176 derivative. Also named JL30	(Lin *et al.* 2002)
DC2	Ery resistant (MIC = 32μg/ml), NCTC 11168 derivative isolated from chicken treated with growth promoter tylosin	(Lin *et al.* 2007)
DC3	Ery resistant (MIC > 512μg/ml), NCTC 11168 derivative isolated from chicken treated with growth promoter tylosin	(Lin *et al.* 2007)
DC6	Ery resistant (MIC > 512μg/ml), NCTC 11168 derivative isolated from chicken treated with growth promoter tylosin	(Lin *et al.* 2007)
DC26	Ery resistant (MIC= 64μg/ml), isolated from chicken inoculated with DC2 and treated with growth promoter tylosin	this study

[a] Ery: erythromycin

Table 2. PCR primers used in this study.

Name	Sequence	Amplicon size	Source
L4 F L4 R	5'- GTA GTT AAA GGT GCA GTA CCA -3' 5'- GCG AAG TTT GAA TAA CTA CG -3'	767 bp	(Cagliero *et al.* 2006b)
L22 F L22 R	5'- GAA TTT GCT CCA ACA CGC -3' 5'- ACC ATC TTG ATT CCC AGT TTC -3'	568 bp	(Cagliero *et al.* 2006b)
23SribF 23SribR	5'- GTA AAC GGC GGC CGT AAC TA -3' 5'- GAC CGA ACT GTC TCA CGA CG -3'	699 bp	(Jensen & Aarestrup 2001)
MOMPF MOMPR	5'-ATG AAA CTA GTT AAA CTT AGT TTA-3' 5'-GAA TTT GTA AAG AGC TTG AAG -3'	1.3 kb	(Huang *et al.* 2005)

Table 3. Erythromycin MICs of *C. jejuni* isolates from chickens receiving non-medicated feed (Control) or feed supplemented with tylosin (50 mg/kg) (Treatment).

Chicken group [a]	Day of age	Ery MIC (μg/ml)	# of isolates
Control	17	16	9
	22	4 16 32	1 6 2
	29	4	9
	36	1 2 4	4 2 3
	43	1 2 4	6 2 1
	50	1 2 4	4 3 2
Treatment	17	16 32 64	3 3 1
	22	16	9
	29	32 64	6 1
	36	32 64	8 2
	43	16 32 64	2 6 1
	50	16 32	1 9

[a] Each chicken was inoculated with approximately 10^7 CFU of *C. jejuni* DC2 (Ery MIC= 32μg/ml) at 15 days of age.

Table 4. Erythromycin MICs of *C. jejuni* isolates obtained from chickens receiving feed supplemented with tylosin (50 mg /kg).

Inoculum[a]	Day of Age	Ery MIC (µg/ml)	# of isolates
DC2 (MIC= 32µg/ml)	20	32	9
		64	1
	34	16	2
		32	7
		64	1
	48	32	9
		256	1
DC26 (MIC= 64µg/ml)	20	64	7
		128	3
	34	64	8
		128	2
	48	64	5
		128	1

[a] Each chicken was inoculated with approximately 10^7 CFU of either *C. jejuni* DC2 (Ery MIC= 32µg/ml) or DC26 (Ery MIC= 64µg/ml) at 15 days of age.

Table 5. *In vivo* stability of Ery-resistance in *C. jejuni.*

Inoculum	Day of age	Ery MIC (µg/ml)	# of isolates
DC2 (Ery MIC= 32µg/ml)	12	16	2
		32	10
	22	4	3
		16	8
		32	1
	38	4	6
		2	2
		1	4
	47	.5	2
		1	1
		2	2
		4	2
		8	1
DC6 (Ery MIC> 512µg/ml)	12	>512	13
	22	>512	13
	38	>512	13
	47	>512	13

Table 6. Mutations in 23SrRNA and changes in ribosomal protein L22 in *C. jejuni* mutants selected *in vitro*.

Name	Ery MIC (μg /ml)	Selective agent	Mutation in 23S rRNA	Change in protein L22	Change in protein L4	Source
Wild- type NCTC 11168	1		-	-	-	ATCC 700819
JL276	16	Ery	-	-	-	
JL277	8	Ery	-	-	-	
JL278	8	Ery	-	-	-	
JL279	8	Ery	-	-	-	
JL283	64	Ery	-	-	-	
JL284	128	Ery	-	-	-	
JL285	128	Ery	-	-	-	
JL286	128	Ery	-	-	-	
JL287	>512	Ery	A2074C[a]	-	-	
JL288	>512	Ery	A2074C[a]	-	-	
JL289	>512	Ery	A2074C[a]	-	-	
JL290	32	Ery	-	Ins $_{98}$TSH	-	
JL291	32	Ery	-	Ins $_{98}$TSH	-	
JL292	8	Ery	-	-	-	
JL293	8	Ery	-	-	-	
JL294	8	Ery	-	-	-	this
JL295	8	tylosin	-	-	-	study
JL296	8	tylosin	-	-	-	
JL297	8	tylosin	-	-	-	
JL298	8	tylosin	-	-	-	
JL299	64	Ery	-	Ins $_{98}$TSH	-	
JL300	128	Ery	-	Ins $_{98}$TSH	-	
JL301	512	tylosin	-	Ins $_{98}$TSH	-	
JL302	512	tylosin	-	Ins $_{98}$TSH	-	
JL303	256	tylosin	-	Ins $_{98}$TSH	-	
JL304	256	tylosin	-	Ins $_{98}$TSH	-	
JL305	256	Ery	-	Ins $_{98}$TSH	-	
JL306	64	Ery	-	Ins $_{98}$TSH	-	
JL307	256	Ery	-	Ins $_{98}$TSH	-	
JL308	64	Ery	-	Ins $_{98}$TSH	-	

- no observed mutation

[a] A2074C mutation was present in two of the three copies of the 23S rRNA gene.

Table 7. Mutations in 23SrRNA, changes in ribosomal protein L4 of *in vivo*-selected mutants.

Name	Ery MIC (μg /ml)	Selective Agent	Mutation in 23S rRNA	Change in protein L4	Change in protein L22	Source
Wild-type NCTC 11168	1		-	-	-	ATCC 700819
DC1	8	tylosin	-	-	-	(Lin *et al.* 2007)
DC2	32	tylosin	-	G74D	-	
DC3	>512	tylosin	A2074G	-	-	
DC4	8	tylosin	-	-	-	
DC5	8	tylosin	-	G74D	-	
DC6	>512	tylosin	A2074G	-	-	
DC7	8	tylosin	-	G74D	-	
DC8	32	tylosin	-	G74D	-	
DC9	>512	tylosin	A2074G	-	-	
DC10	16	tylosin	-	G**57**D	-	
DC11	64	tylosin	-	-	-	
DC12	64	tylosin	-	G74D	-	
DC13	32	tylosin	-	G74D	-	
DC14	32	tylosin	-	G**57**D	-	
DC15	32	tylosin	-	G74D	-	
DC16	32	tylosin	-	G74D	-	
DC17	8	tylosin	-	G**57**D	-	
DC18	16	tylosin	-	G74D	-	
DC19	16	tylosin	-	G**57**D	-	
DC20	16	tylosin	-	G74D	-	
DC21	32	tylosin	-	G74D	-	this study[a]
DC22	32	tylosin	-	G74D	-	
DC23	64	tylosin	-	G74D	-	
DC24	32	tylosin	-	G74D	-	
DC25	64	tylosin	-	G74D	-	
DC26	64	tylosin	-	G74D	-	
DC27	64	tylosin	-	G74D	-	
DC28	8	tylosin	-	G74D	-	this study[b]
DC29	4	tylosin	-	-	-	
DC30	1	tylosin	-	-	-	

Table 7. Continued.

Name	Ery MIC (μg /ml)	Selective Agent	Mutation in 23S rRNA	Change in protein L4	Change in protein L22	Source
DC31	>512	tylosin	A2074G	-	-	this
DC32	>512	tylosin	A2074G	-	-	study[c]
DC33	>512	tylosin	A2074G	-	-	

- no observed mutation
[a] from individual chicken receiving medicated feed and inoculated with DC2 in the first chicken experiment to determine development of high-level Ery resistance (pages 27- 28)
[b] from individual chicken in experiment to determine *in vivo* stability (page 35)
[c] from individual chicken inoculated with DC6 in chicken experiment to determine *in vivo* stability of Ery resistance (page 35).

Table 8. Summary of ribosome modifications in Ery-resistant *C. jejuni* mutants selected *in vivo* and *in vitro*.

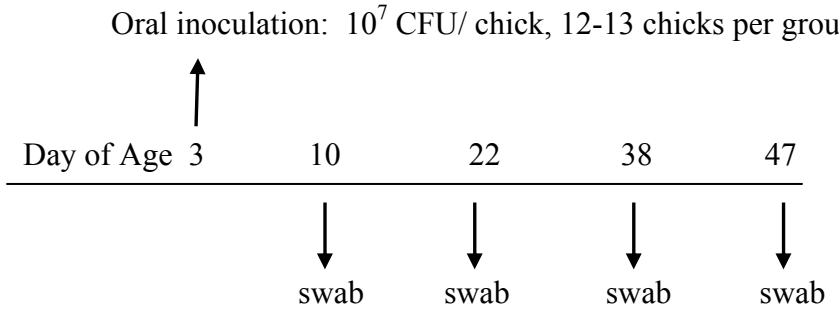
Strain	# of isolates	Ery MIC (µg/ml)	Mutations in 23S rRNA	Change in protein L4	Change in protein L22
Wild type	1	1	-	-	-
in vivo-selected mutants	6	>512	**A**2074**G**	-	-
	18	8-64	-	**G**74**D**	-
	4	8,16,16,32	-	**G**57**D**	-
	4	4,8,8,64	-	-	-
in vitro-selected mutants	3	>512	**A**2074**C**	-	-
	12	32-512	-	-	Ins98TSH
	15	8-128	-	-	-

-, no mutation observed

Table 9. Effect of inactivation of CmeABC multidrug efflux pump on the susceptibility of Ery^R *C. jejuni* mutants to erythromycin.

Strain	**Ery MIC(µg/ml)**	**Mutation in 23S rRNA**	**Change in protein L4**	**Change in protein L22**	**Ery MIC of CmeABC mutant (µg/ml)**
Wild type	1	-	-	-	0.25
in vivo- selected mutants					
DC22	32	-	**G**74**D**	-	0.25
DC27	64	-	**G**74**D**	-	0.25
DC32	>512	**A**2074**G**	-	-	64
in vitro- selected mutants					
JL283	64	-	-	-	0.25
JL284	128	-	-	-	0.5
JL287	>512	**A**2074**C**	-	-	0.5
JL288	>512	**A**2074**C**	-	-	64
JL290	32	-	-	Ins98TSH	0.5
JL301	512	-	-	Ins98TSH	0.5
JL303	256	-	-	Ins98TSH	0.5

***in vivo* test**



***in vitro* test**

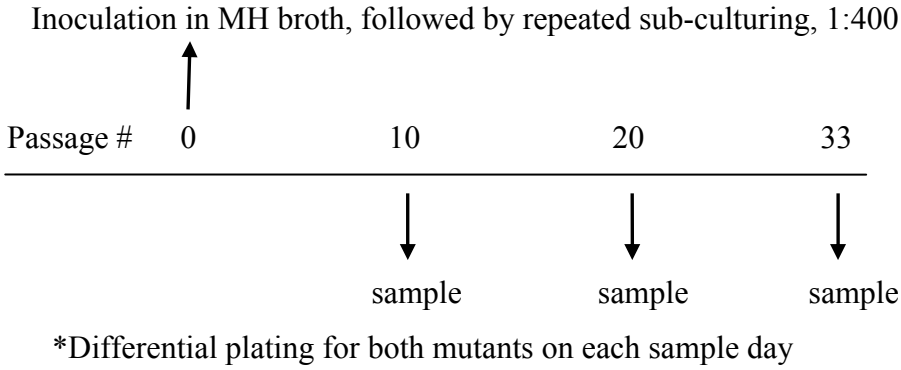


Figure 1. Experimental design of *in-vivo* and *in-vitro* stability test of Ery resistance.

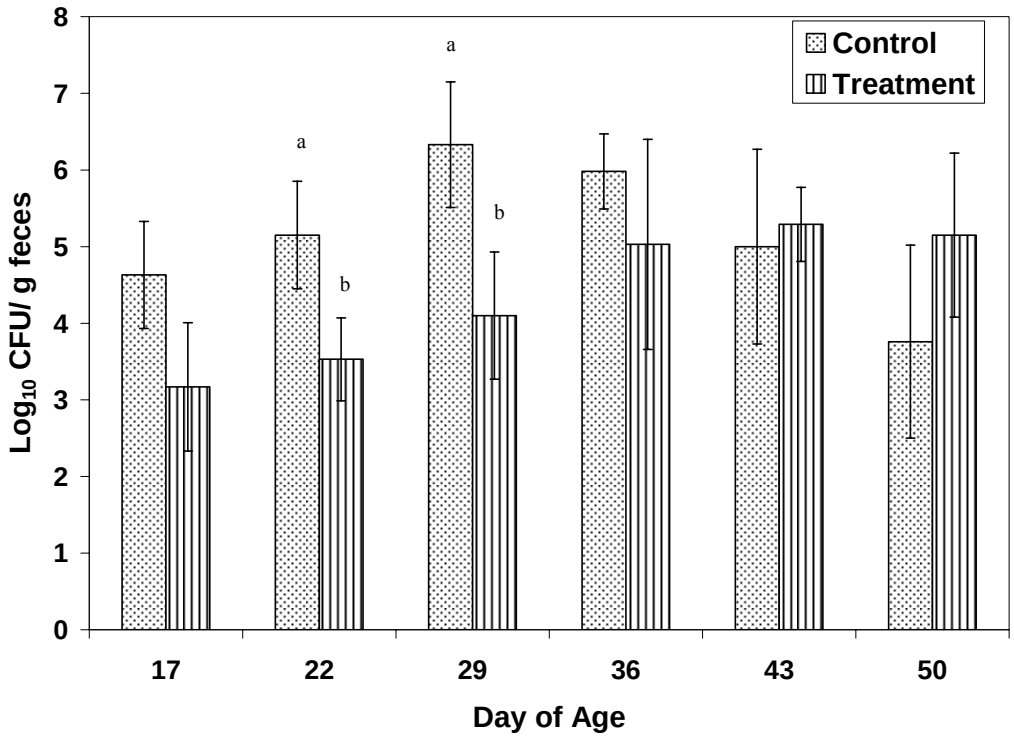


Figure 2. The shedding level of *Campylobacter jejuni* DC2 in chickens receiving non-medicated feed and feed supplemented with growth promoter tylosin (50 mg/kg). Each chicken was inoculated with approximately 10^7 CFU of *C. jejuni* DC2 (Ery MIC= 32μg/ml) via oral gavage at 15 days of age. Control chickens received non-medicated feed. Treatment chickens received feed supplemented with tylosin (50 mg/kg). Each bar represents the mean $\log_{10}$ CFU/g feces +/- standard deviation in each group. Different letters above bars on each sampling day denotes significant difference ($P < 0.01$).

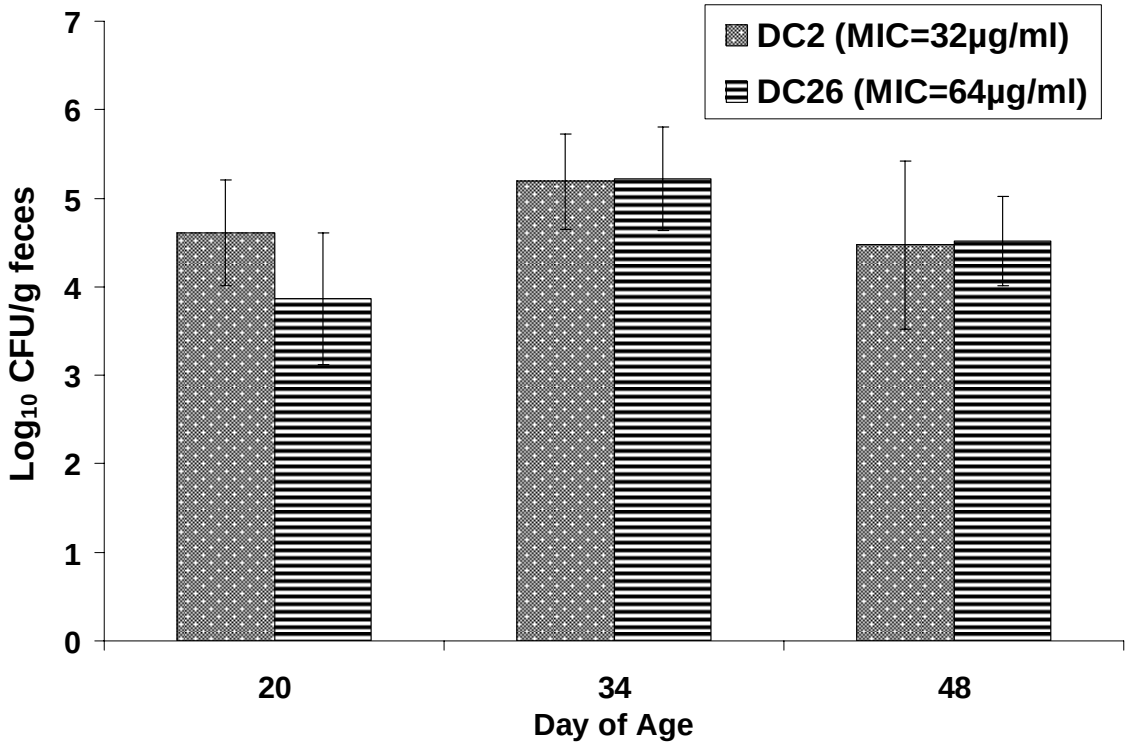


Figure 3. The shedding level of *Campyobacter jejuni* DC2 or DC26 in chickens receiving feed supplemented with growth promoter tylosin (50 mg/kg). Each chicken was inoculated with approximately 10^7 CFU of *C. jejuni* DC2 (Ery MIC= 32µg/ml) or DC26 (Ery MIC= 64µg/ml) via oral gavage at 15 days of age. All chickens received feed supplemented with tylosin (50 mg/kg). Each bar represents the mean $\log_{10}$ CFU/g feces +/- standard deviation in each group.

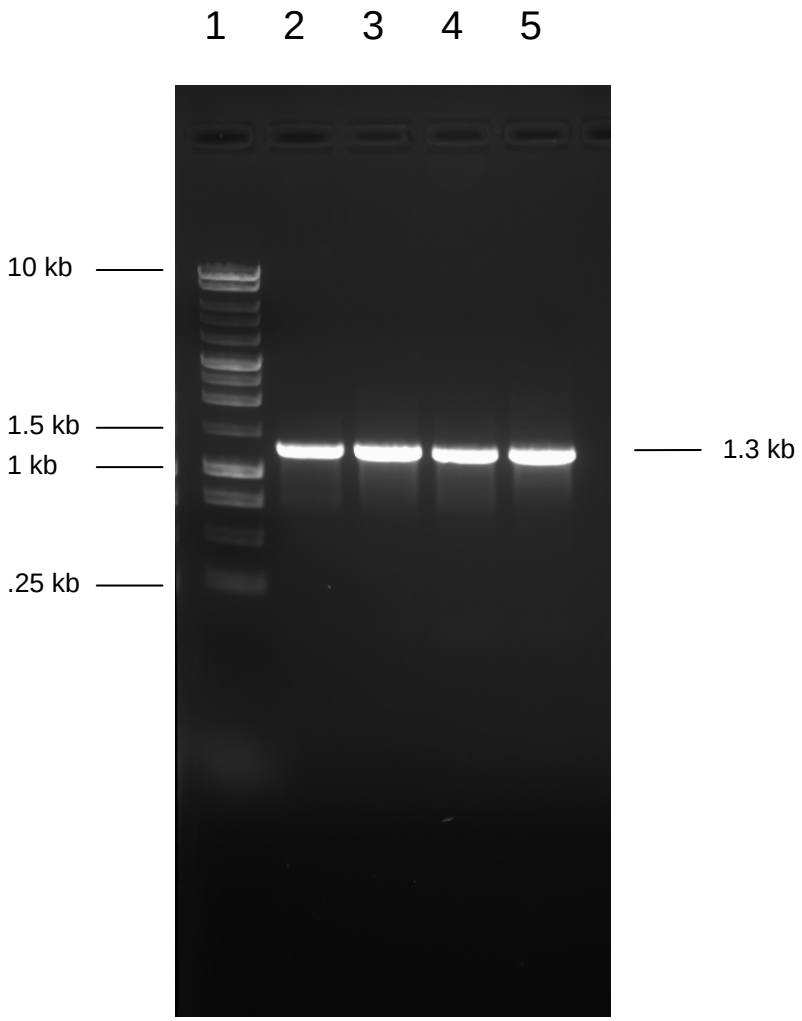


Figure 4. PCR amplification of *Campylobacter* MOMP gene for sequence analysis. Lane 1 is 1kb DNA ladder. *C. jejuni* MOMP gene specific primers were used to amplify MOMP gene from inoculated strain, DC26 (Lane 2), and from three randomly selected isolates from chicken feces (Lanes 3-5).

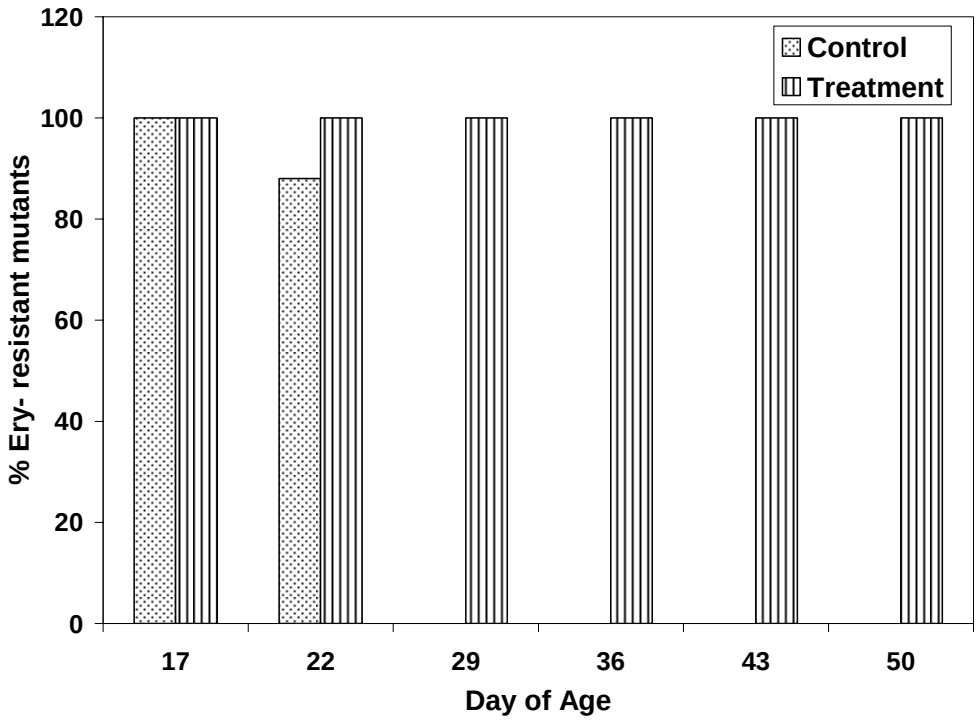


Figure 5. Development of Ery resistance in *C. jejuni* DC2 in response to prolonged low dose tylosin treatment. Each chicken was inoculated with approximately 10^7 CFU of *C. jejuni* DC2 (Ery MIC= 32μg/ml) via oral gavage at 15 days of age. Control chickens received non-medicated feed. Treatment chickens received feed supplemented with tylosin (50 mg/kg). Calculation of % Ery resistant mutants is based on MIC testing using breakpoint 8μg/ml.

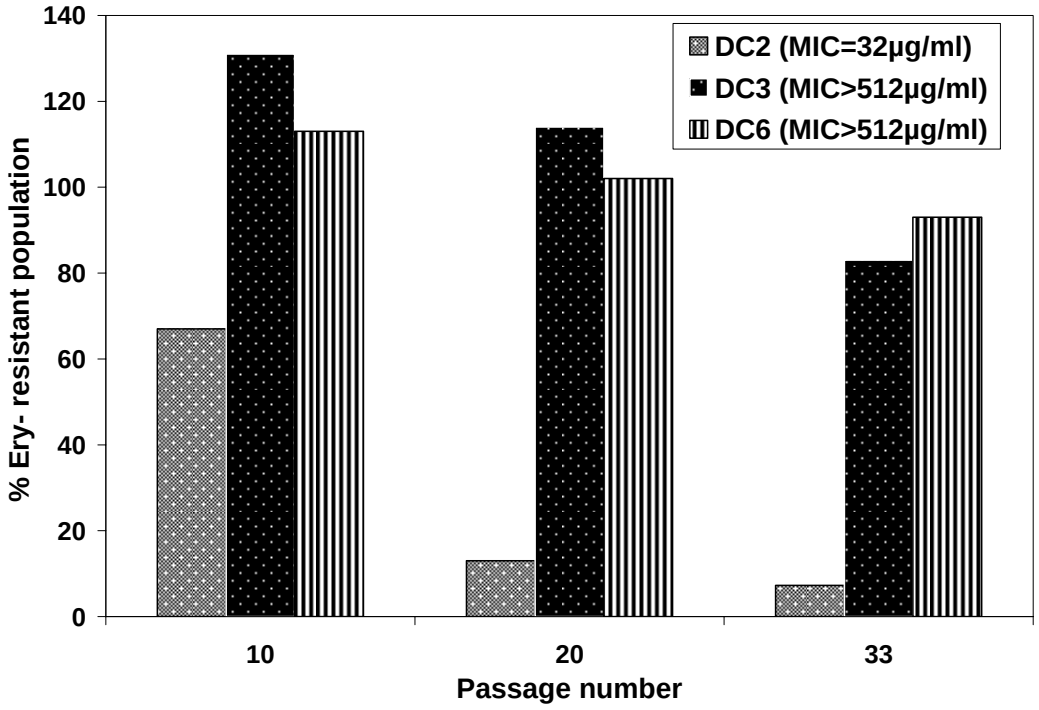


Figure 6. *In vitro* stability of low-level Ery^R *C. jejuni* DC2 (MIC= 32µg/ml) and high-level Ery^R *C. jejuni* DC3 and DC6 (MIC> 512µg/ml). Percentage of Ery^R population was calculated based on differential plating as described in Materials and Methods.

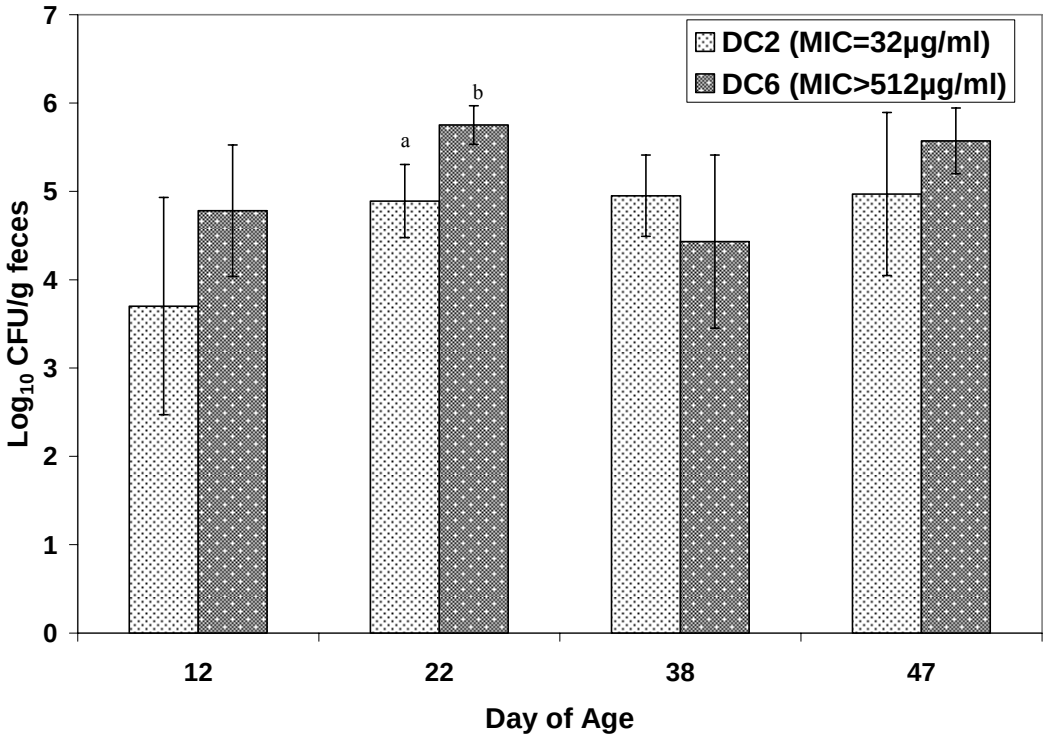


Figure 7. The shedding level of low-level Ery-resistant *C. jejuni* DC2 or high-level Ery resistant *C. jejuni* DC26 in chickens receiving non-medicated feed. Each chicken was inoculated with approximately 10^7 CFU of either *C. jejuni* DC2 (Ery MIC= 32µg/ml) or DC6 (MIC> 512µg/ml) at 3 days of age. All chickens received non-medicated feed. Each bar represents the mean $\log_{10}$ CFU/g feces +/- standard deviation in each group. Different letters above bars on each sampling day denotes significant difference ($P < 0.01$).

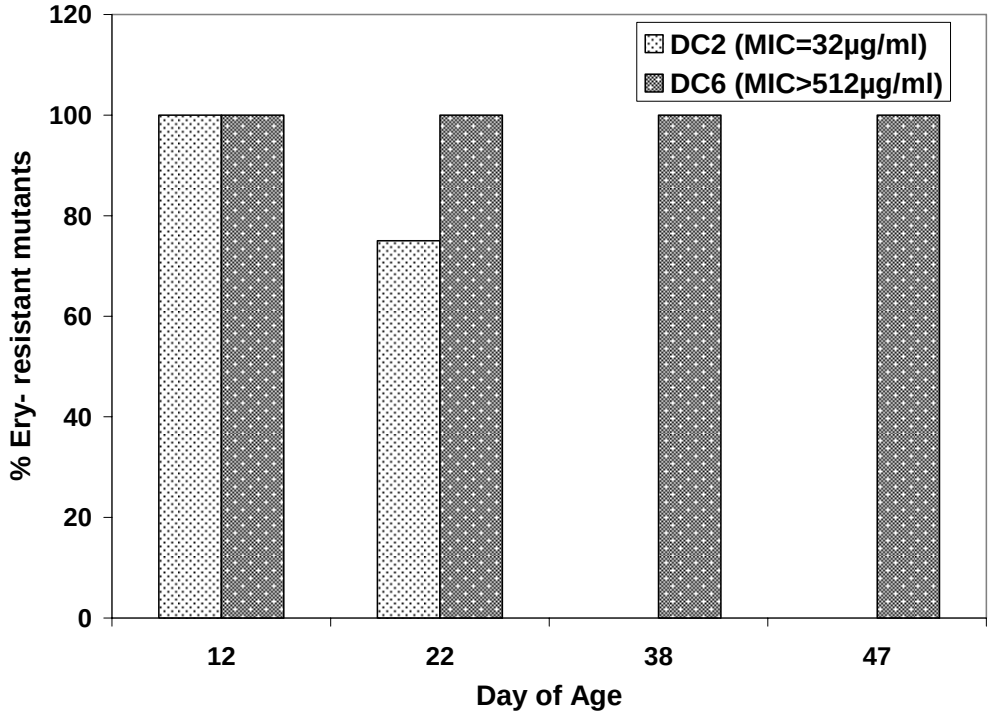


Figure 8. *In vivo* stability of low-level Ery^R *C. jejuni* DC2 and high level Ery^R *C. jejuni* DC6. Each chicken was inoculated with approximately 10^7 CFU of *C. jejuni* DC2 (MIC= 32µg/ml) or DC6 (MIC> 512µg/ml) via oral gavage at 3 days of age. Chickens received non-medicated feed. Calculation of % Ery resistant mutants is based on MIC testing using breakpoint 8µg/ml.

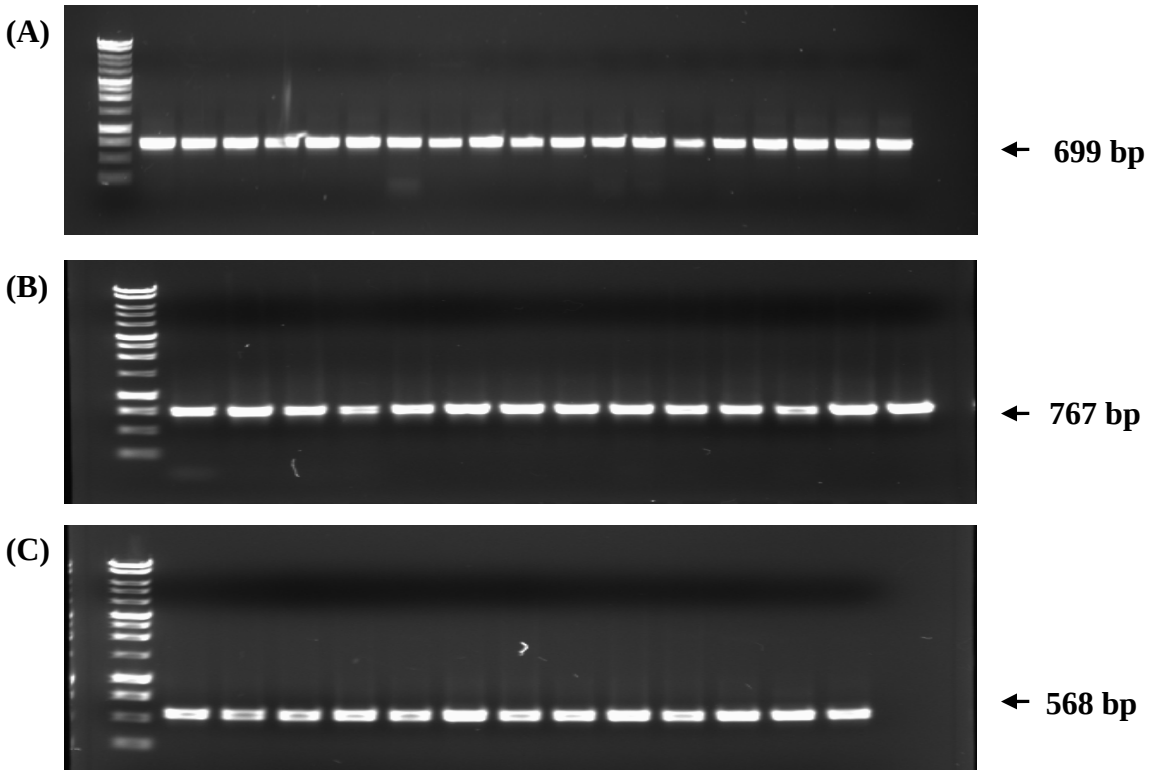


Figure 9. PCR amplification of genes associated with erythromycin resistance in *C. jejuni*: (A) Amplification of 23S rRNA gene using 23S gene specific primers; (B) Amplification of full-length ribosomal protein L4 gene (*rplD*) using *rplD* specific primers; and (C) Amplification of full-length ribosomal protein L22 gene (*rplV*) using *rplV* specific primers.

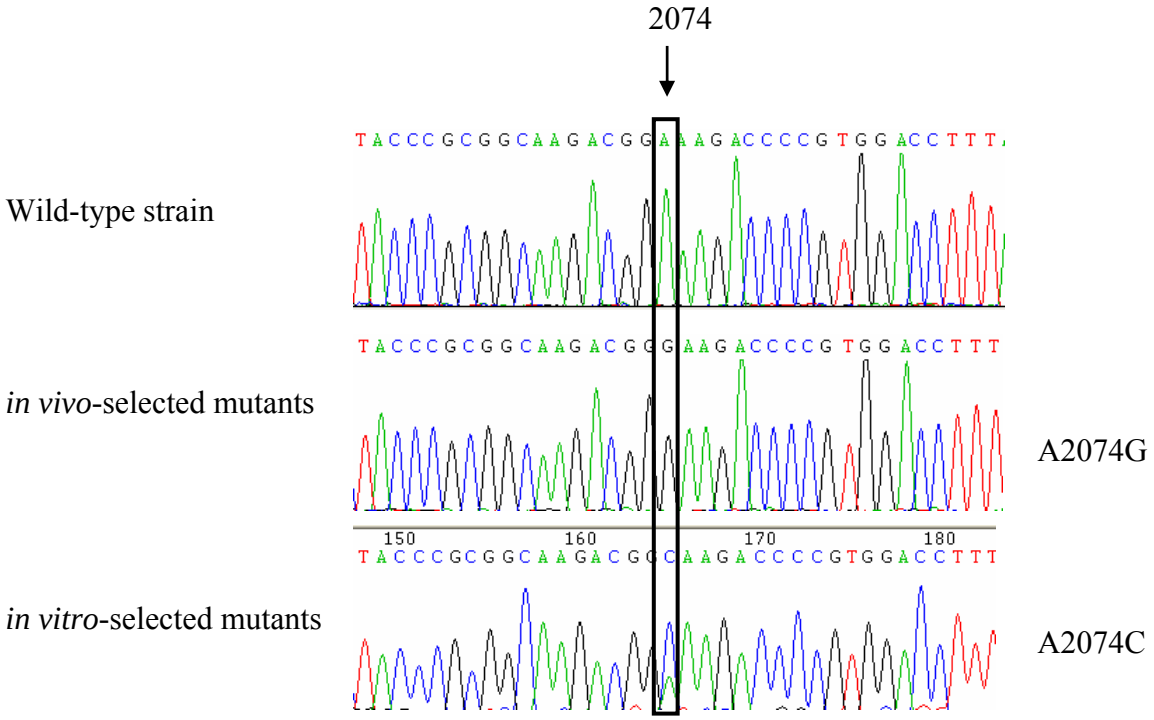
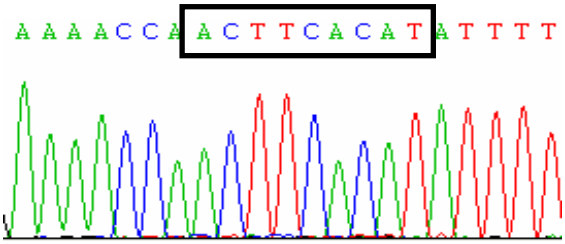


Figure 10. Sequence chromatogram of mutations in 23S rDNA of highly Ery^{R} mutants selected *in vitro* and *in vivo*.

Wild type



in vitro-selected mutants

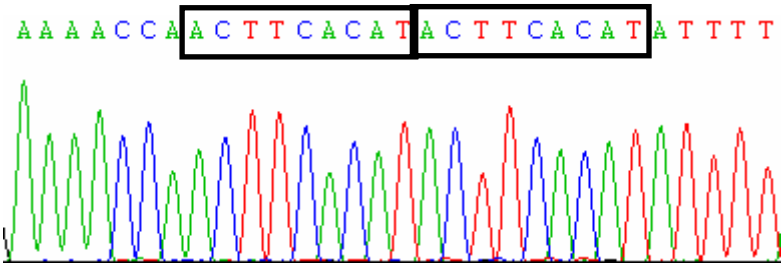


Figure 11. Sequence chromatogram of mutations in ribosomal protein gene L22 in Ery^R mutants selected *in vitro*.

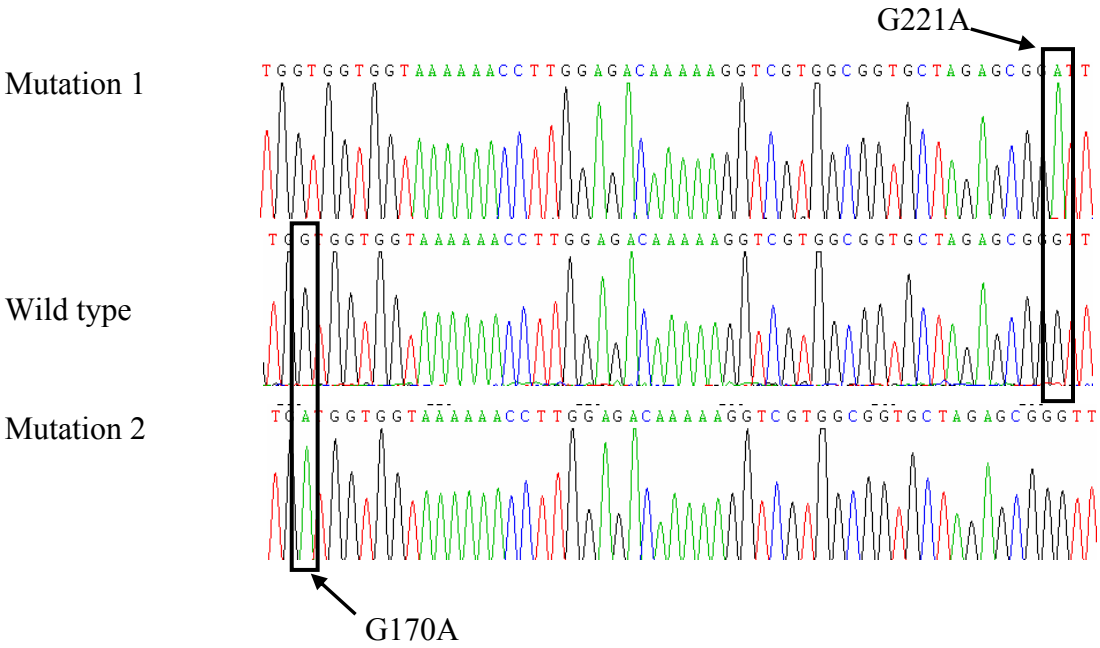


Figure 12. Sequence chromatogram of mutations in ribosomal protein gene L4 in Ery^R mutants selected *in vivo*.

VITA

Dave Bryson Caldwell was born in Asheville, NC on October 29, 1982. He was raised in Waynesville, NC and graduated from Tuscola High School in 2001. He attends the University of Tennessee, Knoxville and received a B.S. in animal science in 2004 and a M.S. in animal science in 2007.

Dave is currently pursuing a D.V.M. at the University of Tennessee College of Veterinary Medicine.