8-2006

# Development of CmeABC Efflux Pump- Based Intervention Strategies Against Campylobacter

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To the Graduate Council:

I am submitting herewith a thesis written by Ad'Lynn Leigh Ensminger entitled "Development of CmeABC Efflux Pump- Based Intervention Strategies Against Campylobacter." 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 .

Jun Lin, Major Professor

We have read this thesis and recommend its acceptance:

Alan Mathew, Mike Davidson

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Vice Chancellor and
Dean of Graduate Studies

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# Development of CmeABC Efflux Pump-Based Intervention Strategies Against Campylobacter

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

Ad'Lynn Leigh Ensminger
August 2006

# DEDICATION

I would like to dedicate this thesis to my husband Michael Martinez. Your love, support, advice and editing skills played an extremely important role in obtaining this degree. Thank you.

# ACKNOWLEDGMENTS

I would like to sincerely thank and acknowledge the may people who helped in the completion of my masters work. First I would like to thank Dr. Jun Lin for his knowledge and guidance and allowing me the opportunity to work with him. I have learned so much from him and it has been a true pleasure. I would also like to thank Dr. Alan Mathew and Dr. Mike Davidson for reviewing my thesis and serving on my committee. A special thanks to Hubbard Hatchery in Pikeville, TN for supplying me with chickens throughout my project; also to Dr. Olga Lomavskova for providing me with new efflux pump inhibitors and Dr. Steve Oliver for giving me clinical isolates to test. Thanks to Nuten Mytle and Janet Chang for all of your assistance in the lab. I would also like to thank all of the staff and faculty of the Animal Science department, particularly Eddie Jarboe and Linda Miller for all of their help and assistance. I would like to thank all of the graduate students for all of their friendship and for making my time here enjoyable. Last but not least I would like to thank my family, especially my husband and my parents for all that they have done to get me to this point.

# ABSTRACT

CmeABC, a multidrug efflux pump, contributes to *Campylobacter* resistance to a broad spectrum of antimicrobial agents and is also essential for *Campylobacter* colonization by mediation of bile resistance. We hypothesize that inhibition of CmeABC will not only control antibiotic resistance but also increase the susceptibility of *Campylobacter* to in vivo bile salts, consequently decreasing the colonization level of *Campylobacter*. Using both in vitro and in vivo systems, we examined the effect of an efflux pump inhibitor (EPI) MC-207,110 on the susceptibility of *Campylobacter* to various antimicrobials. Presence of the EPI resulted in 2- to 2048-fold reduction in the MICs of antimicrobials known to be substrates of CmeABC pump in all *Campylobacter* strains. Particularly, the MICs of selected bile salts were dramatically decreased 64- to 512-fold when the EPI was used. The intrinsic and acquired resistance of *C. jejuni* to macrolide was decreased significantly (32- to 64-fold reduction in the MIC of erythromycin) in the presence of the EPI while the MICs of fluoroquinolones were only slightly decreased (2-4 folds). Investigation of 57 clinical *Campylobacter* isolates of various origins further showed that the EPI decreased the MICs of erythromycin (2- to 512-fold) in all isolates. Compared to wild-type strains, the isogenic CmeB mutants displayed much lower magnitude of reduction in the MICs of antimicrobials in the presence of the EPI. The inhibitory effect of the EPI was does-dependents and as low as 0.5 μg/ml of the EPI resulted in decreased MIC of antimicrobials in *C. jejuni*. Presence of the EPI decreased the frequency of emergence of erythromycin-resistant mutants in *C. jejuni* ($<10^{-11}$), which is well below normal frequency of approximate $10^{-8}$. Notably, MIC

of erythromycin was also greatly decreased (> 4-fold) in CmeB mutants in the presence of EPI, suggesting the existence of other pump(s) involved in macrolide resistance in *C. jejuni*. Chicken colonization study demonstrated that oral administration of EPI dramatically reduced the colonization of *Campylobacter* in the intestine. In addition, anti-CmeC antibodies also enhanced the susceptibility of *C. jejuni* to bile salt, suggesting immune intervention by targeting CmeC may be another effective strategy to inhibit CmeABC efflux pump. Together, these findings indicate that inhibition of CmeABC by specific EPI or antibodies is a promising approach to control antibiotic resistance and colonization of *Campylobacter* in human and animals.

Key Words: *Campylobacter*, efflux pumps, efflux pump inhibitors

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## *Campylobacter* and the Poultry Reservoir

*Campylobacter*, a microaerophilic Gram-negative bacterium, is the leading foodborne human pathogen in the United States and many other industrialized countries (Altekruse et al., 1999; Friedman et al., 2000). There are an estimated 2.1 to 2.4 million reported cases in the United States each year, with even more cases going unreported (Mead et al., 1999). Medical and production costs associated with *Campylobacter* average 1.5 to 8.0 billion dollars each year in the United States (Buzby et al., 1997). This pathogenic organism causes watery diarrhea, hemorrhagic colitis, and even severe abdominal pain (Skirrow and Blaser, 2000). In rare cases *Campylobacter* can also be associated with Guillian-Barre syndrome, which is an autoimmune disease that may lead to respiratory muscle compromise and death (Nachamkin et al., 1998). The infective dose can be as low as 5-800 organisms (Black et al., 1988). Through oral ingestion, *C. jejuni* enters the host intestine via stomach acid barrier and colonizes the distal ileum and cecum. There are more than 14 different species of *Campylobacter*. However, human *Campylobacter* illness are primarily caused by C. *jejuni* (>99%) and secondarily by *C. coli* (Allos et al., 2001). Growth conditions for *C. jejuni* are very specific. *C. jejuni* survive poorly outside of intestine and replication does not occur readily in the environment. *C. jejuni* requires optimal growth temperatures between 37 $^{o}$C and 42 $^{o}$C. In addition, *C.jejuni* grows best in a microaerophilic environment, such as an atmosphere of 5% $O_2$, 10% $CO_2$, and 85% $N_2$. Although these growth conditions are stringent,

*Campylobacter* is still able to cause more infections than *Salmonella*, *Shigella* and *E. coli* O157 (Altekruse et al., 1999).

Although *Campylobacter* is widely present in wild and domestic animals (Jacobs-Reitsma et al., 1997, 2000), poultry are considered the major reservoir of *Campylobacter*, because this organism is highly prevalent in poultry and the majority of human campylobacteriosis are epidemiologically linked to consumption of contaminated poultry products (Allos et al., 2001). *Campylobacter* is also highly prevalent in market-age broiler chickens and turkeys raised on organic or free-range farms (Avrain *et al.*, 2003; Heuer *et al.*, 2001; Rivoal *et al.*, 1999), indicating that different production systems are equally vulnerable to invasion by this organism. Besides chickens and turkeys, *Campylobacter* species also infect ducks, ostriches, and geese (Aydin et al., 2001; Wallace et al., 1998; Yogasundram et al., 1989). *Campylobacter* is carried in the intestinal tract of birds and excreted in feces. The number of *Campylobacter* in intestinal contents of broiler chickens can be as high as $10^8$ CFU $g^{-1}$ feces (Stern et al., 1995; Wempe et al., 1983). Although market-age broilers and turkeys are highly contaminated by *Campylobacter*, the prevalence in individual flocks varies greatly depending on the age of birds (Berndtson et al., 1996; Evans and Sayers, 2000; Kazwala et al., 1990; Shanker et al., 1988). *Campylobacter* is rarely detected in broiler chickens less than 2-3 weeks old under commercial production conditions, although newly hatched chickens can be experimentally infected with this pathogen (Sahin et al., 2002; Stern et al., 1988; Young et al., 1999). For the majority of commercial flocks, *Campylobacter* infection is usually detected after the third week of age (Newell and Fearnley, 2003; Sahin et al.,

2002). Once a flock is infected by *Campylobacter*, most birds soon become colonized and shed large numbers of the organism. Despite persistent and extensive colonization in chickens, this organism does not cause clinical disease in the poultry host under natural conditions (Beery et al., 1988; Meinersmann et al., 1991). In poultry processing plants, carcasses and edible parts are often contaminated with *C. jejuni*, largely through contact with fecal materials. Hence, a large number (up to 100% in summer months) of retail broilers is contaminated with *C. jejuni* (Ge et al., 2003; Willis and Murray, 1997), posing a major threat to public health.

Horizontal transmission, or transmission within a population, is a major mechanism of infection of *Campylobacter* among chicken flocks. Possible sources include feces, untreated drinking water, other farm animals, domestic pets, wildlife species, house flies, insects, equipment, transport vehicles and farm workers (Sahin et al., 2002). However, the most likely source of infection comes from one infected chicken passing it to another in a rapid fire succession. While most evidence supports the theory of horizontal transmission, it is likely that *Campylobacter* infection is also mediated by vertical transmission, which is the transmission from hen to chick at the time of laying and/or hatching. Reasoning behind this theory is that *Campylobacter* can be isolated from chicken eggs and in some cases from newly hatched chicks (Sahin et al., 2002).

**Antibiotic Resistance in *Campylobacter***

Development of antibiotic resistance is inevitable in bacteria, including *Campylobacter*, and every antibiotic that is introduced into market to date has limited time before it is no longer effective (Walsh et al., 2003). In general, *Campylobacter* resistance to antibiotics is mediated by three mechanisms, including 1) synthesis of enzymes (e.g. β-lactamase) that modify or inactivate antibiotics, 2) alteration or protection of targets that results in reduced affinity to antibiotics (e.g. mutations in *gyrA*), and 3) active extrusion of drugs from *Campylobacter* cells through efflux transporters (e.g. CmeABC). Detailed information on resistance mechanisms to different classes of antibiotics has been provided in recent reviews (Taylor and Tracz, 2005; Snelling et al., 2005; Trieber and Taylor, 2000). Antibiotic-resistant *Campylobacter* strains have rapidly increased throughout the world in the past decade, compromising the clinical effectiveness of antimicrobial treatments (Engberg et al., 2001; Taylor and Tracz, 2005). The factors contributing to the rising resistance are complex, and the extensive use of antimicrobial agents in human medicine and agriculture is considered the main driving force for the worldwide progression of antibiotic resistance in *Campylobacter* (Smith et al., 2000).

The increasing resistance of *C. jejuni* to fluoroquinolones (FQs) and macrolides, the two drugs of choice for treating human campylobacteriosis, has become a major concern for public health. FQs target bacterial DNA gyrase and topoisomerase IV, which catalyze the ATP-dependent negative supercoiling of DNA and are involved in DNA

replication, recombination, and transcription (Trieber and Taylor, 2000; Taylor and Tracz, 2005; Zhang et al., 2003). Recent studies indicate that *Campylobacter* displays a hypermutable phenotype in response to *in vivo* treatment of poultry with FQ antibiotics, resulting in rapid emergence of FQ-resistant mutants in poultry (Luo et al., 2003; McDermott et al., 2002). Therefore, on July 28, 2005, the FDA issued a ban on the use of Baytril in poultry (an antibiotic nearly identical to FQ ciprofloxacin) to protect public health (see www.fda.gov/bbs/topics/news/2005/new01212.html). Distinct from other Gram-negative bacteria, acquisition of FQ resistance in *Campylobacter* does not require stepwise accumulation of *gyrA* mutations and overexpression of efflux pumps, and is mainly mediated by single-step point mutations in *gyrA* in the presence of a constitutively expressed multidrug efflux pump (Zhang et al., 2003).

FQs are losing effectiveness in clinical treatments due to the widespread resistance of *Campylobacter* to this class of antimicrobials (Engberg et al., 2001; Smith et al., 1999). Consequently, erythromycin (a macrolide) is considered a best option for treating *Campylobacter* infections. Unfortunately, *Campylobacter* resistance to macrolides is also on the rise (Engberg et al., 2001). Macrolide antibiotics inhibit bacterial protein synthesis by binding to the 50S subunits of ribsome (Engberg et al., 2001; Taylor and Tracz, 2005; Trieber and Taylor, 2000). *Campylobacter* resistance to macrolides results from chromosomal mutations in the 23S RNA gene, which lead to reduced binding of macrolide antibiotics to the 50S subunit of ribosome (Engberg et al., 2001; Taylor and Tracz, 2005). Specifically, the A-2230-G mutation is associated with the majority of erythromycin-resistant *Campylobacter* (Ge et al., 2003; Jensen and

Aarestrup, 2001; Gibreel et al., 2005). Multidrug efflux pump CmeABC also plays an important role in the intrinsic and acquired resistance to macrolides (Mamelli et al., 2005; Lin et al., 2002). Other mechanisms such as methylation, which is involved in macrolide resistance in other bacteria, have not been associated with macrolide resistance in *Campylobacter* (Trieber and Taylor, 2000).

The reported macrolide resistance rates vary with *Campylobacter* species and the animal hosts. In general, *C. coli* has higher erythromycin resistance rates than *C. jeuni*, regardless of the source of isolation (Engberg et al., 2001; Saenz et al., 2000; Van Looveren et al., 2001). Likewise, pigs and turkeys tend to harbor higher numbers of erythromycin-resistant *Campylobacter* than other animal species. For example, a recent study in Italy reported that 3.1% *C. jejuni* and 45% *C. coli* from broilers are resistant to erythromycin, while 42.6% *C. coli* from pigs and 24.1% *C. coli* from humans are resistant to the same antibiotics (Pezzotti et al., 2003). Another study conducted in France showed erythromycin resistance in 0.3% *Campylobacter* from broiler chicken and 31% from turkey (Avrain *et al.*, 2003). A recent survey of retail meats conducted in the U.S. showed that 40-50% *Campylobacter* isolates from chicken and 90-100% *Campylobacter* isolates from turkey were resistant to erythromycin (Ge et al., 2003).

**General Features of MDR Efflux Pumps in Gram-Negative Bacteria**

As a general and important mechanism for antimicrobial resistance, multidrug efflux systems (often named MDR pumps) contribute significantly to antimicrobial

resistance by extruding structurally diverse antimicrobial agents out of bacterial cells (Poole, 2001a; Putman et al., 2000). In pathogenic bacteria, there are many different types of multidrug efflux systems which vary in size, structure, and energy source (proton gradient or ATP hydrolysis (Putman et al., 2000). In general, MDR efflux pumps can be categorized into five different superfamilies, including MFS (major facilitator superfamily), SMR (small multidrug resistance), RND (Resistance-Nodulation-cell Division), MATE (multidrug and toxic compound extrusion), and ABC (ATP-binding cassette) (Putman, 2000). These efflux systems are broadly distributed and a single microorganism can have multiple efflux transporters of different families with overlapping substrate spectra (Poole, 2001a; Putman et al., 2000).

One major and important family of MDR pumps in Gram-negative bacteria is RND efflux system, which consists of an inner membrane transporter, a periplasmic fusion protein, and an outer membrane protein (Zgurskaya and Nikaido, 2000). These three components function together and form a membrane pump to extrude antimicrobials out of cells. Genetically, many of the RND-type MDR efflux systems are encoded by three-gene operons located on bacterial chromosomes (Zgurskaya and Nikaido, 2000; Paulsen et al., 2001). However, some RND-type efflux pumps, such as AcrAB from *Escherichia coli* (Ma et al., 1993), have an outer membrane component that is encoded by a separate gene physically unlinked with the other two members on the bacterial chromosome. More detailed information about molecular properties of various types of bacterial MDR transporters is discussed in an excellent review by Putman et al. (2000).

A key feature of these MDR efflux systems, particularly RND type pump, is their ability to extrude a broad spectrum of substrates including various clinically relevant antibiotics (Poole, 2001a; Putman et al., 2000). Overexpression of RND-type efflux pumps results in a MDR phenotype in bacterial pathogens and is considered a major mechanism of antibiotic resistance in a growing number of pathogenic bacteria (Poole, 2001a; Putman et al., 2000; Van Bambeke et al., 2000). Even without overexpression, constitutively expressed MDR pumps function synergistically with other non-efflux resistance mechanisms (such as target mutations) to confer high levels of antimicrobial resistance in bacteria (Lomovskaya et al., 1999; Oethinger et al., 2000; Wang et al., 2001; Luo et al., 2003). The RND-type efflux systems also play an important role in bacterial resistance to a variety of antimicrobial compounds that are naturally present in animal hosts (e.g. bile salts) (Poole, 2001a; Putman et al., 2000; Gunn, 2000; Lin et al., 2003b; Thanassi et al., 1997; Rosenberg et al., 2003). Major natural function of RND efflux system is proposed to be involved in the *in vivo* adaptation in various host niches (e.g. bile resistance in intestine) during infections (Poole, 2001a; Lin et al., 2003b; Lin et al., 2005b; Webber and Piddock, 2003).

The expression of bacterial MDR efflux pumps is usually controlled by transcriptional regulators that either repress or activate the transcription of the MDR efflux genes (Grkovic et al., 2002; Poole, 2001a). Many of these regulators are local repressors that directly interact with the promoter regions of MDR efflux genes or operons. The operator sequences that interact with the repressor molecules usually consist of inverted repeats (Grkovic et al., 2002). Mutations in the repressors or

repressor-binding regions impede the repression and result in overexpression of efflux pumps, which consequently increases bacterial resistance to structurally unrelated antimicrobial agents (Grkovic et al., 2002; Poole, 2001a; Putman et al., 2000). Some MDR efflux systems are also controlled by global regulators, such as the *marRAB* regulon in *E. coli* (Alekshun and Levy, 1999b). MarR is a repressor of *marRAB*, while *marA* encodes an activator that not only positively regulates *marRAB* but also activates a variety of genes (including *acrAB*) associated with resistance to antibiotics and oxygen stress (Alekshun et al., 2000; Alekshun and Levy, 1999b). Mutations in *marR* substantially increase the expression of *acrAB* and confer *E. coli* resistance to a variety of antimicrobial agents (Alekshun and Levy, 1999a; Kern et al., 2000; Okusu et al., 1996).

In addition to the mutation-based mechanisms that result in sustained overexpression of MDR efflux pumps in bacteria, the production of some MDR efflux pumps can be conditionally induced by structurally diverse substrates or by stress signals (Ahmed et al., 1994; Brooun et al., 1999; Grkovic et al., 1998; Kaatz and Seo, 1995; Kato et al., 1992; Ma et al., 1995; Masuda et al., 2000). This induction is usually due to the direct interaction of the substrates with repressor molecules, which interferes with the binding of repressors to operator DNA and results in increased expression of MDR genes. Two repressors (QacR and AcrR) of such inducible MDR efflux pumps belong to the TetR family of transcriptional regulators, which share a conserved helix-turn-helix DNA-binding motif at their N-terminal regions and have divergent C-terminal sequences that are involved in the binding to inducing compounds (Grkovic et al., 1998; Hillen and Berens, 1994).

**Multidrug Efflux Systems in *Campylobacter***

In contrast to the situation with specific resistance mechanisms, such as target mutations and enzyme modifications, little is known about the general antibiotic efflux mechanisms in *Campylobacter*. In an early study (Charvalos et al. 1995), MDR *C. jejuni* isolates were selected by *in vitro* passage of the organism on pefloxacin-containing plates. These isolates were not only resistant to FQs, but also resistant to other structurally unrelated antibiotics. The results showed that resistant strains had less accumulation of antimicrobial substances than did wild-type strains, indicating the presence of MDR efflux pump systems (Charvalos et al., 1995). However, the identities of putative MDR pumps and their role in efflux of antibiotics were not determined (Charvalos et al., 1995). The genomic sequence of *C. jejuni* NCTC 11168 revealed several genes that share sequence homology with known MDR efflux pumps in bacterial pathogens (Parkhill et al., 2000). Recently, two RND type multi-drug efflux pumps CmeABC and CmeDEF have been identified and functionally characterized (Akiba et al., 2006; Lin et al., 2002, 2003, 2005a, b; Luo et al., 2003; Guo et al., 2005).

MDR efflux pump CmeABC is encoded by a three-gene operon which encodes for CmeA (periplasmic linker protein), CmeB (inner membrane protein) and CmeC (outer membrane protein) (Lin et al., 2002). Inactivation of the CmeABC pump by insertional mutagenesis substantially increased the susceptibility of *C. jejuni* to structurally diverse antimicrobial agents, including FQs, β-lactams, erythromycin, rifampin, ethidium bromide, heavy metals, detergents and various bile salts (Lin et al., 2002, 2003; Pumbwe

and Piddock, 2002). This phenotypic change can be observed in different strain backgrounds. Insertional mutation in *cmeC* resulted in the same changes in antibiotic susceptibility as those caused by *cmeB* mutation (Lin et al., 2003), indicating that inactivation of a single component in the system would cause malfunction of the CmeABC pump and further supporting the notion that the three members of CmeABC function together in the efflux of substrates (Lin et al., 2002). The dramatic change in antibiotic susceptibility caused by inactivation of CmeABC in different *Campylobacter* strains suggests that this pump is a main player in mediating intrinsic resistance to antibiotics. Although the role of CmeABC in the acquired antibiotic resistance in *Campylobacter* has not been well understood, partly because overproduction of CmeABC has not been linked to antibiotic resistance in clinical isolates, several studies (Lin et al., 2002; Luo et al., 2003; Mamelli et al., 2005) provide compelling evidence that CmeABC can work synergistically together with other nonefflux mechanisms to confer high level of resistance to clinically important antibiotics, such as FQs and macrolides. PCR and immunoblotting analysis showed that *cmeABC* was broadly distributed in various *C. jejuni* isolates and constitutively expressed in wild-type strains.

One striking feature of CmeABC is its essential role in *C. jejuni* resistance to bile, a group of bactericidal detergents present in the intestinal tracts of animals (Lin et al., 2003, 2005b). This is a very important aspect because the efflux of these bile salts can allow *Campylobacter* to establish colonization without hindrance from the body's natural defense mechanisms. Inactivation of CmeB or CmeC resulted in malfunction of the CmeABC pump and drastically increased susceptibilities of *C. jejuni* to various bile salts.

Addition of choleate (2 mM) in culture media impaired the *in vitro* growth of the *cmeC* mutant, but had no effect on the growth of the wild-type strain. Bile concentration varied in duodenum, jejunum, and cecum of chicken intestine and the inhibitory effect of the intestinal extracts on the *in vitro* growth of *Campylobacter* was well correlated with the total bile concentration in the individual sections of chicken intestine. When inoculated into chickens, the wild-type strain colonized the birds as early as day 2 post-inoculation with a density as high as $10^7$ CFU/g feces. In contrast, both *cmeB* and *cmeC* mutants failed to colonize any of the inoculated chickens throughout the study. The minimum infective dose for CmeABC mutant is at least $2.6 \times 10^4$ fold higher than that of the wild-type strain. Complementation of the *cmeABC* mutants with a wild-type *cmeABC* allele in trans fully restored the *in vitro* growth in bile-containing media and the *in vivo* colonization to the levels of the wild-type strain. These results provide compelling evidence that CmeABC, by mediating resistance to bile salts in the intestinal tract, is required for successful colonization of *C. jejuni* in host.

A recent study demonstrated that CmeABC efflux system is subject to regulation by CmeR, a transcriptional repressor encoded by a gene immediately upstream of cmeABC operon (Lin et al., 2005a). CmeR represses the transcription of CmeABC by directly binding to the promoter region of the CmeABC operon. Mutations either in CmeR or in the inverted repeat impedes the repression and leads to overexpression of CmeABC in *Campylobacter* (Lin et al., 2005a). In addition to mutation-based mechanisms that resulted in sustained overexpression of CmeABC efflux pump as described above, CmeABC efflux pump can be conditionally induced by bile salts, a

group antimicrobial agents naturally present in intestine (Lin et al., 2005b). The induction is in a dose- and time-dependent manner and is mediated by the direct interaction of bile salts with repressor CmeR. Since CmeABC is essential for bile resistance, the inducible expression of *cmeABC* by bile salts provides a flexible mechanism for *Campylobacter* to adapt in the intestinal environments. This notion is further supported by a recent study (Stintzi et al., 2005) in which the expression of *cmeABC* was found to be highly up-regulated in rabbit ileal loops as determined by whole genome microarray and real-time RT-PCR. The elevated transcription of *cmeABC* in the rabbit ileal loops was likely the direct result of bile induction, although non-bile inducers for *cmeABC* may also exist in the gut. Together, these findings highlight the significance of CmeABC in *Campylobacter* adaptation to the intestinal environment in hosts and CmeABC efflux system is an attractive therapeutic and vaccine target to prevent and control *Campylobacter* infections.

Another RND type efflux system, CmeDEF, has also been identified recently in *C. jejuni* (Pumbwe et al., 2005; Akiba et al., 2006). Mutation in CmeDEF only resulted in moderate decrease in the resistance to antibiotics (Pumbwe et al., 2005; Akiba et al., 2006). The *cmeB*/*cmeF* double mutation, not the single mutations, impaired cell viability in *Campylobacter* (Akiba et al., 2006). These findings indicate that CmeABC is the predominant efflux pump in *C. jejuni* and CmeDEF interacts with CmeABC in conferring antimicrobial resistance and maintaining cell viability in *C. jejuni*.

Based on the complete genome sequence of *C. jejuni* NCTC 11168 (Parkhill et al., 2000) and comparative genomic analysis of MDR transporters (Paulsen et al., 2001), bioinformatics analysis revealed 11 additional putative drug efflux proteins in *C. jejuni*, including four putative MFS efflux pumps, four putative SMR type efflux pumps, one putative ABC type efflux pump, and two putative MATE (Lin et al., 2005c). These eleven putative efflux systems have not been characterized in detail. Recent study by Ge et al. (2005) showed that mutations in most of these efflux pumps did not affect susceptibility of *C. jejuni* to major antibiotics, suggesting that these putative efflux pumps are of minimal importance in mediating antibiotic resistance and that CmeABC is the main efflux system in *Campylobacter* species.

**Efflux Pump Inhibitor**

Efflux pump systems are increasingly recognized as a major and important mechanism contributing to antibiotic resistance, particularly multi-drug resistance (MDR) (Poole, 2005), which provide a strong rationale for industries to preserve and significantly potentiate the efficacy of antimicrobial agents by interefering with efflux pumps through small molecular inhibitors, also called efflux pump inhibitors (EPIs) (reviewed in (Lomovskaya and Watkins, 2001b; Lomovskaya and Bostian, 2006; Kaatz, 2005)). It has been proposed that inhibiting MDR efflux systems by application of EPIs is one approach to enhance drug accumulation inside the bacterial cell, thereby increasing bacterial susceptibility to antimicrobials (Poole, 2001b; Lomovskaya and Watkins, 2001b). Since RND-type efflux pump is the most common and important MDR pump

implicated in clinically relevant resistance in gram-negative bacteria, the EPIs discussed in this session are focused on those targeting RND-type efflux pumps. The information regarding the EPIs inhibiting other types of efflux pumps can be found in recent reviews (Lomovskaya and Watkins, 2001; Kaatz, 2005).

With the development of high-throughput screening system in the late 1990s, Microcide and Daiichi Pharmaceuticals Co. identified the first EPI, MC-207,110 (Phe-Arg β-naphthyl-amide dihydrochloride), that could effectively inhibit RND type efflux pumps in *P. aeruginosa* (Lomovskaya et al., 2001a). This compound was subjected to several growth assays, using *Pseudomonas* species, and was determined to be effective at inhibiting the function of four clinically relevant RND type efflux pumps within those species. Interestingly, there was a difference in the strength of the inhibitory effect of EPI, had depending on the substrate. This information supports the idea that different antimicrobials have different binding sites on the efflux pump (Lomovskaya et al., 2001a). Further testing found that MC-207,110 was itself effluxed out of cells, indicating that there is definitely an interaction between the efflux pump and MC-207,110 (Lomovskaya et al., 2001a). MC-207,110 appears to be a much better substrate of efflux pumps than other antimicrobials and in a given instance, MC-207,110 would be preferentially effluxed before other substrates, thus allowing antimicrobials more time to exert their bactericidal effect (Lomovskaya et al., 2001a). In addition to the effect of increasing the susceptibility to clinical antibiotics in wild-type strains of *P. aeruginosa*, EPI MC-207,110 was also shown to reverse acquired resistance back to susceptible phenotypes and decrease the frequency of emergence of antibiotic resistant strains

(Lomovskaya et al., 2001a). Other studies showed that MC-207,110 is also effective against a variety of Gram-negative bacteria, including *E. coli* (Saenz et al., 2004), *Enterobacter aerogenes* (Chollet et al., 2004), *Klebsiella pneumoniae* (Hasdemir et al., 2004), and *Campylobacter* (Mamelli et al., 2003). Together, these findings indicate that MC-207,110 is very effective against RND-type efflux pumps, and is a promising agent in combating MDR in Gram-negative bacteria.

Although there are a number of beneficial consequences of inhibition of efflux pumps against MDR resistance, development of clinically useful EPIs is still in the early stage (Lomovskaya and Bostian, 2006; Kaatz, 2005). Similar to all infectious disease drug development, development of a promising EPI, such as MC-207,110, into a clinically useful therapeutic agent must address key issues, such as stability, bioavailability, production cost, etc. (Lomovskaya and Watkins, 2001; Lomovskaya and Bostian, 2006; Kaatz, 2005). Mpex Pharmaceuticals, Inc. recently announced a Phase Ib clinical trial to study an aerosol EPI candidate in Cystic Fibrosis patients, which is the first time that a bacterial efflux pump inhibitor has been evaluated in humans for controlling drug resistance (http://www.mpexbio.com/).

### *Campylobacter jejuni* Vaccine Development

Despite growing need for new antibiotics owing to the inevitable development of drug resistance in bacteria, pharmaceutical companies have been phasing out of the antibiotic discovery field recently (Projan, 2003; Walsh, 2003). Therefore, in addition to

searching potent EPIs for combination therapy as discussed above, development of other intervention strategies, such as vaccination, to prevent and control *Campylobacter* infections is also urgently needed. Host immunity plays an important role in anti-*Campylobacter* infection in humans and animals. *C. jejuni* specific serum IgG, IgA, and IgM, and mucosal IgG, IgA were rapidly increased after oral infection with *C. jejuni* (Baqar et al., 2001; Wallis, 1994; Cawthraw et al., 1994). Among the different classes of immunoglobulins, anti-*Campylobacter* intestinal or serum IgA is a major factor contributing to the intestinal mucosal resistance to *Campylobacter* colonization (Wallis, 1994; Burr et al., 1988). Maternal IgA from human breast milk showed protective immunity against enteric *Campylobacter* spp. (Renom et al., 1992). In a volunteer study, re-challenge of previously infected volunteers could not reproduce the disease via ingestion of *C. jejuni* 81-176, suggesting that immunity can be induced to protect *Campylobacter* infections in humans (Black et al., 1988). Laboratory challenge experiments indicated that anti-*Campylobacter* maternal antibodies partly contribute to the lack of *Campylobacter* infection in young broiler chickens in natural environments in the first two weeks (Sahin et al., 2001; Sahin et al., 2003). These findings strongly support the feasibility of development of immunization-based approaches to control *Campylobacter* infections.

There is no vaccine available to date to control *Campylobacter* infections. The following three approaches have been explored for developing effective and safe *C. jejuni* vaccine: 1) Live attenuated vaccines. Since challenge with wild-type *C. jejuni* strain produced solid protective immunity in volunteers (Wallis, 1994), it is likely that

live attenuated vaccine can confer protective effect. However, the paucity of information on the pathogenesis of the organism complicates this strategy. On the other hand, some *C. jejuni* strains that exhibit mimicry of gangliosides in their lipooligosaccharides are associated with development of Guillain-Barre Syndrome, which makes live vaccine potentially unsafe. *C. jejuni* strain 81-176 was proposed as a safe candidate for vaccine design because of absence of persistent antiganglioside antibodies after experimental infection with the strain (Prendergast et al., 2004; Scott and Tribble, 2000). 2) Killed whole-cell vaccines. This type of vaccines could induce high protective immunity without serious toxicity to the hosts. Vaccination of killed *C. jejuni* whole cell enhanced the mucosal immune responses in human and chickens (Baqar et al., 1995; Baqar et al., 1995; Rice et al., 1997; Widders et al., 1996; Prendergast et al., 2004) and partly reduced colonization of *C. jejuni* (Widders et al., 1996; Baqar et al., 1995). 3) Subunit vaccine. Subunit vaccine would have significantly less risk of post-vaccination sequelae than a live attenuated or killed vaccine. However, few studies have been conducted to characterize the immunological properties of protective antigens in *C. jejuni*, primarily due to a lack of understanding of pathogenesis mechanisms and the antigenic complexity of *Campylobacter*. Motility-mediating flagellum (Fla) is an immunodominant antigen and is protective against *C. jejuni* infection in animal models (Guerry, 1997; Morooka et al., 1985; Nachamkin et al., 1993; Pavlovskis et al., 1991; Wassenaar et al., 1993). Fla is modified by glycosylation and undergoes both phase and antigenic variation, which complicates the use of Fla for vaccination (Caldwell et al., 1985; Logan et al., 1989; Szymanski et al., 1999; Doig et al., 1996). However, a truncated recombinant FlaA subunit vaccine showed protection in an animal model (Lee et al., 1999). Oral

immunization of chickens with CjaA that was expressed in a carrier strain elicited specific immune response associated with protection against challenge with wild-type *C. jejuni* (Wyszynska et al., 2004). However, the function of CjaA is still not clear. These findings suggest that subunit vaccine may be a safe and feasible approach for immunization against *Campylobacter* infections.

# 2. INTRODUCTION

*Campylobacter jejuni* is the leading bacterial cause of human enteritis in many industrialized countries (Slutsker et al., 1998; Friedman et al., 2000). The estimated cases of campylobacteriosis in the United States are more than 2 million per year (Mead et al., 1999). The medical costs and productivity losses resulting from *C. jejuni* infection are estimated at 1.5 to 8.0 billion dollars each year in the United States (Buzby et al., 1997; Buzby and Roberts, 1997). *Campylobacter* infections in humans vary from mild diarrhea to severe cramping and abdominal pain (Skirrow and Blaser, 2000). This pathogenic organism is also associated with Guillain-Bare syndrome, an auto immune disease that may lead to respiratory muscle compromise and death (Nachamkin et al., 1998). The majority of human *C. jejuni* infections are epidemiologically linked to ingestion of contaminated poultry meat (Friedman et al., 2000; Tauxe, 2002), and the infective dose can be as low as 5-800 organisms (Black et al., 1988; Mentzing, 1981). In parallel to its increased prevalence, *C. jejuni* has become increasingly resistant to antibiotics including macrolides and fluoroquinolones, the drugs of choice for treating human campylobacteriosis, thus greatly compromising the effectiveness of antibiotic treatments and posing a serious threat to public health (Engberg et al., 2001; Taylor and Tracz, 2005). Therefore, development of effective strategies to prevent or eliminate *Campylobacter* infections is urgently needed. To achieve this goal, it is essential to study the mechanisms contributing to antibiotic resistance in *Campylobacter* and, on the other hand, to develop an understanding of host-pathogen interaction, such as the mechanisms utilized by *Campylobacter* to adapt in the intestinal environment in the presence of

various antimicrobial agents (e.g. bile salts). Understanding the antibiotic resistance mechanisms and the *in vivo* adaptation mechanisms may facilitate the development of effective means to prevent and control *Campylobacter* infections in humans and animal reservoirs.

As general and important mechanisms for antimicrobial resistance, multidrug efflux systems (often named MDR pumps) contribute significantly to the intrinsic and acquired resistance to antibiotics in bacterial organisms (Poole, 2001a; Putman et al., 2000). In addition to being key players in antibiotic resistance, MDR pumps also facilitate bacterial adaptation to deleterious environments where toxic compounds or metabolites are present. Recently, a *Campylobacter* multidrug efflux pump (CmeABC) contributing to antimicrobial resistance was characterized (Lin et al., 2002, 2003, 2005a,b; Luo et al., 2003; Pumbwe and Piddock, 2002). The CmeABC efflux system is composed of three essential units, including an outer membrane protein (CmeC), an inner membrane drug transporter (CmeB) and a periplasmic fusion protein (CmeA). These three proteins are encoded by a three-gene operon (*cmeABC*) and function together to form a membrane channel that extrudes toxic substrates directly out of *Campylobacter* cells (Lin et al., 2002). CmeABC contributes significantly to the intrinsic and acquired resistance of *Campylobacter* to structurally diverse antimicrobials (Lin et al., 2002; Luo et al., 2003; Pumbwe and Piddock, 2002). In addition, CmeABC plays a key role in bile resistance and is essential for *Campylobacter* growth in bile-containing media and colonization in animal intestinal tract (Lin et al., 2003). These findings have defined the importance of CmeABC in antimicrobial resistance and pathophysiology of

*Campylobacter*. Notably, the CmeABC efflux pump can be dramatically induced by bile salts, a group of antimicrobial agents naturally present in intestine (Lin et al., 2005b). This notion is further supported by a recent study by Stintzi et al (2005), in which the expression of *cmeABC* was found to be highly up-regulated in rabbit ileal loops as determined by whole genome microarray and real-time RT-PCR. Together, these findings highlight the significance of CmeABC in antibiotic resistance and in *Campylobacter* adaptation to the intestinal environment in hosts. Thus, CmeABC efflux system is an attractive target for the development of intervention strategies against *Campylobacter* infections in humans and animal reservoirs.

It has been proposed that inhibition of MDR efflux systems by efflux pump inhibitor (EPI) is a novel approach to enhance drug accumulation inside the bacterial cell, thereby increasing bacterial susceptibility to antimicrobials (Ryan et al., 2001; Lomovskaya and Watkins, 2001b). Recently, promising EPIs targeting the inner membrane drug transporter of MDR efflux pumps have been discovered and demonstrated to potentiate the activity of antimicrobial agents against a range of Gram-negative bacteria (Lomovskaya et al., 2001a). The presence of such inhibitors also resulted in a decreased frequency of emergence of fluoroquinolone resistant mutants (Lomovskaya et al., 2001a). Based on the unique features of CmeABC efflux pump as described above, we speculate that inhibitors targeting the CmeABC efflux pump may not only control antibiotic resistance but also increase the susceptibility of *C. jejuni* to *in vivo* bile salts, consequently decreasing the colonization level of *Campylobacter* the in host. Such pump inhibitors could be directly used as novel antimicrobials for therapeutic

intervention of *Campylobacter* infection. The outer membrane component ,CmeC, is essential for the function of CmeABC, immunogenic and inducible *in vivo* (Lin et al., 2003; Lin et al., 2005b), it is likely CmeC antibodies will work similarly to EPI to inhibit the functions of CmeABC pump in *C. jejuni* and provide immunity by targeting CmeC, which may provide an alternative way to fight *Campylobacter* infections. To test our hypothesis and achieve the goal of developing CmeABC-based intervention strategies against *Campylobacter*, the following objectives were pursued in this study:

1) Determine the inhibitory effect of an EPI on the function of CmeABC efflux pump and antibiotic resistance in *Campylobacter*.
2) Examine the effect of inhibition of CmeABC pump by an EPI on the susceptibility of *Campylobacter* to bile salts.
3) Evaluate the *in vivo* efficacy of EPIs on the colonization of *Campylobacter* in the host using chicken model system.
4) Determine the effect of anti-CmeC antibodies on the susceptibility of *C. jejuni* to bile salts.

# 3. MATERIALS AND METHODS

## Bacterial Strains and Culture Conditions

The key *Campylobacter jejuni* strains used in this study are listed in Table 1 (all tables and figures are located in the appendix). Other *Campylobacter* clinical isolates tested in this study are described in Table 2. Both *C. jejuni* 81-176 (a human isolate) and S3B (a chicken isolate) have been used for the characterization of CmeABC efflux system in the previous studies (Lin et.al., 2002, 2003, 2005a,b; Luo et.al., 2003, 2005). All strains were routinely grown in Mueller-Hinton (MH) broth or agar at 42° C under microaerophilic conditions which were generated using a *Campy*pak gas pack (Oxoid) in an enclosed jar. When needed, MH media was supplemented with 30 μg/ml of kanamycin, or appropriate concentrations of ciprofloxacin or erythromycin. All media were purchased from Difco.

## Antimicrobial Stock Preparation

Antimicrobial stocks were prepared by completely dissolving specific antimicrobial (in powder form) in appropriate solvent. Specifically, tetracycline (10 mg/ml), ampicillin (50 mg/ml), ciprofloxacin (10 mg/ml), cefotaxime (25 mg/ml), sodium dodecyl sulfate (50 mg/ml), nalidixic acid (100 mg/ml), novobiocin (50 mg/ml), fusidic acid (50 mg/ml), cetylpyridinium chloride (20 mg/ml) and trisodium phosphate (10 mg/ml) were solublized in deionized distilled $H_2O$ followed by sterilization through

membrane filtration (0.22 μm filter). Norfloxacin (25 mg/ml) was solublized in acetic acid while rifampin (25 mg/ml) and erythromycin (25 mg/ml) were dissolved in dimethyl sulfoxide (DMSO) and ethanol, respectively. The above stock solutions were then aliquoted into sterile 2 ml tubes and stored in a -20ºC freezer.

Bile salts including cholic acid (32 mg/ml), taurocholic acid (64 mg/ml), chenodeoxy choic acid (32 mg/ml) and glycocholic acid (32 mg/ml) were freshly prepared by dissolving the bile salts in MH broth. The solutions were sterilized through membrane filtration (0.22 μm filter) and used immediately. All bile salts used in this study are sodium salts, and their pH is approximately 7.0 after solubilization in MH broth for susceptibility and growth assays.

The antibiotics and other antimicrobials used in this study were purchased from Sigma Chemical Co. (nalidixic acid, norfloxacin, erythromycin, cefotaxime, rifampicin, ampicillin, tetracycline, cholic acid, chenodeoxycholic acid, taurocholic acid, glycocholic acid, cetylpyridinium chloride, trisodium phosphate), ICN Biomedicals Inc (ciprofloxacin), and Bio-Rad (sodium dodecyl sulfate, ethidum bromide).

**Construction of CmeB Isogenic Mutants**

*C. jejuni* 81-176 CmeB isogenic mutant was originally created using EZ::TN <KAN-2> Tnp Transposome and was used in previous studies (Lin et al., 2002, 2003, 2005 a, b). The *cmeB* insertional mutation in 81-176 was introduced to S3B by standard

biphasic natural transformation (Wang and Taylor, 1990) to create JL 141 (Table 1). To perform natural transformation, genomic DNA was extracted from 9B6 using a Wizard Genomic Purification Kit (Promega). Wild-type strain S3B cells grown overnight on MH plates were harvested with MH broth and adjusted to the approximate concentration of 3 x $10^9$ CFU $ml^{-1}$ (corresponding to $OD_{600}$ = 0.5). 0.5 ml of S3B cells were then added to 15 ml polypropylene tubes containing 1 ml MH agar and the tubes were incubated 3 h at 42$^o$C under microaerophilic conditions. Following incubation, 1µl of 9B6 genomic DNA (~ 0.2 µg) was mixed with cells by pipetting up and down. Following another 3 h of incubation under microaerophiilic conditions, cells were plated on MH plates with 30µg/ml of kanamycin and the plates were incubated at 42° C under microaerophilic conditions for two days. A single $Kan^R$ colony was selected, and the insertional mutation in *cmeB* in this isolate was confirmed by PCR using a pair of *cmeB* specific primers ES5 and ERP-2 as described below. Further quick phenotypic confirmation for *cmeB* mutation was performed by disk diffusion method. Briefly, wild-type S3B and its putative isogenic *cmeB* mutant cells were spread onto MH agar plates. A sterile disk (6 mm wide) was placed in the center and 10µl of 5% cholic acid was placed on disk. Plates were incubated at 42° C under microaerophilic conditions for two days and the inhibitory zones were recorded.

**Polymerase Chain Reaction (PCR)**

PCR was performed in a volume of 50µl containing 200µM concentration of each of the deoxynucleoside triphosphates, 200nM concentrations of primers, 2.5mM $MgSO_4$,

50ng of *Campylobacter* genomic DNA, and 5U of *Taq* DNA polymerase (Promega). Cycling conditions varied according to the estimated annealing temperatures of primers and the expected size of the product. To confirm insertional mutation in *cmeB* gene in mutants, primers ES5 (5'- CGATCCCTATGGCTAAATT -3') and ERP-2 (5'- AAAAATTCAAGTTGGTAGCGAAGT -3') were designed from the sequence of *cmeABC* operon in *C. jejuni* 81-176 (Lin et al., 2002) and used in PCR with genomic DNA samples from wild-type strains or mutants. To determine if macrolide resistance-related point mutation occurred in domain V of the 23 S rRNA gene, a 508-bp specific fragment of 23S rDNA of resistant and sensitive strains were amplified by PCR using specific primers 23SF (5'- AAGAGGATGTATAGGGTGTGACG -3') and 23SR (5'- AACGATTTCCAACCGTTCTG -3') (Vacher et al., 2003). A pair of specific primers MOMPF (5'- ATGAAACTAGTTAAACTTAGTTTA- 3') and MOMPR (5'- GAATTTGTAAAGAGCTTGAAG -3') were used to amplify *Campylobacter* major outer membrane protein gene *cmp* that is a good target for molecular typing (Huang et al., 2005). In some PCR reactions, boiling samples were used as template. Briefly, 1ml of *C. jejuni* cells are centrifuged for 4 minutes at 10,000 x g and the resulting pellet was re-suspended in 100µl sterile deionized distilled water. The tubes containing cell suspensions were then placed in boiling water for 10 minutes, followed by centrifugation at speed 10,000 x g for 4 minutes. The supernatant was removed to clean microcentrifuge tube and used as a DNA template for PCR. The PCR products were run along with 1kb DNA ladder (Promega) on a 1.0% agarose gel that was stained by ethidium bromide and visualized by using FluoChem 5500 digital imaging system (Alpha Innotech).

## Sequence Analysis

The amplified PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) prior to sequencing. DNA sequencing was carried out using an automated DNA sequencer (model 377; Applied Biosystems). Sequences were aligned with Pairwise BLAST at the NCBI website (http://www.ncbi.nih.gov/BLAST/).

## Efflux Pump Inhibitors (EPIs)

Efflux pump inhibitor MC-207,110 (Phe-Arg β-naphthyl-amide dihydrochloride) (Figure 1) is commercially available from Sigma (catalog no. P4157). MC-207,110 is the first effective EPI targeting RND efflux pumps and was identified by screening a large synthetic compounds and natural product libraries (Lomovskaya et al., 2001a). Stock solutions were made using deionized distilled water (dd $H_2O$) to final concentration of 25 mg/ml followed by sterilization through membrane filtration. In addition to MC-207,110, MC-04,124 (Figure 1) was also used in this study. MC-04,124 is an analogue of MC-207,110 and is less toxic *in vivo* (Renau et al., 2003). The MC-04,124 was kindly provided by Olga Lomovskaya (MPex Pharmaceuticals, CA). Stock solutions of MC-04,124 were made at a concentration of 20 mg/ml using dd $H_2O$ and sterilized by membrane filtration.

**Susceptibility Tests**

Minimum inhibitory concentration (MIC) tests were performed to determine the susceptibility of *Campylobacter* to a variety of antimicrobials with or without EPI MC-207,110. Standard microtiter broth dilution method was used to determine MICs as described by Jorgensen and Turnidge (2003) with slight modifications. Briefly, antimicrobials stock solutions were completely thawed, vigorously vortexed and diluted in MH broth (with or without 10 μg/ml of EPI MC-207,110) to initial testing concentrations (the highest testing concentration). Then, 240μl of diluted antimicrobial solution was placed into the wells in the first lane of a 96 well plate (Nunc) with duplicate for each antimicrobial. 120μl of plain broth was put into the remaining wells using an 8-channel pipette. Antimicrobial solutions in the first lane were serially diluted using a 2- fold dilution scheme in which 120μl from the wells in the first lane was transferred into the wells in the second land and mixed by pipetting up and down five times. This procedure was repeated for the rest of the wells with the exception of the wells in the last lane, which were left free of antimicrobials as a positive control. To prepare inoculum for MIC test, *C. jejuni* was grown in MH broth to late-log phase (~ $8x10^8$ cfu/ml; grown at 42°C for 24-36 h under microaerophilic conditions). The cultures were then 20-fold diluted in MH broth to make inoculum with appropriate concentration of $4x10^7$ cfu/ml. 5 μl of the above inoculum was transferred to each well in 96-well plate using a 12-channel pipette, resulting in a final inoculum of $1.6 \times 10^5$ cfu/well. Microtiter plates were incubated for 2 days under microaerophilic conditions at 42° C. The MIC was

determined by the lowest concentration of antimicrobial agent that completely inhibited the growth of *Campylobacter*.

**Checkerboard Titration Assay**

To determine if the effect of EPI MC-207,110 on increasing the susceptibility of *C. jejuni* to antimicrobials is dose-dependent, checkerboard assays were performed using 96-well plates as described previously (Lomovskaya, et al., 2001a). Representative antimicrobials erythromycin and cholic acid were tested at 11 concentrations ranging from 0.004 to 4µg/ml and from 0.03 to 30 mg/ml, respectively. The EPI MC-207,110 was tested at 7 concentrations ranging from 0.25 to16 µg/ml. Briefly, in one 96-well plate specific antimicrobial was serially diluted from its starting concentration across the rows as described above for standard MIC tests. The last column was left free of antimicrobial and the final volume of all wells was 50µl. A second plate was prepared for EPI MC-207,110 which was serially diluted in MH broth from its starting concentration down each column. The last row was left free of the EPI and the final volume of all wells was 50µl. A multi-channel pipet was used to transfer the 50µl in each well of the EPI plate into the corresponding well in the antimicrobial plate. The final volume in each well was 100µl. The plate was inoculated with one *C. jejuni* strain (81-176, S3B, 9B6, or JL141) as described above in Susceptibility Test session. Microtiter plates were incubated for 2 days under microaerophilic conditions at 42° C. Bacterial growth in each well was recorded and MICs were determined.

## *In vitro* Selection of Fluoroquinolone(FQ)- and Erythromycin(Ery)-Resistant Mutants

Two different *in vitro* selection experiments were conducted to address different objectives in this study. In the first experiment, FQ-resistant and Ery-resistant mutants were obtained *in vitro* and were used to determine if EPI MC-207, 110 can reverse acquired resistance in these mutants. Briefly, 100µl of wild-type 81-176 or S3B cells were plated on MH plates and grown overnight at 42° C under microaerophilic conditions. The fresh cells were then harvested from plates using fresh MH broth. Cell suspensions were spread on MH agar plates containing antibiotic Ery (8 µg/ml) or ciprofloxacin (4 µg/ml). Following 2-day incubation under microaerophilic conditions at 42° C, single FQ-resistant or Ery-resistant colony on selective plates were selected and inoculated in MH broth. The selected antibiotic resistant mutants (detailed in Table 1) were used for MIC test in MH broth with or whithout EPI MC-207,110. In addition, *cmeB* insertional mutation was also transferred to JL157-160 strains by natural transformation as detailed above, resulting in strains JL161-164 (Table 1). These isogenic *cmeB* mutants were also used for testing MIC of Ery in the presence or absence of EPI MC-207,110.

In the second experiment, the effect of EPI MC-207,110 on the frequency of *in vitro* emergence of Ery-resistant and FQ-resistant mutants in *C. jejuni* was determined. The cells were prepared as described above. The cells were then centrifuged for 4 minutes at 10,000 rpm and pellets were resuspended in small volume of fresh MH broth.

100 μl of the highly concentrated *C. jejuni* suspension was plated onto MH plates containing specific antibiotic (4 μg/ml of Ery or 4 μg/ml of ciprofloxacin) and onto MH plates containing both specific antibiotic and MC-207,110 (10 μg/ml). In the mean time, the *C. jejuni* suspensions were serially diluted in MH broth (1:10) and plated on plain MH plates to determine the concentration of the *C. jejuni* suspension. After a 2-day incubation at 42°C under microaerophilic conditions, colonies on each plate were enumerated. Frequencies of emergence of resistant mutants were determined as ratios between the number of colonies that grew on MH plates containing specific antibiotic (with or without MC-207,110; expressed as numbers of CFU per ml) to the number of colonies showing on MH plates without antibiotics or MC-207110.

**Effect of EPIs on the Colonization of *C. jejuni* in Chickens**

Two animal challenge experiments were conducted in this study: A) To evaluate the effect of treatment of EPI (MC-207,110 or MC-004,124) on the colonization of *C. jejuni* in chickens; and B) To evaluate the effect of multiple dosing and treatment of MC-207,110 on the colonization of *C. jejuni* in chickens. The experimental design is detailed in Table 3. *C. jejuni* strain S3B was chosen for challenge studies based on following reasons. First, *C. jejuni* strain S3B was originally isolated from chickens in our laboratory. This strain has been fully characterized in our laboratory by molecular methods and its genetic fingerprints are fully known, facilitating the differentiation of this strain from other isolates (Huang et al., 2005; Luo et al., 2003, 2005; Zhang et al., 2000). Second, S3B colonizes chickens effectively and has been used in our previous chicken

studies (Luo et al., 2003, 2005; Sahin et al., 2003). In addition, *in vitro* analysis has demonstrated that EPI MC-207,110 dramatically increased susceptibility of S3B to bile salts. These features of S3B have made it an ideal strain for the challenge experiments. For both trials, one-day-old broiler chickens were obtained from Hubbard Hatchery, a commercial hatchery company in Pikeville, Tennessee. Prior to use, these chickens were screened for *Campylobacter* by culturing cloacal swabs, which were plated onto MH agar plates containing *Campylobacter*-specific growth supplements (SR084E and SR117E; Oxoid). All of the birds were negative for *Campylobacter*. For both experiments, 5-day-old chickens were weighed (~62 g/chick) and all chicks were inoculated via oral gavage one time with *C. jejuni* S3B at dose of $10^5$ CFU/chick. In the first experiment, specific EPI were administered into each chick at appropriate dose 30 min after chickens were inoculated with *C. jejuni* S3B (Table 3). In the second experiment, a wider dose range was used for MC-207,110 (3 mg/kg – 75 mg/kg) and an additional two EPI treatments were given at 24 h and 48 h post-inoculation. Each group was maintained in a sanitized wire-floored cage and provided with unlimited access to feed and water. Cloacal swabs were taken at days 2, 4, 7 and 9 days post-inoculation. Swabs were placed in 1ml MH broth, briefly vortexed and 100µl suspensions were spread onto MH plates containing *Campylobacter*-specific selective supplements. The plates were then incubated for two days at 42° C under microaerophilic conditions and the number of colonies was counted. Some of selected colonies were tested by PCR to ensure that the output *Campylobacter* populations were the same as the inoculum and that there was no contamination of the chickens by other sources. The percentage of chickens colonized by *C. jejuni* and the shedding level of *Campylobacter* in chickens colonized by *C. jejuni* after inoculation and

treatment were determined. Chi square analysis (SAS 9.1) was used to measure the significant differences in the percentage of colonized chickens at each time point between groups. One-way analysis of variance followed by a least-significant difference was used to calculate the significant differences in shedding level (log transformed). All animal studies were conducted under IACUC standard protocols (University of Tennessee IACUC protocol number: 1428).

**Preparation of Chicken Intestinal Extracts**

Chicken intestinal extracts were prepared from three different sections of the intestine (duodenum, jejunum and cecum) as described previously (Lin et al., 2003). Six 21-day-old chickens were used for the preparation of chicken intestinal extracts. All birds tested negative for *Campylobacter* by culturing cloacal swabs. The intestinal contents from each section were pooled from six chickens. The same volume of MH broth was thoroughly mixed with each pool of chicken intestinal extracts, followed by centrifugation at 10,000 x g at 4 $^{o}$C for 30 min. The supernatant was carefully pipetted out and passed through a series of membrane filtrations for sterilization (prefilter, 1.2µm, 0.45µm, and 0.22 µm; Millipore Co.). The sterilized extracts were then aliquoted into 2 ml sterile tubes and stored in -20° freezer.

**Effect of CmeC Antibodies on the Susceptibility of *C. jejuni* to Bile Salts**

Polyclonal rabbit anti-CmeC serum directed against a portion of CmeC (aa 41 to 248 of total 492 aa in length) was produced in a previous study (Lin et al., 2002). Prior to any assays, both rabbit control serum (pre-immune serum, negative for CmeC) and anti-CmeC serum was incubated at 56 $^{o}$C for 30 min to abolish complement activity. Susceptibilities of *C. jejuni* to bile salts in the presence of specific CmeC antibodies or control antibodies were performed in 96-well microtiter plates (Corning). Prior to assays, *C. jejuni* 81-176 was grown for 16 hr at 42$^{o}$C in MH broth containing sublethal concentration of bile salt taurochloric acid (2mg/ml) to induce CmeC expression. The cultures were then washed twice in MH and diluted to approximately 1 x $10^{7}$ CFU/ml in MH broth. For the susceptibility assay, the total volume in each well was 250 µl in which 25 µl of diluted culture and 25 µl specific serum (anti-CmeC serum or control serum) were inoculated in 200 µl of MH broth containing 4 µg/ml of cholic acid or 200 µl of chicken intestinal extract. After 6 hr of incubation at 42$^{o}$C under microaerophilic condition, 20 µl of bacterial culture were taken and serially diluted in ice-cold MH broth and plated onto MH agar plates. The number of colony forming units (CFU) was enumerated after a 2-day incubation under microaerophilic conditions at 42$^{o}$C. All assays were done in triplicate. The CFU mean comparisons between treatment groups were analyzed using Student T test (SAS 9.1).

As a long term effort to quantitatively evaluate specific anti-CmeC IgG on the susceptibility of *C. jejuni* to bile salts, the anti-CmeC IgG were purified from the rabbit

anti-serum by protein G affinity chromatography using ImmunoPure (G) IgG Purification Kit (Pierce). SDS-PAGE with 12% (wt/vol) polyacrylamide separating gel was used to determine the purity and molecular mass of purified IgG. The IgG concentration was measured by Bicinchonic Acid Protein Assay (Pierce).

# 4. RESULTS

## EPI MC-207,110 Increases the Susceptibility of *C. jejuni* to Various Antibiotics

As shown in Table 4, the presence of EPI MC-207,110 significantly reduced MICs of different antimicrobial compounds. Of most interest, the MIC of erythromycin, the current antibiotic of choice for treating *Campylobacter* infections, was dramatically decreased 32-fold in 81-176 and 64-fold in S3B strain in the presence of the EPI. When *C. jejuni* 81-176 was grown in MH broth with MC-207, 110, the MICs of fluoroquinolones were decreased 2-fold (ciprofloxacin) and 4-fold (nalidixic acid); the MICs of β-lactams (cefotaxime and ampicillin) were decreased 2-fold; the MICs of rifampin, tetracycline, ethidium bromide, novobiocin and fusidic acid were decreased 1024-fold, 2-fold, 4-fold, 128-fold and 64-fold, respectively (Table 4). Similar significant MIC reductions were also observed for *C. jejuni* S3B grown in MH broth containing MC-207,110 (Table 4). Interestingly, presence of MC-207,110 also significantly increased susceptibility of both *C. jejuni* strains to cetylpyridinium chloride (8-fold MIC reduction), a recently approved food antimicrobial used by the poultry industry to reduce *Campylobacter* spp. during processing (Oyarzabal, 2005). However, MC-207,110 had only moderate or no effect on the susceptibility of *C. jejuni* to other two commonly used food antimicrobials trisodium phosphate and sodium hypochlorite (Table 4).

**Effect of MC-207,110 on the Susceptibility of *cmeABC* Mutants to Various Antibiotics**

Isogenic cmeABC mutants were constructed for MIC test to determine if the effect of MC-207-110 is primarily mediated by CmeABC efflux pump. As shown in Figure 2A, we purified genomic DNA from 9B6 (lane 2), an isogenic *cmeB* mutant of 81-176, for natural transformation. The insertional mutation *cmeB* was successfully transferred to wild-type S3B strain, resulting in the putative *cmeB* mutant JL141 with kanamycin resistance. PCR analysis using *cmeB* specific primers resulted in ~1.25 kb fragment for wild-type 81-176 while a larger size fragment (~3.0 kb) in 9B6 was generated, indicating *cmeB* gene was interrupted by transposon insertion in mutant 9B6 (Figure 2A). However, the *cmeB* specific primers did not result in visible bands for both S3B and JL 141 strains (Figure 2A, lanes 5 and 6), likely due to *cmeB* sequence variations between 81-176 and S3B. Since *cmeB* mutant is hypersusceptible to bile salts (Lin et al., 2002, 2003), a disk diffusion assay was performed to confirm if JL 141 contained the desired mutation in *cmeB* and corresponding phenotype change. Following a 2-day incubation, no inhibition zone was observed for wild-type S3B (Figure 2B) while putative *cmeB* mutant JL 141 showed greatly increased sensitivity to cholic acid, as indicated by a distinct zone of inhibition (diameter of 29 mm) (Figure 2B).

As shown in Table 5, EPI MC-207,110 still resulted in significant MIC reductions in both *cmeB* mutants for most antibiotics. However, for many antibiotics, the magnitude of MIC reduction is smaller than that in wild-type strains (Table 4). For example,

presence of MC-207,110 in broth led to 128-fold and 512-fold reduction in the MIC of novobiocin in 81-176 and S3B, respectively (Table 4). When *cmeB* was knocked out in 81-176 and S3B, MC-207,110 still resulted significant reduction in the MIC of novobiocin (Table 5) but the magnitude of MIC reduction (4- fold) is much smaller than that in wild-type parent strains (128- and 512- fold).

**Effect of MC-207,110 on the Susceptibility of *C. jejuni* to Antibiotics is Dose-Dependent**

To determine if the effect of EPI MC-207,110 on reducing MICs of antibiotics observed above was dose dependent, standard checkerboard assays were performed using clinically important erythromycin as a representative antibiotic. As shown in Table 6, 8 μg/ml of MC-207,110 led to the most significant reduction of MIC in *C. jejuni* 81-176 (256-fold) and in S3B (64-fold) compared to the MICs tested in MH broth without the EPI. With a decrease of MC-207,110 concentration in MH broth for MIC test, the MIC reduction also declined (Table 6). However, as low as 0.25μg/ml of the EPI in MH broth still resulted in significant MIC reductions in most strains (Table 6).

**MC-207,110 Reversed Acquired Antibiotic Resistance in *C. jejuni***

To determine if MC-207,110 reversed acquired antibiotic resistance, both Ery-resistant and Cipro-resistant mutants were first selected in vitro using wild-type susceptible strains 81-176 and S3B. As shown in Table 7, presence of MC-207,110 not

only reduced MICs of Ery in wild-type strain 81-176 and S3B but also resulted in dramatic MIC reduction in all Ery-resistant mutants (JL154 to JL160) with reduction range from 16-fold to >64-fold. In other words, in the presence MC-207,110, all Ery-resistant phenotypes of mutant strains were reversed to susceptible phenotypes. To determine if macrolide resistance-related point mutation also occurred in domain V of the 23 S rRNA gene, a specific 508-bp fragment was amplified from each Ery-resistant mutant and used for sequencing (Figure 3A). Interestingly, sequence analysis showed that none of the mutants had expected A2230G point mutation, which agrees with recent finding that spontaneous Ery-resistant *C. coli* had relatively low MICs (32 to 64 μg/ml) and lacked the A2230G mutation in the 23S rRNA gene (Kim et al, 2006).

The *cmeB* mutation was also introduced to Ery-resistant mutants JL157 to JL160, generating isogenic *cmeB* mutants (JL161-JL164) for evaluating the role of CmeABC pump in acquired Ery-resistance (Table 7). As shown in Table 7, an insertional mutation in *cmeB* (JL161-164) resulted in significantly lower MIC of Ery in MH broth compared to their parent strains (Jl157-160). Supplementation of MC-207,110 in MH broth led to further MIC reductions (4-8 folds) in these *cmeB* mutants.

MC-207,110 also affected the MIC of Cipro in Cipro-resistant mutants (Table 8). However, unlike its effect on Ery-resistant mutants, MC-207,110 only reduced the MIC of Cipro in the mutants with high resistance to Cipro (JL152 and JL153) but had no effect on the mutants with intermediate resistance (JL150 and JL151).

**MC-207,110 Decreased the Frequency of Emergence of Ery-Resistant *C. jejuni***

To determine if the presence of MC-207,110 could also reduce the frequency of emergence of antibiotic resistance in *C. jejuni*, emergence tests were performed on MH plates containing specific antibiotic and MC-207,110 using wild-type susceptible strain 81-176 and S3B. When tests were performed with Ery alone, the frequency of emergence of Ery resistance was approximately $10^{-8}$ level in both strains (Table 9). However, when plates were supplemented with 10 μg/ml of MC-207,110, no single Ery-resistant colony emerged on the plates and the frequency was less than 1.6 $X10^{-10}$ in 81-176 and 5 X $10^{-11}$ in S3B (Table 9). With respect to FQ, presence of MC-207,110 did not result in significantly decreased frequency of emergence of Cipro-resistant *C. jejuni* mutants. The frequency of emergence of Cipro-resistant *C. jejuni* was approximately 2.1 $X10^{-8}$ *in vitro*.

**MC-207,110 Increased Susceptibilities of Various Clinical *Campylobacter* Isolates to Ery**

To determine if MC-207,110 not only increases the susceptibilities of clinical strain *C. jejuni* 81-176 and S3B but also reduces the MICs for other clinical isolates from various sources, MICs of Ery were measured on 57 clinical isolates of different origins (Table 2). The EPI significantly increased the susceptibilities of all clinical isolates to Ery (Figure 4). MIC reduction was as high as 512-fold (Figure 4). The majority of the isolates displayed a 4- to 32- fold reduction in the MIC of Ery in the presence of EPI MC-

207,110 (Figure 4). Importantly, five isolates were highly resistant to Ery and displayed significant Ery MIC reductions ranging from 8- fold to 62- fold.

**EPI MC-207,110 Increases the Susceptibility of *Campylobacter jejuni* to Various Bile Salts**

As shown in Table 10, the MICs of detergent SDS and all four bile salts were dramatically decreased in the presence of the EPI. The MIC reduction ranged from 16-fold to 512-fold. As expected, insertional mutation in *cmeB* drastically decreased MICs of bile salts and detergent in both mutants in MH broth (Table 11). Although presence of MC-207,110 resulted in further MIC reduction for both the two *cmeB* mutants, the magnitude of MIC reduction for bile salts (2- to 32-fold) was much smaller than that observed for wild-type strains (Table 10 and Table 11). The checkerboard titration assay also demonstrated the dose effect of MC-207,110 on increasing susceptibility of *C. jejuni* to the bile salt cholic acid (Table 12).

We also determined the effect of another EPI MC-04,124 on the susceptibilities of *C. jejuni* to different antimicrobials including four bile salts. The EPI MC-04,124 resulted in comparable MIC reductions for all antimicrobials as those for MC-207,110 (Table 4 and 10). However, higher concentrations of MC-04,124 (50 μg/ml) was used in MIC test when compared to MC-207,110 (10 μg/ml), indicating *in vitro* activity of MC-04,124 is not as potent as MC-207,110.

**EPIs Reduced Colonization of *C. jejuni* in Chickens**

As shown in Figure 5, colonization levels of *C. jejuni* in EPI treatment groups were lower than that in the control group. Specifically, the control group without EPI treatment had a 60% colonization rate 2 days post inoculation. However, there was no colonization in any chicken in the group treated one time with low-dose of MC-207,110 and high-dose of MC-04,124 at 2 days postinoculation (Figure 5). EPI MC-04,124 treatment doses also resulted in lower colonization levels compared to the control group (Figure 5). As the study continued, the differences between treatment and control groups lessened (Figure 5), most likely due to horizontal transmission of *C. jejuni* among chickens within a group and the single administration of EPI at day 0. Surprisingly, we observed a reversed dose effect for MC-207,110 in this experiment (Figure 5). To further define the dose effect of MC-207,110 in vivo and examine the effect of EPI administration regimen, we conducted another chicken experiment with modification of using three different EPI dose and administered EPI for three times. The second chicken study (Figure 6A) also showed that MC-207,110 reduced the percentage of chickens colonization by *C. jejuni* S3B when compared to the control group (90%). Similar to the finding in the first chicken study (Figure 5), the highest dose of MC-207,110 resulted in highest percentage of colonization among three EPI-treatment groups (Figure 6A). Despite MC-207,110 were administered for three consecutive days, inhibition of colonization of *C. jejuni* by MC-207,110 lessened throughout the study and by day 9 postinoculation *C. jejuni* S3B colonized 80 – 90 % of chickens for all groups. Shedding

levels of chickens colonized with *Campylobacter* were also evaluated and no significant difference was observed among four groups.

To confirm that the isolates recovered from the experimental chickens were derived from the inoculated S3B, the *cmp* gene encoding the major outer membrane protein was PCR amplified with representative *Campylobacter* isolates obtained from the chickens. The sequence data showed that the *cmp* sequences were identical to that of S3B, indicating that the output *Campylobacter* populations was the same as the inoculum and there was no contamination of chickens by other sources.

**Effect of Anti-CmeC on the Growth of *C. jejuni***

To determine if the function of CmeABC can also be inhibited by immune intervention, the effect of anti-CmeC serum on the *C. jejuni* growth in bile-containing media was investigated. As shown in Figure 7, compared to the growth in the presence of control serum, supplementation of anti-CmeC serum in MH broth containing sublethal concentrations of bile salt cholic acid resulted in moderate but significant growth reduction (~ 0.65 log unit), suggesting anti-CmeC antibodies specifically inhibit the function of CmeABC pump and increased susceptibility of *C. jejuni* to bile salts. Despite above difference resulting from CmeC antibodies, anti-CmeC serum did not led to significant growth reduction of *C. jejuni* in chicken intestinal extracts (data not shown).

**Purification of Anti-CmeC IgG**

To further define the role of anti-CmeC IgG on the function of CmeABC pump in the future, we initiated the purification of rabbit anti-CmeC IgG using protein G affinity chromatography. As shown in Figure 8, highly purified anti-CmeC IgG were successfully obtained using protein G affinity chromatography. Most of the antibodies were eluted in the fourth fraction (lane 6 in Figure 8B) with the highest absorbance (2.22) at 280 nm. Based on BCA assays, a total of ~10 mg of IgG were purified from 2.5 ml of crude rabbit anti-CmeC serum.

# 5. DISCUSSION

The results of this study clearly demonstrate the feasibility of developing a CmeABC efflux pump- based intervention strategy against *Campylobacter*. This conclusion is supported by the following evidence. First, EPI MC-207,110 greatly potentiated the efficacy of various antibiotics against *C. jejuni*, primarily mediated by inhibition of CmeABC efflux pump. Specifically, MC-207,110 dramatically increased the susceptibilities of *C. jejuni* to structurally unrelated antibiotics including the clinically important macrolide Ery (Table 4 and 5) in a dose-dependent manner (Table 6). MC-207,110 also effectively inhibited the CmeABC efflux pump in drug-resistant *C. jejuni* strains and significantly reversed acquired resistance (Table 7 and 8; Figure 4). More importantly, presence of MC-207,110 drastically reduced the frequency of emergence of macrolide-resistant mutants in *C. jejuni* (Table 9). Second, inhibition of the CmeABC efflux system by EPI MC-207,110 made *C. jejuni* strains hypersusceptible to bile salts, which can act as bactericidal antimicrobials in intestine (Tables 10 and 11), and consequently reduced *C. jejuni* colonization in the host, as shown by this experiment using a chicken challenge model system (Figure 5). Finally, antibodies directed against partial region of outer membrane protein CmeC also displayed an inhibitory effect on the function of CmeABC pump and decreased the survival of *C. jejuni* in the presence of bile salts (Figure 7). Together, these findings provide compelling evidence that inhibiting *C. jejuni* CmeABC efflux pump by EPIs or specific CmeC antibodies may be a novel approach to combat antibiotic resistance and prevent and control *Campylobacter* infection in humans and animal reservoirs.

EPI MC-207,110 has been demonstrated to be effective against a variety of Gram-negative bacteria (Chollet et al., 2004; Hasdemir et al., 2004; Lomovskaya et al., 2001a ; Mamelli et al., 2003,2005; Saenz et al., 2004) since it was first discovered by Lomovskaya et al. (2001a). Unlike those studies in which the efficacy of the EPI was only investigated with respect to very limited antimicrobials, our study comprehensively examined the effect of EPI MC-207,110 to a wide spectrum of structurally unrelated antimicrobials. In conjunction with the use of genetic approaches for identified CmeABC efflux pump, findings from this study clearly indicated that inhibition of the CmeABC efflux system greatly increased susceptibility of *C. jejuni* to different antibiotics including a macrolide (erythromycin) and FQ, the major drug of choice for treating human campylobacteriosis. Findings from this study also revealed two unique groups of antimicrobials that are also affected by EPI, which has never been reported in other bacteria. The first group of antimicrobials is bile salts that are the substrate of CmeABC efflux pump and are naturally present in the intestine forming a barrier to limit enteric pathogen infections (Lin et al., 2003, 2005b). The unique role of CmeABC in bile resistance and the potent effect of EPI MC-207,110 on increasing susceptibility of *Campylobacter* to bile salts *in vitro* and *in vivo* strongly suggest that EPI could be directly used as therapeutic intervention of *Campylobacter* infection. The second group of novel antimicrobials consists of commercial food antimicrobials commonly used by the poultry industry to reduce *Campylobacter* spp. during processing (Oyarzabal, 2005). We observed that the presence of MC-207,110 significantly decreased the MIC of two food antimicrobials (Table 4), particularly for cetylpyridinium chloride, a recently approved food antimicrobial. This finding suggests that EPI may be also used as a novel

agent in post-harvest food safety to potentiate commonly used food antimicrobials during processing. However, EPI did not potentiate the efficacy of sodium hypochlorite (Table 4), a commonly used antimicrobial in food processing. The mode of action of specific food antimicrobials may determine its sensitivity to EPI. For example, disruption of cell membrane is a suggested mechanism for cetylpyridinium chloride and trisodium phosphate (Oyarzabal, 2005) and notably, is also a mechanism for detergents and bile salts that are the substrates of CmeABC efflux pump (Lin et al., 2002, 2003, 2005b).

Macrolides such as Ery are considered the antibiotic of choice for treating *Campylobacter* infections because FQs are losing effectiveness in clinical treatments due to the widespread resistance of *Campylobacter* to this class of antibiotics (Enberg et al., 2001; Smith et al., 1999). Unfortunately, *Campylobacter* resistance to Ery is also on the rise (Enberg et al., 2001). Therefore, in this study we placed extensive efforts on determining the effect of EPI MC-207,110 on Ery-resistance in *C. jejuni* and our findings strongly suggest that EPI is a novel agent for fighting Ery resistance in *C. jejuni*. We have demonstrated that inhibition of CmeABC efflux pump (i) decreased the level of intrinsic Ery resistance significantly, (ii) revered acquired Ery resistance, and (iii) resulted in a decreased frequency of emergence of *C. jejuni* mutants that are resistant to Ery. A similar effect resulting from MC-207,110 was observed by Lomovskaya (2001a) for FQ-resistance in *P. aeruginosa*. Although MC-207,110 has also been used to study Ery-resistance in *Campylobacter* by other groups recently (Gibreel et al., 2005; Mamelli et al., 2003, 2005), our study provided much more comprehensive information of the effect of MC-207,110 on the function of CmeABC efflux pump and on the development

of Ery-resistance in *C. jejuni*. In particular, to test the effect of EPI on Ery resistance in *Campylobacter* isolates, we used standard broth dilution method instead of disk diffusion method in which EPI MC-207,110 was supplemented in agar plates in other reports (Gibreel et al., 2005; Mamelli et al., 2003, 2005). Disk diffusion method is not a standard MIC test for *Campylobacter* and is suitable for providing qualitative results but not for a good quantitative result. However, the broth dilution method is a more reliable reference method for susceptibility testing and provides better qualitative results than disk diffusion methods (Jorgensen and Turnidge, 2003). Furthermore, to fully determine the interaction between EPI MC-207,110 and efflux pumps in all *C. jejuni* cells in culture, addition of EPI MC-207,110 in liquid MH broth medium in our study is a better assay system than the supplementation of the EPI in agar plates, as described by Gibreel et al.(2005) and Mamelli et al.(2003, 2005). Because of these methodological differences, findings from our study demonstrated that presence of MC-207,110 greatly reduces the MIC of Ery in all Ery-sensitive and Ery-resistant *Campylobacter* strains, which is in contrast of recent observations that the pattern of most Ery resistant *C. jejuni* isolates was not affected by MC-207,110 (Gibreel et al., 2005; Mamelli et al., 2005). Together, our study demonstrated that EPI is an attractive antimicrobial agent to combat increasing antibiotic resistance in *Campylobacter* and significantly potentiates the efficacy of currently available antimicrobial agents. The *in vivo* efficacy of EPI on antibiotic resistance (e.g. Ery-resistance) in *Campylobacter* needs to be investigated in the future.

Previous studies indicated that CmeABC is a primary MDR efflux pump contributing to antibiotic resistance in *C. jejuni* ( Akiba et al., 2006; Ge et al., 2005; Lin

et al, 2005c). According to whole genome analysis (Lin et al., 2005c), there are only two RND-type efflux pumps in *C. jejuni*: CmeABC and CmeDEF. Recently, CmeDEF has been characterized and found to play a minor role in contributing resistance to various antimicrobials including Ery (Pumbwe et al., 2005; Akiba et al., 2006). Notably, in this study, the EPI still resulted in further MIC reductions in isogenic *cmeB* mutants for most antimicrobials although the magnitude of MIC reductions was smaller (Table 5, 6, 11, 12), which suggested that, in addition to CmeABC efflux system, other unknown efflux system(s) also contributed to *Campylobacter* resistance to antimicrobials including Ery. Ge et al. (2005) has made isogenic mutants of seven putative efflux pumps in addition to CmeABC and CmeDEF and evaluated the role of these efflux pumps in antibiotic resistance. Those investigators observed that CmeABC is the only efflux system that influences antimicrobial resistance in *Campylobacter*. Bioinformatics analysis revealed a total 13 drug efflux systems in *C. jejuni*, including two RND-type systems (CmeABC and CmeDEF),four putative MFS efflux pumps, four putative SMR type efflux pumps, one putative ABC type efflux pump, and two putative MATE (Lin et al., 2005c). It is likely that other unidentified gene loci (e.g. Cj1687, Cj1375 and Cj1187c) contribute to *C. jejuni* resistance to clinically important antibiotics, particularly Ery. We have initiated construction of isogenic mutants of unidentified efflux pumps and will determine MICs of various antibiotics for those mutants. We will also reevaluate all efflux pumps studied by Ge et al (2005) using standard broth dilution method rather than agar dilution method used in Ge's study (2005) because agar dilution method may not be as sensitive as broth dilution to differentiate small MIC difference with respect to specific antibiotics, such as Ery (Personal communication with Dr. Zhang, Iowa State University). After completing

above studies, we anticipate identifying and characterizing other efflux system(s) involved in Ery resistance in *C. jejuni*.

One striking and unique feature of CmeABC is its essential role in bile resistance and *in vivo* colonization (Lin et al., 2003). Recent findings (Lin et al., 2005b) also demonstrated that CmeABC efflux pump is dramatically induced by bile salts, further highlighting the role of CmeABC in pathogenesis and supporting the hypothesis that the bile resistance is a natural function of CmeABC. This notion is also supported by a recent study (Stintzi et al., 2005) in which expression of *cmeABC* was found to be highly up-regulated (up to 300-fold) in rabbit ileal loops. These unique features of CmeABC make the EPI application even more appealing. Inhibition of CmeABC efflux pump by EPI may directly increase the susceptibility of *C. jejuni* to *in vivo* bile salts, consequently decreasing the colonization level of *Campylobacter* in intestine. In this study, presence of MC-207,110 resulted in dramatic MIC reduction (up to 512-fold) for various bile salts in *C. jejuni* strains (Table 10). The reductions in MICs of bile salts are primarily mediated by the CmeABC efflux pump (Table 11). Theses findings clearly support the feasibility of using EPI directly as a therapeutic agent against *Campylobacter*. In *E. coli* and *Salmomella*, the AcrAB-TolC efflux pump (a homolog of the CmeABC pump) also contributes to bile resistance and inducible by bile salts (Prouty et al., 2004; Rosenberg et al., 2003). Thus, it is likely that inhibiting bacterial efflux of bile salts with EPIs may be a general approach for developing therapeutic measures for enteric pathogens. However, the effect of EPI in other enteric pathogens may not be as significant as that observed for *C. jejuni* because expression of AcrAB-TolC pump in *E. coli* and *Salmonella* was only

moderately induced by bile salts and the magnitude of MIC reduction in bile due to the mutation in pump is much smaller in *E. coli* and *Salmonella* than that in *Campylobacter* (Prouty et al., 2004; Rosenberg et al., 2003).

From the standpoint of practical application, it is important to ensure that the EPIs not only work well *in vitro*, but also *in vivo*, where numerous confounding factors may affect EPI. MC-207,110 and its derivatives have been evaluated for *in vivo* toxicity using mouse model system (Renau et al., 2003). When administered via intravenous (iv) bolus injection, MC-207,110 displayed appreciable toxic effects (minimum dose causing lethality to > 66% of the animals tested is < 25 mg/kg). However, a compelling derivative MC-04,124 was less toxic in rodents (Minimum dose causing lethality to > 66% of the animals tested is >150 mg/kg) (Renau et al., 2003). It has been observed that MC-207,110 was not stable in serum but MC-04,124 displayed high stability in serum (Personal communication with Dr. Olga Lomovskaya). Both MC-207,110 and MC-04,124 were used for evaluation of *in vivo* efficacy of EPI using chicken challenge model in this study. Although the EPI was administered to chickens via route (oral delivery) different from that used in mice (iv) (Renau et al., 2003) in the first chicken study, a dose of 25 mg/kg and 150 mg/kg was chosen as highest dose for EPI MC-207,110 and MC-04,124, respectively (Table 3). Results showed that both MC-207,110 and MC-04,124 reduced the percentage of colonization of chickens by *C. jejuni* S3B on day 2 post inoculation when compared to control animals, regardless of high or low dose. It was pleasantly surprising to find that MC-207,110 also displayed inhibitory effect on *C. jejuni* colonization, which indicates that this EPI is stable and functional in the intestinal tract.

In addition, data also indicated that *in vivo* toxicity of EPI also depends on administration route. Both EPIs were administered orally to chickens in this study and none of chickens treated with EPI (regardless of the dose) died throughout the study. It is possible that physiological factors of gastrointestinal tract were responsible for the chickens being able to tolerate high doses of EPIs. The major limitations of the first chicken study were the small number of chickens used in each group and that EPI was only administered a single time to chickens following inoculation of *C. jejuni*. In the second chicken experiment, each group contained 9 or 10 chickens and three different EPI doses (3 mg/kg, 15 mg/kg, and 75 mg/kg for MC-207,110) were administered for three consecutive days following inoculation of *C. jejuni*. However, MC-207,110 did not exert further reduction on the colonization of *C. jejuni* in chickens throughout the study following three consecutive EPI treatments (Figure 6). It is likely that MC-207,110 is effective when it is in contact with *Campylobacter* in the upper and middle sections of the chicken intestinal tract, such as duodenum and jejunum, in the first day of inoculation. However, after being inoculated into chicks, *C. jejuni* gradually established long-term colonization in two ceca, the predominant site for *Campylobacter* colonization in chickens that are immediately after long small intestine and before cloaca. Thus, MC-207,110 may lose its efficacy after it passes through long small intestine and reaches the cecum, possibly due to degradation or binding by host factors. We also consistently observed that high dose of MC-207,110 treatments led to increased *C. jejuni* colonization compared to low dose, suggesting that high dose of EPI MC-207,110 may affect the physiology of chicken intestine and promote *C. jejuni* colonization. Overall, our chicken study strongly indicated that inhibition of *Campylobacter* efflux pump by EPIs is a potential mean for

therapeutic intervention to reduce colonization of *C. jejuni* in human and animal reservoirs. To develop clinically useful EPI compound, more studies using new lead series in conjunction with pharmacokinetics/pharmacodynamic analysis are needed in the future.

Several key issues (e.g. toxicity, *in vivo* stability, production cost) challenge the clinical application of EPIs. Thus, alternative ways to inhibit CmeABC efflux pump should also be explored. Since CmeC is inducible *in vivo* and is an essential OMP component of CmeABC efflux pump, anti-CmeC antibodies may function similarly to EPI in inhibiting CmeABC pumps in *C. jejuni*, as observed in this study. Immune intervention by targeting CmeC may be an alternative way to inhibit the CmeABC efflux pump and avoid problems with EPI. To test this hypothesis, we examined the effect of anti-CmeC peptide antibodies on the susceptibility of *C. jejuni* 81-176 to cholic acid, a representative bile salt. As shown in Fig. 7, anti-CmeC antibodies are only directed against a portion of CmeC (aa 41to 248 of total 492 aa in length) (Lin et al., 2002). When compared to the growth in the presence of control serum (pre-immune serum, negative for CmeC), supplementation of anti-CmeC serum in MH broth containing sublethal concentration of cholic acid resulted in moderate but significant growth reduction (~ 0.6 log unit), suggesting anti-CmeC antibodies specifically inhibit the function of CmeABC efflux pump and increase the susceptibility of *C. jejuni* to bile salts. The inhibitory effect of the serum is not attributed to complement because both sera were inactivated at 56$^{o}$C for 30 min prior to use to abolish complement activity. Since CmeC antibodies only target a partial fragment of CmeC that may only contain partial protective epitopes, the

antibodies against full CmeC antigenic structure likely exerts a more significant inhibitory effect on the *Campylobacter* grown in the presence of bile salts. Findings of this study plus other compelling evidences, reported recently (Lin et al., 2003, 2005 ab; Stintzi et al., 2005) strongly suggest that CmeC could be a novel subunit vaccine against *Campylobacter*. More excitingly, since CmeC is an essential component in CmeABC efflux pump that contributes *Campylobacter* resistance to clinically important antibiotics, immunization of host animals with CmeC subunit vaccine may enhance the activity of clinical antibiotics against *C. jejuni* and CmeC may represent the first vaccine that can combat antibiotic resistance in bacteria.

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# APPENDIX

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

| Strain | Description[a] | Source or Reference |
|---|---|---|
| 81-176 | Wild-type; isolated from human | Black et al., 1988 |
| S3B | Wild-type; isolated from chicken | Luo et al., 2003 |
| 9B6 | 81-176 derivative; *cmeB::kan* | Lin et al., 2002 |
| JL 141 | S3B derivative; *cmeB::kan* | This Study |
| JL 150 | 81-176 derivative; *in vitro* selected Cipro-resistant mutant | This Study |
| JL 151 | 81-176 derivative; *in vitro* selected Cipro-resistant mutant | This Study |
| JL 152 | 81-176 derivative; *in vitro* selected Cipro-resistant mutant | This Study |
| JL 153 | 81-176 derivative; *in vitro* selected Cipro-resistant mutant | This Study |
| JL 154 | S3B derivative; *in vitro* selected Ery-resistant mutant | This Study |
| JL 155 | S3B derivative; *in vitro* selected Ery-resistant mutant | This Study |
| JL 156 | S3B derivative; *in vitro* selected Ery-resistant mutant | This Study |
| JL 157 | 81-176 derivative; *in vitro* selected Ery-resistant mutant | This Study |
| JL 158 | 81-176 derivative; *in vitro* selected Ery-resistant mutant | This Study |
| JL 159 | S3B derivative; *in vitro* selected Ery-resistant mutant | This Study |
| JL 160 | S3B derivative; *in vitro* selected Ery-resistant mutant | This Study |
| JL 161 | JL 157 derivative; *cmeB::kan* | This Study |
| JL 162 | JL 158 derivative; *cmeB::kan* | This Study |
| JL 163 | JL 159 derivative; *cmeB::kan* | This Study |
| JL 164 | JL 160 derivative; *cmeB::kan* | This Study |
| JL 187 | S3B derivative; *in vitro* selected Ery-resistant mutant | This Study |
| JL 188 | S3B derivative; *in vitro* selected Ery-resistant mutant | This Study |

[a] Cipro: ciprofloxacin
Ery: erythromycin

**Table 2.** *Campylobacter* clinical isolates used in this study

| Strain | Source[a] | Strain | Source[a] |
|---|---|---|---|
| CJ 1 | Fecal swab | CJ 30 | Feed |
| CJ 2 | Fecal slurry | CJ 31 | Water from lagoon |
| CJ 3 | Fecal slurry | CJ 32 | Water from lagoon |
| CJ 4 | Water from lagoon | CJ 33 | Water from lagoon |
| CJ 5 | Water from lagoon | CJ 34 | Water from lagoon |
| CJ 6 | Water from lagoon | CJ 35 | Water from lagoon |
| CJ 7 | Water from lagoon | CJ 36 | Water from lagoon |
| CJ 8 | Water from lagoon | CJ 37 | Floor |
| CJ 9 | Water from lagoon | CJ 38 | Floor |
| CJ 10 | Calf fecal swab | CJ 39 | Water from lagoon |
| CJ 11 | Calf fecal swab | JL 10 | Human |
| CJ 12 | Fecal slurry | JL 12 | Bovine |
| CJ 13 | Fecal slurry | JL 20 | Swine (*C. coli*) |
| CJ 14 | Fecal slurry | JL 21 | Swine (*C. coli*) |
| CJ 15 | Water from lagoon | JL 22 | Swine (*C. coli*) |
| CJ 16 | Water from lagoon | JL 25 | Swine (*C. coli*) |
| CJ 17 | Bird dropping | JL 36 | Chicken |
| CJ 18 | Bedding | JL 78 | Human |
| CJ 19 | Floor | JL 88 | Swine |
| CJ 20 | Bedding | JL 93 | Human |
| CJ 21 | Bedding | JL 95 | Ovine |
| CJ 22 | Fecal slurry | JL 114 | Human |
| CJ 23 | Fecal slurry | JL 115 | Human |
| CJ 24 | Fecal slurry | JL 116 | Chicken |
| CJ 25 | Water from lagoon | JL 117 | Chicken |
| CJ 26 | Calf fecal swab | JL 118 | Chicken |
| CJ 27 | Calf fecal swab | JL 170 | Turkey (*C. coli*) |
| CJ 28 | Bedding | JL 171 | Turkey (*C. coli*) |
| CJ 29 | Bedding | | |

[a] Strains CJ 1 to CJ 39 were isolated from dairy farms in east Tennessee (kindly provided by Dr. Steve Oliver, University of Tennessee). If not specified, all isolates are *Campylobacter jejuni*.

**Table 3.** Experiment design of the chicken studies (experiments A and B)

| Expt | group | EPI usage/name | EPI Dose (mg/kg) | EPI treatment[a] | # per Group |
|---|---|---|---|---|---|
| A | 1 (control) | No | N/A | N/A | 5 |
| | 2 | Yes/MC-207,110 | 25 | Single treatment: 30 min after *C. jejuni* inoculation | 7 |
| | 3 | Yes/MC-207,110 | 5 | | 5 |
| | 4 | Yes/MC-004,124 | 150 | | 6 |
| | 5 | Yes/MC-004,124 | 30 | | 5 |
| B | 1 (control) | No | N/A | N/A | 10 |
| | 2 | Yes/MC-207,110 | 75 | Three treatments: 30 min, 24 h, 48 h after *C. jejuni* inoculation | 10 |
| | 3 | Yes/MC-207,110 | 15 | | 10 |
| | 4 | Yes/MC-207,110 | 3 | | 9 |

[a] Five-day-old chickens were inoculated with $10^5$ CFU of *C. jejuni* S3B via oral gavage, followed by oral administration of EPI.

**Table 4**. Susceptibilities of wild-type *C. jejuni* strains to antimicrobials in MH broth with or without efflux pump inhibitor MC-207,110

| Antimicrobial | Minimum Inhibitory Concentration (μg/ml) | | | |
|---|---|---|---|---|
| | *C. jejuni* 81-176 | | *C. jejuni* S3B | |
| | MH | MH + MC[a] | MH | MH + MC[a] |
| Ciprofloxacin | 0.063 | 0.031 (2) | 0.125 | 0.125 (-) |
| Nalidixic Acid | 8 | 2 (4) | 8 | 2 (4) |
| Erythromycin | 0.125 | 0.004 (32) | 0.5 | 0.009 (64) |
| Ampicillin | 1 | 0.5 (2) | 16 | 4 (4) |
| Cefotaxime | 0.125 | 0.063 (2) | 8 | 2 (4) |
| Rifampin | 128 | 0.125 (1024) | 128 | 0.063 (2048) |
| Tetracycline | 0.063 | 0.031 (2) | 32 | 0.063 (512) |
| Ethidium Bromide | 0.25 | 0.063 (4) | 0.5 | 0.25 (2) |
| Fusidic Acid | 128 | 2 (64) | 512 | 2 (256) |
| Novobiocin | 16 | 0.125 (128) | 64 | 0.125 (512) |
| Cetylpyridinium Chloride | 2 | 0.25 (8) | 2 | 0.25 (8) |
| Trisodium Phosphate | 12,500 | 12,500 (-) | 25,000 | 6,250 (4) |
| Sodium Hypochlorite | 62.5 | 62.5 (-) | 15.6 | 15.6 (-) |

[a] The numbers in parentheses indicate the fold reductions in MICs for *C. jejuni* strain grown in MH broth containing 10 μg/ml of EPI MC-207,110 (MH + MC) compared to growth in plain MH broth (MH). "-" represents no observed MIC difference.

**Table 5**. Susceptibilities of *cmeB* isogenic mutants to antimicrobials in MH broth with or without efflux pump inhibitor MC-207,110

| Antimicrobial | Minimum Inhibitory Concentration (μg/ml) | | | |
|---|---|---|---|---|
| | 81-176, *cmeB*::*kan* | | S3B, *cmeB*::*kan* | |
| | MH | MH+ MC[a] | MH | MH+ MC[a] |
| Ciprofloxacin | 0.031 | 0.016 (2) | 0.031 | 0.156 (2) |
| Nalidixic Acid | 2 | 1 (2) | 2 | 1 (2) |
| Erythromycin | 0.016 | 0.004 (4) | 0.063 | 0.002 (32) |
| Ampicillin | 0.125 | 0.031 (4) | 4 | 0.5 (8) |
| Cefotaxime | 0.031 | 0.016 (2) | 0.063 | 0.016 (4) |
| Rifampin | 8 | 0.016 (512) | 1 | 0.004 (256) |
| Tetracycline | 0.031 | 0.031 (-) | 8 | 4 (2) |
| Ethidium Bromide | 0.25 | 0.031 (8) | 0.031 | 0.031 (-) |
| Fusidic Acid | 0.125 | 0.031 (4) | 0.125 | 0.063 (2) |
| Novobiocin | 0.063 | 0.016 (4) | 0.063 | 0.016 (4) |

[a] The numbers in parentheses indicate the fold reductions in MICs for *C. jejuni* strain grown in MH broth containing 10 μg/ml of EPI MC-207,110 (MH + MC) compared to growth in plain MH broth (MH). "-" represents no observed MIC difference.

**Table 6**. Effects of EPI MC-207,110 combined with erythromycin against *C. jejuni* at various concentrations

| Strain | Genotype | Erythromycin MIC reduction (n-fold) in the presence of MC-207,110 at a concentration (μg/ml) of: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 |
| 81-176 | wild-type | 1 | 4 | 8 | 32 | 64 | 64 | 256 | NA |
| 9B6 | 81-176, *cmeB*- | 1 | 2 | 2 | 4 | 4 | 16 | NA | NA |
| S3B | wild-type | 1 | 1 | 2 | 4 | 8 | 32 | 64 | NA |
| JL141 | S3B, *cmeB*- | 1 | 2 | 4 | 4 | 8 | 16 | NA | NA |

**Table 7**. Susceptibilities of erythromycin-resistant ($Ery^R$ ) *C. jejuni* strains to erythromycin in the presence of efflux pump inhibitor MC-207,110 or absence of functional CmeABC efflux pump

| Strain | Genotype | MIC of erythromycin (μg/ml) | |
|---|---|---|---|
| | | MH | MH + MC [a] |
| *C. jejuni* 81-176 | wild-type | 0.125 | 0.004 (32) |
| JL 157 | 81-176 derivative, $Ery^R$ | 0.5 | <0.031 (>16) |
| JL158 | 81-176 derivative, $Ery^R$ | 4 | 0.125 (32) |
| *C. jejuni* S3B | wild-type | 0.5 | 0.009 (64) |
| JL 154 | S3B derivative, $Ery^R$ | 2 | <0.031 (>64) |
| JL 155 | S3B derivative, $Ery^R$ | 2 | <0.031 (>64) |
| JL 156 | S3B derivative, $Ery^R$ | 2 | <0.031 (>64) |
| JL159 | S3B derivative, $Ery^R$ | 16 | 0.25 (64) |
| JL160 | S3B derivative, $Ery^R$ | 4 | 0.125 (32) |
| JL 161 | JL 157 derivative; *cmeB::kan* | 0.063 | 0.008 (8) |
| JL 162 | JL 158 derivative; *cmeB::kan* | 0.063 | 0.002 (32) |
| JL 163 | JL 159 derivative; *cmeB::kan* | 0.25 | 0.031 (8) |
| JL 164 | JL 160 derivative; *cmeB::kan* | 0.125 | 0.031 (4) |

[a] The numbers in parentheses indicate the fold reductions in MICs for *C. jejuni* strain grown in MH broth containing 10 μg/ml of EPI MC-207,110 (MH + MC) compared to growth in plain MH broth (MH).

**Table 8**. Susceptibilities of ciprofloxacin-resistant *C. jejuni* strains to ciprofloxacin in the presence of efflux pump inhibitor MC-207,110

| Strain | Genotype | MIC of erythromycin (μg/ml) | |
|---|---|---|---|
| | | MH | MH + MC [a] |
| *C. jejuni* 81-176 | wild-type | 0.063 | 0.031 (2) |
| JL 150 | 81-176 derivative, Cipro$^R$ | 8 | 8 (-) |
| JL 151 | 81-176 derivative, Cipro$^R$ | 8 | 8 (-) |
| JL 152 | 81-176 derivative, Cipro$^R$ | 32 | 16 (2) |
| JL 153 | 81-176 derivative, Cipro$^R$ | 16 | 8 (2) |

[a] The numbers in parentheses indicate the fold reductions in MICs for *C. jejuni* strain grown in MH broth containing 10 μg/ml of EPI MC-207,110 (MH + MC) compared to growth in plain MH broth (MH).

**Table 9**. Effect of presence of EPI MC-207,110 (10 μg/ml) on the frequency of emergence of erythromycin-resistant mutants in *C. jeuni*.

| *C. jejuni* strain | Erythromycin MIC (μg/ml) | Frequency of Erythromycin-resistant mutants (MH agar plates + 4 μg/ml of Erythromycin) | |
|---|---|---|---|
| | | MC-207,110 (−)[a] | MC-207,110 (+) [a] |
| 81-176 | 0.125 | $1.7 \times 10^{-8}$ | $<1.6 \times 10^{-10}$ |
| S3B | 0.5 | $8 \times 10^{-8}$ | $<5 \times 10^{-11}$ |

[a] The data represent the mean from two treatments.

**Table 10**. Susceptibilities of wild-type *C. jejuni* to detergent and various bile salts in the presence and absence of EPI

| Antimicrobial | Minimum Inhibitory Concentration (μg/ml) | | | |
|---|---|---|---|---|
| | *C. jejuni* 81-176 | | *C. jejuni* S3B | |
| | MH | MH + MC[a] | MH | MH + MC[a] |
| Sodium Dodecyl Sulfate | 256 | 4 (64) | 256 | 4 (64) |
| Cholic Acid | 4,000 | 250 (16) | 8,000 | 250 (32) |
| Taurocholic Acid | 64,000 | 125 (512) | 64,000 | 500 (128) |
| Chenodeoxy Cholate | 4,000 | 62.5 (64) | 8,000 | 125 (64) |
| Glychocholate | 32,000 | 250 (128) | 32,000 | 250 (128) |

[a] The numbers in parentheses indicate the fold reductions in MICs for *C. jejuni* strain grown in MH broth containing 10 μg/ml of EPI MC-207,110 (MH + MC) compared to growth in plain MH broth (MH). "-"represents no observed MIC difference.

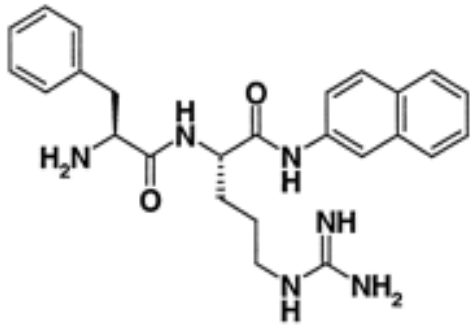
**Table 11**. Susceptibilities of *cmeB* isogenic mutants of *C. jejuni* to detergent and various bile salts in the presence and absence of EPI

| Antimicrobial | Minimum Inhibitory Concentration (μg/ml) | | | |
|---|---|---|---|---|
| | 81-176, *cmeB::kan* | | S3B, *cmeB::kan* | |
| | MH | MH + MC[a] | MH | MH + MC[a] |
| Sodium Dodecyl Sulfate | 64 | 1 (64) | 128 | 4 (32) |
| Cholic Acid | 125 | 15.625 (8) | 250 | 31.25 (8) |
| Taurocholic Acid | 1000 | 31.25 (32) | 1000 | 62.5 (16) |
| Chenodeoxy Cholate | 15.6 | 7.8 (2) | 31.3 | 15.6 (2) |
| Glychocholate | 1000 | 125 (8) | 1000 | 250 (4) |

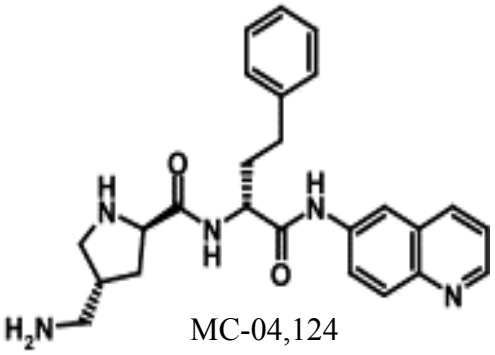
[a] The numbers in parentheses indicate the fold reductions in MICs for *C. jejuni* strain grown in MH broth containing 10 μg/ml of EPI MC-207,110 (MH + MC) compared to growth in plain MH broth (MH).

**Table 12**. Effects of EPI MC-207,110 combined with cholate against *C. jejuni* at various concentrations

| Strain | Genotype | Cholate MIC reduction (n-fold) in the presence of MC-207,110 at a concentration (μg/ml) of: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 |
| 81-176 | wild-type | 1 | 1 | 1 | 1 | 8 | 16 | 64 | NA |
| S3B | wild-type | 1 | 1 | 1 | 2 | 4 | 16 | 64 | NA |
| JL141 | S3B, *cmeB*- | 1 | 1 | 1 | 1 | 2 | 4 | NA | NA |




MC-207,110




MC-04,124

**Figure 1**. Structures of efflux pump inhibitors used in experiments. MC-207,110 is available from Sigma and the formal name is Phe-Arg β-naphthyl-amide dihydrochloride. MC-207,110 was used throughout all *in vitro* experiments and also the *in vivo* experiment; MC-04,124 was primarily used for the *in vivo* experiment.

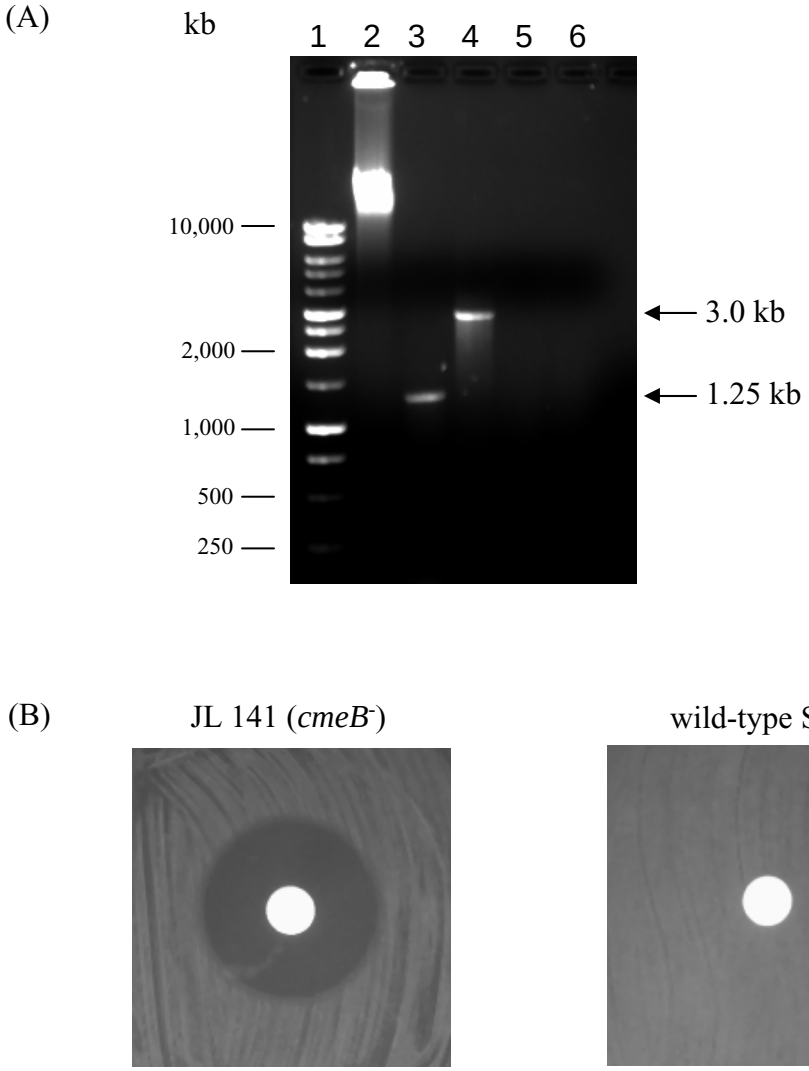



**Figure 2.** Construction and confirmation of isogenic *cmeB* mutant. (A) Construction of *cmeB* mutant and PCR confirmation. Purified genomic DNA from 9B6 (lane 2) was used for natural transformation. *cmeB* specific primers ES5 and ERP-2 were used in PCR with DNA samples from 81-176 (lane 3), 9B6 (lane 4), S3B (lane 5) and JL 141 (lane 6). Lane 1 is standard 1kb ladder (Promega). (B) Susceptibility of JL 141 and S3B to bile salt using disk diffusion assay. Wild-type S3B (right) and its putative isogenic *cmeB* mutant cells were spread onto MH agar plates. A sterile disk was plated in the center and 10µl of 5% cholic acid was placed on disk. Plates were incubated at 42°C under microaerophilic conditions for two days.

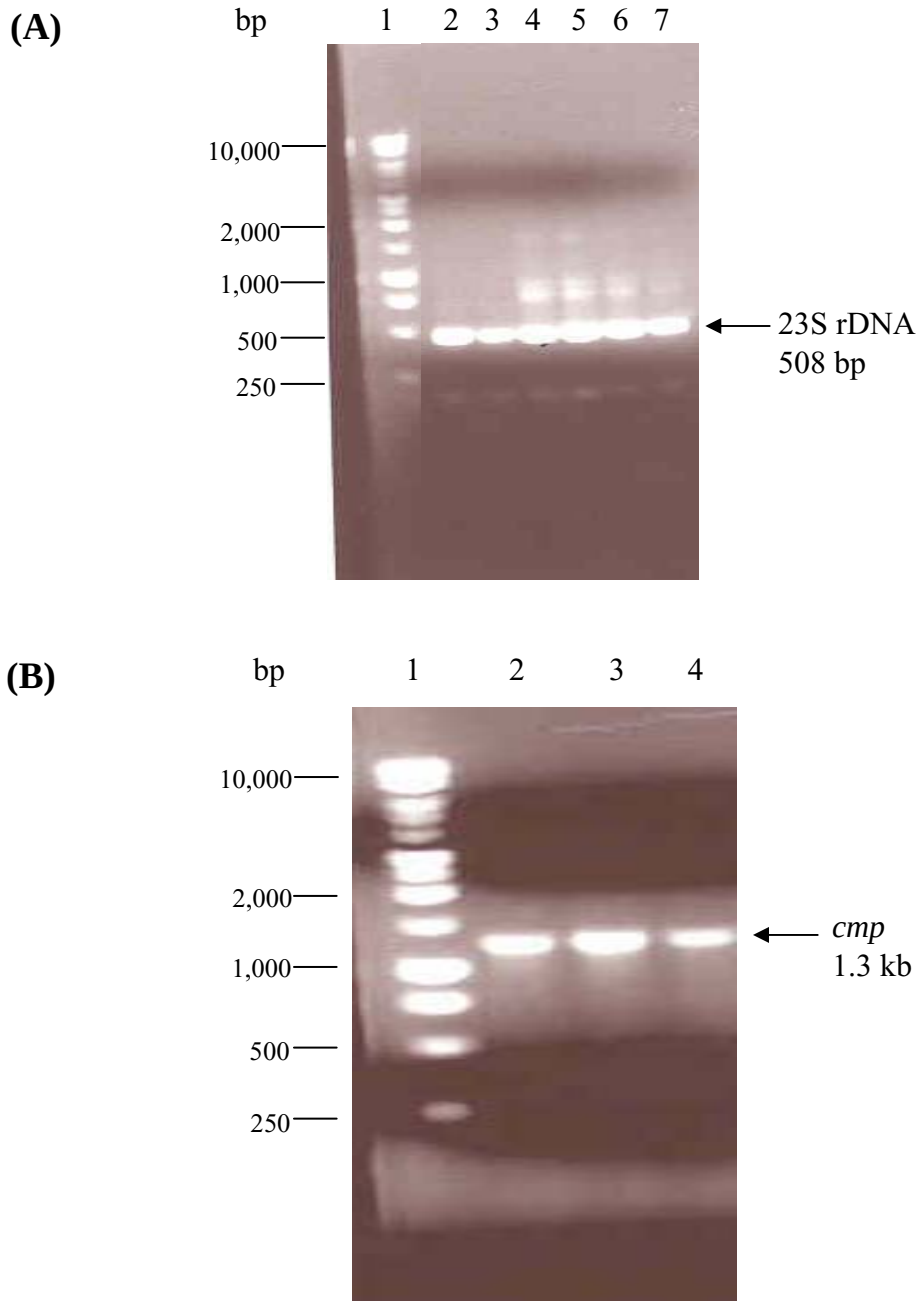



**Figure 3**. PCR amplification of specific *C. jejuni* gene fragments for sequence analysis. A) Amplification of 23S rRNA gene. 23S rRNA gene specific primers were used in PCR with DNA samples from JL155 (lane 2), JL 156 (lane 3), JL 159 (lane 4), JL 160 (lane 5), JL 187 (lane 6) and JL 188 (lane 7). B) Amplification of *Campylobacter* outer membrane protein gene (*cmp*). *cmp* specific primers were used in PCR with DNA samples from inoculated S3B (lane 2), and two isolates from feces (output; lane 3, 4).

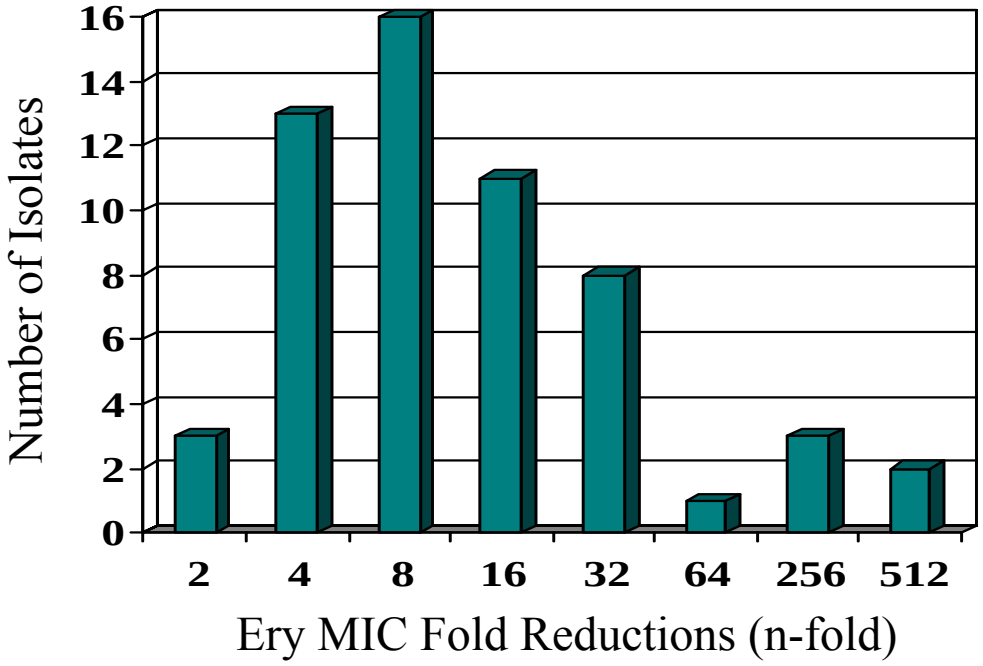



**Figure 4**. Effects of MC-207,110 on the susceptibilities of 57 clinical *Campylobacter* isolates to erythromycin (Ery).

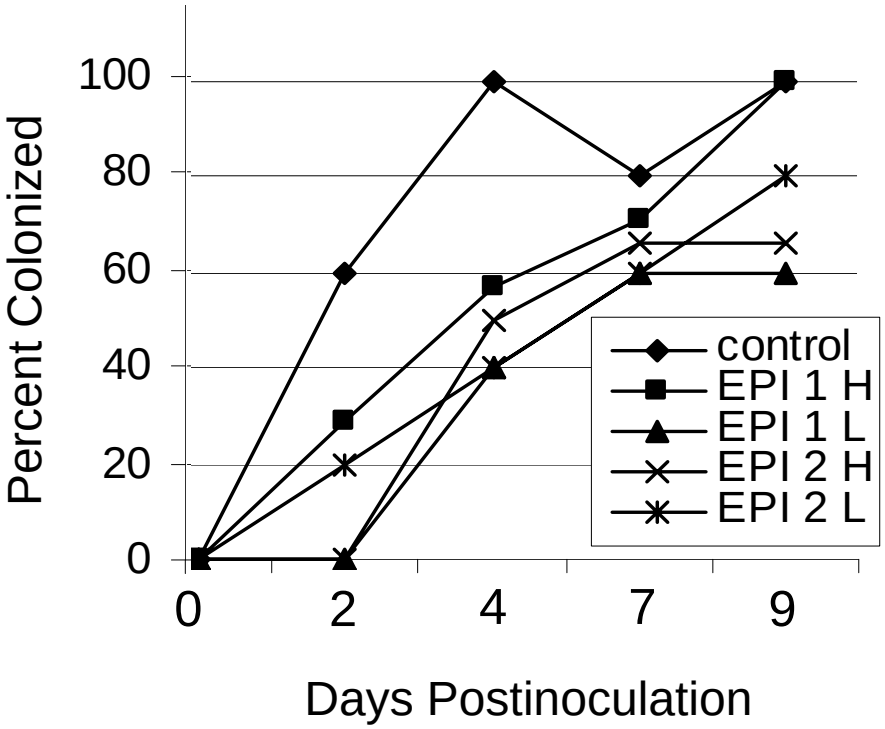



**Figure 5**. Effect of EPI treatment on the colonization of *C. jejuni* S3B in chickens. Five-day-old chickens in each group were inoculated with $10^5$ CFU of *C. jejuni* S3B via oral gavage. Thirty minutes following *C. jejuni* inoculation, MC-207,110 (EPI 1) or MC-04,124 (EPI 2) were orally administered one time at either high (H) or low (L) dose. The groups were as follows: A) control- no EPI administered; B) EPI 1 at a high dose of 25 mg/kg; C) EPI 1 at a low dose of 5 mg/kg; D) EPI 2 at a high dose of 150 mg/kg; and E) EPI 2 at a low dose of 30 mg/kg.

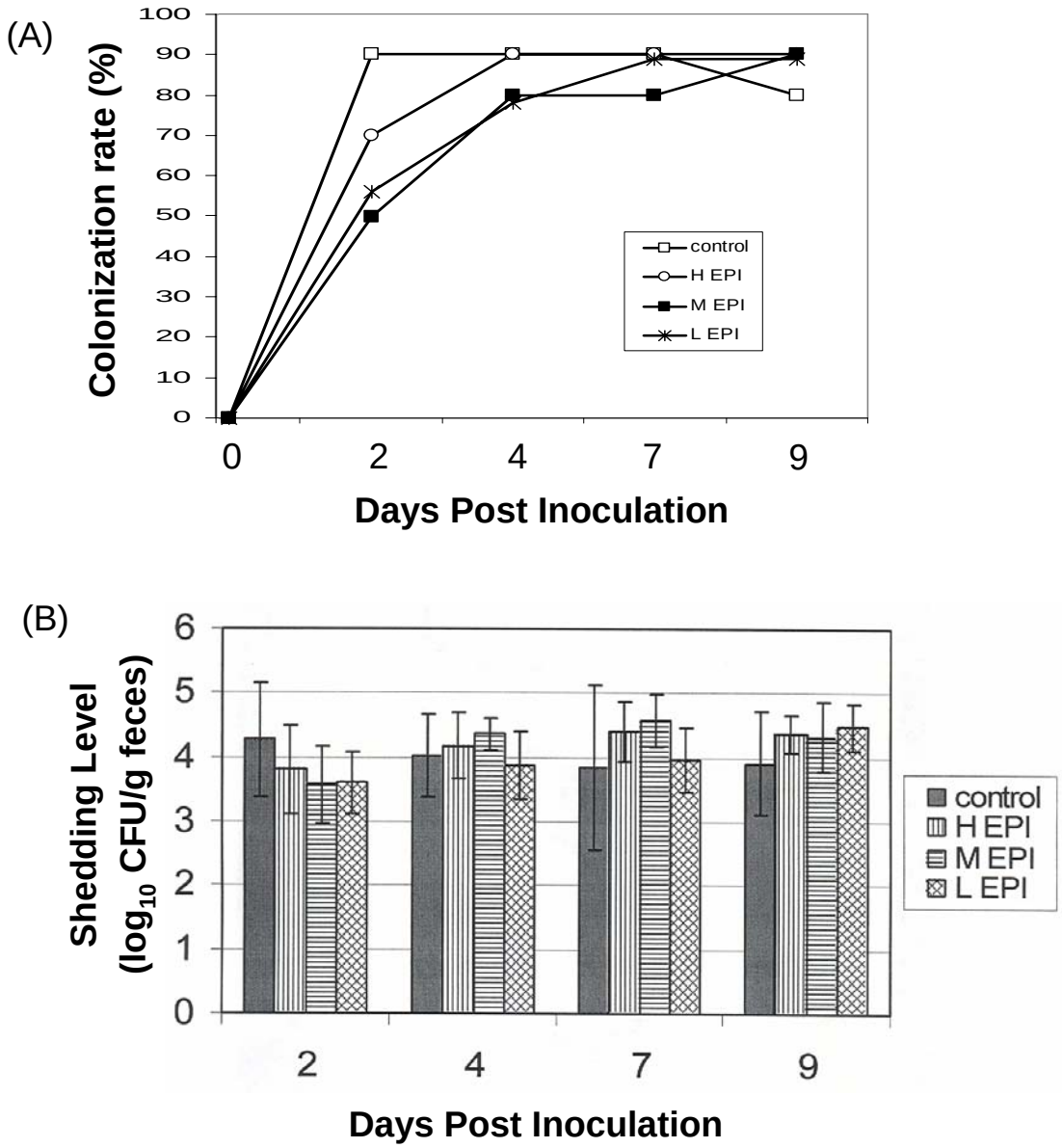



**Figure 6**. Effect of multiple EPI treatment on the colonization of *C. jejuni* S3B in chickens. Five-day-old chickens in each group were inoculated with $10^5$ CFU of *C. jejuni* S3B via oral gavage. 30 min., 24 hr. and 48 hr. following *C. jejuni* inoculation, MC-207,110 was orally administered at either high (H), medium (M) or low (L) dose. The groups were as follows: 1) control- no EPI administered; 2) EPI at a high dose of 75 mg/kg; 3) EPI at a medium dose of 15 mg/kg; 4) EPI at a low dose of 3 mg/kg. A) Percent of chickens colonized with *Campylobacter* following treatment. B) Shedding levels of chickens colonized with *Campylobacter* following treatment.

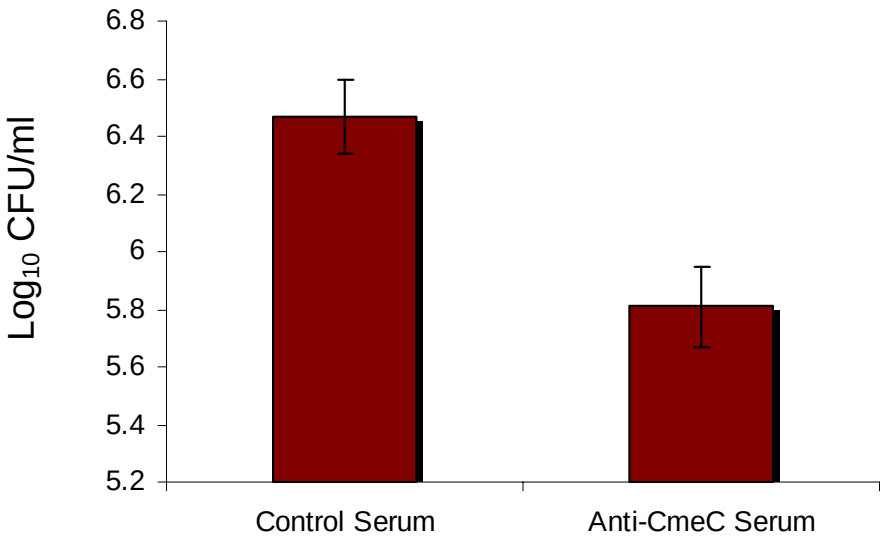



**Figure 7**. Growth response of *C. jejuni* 81-176 to CmeC-negative and CmeC-positive serum. The log-phase culture of 81-176 was diluted to approximately 2 x $10^6$ cfu/ml in MH broth containing sublethal concentrations of cholic acid (2 mg/ml). Anti-CmeC and control sera were added to cells with 1:10 dilution and cells were incubated for 6 hours at 42°C under microaerophilic conditions. Samples were diluted in MH broth and plated on MH agar plates to determine bacterial viability. Each bar represents the mean value obtained from triplicate assays.

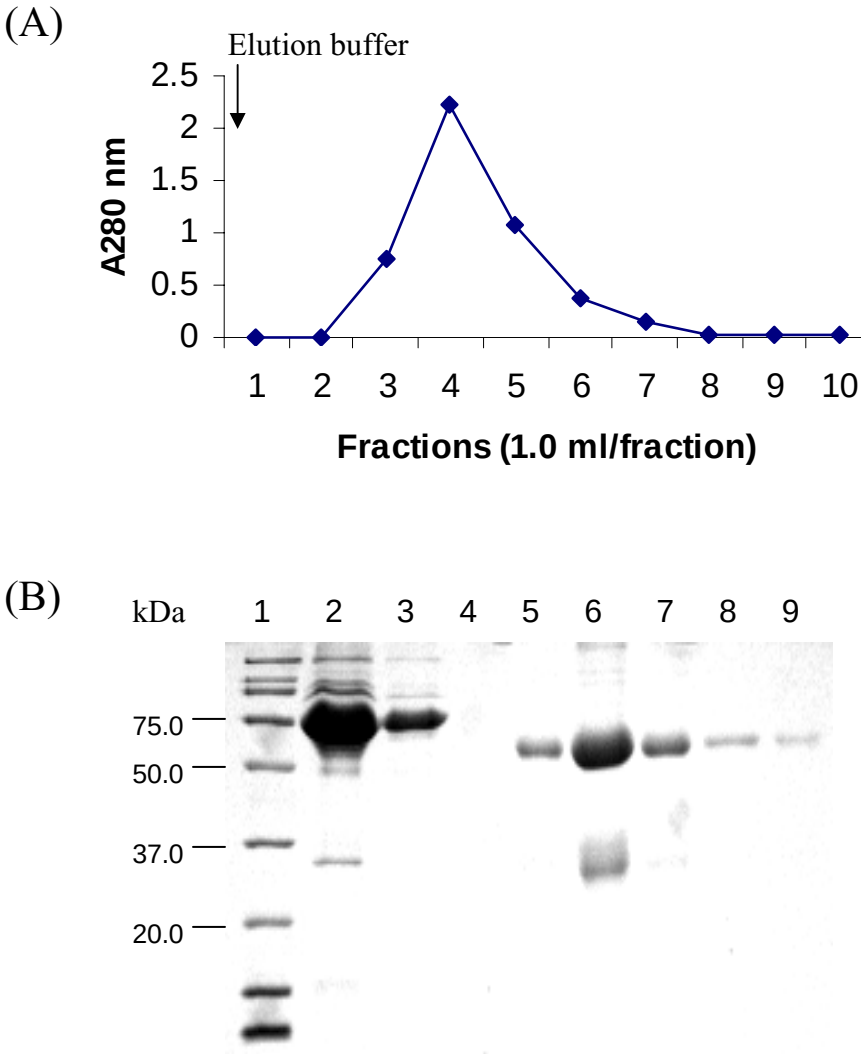



**Figure 8.** Purification of rabbit IgG by protein G affinity chromatography. (A) Chromatography of purified rabbit IgG with ImmunoPure (G) IgG Purification Kit (Pierce). Following collection of unbound protein using 10 ml of binding buffer (pH 5.0), the bound rabbit IgG was eluted by low pH of elution buffer (pH 2.8). (B) Analysis of the purification of IgG from rabbit serum by SDS-PAGE. Proteins were stained with 0.1% Comassie blue. Lane contents were 1) molecular mass standard; 2) crude rabbit serum, 1:50 dilution in PBS; 3) unbound protein which was eluted in the protein G column flowthrough; 4-9) aliquots from protein G column, corresponding to fraction number 2, 3, 4, 5, 6 and 7, respectively in panel (A).

# VITA

Ad'Lynn Leigh Ensminger was born in Cookeville, Tennessee on February 20, 1982, but spent most of her life in Delano, TN, where she considers home. Upon graduating from Central High School of McMinn County, she attended Brigham Young University in Provo, UT where she obtained her Bachelor of Science degree in Animal Science in August 2004. After obtaining her Bachelors degree, Ad'Lynn returned back to Tennessee and was accepted into the Department of Animal Science at the University of Tennessee in Knoxville as a graduate research assistant under the leadership of Dr. Alan Mathew. Upon obtaining her Masters degree she would like to pursue a career in microbiological research.