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Mechanisms of Antimicrobial Peptide Resistance in Campylobacter

Ky Van Hoang
The University of Tennessee, vhoang1@utk.edu

To the Graduate Council:

I am submitting herewith a dissertation written by Ky Van Hoang entitled "Mechanisms of Antimicrobial Peptide Resistance in Campylobacter." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

Jun Lin, Major Professor

We have read this dissertation and recommend its acceptance:

Kelly R. Robbins; Arnold M. Saxton; Gina M. Pighetti; Chunlei Su

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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MECHANISMS OF ANTIMICROBIAL PEPTIDE RESISTANCE IN *CAMPYLOBACTER*

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Ky Van Hoang
December 2010

DEDICATION

To

my father and mother

Thuc Van Hoang and Hoi Thi Nguyen,

Who have made my dream come true;

And also to

my brothers and sisters,

Who make my life meaningful.

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I am grateful to the Department of Animal Science, The University of Tennessee for offering graduate assistantship. I would like to express sincere appreciation to my major professor, Dr. Jun Lin, the best mentor and professor that any graduate student can have, for his extreme patience, professional guidance, financial support in dissertation research. Without his assistance, I would never have obtained my scientific goals from this project.

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Last, but not least, I thank and honor my parents who had worked extremely hard for my better education; my brothers, sisters, and my girlfriend for their unconditioned support, encouragement, patience, and love.

ABSTRACT

Campylobacter is the major bacterial cause of human gastroenteritis in the United States and other developed countries. Poultry are considered a main source of human *Campylobacter* infections. Thus, reduction of *Campylobacter* load in poultry is significant in food safety and public health. However, no effective measure is commercially available to prevent *Campylobacter* colonization in poultry to date. Antimicrobial peptides (AMPs) are short and bactericidal peptides widely present in intestine to limit bacterial infections. Recently, AMPs have been increasingly recognized as a novel class of antibiotics (peptide antibiotics) to control foodborne pathogens. Notably, several potent anti-*Campylobacter* bacteriocins, a group of AMPs produced by commensal bacteria, dramatically reduced *C. jejuni* colonization in chickens and are being directed toward on-farm control of this pathogen to protect public health. As an important strategy to evade killing by potential peptide antibiotics and by host innate defense, AMP resistance mechanisms in *C. jejuni* are critical to understand, but are still unknown. In this dissertation, molecular basis of *Campylobacter* resistance to polymyxin B, the anti-*Campylobacter* bacteriocins (BCNs), and a chicken host defense AMP (fowlicidin-1) was comprehensively examined using both *in vitro* and *in vivo* systems. Although polymyxin B has been successfully used as a model peptide to study AMP resistance in other Gram-negative bacteria, functional genomics examination in this study suggested that polymyxin B is not a good surrogate to study *Campylobacter* resistance to physiologically relevant AMPs. *Campylobacter* only developed low-level BCN resistance with low frequency *in vitro* and *in vivo*; the acquired BCN resistance was not

stable in *Campylobacter*. Genomic examination of two BCN resistant mutants using DNA microarray and random transposon mutagenesis revealed that the multidrug efflux pump CmeABC contributes to both intrinsic and acquired resistance of *Campylobacter* to the BCNs. Random transposon mutagenesis and targeted site-directed mutagenesis identified four genes (*cbrR*, *tig*, *cjaB*, and *cj1583c*) involved in *Campylobacter* resistance to fowlicidin-1. These genes were also required for optimal colonization of *Campylobacter* in chickens. Together, the findings from this dissertation revealed uniqueness and complexity of AMP resistance in *Campylobacter* and will enable us to develop more sustainable peptide antibiotics and novel intervention strategies to prevent and control *Campylobacter* infections in humans and animal reservoirs.

Key words: *Campylobacter*, antimicrobial peptide resistance, polymyxin B, bacteriocins, fowlicidins

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INTRODUCTION

Campylobacter has emerged as the leading bacterial cause of human gastroenteritis in the United States and other industrialized countries (Ruiz-Palacios 2007). This pathogenic organism is also associated with Guillain-Bare syndrome, an acute flaccid paralysis that may lead to respiratory muscle compromise and death. In the United States, the estimated cases of campylobacteriosis are more than 2 million, which have resulted in economical and medical costs of 1.5 to 8 billion US dollars each year (Buzby et al. 1997). Poultry and their products are the major sources for human *Campylobacter* infections (Kassenborg et al. 2004). In parallel to its increased prevalence, *Campylobacter* has become increasingly resistant to clinical antibiotics, posing a serious threat to public health (Allos 2001). Therefore, development of effective intervention strategies is urgently needed to prevent and control *Campylobacter* infections in humans and animal reservoirs.

Endogenous antimicrobial peptides (AMPs) belong to the most ancient and efficient components of host defense. Defensins and cathelicidins are two major groups of host AMPs that limit bacterial infections at the gastrointestinal mucosal surface (Bulet et al. 2004; Wehkamp et al. 2007). In addition to their significant role in host innate defense, AMPs including chicken cathelicidin fowlicidin-1 (F-1) have been increasingly recognized as a novel class of antibiotics (peptide antibiotics) to control pathogens (Xiao et al. 2006; Bommineni et al. 2007). Given the wide prevalence and successful colonization of *C. jejuni* in intestinal tract, *C. jejuni* should have acquired mechanisms to

resist AMPs widely present in the host. However, as an important strategy to evade killing by innate immunity and by potential peptide antibiotics AMP resistance mechanisms are still largely unknown in *Campylobacter*. Availability of this information will provide insights into the delicate host-pathogen interactions and reveal novel intervention targets to control *Campylobacter* infections in humans and animal reservoirs.

Some commensal bacteria also produce defense peptides, called bacteriocins (BCNs), to inhibit growth of other bacteria and gain survival advantage in specific niche in intestine (Riley et al. 2002). The natural and low-toxic BCNs have been proposed as promising candidates for novel antimicrobials (Cotter et al. 2005). Notably, several anti-*Campylobacter* BCNs have been characterized (Stern et al. 2005; Svetoch et al. 2005; Stern et al. 2006). These BCNs have displayed potent killing effect against *Campylobacter* and dramatically reduced *C. jejuni* colonization in the intestine. Therefore, these natural anti-*Campylobacter* BCNs are being directed toward on-farm control of *Campylobacter* (Lin 2009). However, several critical issues of BCN applications (e.g. resistance development and mechanism) need to be addressed for future regulatory approval and public acceptability of this intervention measure.

Polymyxin B (PM), a peptide antibiotic, has been used as a model peptide to study AMP resistance in many Gram-negative bacteria (Ernst et al. 2001; Finlay et al. 2004). Gram-negative bacterial pathogens utilize multiple mechanisms to resist killing by PMs and other AMPs (Groisman et al. 1997; Ernst et al. 1999; Guina et al. 2000; Ernst et al. 2001; Groisman 2001; Lee et al. 2004; Moon et al. 2009). Inactivation of the genes responsible for PM resistance usually results in increased susceptibility of bacteria to a

variety of innate AMPs, leading to the reduced virulence of the mutants in animal model system (Ernst et al. 2001). Thus, investigation of PM resistance should be highly relevant to resistance to physiologically relevant AMPs and pathogenesis of *Campylobacter*.

The main objective of this project is to examine AMP resistance in *Campylobacter* using both *in vitro* and *in vivo* systems. The following specific objectives were persued in this dissertation research:

1) To identify and characterize genes involved in *C. jejuni* resistance to polymyxin B.

2) To determine the prevalence, development, and molecular mechanisms of BCN resistance in *Campylobacter*.

3) To determine the *in vivo* development and stability of BCN resistance in *Campylobacter* using chicken model system.

4) To systemically identify genes required for *Campylobacter* resistance to fowlicidin-1, a chicken host defense peptide.

CHAPTER I

LITERATURE REVIEW

BIOCHEMICAL PROPERTIES AND STRUCTURES

Antimicrobial peptides (AMPs), short peptides with length ranging from 15 to 50 amino acid residues, are produced virtually by all classes of lives from prokaryotes to human and play an important role in innate immunity due to their potent antimicrobial activities against microorganisms (Bulet et al. 2004; De Smet et al. 2005). Based on the sequence and structural analysis, more than 900 AMPs have been predicted and characterized to date. AMPs exert killing effects against many microorganisms including bacteria, viruses, and fungi. Recently, some AMPs (e.g. defensin, lactoferin, cecropin, magainin and melitin) were also found to display anticancer function (Hoskin et al. 2008).

AMPs are cationic and amphipathic molecules (Yeaman et al. 2003). Cationic characteristic is an important feature of AMPs, which could initiate electrostatic interaction of AMPs with negatively charged phospholipid membrane of bacteria and many other microorganisms (Shai 1999; Yeaman et al. 2003; Toke 2005). Most AMPs have high content of positively charged amino acids such as arginine and lysine rather than negatively charged amino acids such as aspartic and glutamic acids. Thus, the overall charge of AMPs is usually cationic, ranging from +2 to +9 at physiological pH (Yeaman et al. 2003; Bulet et al. 2004; Otvos 2005). Hydrophobicity of antimicrobial peptides is determined by the percentage of hydrophobic residues in peptides (Yeaman et al. 2003), which facilitates the interaction of AMPs with hydrophobic regions of microbial cell membrane. Increasing hydrophobicity significantly increases the ability of AMPs to bind and permeate bacterial membrane (Yeaman et al. 2003). Together, the

cationic and amphipathic properties of AMPs are critical for killing microorganisms. However, the amphipathicity of AMPs could cause toxicity to host cells whose membrane is composed of neutral phospholipids (Yeaman et al. 2003). Based on the sequence and structure analysis, AMPs have been classified into four groups (Oren et al. 1998; Weinberg et al. 1998):

(1) Linear α-helical AMPs. This group contains AMPs with simple linear, helical structure lacking disulfide bonds. They can be virtually found in many classes of living organisms including virus, bacteria, insects, fish, and human (Oren et al. 1998; Tossi et al. 2000). For example, cecropins, ceratotoxins, and metilins, which are produced by moths, flies, and bees, respectively, all belong to this group (Saito et al. 2005; Brown et al. 2008). Despite great differences on the origins, length, charge distribution, and hydrophobicity, the AMPs in this group contain positively charged regions and prone to adopt alpha helical conformation. Notably, many such AMPs only become alpha helical when they interact with amphipathic phospholipids of cell membrane (Yeaman et al. 2003).

(2) Linear α-helical peptides without cystein and with high portion of certain amino acid residues such as proline, arginine and tryptophan (Dathe et al. 1999; Yeaman et al. 2003). These AMPs with high content of proline, arginine, or triptophan have distinctive cyclic structure that typically form extended conformation in aqueous solution and more compact in hydrophobic solvents (McCafferty et al. 1996). Many proline-rich AMPs have been identified such as apidaecin from honey bee, pyrrocidin

from insect, drocins from flies, bac-5 and bac-7 from bovine, and buforin II from frog (Bulet et al. 2004).

(3) β-sheet AMPs: This group of AMPs is characterized by the β-hairpin structure containing disulfide linkages (Weinberg et al. 1998; Reddy et al. 2004). Beta-sheet AMPs can be isolated from a large numbers of different plants, animals and from various tissues such as trachea, intestine, tongue, plasma, and skin (Weinberg et al. 1998; Thomma et al. 2002; Higgs et al. 2007). Defensins, the representative of this group found in mammals, have three subfamilies sharing common genetic origin including α-defensins, β-defensins, and ɵ-defensins. All of these mammalian defensins are small molecules (18–45 residues) that are cysteine and arginine-rich, and have a predominantly β-sheet structure that is stabilized by three intramolecular disulfide bonds (Sugiarto et al. 2004).

(4) ***Cysteine-rich peptides***. This group of AMPs contains several cysteine residues near C-terminal and N-terminal facilitating intramolecular disulfide bonds (Weinberg et al. 1998). For example, human neutrophil HNP-1, -2, and -3 from human granules (Ganz et al. 1985), drosomocin from drosophila, bacternectin from bovine neutrophill, brevinins and esculetin from frog skin (Ganz et al. 1985; Weinberg et al. 1998) have three to four disulfide bonds making up complex structure containing alpha and anti-parallel beta sheets (Reddy et al. 2004).

MODES OF ACTION

AMPs have very diverse sequence composition, conformation structures, and biophysical characteristics. The modes of action of AMPs are not fully understood. However, it has been widely accepted that AMPs exert antimicrobial activities through their interactions with the target membrane and interfering with the intracellular components of the microorganisms (Park et al. 1998; Shai 1999; Reddy et al. 2004; Otvos 2005; Brown et al. 2006; Meade et al. 2009).

Bacterial membrane targets. Fundamental differences between the mammalian cell membrane and bacterial cell membrane are the constituent compositions and architectures of the cell membrane. The normal mammalian cell membrane lipid bilayer is composed of neutrally charged lipid such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingo myline (SM). However, the lipid bilayers of bacterial cell membrane consist of positively charged components such as phosphatidylglycerol (PG), phosphatidylserine (PS), and cardiolipin (Yeaman et al. 2003). The surface peptidoglycan is also negatively charged with the presence of techoic or terichuronic acids. In addition, Gram-negative bacteria have outer membrane composed of lipopolysaccharides (LPS) with negatively charged constituents such as phosphate group. These features make bacterial cell membrane have higher overall negative charge than normal mammalian cell membrane. Therefore, the negative charge together with the amphipathic feature of bacterial cell membrane facilitates the direct interaction between AMPs and bacterial envelope.

AMPs interact with bacterial cell membrane via electrostatic and/or hydrophobic interactions. Briefly, cationic AMPs contact with negatively charged phosphate lipid

group of membrane via electrostatic force. Following this process is the interaction of hydrophobic domains of AMPs with hydrophobic groups of lipid chains in membrane. These interactions facilitate the AMP structure transit to favorable conformation, leading to the membrane pore formation, following by permeation, and transfer of AMPs into the cells. Three models of bacterial cell membrane pore formation have been proposed including barrel-stave mechanism, carpet-like mechanism, and toroidal pore mechanism (Oren et al. 1998; Park et al. 1998; Shai 1999; Yeaman et al. 2003; Bulet et al. 2004; Sengupta et al. 2008). Although AMPs commonly interact with bacterial cell membrane without requirement of AMP specific receptor, recent research demonstrated that some AMPs interact with bacterial cell membrane through specific receptors. For example, some AMPs, such as nisin, could target on lipid II, the precursor required for membrane peptidoglycan synthesis during cell division, and cause cell death (Hasper et al. 2004).

Intracellular targets. The membrane perturbation of AMPs has been widely examined and is a major mechanism of AMP actions. However, there is increasing evidence to prove that AMPs have intracellular targets (Kragol et al. 2001; Xiong et al. 2002; Yeaman et al. 2003; Otvos 2005; Pujals et al. 2008; Cho et al. 2009). Some AMPs could inhibit DNA replication. For instance, buforin II directly binds to DNA (Cho et al. 2009). Thrombin-induced palette protein tPMP-1, human neutrophil defensin HNP-1, and bacterial peptide microcin B17 have been reported to interfere with DNA gyrase, an essential enzyme for relaxing the DNA scaffold during DNA replication (Xiong et al. 1999; Heddle et al. 2001; Otvos 2005; Cho et al. 2009). Demaseptin, a chiramic peptide, could inhibit RNA synthesis (Balaban et al. 2004). Several AMPs also interfere with

protein translation and functions. For example, tPMP-1 binds to both 30S and 50S ribosomal subunits while HNP-1 only binds to 50S subunit (Xiong et al. 1999); proline-rich peptides pyrrhocoricin, drocin, and apidaecin could interact with bacterial heat shock protein DnaK, an important protein responsible for protein folding (Kragol et al. 2001). Human histatins have high affinity to fungi mitochondrion and inhibit respiration process (Helmerhorst et al. 1999). However, the mechanisms of translocation of AMPs from surface to cytoplasm are not fully understood. It is important to note that a single AMP may have multiple surface as well as intracellular targets.

AVIAN ANTIMICROBIAL PEPTIDES

Recently, a full panel of avian AMPs have been identified and characterized from chicken, turkey, penguin, and mallard duck (Sugiarto et al. 2004; Lynn et al. 2007). Based on their sequences and structures, these AMPs fall into two groups, β- sheet and α-helical known as β-defensins and fowlicidins respectively (van Dijk et al. 2005; Lynn et al. 2007).

β-defensins are cystein-rich with a triple-stranded β-sheet structure connected by a loop of β-hairpin and are evolutionary conserved in humans and various animals (Sugiarto et al. 2004). Avian β-defensins (AvBDs), produced by heterophils and epithelial cells also called 'gallinacins' in chicken, include 14 homologous peptides encoded by cluster of genes located on chromosome 3 (Xiao et al. 2004; Lynn et al. 2007). Avian heterophil β-defensins include Gal -1, Gal-1α, Gal-2 isolated from chicken; THP-1, THP-2 isolated from turkey; Osp-1 isolated from ostrich (Sugiarto et al. 2004;

Lynn et al. 2007). Avian non-heterophil β-defensins are produced by epithelial cells at different locations in the body. Chicken Gal-3 and turkey GVP-1 both containing 39 amino acid residues and are produced by tracheal epithelial cells (Zhao et al. 2001). Penguin sphenicin was isolated from stomach content of penguin (Thouzeau et al. 2003). Gal-3 was also expressed in large intestine, air sac esophagus, skin, bursa of fabricius, and kidney (Sugiarto et al. 2004). Different avian β-defensins and nomenclatures are summarized in Table 1.

Fowlicidin family consists of four members including CTHL-1, CTHL-2, CTHL-3 and CTHL-B1 encoded by cluster of genes located on chromosome 2 (van Dijk et al. 2005; Xiao et al. 2006). Phylogenetic analysis demonstrated that CTHL-1, CTHL-2, and CTHL-3 are expressed by heteotrophil while CTHLB1 is expressed by mucosal epithelial M cells (van Dijk et al. 2005; Xiao et al. 2006).

Avian AMPs could be constitutively expressed under normal physiological conditions to resist microbial infections (Meade et al. 2009). Some chicken AMPs are expressed at different levels at different locations during early period of embryo development. Avian β-defensins 9, 10 were highly expressed at day 10 and day 12 in embryo abdomen while β- defensin 14 was highly expressed in the head of embryo (Meade et al. 2009). Avian defensins were found to be expressed in liver, intestine, and reproductive tracts (Silphaduang et al. 2006; Li et al. 2007; van Dijk et al. 2007). The expression of avian AMPs increases upon microbial infection. The β-defensin 1, 2, 4, 6 and fowlicidins were highly expressed in chicken infected with *Salmonella* enterica serova *Typhimurium* (Akbari et al. 2008).

AMPs play an important role in innate and specific immune response. The antimicrobial activities are more important in avian than mammals because avian heterophils lack superoxide ion and myeloperoxidase (Penniall et al. 1975; Sugiarto et al. 2004). Avian AMPs have a wide range of antimicrobial activities against Gram-negative, Gram-positive bacteria, and fungi (Sugiarto et al. 2004). Chicken heterophil β- defensin Gal-1, Gal-1α, and Gal-2 displayed antimicrobial activities against *E. coli* and *Listeria monocytogenes*; turkey defensins have broader antimicrobial spectrum against various pathogens including *Mycoplasma* and fungi (Sugiarto et al. 2004). Antimicrobial activities of fowlicidins have been well studied. CTHL-1, and 2 display potent and broad antimicrobial activities against both Gram-negative and Gram-positive bacteria including those with antibiotic resistance (Xiao et al. 2006). These fowlicidins also have ability to neutralize bacterial LPS, and consequently reduce inflammation response (Xiao et al. 2006). However, at high concentration, these fowlicidins exert cytotoxicity toward mammalian erythocytes and epithelial cells (Xiao et al. 2006).

BACTERIOCINS: STRUCTURE AND CLASSIFICATION

Development and spread of antibiotic resistance in bacterial pathogens have been the driving force for scientists to seek antibiotic alternatives. Great attention has been focused on bacteriophages, probiotics, and antimicrobial peptides including bacteriocins (Asaduzzaman et al. 2009; Lin et al. 2009). Bacteriocins (BCNs) are ribosomally synthesized bacterial proteins or peptides with narrow or broad antimicrobial activity spectrum against other bacteria. BCNs are produced by both Gram-negative and Gram-

positive bacteria and up to 99% of bacteria may make at least one BCN for their microbial defense system (Klaenhammer 1988; Riley et al. 2002).

BCNs have a large degree of structural and biochemical diversity. Although a large numbers of bacteriocins have been identified and characterized, new BCNs are still being discovered and documented. Based on the physiochemical properties, BCNs have been classified into the following classes:

Class I bacteriocin (also named 'lantibiotics'). Lantibiotics are ribosomally synthesized peptides containing unusual amino acid lanthionine produced by Gram-positive bacteria (Willey et al. 2007). To date, about 50 lantibiotics with average size of 19-38 amino acid residues have been identified and characterized. To synthesize lantibiotics, the prepeptides of lantibiotic undergo extensive posttranslational modifications (e.g., dehydration of Ser and Thr), followed by the intramolecular addition of Cys residue onto the modified unsaturated amino acid residues (Willey et al. 2007; Smith et al. 2008). More than 15 different posttranslational modifications have been found in lantibiotics. The different posttranslational modifications result in structurally diverse peptides derived from the same original pre-peptide sequences (Willey et al. 2007).

Class II bacteriocins. Class II BCNs are the heat stable peptides with molecular weight less than 10 kDa, and do not contain lanthionine as observed in Class I BCNs. They have not undergone posttranslational modifications (Cotter et al. 2005). Based on

the structure, antimicrobial activities, and amino acid composition, class II BCNs have been divided into following three groups.

Class IIa bacteriocins. More than 25 different class IIa BCNs with size ranging from 37 to 48 amino acid residues have been characterized (Drider et al. 2006). The most important characteristic of class IIa BCNs is that cysteine residues are usually located at N-terminal and C-terminal that form disulfide-bridge and stabilize their structure. The BCNs with higher numbers of disulfide bridges seem to have greater antimicrobial activities in comparison with those having less disulfide bonds (Eijsink et al. 1998). Class IIa BCN consists of an N-terminal β-sheet-like domain that is stabilized by the conserved disulfide bridge and a C-terminal domain consisting of one or two α-helices of which the central α-helix is usually hydrophilic and the C-terminal α-helix is hydrophobic/amphipathic (Fimland et al. 2005). Most of BCNs in this group have extended C-terminal tail structure containing an additional sulfide bridge to stabilize the 3-D structure. N-terminal β-sheet-like domain contains the conserved YGNGVXaaC β-turn motif. This conformation gives N-terminal of class II bacteriocins amphiphilic characteristics (Fimland et al. 2005). It has been suggested that YGNGVXaaC β-turn motif is a recognition sequence for membrane receptor which allows correct positioning of the BCN in the membrane surface (Chen et al. 1997). Previous studies have supported that the changes in YGNGVXaaC motif could lead to the change of the N-terminal β-sheet-like domain, ultimately affecting the antibacterial activities of class IIa BCNs (Quadri et al. 1997). Furthermore, most positively charged residues are located in the N-terminal of the peptides. Thus, they are of importance for the initiation of electrostatic

interaction with the cell membrane (Chen et al. 1997). The central hydrophilic or slightly amphipathic α-helix is believed to have oblique orientation creating 30-60^0 with cell interface when class IIa bacteriocin interacts with bacterial membrane. During the interaction, central hydrophilic α- helix interacts with hydrophilic part of the cell membrane and spans onto membrane but does not inserts. This interaction facilitates the insertion of the C-terminal α- helix into the cell membrane (Bhugaloo-Vial et al. 1996). The C-terminal hydrophobic/amphiphilic α-helix also plays a very important role in class IIa bactriocin modes of action. It is believed that C-terminal hydrophobic/amphiphilic α-helix is a putative transmembrane helix and inserts into bacterial cell membrane with the help of upstream oblique α-helix to form "barrel-stave" (Bhugaloo-Vial et al. 1996).

Class IIb bacteriocins are the BCNs containing two peptides that form a bacteriocin. To date, about 15 two-peptide bacteriocins have been identified and characterized (Nissen-Meyer et al. 2010). A peptide (15-30 residues) from a two-peptide bacteriocin displays high antimicrobial activity only when it is combined with a complementary peptide. It has been proposed that the bacteriocins form a membrane-penetrating helix-helix structure involving helix-helix-interacting GxxxG-motifs that are present in all characterized two peptide bacteriocins. The membrane and two-peptide bacteriocin interaction results in membrane pore formation that renders the membrane of sensitive bacteria permeable to a specific group of ions (Nissen-Meyer et al. 2010).

Class IIc bacteriocins consist of BCNs whose N- and C-terminals are covalently linked, forming cyclic structure. Most BCNs of class IIc BCNs contain D-amino acids and the mechanisms of action of this bacteriocin group are not been well understood.

However, there is evidence that the bacteriocins exert killing bacteria via increased potassium ion efflux (Kawai et al. 2004).

Class III bacteriocins. This group of BCNs also named 'bacteriolysins' (Cotter et al. 2005) are large and heat-labile AMPs with different domains responsible for different functions such as translocation, receptor binding, and killing activity (Cotter et al. 2005). Less than 10 class III BCNs have been identified and characterized including those from non-lactic acid bacteria (Cotter et al. 2005). The mode of action of this group of BCNs is different from class I and class II BCNs. They display antimicrobial activities toward sensitive cells by catalyzing cell wall hydrolysis (Cotter et al. 2005). For this group of BCNs, N-terminal domain is a catalytic domain homologous to endopeptidase while C-terminal domain is responsible for recognizing the targets (Valdes-Stauber et al. 1994; Lai et al. 2002; Johnsen et al. 2005).

GENETIC ORGANIZATION OF BACTERIOCIN SYNTHESIS

The genes involved in BCN synthesis are generally found in clusters which can be located on bacterial chromosomes or on plasmids (Peschel et al. 2006; Willey et al. 2007; Asaduzzaman et al. 2009). The class I BCN producers have a panel of genes involved in BCN synthesis, which typically consist of genes encoding pre-peptide, posttranslational modification enzymes, transporters, accessory proteins, immunity proteins, and regulator. Class II and III BCN producers, however, do not have the enzymes required for posttranslational modification.

The structural gene encoding pre-peptide consists of N-terminal leader sequence and the BCN peptide sequence. The leader sequence contains two glycine residues which are believed to serve as signal recognition motif for the maturation and membrane translocation of the peptide across membrane. BCN transport system includes two distinct proteins: an ABC transporter consisting of 2-3 subunits located on the cell membrane, and an accessory protein. ABC translocator protein has conserved ATP-binding domain at C-terminal and hydrophobic integral membrane N-terminal domain which was shown to cleave off the leader peptide sequence at double glycine motif. C-terminal cytosolic domain contains the ATP-binding cassette which, upon binding to peptide, triggers ATP hydrolysis, consequently changing the ABC conformation leading to pre-peptide removal and translocation across the cytoplasmic membrane (Havarstein et al. 1995). It is suggested that the recognition between the N-terminal proteolytic domain of ABC transporter and the immature BCNs occurs by the interactions between hydrophobic residues on the amphiphilic α-helix pre-sequence and hydrophobic residues on or near the catalytic site in the N-terminal proteolytic domain of the ABC transporter protein (Aucher et al. 2005). Accessory protein bearing a hydrophobic N-terminal region and a large hydrophilic C-terminal section is postulated to be involved in processing membrane translocation and helping cleaving off peptide leader sequence (Havarstein et al. 1995).

The BCN-producers use two distinct mechanisms to protect themselves from killing by the BCNs produced by themselves, production of immunity protein and specific ABC transporter. The ABC membrane efflux transporters are encoded by genes

adjacent to BCN synthesis genes. Different class bacteriocin producers use different protective strategies. Class I bacteriocin producers use both immunity protein and ABC type transporter, meanwhile class IIa and IIb producers use only immunity proteins, and class IIc producers rely only on ABC type transporter (Cotter et al. 2005; Stein et al. 2005). Immunity proteins are highly charged cytosolic proteins associated with cell membrane (Dayem et al. 1996). Immunity proteins interact with specific membrane proteins to confer resistance to cognate BCN products (Ennahar et al. 2000; Vincent et al. 2009). Immunity proteins display high specificity towards their BCNs and exert protection only against their cognate BCNs, or under rare situations, against one or two other closely related BCNs (Fimland et al. 2002).

REGULATION OF BACTERIOCIN SYNTHESIS

Bacteriocin synthesis has long been proven to be regulated by quorum-sensing mechanism which includes production of autoinducer and the corresponding two-component regulatory systems (Quadri 2002; Sturme et al. 2002). In particular, the regulation of class II and III BCN synthesis by quorum sensing has been extensively studied (Quadri 2002; Riley et al. 2002; Stein et al. 2002). The regulatory system is composed of three gene products, also termed three–component regulatory system which consists of an autoinducer peptide-pheromone (AIP), a transmembrane histidine kinase (pheromone receptor), and a cytoplasmic response regulator (Drider et al. 2006). Similar to the BCN structural genes, the genes encoding for autoinducers have two parts: the leader sequence and inductive sequence (Ennahar et al. 2000). The processing of

immature inductive peptide including cleavage of leader sequence and membrane translocation is similar to the process of BCN maturation and export (Ennahar et al. 2000). Once outside the cell, induction peptides act as autoinducers for quorum sensing to regulate BCN synthesis. In addition to depending on cell density, production of class II and III BCNs is affected by temperature, ionic strength, and pH (Leroy et al. 1999; Fimland et al. 2000). Class I BCN producers do not have specific autoinducer genes in BCN synthesis cluster. Interestingly, some class I BCNs also could function as an autoinducer in addition to antibacterial activity. For example, Nisin A has unique sequences at N- and C-terminal that serve as quorum sensing signal to interact with its corresponding two-component regulatory systems (Miller et al. 2001; Quadri 2002; Sturme et al. 2002). However, some evidence suggests that class I bacteriocin producers also have other autoinducer genes separating from bacteriocin synthesis cluster genes (Sturme et al. 2002).

APPLICATION OF BACTERIOCINS

BCNs have been widely used in food processing and preservation (Cotter et al. 2005; Drider et al. 2006). Recently, BCNs have been increasingly recognized as novel antibiotic alternatives to clinical antibiotics to control pathogens (Ennahar et al. 2000; Drider et al. 2006). Following are brief reviews for the application of BCNs in different areas.

For decades, BCNs have been used in food processing and preservation. Most BCNs are produced by food-grade microorganisms such as lactic acid bacteria (LAB). In

addition, most BCNs are heat and pH stable, low toxicity to eukaryotic cells and resistant to digestive protease (Cotter et al. 2005; Drider et al. 2006). Thus, application of BCNs in food processing and preservation have benefits such as extended shelf life of foods, reducing risks of foodborne pathogen transmission and reducing chemical use as preservatives (Settanni et al. 2008). Bacteriocins have been applied in food to either control the microbial succession during fermentation or inhibiting the uncontrolled growth of bacterial spoilage during storage of both animal and vegetable products (Aymerich et al. 2002; Settanni et al. 2008). For example, nisin A and Z have been accepted worldwide as powerful and safe food additives in controlling food spoilage in almost 50 countries for over 40 years (Asaduzzaman et al. 2009). Inoculation of a leucocin, a BCN produced by *Leuconostoc gelidum*, to meat can preserve vacuum-packed meat up to 8 weeks by inhibiting the growth of *Lactobacillus sakei* which spoils meat and generates distinct sulfur odors (Leisner et al. 1996).

Food-borne pathogens are still major problem throughout the world. The leading human pathogens associated with foods include *Campylobacter* spp, *Salmonella* spp, *E. coli* spp, and *Listeria* spp. One potential advantage of BCNs or their producer organisms is their ability to inhibit the growth or kill foodborne pathogens (Drider et al. 2006). On-farm control of food borne pathogens is a major concern in the food chain due to the high probability of pathogens originating from farms. For example, human campylobacateriosis is mostly due the consumption of contaminated poultry meat in which *Campylobacter* colonize commensally. Reduction of *Campylobacter spp* on farms would significantly reduce *Campylobacter* in meat, and thus reduce human

Campylobacter infections (Lin et al. 2009). Several anti-*Campylobacter* BCNs were successfully isolated and characterized from chicken commensal bacteria, which include OR-7 from *Lactobacillus salivarius* (Stern et al. 2006), E-760 and E50-52 from *Enterococcus faecium* (Line et al. 2008; Svetoch et al. 2008), and SRCAM from *Paenibacillus polymyxa (Stern et al. 2005)*. Animal studies have demonstrated that these BCNs dramatically reduced *C. jejuni* colonization in chicken intestine and these bacteriocins are being developed toward on-farm control of *Campylobacter* to protect public health (Stern et al. 2005; Cole et al. 2006; Svetoch et al. 2008; Lin et al. 2009).

BCNs and BCN-producers have been also used to control food borne pathogens during food processing. For example, supplementation of class IIa bacteriocin producing LAB to fresh and processed meat resulted in temperature-dependent inhibition of the growth of *Listeria monocytogenesis* and *Listeria innocua* (Hugas et al. 1998). Addition of purified piscicolin 126 and enterocin CCM4231 to cheese and soy milk during processing inhibits the growth of *L. monocytogenesis* (Wan et al. 1997; Laukova et al. 1999). Also addition of piscicolin 126 to ham paste resulted in reduction of *L. monocytogenesis* below detectable levels (Jack et al. 1996). Recently, a number of BCNs isolated from different bacterial strains display antimicrobial activity against *L. monocytogenesis* and could be used to control this food borne pathogen in food products (Drider et al. 2006). BCNs can be used in combination with other methods which will increase the antimicrobial activity and efficiency of BCNs. These methods include chemical substances, pulse electric field, heat treatment, hydrostatic pressure, modified atmosphere packaging, and irradiation (Galvez et al. 2007)

The rapid development and spread of multi-drug resistance to traditional antibiotics are the driving force for seeking alternative methods for infection treatment and prevention (Davies 1997). In addition, the biggest limitation of conventional antibiotics is their broad spectrum of activities which kill not only targeted bacteria but also untargeted microorganisms in various environmental and host niches. Thus, frequent use of those antibiotics results in intense selection pressure on sensitive microorganisms in the ecosystem (Walker et al. 2001). The current attempt to solve this problem, perhaps, is to develop new antibiotics that have narrow spectrum of activity, least likely to facilitate resistance, and can combat multi-drug resistant bacteria. Given its narrow killing spectrum and diverse modes of action, BCN is a novel and effective alternative to conventional antibiotics. The use of BCNs in human and veterinary medicine is still in the preclinical stages (Breukink et al. 2006). *In vivo* trials with animal models have demonstrated the success of bacteriocins in treating infections caused by *S. pneumonia*, and multi drug resistant *Staphylococcus aureus* (Goldstein et al. 1998; Kruszewska et al. 2004). Nisin, lacticin 3147, mersacin, and actagardine have been demonstrated to be effective against bacterial mastitis, oral decay, enterococcal infection, and septic ulcer (DelvesBroughton et al. 1996; Goldstein et al. 1998; Galvin et al. 1999).

THE ROLE OF ANTIMICROBIAL PEPTIDES IN INNATE IMMUNITY AND GASTROINTESTINAL INFECTION

Antimicrobial peptides (AMPs) are a major component of host innate immunity systems (Mallow et al. 1996; Bals et al. 2003; Bevins 2005; De Smet et al. 2005;

Hasenstein et al. 2006). Defensins and cathelicidins (α-helical AMPs) are two major families of AMPs in humans and animals (Wehkamp et al. 2007). Defensins are broadly present in mammalian epithelial cells and phagocytes with concentrations as high as millimolar levels. Cathelicidins are present in the granules of phagocytes and mucosal epithelial cells (Wehkamp, J., J. Schauber, and E. F. Stange. 2007. In the human intestinal tract both defensins and cathelicidins are important AMPs expressed by the gastrointestinal epithelium and protect host against pathogens (Wehkamp 2007). α-defensins and β-defensins exert potent killing effect against various pathogens such as *E. coli, Staphylococcus aureus, Salmonella enteritica typhimurium, Pseudomonas aeruginosa, Listeria monocytogenes* ,and fungus *Candida albicans* (Porter et al. 1997; Ghosh et al. 2002). The widespread expression of defensins in the GI tract suggests their important role in the maintenance of a stable microbial population in the intestine. This could, on the one hand, prevent invasion of host tissues by luminal flora and ingested pathogenic bacteria, and on the other hand, maintain relative sterility in certain areas of GI tract (Cunliffe et al. 2004). Mice lacking defensins were found to be sensitive to lethal infection with *S. enteritica serovar Typhimurium*, but transgenic mice that expressed human defensins could resist oral infection with *S. enteritica serovar Typhimurium* at dose lethal to nontransgenic animal control (Salzman et al. 2003). Moreover, in wild type mice the antimicrobial activity was largely neutralized by defensin-specific antibodies, indicating that defensins were responsible for most of the activity. Taken together, these experiments provided important evidence for the protective role of defensins in the early stage of infection. Several studies also demonstrated that defensins take part in adaptive

immunity by acting as chemo-attractants for dendritic cells (DCs), monocytes, and T cells (Durr et al. 2002).

Cathelicidins are widely expressed in various tissues including myeloid cells, mucosal surface of skin, respiratory epithelia, GI tract, reproductive tract, eccrine and salivary glands (Zanetti 2005). Cathelicidins display antimicrobial activities against many microorganisms including Gram-negative bacteria such as *P. aeruginosa, S. typhimurium, E. coli* and Gram-positive bacteria such as *S. aureus* and *S. epidermidis* (Smeianov et al. 2000). Cathelicidins-deficient mice developed much larger areas of subcutaneous infection with Group A *Streptococcus* than heterozygous or wild type mice (Nizet et al. 2001). Other effects of cathelicidin (LL-37) to the innate immune response is the ability to bind and neutralize the biological activities of LPS, a septic shock component of gram negative bacteria (Larrick et al. 1995). Recruitment of immune cells to the site of infection is one process in the innate and adaptive defense. Cathelicidins are chemotactic for neutrophils, monocytes, mast cells, and T-cells. In addition, they induce degranulation of mast cells, alter transcriptional responses in macrophage, stimulate wound vascularization and re-epithelialization of the healing skin, and antitumor activities (Okumura et al. 2004; Zanetti 2004).

Other host AMPs such as histatins and lysozyme that are produced by mammalian parotid glands also play an important role in innate immunity. Histatins have antimicrobial activity against pathogenic fungi such as *Candida albicans, Cryptococcus neoformans,* and *Aspergillus fumigatus* (Helmerhorst et al. 1999). Histatins can bind to a receptor and enter the cell, leading to cell cycle arrest, efflux of ATP out of the cell, and

release of the reactive oxygen radicals (Kavanagh et al. 2004). Lysozyme displays bactericidal activity by hydrolyzing β1-4 glycosidic bonds between *N-acetyl* glucosamine and *N-acetyl* muramic acid of Gram-positive bacterial cell wall and subsequently causes cell death. Lysozyme is active against *Enterococcus faecalis* and *Staphylococcus aureus* (Dommett et al. 2005). Moreover, lysozyme is also able to enhance the phagocytic activity of both polymorphonuclear leukocytes and macrophages and bind to free LPS and lipoteichoic acid of Gram-negative and Gram-positive bacteria respectively (Kokoshis et al. 1978; Ginsburg 2002). PLA2, cytokine CCL20 expressed in GI tract, has strong antimicrobial activity against a variety of microorganisms such as *Salmonella, E. coli, Listeria,* and *C. albicans* (Beers et al. 2002; Yang et al. 2003). Taken together, AMPs play indispensible roles in innate immunity in gastrointestinal tracts.

ANTIMICROBIAL PEPTIDE RESISTANCE IN GRAM-NEGATIVE BACTERIA

Bacterial pathogens have co-evolved with host innate defense and developed means to curtail the effects of endogenous AMPs such as defensins, cathelicidins and bacteriocins (Riley et al. 2002; Peschel et al. 2006). The best studied AMP resistance mechanisms in Gram-negative bacteria involve 1) electrostatic repulsion of AMPs by modification of cell surface, 2) proteolytic cleavage of AMPs, and 3) active extrusion of AMPs by drug efflux pumps (Guo et al. 1998; Yeaman et al. 2003; Campos et al. 2004). Other mechanisms, such as capsule production, changes in the composition of lipid fatty acids in membrane and maintenance of appropriate membrane potential have also been implicated in AMP resistance (Campos et al. 2004). Gram-negative bacteria capable of

surviving exposure to AMPs utilize two distinct strategies: constitutive resistance and inducible resistance (Guo et al. 1998; Yeaman et al. 2003; Yount et al. 2005). These two strategies are briefly reviewed below.

Constitutive mechanisms of resistance are defined as intrinsic or inherent properties of bacteria that confer basal level of AMP resistance and are constitutively expressed. In general, there are five mechanisms contributing to constitutive resistance to AMPs: *1) Inherent cell wall composition or architecture.* Most AMPs interact with bacterial cell wall which is composed of glycocalyx or phospholipids. Thus, bacteria possessing unusual glycocalyx or phospholipid composition inherently may lack electrostatic affinity or even repel cationic AMPs, leading to significant intrinsic AMP resistance (Yount et al. 2005). *2) Influence of transmembrane potential*. Transmembrane potential is electrochemical gradient between inner and outer bilayers of the cytoplasmic membrane, resulting from differing degrees and rates of proton flux across the membrane. This significant difference in membrane electrochemistry has been postulated to be crucial in defining selective toxicity of AMPs through self-promoted uptake mechanism (Hancock 1997). Transmembrane potential or proton motive force of energized membrane facilitates the interaction with antimicrobial peptides. Thus, reduction in transmembrane potential may help bacteria resistance to AMPs (Yeaman et al. 1998). *3) Electrostatic shielding.* Capsule, glycocalyx or biofilm of certain bacteria containing an anionic complex of carbon hydrate and phosphate including anionic acid alginate plays very important role in adherence of bacteria to the target cells and helping them to avoid opsonic phagocytes (Campos et al. 2004; Bishop et al. 2008). *In vitro* study

showed that the cationic peptides are more antagonized by polyanion alginate (Friedrich et al. 1999). Capsule of some bacterial pathogens such as *P. aeruginosa, E. coli, Klebsiella* has been demonstrated to have electronegative alginate which is thought to sequester cationic antimicrobial peptides, consequently preclude peptide access to the bacterial membrane (Chan et al. 2004). *4) Multidrug efflux pumps.* There three major families of multidrug efflux pumps have been characterized in Gram-negative bacteria, which include the Major Facilator Superfamily (MFS), the ATP-binding cassette (ABC) superfamily, and the Resistance Nodulation Division (RND) family (Piddock 2006). The constitutively expressed efflux pumps have been demonstrated to play a role in resistance to AMPs such as protamine and human β-defensins (Lin et al. 2002; Moskowitz et al. 2004; Eswarappa et al. 2008). *5) Niche-specific resistance.* In certain anatomical and physiological niches, bacteria can resist killing by AMPs due to the expression of specific components in specific niches (Yeaman et al. 2003). Some bacteria have potential to exploit specific anatomical settings as a means of circumventing the effect of antimicrobial peptides. For instance, opportunistic Gram-negative bacterium *P. aruginosa* preferentially colonizes tissues such as respiratory airway, the mucosal surface having abnormal osmotic or ionic strength. The lipid A structures in cystic fibrosis (airways) clinical isolates that are more resistant to antimicrobial peptides are distinct from those seen in acute clinical infections and isolates from environments (Ernst et al. 1999). This phenomenon suggests that the anatomical and physiological niches facilitate AMP resistance.

With respect to inducible AMP resistance mechanism that is triggered by environmental signals, modifications of LPS in outer membrane via two-component regulatory systems (e.g. PhoP/PhoQ) have been identified as major mechanisms to APMs in Gram-negative bacteria (Guo et al. 1998; Yount et al. 2005). The negatively charged LPS involves the electrostatic interaction of cell surface with cationic AMPs in Gram-negative bacteria. Thus, Gram-negative bacteria can add covalent modification to LPS and consequently reduce the negative charge and/or membrane fluidity of LPS and protect themselves from attacks by AMPs. AMP resistance mediated by LPS modifications has been well studied in *Salmonella typhimurium* (Gunn et al. 1996; Guo et al. 1998; Guina et al. 2000) and in other bacteria including *Escherichia coli (Trent et al. 2001; Yan et al. 2007; Herrera et al. 2010), Pseudomonas aeruginosa* (Dorrer et al. 1977; Ernst et al. 1999), *Proteus* mirabilis (McCoy et al. 2001; Wang et al. 2008), *Bordetella bronchiseptica* (Banemann et al. 1998; West et al. 2000). In *Salmonella*, two-component regulatory systems including PhoP/PhoQ and PmrA/PmrB contributed significantly to LPS modifications and AMP resistance by modulating the transcription of genes involved in the modification of lipid A, the bioactive component of LPS (Gunn et al. 1996; Gunn et al. 1998). In response to low extracellular Mg^{2+} or Ca^{2+}, PhoP/PhoQ directly activated *pagP* and *ugtL* genes for LPS modification (Guina et al. 2000). PagP is an outer membrane protein essential for addition of palmitate to *Salmonella* lipid A, leading to the enhanced resistance of *S. typhimurium* to α-helical AMPs (Guina et al. 2000). UgtL is an inner membrane protein that promotes the formation of monophosphorylated lipid A in the LPS and contributes resistance to polymyxin B (PM) and α-helical AMP magainin (Shi et al. 2004). By mediating activation of the second

two-component system PmrA/PmrB under low Mg^{2+} conditions, PhoP/PhoQ also indirectly activated genes required for modification of lipid A with aminoarabinose (Gunn et al. 1998; Guina et al. 2000) and phosphoethanolamine (Gunn 2001). Specifically, the *pbgP* operon and *ugd* genes were essential for both the biosynthesis and incorporation of aminoarabinose into lipid A and are required for PM resistance (Lee et al. 2004). The *pmrC* gene encoding a putative aminotransferase mediated phosphoethanolamine modification of lipid A and confers PM resistance in *S. typhimurium* (Lee et al. 2004).

In addition to LPS modification, Gram-negative bacteria also have other inducible mechanisms to resist AMPs via two-component or other regulatory systems. Direct degradation of AMPs by proteases is important for α-helical AMP resistance in some bacteria. *Salmonella* outer membrane endopeptidase PgtE inactivated a panel of α-helical but not β-sheet AMPs and inactivation of PgtE resulted in increase *in vitro* sensitivity of *Salmonella* to many α-helical AMPs (Guina et al. 2000). PgtE homologies were found in outer membrane of *E. coli* (OmpT), and *Yersnia* (Pla) (Sugimura et al. 1988; Sodeinde et al. 1992), suggesting that this is a common AMP resistance mechanism in bacteria. Expression of *E. coli* OmpT increased survival of *E. coli* growth in the presence of protamine. OmpT degraded protamine at the extracellular facet of the outer membrane (Yeaman et al. 2003). Strains with deletion of this gene became hyper susceptible to protamine. Some Gram-negative bacteria can also secrete peptidase to inactivate AMPs. Secreted metallopeptidases ZmpA and AmpB were found in *Burkholderia cenocepacia*. These enzymes were shown to cleave human cathelicidin LL-37 and β-defensin-1,

respectively (Kooi et al. 2009). In addition to AMP enzymatic degradation, there also have been numbers of studies on the role of efflux pumps in inducible AMP resistance. In *Neisseria gonorroeae*, efflux pump MtrCDE expression was increased in the presence of protegrin-1 and human cathelicidin LL -37 (Shafer et al. 1998). Similarly, the efflux pump RosA/RosB in *Yersinia* was found to be induced in the presence of AMPs such as polymyxin B (Bengoechea et al. 2000). Collectively, resistance to AMPs in bacteria is mediated by membrane potential, modification of bacterial membrane, production of enzymatic activities, and efflux pumps.

APPENDIX

Table 1. Nomenclature of chicken β-defensins

No.	New gene/protein name	RefSeq definition	Lynn/Higgs et al. definition	Xiao et al. definition
1	Avian beta-defensin 1 (AvBD1)	Gallinacin 1 (GAL1)	Gallinacin 1 (GAL1)	Gallinacin 1 (GAL1)
2	Avian beta-defensin 2 (AvBD2)	Gallinacin 2 (GAL2)	Gallinacin 2 (GAL2)	Gallinacin 2 (GAL2)
3	Avian beta-defensin 3 (AvBD3)	Beta-defensin prepropeptide (GAL3)	Gallinacin 3 (GAL3)	Gallinacin 3 (GAL3)
4	Avian beta-defensin 4 (AvBD4)	GAL4 (GAL4)	Gallinacin 7 prepropeptide (GAL7)	Beta-defensin 4 (AvBD4)
5	Avian beta-defensin 5 (AvBD5)	GAL5 (GAL5)	Gallinacin 9 prepropeptide (GAL9)	Beta-defensin 5 (AvBD5)
6	Avian beta-defensin 6 (AvBD6)	GAL6 (GAL6)	Gallinacin 4 prepropeptide (GAL4)	Beta-defensin 6 (AvBD6)
7	Avian beta-defensin 7 (AvBD7)	GAL7 (GAL7)	Gallinacin 5 prepropeptide (GAL5)	Beta-defensin 7 (AvBD7)
8	Avian beta-defensin 8 (AvBD8)	GAL8 (GAL8)	Gallinacin 12 prepropeptide (GAL12)	Beta-defensin 8 (AvBD8)
9	Avian beta-defensin 9 (AvBD9)	GAL9 (GAL9)	Gallinacin 6 prepropeptide (GAL6)	Beta-defensin 9 (AvBD9)
10	Avian beta-defensin 10 (AvBD10)	GAL10 (GAL10)	Gallinacin 8 prepropeptide (GAL8)	Beta-defensin 10 (AvBD10)
11	Avian beta-defensin 11(AvBD11)	GAL11(GAL11)		Beta-defensin 11(AvBD11)
12	Avian beta-defensin 12(AvBD12)	Beta-defensin 12 (GAL12)	Gallinacin 10 prepropeptide (GAL10	Beta-defensin 12(AvBD12)
13	Avian beta-defensin 13(AvBD13)	Beta-defensin 13 (GAL13)	Gallinacin 11 prepropeptide (GAL11)	Beta-defensin 13(AvBD13)
14	Avian beta-defensin 14(AvBD14)	Gallinacin 14 (GAL14)		Beta-defensin 14(AvBD14)

CHAPTER II

MECHANISMS OF POLYMYXIN B RESISTANCE IN *CAMPYLOBACTER JEJUNI*

ABSTRACT

Antimicrobial peptides (AMPs) function as critical innate barriers limiting bacterial infections at the gastrointestinal mucosal surface. Recently, AMPs also became recognized as a novel class of antibiotics to control pathogens. As an important strategy to evade killing by host innate immunity and by novel peptide antibiotics, the mechanisms of AMP resistance in *C. jejuni* are critical to understand, but are still unknown. In this study, Polymyxin B (PM), a peptide antibiotic, was used as a model peptide to study mechanisms of AMP resistance in *Campylobacter*. Using bioinformatic and microarrays in conjunction with molecular approaches, we observed that several lipooligosaccharide (LOS) synthesis genes (*galU*, *waaC*, *waaF*, *neuB*, and *cgtA*) were involved in PM resistance in *Campylobacter*. Inactivation of GalU also resulted in significantly reduced colonization ability of *C. jejuni* in chickens. Examination of the sequences encoding various two-component regulatory systems in two PM resistant mutants did not identify any mutations, which is different from what has been observed in other Gram-negative bacteria. Transcriptome analysis of PM resistant mutant using microarray showed that Cj0811, which is likely involved in lipid A synthesis, contributed to PM resistance. However, the identified genes were not involved in resistance of *Campylobacter* to physiologically relevant AMPs such as chicken host AMPs and anti-*Campylobacter* bacteriocins (BCNs). Therefore, PM may not be an ideal surrogate to study *Campylobacter* resistance to physiologically relevant AMPs. Natural AMPs should be directly used to identify *Campylobacter* genes contributing to AMP resistance.

INTRODUCTION

Campylobacter has emerged as the leading bacterial cause of human gastroenteritis in the United States and other industrialized countries (Ruiz-Palacios 2007). This pathogenic organism is also associated with Guillain-Bare syndrome, an acute flaccid paralysis that may lead to respiratory muscle compromise and death. In the United States, the estimated cases of campylobacteriosis are more than 2 million. Total economical and medical costs were estimated 1.5 to 8 billion US dollars each year (Buzby et al. 1997). Poultry and their products are the major sources for human *Campylobacter* infections (Kassenborg et al. 2004). In parallel to its increased prevalence, *Campylobacter* has become increasingly resistant to clinical antibiotics, posing a serious threat to public health (Allos 2001). Therefore, development of effective intervention strategies is urgently needed to prevent and control *Campylobacter* infections in humans and animal reservoirs.

Antimicrobial peptides (AMPs) are a major component of host defense and function as critical innate barriers limiting microbial infections at the gastrointestinal mucosal surface in animals (Yeaman et al. 2003; De Smet et al. 2005; Brown et al. 2006). Recently, AMPs also became recognized as a novel class of antibiotics (peptide antibiotics) and have been proposed as future anti-infective products to control zoonotic pathogens in the avian intestinal tract. Notably, some chicken commensal bacteria-produced AMPs, also called “bacteriocins” (BCNs), dramatically reduced *C. jejuni* colonization in chickens and turkeys and are being directed toward on-farm control of this pathogen (Stern et al. 2005; Svetoch et al. 2005; Stern et al. 2006). As an important

strategy to evade killing by avian innate immunity and by novel peptide antibiotics, the mechanisms of AMP resistance are still unknown in *C. jejuni*. Availability of this information is not only critical to reveal novel virulence determinants contributing to *in vivo* survival of *C. jejuni* but also is important to better depict the modes of AMP action and facilitate the development of potent peptide antibiotics to reduce *C. jejuni* load in poultry. The recent completion of several *C. jejuni* whole genome sequences allows us to comprehensively identify genetic loci required for AMP resistance using functional genomic approaches.

Polymyxin B (PM), an AMP produced by bacterium *Paenibacillus polymyxa*, has been widely and successfully used for studying AMP resistance in bacteria including different enteric pathogens (Ernst et al. 2001; Finlay et al. 2004). Although PM bears little structural resemblance to the host defense AMPs, inactivation of the genes responsible for PM resistance usually resulted in increased susceptibility of bacteria to a variety of innate AMPs, leading to reduce virulence of the mutants in animal model system (Ernst et al. 2001). Therefore, studying mechanisms of PM resistance in *Campylobacter* may reveal novel virulent determinants required for *in vivo* adaptation of *Campylobacter*. Furthermore, PM is easily available compared to most natural AMPs and is ideal for large-scale functional genomics studies. Thus, investigation of PM resistance likely reveals genes important for *Campylobacter* resistance to physiologically relevant AMPs.

In our recent study, using random transposon mutagenesis, we identified several genetic loci contributing to PM resistance including GalU, an enzyme involved in LOS

synthesis (Lin et al. 2009). In this study, we continue to evaluate the role of *galU* in AMP resistance and *in vivo* colonization of *Campylobacter* in chickens. The contribution of other LOS synthesis genes (*waaC*, *waaF*, *neuB*, and *cgtA*) to AMP resistance was also determined. In addition, several molecular approaches including microarray and sequence comparison were used to further determine the mechanisms of *Campylobacter* resistance to PM and other AMPs.

MATERIALS AND METHODS

Major bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 2. *Campylobacter* strains were routinely grown in Mueller-Hinton (MH) broth (Difco) or on MH agar at 42^0C under microaerobic conditions, which was generated by CampyGen Plus (Oxoid) gas pack in an enclosed jar. When needed antibiotics kanamycin (Kan) or chloramphenicol (Cm) were added into MH broth or agar to desired concentrations. *E. coli* strains were grown at 37^0C in Luria-Bertani (LB) medium with shaking and supplemented with 30 μg/ml of Kan or 20 μg/ml of Cm.

***in vitro* selection of PM^r mutants.** The PM^r mutants were obtained by single or stepwise *in vitro* selection methods. Briefly, to obtain the PM^r mutant JL216 (MIC = 64 μg/ml), an overnight culture of *C. jejuni* 81-176 grown on MH agar plates was harvested and approximately 10^{12} cells were spread on MH agar plate containing 128 μg/ml of PM. The plates were incubated for two days under microaerophilic conditions at 42^0C. The resistant mutants were collected and confirmed by MIC test with PM. The spontaneous

PM^r mutant JL148 (MIC = 64 µg/ml) was obtained from stepwise selection. Briefly, approximately 10^{12} CFU of *C. jejuni* 81-176 were spread on MH agar containing 32 µg/ml of PM. The resistant mutants selected after 2 days of incubation were subsequently used for additional rounds of selection on plates containing increasing concentrations of PM. One PM^r mutant named JL148 (MIC = 64 µg/ml) was obtained from the third step of selection using plates with 128 µg/ml of PM.

PCR, qRT-PCR and sequence comparison. Key primers used in this study for PCR and sequencing are listed in Table 3. PCR was performed in a 50 µl mixture containing each deoxynucleosite triphosphate at a concentration of 200 nM, each primer at a concentration of 200 nM, 2.0 nM $MgCl_2$, 50 ng of *C. jejuni* genomic DNA, and 2.5 U of Platinum *Taq* DNA *polymerase* (Invitrogen). PCR products were purified by QIAquick PCR Purification Kit (Qiagen), when needed, for cloning or sequencing analysis. Real-time quantitative PCR was performed as described previously (Guo et al. 2008) using gene specific primers (data not shown). Primers for real time qRT-PCR were designed using Primer3 online interface (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Each primer was subsequently analyzed with MFold (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) to avoid secondary RNA structures and hairpin loops. 16S RNA primers were set for internal control. The qRT-PCR was performed as described previously (Lin et al. 2005). The relative expression of different genes in wild type and PM resistant mutant was normalized with 16S RNA gene amplified from the corresponding sample. Differential expression was determined by

threshold cycle (CT) method (Guo et al. 2008) and two independent experiments with triplicates conducted for each independent experiment for each sample.

Antibiotic susceptibility test. The susceptibilities of *C. jejuni* strains to PM and other antimicrobials were determined by standard microtiter broth dilution method with an inoculum of 10^6 bacterial cells/ml as described previously (Lin et al. 2002). Minimum inhibitory concentrations (MICs) were determined by the lowest concentration of a specific antimicrobial showing complete inhibition of bacterial growth after two days of incubation at 42^0C. The tested AMPs were purchased from Sigma (St. Louis, MO; PM, magainin, cecropin A) or kindly provided by Dr. Norman Stern (USDA; bacteriocin OR-7 and E-760).

DNA isolation and natural transformation. Chromosomal DNA was isolated from *Campylobacter* using the Wizard Genomic Purification Kit (Promega) according to the manufacturer's instructions. Biphasic natural transformation method was performed as described previously (Davis et al. 2008). Briefly, *C. jejuni* cultures grown overnight on MH agar plates were collected and re-suspended in MH broth to OD_{600} of 0.5. Bacterial suspensions (0.5ml) were transferred to sterilized tubes and incubated at 42^oC under microaerophilic conditions for three hours. One μg DNA of donor strain which carries the chloramphenicol resistant gene (*cm*) or kanamyxin resistance gene (*kan*) marker was added to culture. After five hour incubation, bacterial cultures were spread on MH agar containing Cm or Kan to select transformants. The transformants were confirmed by PCR using specific primers.

Whole genome microarray analysis. We used microarrays to compare the transcriptome of wild-type *C. jejuni* 81-176 with that of PM resistant mutant JL216 (Table 2). The microarray glass slides (*C. jejuni* OciChip^{TM}) were purchased from Ocimum Biosolutions (Indiana). The bacterial RNA isolation, cDNA synthesis and labeling, microarray hybridization, and data collection and analysis were detailed in previous publication (Guo et al. 2008). Briefly, RNAprotect^{TM} Bacteria Reagent (Qiagen) was immediately added to mid-log phase *C. jejuni* culture (OD of approximately 0.12) to keep the bacterial transcriptome intact. Total RNA was then extracted and purified using RNeasy Mini Kit (Qiagen). Twelve μg of total RNA was reverse transcribed and fluorescently labeled with Cy3 or Cy5 (Amersham) using SuperScript Indirect cDNA labeling System (Invitrogen). Equal volumes of Cy3 and Cy5-labeled cDNA were mixed, dried under vacuum and then suspended in 120 μl of hybridization buffer (Ocimum Biosolutions). A second microarray slide was hybridized with dye swap to generate technical replicates. The experiment was replicated five times using RNA from five independent experiments. Hybridization slides were scanned at 650 nm for Cy5 and 550 nm for Cy3 using Axon GenePix 4000 Scanner at 10-μm resolution. The fluorescent signal intensities were collected using GenePix Pro4 software and the data was normalized with removal of outliers and loess smoothing of ratio-intensities plots for each array. The data was then analyzed using mixed linear models for each gene, accounting for array variation, dye swap bias, treatment effects. *P*-value was adjusted by a False Discovery Rate of 5%. R-package (version 0.5.12 2003) was used for data normalization and statistical analysis. For this study, we chose a *P* value of < 0.05 and a

change ≥ 2.0-fold as the cutoff for significant differential expression between *C. jejuni* 81-176 and PM resistant mutant JL216.

Construction of isogenic *Cj1506c* and *Cj0811* mutants. A 1.3-kb fragment was amplified from JL28 genomic DNA using primers Cj1506c-R and Cj1506c-F (Table 3). The PCR product was purified and ligated into TOPO TA cloning® vector, resulting in pCj1506c. A 0.8-kb fragment of chloramphenicol resistant cassette (*cm*) with an *XbaI* restriction site was amplified from plasmid pUOA18 (Wang et al. 1990) using primers CmXbaI-R and CmXbaI-F (Table 3). The PCR product, which was digested with *XbaI* was ligated into pCj1506c, which was also digested with *XbaI* prior to ligation. The ligation mix was transformed into *E. coli* DH5α, and one transformant bearing construct pcmCj1506c was selected on LB agar containing 20 μg/ml of Cm. The construct pcmCj1506c containing chloramphenicol resistant cassette was introduced into JL28 by biphasic natural transformation method. One transformant was selected on MH plus 6 μg/ml of Cm and confirmed by PCR.

The same strategy was used to generate a *Cj0811* isogenic mutant of *C. jejuni* JL216. Briefly, an approximately 1.2-kb fragment of *Cj0811* along with 200-bp of its upstream and downstream regions was amplified using genomic DNA of JL216 and primers Cj0811-F and Cj0811-R (Table 3). The PCR product was purified and ligated into a TOPO TA cloning® vector (Invitrogen) resulting in plasmid pCj0811. The chloramphenicol resistant cassette (*cm*) with introduced *PflmI* restriction sites at two ends was amplified from pUOA18 (Wang et al. 1990). The *PflmI* digested *cm* cassette was inserted into pCj0811 which was digested by *PflmI* prior to ligation. One mutant

construct designated as pcmCj0811 was obtained and used for natural transformation using JL216 as the recipient strain. One transformant (JL417) bearing *cm* inserted in *Cj0811* was selected on MH agar plate containing 6 μg/ml of Cm and confirmed by PCR using primers Cj0811-F and Cj0811-R.

Chicken experiment. One day old chickens (a kind gift from Hubbard Hatchery, Pikeville, TN) were randomly assigned into four groups (5 chickens per group). All birds were placed in sanitized wire cages with unlimited access to feed and water. All antibiotic-free feed was prepared by the feed mill at the Johnson Animal Research and Teaching Unit, University of TN. Prior to inoculation with *C. jejuni* strains, all birds were confirmed to be free of *Campylobacter* by culture of cloacal swabs and plated on MH agar containing selective supplements that inhibit majority of intestinal microflora. At day four of age, chickens in each group were inoculated with one of following 4 individual *Campylobacter* strains via oral gavage with inoculums of 10^7 CFU per chick: wild type *C. jejuni* 81-176, PM^r mutant JL280, isogenic *galU* mutants JL281 or JL282 (Table 2). Fecal samples were collected at 7 and 11 days post inoculation (DPI) and plated on MH agar containing selective supplements. *Campylobacter* colonies were enumerated after two days of incubation under microaerophilic conditions at 42^0C. The detection limit of plating method was approximately 100 CFU/gram feces. The significant difference in *Campylobacter* colonization levels ($\log_{10}$ transformed CFU/g of feces) at each sampling point between groups was calculated using Student's *t* test. A *P*-value of <0.01 was considered significant.

RESULTS

The gene *galU* is responsible for PM and other AMP resistance in *C. jejuni*. In our previous study, *galU* was observed to be responsible for PM resistance in PM^r mutant JL148. In this study, we further tested sensitivity of *galU* mutant to AMPs from various sources. As shown in Table 4, inactivation of the *galU* also led to increased sensitivity to other AMPs such as colistin, magainin, cecropin-A, and bacitracin. Complementation of *galU* (JL230) fully restored resistance levels back to its parent strain JL148. Although *galU* was found contributing to several AMPs, it was not responsible for resistance to chicken AMPs such as fowlicidin-1 and -2, and bacteriocins OR-7 and E-760.

LOS required for PM resistance in *Campylobacter*. We further examined if other LOS synthesis genes are involved in PM resistance, including *neuB, ctgA, waaC, and waaF*. As shown in Table 5, inactivation of these genes in PM resistant strain JL216 resulted in increased susceptibility to PM (8 to 32-fold reductions in MIC).

GalU is essential for colonization of *Campylobacter* in chickens. Because mutation in *galU* resulted in significant truncation of LOS of *C. jejuni* and led to increased susceptibility to various AMPs (Lin, 2009), GalU may play an important role in the *in vivo* colonization of *C. jejuni* by mediating AMP resistance. To test this hypothesis, strains wild type *C. jejuni* JL242, derivative spontaneous PM^r mutant JL280 and their corresponding isogenic *galU* mutants were inoculated into four chicken groups. As shown in Fig 1, wild-type *C. jejuni* JL242 colonized in 80% of the chickens at day 7 post

inoculation, and by day 11 post inoculation, all of the chickens were colonized by JL242 with a shedding level of about 10^5 CFU/gram feces. In contrast, *C. jejuni* was not detected throughout the study in any of cloacal swabs collected from the chickens inoculated with JL280 or *galU* mutants JL281 and JL282.

Sequence comparison of signal transduction systems between wild type and PM resistant mutants. Many studies have demonstrated that Gram-negative bacteria can govern inducible antimicrobial peptide (AMP) resistance via surface modifications that are controlled by two-component regulatory systems (Groisman et al. 1997; Ernst et al. 2001; Groisman 2001; Cheng et al. 2010; Fernandez et al. 2010). Point mutations occurring in such two-component systems (e.g. PhoP/PhoQ, PmrA/PmrB) could result in acquired resistance to PM and other AMPs. We hypothesized that the mutants (JL216 and JL148) with acquired PM resistance may have mutations in two-component regulatory systems; identification of such two-component system(s) is critical to study the regulatory mechanisms of AMP resistance in *Campylobacter*. According to the *C. jejuni* NCTC 11168 genome sequence (Parkhill et al. 2000), twelve putative two-component regulators and seven two component sensors (histidine kinase) exist. In this study, nine regulators, five histidine kinases, and six signal transduction systems (Table 6) were PCR amplified from wild-type 81-176 and its two PM resistant mutants JL148 and JL216 using specific primers (Table 3). The PCR fragments were sequenced and compared using DNAStar. No sequence difference was found in these regulatory genes between wild-type 81-176 and its PM resistant derivatives.

Transcriptional profiling of PM resistant mutant JL216. A microarray experiment was performed to compare the transcriptome of PM resistant mutant JL216 with that of its parent strain *C. jejuni* 81-176. The microarray result in conjunction with qRT-PCR revealed that the gene (*Cj0811*) encoding putative tetraacyldisaccharide 4`-kinase LpxK, which is likely involved in lipid A synthesis, was up-regulated (2 fold) and two other genes down-regulated *Cj0403c* (4-fold) and *Cj1506c* (16-fold) (Table 7). The *Cj1506c* and *Cj0811* isogenic mutants were constructed by site-targeted mutagenesis. Only isogenic *Cj0811* mutant showed increased sensitivity to PM (2-fold) and inactivation of *Cj1506c* did not affect MIC of PM compared with its parent strain (data not shown).

DISCUSSION

Modifications of LPS in conferring resistance to PM and other AMPs have been found in many Gram-negative bacteria such as *Salmonella*, *Klebsiella*, *Proteus miralitis*, and *E. coli* (Guo et al. 1998; Gunn et al. 2000; Campos et al. 2004; Moon et al. 2009; Cheng et al. 2010). Unlike other Gram-negative bacteria, *Campylobacter* only has LOS (not LPS) and capsule polysaccharide (CPS) on the surface (Karlyshev et al. 2005). The LOS comprises two main components: the hydrophobic lipid A anchor and an oligosaccharide consisting of a conserved inner core and a hyper variable outer core (Golec 2007). LOS plays multiple roles in pathogenesis of *Campylobacter* including acting as endotoxin, adherence factor, and a factor that maintains stability of the outer membrane and protects the cells from environmental stress (Kanipes et al. 2008; Jeon et al. 2009). The enzymes responsible for LOS production in *Campylobacter* include GalU,

WaaC, WaaF, WaaM, WaaV (inner core), LgtF, AgtA, and GalT (outer core) (Kanipes et al. 2008; Naito et al. 2010). In our study, we demonstrated that LOS production in *C. jejuni* is required for PM resistance.

Lipid A core modifications including addition or alteration of fatty acid chains, or addition of polar groups such as phosphoethanolamine, 4-amino-4-deoxy-L-arabinose, and/or palmitate can confer resistance to AMPs including PM in many Gram-negative bacteria (Wang et al. 2010). In our study, inactivation of the gene *Cj0811* which encodes putative tetraacyldisaccharide 4`-kinase (LpxK) responsible for addition of fatty acid chains to a disaccharide backbone resulted in slightly increased susceptibility to PM and magainin. However, there were no MIC difference caused by Cj0811 with respect to chicken cathelicidins (F-1 and F-2), or bacteriocins (OR-7 and E-760) (data not shown). Recently, it has been reported that addition of a polar group phosphoethanolamine at the 1 or 4-position in the disaccharide backbone of lipid A resulted in increased resistance of *C. jejuni* to PM (Cullen et al. 2010). Wosten *et al* (2010) reported that alteration of the disaccharide backbone in *Campylobacter* lead to mildly increased sensitivity to PM and other AMPs including chicken cathelicidin-1 and colistin (van Mourik et al. 2010).

The gene *galU* is critical for production of uridine diphosphate (UDP)-glucose, a sugar precursor required for polysaccharide formation and LOS outer core synthesis in *Campylobacter* and other Gram-negative bacteria (Weickert et al. 1993). This study demonstrated that defects in the outer core of LOS due to GalU mutation significantly *in vivo* colonization of *Campylobacter* in chickens. It is not surprising that the *in vitro*-selected PM resistant mutant JL280 also reduced colonization levels in chickens because

the mutation(s) which occurred in JL280 may exert pleiotropic effects on the phenotype of *C. jejuni* in addition to PM resistance, consequently leading to an overall negative impact on the colonization ability of the mutant. Similar findings also were observed in that both an AMP-sensitive PhoP-null mutant and an AMP-resistant mutant with a mutation in the two-component regulatory system PhoP/PhoQ were avirulent in a mouse model and displayed pleiotropic mutant phenotypes (Gunn et al. 1996; Groisman 2001). Our result was also consistent with the recent report that inactivation of genes contributing to synthesis of either inner core (WaaF) or outer core (LgtF) of LOS abolished *Campylobacter in vivo* colonization using a chicken model system (Naito et al. 2010).

Mutations occurring in two-component regulatory systems could result in acquired resistance to PM as well as AMPs in other bacteria (Roland et al. 1993; Gunn et al. 1996; Guo et al. 1998). Sequence analysis of multiple two-component regulatory systems in PM resistant mutants did not reveal any mutations when compared to its wild-type PM-sensitive parent strain, suggesting that *Campylobacter* may use different mechanisms to acquire AMP resistance. These sequence analyses were also consistent with the microarray data comparing the transcriptome of wild type *C. jejuni* 81-176 and its derivative PM^r mutant JL216. If the mutation occurred in the two-component regulatory systems, many genes would have been found differentially expressed. In fact, very small numbers of genes (three genes) were found differently expressed between wild type and PM resistant mutant from microarray data analysis. Comparative genomics revealed small differences between *C. jejuni* 11168 and 81-176 (Pearson et al. 2003), and

because of the unavailability of microarray glass slides designed specifically for *C. jejuni* 81-176, we could not rule out the possibility that using *C. jejuni* 11168-based microarray glass slides for hybridization of *C. jejuni* 81-176 may fail to identify the genes that are not present in *C. jejuni* 11168.

Despite discovery of genes involved in PM resistance in this study, these genes are not required for *Campylobacter* resistance to physiologically relevant AMPs (e.g. chicken host AMP fowlicidins) and to the anti-*Campylobacter* BCNs. It is likely that resistance to PM in *Campylobacter* is unique, possibly mediated by mutations in the specific targets of PM. The results from this study suggested that PM is not an ideal surrogate for studying *Campylobacter* resistance to physiologically relevant AMPs such as bacteriocins and fowlicidins. More studies are needed to reveal both common and unique mechanisms of *Campylobacter* resistance to natural AMPs such as bacteriocins and fowlicidins, which are described in following chapters in this dissertation.

APPENDIX

Table 2. Major bacterial strains and plasmids used in this study (Chapter II)

Strains or plasmids	Description	Sources or references
Strains		
JL28	81-176 human isolate with low motility	(Black et al. 1988)
JL148	81-176 derivative PM resistant mutant obtained by stepwise selection *in vitro*, MIC = 64 μg/ml	(Lin et al. 2009)
46B1	JL148 derivative, *galU::kan*	(Lin et al. 2009)
JL230	46B1 /pGalU	(Lin et al. 2009)
JL216	81-176 derivative, PM resistant mutant obtained by single step selection *in vitro*, MIC = 64 μg/ml	This study
JL320	81-176 derivative, *waaC::cm*	(Kanipes et al. 2006)
JL321	81-176 derivative, *waaF::cm*	(Kanipes et al. 2004)
JL322	81-176 derivative, *ctgA::cm*	(Guerry et al. 2002)
JL323	81-176 derivative, *neuB::cm*	(Guerry et al. 2002)
JL328	JL216 derivative, *neuB::cm*	This study
JL329	JL216 derivative, *cgtA::cm*	This study
JL330	JL216 derivative, *waaC::cm*	This study
JL331	JL216 derivative, *waaF::cm*	This study
JL317	JL216 derivative, *galU::kan*	This study
JL388	81-176 derivative, *Cj1506c::cm*	This study
JL242	81-176 human isolate, highly motile	(Black et al. 1988)
JL281	JL242 derivative, *galU::kan*	This study
JL280	JL242 derivative spontaneous PM^r mutant	This study
JL282	JL280 derivative, *galU::kan*	(Lin et al. 2009)
JL417	JL216 derivative, *Cj0811::cm*	This study
Plasmids		
pGEM-T	PCR cloning vector, Amp^r	Promega
pCj1506c	pGEM-T Easy containing 1.3-kb gene fragment *Cj1506c* of JL28	This study
pcmCj1506c	pCj1506c with *cm* cassette inserted in *Cj1506c*	This study
pCj0811	pGEM-T Easy containing 1.2-kb gene fragment *Cj0811* of JL216	This study
pcmCj0811	pCj0811 with *cm* cassette inserted in *Cj0811*	This study
E.coli		
$DH5_\alpha$	F^- ϕ80*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_k^-, m_k^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Invitrogen

Table 3. Key primers used in this study (Chapter II)

Primers	Sequence (3'-5')[a]	Target genes
Cj1222c-R	TGCCAGATATGAGAGGTATG	Cj1222c
Cj1222c-F	AAGAGGCAGGGGATATTGCT	
Cj1223c-R	TTTTGATACCATAATGCAAAAA	Cj1223c
Cj1223c-F	TTGATAAGAATTTTGATACAATGAT	
Cj1226c-R	AAAACAAATTTACCCCATCTTCA	Cj1226c (CprR)
Cj1226c-F	GGGTGGAGTGGTAAGTCGTG	
Cj1227c-R	CACTAACCCCAGCAAAAATAAAA	Cj1227c (CprS)
Cj1227c-F	TGCGACTTTGCTTGTGCTTA	
Cj1261-R	TCCCTTCTATTTTTAAAAGAACGGATAA	Cj1261c (RacR)
Cj1261-F	AAAGACTACATAAAAATTTACAAGGAC	
Cj1262-R	GTGCGAGTTTCATACCATCG	Cj1262c (RacS)
Cj1262-F	CATCGGTCGCTTAAGGGTAA	
Cj0246c-R	AAAACCTTTCCGGTTGAAGC	Cj0246c
Cj0246c-F	TTTGCAAAAATTGCAGAGG	
Cj1189c-R	CAAGATGAGAGAGCTCAAGGTG	Cj1189c
Cj1189c-F	AAGCCTTGTTGCTGTTCTGC	
Cj1191c-R	TTTTGTAATTCAGTTTGCTTTTTG	Cj1191c
Cj1191c-F	TGACACCTAAAACACCGATAACA	
Cj1492c-R	TGGCGTTATTGTTTGTTACGG	Cj1492c
Cj1492c-F	CCCTCATCTCCATTTTGAGC	
Cj0951c-R	GTACTAGCACAACCCGCAAA	Cj0951c
Cj0951c-F	GTACTAGCACAACCCGCAAA	
Cj1491c-R	TGTTTTATATTTTAAGGCTAAATCAGT	Cj1491c
Cj1491c-F	ATGGAAAAGACGGGGCTTG	
Cj0889c-R	CCCTCGTTTGCGCTAAAAT	Cj0889c
Cj0889c-F	AAGTGTTAGGGGTATTGGCTCTAAA	
Cj0890c-R	AACCCAAAAATCAAAGCCAAT	Cj0890c
Cj0890c-F	GAGGCGTGTGTATTTGTCCA	
Cj0643-R	GCAATGCGTATCAACAATCC	Cj0643 (CbrR)
Cj0643-F	AAAAATTTCCTTTCTTTTGAAAAC	
Cj1608-R	GCAAGTGCCTAGCAAACCTT	Cj1608
Cj1608-F	AAGCCACAACAACGGAAAAA	
Cj0355c-R	CCGAAGGTGCAAAATTGTTA	Cj0355c
Cj0355c-F	TTTTCTTAATAAGGATAGAAACAAATGA	
Cj1262-R	TGAAAAGGTGATAAAGCAAGC	Cj1262
Cj1262-F	CATCGGTCGATTAAAGGTAA	
Cj0793-R	CGTTTTGGGGTGAAAAGAAA	Cj0793
Cj0793-F	TGTGAACAGAATTTGCTTTTACTTG	
Cj0448c-R	GCTTCATGTAAAAGATGAACTTAGC	Cj0448c
Cj0448c-F	GCCTTTGCTTGATTTTGCTT	
Cj1506c-R	GGCTGAGGACAAGATAGATTGC	Cj1506c
Cj1506c-F	GCAAAAAGACATAGAAGA	
CmXbaI-R	TTT**TCTAGA**TGATCGGCGGTGTTCCTTT	cm^r cassette
CmXbaI-F	TTT**TCTAGA**GCGCCCTTTAGTTCCTAAT	
Cj0811-R	GCGAATTGCTTTTAGGTTATGG	LpxK
Cj0811-R	GCTGTTTTAGGGACGGAAGA	
CmPflmI-R	TTT**CCATTTTTTGG**GCGCCCTTTAGTTC	cm^r cassette
CmPflmI-F	TTT**CCATTTTTTGG**TGCTCGGCGGTGTT	

[a]bold and underlined sequences are enzyme restriction sites

Table 4. Susceptibilities of *C. jejuni* JL148, isogenic *galU* mutant 46B, and complemented construct JL230 to different AMPs

Antimicrobial peptides	**MIC (µg/ml)**		
	JL148	46B1	JL230
Polymyxin B	64	4	64
Colistin	64	2	64
Magainin	32	16	32
Cecropin A	4	2	4
Bacitracin	1,024	512	1,024
Fowlicidin-1	4	4	ND[a]
Fowlicidin-2	8	8	ND
OR-7	1	1	ND
E-760	1	1	ND

[a]ND: Not determined

Table 5. LOS required for PM resistance in *Campylobacter*

Strains	**Isogenic mutants**	**Function of inactivated genes**	**MIC (µg/ml)**
JL216	N/A	N/A	64
JL328	JL216 derivative, *neuB::cm*	N-acetylneuraminic acid synthetase	2
JL329	JL216 derivative, *cgtA::cm*	Beta-1,4-N-cetylgalactosaminyltransferase	8
JL330	JL216 derivative, *waaC::cm*	Lipopolysaccharide heptosyltransferase I	8
JL331	JL216 derivative, *waaF::cm*	ADP-heptose--LPS heptosyltransferase II	8
JL317	JL216 derivative, *galU::kan*	UTP-glucose-1-phosphate uridylyltransferase	4

N/A, Not applicable

Table 6. Putative two-component regulatory and signal transduction systems of *C. jejuni* 11168 used for sequence comparison

Gene name	**Putative functions**
Cj1222c	Two-component sensor histidine kinase (DccS)
Cj1223c	Two-component regulator (DccR)
Cj1226c	Putative two-component sensor histidine kinase (CprR)
Cj1227c	Putative two-component regulator (CprR)
Cj1262	Two-component sensor histidine kinase (RacS)
Cj1261	Two-component regulator (RacR)
Cj1492c	Two-component sensor histidine kinase
Cj1491c	Putative two-component regulator
Cj0889c	Putative sensory transduction histidine kinase
Cj0643	Two-component regulator (CbrR)
Cj1608	Putative two-component regulator
Cj0355c	Putative two-component regulator
Cj1191c	PAS domain containing signal-transduction sensor protein
Cj0448c	Putative MCP-type signal transduction protein
Cj1506c	Putative MCP-type signal transduction protein
Cj0246c	Putative MCP-domain signal transduction protein
Cj1189c	Bipartate energy taxis response protein CetB
Cj0951c	Putative MCP-domain signal transduction protein
Cj0793	Signal transduction histidine kinase

Table 7. Transcriptome profiling of PM resistant mutant JL216

Gene ID	**Function description**	**Fold change (microarray)**	**Fold change (qRT-PCR)**	***P*-value**
Cj0811	LpxK; putative tetraacyldisaccharide 4`-kinase	1.84	2.1	0.02
Cj0403c	Hypothetical protein	- 2.0	- 4	5.10E-06
Cj1506c	Putative MCP-type signal transduction protein	- 4.3	-16	6.60E-06

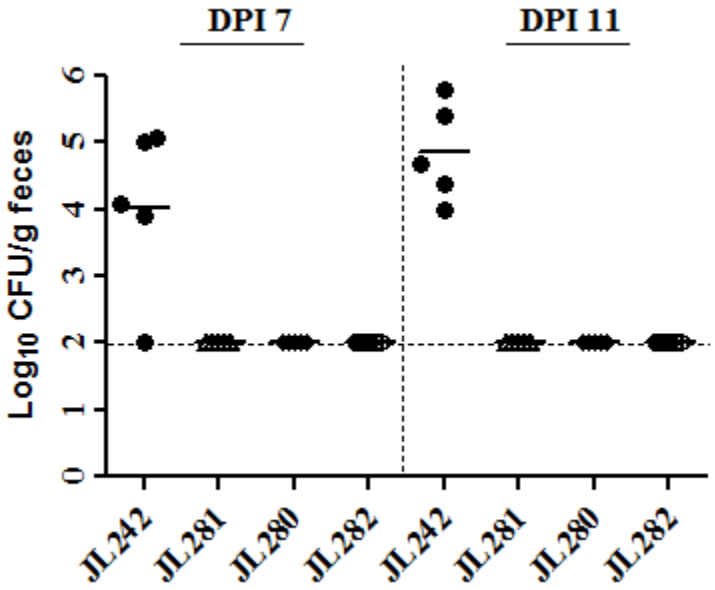

Figure 1. GalU is required for chicken colonization. Colonization of *C. jejuni* JL242, its derivative PM^r mutant JL280, and their isogenic *galU* mutants JL281 and JL282 respectively in chickens. The shedding levels of *Campylobacter* in chicken colonized by *C. jejuni* at 7 and 11 days postinoculation (DPI). Each data point represents the mean shedding level of *Campylobacter* of colonized in chickens (10 chickens) of each group.

CHAPTER III

PREVALENCE, DEVELOPMENT, AND MOLECULAR MECHANISMS OF BACTERIOCIN RESISTANCE AMONG *CAMPYLOBACTER* SPP

ABSTRACT

Bacteriocins (BCNs) are antimicrobial peptides produced by bacteria with narrow or broad spectrum of antimicrobial activity. Recently, several unique anti-*Campylobacter* BCNs have been identified in commensal bacteria isolated from chicken intestine. These BCNs dramatically reduced *C. jejuni* colonization in poultry and are being directed toward on-farm control of *Campylobacter*. However, no information exists concerning prevalence, development, and mechanisms of BCN resistance in *Campylobacter*. In this study, susceptibilities of 137 *C. jejuni* and 20 *C. coli* isolates to the anti-*Campylobacter* BCNs OR-7 and E-760 were examined. Only one *C. coli* strain displayed resistance to the BCNs (MIC = 64 μg/ml) while others were susceptible with MIC ranging from 0.25 to 4 μg/ml. BCN resistant (BCN^r) *C. coli* mutants also were obtained by *in vitro* selection but all of them displayed low-level resistance to the BCNs (MIC = 8 to 16 μg/ml). The acquired BCN resistance in *C. coli* could be transferred at intra- and inter-species levels in *Campylobacter* by biphasic natural transformation. Genomic examination of the BCN^r mutants using DNA microarray and random transposon mutagenesis revealed that the multidrug efflux pump CmeABC contributes to both intrinsic and acquired resistance to the BCNs. Together, this study represents the first report and a major step forward in understanding BCN resistance in *Campylobacter*, which will facilitate the development of effective BCN-based strategies to reduce *Campylobacter* load in poultry.

INTRODUCTION

Campylobacter species including *C. jejuni* and *C. coli* are the most common bacterial causes of human gastroenteritis in the United States (Ruiz-Palacios 2007). Human *Campylobacter* illnesses are caused primarily by *C. jejuni* (~90%) and secondarily by *C. coli* (~10%). This group of pathogenic organisms causes watery diarrhea and/or hemorrhagic colitis in humans and is associated with Guillain-Barre syndrome, an acute flaccid paralysis that may lead to respiratory muscle compromise and death (Nachamkin et al. 1998). There are more than two million estimated cases of human campylobacteriosis in the United States each year, and the annual medical and productivity costs resulting from *Campylobacter* infection are estimated at 1.5 to 8.0 billion dollars (Buzby et al. 1997). Poultry, particularly chickens, are considered a major source of human campylobacteriosis (Kassenborg et al. 2004). Thus, on-farm control of *Campylobacter* in poultry would reduce risk of human exposure to this pathogen and have a significant impact on food safety and public health (Lin 2009). In particular, at the same time that prevalence of infection is increasing, *Campylobacter* has become increasingly resistant to antibiotics, including fluoroquinolones and macrolides, the major drugs of choice for treating human campylobacteriosis (Luo et al. 2005), which raises an urgent need for novel strategies to prevent and control *Campylobacter* colonization in poultry (Lin 2009).

To date, three general strategies have been proposed to control *Campylobacter* in poultry on the farm, including 1) reduction of environmental exposure (biosecurity measures); 2) an increase in poultry's host resistance to reduce *Campylobacter* carriage in the gut (e.g. competitive exclusion, vaccination, and host genetic selection); and 3) the use of antimicrobial alternatives to reduce and even eliminate *Campylobacter* from colonized chickens (e.g. bacteriophage therapy (Lin 2009)). Effective implementation of biosecurity measures relies on a better understanding of risk factors and sources of *Campylobacter* for poultry (van Gerwe et al. 2009). Except for biosecurity measures, the other two general intervention approaches are currently not commercially available and are still under development. Notably, recent breakthroughs in the discovery and characterization of potent anti-*Campylobacter* bacteriocins (BCNs) may lead to an effective measure for on-farm control of *Campylobacter* in poultry (Lin 2009). BCNs are short antimicrobial peptides (AMPs) produced and exported by most bacterial species examined to date for the apparent purpose of destroying their competitors (Riley et al. 2002). Many BCN-producing bacteria (e.g. lactic acid bacteria) are commensals in the intestine. Therefore, the intestinal BCN-producing bacteria may achieve competitive advantage and function as an innate barrier against pathogens in the host. The natural BCNs have been proposed as promising candidates for novel antimicrobials (Cotter et al. 2005). Several anti-*Campylobacter* BCNs produced by chicken commensal bacteria, such as OR-7 from *Lactobacillus salivarius* (Stern et al. 2006), E-760 and E50-52 from *Enterococcus faecium* (Svetoch et al. 2008), and SRCAM from *Paenibacillus polymyxa* (Svetoch et al. 2005), have displayed potent killing effect *in vitro*. Oral administration of these BCNs dramatically reduced *C. jejuni* colonization in poultry intestine (5-8 $\log_{10}$

CFU/gram feces reductions). Thus, these natural anti-*Campylobacter* BCNs have been proposed as effective alternatives to therapeutic antibiotics and are being developed for on-farm control of *Campylobacter* (Lin 2009).

Although the above anti-*Campylobacter* BCNs have been demonstrated to be very effective in reducing *C. jejuni* colonization in poultry, several critical issues (e.g. production and resistance development) need to be addressed for future regulatory approval and public acceptability of this intervention measure. In this study, we examined prevalence, development, and molecular mechanisms of BCN resistance in *Campylobacter* using molecular and genomic approaches. Our findings strongly suggest that there is less resistance development for the anti-*Campylobacter* BCNs, such as OR-7 and E-760 compared to traditional antibiotics. In addition, microarray and random transposon mutagenesis studies indicated that the multidrug efflux pump CmeABC contributes to both intrinsic and acquired resistance of *Campylobacter* to the BCNs.

MATERIALS AND METHODS

Bacteria strains, plasmids, and growth conditions. The major bacterial strains and plasmids used in this study are listed in Table 8. Among the 157 strains used for the prevalence survey in this study, 137 isolates are *C. jejuni* while 20 isolates are *C. coli*. These *Campylobacter* strains were isolated from different hosts including human (15), bovine (5), chicken (111), turkey (1), pigs (4), as well as from the environment including mouse traps (5), bird droppings (5), and lagoons (1). All of these strains were collected from 16 geographically diverse areas in the United States. These *C. jejuni* and *C. coli*

strains were routinely cultured in Mueller-Hinton (MH) broth (Difco) or on MH agar at 42^0C under microaerophilic conditions, which was generated by CampyGen Plus (Oxoid) gas pack in an enclosed jar. When needed, MH media were supplemented with kanamycin (Kan), chloramphenicol (Cm), or BCNs at desired concentration. *E. coli* cells were grown at 37^0C in Luria-Bertani (LB) medium with shaking and supplemented with 30 μg/ml of Kan or 20 μg/ml of Cm.

Bacteriocins. The BCNs OR-7 and E-760 were purified from *L. salivarius* NRRL B-30514 and *E. faecium* NRRL B-30745, respectively, as described in recent publications (Stern et al. 2006; Line et al. 2008). These BCNs were dissolved in sterile H_2O and stored at -20^0C prior to use.

BCN susceptibility test. The susceptibilities of *C. jejuni* and *C. coli* isolates to BCNs OR-7 and E-760 were determined by standard microtiter broth dilution method with an inoculum of 10^6 bacterial cells/ml as described previously (Lin et al. 2002). Minimum inhibitory concentrations (MICs) were determined by the lowest concentration of specific BCN showing complete inhibition of bacterial growth after two days of incubation at 42 ^{0}C.

in vitro* selection of BCN^r *C. jejuni* and *C. coli. BCN OR-7 was used as a selective agent for generating spontaneous BCN^r mutants *in vitro*. Briefly, *C. jejuni* and *C. coli* strains were grown in BCN-free MH broth to late log phase. The cultures were centrifuged and the pellets were suspended in MH broth to a final concentration of approximately 10^{10} cells/ml. The cell suspensions were plated in duplicate on MH agar

plates containing 4, 8, or 16 μg/ml of OR-7. After two days of incubation, the BCN^r colonies were enumerated and the frequency of emergence of BCN resistance was calculated, which is the ratio of CFU on BCN containing plates to CFU on BCN-free MH agar plates. The single-step *in vitro*-selected BCN^r mutants were randomly selected and used for MIC test together with the corresponding parent strain. The experiment was repeated twice with triplicate measurements in in independent experiment.

DNA isolation and natural transformation. Chromosomal DNA was isolated from *Campylobacter* using the Wizard Genomic Purification Kit (Promega) according to the manufacturer's instructions. Natural transformation (biphasic method) was performed as described previously (Davis et al. 2008). The natural transformation efficiency was expressed as transformants per μg DNA per recipient CFU.

Whole genome microarray analysis. We used microarrays to compare the transcriptome of wild-type *C. jejuni* NCTC 11168 with its BCN^r derivative JL341 (OR-7 MIC = 8 μg/ml) (Table 8). The microarray glass slides (*C. jejuni* OciChip™) were purchased from Ocimum Biosolutions (Indiana). The bacterial RNA isolation, cDNA synthesis and labeling, microarray hybridization, and data collection and analysis were detailed in our previous publication (Guo et al. 2008). The hybridization experiments were repeated five times (biological replicate, n = 5) by using total RNA isolated from five independent experiments. In this study, we chose a P value of < 0.05 and a change $\geq$ 2.0-fold as the cutoff value for significant differential expression between *C. jejuni* NCTC11168 and its derivative BCN^r mutant JL341.

***in vivo* random transposon mutagenesis.** The BCN^r *C. jejuni* JL358, a 81-176 derivative (Table 8), was subjected to *in vivo* transposon mutagenesis using EZ::Tn5™ <KAN-2> Transposome (Epicentre) as detailed in our previous publication (Lin 2009). Briefly, one microlitter of EZ::Tn5™<KAN-2> Transposome was used to electroporate *C. jejuni* JL358 competent cells. The Kan^r transformants were individually picked and inoculated in 96-well microplates. Following 2 days of incubation, cultures of mutants were replicated into microtiter plates containing 4 μg/ml of OR-7 (4-fold reduction of MIC of parent strain). Those mutants that could not grow in OR-7-containing medium were selected from initial plates and subjected to second screening to confirm increased sensitivities of the mutants to OR-7. To confirm specific genetic associated with the transposome insertion and the increased susceptibility of each mutant to BCN, backcrossing of the transposon mutations into the parent strain was performed using natural transformation (Davis et al. 2008). The MICs of BCN for the backcrossed mutants were determined together with parent strain *C. jejuni* JL358. Specific transposon insertion site of each mutant was determined by direct sequencing of the genomic DNA (Lin et al. 2009). Sequence analysis was performed using DNAStar software package.

PCR and Real-time qRT-PCR. Key PCR primers used in this study were listed in Table 9. PCR was performed in a 50 μl mixture containing each deoxynucleotide triphosphate at a concentration of 200 nM, each primer at a concentration of 200 nM, 2.0 nM $MgCl_2$, 50ng of *C. jejuni* genomic DNA, and 2.5 U of Platinum *Taq* DNA *polymerase* (Invitrogen). Real-time quantitative RT-PCR was performed as described previously (Guo et al. 2008) using gene specific primers.

Isogenic mutants construction and complementation *in trans*. To construct isogenic *Cj0035c* (a putative efflux pump gene) mutant of JL341, genomic DNA of *Cj0035c* isogenic mutant of 81-176 (Ge et al. 2005) (Table 8) was extracted and used for natural transformation with JL341 as a host strain, creating mutant JL377 with insertional inactivation in *Cj0035c*.

Isogenic *Cj1125c* and *Cj1687* mutants of JL341 were constructed by insertional mutagenesis as described in a previous publication (Lin et al. 2005). Briefly, to construct *Cj1125c* isogenic mutant of JL341, an approximately 1.7-kb fragment was PCR amplified from genomic DNA of JL341 using primer pairs of Cj1125cF and Cj1125cR (Table 9). The PCR product was cloned into pGEM T-easy vector (Promega), resulting in construct pCj1125c. The chloramphenicol (*cm*) resistance gene cassette was PCR amplified from plasmid pUOA18 (Wang et al. 1990) using *pfu* polymerase (Stratagene) and primers CmAfeI-F and CmAfeI-R (Table 9). The resulting blunt-ended PCR product was purified and ligated into pCj1125c vector, which was digested with *SwaI* prior to ligation, to generate mutant construct pcmCj1125c. The construct pcmCj1125c, which serves as a suicide vector, was introduced into JL341 by natural transformation (Van Vliet et al. 1998). One transformant designated as JL416 was selected on MH agar plate containing 5 μg/mL of chloramphenicol and the insertion of Cm-resistant cassette was confirmed by PCR using the specific primers Cj1125cF and Cj1125cR (Table 9). Similarly, to construct the isogenic *Cj1687* mutant of JL341, primers Cj1687-F and Cj1687-R (Table 9) were used to amplify a 2.0-kb fragment from genomic DNA of JL341 using *Taq* polymerase. The PCR product was cloned into a pGEM T-easy vector,

resulting in the pCj1687 plasmid. The plasmid was then digested by *SwaI* and ligated to the PCR product of the Cm-resistant gene cassette as described above, generating plasmid pcmCj1687. The pcmCj1687 served as a suicide vector to generate an isogenic *Cj1687* mutant in the JL341 background as described above. The *Cj1687* mutation in JL372 was confirmed by PCR using the specific primers Cj1687-F and Cj1687-R (Table 9). A similar site-directed approach was used to create isogenic *Cj0630c (DNA polymerase III, delta subunit)* and *Cj1116c* (a putative membrane bound zinc metallopeptidase) mutants of JL341.

To complement *perR* mutation in K15A2, an 81-176 derivative obtained by random transposon mutagenesis, the complete *perR* gene together with its 200 -bp upstream and 100 -bp downstream regions was amplified using PerRF and PerRR primers (Table 9) in conjunction with *Pfu* polymerase (Stratagene). The blunt -ended PCR product was purified and ligated to the *SmaI*–digested shuttle vector pRY111 (Yao et al. 1993). The ligation mixture was then introduced into *E.coli* DH5α, creating construct JL402. The pPerR plasmid from JL402 was transferred into K15A2 by triparental conjugation using DH5α/pRK2013 as a helper strain (Akiba et al. 2006). The complemented strain JL412 was tested for BCN susceptibility together with other related strains. To complement the *cmeB* mutation in JL360 (JL341 background), the plasmid pCME bearing *cmeABC* operon (Lin et al. 2003) was extracted and transferred into JL360 by natural transformation, creating complemented strain JL424.

RESULTS

Prevalence of BCN resistance in *C. jejuni* and *C. coli* isolates from various source. Total of 137 *C. jejuni* and 20 *C. coli* isolates were subjected to susceptibility test for BCN OR-7 and E-760. As shown in Fig 2, all *C. jejuni* isolates were susceptible to both OR-7 and E-760 with MICs ranging from 0.25 to 1.0 μg/ml. *C. coli* appeared to display higher intrinsic BCN resistance than *C. jejuni* with majority of *C. coli* showing MIC ≥ 1 μg/ml. Only one *C. coli* human isolate, designated as JL106, was resistant to both OR-7 and E-760 with MIC of 64 μg/ml.

Frequency of emergence of BCN^r resistant *Campylobacter in vitro.* Three *C. jejuni* strains (NCTC 11168, 81-176, S3B) and two *C. coli* isolates (JL20, JL25) that displayed the same MIC of BCN OR-7 (0.5 μg/ml) were chosen for examination of frequency of *in vitro* emergence of BCN resistance. As shown in Table 10, BCN^r mutants were not obtained using *C. jejuni* strains under the tested selection pressures. In contrast, *C. coli* strains displayed higher frequencies of emergence of BCN resistance (1.5×10^{-8} to 8.7×10^{-7}) in the presence of different concentrations of OR-7 by a single-step selection; the MICs of selected BCN^r mutants ranged from 8 to 32 μg/ml.

The acquired BCN resistance could be transferred at intra- and inter-species levels in *Campylobacter*. *C. jejuni* and *C. coli* are well-known for the exceptional ability to acquire exogenous DNA by natural transformation, which is considered a major mechanism mediating horizontal transfer of antibiotic resistance in *Campylobacter*. Thus, we examined if natural transformation contributes to horizontal transfer of BCN

resistance in *Campylobacter*. Genomic DNA of *C. coli* JL106 (clinical isolate, MIC = 64 μg/ml) and JL349 (obtained from above one-step selection *in vitro*, MIC = 32 μg/ml) were extracted and transferred into several *C. jejuni* and *C. coli* strains by natural transformation. As shown in Table 11, the acquired BCN resistance in *C. coli* JL349 could be transformed to *C. coli* isolates such as JL20 and JL25 with transformation frequency about $4x10^{-7}$ CFU/μg DNA/recipient cell. Interestingly, the BCN resistance of JL106 and JL349 also could be easily transferred into different species such as *C. jejuni* 81-176 and NCTC 11168 with transformation with frequency of 10^{-8} CFU/μg DNA/recipient cell. The MICs of BCN for the selected BCN^r transformants were up to16 μg/ml (Table 11).

Transcriptional profiling of BCN^r mutant JL341. DNA microarray analysis was performed to compare the transcriptome of the BCN^r mutant JL341 to that of its parent strain NCTC 11168. The microarray analysis revealed that 9 genes were up-regulated and 10 genes were down-regulated in BCN^r mutant JL341 (Table 12). Since up-regulation of some genes such as those involved in LPS modification and peptide degradation is a common mechanism used by enteric pathogens to confer resistance to AMPs (Yeaman et al. 2003), the up-regulated genes from this microarray analysis are of particular concern, which include those encoding multidrug efflux pump CmeABC, putative drug efflux pump Cj1687 and Cj0035c belonging to a major facilitator super family, putative membrane bound zinc metallopeptidase Cj1125c, galactosyltransferase PglA, and a putative DNA polymerase III Cj0630c (Table 12). Up-regulation of these genes was also confirmed by qRT-PCR using specific primers as described previously

(Guo et al. 2008) (data not shown). The overexpression of CmeABC in JL341 was further confirmed by a β-galactosidase promoter fusion assay and immunoblotting using specific blotting antibodies against CmeB and CmeC as described in our previous publication (Lin et al. 2002) (data not shown).

The isogenic *cmeB*, *Cj0035c*, *Cj1125c*, and *Cj1687* mutants of JL341 were obtained using natural transformation or site-directed mutagenesis. However, our extensive efforts to construct isogenic *Cj0630c* (DNA polymerase III) and *Cj1116c* (membrane Zn metallopeptidase) mutants of JL341 were unsuccessful, probably due to the essential role of these gene products in *Campylobacter* growth. Except for the *cmeB* mutant, none of the generated isogenic mutants (*Cj0035c*, *Cj1125c*, and *Cj1687*) displayed increased susceptibilities to OR-7. Inactivation of *cmeB* in JL341 led to 4-fold MIC reduction for BCN OR-7 (Table 13). Complementation of the *cmeB* mutant with pCME plasmid fully restored MIC back to the level of the parent strain (Table 13). To test if CmeABC contributes to intrinsic resistance of *C. jejuni* NCTC 11168 to BCN, the isogenic *cmeB* mutant was compared to wild-type strain for susceptibility to OR-7. As shown in Table 13, inactivation of CmeB in wild-type strain also significantly increased susceptibility of the mutant (JL199) to OR-7. To determine whether the BCN resistance in JL341 is partly attributed to the overexpression of CmeABC, the *cmeR* mutant of 11168, which overexpresses CmeABC (Lin et al. 2005), was also subjected to MIC test. Compared to its parent strain, overexpression of CmeABC in JL4 due to *cmeR* mutation led to slight but consistent increase in the MIC of BCN (1 μg/ml) (Table 13).

Identification of genes contributing to BCN resistance by random transposon mutagenesis. A complementary genomic approach to microarray, random transposon mutagenesis, was used to identify genes involved in BCN resistance in this study. A library containing 2496 Kan^r mutants were generated for screening mutants with an increased susceptibility to BCN. Six mutants displaying higher susceptibility to BCN OR-7 than parent strain JL358 were identified (Table 14). Backcrossing of the transposon mutations into the parent strain by natural transformation further confirmed that the BCN-sensitive phenotype in each mutant was linked to the gene with a specific transposon insertion. Direct sequencing of the mutant genomic DNA using transposon-specific primers (Lin et al. 2009) revealed a specific transposon insertion site in each mutant (Table 14). All the transposon insertions were in coding regions of corresponding genes. Five of the six mutants had transposons inserted in different sites of the genes encoding the multidrug efflux pump CmeABC, which has been characterized in our previous study (Lin et al. 2002). This finding clearly indicated that CmeABC is involved in acquired BCN resistance in JL358. However, the expression level of CmeABC in JL358 is comparable to its BCN^s parent strain 81-176 as demonstrated by immunoblotting and LacZ-promoter fusion assay (data not shown). Similar to the finding in NCTC 11168 (Table 13), inactivation of CmeABC in 81-176 also led to increased susceptibility to OR-7 (4-fold MIC reductions) (data not shown).

The remaining mutant K15A2 has a transposon insertion in *perR*, a gene encoding the Fur family regulator. Complementation of *perR* mutation in K15A10 (construct JL412) partially restored BCN resistance to 8 μg/ml for OR-7. In addition, inactivation of

PerR in wild type *C. jejuni* 81-176 resulted in increased susceptibility to OR-7 (2-fold MIC reduction), indicating that PerR is also involved in intrinsic resistance to BCN.

DISCUSSION

The comprehensive survey in this study using *Campylobacter* strains from various origins and geographically diverse regions provides more compelling evidence demonstrating the potent killing activity of BCNs OR-7 and E-760 against *Campylobacter*, which has been shown in previous publications (Svetoch et al. 2005; Svetoch et al. 2008). The survey also clearly indicated that BCN *Campylobacter* is rarely detected in clinical and environmental isolates, suggesting that *Campylobacter* has difficulty developing BCN resistance in hosts even if *Campylobacter* may frequently encounter various BCNs produced by commensals in the intestine (Svetoch et al. 2005; Svetoch et al. 2008). The finding from this survey is consistent with the examination of *in vitro* emergence of BCN^r *Campylobacter* described in this study (Table 10), in which no BCN^r *C. jejuni* mutants were selected under selection pressure while only *C. coli* mutants could develop BCN resistance *in vitro* with all mutants only displaying low-level resistance to BCN. Although it is still unknown why *C. coli* develops BCN resistance more easily than *C. jejuni*, our findings strongly suggest that there is less in the way of resistance development for the anti-*Campylobacter* BCNs, such as OR-7 and E-760. These findings support a recent theory that bacteria have not developed highly effective mechanisms to resist BCNs and other endogenous AMPs during evolution, which is likely due to multiple activities of natural AMPs (Peschel et al. 2006). The best studied

BCN is nisin which is ribosomally produced by *Lactococcus lactic*. Nisin exerts bactericidal effects via at least two modes of action: targeting the membrane-bound cell wall precursor lipid II, consequently resulting in inhibition of peptidoglycan synthesis, and membrane pore formation resulting in membrane damage and depolarization (Hasper et al. 2006). Resistance to nisin has been reported in some bacteria (Margolles et al. 2006; Sun et al. 2009); however, high levels of nisin resistance in bacteria were not observed in bacteria eventhough it has been used as a food preservative for a half century (Enserink 1999). It has been also reported that the acquired bacterial resistance to BCNs, such as pediocin PA-1 and nisin A, is unstable and has associated with a fitness cost (Gravesen et al. 2002). The stability of the acquired resistance to anti-*Campylobacter* BCNs in *Campylobacter* is still unknown and needs to be examined in future studies**.**

AMPs including various BCNs have been considered potential natural 'peptide antibiotics' to combat bacterial infections (Hancock 1997; Ennahar et al. 2000; Cotter et al. 2005; Asaduzzaman et al. 2009). Elucidating the underlying mechanisms of AMP resistance in bacteria could help us to develop 'smarter' antibiotics (Peschel et al. 2006). Therefore, in this study, we also determined genetic loci involved in BCN resistance in *Campylobacter* using complementary genomic approaches. Both microarray and random transposon mutagenesis demonstrated the role of the multidrug efflux pump CmeABC in resistance of *Campylobacter* to BCN OR-7. Active extrusion of antimicrobials by multidrug resistant (MDR) efflux pumps plays vital roles in antimicrobial resistance in many Gram-negative bacteria (Poole 2000). It has been observed that MDR efflux pumps are also involved in AMP resistance in several bacteria (Yeaman et al. 2003; Tzeng et al.

2005; Otto 2009). The best studied bacterium is *Neisseria gonorrhoeae* whose multidrug efflux pump *mtrCDE* contributes to both intrinsic and acquired AMP resistance; overexpression of MtrCDE mildly increased resistance to human AMPs (2 to 4 fold increase in MIC) (Shafer et al. 1998). In *Campylobacter*, the CmeABC MDR efflux pump contributes to resistance to various antimicrobials, including both structurally diverse antibiotics and natural antimicrobials present in the intestine, such as bile salts (Lin et al. 2002; Lin et al. 2003; Yan et al. 2006). In this study, we observed that CmeABC also contributes to both intrinsic and acquired BCN resistance in *Campylobacter*. Notably, the findings from this study indicated that involvement of CmeABC in BCN resistance does not require overexpression of CmeABC although overexpression of CmeABC could lead to increased BCN resistance. Together, this study provides further evidence explaining why CmeABC is essential for *Campylobacter* colonization in the intestine (24) and further highlighting the multi-function nature of CmeABC and critical role of CmeABC in *Campylobacter* pathobiology.

Although CmeABC plays an important role in BCN resistance, inactivation of CmeABC alone in the BCN^r strain JL360 did not lead to a susceptibility level comparable to its parent strain JL241 (Table 13), strongly suggesting that other factors work together with CmeABC to contribute to the acquired BCN resistance observed in JL360. Based on microarray work in this study, several up-regulated genes are of particular interest. In addition to the putative efflux transporters Cj1687 and Cj0035c, Cj1116c, a putative peptidase, may involve proteolytic cleavage of BCNs because degradation of AMPs by protease is one of mechanisms used by bacteria to resist endogenous AMPs (Yeaman et

al. 2003). In *Salmonella*, the membrane protease PgtE, which is regulated by the two component regulatory system PhoP/PhoQ, is responsible for degrading cationic AMPs (Guina et al. 2000). The zinc-dependent membrane metalloprotease ZmpA and ZmpB in *B. ennocepacia* degrade antimicrobial peptides protamin and human cathelicidin LL-37 as well as other alpha helical cationic AMPs (Kooi et al. 2009). The PglA (Cj1125c) is a galactosyltransferase that is involved in *N*-linked protein glycosylation, a unique surface carbohydrate modification mechanism that was observed in *C. jejuni* (Linton et al. 2005); such surface modification may be required for BCN resistance in *Campylobacter*. In this study, we have successfully obtained mutants with mutations in most of these interested genes. However, none of these mutants displayed increased susceptibilities to BCN compared to their parent strain JL341. We were not able to generate a mutation in Cj1116c, strongly suggesting that Cj1116c is an essential gene for *Campylobacter* physiology. Additional work, such as production and purification of recombinant Cj1116c, are needed to determine whether Cj1116c could function as a peptidase to degrade the anti-*Campylobacter* BCNs.

Random transposon mutagenesis also identified another gene *perR* that is involved in BCN resistance. PerR is a transcriptional regulator controlling transcription of genes encoding oxidative stress resistance proteins (such as catalase KatA, superoxide dismutase SodB, and alkyl-hydroxyperoxidase AhpC) (Palyada et al. 2009). Transcriptional profiling analysis of an isogenic *perR* mutant identified 104 genes that belong to the PerR regulon (Palyada et al. 2009). PerR activates several genes encoding proteins responsible for capsule biosynthesis, which include acetyl-CoA carboxylase

AccA, 3-oxoacyl-synthase FabH, and fatty acid/phospholipid synthesis protein PlsX (Palyada et al. 2009). Thus, inactivation of *perR* in BCN^r JL358 may affect capsule synthesis and such surface remodeling may increase the susceptibility of JL358 to the BCN OR-7. This speculation needs to be examined in the future.

The results obtained from this study provide helpful information for risk assessment of on-farm control of *Campylobacter* using anti-*Campylobacter* BCNs, and represent the first and major step forward in understanding the genetic mechanisms of *Campylobacter* resistance to BCNs. We have observed that *Campylobacter* can develop low-level BCN resistance *in vitro*. Clinical survey also suggests that *Campylobacter* may develop BCN resistance at very low frequency in the host. However, it is unknown if higher selection pressure (e.g. therapeutic usage of bacteriocins) will promote emergence of BCN^r *Campylobacter* mutants *in vivo*. If so, can *Campylobacter* develop high-level bacteriocin resistance in response to therapeutic treatment with BCN? In addition, it is unclear if the BCN^r *Campylobacter* can persist in the absence of selection pressure. To obtain solid answers to these questions, multiple laboratory experiments using chickens should be performed to examine the dynamic changes of the *Campylobacter* population in response to BCN treatment and to determine *in vivo* stability of BCN resistance in *Campylobacter*. These studies are expected to provide important information that may help avoid a rapid loss of efficiency of BCN and to design more sustainable and 'smarter' peptide antibiotics. In addition, examination of the molecular basis of BCN resistance in *Campylobacter* may help us to develop more sustainable and effective BCN-based intervention strategies against *Campylobacter* colonization in chickens. In this study, we

revealed that active transport of multidrug efflux pump CmeABC confers resistance to BCN in *Campylobacter*. Thus, inhibition of this pump by efflux pump inhibitors will significantly increase susceptibility of *Campylobacter* to BCN. Previous studies (Lin et al. 2006; Hannula et al. 2008) have shown that inhibition of efflux pump CmeABC in *Campylobacter* spp resulted in increased susceptibility to various antimicrobials. Notably, such an efflux pump inhibitor also could dramatically increase *Campylobacter* susceptibility to intestinal bile salts by inhibiting CmeABC, leading to reduced colonization of *Campylobacter* in chickens (Lin et al. 2006; Hannula et al. 2008). Therefore, oral administration of the anti-*Campylobacter* BCNs together with such efflux pump inhibitors using an appropriate delivery system (e.g., encapsulation) would enhance the therapeutic effect of anti-*Campylobacter* BCNs. This speculation needs to be determined in future studies.

APPENDIX

Table 8**.** Major bacterial strains and plasmids used in this study (Chapter III)

Strains or plasmids	**Description**	**Source or reference**
Strains		
Campylobacter		
JL106	*C. coli* strain isolated from human	Human [a]
JL20	*C. coli* strain isolated from pig	This study
JL349	JL 20 derivative, BCN^r mutant obtained from single step selection *in vitro*	This study
JL241	*C. jejuni* NCTC 11168 strain isolated from human	(Parkhill et al. 2000)
JL28	*C.jejuni* 81-176 isolated from human	(Black et al. 1988)
JL199	NCTC 11168 derivative, *cmeB::kan*	(Lin et al. 2005)
JL341	NCTC11168 derivative, BCN^r mutant generated by natural transformation using genomic DNA of *C. coli* JL106	This study
JL4	NCTC 11168 derivative, *cmeR::cm*	
JL360	JL341derivative, *cmeB*::*kan*	This study
JL199	NCTC 11168 *cmeB::kan*	(Lin et al. 2002)
JL424	JL360 containing shuttle vector pCME	This study
JL416	JL341derivative, *Cj1125c*::*cm*	This study
JL372	JL341derivative, *Cj1687*::*cm*	This study
JL3	81-176 derivative, *cmeB::kan*	(Lin et al. 2002)
JL219	81-176 derivative, *Cj0035c::cm*	(Ge et al. 2005)
JL377	JL341derivative, *Cj0035c::cm*	This study
JL358	81-176 derivative, BCN^r mutant generated by natural transformation using JL349 genomic DNA	This study
K15A2	JL358 *perR::kan*	This study
JL412	K15A2/pPerR	This study
Plasmids		
pGEM-T Easy	PCR cloning vector, Amp^r	Promega
pCj1125c	pGEM-T Easy containing 1.7 kb *Cj1125c* gene of JL241	This study
pcmCj1125c	pCj1125c with Cm resistance gene inserted in *Cj1125c* gene	This study
pCj1687	pGEM-T Easy containing 2.0 kb *Cj1687* gene of JL241	This study
pcmCj1687	pCj1687 with Cm resistance gene inserted in *Cj1687* gene	This study
pRY111	*E.coli-C.jejuni* shuttle vector, cm^r	(Lin et al. 2002)
pPerR	pRY111 derivative containing a 1.45-kb *perR* gene plus its promoter region	This study
pCME	pUOA18 shuttle vector derivative containing a wild-type *cmeABC* operon	(Lin et al. 2003)
pRK2013	IncP Tra $RK2^+$ Δ*repRK2 repE1*$^+$, Kan^r	(Ditta et al. 1980)
E.coli		
$DH5_\alpha$	F^- ϕ80*lac*ZΔ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_k^-, m_k^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Invitrogen
JL402	*E.coli* $DH5_\alpha$ containing pPerR	This study
JL48	Conjugation helper strain, $DH5_\alpha$ containing plasmid RK2013	(Akiba et al. 2006)

[a] Isolated from human feces and kindly provided by Qijing Zhang (Iowa State University).

Table 9. Key oligonucleotide primers used in this study (Chapter III)

Primer	DNA Sequences (5' - 3') [a]	Product size	Gene amplified
Cj1125cF	GCCCGCTAGAATGTCTTTGA	1733	*Cj1125c*
Cj1125cR	ATCTAACCCGGGACGATTTT		
Cj1116cF	GGAACTCATTGATGAAATGCAA	1982	*Cj1116c*
Cj1116cR	CCCTACCATCTATAGGTGCAAAA		
CmAfeI-F	GCG*AGCGCT*TGCTCGGCGGTGTTCCTTT	811	*cm*^{r} cassette
CmAfeI-R	GCG*AGCGCT*GCGCCCTTTAGTTCCTAAAG		
Cj1687F1	TCTTTGGCATCTTTGGCTTT	2000	*Cj1687*
Cj1687R1	TGCGATTTTGATGTTTCC		
Cj0630cF	CAACGAAAAACAAAGCAA	1350	*Cj0630c*
Cj0630cR	TGTTTTTAAGTTCTTCGATTTTTGC		
PerRF2	AAACAAGTAAGGTGGAA	1662	*Cj0032* (*perR*)
PerRR2	AGTGCAATCAGATAGTAAA		

[a] Restriction sites are underlined in the primer sequences

Table 10. Frequency of emergence of BCN resistant *Campylobacter in vitro*

BCN conc. (µg /mL)	**Frequency of emergence of BCN resistance for each strain[a]**				
	C. jejuni			***C. coli***	
	81-176	**11168**	**S3B**	**JL20**	**JL25**
4	$<9.2 \times 10^{-9}$	$<2 \times 10^{-10}$	$<1.5 \times 10^{-10}$	$(3.5 \pm 1.0) \times 10^{-7}$	$(8.7 \pm 5.6) \times 10^{-7}$
8	$<9.2 \times 10^{-9}$	$<2 \times 10^{-10}$	$<1.5 \times 10^{-10}$	$(1.8 \pm 1.6) \times 10^{-7}$	$(1.1 \pm 1.0) \times 10^{-7}$
16	$<9.2 \times 10^{-9}$	$<2 \times 10^{-10}$	$<1.5 \times 10^{-10}$	$(1.5 \pm 1.4) \times 10^{-8}$	$(1.7 \pm 1.4) \times 10^{-8}$

[a] The values are means of two independent experiments with triplicate measurements.

Table 11. Horizontal gene transfer of BCN resistance in *Campylobacter* spp

Donor strains (*C. coli*)	**Recipient strains**	**Transformation frequency**[a] (transformants/µg DNA/ recipient CFU)	**Highest MIC of selected transformants** (µg/ml)
C. coli JL106 (MIC = 64)	*C. jejuni* NCTC11168	1.2 x10^{-8}	8 (JL341)
C. coli JL349 (MIC = 32)	*C. jejuni* 81-176	1.0 x10^{-8}	16 (JL358)
	C. coli JL20	4 x10^{-7}	16
	C. coli JL25	4 x10^{-7}	16

[a] The values are from a representative experiment with duplicate measurements within each independent experiment.

Table 12**.** Differentially expressed genes (≥ 2-fold change) in NCTC 11168 BCN^r mutant JL341 identified by microarray

Gene	**Function description**	**Fold change (microarray)**	***P*-value**
Up-regulated genes			
Cj0366c (*cmeB*)	Inner membrane transporter of CmeABC efflux system	4.9	5.93E-06
Cj0365c (*cmeC*)	Outer membrane component of CmeABC efflux system	4.0	2.72E-05
Cj0630c	DNA polymerase III, delta subunit	13.5	4.63E-04
Cj1726c	Putative homoserine O-succinyltransferase	2.0	8.31E-04
Cj1116c	Putative membrane bound zinc metallopeptidase	4.8	4.00E-03
Cj1125c	Putative galactosyltransferase (wlaG/pglA)	12.8	5.50E-03
Cj0176c	Putative lipoprotein	2.0	7.05E-03
Cj1687	Putative efflux transporter (MFS family)	2.0	2.14E-02
Cj0035c	Putative efflux transporter (MFS family)	3.4	2.19E-02
Down-regulated genes			
Cj0508	Penicillin-binding protein	-4.5	2.37E-05
Cj0093	Putative periplasmic protein	-2.5	1.49E-04
Cj0628	Putative lipoprotein	-2.9	1.57E-04
Cj0045c	Putative iron-binding protein	-2.0	6.60E-04
Cj0091	Putative lipoprotein	-2.5	8.90E-04
Cj0629	Putative lipoprotein	-2.7	1.16E-03
Cj1423c	Putative sugar-phosphate nucleotidyltransferase	-2.7	1.49E-03
Cj1650	Hypothetical protein	-17.9	1.80E-03
Cj1714	Small hydrophobic protein	-2.4	4.56E-03
Cj1539c	Putative anion-uptake ABC-transport system protein	-2.1	9.39E-03

Table 13**.** CmeABC contributes to both intrinsic and acquired BCN resistance in *C. jejuni* NCTC 11168

Strains	**Description**	**MICs of OR-7 (µg/ml)**
JL241	Wild type NCTC 11168	0.5
JL199	NCTC 11168 derivative, *cmeB::kan*	0.125
JL4	NCTC 11168 derivative, *cmeR::cm*	1
JL341	NCTC 11168 derivative, BCN^r mutant	8.0
JL360	JL341 derivative, *cmeB::kan*	2
JL424	JL360 containing pCME for complementation of *cmeB*	8

Table 14. Identification of transposon mutants with increased sensitivity to BCN compared to MIC of 16 µg/ml for parent strain JL358

Strain	**MICs of BCN (µg/ml)**	**Locus designation**	**Tn location (ORF size, bp)[a]**	**Function of inserted gene product**
K15A2	4	*Cj0322* (*perR*)	54 (411)	Fur family regulator
K17E10	4	*Cj0367c* (*cmeA*)	557(1104)	Component of CmeABC pump
K16H6	4	*Cj0366c* (*cmeB*)	790(3123)	Component of CmeABC pump
K11A10	4	*Cj0365c* (*cmeC*)	107(1479)	Component of CmeABC pump
K1H1	4	*Cj0366c* (*cmeB*)	1679(3123)	Component of CmeABC pump
K15G10	4	*Cj0365c* (*cmeC*)	1213(1479	Component of CmeABC pump

[a]The number indicates the nucleotide before which the transposon (Tn) is inserted.
ORF, open reading frame.

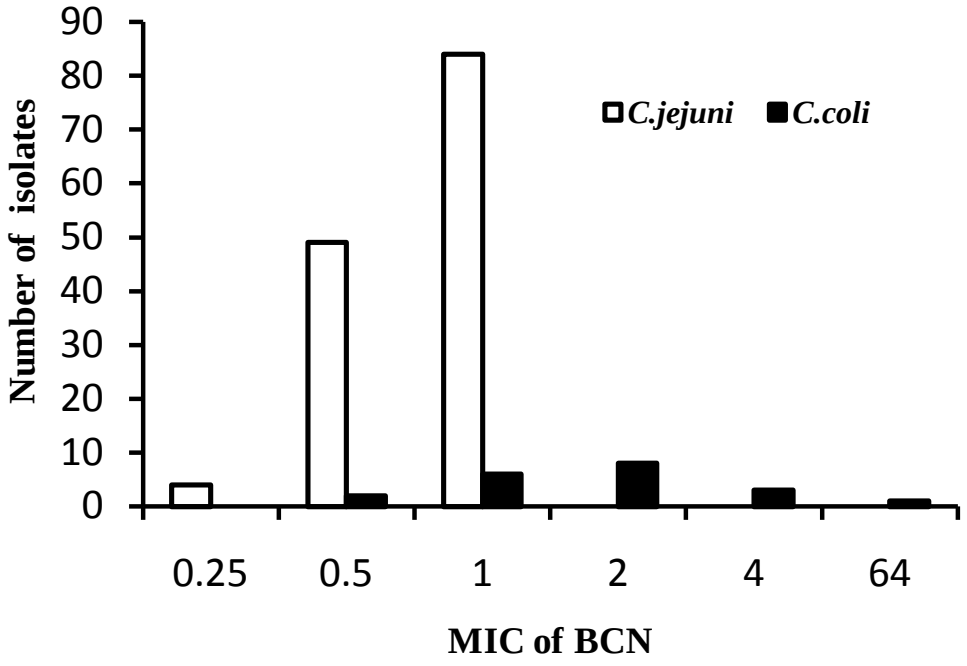

Figure 2**.** Prevalence of *Campylobacter spp* isolates resistance to bacteriocin. 137 *C. jejuni* and 20 *C. coli* isolates were examined for the susceptibility to BCN OR-7 and E-760 using MIC tests. Open bars represent *C. jejuni* isolates. Black bars represent *C. coli* isolates.

CHAPTER IV

DEVELOPMENT AND STABILITY OF BACTERIOCIN RESISTANCE IN *CAMPYLOBACTER*

ABSTRACT

Bacteriocins (BCNs) are antimicrobial peptides produced by bacteria with narrow or broad ranges of antimicrobial activity. Recently, several unique anti-*Campylobacter* BCNs have been identified in commensal bacteria isolated from chicken intestines. These BCNs dramatically reduced *C. jejuni* colonization in poultry and are being directed toward on-farm control of *Campylobacter*. Our previous study in Chapter III demonstrated that *Campylobacter* could develop resistance to BCNs *in vitro*. However, no information exists concerning *in vivo* development and stability of BCN resistance in *Campylobacter*, an important issue needing to be addressed for practical application of BCN-based intervention measures. In this study, development and stability of BCN resistance were examined. For the *in vivo* development of BCN resistance, chickens that have been colonized by *C. jejuni* NCTC 11168 were fed with BCN E-760 at dose of 5 mg/kg body weight for three consecutive days. For *in vivo* stability of BCN resistance, groups of chickens were fed one of three BCN^r *Campylobacter* strains obtained from different sources. Total and BCN^r *C. jejuni* populations in fecal samples from both studies were determined by differential plating using selective plates with or without BCN E-760. In addition, *in vitro* stability was tested by repeated subculturing of the BCN^r mutants in BCN E-760-free broth medium followed by differential plating. Our results showed that *C. jejuni* could develop resistance to BCN *in vivo*. However, the BCN^r mutants only accounted for 0.0005% of the total *C. jejuni* population in the feces. MIC tests indicated all mutants displayed low-levels of resistance to E-760 with MIC

values ranging from 2 to 8 µg/ml. Inactivation of the CmeABC efflux pump of the BCN^r mutants led to increased susceptibility to E-760. The low-level of BCN resistance was not stable *in vivo*, and BCN^r mutants were not detected at day 42 after inoculation. BCN resistance was also not stable after 10 passages *in vitro*; more than 95% of BCN^r *Campylobacter* spp were sensitive to the BCN after 35 passages. This study highlights the significance of *in vivo* usage of BCN for *Campylobacte*r control in poultry. BCN selected only low-level BCN^r *C. jejuni* mutants *in vivo* and the low-level BCN resistance was not stable both *in vivo* and *in vitro*.

INTRODUCTION

Campylobacter species, the epsilon class of proteobacteria, are the leading bacterial causes of human gastroenteritis in developed countries (Allos 2001). In addition to watery diarrhea and/or hemorrhagic colitis, infection with *Campylobacter* spp can result in post-infectious manifestations such as Guillain-Barre' syndrome, an acute immune mediated disorder that may lead to respiratory muscle compromise and death (Nachamkin et al. 1998). *Campylobacter* spp are considered to be commensal organisms in the intestinal tract of wild and domestic animals including chicken and other avian species (Diker et al. 2000). Epidemiological studies demostrated that consumption of contaminated poultry meat is the major cause of human campylobacteriois (Stern et al. 2001; Cox et al. 2002; Stern et al. 2004). Thus, on-farm control of *Campylobacter* spp could reduce risk of human *Campylobacter* infection. Of the several proposed strategies to reduce this risk, anti-*Campylobacter* bacteriocins (BCNs) are considered a promising strategy to protect food safety and public health (Lin, 2009).

BCNs are short cationic antimicrobial peptides naturally produced by diverse microbes in different environments (Willey et al. 2007). Despite significant structural and characteristic differences, BCNs display potent antimicrobial activities against a wide range of viruses, bacteria, and fungi with diverse modes of action and have been recognized as a novel class of antimicrobials to control foodborne pathogens (Hugas et al. 1998; Zasloff 2002; Galvez et al. 2007; Settanni et al. 2008). As a group of natural antimicrobials, some BCNs, such as nisin, have long been applied for food preservation.

More than 99 percent of bacteria including intestinal commensals can make at least one bacteriocin (Klaenhammer 1988; Riley et al. 1992). Therefore, intestinal BCN-producing bacteria may achieve a competitive advantage and function as an innate barrier against pathogens in the hosts. The natural BCNs have been proposed as promising candidates for novel antimicrobials (Joerger 2003; Asaduzzaman et al. 2009).

Several anti-*Campylobacter* BCNs were successfully isolated and characterized from chicken commensal bacteria, which includes OR-7 from *Lactobacillus salivarius* (Stern et al. 2006), E-760 and E50-52 from *Enterococcus faecium* (Line et al. 2008; Svetoch et al. 2008), and SRCAM from *Paenibacillus polymyxa* (Stern et al. 2005). Animal studies have demonstrated that these BCNs dramatically reduced *C. jejuni* colonization in the intestine and these BCNs are being developed toward on-farm control of *Campylobacter* to protect public health. Although the anti-*Campylobacter* BCNs are very effective in reducing *C. jejuni* colonization in poultry, several important issues (e.g. production, safety, and development and stability of resistance) need to be addressed for future regulatory approval and public acceptability of this intervention measure. The study in chapter III has demonstrated that *Campylobacter* could develop low-level BCN resistance *in vitro*. However, no information exists concerning *in vivo* development and stability of BCN resistance in *Campylobacter*. Using both *in vitro* and *in vivo* systems, we showed the *in vivo* usage of BCN selected only low-level BCN^r *C. jejuni* mutants and the low-level BCN resistance was not stable under both *in vitro* and *in vivo* conditions. This study provides helpful information for risk assessment of the future practical application of the anti-*Campylobacter* BCNs in poultry.

MATERIALS AND METHODS

Bacteriocin E-760, bacterial strains and growth conditions. Bacteriocin E-760 (Line et al. 2008) was kindly provided by Dr. Norman J. Stern (USDA/ARS). E-760 was dissolved in distilled water and stored at -20^{0}C prior to use. The major bacterial strains used in this study are listed in the Table 15. All strains were routinely grown in MH broth or agar at 42^{0}C under microaerophilic conditions generated using a CampyGen Plus gas pack (Oxiod, Lenxa, KS) in an enclosed jar. When needed, E-760 was added in MH agar at desired concentrations.

***in vivo* development of BCN resistance in *Campylobacter*.** The *in vivo* development of BCN resistance in *Campylobacter* was examined using a chicken model system. In this experiment, day-old broiler chicks (a kind gift from commercial company Hubbard Hatchery, Pikeville, TN) were randomly assigned to either treatment (10 chicks) or control groups (10 chicks). All birds were placed in sanitized wire cages with unlimited access to feed and water. Nutritionally complete feed was prepared in the feed mill at the Johnson Animal Research and Teaching Unit, the University of Tennessee. Prior to inoculation with *C. jejuni* NCTC 11168, all birds were confirmed to be free of *Campylobacter* by culture of cloacal swabs. At 2 days old, all birds in treatment and control groups were inoculated with fresh *C. jejuni* NCTC 11168 cultures (10^7 CFU/bird) via oral gavages. Three days after *Campylobacter* inoculation, all birds were examined to ensure colonization by *Campylobacter*. For the treatment group, at 9 days of age, all birds were treated with BCN E-760 at the dose of 5 mg/kg body weight/day via oral gavages for three consecutive days. Birds in the control group were gavaged with water. Cloacal

swabs were collected from all birds in both groups at day 1, 2, 3, 5, and 7 after the initial BCN treatment. Samples from each bird were serially diluted and spread onto two different plates: MH agar selective supplement (normal selective plates) to recover all *Campylobacter* populations, and MH agar selective supplement plus E-760 with final concentration of 16 µg/ml to recover BCN resistant populations. *Campylobacter* colonies were counted following 48 hours of incubation at 42^0C under microaerophilic conditions. Individual colonies from E-760 containing plates were randomly picked to identify level of BCN resistance using MIC testing as described below. In addition, representative colonies from BCN-free plates were also chosen for MIC test.

The detection limit of the plating method was approximately 100 CFU/g of feces. The significant difference in *Campylobacter* colonization levels ($\log_{10}$ transformed CFU/g of feces) at each sampling point between groups was calculated using Student's *t* test. A *P*-value of <0.05 was considered significant.

***in vitro* stability of BCN resistance.** Three BCN resistant mutants were examined for *in vitro* stability of acquired BCN resistance, which include a human clinical isolate JL106 (E-760 MIC = 64 µg/ml), the *in vitro*-selected mutant JL341 (E-760 MIC = 8 µg/ml), and the *in vivo*-selected strain K58 (MIC = 8 µg/ml) (Table 15). Briefly, the three strains were inoculated in E-760-free MH broth and grown under microaerophilic conditions at 42^0C. The *Campylobacter* cultures were sub-cultured every 2 to 3 days in fresh MH broth (1:400 dilutions) for 70 days in the absence of any antimicrobials. Following passages 10, 15, 20, 25, 30, and 35, the cultures were serially diluted (10-fold dilutions) in MH broth and plated onto both MH agar plates and MH

agar plates supplemented with E-760 at a concentration of 16 μg/ml. The plates were then incubated in microaerophilic conditions at 42^0C for two days. Total numbers of colonies on each type of plate were counted and compared for each time point. In addition, following passage 35 differential plating, 20 colonies for each mutant were randomly picked up from bacteriocin-free MH agar plates and were subjected to E-760 MIC test as described below.

***in vivo* stability of BCN resistance using a chicken model system.** The same BCN resistant mutants were used for an *in vivo* stability test using a chicken model system. Bird source and maintenance were the same as those used in the *in vivo* development of BCN resistance experiment described above. Forty one-day old chickens were randomly assigned into four groups (10 chickens each group). Prior (3 days) to inoculation with *Campylobacter*, all birds were confirmed free of *Campylobacter* by cultured cloacal swabs. Birds in each group received the corresponding *Campylobacter* strain at a dose of 10^7 CFU/bird via oral gavage at 3 days old. Birds in the control group were treated with wild-type, E-760 sensitive strain *C. jejuni* NCTC 11168. Birds in the other three treatment groups were inoculated with JL106, JL341, and K58, respectively (all strains displayed comparable growth level *in vitro*). Birds in all groups received BCN-free feed and water throughout the trial. Cloacal swabs were collected from birds in all groups at day 6, 22, and 42 after *Campylobacter* inoculation. Isolation of *Campylobacter* spp and differential plating for enumerating the proportion of BCN resistance mutants in the populations in each group were identified the same as the chicken experiment described above. At each time point, representative colonies (40

colonies) from chickens in each group were chosen for E-760 MIC test. Multiple isolates with different E-760 MICs were analyzed by PCR to confirm their genetic identities. The PCR was done using primers specific for the *cmp* gene encoding the major outer membrane protein as described previously by Huang *et al*. (2005), which revealed no difference between input strain and output isolates.

E-760 susceptibility test. The susceptibility of *Campylobacter* spp to E-760 was determined by standard micro titer broth dilution method with an inoculum of 10^6 bacterial cells/ml that was described previously (Lin et al. 2002). Minimum inhibitory concentration (MIC) was determined by the lowest E-760 concentration that completely inhibited *Campylobacter* growth after 24 hours under microaerophilic condition at 42^0C.

Insertional mutagenesis of the *cmeB* gene in BCN resistant mutants. To construct an isogenic *cmeB* mutant in *Campylobacter in vivo*-selected BCN resistant mutants, genomic DNA of JL3 (Table 15), a *cmeB* mutant, was extracted and was used for natural transformation by biphasic natural transformation as described previously (Davis et al. 2008).

RESULTS

Effect of E-760 treatment on the emergence of BCN resistant *Campylobacter* in chickens. In this experiment, all chickens in both groups were successfully colonized by *C. jejuni* NCTC 11168 prior to E-760 treatment. The shedding levels of *C. jejuni* in both groups were similar (approximately 5.5 $\log_{10}$ units). Throughout the trial *C. jejuni* consistently colonized all chickens regardless of BCN treatment (Fig 3). E-760 resistant

mutants were selected from one chicken in the BCN treated group one day after the first treatment. Six days after the first treatment, 80% of chickens were found to shed E-760^{r} mutants (Fig 4). However, the E-760 resistant mutants only accounted for a small portion of *Campylobacter* population in individual chicken (0.0005%). MIC test of 17 randomly selected E-760 resistant *Campylobacter* isolates generated *in vivo* indicated that mutants displayed low-level resistance to E-760 with MIC ranging from 2 µg/ml to 8 µg/ml (Table 16). No chickens in the control group shed E-760 resistant mutants.

CmeABC contributes to E-760 resistance in the *in vivo*-selected *Campylobacter* isolates. Studies in chapter III have shown the multidrug efflux pump CmeABC contributed to both intrinsic and acquired BCN resistance in *Campylobacter*. However, it is unknown if CmeABC plays a role in E-760 resistance in the *in vivo*-selected mutants obtained from this study. As shown in Table 16, regardless of resistance level of a specific mutant, inactivation of *cmeB* significantly reduced E-760 MIC to 0.125 µg/ml, which is lower than the E-760 MIC for the wild-type strain NCTC 11168.

Bacteriocin E-760 resistance is not stable *in vitro* in the absence of BCN selection pressure. As shown in Fig 5, less than 10% of JL341 populations could be selected on MH agar plates containing 16 µg/ml of E-760 after 10 passages in the absence of BCN selection pressure. Following 35 passages, only a very small population of JL341 (0.0005%) were recovered on an E-760-containing plate. Although JL106 and K58 showed higher stability than JL341 *in vitro*, less than 0.1% and 1% of JL106 and K58, respectively, were selected on BCN-containing plates after 35 passages. The

differential plating results were also confirmed by E-760 MIC tests of representative colonies selected after 35 passages (data not shown).

***in vivo* instability of E-760 resistance in *Campylobacter*.** All chickens were successfully colonized by *Campylobacter* in both control (*C. jejuni* NCTC 11168) and treatment groups inoculated with *C. jejuni* JL341, K58, or *C. coli* JL106 with shedding levels of approximately 7 $\log_{10}$ units per gram feces at 6 days post inoculation (Fig 6A). Unexpectedly, the percentage of chickens colonized with E-760 resistant strains decreased after 22 days (JL106) and 42 days (JL341, K58, and JL106) of inoculation (Table 17). The shedding levels of *Campylobacter* in colonized chickens were also slightly reduced in both control and treatment groups at 22 and 42 days postinoculation when compared to 6 days postinoculation (Fig 6A).

The *in vivo* stability of BCN resistance was monitored by differential plating as well as E-760 MIC tests of randomly selected colonies (40 colonies per time point per group). As showed in Fig 6B, percentage of E-760 resistant mutants decreased for all three *Campylobacter* strains with time. By 42 days post inoculation, E-760 resistant populations were less than 7% for all three resistant strains. The resistance levels were also reduced to < 2 µg/ml for randomly selected resistant colonies (data not shown).

DISCUSSION

Several anti-*Campylobacter* BCNs have been successfully identified and characterized from chicken commensal bacteria (Stern et al. 2005; Svetoch et al. 2005; Stern et al. 2006; Line et al. 2008; Svetoch et al. 2008). Feeding these anti-

Campylobacter BCNs to poultry pre-slaughter eliminated *Campylobacter* colonization (Stern et al. 2005; Svetoch et al. 2005; Stern et al. 2006; Line et al. 2008; Svetoch et al. 2008). Although these BCNs are very effective in reducing *Campylobacter* spp colonization in poultry, the use of these anti-*Campylobacter* BCNs may lead to emergence of BCN resistant isolates resulting in problems in the treatment of resistant *Campylobacter* infection. Therefore, studying BCN resistance including development and stability of resistance are crucially important for future regulatory approval and public acceptability of this intervention measure. In our previous study discussed in Chapter III, we demonstrated that *Campylobacter* spp could develop low levels of BCN resistance *in vitro*. In this study, we examined the *in vivo* development BCN resistance in *Campylobacter* using a chicken model system. Because BCNs have been proposed to be fed to chickens for three consecutive days pre-slaughter to control *Campylobacter* (Cole et al. 2006), a treatment regimen mimic clinical (3 consecutive days) with BCN was applied in this study. Our data showed that in response to BCN treatment, BCN resistant *C. jejuni* quickly emerged as early as one day after the first BCN treatment. However, the BCN resistance in *Campylobacter* was low in terms of MIC (1 µg/ml to 8 µg/ml, even after the third BCN treatment) and frequency (0.0005%) during the whole experiment. This *in vivo* finding is consistent with the *in vitro* results described in Chapter III. *In vivo* resistance development of *Campylobacter* to several antibiotics used as either therapeutic or growth promotants has been well studied (Luo et al. 2003; Lin et al. 2007; Han et al. 2008; Lindow et al. 2010). A previous study (Lin, Yan et al. 2007) showed that development of macrolide resistance *in vivo* was only observed after long-term exposure to tylosin. Short-term treatment of *Campylobacter*-infected chickens with a high dose of

macrolide also did not result in selection of Erythromycin resistance. However, long-term treatment with a low dose of tylosin in feed resulted in the emergence of resistance mutants with high levels of MIC (12 to 100% of tested isolates). The result in this study revealed different patterns of resistance development for tylosin and BCN usage. The BCN resistance appears to develop quickly but the MIC resistance levels of the mutants are low in *Campylobacter*. However, the results should be interpreted cautiously because the experiment was conducted in a laboratory environment using a small number of chickens which does not represent the production conditions in most poultry farms. In addition, a dose experiment should be performed to determine if greater selection pressure would promote the development of mutants with high-level BCN resistance. Finally, antibiotic resistance development on farms is very complex and influenced by multiple factors such as animal species, production environment, genetic background of bacterial species, and management practices.

In addition to demonstrating the development of BCN resistance, we also observed dynamic changes in BCN resistance of individual chickens in this study. One day after the first treatment, only 10% of the chickens in the treated group were positive with BCN resistant *Campyloabcter*. Then, the number of chickens colonized by resistance *Campylobacter* steadily increased during the course of treatment eventually 80% of chickens were found positive with BCN resistant *Campylobacter* two days after the third treatment, and quickly dropped to 40% two days afterward (Fig 4). This finding showed the 3-day BCN treatment exerted continuous selection pressure and resulted in an increased number of chickens shedding BCN resistant mutants by 5 days after the first E-

760 treatment. The resistance in *Campylobacter* is likely not stable due to fitness cost, consequently resulting in clearance of BCN resistant *Campylobacter* after cessation of BCN treatment. These speculations need to be further examined in future studies.

Multidrug resistance efflux pump CmeABC plays a prominent role in resistance to various antimicrobials and chicken colonization (Lin et al. 2002; Lin et al. 2003). It was shown in Chapter III that CmeABC contributed to both intrinsic and acquired resistance to BCN for the mutants selected *in vitro*. This result suggests that CmeABC plays a role in low level BCN resistance in *Campylobacter* in both *in vivo* and *in vitro*. Thus, a combination of anti-*Campylobacter* BCN and CmeABC pump inhibitors could be a feasible approach to eliminate *Campylobacter* colonization and reduce BCN resistance in chickens.

Antibiotic resistance in bacteria is occurring as the result of either chromosomal mutations or horizontal transfer of mobile genetic elements, such as plasmids, phages, transposons, and integrons. These genetic changes may influence physiological processes in microorganisms. Both chromosomal mutations and plasmid-mediated antibiotic resistance, generally result in a fitness cost creating growth impairment and/or less decreased competitiveness with antibiotic sensitive strains. The stability of antibiotic resistance and fitness cost due to acquisition of antibiotic resistance are unique in *Campylobacter* compared to other microorganisms. Our previous studies demonstrated that low levels of macrolide resistance in *Campylobacte*r associated with mutations in ribosomal proteins L4 and L22 were unstable *in vitro*. In contrast, high levels of macrolide resistance due to mutations at A2074G and A2075G in 23S rRNA were stable

in vitro in the absence of macrolide selection pressure (Lin et al. 2007; Caldwell et al. 2008). Luo et al. (Luo et al. 2005) reported that fluoroquinolone (FQ) resistance in *Campylobacter* associated with *gyrA* mutation is very stable *in vivo* and *in vitro*, and FQ-resistance strains did not show any fitness costs. FQ-resistance even enhances ecological fitness and outcompetes FQ-sensitive strains when coinoculated in chickens. In our study, both *in vivo* and *in vitro* experiments clearly showed that BCN resistance was not stable regardless of *Campylobacter* species and resistance levels. This information is also important for the risk assessment of using BCN in practical applications. For example, it is likely that some BCN resistant *Campylobacter* mutants may be transmitted to a new flock. However, based on the finding from this study, the BCN resistance trait will be lost when these mutants colonize new a flock that receives BCN-free feed, consequently having little effect on BCN treatment at pre-slaughter.

APPENDIX

Table 15. Major bacterial strains used in this study (Chapter IV)

Strains	**Description**	**Source and reference**
JL3	NCTC 11168 derivative, *cmeB::kan*	(Lin et al. 2002)
JL106	E-760^{r} *C. coli*; clinical strain isolated from	This study
NCTC 11168	Human isolate, genome available	(Parkhill et al. 2000)
JL341	NCTC 11168 derivative, E-760^{r} mutant generated by transformation using genomic	This study
K58	NCTC 11168 derivative, E-760^{r} mutant selected from *in vivo* experiment	This study

Table 16. E-760 MICs of the *in vivo*-selected E-760^{r} *C. jejuni* isolates and their isogenic *cmeB* mutants

Strain	**E-760 MIC (µg/ml)**[a]	**E-760 MIC of isogenic *cmeB* mutant(µg/ml)**
C. jejuni NCTC 11168	0.5	0.125
E-760^{r} mutants selected *in vivo*	2 (3)	0.125
	4 (4)	0.125
	8 (10)	0.125

[a] Numbers in parentheses indicate the total number of isolates corresponding to each MIC.

Table 17. Percentage of chickens colonized with *Campylobacter* spp

Strains	**Day 6 (%)**	**Day 22 (%)**	**Day 42 (%)**
NCTC 11168	100	100	100
JL341	100	100	60
K58	100	100	60
JL106	100	30	20

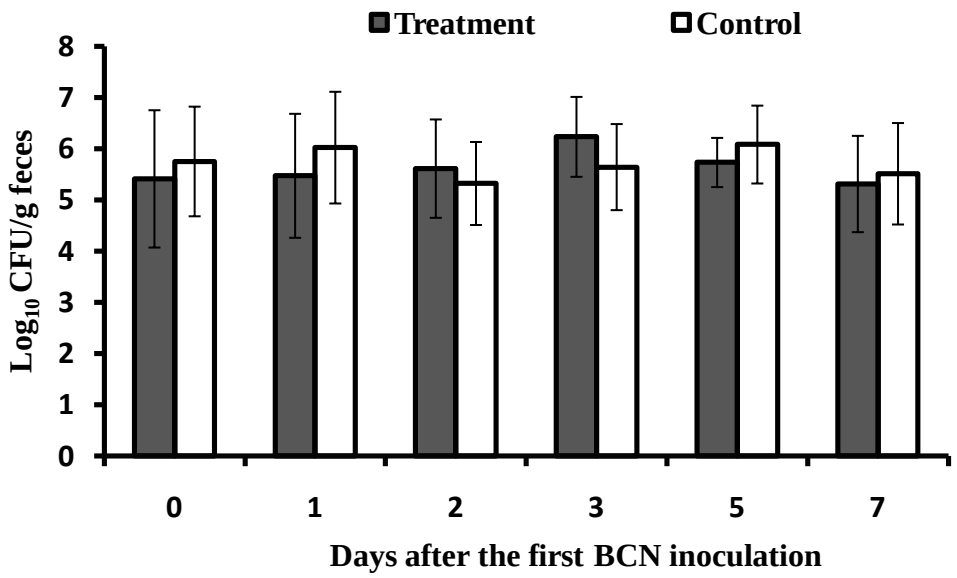

Figure 3. Shedding levels of *C. jejuni* NCTC 11168 in chickens orally gavaged with water (control) or BCN E-760 (treatment). In the treatment group, all birds were treated with BCN E-760 at 5 mg/kg body weight/day via oral gavage for three consecutive days. Each bar represents the mean $\log_{10}$ CFU/gram feces ± standard deviation in each chicken group.

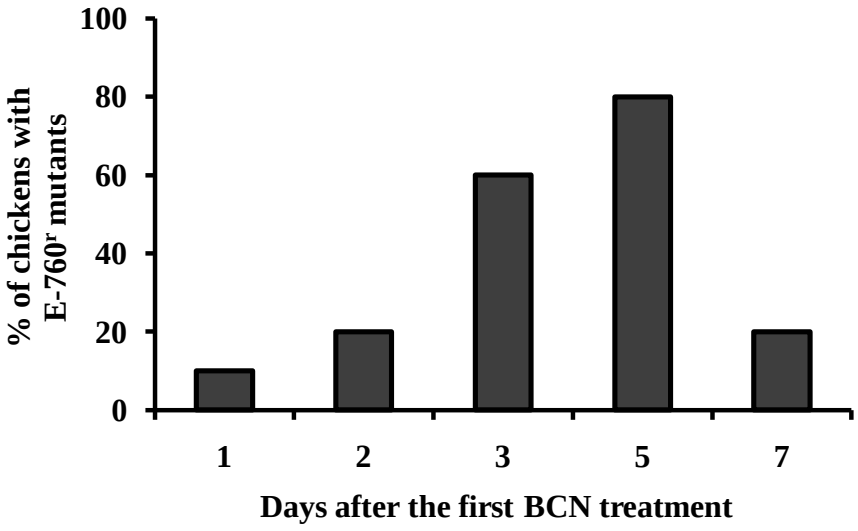

Figure 4. Percentage of chicken colonized with E-760 mutants following E-760 treatment. Each bar represents the percentage of chickens that shed E-760 resistant mutants at each sampling point (n = 10).

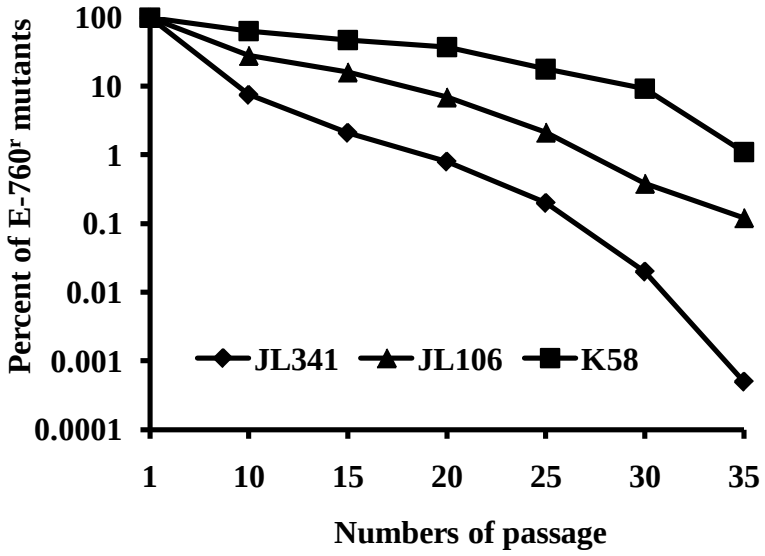

Figure 5. Stability of E-760 resistance *in vitro*. Three strains were passed in MH broth without E-760 selection pressure as described in Materials and Methods. The percentage of BCN^r population was calculated based on differential plating using plates with or without 16 μg/ml of E-760.

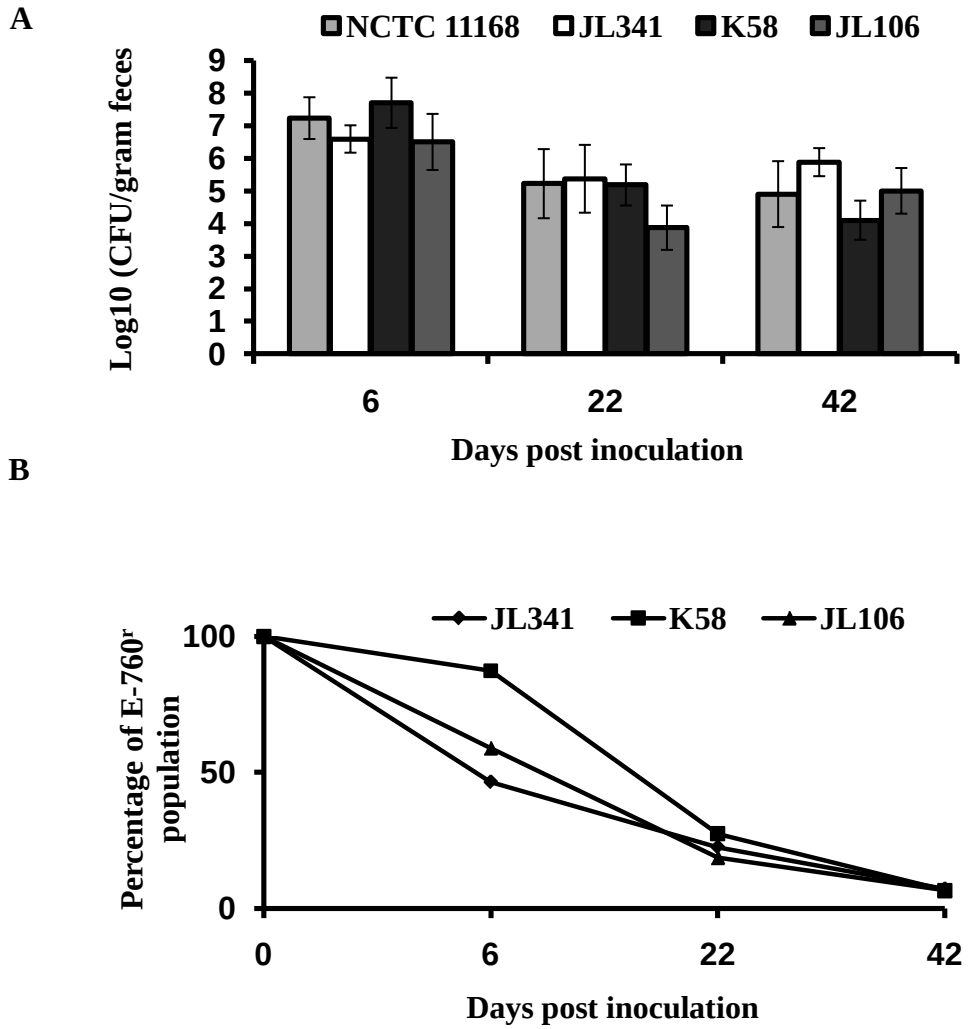

Figure 6. Stability of BCN resistance *in vivo*. (A) Shedding levels of BCN resistant *Campylobacter* strains in chickens colonized with *Campylobacter*. Chickens in each group (10 birds/group) were inoculated with BCN sensitive strain NCTC 11168, or one of E-760 resistant mutants (JL341, K58, or JL106). All chickens received nonmedicated feed throughout the study. Each bar represents the mean of $\log_{10}$ CFU/gram feces ± standard deviation of *Campylobacter* colonized chickens in each group; (B) The E-760 resistance was not stable *in vivo*. The percentage of BCN^r population was calculated based on differential plating using plates with or without 16 μg/ml of E-760.

CHAPTER V

IDENTIFICATION AND CHARACTERIZATION OF GENES REQUIRED FOR *CAMPYLOBACTER* RESISTANCE TO FOWLICIDIN-1, A CHICKEN HOST DEFENSE PEPTIDE

ABSTRACT

Antimicrobial peptides (AMPs) are critical components of host defense limiting bacterial infections at the gastrointestinal mucosal surface. Bacterial pathogens have co-evolved with host innate immunity and developed means to counteract the effect of endogenous AMPs. However, AMP resistance mechanisms are still unknown in *C. jejuni*, an important human foodborne pathogen with poultry as a major reservoir. In this study, random transposon mutagenesis and targeted site-directed mutagenesis approaches were used to identify genes contributing *Campylobacter* resistance to fowlicidin-1, a representative AMP in chickens. In addition, a chicken experiment was performed to determine the role of candidate genes in *Campylobacter* colonization in the intestine. An efficient transposon mutagenesis approach (EZ::TN^{TM} <KAN-2> Transposome) in conjunction with microtiter plate screening identified three mutants whose susceptibilities to fowlicidin-1 were significantly increased. Backcrossing of the transposon mutations into parent strain confirmed that the AMP-sensitive phenotype in each mutant was associated with the specific transposon insertion. Direct sequencing showed that these mutants have a transposon inserted in the genes encoding the two-component regulator CbrR, transporter CjaB, and a putative trigger factor (Tig). Based on the analysis of 12 *Campylobacter* genomes, a conserved gene *cj1583c* displayed high homology (up to 46% similarity at the amino acid level) to *sap*, an AMP resistance gene identified in other pathogens. Insertional inactivation of Cj1583c also significantly increased susceptibility of *Campylobacter* to fowlicidin-1 in diverse strain backgrounds. In vivo administration of CbrR, Tig, and SapB mutants showed a reduction in *in vivo* colonization. Together,

these results have defined four *C. jejuni* genetic loci that will be useful for characterizing the molecular basis of *Campylobacter* resistance to AMPs, a significant knowledge gap in *Campylobacter* pathogenesis.

INTRODUCTION

Campylobacter is a major causative agent of human bacterial gastrointestinal diseases worldwide (Allos 2001). *Campylobacter* is commonly found as a commensal organism in the digestive tracts of a variety of wild and domestic animals. Commercial poultry are considered the major reservoir for human campylobacteriosis (Moran et al. 2009). Most *Campylobacter* infections are epidemiologically associated with consumption of undercooked poultry meats; the remaining cases are due to other risk factors including contaminated milk, water, and direct contact with infected pets (Lin 2009). To successfully colonize and persist in intestinal tracts of many animals and humans, *Campylobacter* must have evolved a variety of mechanisms to counteract and adapt to harsh conditions. Examination of how this important human pathogen adapts to and evades host innate immunity may reveal targets for developing novel vaccine and therapeutics to prevent and control *Campylobacte*r infections in humans and animal reservoirs.

Endogenous antimicrobial peptides (AMPs) belong to the most ancient and efficient components of host defense. Defensins and cathelicidins are two major groups of host AMPs that limit bacterial infections at the gastrointestinal mucosal surface (Bulet et al. 2004; Wehkamp et al. 2007). Epithelial AMPs, such as β-defensins, have been

observed to be induced in human intestinal epithelia upon infection by *C. jejuni* (Zilbauer et al. 2005; Wehkamp et al. 2007). In addition to their significant role in host innate defense, AMPs including chicken cathelicidin fowlicidin-1 (F-1) have been increasingly recognized as a novel class of antibiotics (peptide antibiotics) to control pathogens (Xiao et al. 2006; Bommineni et al. 2007). It is not surprising that many bacterial pathogens have co-evolved with the host and developed means to counteract the effect of AMPs (Yeaman et al. 2003; Peschel et al. 2006). Given the wide prevalence and successful colonization of *C. jejuni* in intestinal tracts, *C. jejuni* should have acquired mechanisms to resist AMPs widely present in the host. However, as an important strategy to evade killing by innate immunity and by potential peptide antibiotics, AMP resistance mechanisms are still largely unknown in *C. jejuni*. Availability of this information will not only help us to develop more sustainable peptide antibiotics but also provide insights into the delicate host-pathogen interactions and reveal novel intervention targets to control *Campylobacter* infections in humans and animal reservoirs.

In this study, using chicken host defense peptide F-1 as a model peptide in conjunction with random transposon mutagenesis as well as targeted mutagenesis, we identified four genetic loci required for F-1 resistance in *Campylobacter*, which include a two-component regulator CbrR, transporter CjaB, a putative trigger factor Tig, and Sap that is homologous to the previously described transporter conferring resistance to AMPs in other Gram-negative bacteria. Administration *in vivo* also demonstrated that these genes play an important role in the colonization of *Campylobacter* in the intestine.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. The major bacterial strains and plasmids used in this study are listed in Table 18. Among 174 *Campylobacter* isolates used for testing susceptibility to chicken AMP F-1, 154 isolates were *C. jejuni* and 20 isolates were *C. coli*. These *Campylobacter* strains were isolated from different hosts and geographically diverse locations. *Campylobacter* strains were routinely grown in MH broth (Difco) or MH agar at 42^0C under microaerophilic conditions generated by using a CampyGen Plus gas pack in an enclosed jar or by using a tri-gas incubator. *E. coli* strains were grown in Luria-Bertani (LB) broth with shaking (250 rpm) or on agar at 37ºC overnight. Antibiotics Kanamycin (Kan) or Chloramphenicol (Cm) was added in medium at a desired concentration when needed.

AMP susceptibility testing. The susceptibilities of *C. jejuni* and *C. coli* isolates to the chicken cathelicidin F-1 were determined by a standard microtiter broth dilution method with an inocula of 10^6 bacterial cells/ml as described previously (Lin et al. 2002). Minimum inhibitory concentrations (MICs) were determined by the lowest concentration of specific antimicrobial showing complete inhibition of bacterial growth after two days of incubation at 42^0C. Chicken cathelicidin F-1A (F-1) (22 amino acids in length with > 95% purity) was synthesized by Bio-Synthesis (Lewisville, TX).

in vitro* selection of F-1 resistant *C. jejuni. F-1 was used as the selective agent to obtain spontaneous F-1 resistant mutants *in vitro*. Briefly, 0.1 ml of *Campylobacter* cells (OD ~1.2) harvested from MH agar plates incubated overnight were spread on MH agar

plates supplemented with different concentrations of F-1 (32 and 64 μg/ml). To increase the chance to get F-1 resistant mutants, the *C. jejuni* cells were also grown and passed five times (2-days for one passage) in MH broth containing sub-lethal concentration of F-1 (4 μg/ml). After five passages, the bacterial cells then were spread on MH plates containing a higher concentration of F-1 (64 μg/ml). F-1 resistant mutants were selected from the plates after 4 days of incubation at 42^0C under microaerophilic conditions. All these selected mutants were grown in MH broth and subjected to MIC test together with the parent strain *C. jejuni* 81-176.

Random transposon mutagenesis. *C. jejuni* 81-176 (JL28 in Table 18) was subjected to *in vivo* transposon mutagenesis using EZ::Tn5™<KAN-2> Transposome (Epicentre) as detailed in our previous publication (Lin et al. 2009). Briefly, one microliter EZ-Tn5 <KAN-2> TnP transposome complex containing 25 ng transposon was used to electroporate *C. jejuni* JL28 competent cells. The Kan^r transformants were individually picked and inoculated in 96-well microplates. Following 2 days of incubation, cultures of mutants were replicated into microtiter plates containing 4 μg/ml of F-1 (2-fold reduction of MIC of the parent strain). Those mutants that could not grow in F-1-containing media were selected from the initial plates and subjected to a second screening to confirm increased sensitivities of the mutants to F-1. To confirm specific genetic linkage between the transposon insertion and increased BCN susceptibility of each mutant, backcrossing of the transposon mutations into the parent strain was performed using natural transformation (Wang et al. 1990). The MICs of F-1 for the backcrossed mutants were determined together with parent strain *C. jejuni* JL28. The

specific transposon insertion site of each mutant was determined by directly sequencing the genomic DNA (Lin et al. 2009). Sequence analysis was performed using DNAStar software package.

Targeted mutagenesis of *C. jejuni* and complementation *in trans*. An isogenic *Cj1583c* (designated as *sapB*) mutant of *C. jejuni* NCTC 11168 was constructed by insertional mutagenesis according to our previous publication (Lin et al. 2005). Briefly, approximately 2-kb of the complete Cj1583c was PCR amplified from *C. jejuni* JL241 genomic DNA using primer pairs of Cj1583c-R and Cj1583c-F (Table 19) and *Taq* DNA polymerase (Promega). The PCR product was cloned into a pGEM-T Easy vector (Promega) to generate pSapB. The chloramphenicol resistance gene cassette (*cm*) was PCR amplified from plasmid pUOA18 (Wang et al. 1990) using *PfuUltra*® High-Fidelity DNA polymerase (Stratagene) and primers of CmF and CmR (Table 19). The resulting blunt-ended PCR product was purified and ligated into pSapB vector, which was digested with *Hind* III prior to ligation, to generate the mutant construct pcmSapB (Table 18). The construct pcmSapB, which serves as suicide vector, was then introduced into *C. jejuni* JL241 and JL242 by biphasic natural transformation or electroporation (Wang et al. 1990; Lin et al. 2009). The putative *sapB* isogenic mutants of corresponding strains were selected on MH agar containing 5 μg/ml of Cm. The inactivation of putative *sapB* (Cj1583c) in mutant JL697 was confirmed by PCR (data not shown).

To complement *cjaB* mutation in JL657, a 2.7-kb fragment including 174 bp upstream of *cjaA* covering promoter region to 193 bp downstream of *cjaB* stop codon was PCR amplified from NCTC11168 using primer pairs of CjaAB-F and CjaAB-R

(Table 19). The PCR was performed using *pfu* DNA polymerase (Stratagene) and the blunt-ended PCR product was purified and ligated to shuttle vector pRY111 (Yao et al. 1993), which was digested with *SmaI* prior to ligation. The ligation mix was introduced into DH5α by transformation. One transformant (JL690) with a plasmid bearing an intact *cjaAB* operon was created. The pCjaAB from JL690 was then transferred to JL657, a *cjaB* isogenic mutant, by tri-parental conjugation using DH5α/pRK2013 as a helper strain (Lin et al. 2005). A similar approach was used to complement *tig* and *cbrR* mutations using primer pairs of Td-F/Td-R and Cj0643-F/Cj0643-R (Table 19), respectively. The insertion of *cjaAB, tig,* or *cbrR* sequence in shuttle vector pRY111 was confirmed by sequencing using specific primers.

Prevalence of *sap* genes among *Campylobacter* species. To examine the prevalence of *sapA* and *sapB* in *C. jejuni* and *C. coli*, *sapA* and *sapB* sequences of 12 *Campylobacter* strains in *Campylobacter* a data bank (*CampyDB*, http://xbase.bham.ac.uk/campydb) were obtained and aligned using the *Clustalw* program. The conserved domains of *sapA* and *sapB* were used to design specific primers for PCR amplification of *sapA* and *sapB*, respectively (Table 19**)**. The primers were used to examine the prevalence of *sapA* and *sapB* in nineteen *Campylobacter* strains including fifteen *C. jejuni* strains and four *C. coli* strains.

F-1 killing assay. The F-1 killing assay was performed in a 96-well plate as described previously (Mount et al. 2010) with minor modifications. Briefly, *Campylobacter* strains were grown in MH broth to mid-log phase, and the cells were washed with MH broth and diluted to approximately 10^6 CFU/ml in MH broth. A volume

of 180 µl of the diluted cells was mixed with 20 µl MH broth (control) or F-1 stock (80 µg/ml) (treatment). The plates were incubated under microaerophilic conditions at 42^0C for 2 hours. After 2-hour incubation, 20 µl of bacterial culture were taken and serially diluted in MH broth and plated onto MH agar plates. The number of CFU was enumerated after 2 days of incubation under microaerophilic conditions at 42^0C. Percentage survival was calculated by dividing the CFU of bacteria incubated with F-1 relative to those incubated in the presence of MH broth and then multiplied by 100. All assays were done in triplicate and two independent experiments were performed. The significance of differences in susceptibility was determined using the Student's *t*-test.

Chicken colonization experiment. One-day-old broiler chickens were obtained from a commercial hatchery (Hubbard Hatchery, Pikesville, TN). The chickens were negative for *Campylobacter* as determined by culturing cloacal swabs prior to use in this study. These chickens were randomly assigned into three treatment groups (10 or 11 chicks/group). At 4 days of age, each chicken was orally inoculated with a 1:1 mixture of wild type 81-176 (JL242) and its isogenic *cjaB* mutant (group I), *tig* mutant (group II), or *sapB* mutant (group III), with a dose of approximately 10^7CFU of bacteria per chick. For each group, five birds were euthanized and cecal content were collected at 3 and 10 days postinoculation (DPI). The cecal content from each bird were weighed and diluted in MH broth. The cecal suspensions were duplicate plated onto MH agar plates with *Campylobacter*-specific selective supplements (Oxoid, United Kingdom) for total *Campylobacter* enumeration and onto selective plates supplemented with appropriate antibiotics (30 µg/ml of Kan or 6 µg/ml of Cm) for the specific mutant numbers in each

sample. The plating media were tested prior to use to ensure that they supported the growth of the mutant strains. Notably, before inoculation, the motility of the wild-type and its isogenic mutants were confirmed to be at a comparable level. The number of CFU per gram of cecal contents was calculated for each chicken and was used as an indicator of the colonization level. The detection limit of the plating methods was 100 CFU/g of cecal contents. The bird from which no *Campylobacter* colonies were detected was assigned a conservative value of 99 CFU/g of cecal content for the purpose of calculating means and for statistical analysis. The significant difference in *Campylobacter* colonization levels ($\log_{10}$ transformed CFU) at each sampling point within group was calculated using Student's *t* test. A *P*-value of < 0.01 was considered significant.

RESULTS

Susceptibilities of *Campylobacter* to chicken cathelicidin F-1. As shown in Table 20, the majority of *C. jejuni* and *C. coli* isolates exhibited susceptibility to chicken cathelicidin F-1 with MICs ranging from 4 μg/ml to 8 μg/ml. Only one *C. jejuni* strain showed a slightly higher MIC of 16 μg/ml.

***in vitro* selection of F-1 resistant mutants**. Different *in vitro* selection methods led to the emergence of F-1 resistant mutants on the selective plates. A total of 8 mutants were selected for MIC testing together with the parent strain 81-176. However, after being cultured in F-1 free MH broth, none of these mutants displayed a higher MIC than 81-176 for F-1, indicating all these mutants were false-positive. Despite extensive efforts, no F-1 resistant mutants were selected *in vitro*.

Identification of genetic loci contributing to F-1 resistance by random transposon mutagenesis. A library containing 4,800 Kan^r mutants were generated to screen for increased susceptibility to F-1. Three mutants displaying increased sensitivity to chicken cathelicidin F-1 compared to the parent strain JL28 were identified (Table 21). Backcrossing of the transposon mutations into the parent strain by natural transformation further confirmed that the F-1-sensitive phenotype in each mutant was linked to the gene with a specific transposon insertion. Direct sequencing of the mutant genomic DNA using transposon-specific primers revealed a specific transposon insertion site in each mutant (Table 21). All the transposon insertions occurred in the coding regions of corresponding genes. The orientation of Kan^r cassette within the transposon of each mutant was the same as the corresponding disrupted gene.

F-1 killing assays were further performed to confirm if these three transposon mutants displayed higher susceptibility to F-1 than 81-176. As shown in Fig 8A, inactivation of CbrR, CjaB, or Tig led to increased susceptibility (5 to 12-fold) to F-1 compared with their parent strain 81-176. However, only complementation of CjaB mutant with wild-type CjaB restored F-1 resistance back to the level of parent strain; complementation of CbrR and Tig did not affect F-1 sensitivity of the mutants. In addition, inactivation of CbrR, Tig, or CjaB alone in other strain background (NCTC 11168 and S3B) also led to increased susceptibilities of the mutants to F-1 (Fig 8B).

SapB is required for F-1 resistance in *C. jejuni*. The *sap* genes in *Haemophilus influenza* confer AMP resistance in this bacterium (Mason et al. 2005). A BLAST search of this study identified genes homologous to the *sap* genes identified in *H. influenza* 86-

028NP (Mason et al. 2005), which include periplasmic solute peptide binding protein Cj1584c (25% aa identity to SapA), membrane permease proteins Cj1583c (20% aa identity to SapB), Cj1582c (21% identity to SapC). Cj1581c (25% aa identity to SapD), and Cj1580c (26% aa identity to SapF). However, the *sapZ* identified in *H. influenza* is not located on the *sap* opreron in *Campylobacter*; the SapZ homolog Cj0999c (32% aa identity) was identified in a separate region in *C. jejuni* NCTC 11168 genome. Isogenic SapB mutants were created in *C. jejuni* 81-176 and NCTC 11168. As shown in Fig 8C, inactivation of SapB resulted in increased sensitivity to F-1 (~4-fold) compared with their parent strains.

Two pairs of primers (Sap4-F, Sap4-R) and (Sap5-F, Sap5-F) (Table 19) were used to amplify a conserved fragment of *sapB* and *sapA*, respectively, in 19 *Campylobacter* strains. The PCR survey showed that majority of strains contains *sapB* (17 out of 19) and *sapA* (15 out of 19) genes.

Genomic organization of the genes involved in F-1 resistance. As shown in Fig 7A, *cjaB* operon consists of two genes *cjaA* and downstream *cjaB* (Wyszynska et al. 2006). The *tig* operon contains two genes encoding trigger factor (Tig) which may function as a chaperon facilitating folding of nascent peptides (Martinez-Hackert et al. 2009) and an ATP-dependent Clp protease, proteolytic subunit ClpP which was demonstrated to be involved in stress tolerance and virulence in *Campylobacter* and other bacteria (Cohn et al. 2007; Frees et al. 2007; Ingmer et al. 2009) (Fig 7B). Sequence analysis showed that both trigger factor and ClpP are highly conserved in *Campylobacter* spp (data not shown). The *clpP* is separated from its downstream gene *def* by a 28-bp

intergenic region which is predicted to contain a promoter sequence (*http://www.fruitfly.org/seq_tools/promoter.html*). Thus, *tig* operon probably contains two genes, trigger factor and *clpP*. Similarly, genetic organization of the *cbrR* operon was analyzed and presented in Fig 7C. The *cbrR* operon contains ten genes and the functions of the majority of these genes are not understood. The gene *cbrR* encoding a response regulator was demonstrated to be involved in bile salt resistance and *in vivo* colonization in *Campylobacter* (Raphael et al. 2005). Finally, in Fig 7D**,** putative *sap* operon contains five genes *cj1584c* (*sapA*), *cj1583c* (*sapB*), *cj1582c* (*sapC*), *cj1581c* (*sapD*), and *cj1580c* (*sapF*).

Tig, CjaB, and SapB are required for *Campylobacter* optimal colonization in chickens. Since the identified genes confer resistance to F-1 *in vitro*, it is likely that these genes also contribute to colonization of *Campylobacter* in chicken by mediating resistance to F-1. It has been demonstrated that *cbrR* is required for optimal colonization of *Campylobacter* in chickens (Raphael et al. 2005). Here we examined the role of *cjaB*, *tig* and *sapB* in colonization of *Campylobacter* in chickens. As shown in Fig 9A, B, and C**,** inactivation of *cjaB*, *tig*, or *sapB* greatly impaired the colonization ability of *C. jejuni* 81-176 in chickens. Specifically, when the wild-type and its specific isogenic mutant were coinoculated into a group of chickens, the colonization level of the specific mutant was significantly ($P < 0.01$) lower than that of the corresponding wild-type strain at 10 days post-inoculation (DPI). In particular, by 10 DPI, the specific mutant of *C. jejuni* 81-176 (*tig*$^-$, *cjaB*$^-$, or *sapB*$^-$ mutant) was no longer detected in any of the cecal samples collected from the chickens inoculated with a mixture of JL242 and its isogenic mutant.

Notably, both the wild-type and its isogenic mutants showed comparable growth patterns in MH broth (data not shown).

DISCUSSION

Our comprehensive survey using *Campylobacter* strains demonstrates the potent killing activity of F-1 against *Campylobacter* from various origins and geographically diverse regions. The unsuccessful generation of F-1 resistant *Campylobacter* mutants, using cells grown in MH broth that was free of F-1 or containing sublethal concentrations of F-1 strongly suggest that it is difficult for *Campylobacter* to develop acquired resistance to chicken host defense peptide F-1. Recently, it has been proposed that AMPs exert killing activity via interaction with multiple low-affinity targets. Therefore, conceptually it is very difficult for bacteria to develop resistance to AMPs while high-level resistance to most clinical antibiotics (e.g. fluroquinolone) would be developed quickly due to their specific and high affinity target (Peschel et al. 2006). The findings from this chapter and the BCN studies in chapter III and IV strongly support this theory that bacteria have not developed highly effective mechanisms to resist various AMPs during evolution (Peschel et al. 2006). Consequently, either BCNs or endogenous AMPs are promising antibiotic alternatives (peptide antibiotics) to control bacterial pathogens.

Mechanisms of *Campylobacter* resistance to endogenous defense AMPs are largely unknown. Alteration of LOS in the *Campylobacter* outer membrane led to altered susceptibility to the host antimicrobial peptide cathelicidin (Naito et al. 2010; van Mourik et al. 2010). In *Campylobacter* LOS synthesis, two genes *gnnA* and *gnnB* facilitate the

formation of a 2, 3-diamino-2,3-dideoxy-D-glucose (GlcN3N) disaccharide lipid A backbone when compared with the -1-6-linked D-glucosamine (GlcN) disaccharide observed in *E. coli* lipid A. *van Mourik et al* 2010 demonstrated that inactivation of *gnnA* and *gnnB* resulted in lipid A alternation and consequently increased sensitivity of the mutants to chicken cathelicidin F-1 (van Mourik et al. 2010). Truncation of LOS core in different locations resulted in altered sensitivity to human cathelicidin LL-37 (Naito et al. 2010).

In this study, inactivation of *Campylobacter* bile salt resistance regulator (CbrR) resulted in increased sensitivity to F-1 and reduced colonization ability in chickens (Raphael et al. 2005). However, the *CbrR* complemented strain did not restore resistance back to wild type. Transposon insertion in *cbrR* may yield polar effects on downstream genes. Notably, the gene *Cj0649* encodes a beta-barrel OstA protein, known as LptD which functions in the assembly of LPS in the outer leaflet of the outer membrane in Gram-negative bacteria (Srinivas et al. 2010) and LOS modification in *Campylobacter* altered host cathelicidin susceptibility (Naito et al. 2010; van Mourik et al. 2010). Thus, it is likely that LOS synthesis defect may result in increased susceptibility to F-1.

The gene encoding efflux pump CjaB is widely distributed in *Campylobacter* species and is located downstream of *cjaA*, and likely these two genes are organized in the same operon (Wyszynska et al. 2006). Classification of transporter CjaAB is intriguing at this stage (Wyszynska et al. 2006). In this study, we identified *Campylobacter* transporter CjaB that contributes to F-1 resistance *in vitro* and colonization of *Campylobacter* in chickens. In the chicken digestive tracts,

Campylobacter has to interact with multiple antimicrobials and toxic substances including chicken antimicrobial peptides (cathelicidin known as fowlicidin, and defensins). Using efflux pumps (Lin et al. 2002; Akiba et al. 2006; Lin et al. 2006) to extrude toxic compounds is critical for *Campylobacter* persistence and colonization in chickens. At this point, the fowlicidin concentration in chicken intestine and comprehensive *in vivo* information on substrates for the CjaAB efflux transporter are still unknown. Thus, a defect in the colonization ability of the *Campylobacter* CjaB mutant is likely the result of multiple actions of other unknown toxic compounds in the digestive tract. This speculation needs more studies to identify and characterize the transporter CjaAB and its cognate substrates.

Trigger factor (Tig) was found highly conserved in eubacteria, functioning as a chaperon to interact with newly synthesized polypeptides and assist their folding as they emerge from the ribosome (Rassow et al. 1996). In *Campylobacter*, the gene encoding trigger factor (Tig) is located upstream of *clpP* which has proteolytic activity to degrade misfolded proteins and plays a critical role in protein quality control in bacteria (Frees et al. 2007). Inactivation of Tig by transoposon resulted in increased sensitivity to F-1 in *Campylobacter*. However, complementation of Tig did not restore F-1 resistance back to wild type strain, suggesting the *tig* downstream gene *clpP* may be indeed responsible for F-1 resistance. ClpP mutant resulted in increased sensitivity of *Campylobacter* to stress conditions such as heat and reduced virulence (Cohn et al. 2007). Shauna M. Mc Gillivray (ASM 2010 meeting) demonstrated that a ClpP mutant resulted in increased sensitivity of *Bacillus anthracis* to antimicrobialsl, including human cathelicidin LL-37

(ASM 2010 meeting). This information strongly suggests the importance of the *tig* operon in F-1 resistance and *in vivo* colonization of *Campylobacter* in chickens. It is noteworthy that defective colonization of *Campylobacter* Tig mutant in chickens might not be solely the result of increased susceptibility to F-1, but as a consequence of increased susceptibility to overall stress conditions in the chicken intestine such as temperature, bile salts, and other toxic compounds.

Our random transposon mutagenesis library (4,800 mutants) was not large enough to identify all gene candidates, and thus failed to identify previously known genes contributing to fowlicidin resistance such as relevant LOS genes (Naito et al. 2010; van Mourik et al. 2010). Bacteria have co-evolved resistance to host AMP and resistance to AMP as a combination of multiple components (Peschel et al. 2006). Thus, technical limitation of random transposon mutagenesis capable of selecting the mutants with at least two fold increased sensitivity could not identify all mutants with mildly increased susceptibility (less than two-fold reduction in MIC) to F-1.

Sap transporters that belong to the ABC-type influx transporter have been demonstrated to be required for AMP resistance in various Gram-negative bacteria including *Salmonella enterica* serovar Typhimurium, *Proteus mirabilis*, *Erwinia chrysanthemi*, and *Haemophilus ducreyi* (Parra-Lopez et al. 1993; Lopez-Solanilla et al. 1998; McCoy et al. 2001; Mason et al. 2005; Mount et al. 2010). Inactivation of the Sap transporter resulted in defects in colonization in animal models (Lupp et al. 2002; Mason et al. 2005; Mason et al. 2006; Mount et al. 2010). However, the Sap transporter does not always confer AMP resistance in all bacteria expressing *sap* genes (Lupp et al. 2002).

Thus, Sap transporters may display distinct functionality within bacterial species to comply with the demands of that particular pathogen. In our study, a homolog to SapABCDFZ of non-typeable *Haemophilus influenza* 86-028NP was identified among *Campylobacter* species. Inactivation of SapB resulted in increased susceptibility to F-1 (but not to other AMPs such as bacteriocins OR-7, E-760, magainin and cecropin A, using MIC tests, data not shown) and caused colonization defects in a chicken model system. The gene *sapB* and *sapA* were amplified from a majority of *Campylobacter* species including *C. jejuni* and *C. coli*. These results suggest the specific functionality of Sap transporter in resistance to F-1A and *in vivo* colonization. The mechanisms of how Sap transporters protect the cells from AMP attack are still unknown. It is generally believed that AMPs exert bacterial killing activities by inserting cationic peptides into the cytoplasmic membrane and form channels that result in leakage of cytoplasmic contents (Hancock et al. 2006; Peschel et al. 2006). Because Sap transporters function as influx pump, it is likely that Sap transporters reduce AMP localization in the periplasmic space by pumping these AMPs into bacterial cytosol and consequently subjecting them to enzymatic degradation. More studies to compare the fate of AMP in wild type and different *sap* isogenic mutants could shed light on mechanisms of Sap-mediated resistance to different AMPs in *Campylobacter*. We are currently examining functions of each individual *sap* gene.

In this study, using F-1 as a model peptide to study chicken AMP resistance in *Campylobacter*, four genes were identified contributing to F-1 resistance. However, at this time, there are at least eighteen chicken AMPs (fourteen defensins and four

cathelicidins) that have been identified and characterized (Xiao et al. 2006; Lynn et al. 2007). Thus, more studies are needed to test if these identified genes are also required for resistance to other chicken AMPs such as defensins. Taken together, our study provided comprehensive information on mechanisms of F-1 resistance in *Campylobacter*. *Campylobacter* has utilized various strategies in conferring resistance to chicken host antimicrobial peptide F-1 to persist and colonize in chickens. Although our results did not pinpoint the exact mechanisms which *Campylobacter* utilizes to resist killing by F-1, the information in this study provides a solid foundation to further characterize the mechanisms of F-1 resistance in *Campylobacter* that will help us better understand *Campylobacter*-chicken host interaction and facilitate development of effective measures to reduce *Campylobacter* loads in chickens.

APPENDIX

Table 18. Major bacterial strains and plasmids used in this study (Chapter V)

Strains or plasmids	Description	Source and reference
C. jejuni		
JL241	NCTC 11168, human isolate	(Parkhill et al. 2000)
JL28	81-176, human isolate, unable to colonize in chicken	(Black et al. 1988)
JL242	81-176, human isolate, can colonize in chickens	(Black et al. 1988)
JL599	JL28 derivative, *cbrR*::*kan*	This study
JL628	JL599/pCbrR	This study
JL623	JL241 derivative, *cbrR*::*kan*	This study
JL656	JL242 derivative, *cbrR*::*kan*	This study
JL601	JL28 derivative, *cjaB*::*kan*	This study
JL665	JL241derivative, *cjaB*::*kan*	This study
JL657	JL242 derivative, *cjaB*::*kan*	This study
JL602	JL28 derivative, *tig*::*kan*	This study
JL629	JL241derivative, *tig*::*kan*	This study
JL658	JL242 derivative, *tig*::*kan*	This study
JL668	JL656/pCbrR	This study
JL694	JL657/pCjaAB	This study
JL695	JL658/pTig	This study
JL624	*C. jejuni* S3B derivative, *cbrR::kan*	This study
JL631	*C. jejuni* S3B derivative, *tig::kan*	This study
JL697	JL242 derivative, *cj1583c*::*cm*	This study
JL706	JL241 derivative, *cj1583c*::*cm*	This study
Plasmids		
pRY111	*E.coli-C.jejuni* shuttle vector, Cm^r	(Yao et al. 1993)
pCbrR	pRY111 containing *cbrR*	This study
pTig	pRY111 containing *tig*	This study
pCjaAB	pRY111 containing *cjaAB*	This study
pGEM-T Easy	Cloning vector, Amp^r	Promega
pSapB	pGEM-T Easy containing *sapB*, Amp^r	This study
pcmSapB	pSapB with *cm* inserted *sapB*	This study
E.coli		
$DH5_\alpha$	F^- ϕ80*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_k^-, m_k^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Invitrogen
JL690	DH5α containing pCjaAB operon	This study
JL691	DH5α containing pTig	This study
JL652	DH5α containing pCbrR	This study
JL692	DH5α containing pGEM-T Easy plus C*j1583c*::*cm*	This study
JL48	Conjugation helper strain, $DH5_\alpha$ containing plasmid RK2013	(Akiba et al. 2006)

Table 19. Key primers used for PCR in this study (Chapter V)

Primer	DNA Sequence (5'-3')[a]	Product size (kb)	Target gene or functional description
Tf-F Tf-R	TCATGAATTTCACCACTTAGCA TGCTATCATTGAAGGCAAATTTTA	1.6	Trigger factor (*tig*)
CjaAB-F CjaAB-R	TCGCCTAATGCCAAAGTTTC TCACCATCTGCATTGCATTTA	2,7	Complete *cjaAB*
Cj0643-F Cj0643-R	GCAATGCGTATCAACAATCC AAAAATTTCCTTTCTTTTGAAAAC	1.5	*cbrR*
Cj1583c-F Cj1583c-R	AAAAAGCCGAGGATTTGCTT CTGTGGCTATAGCATGAACGA	2,0	*Cj1583c*
CmF CmR	CGATTTAAATGCTCGGCGGTGTTCCTTT CGATTTAAATGCGCCCTTTAGTTCCTAAAG	0.8	cm^r cassette
Sap4-F Sap4-R	GTG CTA AAA CGCTTA GTTTTTAGTATT AATCAAATGCTCTAAACGATTTAA AAA	0.6	Conserved *sapB*
Sap5-F Sap5-R	GATGCAGTG ATTAATCTTGTATTT TCAGG TCCATTTTACAAATTTATAAGGACCTG	0.5	Conserved *sapA*

Table 20. Susceptibilities of diverse *Campylobacter* spp. to F-1

Species	**Number of isolates with MIC of F-1 (µg/ml)**						**Total**
	0.5	1	2	4	8	16	174
C. jejuni	0	2	8	58	85	1	154
C. coli	1	3	3	8	5	0	20

Table 21. Mutants with increased sensitivity to F-1

Strains	**MICs of F-1 (µg/ml)**	**Insertion site/ORF size**	***Cj* number NCTC11168**	**Functions or description**
JL28	8	N/A		
JL602	4	101/1335	*Cj0193c*	Trigger factor (Tig)
JL599	4	910/1245	*Cj0643*	Response regulator (CbrR)
JL601	4	258/1248	*Cj0981c*	Putative transporter (CjaB)

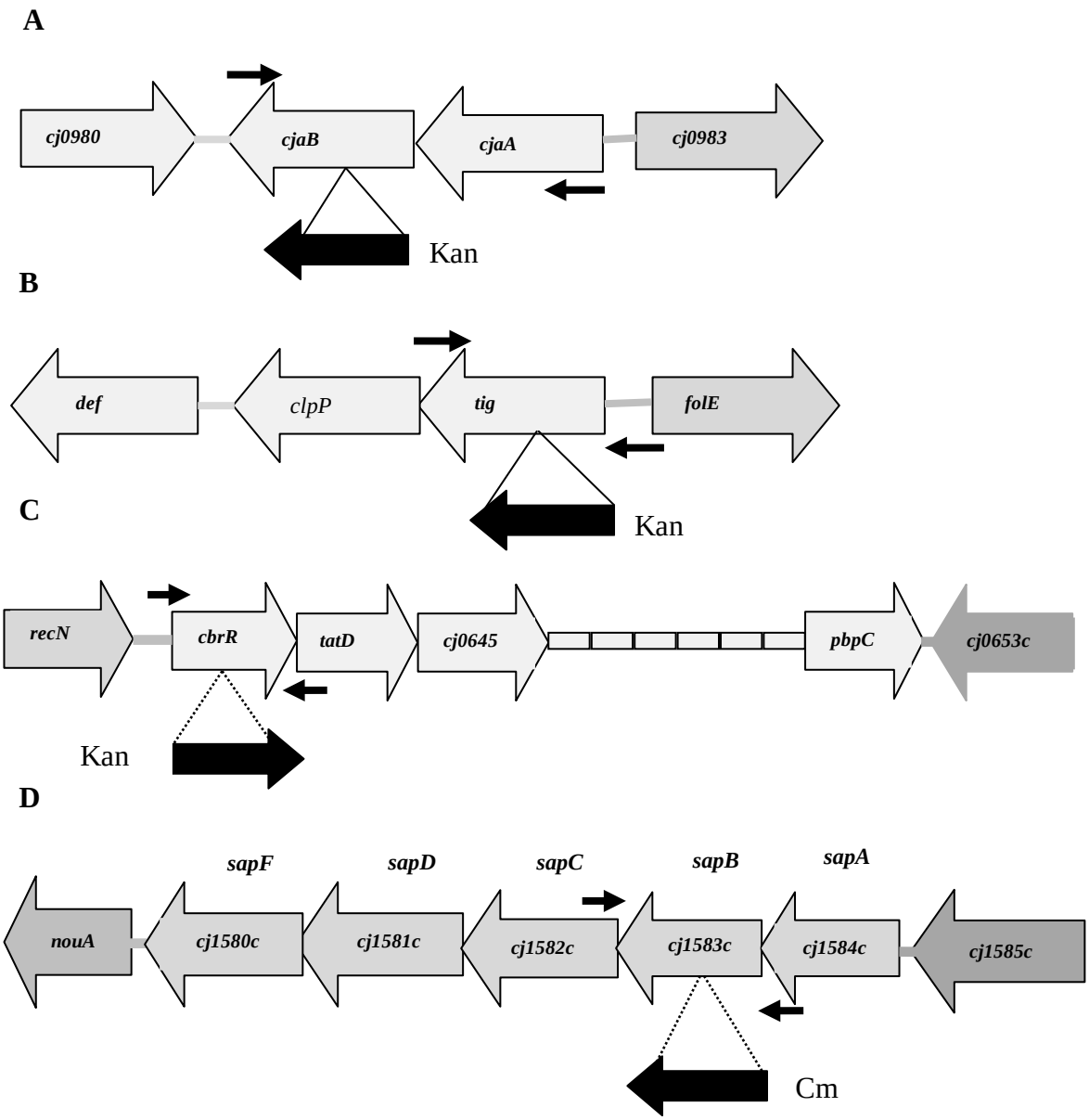

Figure 7. Genomic context of *cjaB* (A), *tig* (B), *cbrR* (C), and *cj1583c* (D). The locations of various major primers used in this study are indicated by arrows. The location and orientation of antibiotic resistance cassette are indicated below each candidate gene

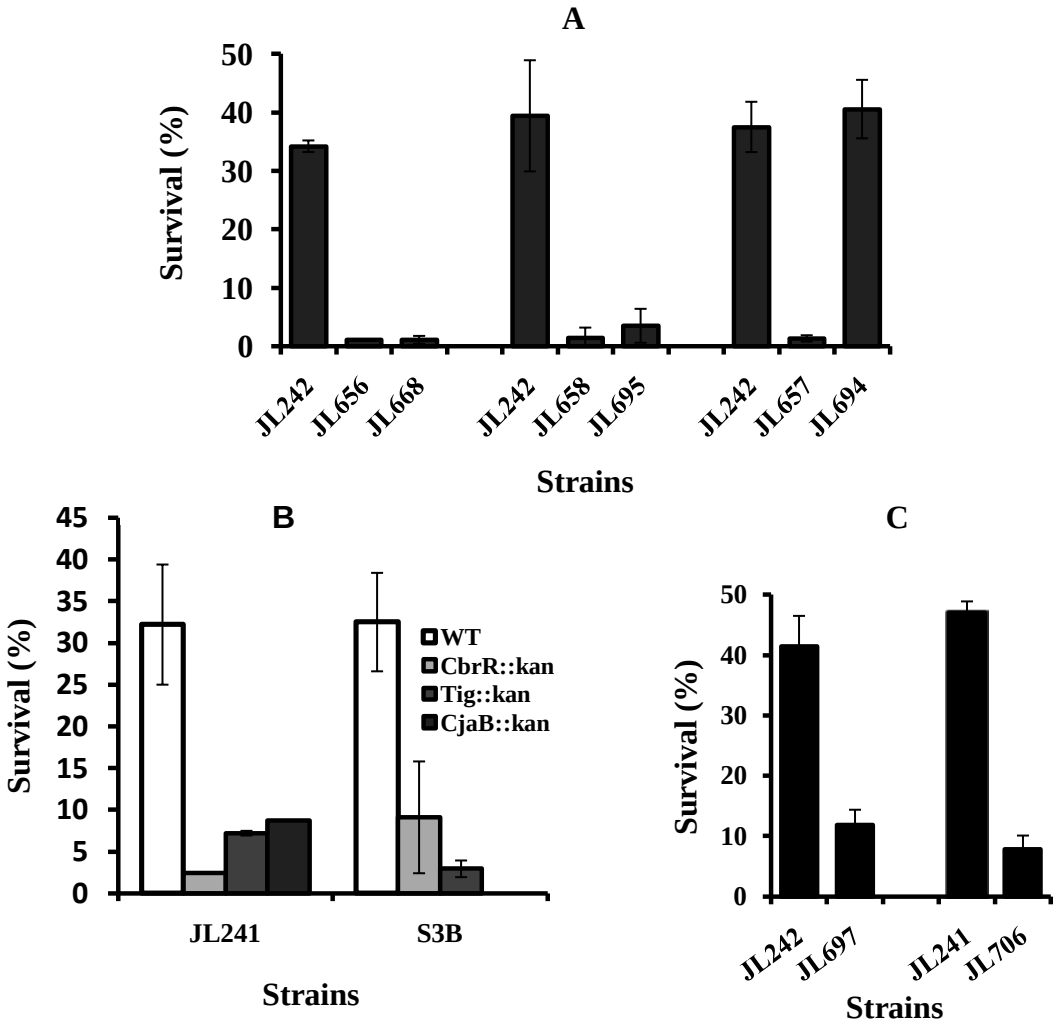

Figure 8. Role of *cbrR*, *tig*, *cjaB* and *sapB* in F-1 resistance in different *C. jejuni* strains. The F-1 was added to washed bacteria and incubated for 2 hrs at 42°C under microaerophic condition. Survival values are relative to original inoculumn (details of bacterial strains in Table 18). (**A**) Susceptibility of wild-type 81-176 (WT), its *cbrR* (JL656), *tig* (JL658) and *cjaB* (JL657) derivative mutants, and their corresponding complementation construct JL668, JL695, and JL694 respectively to F-1. (**B**) Effects of mutations in *cbrR*, *tig*, and *cjaB* on the susceptibility of NCTC 11168 and S3B to F-1. (**C**) Inactivation of SapB increased susceptibility of 81-176 and NCTC 11168 to F-1. JL697 and JL706 are isogenic SapB mutants of 81-176 and NCTC 11168 respectively. Each data represents the mean value obtained from two independent experiments with triplicate measurements in each independent experiment.

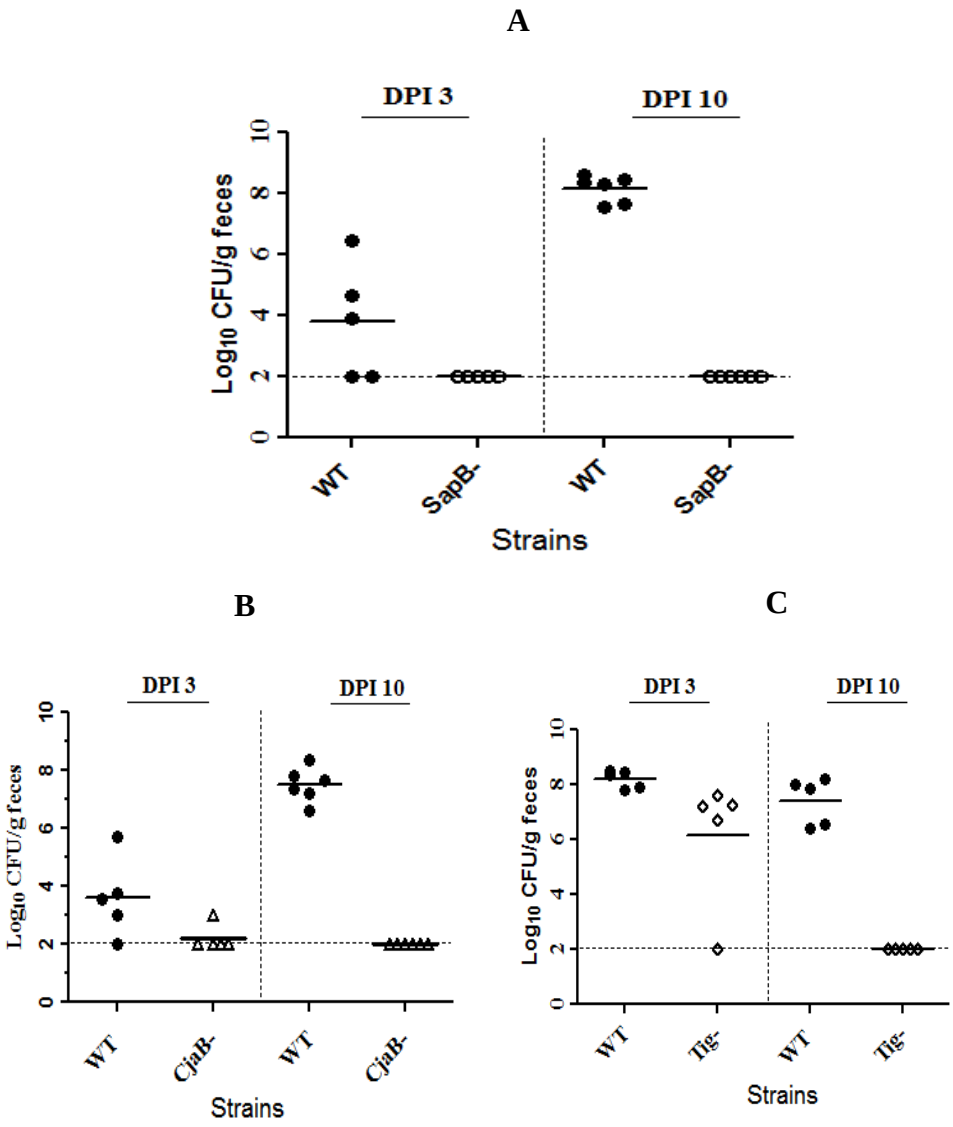

Figure 9. Inactivation of Tig, CjaB, and SapB reduced *C. jejuni* 81-176 colonization of chickens. Three chicken groups (10 or 11 birds/group) were inoculated with a 1:1 mixture of wild type 81-176 and its SapB isogenic mutant (A), CjaB isogenic mutant (B), or Tig isogenic mutant (C). For each group, five to six birds were euthanized and the cecal contents were collected and used for CFU enumeration at the indicated day postinoculation (DPI). Each symbol indicates the $\log_{10}$ number of CFU/g of cecal contents for a single chicken. The horizontal bars indicate the means of groups at the indicated times. Details were described in Materials and Methods.

CHAPTER VI

CONCLUSIONS

Campylobacter, commonly found as a commensal organism in the digestive tracts of a variety of wild and domestic animals, is a major causative agent of human bacterial gastrointestinal diseases in developed countries worldwide (Ruiz-Palacios 2007). Poultry are the major reservoir for human *Campylobacter* infections (Kassenborg et al. 2004). Thus, reduction of *Campylobacter* load in poultry is significant to food safety and public health. In particular, at the same time that prevalence of infection is increasing, *Campylobacter* has become increasingly resistant to antibiotics, including fluoroquinolones and macrolides, the major drugs of choice for treating human campylobacteriosis (Luo et al. 2005), which raises an urgent need for novel intervention strategies to prevent and control *Campylobacter* colonization in poultry (Lin 2009). Antimicrobial peptides (AMPs) including bacteriocins (BCNs) are major components of host innate immune systems as well as a novel class of antibiotics to control food-borne pathogens. Recently, several anti-*Campylobacter* BCNs and chicken host antimicrobial peptide (AMPs) such as fowlicidin-1 have been proposed to control *Campylobacter* in poultry (Stern et al. 2005; Svetoch et al. 2005; Stern et al. 2006; Xiao et al. 2006). Resistance mechanisms of this pathogen to these AMPs are critical to understand, however, largely unknown. In this dissertation research, resistance mechanisms of *Campylobacter* to PM, BCNs, and fowlicidin-1 were studied comprehensively.

Polymyxin B (PM) has been successfully used as a model to study resistant mechanisms of Gram-negative bacteria to host defense AMPs (Ernst et al. 2001; Finlay et al. 2004). Inactivation of the genes responsible for PM resistance usually results in increased susceptibility of bacteria to a variety of innate AMPs, leading to reduce

virulence of the mutants in an animal model system (Ernst et al. 2001). Functional genomic studies described in Chapter II successfully identified several genes involved in PM resistance in *Campylobacter*. However, these identified genes did not contribute to *Campylobacter* resistance to other AMPs such as anti-*Campylobacter* BCNs and chicken host defense peptides (F-1, 2). Thus, PM is not an ideal surrogate to study *Campylobacter* resistance to physiologically relevant AMPs.

Resistance to the promising anti-*Campylobacter* BCNs was comprehensively examined in this dissertation research. Studies in Chapter III and IV demonstrated that *Campylobacter* only developed low-level BCN resistance with low frequency *in vitro* and *in vivo*. The acquired BCN resistance can be transferred at both inter- and intra-species levels. However, BCN resistance is not stable in *Campylobacter*. Genomic examination of two BCN resistant mutants using microarray, random transposon mutagenesis, and other functional approaches, revealed that the multidrug efflux pump CmeABC contributed to both intrinsic and acquired resistance to BCN (Chapter III). The findings from these two chapters support the feasibility of BCN in controlling *Campylobacter* in poultry at the production level, and provide key information for the development of effective and sustainable BCNs against *Campylobacter* infections.

Campylobacter resistance to F-1, a representative for chicken endogenous AMPs was examined in Chapter V. The study in this chapter revealed that *Campylobacter* spp were highly sensitive to F-1 and it was very difficult for *Campylobacter* to develop resistance to F-1 *in vitro*. Comprehensive examination using random transposon mutagenesis, homolog blast search, and site-targeted mutagenesis identified four genes

(*cbrR*, *cjaB*, *tig*, and *cj1385c*) involved in resistance to F-1. These genes were also required for optimal colonization of *Campylobacter* in chickens. The findings from Chapter V will not only help us to develop more sustainable peptide antibiotics but also provide insights into the delicate host-pathogen interactions and reveal novel intervention targets to control *Campylobacter* infections in humans and animal reservoirs. Together, the findings from this dissertation have revealed uniqueness and complexity of AMP resistance in *Campylobacter*.

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VITA

Ky Van Hoang, the author of this dissertation, was born in Vietnam. He received DVM degree from the Department of Veterinary Medicine at Ha Noi Agricultural University, Vietnam in 2000. Following graduation he involved in teaching activities at the same University and Hue University, Hue City, Vietnam. In the Fall of 2006, he received graduate assistantship for doctoral program from the Department of Animal Science, the University of Tennesee, working under Dr. Jun Lin's instruction. His dissertaion was accomplished in the Fall 2010.