Regulation and function of a cluster of lipoproteins in Campylobacter jejuni

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Regulation and function of a cluster of lipoproteins in *Campylobacter jejuni*

by

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ABSTRACT

Membrane-associated lipoproteins have important functions in bacterial pathogenesis and physiology. According to the available genomic sequences, *C. jejuni* possesses multiple lipoproteins, but the majority of them have not yet been functionally characterized. To better understand the pathogenic mechanisms of *C. jejuni*, in this work we characterized a three-gene operon (*cj0089, cj0090, and cj0091*) encoding a cluster of lipoproteins. It was shown that this lipoprotein-encoding operon is indirectly regulated by CmeR, a transcriptional regulator repressing the expression of the multidrug efflux pump CmeABC in *C. jejuni*. We determined that Cj0091 is an outer membrane associated protein, identified it as a new adhesin in *C. jejuni*, and demonstrated that this protein is involved in *Campylobacter* colonization *in vivo*. This study suggests that Cj0091 may be a promising candidate for the development of vaccines to reduce *Campylobacter* colonization in animal hosts.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

_Campylobacter jejuni_ is a leading cause of acute gastroenteric diseases worldwide. In the United States, it is estimated that 2.4 million cases of human _Campylobacter_ infections occur annually (24). Although _Campylobacter_ enteritis is generally a self-limiting disease in developed countries, it causes significant childhood morbidity and mortality in developing countries. _Campylobacter_ infection can also develop to Guillain-Barré syndrome (GBS), an autoimmune-mediated neurodegenerative complication (71). _Campylobacter_ is also among the most common bacterial causes of diarrhea among travelers returning from developing countries (75). Most of the human _Campylobacter_ infections are caused by consumption of under-cooked chickens (32). In contrast to _Salmonella_ where control in poultry is quite successful, effective intervention strategies remain to be developed for control of _Campylobacter_ infections.

Despite the high incidence of infections caused by _Campylobacter_, virulence factors and mechanisms involved in pathogenesis are still poorly known. A few putative virulence factors have been discovered so far. For example, PEB1 and CadF were identified as adhesion molecules (51, 80). Both are involved in adhesion and invasion of human intestinal epithelial cells. In addition, PEB1 may also be involved in utilization of aspartate and glutamate by binding to these amino acids, which are important sources of carbon and energy in _Campylobacter_ (27). The multidrug efflux pump CmeABC contributes significantly to
*Campylobacter* colonization in the intestinal tract by mediating bile resistance (59). Flagellum, which is one of the best studied virulence factors in *Campylobacter*, is involved in motility, chemotaxis, invasion, and *in vivo* colonization. *Campylobacter* seems to produce a variety of toxic activities, but cytolethal distending toxin (CDT) is the only toxin identified in *Campylobacter* so far (24). Lipooligosaccharide (LOS) is involved in the interaction with host cells and functions as an endotoxin, which induces host intestinal inflammatory responses (39, 67, 100). In addition, LOS mimics human gangliosides, which is considered a key factor in the development of GBS (6, 54). Recently, two lipoproteins, JlpA and CapA, have been characterized and they appear to be involved in adhesion of *Campylobacter* to host epithelial cells (5, 42).

Bacterial lipoproteins have diverse functions in physiology and pathogenesis (8, 44). Based on the genomic sequence of *C. jejuni* NCTC 11168, it is predicted that *C. jejuni* possess twenty two lipoproteins (8). So far, only two of them, JlpA and CapA, have been functionally characterized (5, 42, 43). A previous work conducted by our laboratory revealed that several lipoprotein-encoding genes including *cj0089* and *cj0091* were down-regulated in the *cmeR* mutant background, suggesting that CmeR, a transcriptional repressor for the multidrug efflux pump CmeABC, is a potential activator for the lipoprotein genes. *cj0089* and *cj0091* along with *cj0090* are tandemly positioned on the *Campylobacter* chromosome and likely form an operon. However, the role of this cluster of lipoproteins in *Campylobacter* pathophysiology is unknown. In this project, we characterized the regulation of the lipoprotein operon and examined the function of Cj0089 and Cj0091 in *Campylobacter* adherence and colonization using both *in vitro* and *in vivo* experiments. It was found that
Cj0091 functions as an adhesin and contributes to the early-stage colonization of *Campylobacter* in the intestinal tract of chickens. These findings improve our understanding of *Campylobacter* pathogenesis and suggest the possibility that lipoproteins can be used as vaccine candidates for the control of *Campylobacter* colonization.

**Thesis organization**

This thesis consists of three chapters. The first chapter is a literature review including general overview of *Campylobacter* and *Campylobacter* infections; characteristics of bacterial outer membrane proteins and lipoproteins; and mechanisms and virulence factors involved in the adhesion and invasion of *C. jejuni*. The second chapter describes the study characterizing the three-gene operon encoding the lipoprotein cluster. The third chapter is the general conclusion.
Literature review

Microbiology and genomics

_C. jejuni_ is a gram-negative bacterium with a curved or spiral shape. The organism possesses a single, polar, unsheathed flagellum at one or both ends, which allows a characteristics corkscrew-like motion. _C. jejuni_ grows best at 37 ºC to 42 ºC, which are the body temperatures of animals and birds. The optimal growth of the organism requires a microaerophilic atmosphere consisting of 5 % O₂, 10 % CO₂, and 85 % N₂ (98).

Currently the complete genome sequences of three _C. jejuni_ strains are available including NCTC 11168, RM1221, and 81-176. _C. jejuni_ has a circular chromosome with the size of about 1.6 to 1.8 megabases. Approximately 1,600 proteins are encoded in NCTC11168 and 811-76, and 1,800 in RM1221. The G + C contents of all three chromosomes are low, at approximately 30 %. The average length of ORFs is about 900 bp. The genome of _C. jejuni_ is quite dense and over 90 % of the genome codes for proteins (79). Several unique features were found from the genomic sequences of these three strains of _C. jejuni_. The first one is the general lack of insertion sequence (IS) elements, except for a copy of putative IS605 (31, 79). The second important feature is the presence of hypervariable sequences on the chromosomes. Variations in the length of poly G:C tracts, which is due to DNA polymerase slippage during DNA replication, form several hypervariable regions of the genomes. These variable regions are localized in the genes involved in biosynthesis of lipoooligosaccharide (LOS) and capsular polysaccharide (CAP), flagellar modification, and
DNA restriction-modification (RM) (78). *C. jejuni* is speculated to have no intact DNA repair systems since many of the genes important for direct repair, mismatch repair, and SOS response are missing in the genome. Lack of a DNA repair system may contribute to the rapid phase and sequence variations in this organism. In *Campylobacter*, the frequent variations in components of surface structures may play an important role in the adaptation to the host intestinal environments rather than in avoiding host immune responses (79).

The major difference between *C. jejuni* RM1221 and the other two sequenced strains is the presence of four prophages/genomic islands in the genome of RM1221 (31). CJIE1 is a Mu-like phage and is located upstream of *argC* (CJE0275). CJIE1 has genes encoding Mu-like phage protein (68), although their functions are unknown. CJIE2 and CJIE4 are found in the 3’ end of arginyl- and methionyl-tRNA genes, respectively. These elements include genes predicted to encode phage-related endonucleases, methylases, or repressors. Since no major phage structural proteins (capsid, portal and scaffold protease proteins) are found in CJIE2, it is predicted that CJIE2 may be an intact prophage with novel head structures, a satellite phage, nonfunctional prophage, or genomic island. (31). On the other hand, CJIE4 is similar to a putative prophage found in the genome of *C. lari* RM2100, the integrated elements 1 (CLIE1). In CJIE4, a few genes encoding phage structural proteins were identified (31). CJIE3 is integrated into the 3’ end of an arginyl-tRNA, and is predicted to be a genomic island or integrated plasmid rather than a prophage since no phage-related genes are found (31).
The genome of *C. jejuni* 81-176 is syntenic with the genome of *C. jejuni* NCTC 11168 and RM1221 but is disrupted by some duplications, deletion, or insertion of genes. Some of the genes involved in capsule biosynthesis and posttranslational modification of flagellar proteins are missing from *C. jejuni* 81-176 (38). In contrast, *C. jejuni* 81-176 possess additional genes, which are absent or represented as pseudogenes in *C. jejuni* NCTC 11168 and RM1221. These include genes involved in electron transport, energy metabolism, anaerobic respiratory system, and integrated plasmids (38).

The unique feature of *C. jejuni* 81-176 is that it carries two large plasmids, pTet and pVir. The pTet plasmid carries the *tetO* gene, making *C. jejuni* 81-176 tetracycline resistant. The pVir plasmid contains genes encoding orthologs of type IV secretion proteins found in *Helicobacter pylori* (10). Type IV secretion system is usually involved in conjugation and translocation of DNA and proteins (21). It seems that the pVir plasmid is involved in invasion as well as natural transformation in *Campylobacter* (9).

**Clinical manifestation**

Clinical manifestations of *Campylobacter* infections differ between developing and industrialized countries. In developing countries the infection is often asymptomatic or mild, and non-inflammatory, inducing watery diarrhea (24) and mostly affecting children of less than 2 years of age (22). In industrialized countries, the infection of *Campylobacter* can be very severe. The most prominent features include abdominal pain, diarrhea, and fever (3, 75, 92). The degree of diarrhea varies from loose, watery to bloody. The stools may contain fecal
leukocytes and red blood cells (3), and patients may suffer fever, accompanied by headache, dizziness, and myalgia (3). The divergence in the clinical features of *Campylobacter* infections may be due to differences in serotypes and virulence factors, or host immune responses (24, 75).

The mean incubation period of *Campylobacter* enteritis is 3.2 days with a range of 18 h to 8 days (92). The acute diarrhea usually lasts for 2-3 days and abdominal pain persists for another few days (47, 92). Persistence or relapse of illness may occur occasionally (45). *Campylobacter* can be isolated from patients’ feces for several weeks even after the illness has resolved (47, 92). *Campylobacter* enteritis is usually self-limiting and the development of complications is rare. The most significant complication following *Campylobacter* infection is GBS (Guillain-Barré syndrome). Although it was estimated that 30-40 percent of GBS patients had recent infections with *C. jejuni*, the risk of actually developing GBS after *Campylobacter* infections is quite low, approximately 1 GBS case out of 1,000 *Campylobacter* infections (2, 71). GBS is the most frequent cause of acute neuromuscular paralysis (71, 85). The symptoms include weakness of the limbs and the respiratory muscles, areflexia (loss of reflexes), and raised protein concentrations in cerebrospinal fluid (70, 71). GBS is a clinically diverse disorder, which includes both acute inflammatory demyelinating polyneuropathy (AIDP) and acute motor axonal neuropathy (AMAN) (82). Certain *Campylobacter* serotypes are more frequently associated with GBS. In the United States and Japan, Penner serotype O:19 is the most frequently associated strain with GBS (70, 82), while in South Africa O:41 strains seems to be more associated with disease (82). GBS usually occurs 1 – 3 weeks after the infection (70), which indicates that humoral
immunopathogenic mechanisms are involved (3). Several studies have shown that certain O
serotypes of *C. jejuni* have an LOS core structure homologous to a number of different
glycosphingolipids of the gangliosides on the surface of host cells (7, 70, 71, 82, 104, 105).
This molecular mimicry seems to be an important cause of GBS.

**Epidemiology**

*C. jejuni* resides in the intestinal tracts of many wild and domestic animals as a
commensal organism. The organism is especially found in domestic poultry, such as
chickens, turkeys, ducks, ostriches, and wild birds including cranes, geese (63), and seagulls
(36).

The major route of *C. jejuni* infection for poultry is via horizontal transmission from
environmental sources. Some studies have suggested vertical transmission of *C. jejuni* via
eggs may occur, but evidence proving this hypothesis has not been conclusively documented
(13, 89). Potential factors associated with horizontal transmission include wild birds, rodents,
other livestock on or nearby the farms, pets, and insects.

*Campylobacter* is often found in the intestine of wild birds. It is highly possible that
contamination of poultry farm environments by wild-bird feces contributes to the spread of
this organism (89). Indeed, Barrios *et al.* showed a strong association of vertical ventilation
systems in poultry houses with *Campylobacter* infection in poultry. They speculate that the
vertical fans create an environment to attract wild birds by producing heat, which may lead to the falling of their feces to the houses through the fans (13).

The other sources of infection for poultry include clothing and footwear of farmers, transport crates, and vehicles. Some studies have shown the reduced number of *Campylobacter* infection in poultry by applying hygiene measures including disinfection or changing of boots before entering the poultry houses, and cleaning and disinfection of the houses before repopulation and transport crates (41, 95-97). Therefore, farm workers or visitors and farm equipment may also be involved in the spread of *Campylobacter* between poultry and farms (89).

During processing, broiler carcasses are contaminated by fecal materials due to rupture of intestines (23, 87). Because multiple flocks are processed at a given time, poultry flocks that are negative for *Campylobacter* can be contaminated with *Campylobacter* from *Campylobacter*-positive flocks, leading to high prevalence of *Campylobacter* on chicken carcasses.

In humans, the majority of *Campylobacter* infections occur as sporadic cases rather than outbreaks. The most significant source of sporadic *Campylobacter* infections is the consumption of undercooked chickens or mishandling of the birds during food preparation in the kitchen (32). Contact with poultry, other farm animals, pet, and wild birds can contract the infection as well. In addition, consumption of contaminated water and raw, unpasteurized milk has been associated with both sporadic and outbreak cases of *Campylobacter* infection.
Cross-contamination of other foods by raw chicken meats (in kitchens and other processing sites) is also a risk factor for Campylobacteriosis.

**Treatment**

Most cases of *Campylobacter* infection do not require antibiotic treatment unless patients suffer a persistent high fever, bloody diarrhea, or prolonged diarrhea. Antibiotic treatment is often required for patients who are pregnant, HIV infected, or immunocompromised. Erythromycin or fluoroquinolone are used to treat *Campylobacter* infections. *Campylobacter* has developed high-level resistance to fluoroquinolone antibiotics, but the resistance rate to erythromycin is still low (72). Since erythromycin is of low cost and has low toxicity, is acid-resistant, and remains unabsorbed in the gut, it is now considered the first-choice drug for treating *Campylobacter* infections (3, 18).

Fluoroquinolones are often used to treat patients with the symptoms of bacterial gastroenteritis. When the causative agents responsible for the enteric disease are unknown, fluoroquinolones are considered the first choice because many enteric pathogens are susceptible to this class of antibiotic, but intrinsically resistant to macrolide antibiotics (including erythromycin). Since the symptom of gastroenteritis caused by *Campylobacter* and other organisms, such as *Salmonella* and *Shigella*, are undistinguishable, fluoroquinolones can be prescribed for patients in the lack of a diagnosis (3).
However, there is a rapid increase in fluoroquinolones-resistant strains of *Campylobacter* worldwide (3). Fluoroquinolones, including sarafloxacin and enrofloxacin, were used in drinking water to treat bacterial respiratory infections in poultry (93). The increased resistance to fluoroquinolones in human isolates of *Campylobacter* seems to be correlated with the approval of fluoroquinolones for use in food animals (3, 93). In various countries, isolation of fluoroquinolone-resistant *Campylobacter* from retail chicken products has been documented (93). In addition, studies using PCR-based restriction length polymorphism (PCR-FLA) revealed that strains of fluoroquinolone-resistant *C. jejuni* isolated from both human and from chicken products were shown to be the same (93). Use of fluoroquinolones in veterinary medicine is considered a major cause of the increase in infections with fluoroquinolone-resistant strains.

**Prevention**

An ideal way to control *Campylobacter* infection in humans would be to reduce the prevalence of *Campylobacter* in poultry flocks and the level of cross-contamination during carcass processing (3, 4). According to several studies, application of appropriate hygiene measures seems to reduce the contamination of *Campylobacter* in poultry flocks on farms (41, 95-97). These hygiene procedures include proper cleaning and disinfection of poultry houses, equipment, and transport crates, control of insects and rodents entering in the houses, and disinfection or changing of footwear and clothes at the entrance of each poultry houses. However, even the strict measures only delay the onset of *Campylobacter* colonization, or maintain the reduction of *Campylobacter* contamination level only in a short time (74). The
multi-source feature of flock contamination by *Campylobacter* makes it difficult to control *Campylobacter* using hygiene measures.

During processing in slaughterhouses, air chilling and water chilling along with use of chlorinated water reduce *Campylobacter* concentration on the poultry carcasses (87, 94). In addition, freezing of poultry carcasses reduced the contamination level (87), likely due to the fact that *Campylobacter* is sensitive to the freeze-thaw process.

Irradiation is highly effective in reducing pathogens in food. In poultry and red meat the safe use of irradiation technology was approved and confirmed by Food and Drug Administration (FDA) (84). Low-dose irradiation can reduce the number of *Campylobacter* as well as other food-borne pathogens such as *Salmonella* and *E. coli* O157:H7 significantly (20, 30). However, despite the effectiveness and safe use of irradiation technology, few meat and poultry industry are applying this technology in the U.S. (23).

Vaccination against *Campylobacter* in broiler chickens would be an ideal strategy to prevent chickens from colonization by *Campylobacter*. However, an effective vaccine has not yet been available. The great antigenic diversity of *Campylobacter* between different strains and poor knowledge of antigens conferring protective immunity make the development of vaccines challenging. Most of the reported immunization studies were not able to show a significant level of protection against *Campylobacter* in chicken (48, 74, 86). On the other hand, a study by Wyszyńska et al. (101) showed promising results. In their protection experiment, they immunized chickens orally with an avirulent *Salmonella* vaccine
strain expressing the \textit{C. jejuni} \textit{cjaA} gene, which encodes a protein that is highly immunogenic in chickens and well conserved among different \textit{Campylobacter} serotypes. After the challenge with the wild-type \textit{C. jejuni}, a reduction in bacterial colonization in the ceca of immunized chickens were observed. In contrast, all of the non-vaccinated control chickens were heavily colonized with \textit{C. jejuni}. This study suggests that vaccination is a promising approach to control \textit{Campylobacter} infection in chickens (101).

Limited efforts have been made in the development of vaccines against \textit{Campylobacter} infection in humans. The enteric diseases program at the Naval Medical Research Institute, Bethesda, MD reported a vaccine candidate consisting of killed whole \textit{Campylobacter} cells (CWC) (11, 12). The CWC vaccine showed a high efficacy of protection against \textit{C. jejuni} in non-human primates and mice. In addition, it was demonstrated that the combined use of CWC and the heat-labile enterotoxin of \textit{E. coli} (LT) increased the immune response compared with CWC alone (11, 12). A truncated recombinant flagellin subunit vaccine, which was tested in mice by the nasal route with LT, also showed a high protection against \textit{C. jejuni} (56). In addition, Antex (USA) is developing an oral Traveler’s Diarrhea vaccine (activax) expressing antigens from \textit{Campylobacter}, \textit{Shigella}, and enterotoxigenic \textit{E. coli} (ETEC) (35). Despite these efforts, an effective \textit{Campylobacter} vaccine for human use is still not available.
CmeR and CmeR regulation

CmeR was originally identified as a repressor for the multidrug efflux pump CmeABC in \textit{C. jejuni} (57). The CmeR gene, which encodes a 210-residue protein, is located immediately upstream of the \textit{cmeABC} operon (57). The intergenic region (IT) between \textit{cmeR} and \textit{cmeABC} is 97-bp and contains an inverted repeat (IR) operator site (57). Lin et al. (57) showed that CmeR directly binds to the IR, inhibiting the expression of \textit{cmeABC}. When \textit{cmeR} or the IR was mutated, a drastic increase in the level of \textit{cmeABC} expression was observed, indicating that CmeR acts as a local repressor for \textit{cmeABC}.

CmeABC functions as a multidrug efflux pump (or MDR pump) and is encoded by a three-gene operon. This operon encodes three proteins including the outer membrane protein (CmeC), an inner membrane drug transporter (CmeB), and a periplasmic membrane-fusion protein (CmeA) (57). CmeABC plays a role in the extrusion of various substrates such as antibiotics, as well as ions, and lipophilic compounds such as SDS and bile salts. This indicates that CmeABC contributes to the intrinsic resistance of \textit{C. jejuni} to a broad range of antimicrobials (19, 57, 64, 83, 103). Interestingly CmeABC is also required for the adaptation of \textit{C. jejuni} in the gastrointestinal tract of chickens by mediating resistance to bile salts. These compounds are present in animal intestinal tracts and have bactericidal activities (61).

CmeR has sequence and structural homologies to the members of the TetR family of transcriptional repressors (57). In the N-terminus of CmeR in particular, there is a DNA-
binding domain, which is well conserved within the TetR family, such as QacR of *Staphylococcus aureus*, AcrR of *Escherichia coli*, and MtrR of *Neisseria gonorrhoeae* (57). In TetR, three-helix bundles form the DNA-binding domain and two of the helices form a helix-turn-helix motif, which can also be found in the N-terminal domain of CmeR (57, 77). While the N-terminal regions of TetR family members are conserved, their C-terminal regions vary, which enables them to interact with diverse substrates. A study by Lin et al. (59) showed that expression of the *cme*ABC was greatly induced in the presence of various bile salts. It also showed that bile salts inhibited CmeR binding to the promoter region of *cme*ABC. These findings indicate that bile salts function as inducing ligands for CmeR (59). It has been hypothesized that conformational changes in the N-terminal DNA-binding region of CmeR occur upon binding of inducing ligands to the C-terminal domain, which results in the release of CmeR from its operator DNA allowing the transcription of *cmeABC* (59).

Recently our laboratory also showed that CmeR functions as a pleiotropic regulator in *C. jejuni*. By comparing the transcriptional levels in 11168 and its CmeR mutant using DNA microarrays, multiple genes affected by the CmeR mutation were identified. The majority of the CmeR-regulated genes identified by microarray were confirmed by quantitative real-time RT-PCR. These identified genes encode diverse functions involved in membrane transport, capsular polysaccharides biosynthesis, C₄-dicarboxylate transport/utilization, and metabolism. Several periplasmic proteins and lipoproteins of unknown functions are also regulated by CmeR. For example, *cj0089* and *cj0091*, which encode putative lipoproteins, are down-regulated in the CmeR mutant, suggesting that CmeR acts as an activator for the genes.
Membrane proteins of *Campylobacter*

The cell envelope of gram-negative bacteria consists of an inner membrane (IM) and an outer membrane (OM). While the IM is composed of phospholipids showing a symmetrical structure of the inner and outer leaflet, the OM is asymmetric in that it contains lipopolysaccharides (or lipooligosaccharides) on the outer leaflet. Besides the lipopolysaccharides/lipooligosaccharides and phospholipids, the OM of gram-negative bacteria consists of outer membrane proteins (OMPs), which account for about half of the OM mass (49). OMPs include integral membrane proteins and lipoproteins. Integral OMPs usually have a β-barrel structure, in which hydrophilic residues face outward. These OMPs play essential roles in maintaining the integrity and permeability of bacterial membranes (17, 60), as well as adaptation in host niches such as iron uptake and resistance to antimicrobial peptides, sera, antibiotics, and bile salts (60).

Only a limited number of membrane proteins in *C. jejuni* have been functionally characterized. CadF (*Campylobacter* adhesion to fibronectin) and PEB1 (51, 81) are involved in *Campylobacter* adhesion and/or invasion to epithelial cells. CadF, a 37-kDa OMP, mediates the binding of the organism to fibronectin located at cell-to-cell contact regions in the gastrointestinal epithelium and stimulates the host cell signal transduction pathway (51, 66). PEB1 is a periplasmic protein homologous to the periplasmic-binding protein component of amino acid ABC transporters (80). PEB1 is an adhesin involved in *Campylobacter* adherence and invasion to human cells and colonization in the intestinal guts of mice (81). It was also shown that PEB1 binds to aspartate and glutamate (27). *C. jejuni* is
not able to utilize glucose as a carbon source, nor is it capable of sugar catabolism (99). Therefore, amino acids (especially serine, proline, aspartate, asparagines, glutamine, and glutamate) appear to be the important sources of carbon and energy (46, 55, 99). Thus, the PEB1 transport system appears to have an important role in utilization of aspartate and glutamate. However, how PEB1, a periplasmic protein, functions as an adhesin in *C. jejuni* is unknown. The major outer membrane protein (MOMP) functions as a porin (26) and also appear to be involved in *Campylobacter* adherence to human intestinal cell membranes and fibronectin (69, 99). MOMP is an integral protein (15, 106) and has a molecular mass of 45 kDa (62). As a porin, MOMP has pore-forming activity, which allows the diffusion of a wide range of compounds to bacterial cells (26). The other well-characterized membrane proteins are the multidrug efflux pumps, CmeABC and CmeDEF, both of which belong to the resistance-nodulation-cell division (RND) efflux systems (58, 61). The regulation and function of CmeABC has been described in the previous section. CmeDEF is also encoded by a three-gene operon and composed of an outer membrane channel protein CmeD, a periplasmic fusion protein CmeE, and an inner membrane transporter CmeF (58). In contrast to CmeABC, this efflux pump is not regulated by CmeR. Nonetheless, CmeDEF is also involved in resistance to several antibiotics and toxic compounds. Compared to CmeABC, CmeDEF is expressed at a low level and contributes moderately to antibiotic resistance in *C. jejuni* (1). It appears that CmeABC and CmeDEF interact with each other in conferring antimicrobial resistance and maintaining cell viability (1).
Bacterial lipoproteins

Bacterial lipoproteins are structurally and functionally diverse proteins. A common feature shared by all bacterial lipoproteins is that they contain N-acyldiacylglycerol-cysteine (Cys) at their N-terminal ends (37). This is the result of posttranslational modification, which is a significant characteristic of all the lipoproteins. This modification is very important since the three fatty acyl groups at the N-terminal end enable even highly hydrophilic proteins to anchor to the hydrophobic membranes (8). The precursor of a lipoprotein is a prolipoprotein, which contains a signal sequence of a tripartite structure: n-region, h-region, and c-region. The n-region is positively charged due to the presence of lysine and/or arginine, the h-region is hydrophobic, and the c-region contains lipobox, which is a consensus sequence around the signal cleavage site, comprised of (LVI)(ASTVI)(GAS)-C (8, 44, 73).

The biosynthesis of bacterial lipoproteins involves three steps as described by Sankaran and Wu (90). First, the diacylglyceride of phosphatidylglycerol is transferred to the thiol of Cys in a prolipoprotein by prolipoprotein-phosphatidylglycerol diacylglycerol transferase. Then, the signal peptide of the modified prolipoprotein is cleaved off by signal peptidase II, which is the leading formation of apolipoprotein. Lastly, amino-acylation of the N-terminal Cys of apolipoprotein takes place by phospholipid-apolipoprotein transacylase to form a mature lipoprotein (90). This biosynthetic pathway of bacterial lipoproteins occurs on the periplasmic side of the inner membrane after the precursors have been synthesized in the cytosol (73). Lipoproteins are anchored either on the inner or outer membranes facing toward the periplasmic place. They can also be located on the surface of outer membranes.
able to function correctly, they need to be localized properly on cell membranes. Since the lipid region of lipoproteins is very hydrophobic, protein export machinery, such as the Lol system in *E. coli* is required in order to transfer lipoproteins through the hydrophilic periplasmic space (73). Although the amino acid positions +2 and +3 of lipoproteins were previously thought to be the determinants for their localization to either inner or outer membranes (34, 102), it seems that this is not always the case (91).

Lipoproteins have diverse functions as adhesins, antigens, enzymes, transporters, binding proteins, and toxins. In *C. jejuni* NCTC 11168, the number of predicted lipoproteins is twenty two (8). So far, there are only two lipoproteins in *C. jejuni* that have been characterized. Jin et al. (42) characterized a surface-exposed lipoprotein, JlpA, which has a molecular mass of 42.3 kDa. This lipoprotein is loosely associated with the surface of the bacterial cell and appears to mediate *Campylobacter* adherence to host epithelial cells (42). Further study indicates that JlpA interacts specifically with surface-exposed heat shock protein 90α (Hsp90α) on host cells and triggers signaling pathways leading to the activation of NF-κB and p38 MAP kinase, both of which are components involved in host proinflammatory responses to infections (43). Recently, another lipoprotein, CapA, was characterized in *C. jejuni* (5). CapA also mediates *Campylobacter* adherence to host epithelial cells and is involved in the colonization of chicken gut.
Adhesion and invasion of *Campylobacter jejuni*

*C. jejuni* is able to reside in the mucous layer as a free-living organism and can also invade into host cells (100). Motility is essential for the organism to be able to colonize host intestinal cells. A drastic reduction in motility and intestinal colonization was observed with the *flaA* mutant. *flaA* is the primary structural gene of flagellum in *Campylobacter* (29). The motility provided by flagella enables the organism to penetrate the mucous layer (100). Once the organism reaches the apical surface of the epithelium, it adheres to the host cells via adhesion molecules. So far, the putative adhesins of *C. jejuni* include MOMP (69), lipoooligosaccharide (33, 65), PEB1 (80), CadF (51), JlpA (42), and CapA (5).

Cellular invasion is an important pathogenic mechanism of *C. jejuni*. The invasion of epithelial cells *in vivo* results in cellular damage and function loss, which lead to stimulation of host inflammatory responses and diarrhea (100). Standard assays for studying adhesion and invasion are available. Cell lines such as INT 407, Caco-2, and HEP-2 are commonly used in these assays. The ability of *C. jejuni* to invade host cells seems to vary depending on the strain (28, 47, 50). Clinical strains are more invasive than laboratory strains (50). *C. jejuni* strain 81-176 is highly invasive, and studies on the invasion mechanism of *C. jejuni* are mostly based on this strain.

Many invasive bacterial pathogens seem to enter into the host cells via cytoskeletal rearrangement. The cytoskeleton of eukaryotic cells is composed of various filaments including actin filaments, microtubules (MT), and accessory proteins (53). The invasion
mechanisms of *C. jejuni* vary, but mostly require either actin filaments or MTs (25, 40, 50, 76), or both (14, 76), or neither (88). *C. jejuni* 81-176 invades host cell in a MT-dependent manner (16, 24, 40). It also seems that microtubule motors are involved in the uptake and intracellular motility of this strain (16, 24).

Hu and Kopecko (40) conducted kinetic studies of entry of *C. jejuni* 81-176 into INT 407 cells. According to their study, the invasion of *C. jejuni* is most efficient at the lowest starting multiplicities of infection (MOI) of 0.02, but the number of internalized *C. jejuni* reaches its highest at an MOI of 200, where the efficiency of invasion is lower. This indicates that *C. jejuni* is a “highly efficient solitary invader” when compared to *Salmonella typhi* invasion, which seems to require enough signaling of bacteria to host cells in order for the internalization to occur (40). Using direct visualization, which allowed direct observation of bacteria in infected INT 407 cells, the authors also found that about two bacteria are internalized per infected host cell regardless of MOI (40). The internalization of *C. jejuni* is thus strictly limited (40). This might be due to host factors, such as a limited number of host cell “invasion receptors” and entry sites and host cell modifications to prevent further entry of *C. jejuni*, or due to the fact that the entry of the organism is dependent on host cell cycles (39, 40). The initiation of invasion requires the host cytoskeletal rearrangement triggered by signals from *C. jejuni*. CiaB (52) appears to be an effector of host cell signaling, but its exact role in invasion is still unknown.
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CHAPTER 2. Cj0091, an immunogenic lipoprotein of *Campylobacter jejuni*, is required for the adherence to host epithelial cells and colonization of the intestinal tract of chickens.

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Abstract

Bacterial lipoproteins have important functions in bacterial pathogenesis and physiology. In *Campylobacter jejuni*, a major foodborne pathogen causing gastroenteritis in humans, the majority of lipoproteins have not been functionally characterized. In this work, we identified a three-gene operon (*cj0089*, *cj0090*, and *cj0091*) encoding a cluster of lipoproteins. It was shown that this lipoproteins-encoding operon is regulated by CmeR, a transcriptional regulator repressing the expression of the multidrug efflux pump CmeABC in *C. jejuni*. In contrast to the regulation of CmeABC, CmeR appears to activate the expression of the lipoprotein genes and the regulation is possibly via an indirect mechanism. Analysis of different cellular fractions indicated that Cj0089 was associated with the inner membrane, while Cj0091 was located on the outer membrane. Inactivation of *cj0091*, but not *cj0089*, significantly reduced the adherence of *C. jejuni* to INT 407 cells *in vitro*, indicating that Cj0091 function as an adhesin. When inoculated into chickens, the Cj0091 mutant also
showed a defect in early colonization of the intestinal tract, suggesting that Cj0091 contributes to *Campylobacter* colonization *in vivo*. It was also shown in this study that Cj0091 was produced and immunogenic in chickens that were naturally infected by *C. jejuni*. These results identify Cj0091 as a new adhesin in *C. jejuni* and suggest that it may be targeted to reduce *Campylobacter* colonization in animal hosts.

**Introduction**

*Campylobacter jejuni* is a Gram-negative, curved or spirally shaped bacterium with a single, polar, unsheathed flagellum at one or both ends (62). It is a commensal organism existing in the intestinal tracts of a variety of wild and domestic animals, especially in birds. *Campylobacter jejuni* is a leading cause of acute diarrhea in humans worldwide (22). The typical symptoms of *Campylobacter* infections in humans include watery to bloody diarrhea, abdominal pain, fever, and presence of leukocytes and red blood cells in feces (3, 60). *Campylobacter* infections can also develop to Guillain-Barré syndrome (GBS), an autoimmune-mediated neurodegenerative disorder which causes acute neuromuscular paralysis (44, 53). The most significant source of *Campylobacter* infections for humans is the consumption of undercooked chicken.

The pathogenic process of *C. jejuni* in humans has not been well understood, but can be divided into several stages (25). Once ingested by the host, *C. jejuni* survives the stresses in the stomach and small intestine. Upon reaching the large intestinal tract, *C. jejuni* colonizes mucus layer and adheres to the intestinal cell surface of the host gastrointestinal
tract. The organism produces a cytolethal distending toxin and possibly other toxins, but their role in pathogenesis is not clear (51). Once adhered to the host intestinal epithelial cells, *C. jejuni* may invade into and proliferate within the host cells. The invasion and proliferation of the organism inside host cells are considered the cause of cell damage and induce host inflammatory responses, which result in diarrhea with fecal leukocytes (25). Occasionally *C. jejuni* can spread to extraintestinal sites, such as liver, gallbladder, pancreas, uterus, and fetal tissues (25, 60).

The known putative virulence factors involved in *Campylobacter* pathogenesis include flagella, lipoooligosaccharide (LOS), cytolethal distending toxin (CDT), and outer membrane proteins (25, 64). Flagella aid *Campylobacter* to move through the mucus layer and contribute to colonization and invasion (25). LOS is involved in adherence to host cells and serves as an endotoxin that induces host intestinal inflammatory responses (25, 42, 64). In addition, molecular mimicry of LOS to human gangliosides is considered a key factor in the development of GBS (6, 33). CDT causes cell cycle arrest and host DNA damage, which induce host inflammatory responses (20, 34, 65). The outer membrane proteins of *Campylobacter* are involved in interactions with hosts and play important roles in adherence and colonization. CadF, a 37-kDa surface protein, binds to fibronectins located at cell-to-cell contact regions in the gastrointestinal epithelium. CadF is required for *Campylobacter* colonization of chickens (32, 71). PEB1 is a periplasmic protein homologous to a solute-binding component of amino acid ABC transporters (48). PEB1 is important for *C. jejuni* adherence to human cells and colonization in the intestinal tract of mice (49). The major outer membrane protein (MOMP), a 45-kDa porin, adheres *in vitro* to human intestinal cell
membranes and fibronectin (43), but whether it is involved in \textit{in vivo} adherence is unknown. CmeABC functions as an efflux pump to extrude a variety of substrates such as antibiotics, ions, SDS, and bile salts (12, 37, 38, 52, 68). In addition, CmeABC mediates bile resistance and is required for \textit{C. jejuni} colonization in the gastrointestinal tract of chickens (37).

Bacterial lipoproteins have diverse functions including cell adhesion, transport, nutrient acquisition, stimulation of inflammatory/immune responses in host cells, mating, and serum resistance (8). \textit{C. jejuni} has multiple membrane lipoproteins predicted from the genomic sequences (8). At present, only two of these lipoproteins, JlpA (29) and CapA (5), have been functionally characterized in \textit{C. jejuni}. JlpA, which is a surface-exposed, loosely cell-associated lipoprotein, is involved in the interaction of \textit{C. jejuni} with the surface-exposed heat shock protein 90α (Hsp90α) of host cells and triggers signal transduction, leading to the activation of components (NF-κB and p38 MAP kinase) involved in host proinflammatory responses to infections (30). CapA is also involved in \textit{Campylobacter} adherence to host epithelial cells and colonization in gastrointestinal tract of chicken (5).

In a previous study, we found that several putative lipoproteins in \textit{C. jejuni} were regulated by CmeR (Table 1). CmeR was previously identified as a transcriptional repressor for the multidrug efflux pump CmeABC (36). Using DNA microarray, we also found that CmeR functions as a pleiotropic regulator modulating the expression of multiple genes in \textit{C. jejuni} NCTC 11168. In total, 28 genes showed ≥ 2-fold changes in expression in the CmeR-deletion mutant compared with the wild-type strain. Among the CmeR-regulated targets include two genes (\textit{cj0089} and \textit{cj0091}) encoding putative lipoproteins (Table 1). \textit{cj0089} and
*cj0091*, along with *cj0090* (also encoding a putative lipoprotein) appear to be organized into an operon, but their detailed regulatory mechanisms and role in *Campylobacter* pathophysiology remain unknown. Considering the fact that bacterial lipoproteins have important functions and the majority of lipoproteins in *C. jejuni* have not been characterized, we conducted both *in vitro* and *in vivo* experiments to elucidate the regulation of the lipoproteins-encoding operon and the functions of their encoded products in *Campylobacter* adherence and colonization.

**Materials and methods**

**Bacterial strains, plasmids, and culture conditions.** The various bacterial strains, mutants, and plasmids used in this study are listed in Table 2. *Campylobacter* strains were routinely grown in Mueller-Hinton (MH) broth (Difco) or agar at 42 °C with an atmosphere of 5 % O₂, 10 % CO₂, and 85 % N₂. *Escherichia coli* strains were grown is Luria-Bertani (LB) broth (Difco) or agar at 37 °C with shaking at 250 rpm. Media were supplemented with ampicillin (Amp) (100 μg/ml), kanamycin (Km) (50 μg/ml), or chlamphenicol (Cm) (4 μg/ml for *Campylobacter*, 20 μg/ml for *E. coli*) as needed.

**PCR.** All primers used for PCR are listed in Table 3. PCR amplification was performed in a volume of 100 μl containing 200 μM of each deoxynucleoside triphosphate, 200 nM primers, 2.5 mM MgSO₄, 100 ng of template DNA and 5 U of *Taq* DNA polymerase (Promega) or *pfu Turbo* DNA polymerase (Stratagene). Cycling conditions varied depending on the estimated annealing temperatures of the primers and the expected size of the products.
PCR products were purified with a QIAquick PCR purification kit (QIAGEN). For reverse transcriptase PCR (RT-PCR), total RNA was isolated from *C. jejuni* NCTC 11168 using the RNeasy minikit (QIAGEN). Isolated total RNAs were treated with RNase-free DNase (QIAGEN) to remove contaminating genomic DNA, and this was followed by quantification and qualification of the RNA using a NanoDrop microscale spectrophotometer (NanoDrop Technologies). RT-PCR was conducted using the SuperScript™ III One-Step RT-PCR system with Platinum® Taq DNA Polymerase (Invitrogen). Cycling conditions for the RT-PCR were as follows: synthesis of cDNA at 50 °C for 30 min; denaturation at 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 2 min 30 s; and a final extension at 72 °C for 10 min. The negative control was a RT-PCR mixture with the Taq polymerase (Promega) without the RT step, while the positive control was a reaction with genomic DNA as the template.

**Deletional mutation of *cj0089***. An isogenic *cj0089* mutant was constructed by deletional mutagenesis in *C. jejuni* strain NCTC 11168. To construct the *cj0089* mutant, primers *cj0089*-F5 and *cj0089*-R5 (Table 3) were used to amplify the 1915-bp fragment containing the entire open reading frame (ORF) of *cj0089* and its flanking sequences from *C. jejuni* strain NCTC 11168 chromosomal DNA. The PCR product was digested with *Sph*I and *Sac*I and cloned into pUC19 to form pUC19-89. Inverse PCR was performed using pfu Turbo DNA polymerase (Stratagene) on pUC19-89 using primers *cj89*-U2 and *cj89*-L2 (Table 3), which resulted in a 460-bp deletion within *cj0089*. A kanamycin-resistance (KanR) cassette, amplified from pMW10 by pfu Turbo DNA polymerase (Stratagene), was inserted to the amplified product to obtain pUC19-89K. The suicide vector, pUC19-89K, was introduced
into *C. jejuni* strain NCTC 11168 by electroporation. Transformants were selected on MH agar containing Km (50 µg/ml). Disruption of *cj0089* by deletion of the partial ORF and insertion of the KanR gene with the right orientation was confirmed by PCR, and the *cj0089* mutant was named Cj89\(^{-}\).

**Insertional mutation of *cj0091***. An isogenic *cj0091* mutant was constructed by insertional mutagenesis in *C. jejuni* strain NCTC 11168. The 1724-bp fragment containing the entire open reading frame (ORF) of *cj0091* and its flanking sequences was amplified using primers cj0091-F2 and cj0091-R2 (Table 3) from *C. jejuni* strain NCTC 11168 chromosomal DNA. The PCR product was digested with *Sac*I and *Xba*I and cloned into pUC19, forming pUC19-91. pUC19-91 was digested with *Cla*I followed by Klenow treatment (Takara) to form blunt ends. The KanR cassette, amplified from pMW10 by *pfu* Turbo DNA polymerase (Stratagene), was inserted to the *Cla*I-digested and Klenow-treated pUC19-91 to obtain pUC19-91K. The suicide vector, pUC19-91K, was introduced into *C. jejuni* strain NCTC 11168 by electroporation. Transformants were selected on MH agar containing 50 µg of Km per ml. PCR was performed to confirm the disruption of *cj0091* by the KanR gene with the right orientation, and the *cj0091* mutant was named Cj91\(^{-}\).

**Expression and purification of recombinant Cj0089 and Cj0091**. Histidine (His)-tagged recombinant Cj0089 and Cj0091 (rCj0089 and rCj0091, respectively) were produced in *E. coli* using the pQE-30 vector of the QIAexpress System (QIAGEN). A 1323-bp sequence covering *cj0089* was amplified using primers cj0089F and cj0089R (Table 3). A 567-bp fragment of *cj0091* was also amplified using primers cj0091-F1 and cj0091-R1
(Table 3). The amplified products containing restriction sites at the 5'-ends (Table 3) were purified and digested with appropriate restriction enzymes and separately ligated into pQE-30 vectors. The predicted lipoprotein signal peptides of Cj0089 and Cj0091 were not included in the recombinant products. Thus, the recombinant products represented the mature proteins in C. jejuni. Cloning, expression and purification of rCj0089 and rCj0091 were performed using the procedures provided by the QIAexpress system. The purified proteins were washed with 1 x PBS (containing 10mM benzamidine) to remove imidazole and were concentrated using Ultracel®YM-30 (for Cj0089) and Ultracel®YM-10 (for Cj0091) (Millipore).

**Production of rabbit antisera.** A polyclonal rabbit antiserum against rCj0089 was generated in our laboratory. A New Zealand white rabbit was immunized three times with rCj0089 at a 2-week interval between the injections. Each immunization used 100 µg of rCj0089 emulsified in incomplete Freund’s adjuvant. The animal was sacrificed at week 6 post the first injection to collect the antisera against rCj0089. Polyclonal antiserum against rCj0091 was generated in two rabbits by Pacific Immunology Corp (San Diego, CA, USA). The antisera were stored at -80 °C for further use.

**Electrophoretic mobility shift assays.** To determine whether CmeR directly binds to the operator region of cj0089, electrophoretic mobility shift assays, as described by Alekshun et al. (2), were performed with slight modifications. The 179-bp promoter region of cj0089 was amplified using primers Pcj89-F1 and Pcj89-R1 (Table 3). The amplified product was then labeled at the 3’ end with digoxigenin-11-ddUTP (DIG-11-ddUTP) using the DIG
Oligonucleotide 3’-End Labeling kit (Roche Molecular Biomedical). The DIG-11-ddUTP-labeled DNA (0.2 pmol) was incubated with different amounts of recombinant CmeR (0, 75, and 150 ng) in 20µl binding buffer containing 20 mM HEPES (pH 7.6), 1 mM EDTA, 10 mM(NH₄)₂SO₄, 5 mM dithopthreitol, 0.2 % Tween-20, 30 mM KCl, and 25 ng of poly(dI-dC). The reaction mixtures were incubated at room temperature for 30 min, followed by electrophoresis on a nondenaturing 6 % (w/v) polyacrylamide gel in a 0.25 x TBE buffer (22 mM Tris, 22 mM boric acid, 0.5 mM EDTA, pH 8.0) at 200 V for 40 min. The DNA was transferred from the gel to a positively charged nylon membrane using a vacuum blotter. Alkaline phosphatase-conjugated anti-DIG antibody and the chemiluminescent substrate CDP-star (Roche Molecular Biochemicals) were sequentially applied to the membrane. The chemiluminescence signals on the blots were captured using a digital imaging system (chemiLmager™5500 from Alpha Innotech). The promoter DNA of cmeABC and the intragenic fragment of cmeA were used as the positive and negative controls, respectively, as described in a previous study (36).

Preparation of cell fractions. The cell fractions of cytoplasm, periplasm, inner membrane, and outer membrane were obtained using the method described by Leon-Kempid et al. (17), with a slight modification. In total, 500 ml of an overnight culture of C. jejuni strain NCTC 11168 were harvested by centrifugation at 5000 x g for 30 min, and the cell pellet was resuspended in 20 ml of the ST buffer (20 % w/v sucrose, 30 mM Tris-HCl, pH 8.0) at room temperature. EDTA was added to the cell suspension to a final concentration of 1 mM and the suspension was incubated for 10 min at room temperature with shaking. The cells were then centrifuged at 8000 x g for 10 min at room temperature to collect the cell
pellet. The supernatant was discarded. The pellet was resuspended and stirred in ice-cold 10 mM Tris-HCl (pH 7.5) at 4 °C for 10 min followed by centrifugation at 15,000 x g at 4 °C for 15 min. The supernatant was concentrated by 10 % TCA and kept as the periplasmic fraction. The pellet was again resuspended in 5 ml of ice-cold 10 mM Tris-HCl (pH 7.5), followed by sonication (Virsonic, an SP Industries Company) by three bursts of 30 s at the 6 µm amplitude and centrifugation at 13,000 x g at 4 °C for 15 min. The pellet was discarded. The supernatant was ultracentrifuged at 100,000 x g at 4 °C for 1 hour. The supernatant was then transferred to a new tube and again centrifuged at 100,000 x g at 4 °C for 1 hour. The supernatant was concentrated by 10 % TCA, corresponding to the cytoplasmic fraction. The pellet collected was the total membrane fraction. The isolation of inner and outer membrane was performed using the method of Carlone et al (14). The pellet of membrane fraction was washed three times with ice-cold 10 mM Tris-HCl (pH 7.5) and resuspended in 0.2 ml of 10 mM HEPES buffer (pH 7.4). An equal volume of sarkosol buffer (2 % sodium N-lauroylsarkosine, SIGMA) was added and incubated at room temperature for 60 min, during which the solution was mixed occasionally. The inner membrane proteins were solubilized at this step. The mixture was centrifuged at 15,600 x g for 30 min at 4 °C. The supernatant was kept as the soluble inner membrane fraction. The pellet was washed with 0.5 ml of 10 mM HEPES buffer followed by resuspension in 200 µl of 10 mM HEPES buffer. This fraction was the outer membrane fraction. The fractions were analyzed by SDS-PAGE and immunoblotting.

**Adhesion assays.** Adhesion assays were performed according to the method described by Jin *et. al.* (29), with some modifications. INT 407 cells (ATCC CCL-6) were grown in
Minimum Essential Medium (MEM) (GIBCO) containing 10 % fetal bovine serum (FBS), 100 µg/ml streptomycin, and 100 units/ml penicillin G (GIBCO) at 37 °C under the atmosphere of 5 % CO₂. Once the INT 407 cells become confluent, the monolayers were trypsinized and approximately 1 x 10^5 cells/well in MEM with 10 % FBS without antibiotics were applied to each well of a 24-well tissue culture plate, which was incubated at 37 °C for 18 h under the atmosphere of 5 % CO₂. The monolayers were washed twice with 1 x PBS. NCTC 11168, cj89⁻, and cj91⁻ that grew overnight were added to wells containing monolayers in 1 ml of MEM with 10 % FBS at an INT 407 cells-to-bacteria ratio of 1:100, and incubated at 37 °C under the atmosphere of 5 % CO₂ for 3 h. The monolayers were washed five times with 1 x PBS. The washed monolayers were then lysed with 0.2 ml of 1 % (w/v) Triton X-100 in PBS for 15 min. The *Campylobacter* cells were enumerated by plate counting on MH agar plates. All assays were repeated three times and were done in quadruplicate.

**Colonization in chicken.** To examine whether the mutations of cj0089 and cj0091 affect *C. jejuni* colonization in chicken, 3-day-old commercial broiler chickens (ross x cobb), purchased from a commercial hatchery, were inoculated via oral gavage with approximately 10⁷ CFU of NCTC 11168, Cj89⁻, or Cj91⁻ (each group consists of 15 birds). Cecal samples were collected from 5 birds from each group at 3, 6, and 10 days post the inoculation (DPI), homogenized, serially diluted in 1 x PBS, and plated on MH plates containing *Campylobacter*-specific selective agents and growth supplements (SR084E and SR117E; Oxoid). After two-day incubation at 42 °C under microaerophilic conditions, the *Campylobacter* colonies were counted.
Results

Genomic organization and cotranscription of *cj0089*, *cj0090*, and *cj0091*. The three genes encoding the cluster of lipoproteins are tandemly positioned on the chromosome of *C. jejuni* NCTC 11168 (Fig. 1). *cj0089* and *cj0090* are separated by 9 nucleotides, while *cj0090* and *cj0091* are separated by 23 nucleotides. No predicted stem-loop structures exist between the ORFs. According to the prediction by Petersen *et al.* (50), there is a putative RpoD promoter located upstream of *cj0089*. The TATA-box of this promoter is located 40 nucleotides upstream of the *cj0089* translational initiation codon. No promoter was predicted immediately upstream of *cj0090* or *cj0091* (50). To determine whether *cj0089*, *cj0090*, and *cj0091* are co-transcribed, RT-PCR was performed using primers cj89int-F and cj91int-R (Table 3), which span the three ORFs (Fig.1A). A 1194-bp expected product was amplified in the PCR reaction with the RNA and RT, while no product was obtained in the reaction without RT, suggesting that *cj0089*, *cj0090*, and *cj0091* are co-transcribed and likely organized into an operon (Fig 1B). The operon is flanked by an upstream gene encoding an anaerobic C4-dicarboxylate transporter (*dcuA*) and a downstream gene encoding a hypothetical protein. There is a potential Rho-independent transcriptional terminator forming a 23-nucleotide stem-loop structure located immediately downstream of *dcuA* (Fig. 1A). Another stem-loop structure is present downstream of *cj0091* (Fig.1A). These sequence features further support the notion that the three lipoprotein genes form an operon.
**Predicted features of the lipoproteins.** Analysis of amino acid sequences predicted from DNA sequence analysis indicated that *cj0089*, *cj0090*, and *cj0091* encode putative lipoproteins because each of them has a typical N-terminal lipoprotein signal peptide. LipoP algorithm (31) predicted a signal peptidase II cleavage site at LFLTA↓C for Cj0089, at FLLSA↓C for Cj0090, and at LLFSG↓C for Cj0091. Cj0089 (453 amino acids), Cj0090 (122 amino acids), and Cj0091 (207 amino acids) each encodes a protein with a calculated molecular mass of 51.28 kDa, 13.92 kDa, and 22.32 kDa, respectively. The molecular masses of the mature lipoproteins, after cleavage of the signal peptides, are predicted to be 49.4 kDa, 12.29 kDa, and 20.51 kDa, respectively. BLAST searches showed that *cj0089* bears similarity to tetratricopeptide TPR-2 repeat proteins found in *Shewanella* spp. The functions of these tetratricopeptide TPR-2 repeat proteins have not been characterized. Cj0089 also has a 27.4 % similarity to HP0018, a hypothetical protein in *Helicobacter pylori*. Cj0091 has a 33.5 % similarity to HP1457 and has a conserved domain similar to the collagen-binding surface adhesin, SpaP (antigen I/II family) of *Yersinia pestis* biovar *Orientalis*, where its function has not been determined empirically.

**Production of rCj0089 and rCj0091 and their specific antibodies.** The coding sequences of *cj0089* and *cj0091*, excluding their signal peptides, were cloned into pQE-30 vector, expressed in *E. coli* JM109, and the proteins purified by affinity chromatography. Using the purified proteins, rabbit polyclonal antibodies against rCj0089 and rCj0091 were produced. The anti-Cj0089 antibody recognized a protein with a molecular mass of 49 kDa in NCTC 11168. On the other hand, anti-Cj0091 recognized a protein with a molecular mass of approximately 18 kDa in the organism, slightly smaller than the calculated mass of Cj0091.
(20.51 kDa). These immunoblotting results indicated that both lipoproteins were produced in *C. jejuni* when grown in MH broth.

**Regulation of *cj0089* and *cj0091* by CmeR.** In a previous DNA microarray study by our laboratory, it was found that *cj0089* and *cj0091* were down-regulated in the *cmeR* mutant, suggesting that CmeR is an activator for the lipoprotein genes (Table 1). To confirm the previous finding at the protein level, the expression levels of Cj0089 and Cj0091 in the wild-type strain and in the *cmeR* mutant were analyzed by immunoblotting using anti-Cj0089 and anti-Cj0091. MOMP was used to normalize the loading condition. The immunoblotting results clearly showed that the production of Cj0089 and Cj0091 in the *cmeR* mutant was decreased compared with that in the wild-type (Fig. 2). According to the measurements with the AlphaEase® software (Alpha Innotech), the band intensity of Cj0089 was 2.98 fold higher in the wild-type than in the CmeR mutant and the band intensity of Cj0091 was 1.92 fold higher in the wild-type than in the CmeR mutant. The immunoblotting data further confirmed the results of the DNA microarray and real-time RT-PCR.

To determine if CmeR directly or indirectly regulates the lipoprotein operon, a gel mobility shift assay was performed using rCmeR and the promoter sequence in front of *cj0089*. The promoter DNA was amplified with primers Pcj89-F1 and Pcj89-R1 (Table 3, Fig. 3A). rCmeR did not bind to the promoter region of *cj0089* when different concentrations of rCmeR were used in the assay (Fig. 3B). As a positive control, rCmeR bound to the promoter DNA of *cmeABC*, and as a negative control, rCmeR did not bind to the internal
region of *cmeA*. These findings suggest that the lipoprotein operon is indirectly regulated by CmeR.

**Cj0089 and Cj0091 are membrane-associated proteins.** In order to determine the cellular localization of Cj0089 and Cj0091, *C. jejuni* strain NCTC 11168 was fractionated to isolate proteins from the cytosol, periplasm, inner membrane, and outer membrane. The fractions were examined by immunoblotting with anti-Cj0089 and anti-Cj0091 (Fig. 4). Cj0089 was detected predominantly in the inner membrane fraction, while Cj0091 was found in both the inner and outer membrane fractions, but the majority of Cj0091 was in the outer membrane. Antisera against CmeB, an inner membrane drug transporter of multidrug efflux pump CmeABC (37), and against the major outer membrane protein (MOMP) (27), were used as controls for the membrane fractions. CmeB is exclusively detected in the inner membrane fraction; however, MOMP was detected in both outer membrane and inner membrane fractions, with the majority of MOMP was in the outer membrane fraction. The results from the controls suggested that the inner membrane fraction contained some outer membrane proteins, while the outer membrane fraction was pure. Based on the blotting results and in reference to the controls, it was concluded that Cj0089 is associated with the inner membrane, while Cj0091 is associated with outer membrane of *C. jejuni*.

**Cj0089 and Cj0091 were inactivated in the insertional mutants.** To study the functions of the lipoproteins, insertional mutations of *cj0089* and *cj0091* were generated as described in Materials and methods (Fig. 5A). Correct insertion and orientation of the KanR cassette in Cj89Δ and Cj91Δ were confirmed by PCR (Fig. 5B). The disruptions of *cj0089* and
were also confirmed by immunoblotting using anti-Cj0089 and anti-Cj0091 (Fig. 5C). In Cj89', the expression level of cj0091 was significantly decreased, but was not abolished (Fig. 5C), indicating that the insertion in cj0089 caused a partial polar effect on cj0091.

**Cj0091 is required for Campylobacter adherence to INT 407 cells in vitro.** Some proteins associated with cell membranes in *C. jejuni*, such as JlpA (29), CapA (5), PEB1a (49), CadF (32) and MOMP (43), play roles in adhesion to human cells *in vitro*. Since Cj0091 is an outer membrane-associated protein and has a domain homologous to SpaP involved in surface adherence, it is possible that Cj0091 is involved in the interaction between *C. jejuni* and host cells. To examine this possibility, the ability of NCTC 11168 and the two mutants, Cj89' and Cj91', to adhere to the monolayers of human intestinal cell line INT 407 was measured using a cell culture system (Fig. 6). Cj91' showed a 6.7-fold reduction in adherence compared to the wild-type, a statistically significant reduction (P < 0.05). Cj89' had a 1.64-fold reduction in adherence compared to the wild type; however, the difference was not statistically significant (p > 0.05). These findings indicate that Cj0091, but not Cj0089, contributes significantly to *Campylobacter* adherence to INT 407 cells.

**Cj0091 is involved in intestinal colonization in chicken.** To test whether Cj0089 and Cj0091 has a role in chicken colonization, the wild type, Cj89', and Cj91' were separately inoculated to three groups of chickens. At DPI 3, 6, and 9, cecal contents were collected from 5 chickens from each group and cultured for enumeration of *Campylobacter* using selective plate counting. On DPI 3 and 6, the mean level of colonization by Cj91' was significantly lower (reduced approximately 2.7 and 2.5 log_{10} units, respectively) than that of the wild type
strain (Fig. 7A). The differences were statistically significant (p < 0.05). On DPI 10, no significant differences in colonization between the wild-type and Cj91− were observed (Fig. 7A). Throughout the experiment, no differences in colonization were observed between the wild-type and cj89− (Fig. 7A). The colonization reduction seen with Cj91− was not attributable to the difference in in vitro growth rates or motility because the mutants grew equally well and were equally motile when compared with the wild-type strain (Fig. 7B, Table 4). Together, these results indicate that Cj0091, not Cj0089, contribute to the early colonization of C. jejuni in the intestinal tract of chickens.

**Cj0091 is expressed and immunogenic in chickens.** The contribution of Cj0091 to adherence and in vivo colonization prompted us to determine if Cj0091 is produced and immunogenic in chickens naturally infected by Campylobacter. For this purpose, rCj0091 was used as the antigen on an immunoblot and was probed with chicken sera collected from 5 individual chickens, among which 4 contained natural maternal antibodies against C. jejuni and the other one was a negative control (Campylobacter-free and no anti-Campylobacter maternal antibodies) (59). As shown in Fig. 8, the 21-kDa rCj0091 was detected by the 4 sera, in which high level of C. jejuni-specific maternal antibodies (IgG) were present, but not by the serum in which the C. jejuni-specific maternal antibodies were absent (59) (Fig 8). These findings strongly suggest that Cj0091 was produced and immunogenic during the infection of chickens by C. jejuni.
Discussion

In this study, we characterized an operon encoding three lipoproteins, Cj0089, Cj0090, and Cj0091. It was found that this operon is regulated by CmeR, possibly by an indirect mechanism. We also demonstrated that Cj0091, but not Cj0089, has an important role in mediating *Campylobacter* adherence to INT 407 cells and in *Campylobacter* colonization of the gastrointestinal tract of chickens, especially in the early stage of the infection. These findings identify a new adhesin in *C. jejuni* and provide new insights into the pathogenic mechanism of *C. jejuni*.

BLAST searches showed that Cj0089 is similar to the tetratricopeptide TPR-2 repeat protein of *Shewanella* spp. (E-value = 4e-19 to 4e-16). The tetratricopeptide repeat (TPR) is a structural motif, which mediates protein-protein interactions. TPR-containing proteins have diverse functions. They are, for example, involved in the regulation of cell cycles, protein transport, the regulation of transcription, splicing events, and protein folding (10, 24). Proteins containing the TPR motif have been found in various organisms including bacteria, yeast, plants, insects, and animals (10, 24). At present the function of Cj0089 is unknown. Since *cj0089*, *cj0090*, and *cj0091* form an operon, it is possible that the three encoded products have a functional link. This possibility remains to be defined in future studies. Cj0091 has a conserved domain homologous to the collagen-binding surface adhesin, SpaP (antigen I/II family) of *Yersinia pestis* biovar *Orientalis*. *spaP* was first identified in *Streptococcus mutans* as a gene encoding antigen I/II (55, 70), and is also known as P1 (21), PAc (45, 46, 61), Antigen B (54, 58), IF (26), and MSL-1 (18). The antigen P1, encoded by
spaP, plays a role in adherence of *Streptococcus mutans* to the salivary pellicle of tooth surfaces or to other microorganisms (19, 23, 35, 39).

In this study we confirmed the regulation of the putative lipoprotein operon by CmeR (Fig. 2). Based on the result of electrophoretic gel mobility shift assay, it appears that this operon is indirectly regulated by CmeR (Fig. 3). However, we cannot exclude the possibility that the binding region of CmeR is further upstream of the promoter of *cj0089* and was not included in the DNA fragment used for the gel-mobility shift assay. Analysis of the upstream sequence did not identify the typical inverted repeat found in the promoter region of *cmeABC* recognized by CmeR (36). Since CmeR is expected to serve as an activator for the lipoprotein operon, which is in contrast to the regulation of *cmeABC* (repressed by CmeR), the binding site for CmeR in front of the lipoprotein operon could be different from the one in front of *cmeABC*. It is also possible that CmeR requires a co-factor for binding. Alternatively, CmeR may regulate another regulatory protein, which then directly modulates the transcription of the lipoprotein operon. How CmeR regulates the lipoprotein operon and the biological consequences of the regulation remain to be examined in future studies.

The contribution of Cj0091 to *Campylobacter* adherence was identified using the human intestinal epithelial cell line INT 407 (Fig. 6). So far adhesins characterized in *C. jejuni* include PEB1 (49), CadF (32), MOMP (43), JlpA (29), and CapA (5). All of these are membrane associated-proteins. PEB1 is a periplasmic protein, homologous to the periplasmic solute-binding protein component of amino acid ABC transporters (48). An important function of PEB1 is that it binds to aspartate and glutamate, which are important sources of
carbon and energy for *Campylobacter* (48, 63). CadF, which is a surface protein, is involved in the binding of *C. jejuni* to fibronectin in the host gastrointestinal epithelium and stimulation of the host cell signal transduction pathway (32, 41). MOMP, a trimeric outer membrane protein (11, 28), is involved in pore-forming activity (16), and assists outer membrane structural organization and stabilization (4). Although one study suggested MOMP could be involved in adherence (43), the exact role of MOMP in the interaction between *Campylobacter* and host cells is unknown. JlpA is a lipoprotein and is involved in interaction with host cells *in vitro* (29). Besides functioning as an adhesin, JlpA interacts with surface-exposed heat shock protein 90α (Hsp90α) on host cells, triggering signaling pathways and leading to the activation of NF-κB and p38 MAP kinase (30). CapA, which is an autotransporter, is involved in the colonization of the chicken gut and plays a role in adherence to human epithelial cells (5). Thus, *C. jejuni* appears to possess multiple adhesins and the partial reduction of adherence seen with Cj91⁻ may be explained by the complementary effects of other adhesins.

Although we showed that Cj0091 is an outer membrane associated protein (Fig. 4), it has yet to be determined whether this protein is located on the outer surface or is facing the periplasmic side. Therefore, it is uncertain whether involvement of Cj0091 in the adherence to human epithelial cells is direct or indirect. If it is located on the surface of the cells, it is likely that Cj0091 directly interacts with human cells. If the Cj0091 is located on the periplasmic side, it may act as a structural protein, which supports other protein(s) involved in the direct adherence to epithelial cells. Further localization of Cj0091 is required to confirm that the phenotypic difference observed is indeed due to the direct effect of Cj0091.
*C. jejuni* mainly colonizes in cecal and cloacal crypts (1, 9, 40). Unlike the colonization in mammals, such as mice, swine, rabbits, monkeys, and humans, where the organism commonly invades the host intestinal epithelial cells, *C. jejuni* does not usually invade the intestinal epithelium of chickens (7, 13, 56, 57). Thus, surface colonization is an important feature of *Campylobacter* infection in chickens. The differences in colonization between the wild-type and Cj91− were only seen at the early stage (Fig. 7A). We speculate that this may reflect the possibility that Cj0091 is only required for the initial adherence, or adaptation of the Cj91− mutant occurs in the intestinal tract, overcoming the early defect in colonization. Although it has been reported that *Campylobacter* does not directly interact with intestinal epithelial cells but rather resides in the mucus layer of the crypts moving freely and rapidly in chickens (9, 40), it is highly possible that adherence to gastrointestinal mucosal cell surface is still required for successful colonization in chickens (5, 25). Indeed, independent studies have shown that CapA and CadF are each required for *Campylobacter* colonization in chickens (5, 71). In addition, another adhesion, PEB1, is also required for the optimal colonization of *Campylobacter* in mice (49).

Ceca of chickens are blind-ended sacs filled with small food particles, fluid, and microorganisms. Cecal contents are constantly moving in ceca and evacuated periodically (15). Reduction of adherence to intestinal cells may cause difficulty in the initial multiplication in ceca since most of the organisms will be excreted from ceca before the successful proliferation. However, once *C. jejuni* is able to start multiplying successfully in ceca, it will eventually overcome the initial impairment in colonization. This may be another
reason why Cj91 was deficient only in the initial colonization in chickens. When mutants of PEB1, CapA, and CadF were used for colonization studies, all showed a colonization defect throughout the entire study periods (5, 49, 71), which is in contrast to our results. The reason for the observed difference between Cj0091 and other reported adhesins in the level of colonization reduction is unknown.

It was demonstrated in this study that Cj0089 is an inner membrane protein (Fig. 4). Disruption of \textit{cj0089} did not result in significant changes in adherence and \textit{in vivo} colonization (Fig. 6, 7A). This suggests that Cj0089 is not essential for \textit{Campylobacter} growth in the intestinal tract. Alternatively, it may suggest that Cj0089 may share a redundant function(s) with other genes in \textit{C. jejuni}. Although \textit{cj0089} and \textit{cj0091} are located in the same operon and both are regulated by CmeR, they may have different functions in \textit{Campylobacter}. Interestingly, mutation of \textit{cj0089} reduced, but did not abolish, the expression of \textit{cj0091} due to a polar effect (Fig. 5c). The partial reduction in Cj0091 expression did not cause an apparent phenotypic change, suggesting that partial production of Cj0091 is sufficient for adherence and colonization at the wild-type level.

Another interesting finding in this study is that Cj0091 is abundant in \textit{C. jejuni in vitro} and is apparently immunogenic in chickens. To date, no vaccines against \textit{Campylobacter} are available for humans or poultry. The fact that the \textit{Campylobacter} is commensal in poultry and that it shows high genetic and antigenic diversity among different strains makes the development of an effective vaccine quite difficult. One of the promising vaccine candidates is an attenuated \textit{Salmonella} strain carrying the \textit{C. jejuni} CjaA antigen (67). CjaA is a highly
immunogenic protein that is well conserved among different *Campylobacter* serotypes and induces protective immune responses in chickens (67). Cj0091 is also highly conserved among the 4 sequenced strains of *C. jejuni* (≥ 99 % amino acid identity). This fact plus the findings from this study suggest that Cj0091 may be used as potential vaccine candidate in the control of *C. jejuni* colonization in chickens. This possibility will be examined in future studies.

**Acknowledgements**

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**References**


17. **del Rocio Leon-Kempis, M., E. Guccione, F. Mulholland, M. P. Williamson, and D. J. Kelly**. 2006. The *Campylobacter jejuni* PEB1a adhesin is an


triggers signalling pathways leading to the activation of NF-kappaB and p38 MAP kinase in epithelial cells. Cell. Microbiol. 5:165-174.


72Dz/92 cjaA gene elicits specific humoral immune response associated with protection against challenge with wild-type Campylobacter. Vaccine 22:1379-1389.


TABLE 1. Representative genes regulated by CmeR

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Description</th>
<th>$P$-value</th>
<th>Fold change by microarray</th>
<th>Fold change by real-time RT-PCR*</th>
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</thead>
<tbody>
<tr>
<td><em>cj0366c</em></td>
<td><em>cmeB</em>, RND-type transporter</td>
<td>0.0017</td>
<td>7.8</td>
<td>6.8</td>
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<td><em>cj0089</em></td>
<td>Putative lipoprotein</td>
<td>0.0419</td>
<td>-2.1</td>
<td>-2.0</td>
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<td><em>cj0091</em></td>
<td>Putative lipoprotein</td>
<td>0.0200</td>
<td>-4.3</td>
<td>-4.5</td>
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<td><em>Cj0092</em></td>
<td>Probable periplasmic protein</td>
<td>0.0087</td>
<td>-2.6</td>
<td>-1.9</td>
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*Mean of three independent experiments.*
TABLE 2. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td></td>
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<tr>
<td>NCTC 11168</td>
<td>originally isolated from human</td>
<td>(47)</td>
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<tr>
<td>Cj89</td>
<td>11168 derivative; <em>cj0089::Kan</em></td>
<td>This study</td>
</tr>
<tr>
<td>Cj91</td>
<td>11168 derivative; <em>cj0091::Kan</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em></td>
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<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F−Φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1- hsdR17 (rκ-, mκ+) phoA supE44 thi-1 gyrA96 relA1 ΔendA1 recA1 gyrA96 thi hsdR17 (rκ-, mκ+) relA1- supE44 Δ(lac-proAB)[F’ traD36 proAB lacZΔM15]</td>
<td>Invitrogen</td>
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<td>Promega</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pUC19</td>
<td><em>E. coli</em> cloning vector</td>
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<tr>
<td>pUC19-89</td>
<td>pUC19 containing full-length <em>cj0089, Amp</em></td>
<td>This study</td>
</tr>
<tr>
<td>pUC19-89K</td>
<td>pUC19-89 derivative carrying <em>cj0089::Kan</em></td>
<td>This study</td>
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<td>pUC19-91</td>
<td>pUC19 containing full-length <em>cj0091, Amp</em></td>
<td>This study</td>
</tr>
<tr>
<td>pUC19-91K</td>
<td>pUC19-89 derivative carrying <em>cj0091::Kan</em></td>
<td>This study</td>
</tr>
<tr>
<td>pMW10</td>
<td><em>E. coli</em> - <em>C. jejuni</em> shuttle vector with a promoterless lacZ gene, Kan</td>
<td>(66)</td>
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TABLE 3. Primer sequences used in this study

<table>
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<th>Primer</th>
<th>Sequence*</th>
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<tr>
<td>cj0089-F5</td>
<td>5'-GAGgcatgcCCGACTTTGTTAGGTGCAGTGCAA-3' (Sph I)</td>
</tr>
<tr>
<td>cj0089-R5</td>
<td>5'-GCAgagctcGCTGGAATTCTAAGGGCTTGATAGTCCT-3' (Sac I)</td>
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<tr>
<td>cj89-U2</td>
<td>5'-GCTccatggTGCCTTATCTTGACGCATTAAAACACG-3' (Nco I)</td>
</tr>
<tr>
<td>cj89-L2</td>
<td>5'-CAGccatggGGGAAGCTTCTTTTGCAAATTTAAATATAAATGG-3' (Nco I)</td>
</tr>
<tr>
<td>cj0091-F2</td>
<td>5'-GTGCGTtctagaAGAGGTTTACCAAAAAGTA-3' (Xba I)</td>
</tr>
<tr>
<td>cj0091-R2</td>
<td>5'-CTTTTGGgagctcATCAAATTCCTTAGCATTCGTA-3' (Sac I)</td>
</tr>
<tr>
<td>cj0089F</td>
<td>5'-AGTggatccTGCCTTTTTTTAACAGCTTGTG-3' (BamH I)</td>
</tr>
<tr>
<td>cj0089R</td>
<td>5'-GAGgagctcTTATTTTTCCATGATAGCAAC-3' (Sac I)</td>
</tr>
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<td>cj0091-F1</td>
<td>5'-AGTggatccTGTGCGCAAACAGCTTATACAGATGGAAAG-3' (BamH I)</td>
</tr>
<tr>
<td>cj0091-R1</td>
<td>5'-GAGgagctcTTACCAAGTAACTGATTTACTAGAACCGGTTTTATC-3' (Sac I)</td>
</tr>
<tr>
<td>Pcj89-F1</td>
<td>5'-AGGCTTTGTATTAGCTCCTATGCTTAT-3'</td>
</tr>
<tr>
<td>Pcj89-R1</td>
<td>5'-CAAGCAATACCTGAAAAATCAACCCAA-3'</td>
</tr>
<tr>
<td>cj89int-F</td>
<td>5'-TTTAAAATAATATGCTGCAATGA-3'</td>
</tr>
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<td>cj91int-R</td>
<td>5'-GCCATACCAATGTCCCTTTT-3'</td>
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<tr>
<td>KanNco-F</td>
<td>5'-CTTATCAATATATccatggAATGGGCAAACG-3' (Nco I)</td>
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<tr>
<td>KanNco-R</td>
<td>5'-GATAGAACCacaggATAATGCTAAGACAATCAA-3' (Nco I)</td>
</tr>
</tbody>
</table>

*The restriction sites in the sequences are indicated by lower cases. The corresponding restriction enzymes for the restriction sites were indicated in parenthesis.
TABLE 4. Motility of various *C. jejuni* constructs

<table>
<thead>
<tr>
<th>Strain</th>
<th>Motility (cm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC 11168</td>
<td>7.00 ± 0.20</td>
</tr>
<tr>
<td>Cj89⁻</td>
<td>6.93 ± 0.31</td>
</tr>
<tr>
<td>Cj91⁻</td>
<td>7.03 ± 0.12</td>
</tr>
</tbody>
</table>

*Mean of three independent experiments*
FIG. 1. Genomic organization and co-transcription of *cj0089*, *cj0090*, and *cj0091*. (A) The genes are depicted by boxed arrows. The locations of the primers used for RT-PCR were indicated by arrows. Predicted stem-loop encoded regions are shown by vertical oval arrows. (B) RT-PCR was performed to determine the co-transcription of *cj0089*, *cj0090*, and *cj0091* using primers *cj89int*-F and *cj91int*-R. Lane 1, no RT control; lane 2, RNA with RT and DNA polymerase; lane 3, positive control (DNA was used as the template).
FIG. 2. Effect of *cmeR* mutation on the expression of Cj0089 and Cj0091 as determined by immunoblotting. NCTC 11168 (lane 1) and *cmeR* (lane 2) were separated by SDS-PAGE and probed with anti-Cj0089 (panel I), anti-Cj0091 (panel II), and anti-MOMP (panel III). MOMP, a 45-kDa outer membrane protein, was used as to normalize the loading condition.
FIG. 3. Regulation of *cj0089*, *cj0090*, and *cj0091*, by CmeR. (A) Sequence features of the *cj0089* promoter region. Coding sequences are highlighted with gray and in bold. The stop codon and terminator of *dcuA*, the -10 element, ribosomal binding site (RBS), and start codon of *cj0089* are underlined. (B) Gel mobility shift assay with the positive control DNA, which contains CmeR binding site (lanes 1 and 2), negative control DNA, which is an internal *cmeA* fragment (lanes 3 to 5), or *cj0089* promoter DNA (lanes 6 to 8). In the assay, the DIG-labeled DNA (0.1 pmol) was incubated with 0 (lanes 1, 3, and 6), 75 (lanes 2, 4, and 7), and 150 ng (lanes 5 and 8) of rCmeR, respectively. The position of the DIG-labeled DNA (D) and DNA-rCmeR complex (D/P) are indicated.
FIG. 4. Localization of Cj0089 and Cj0091 in *C. jejuni*. The cytosol fraction (lane 1), periplasm fraction (lane 2), inner membrane fraction (lane 3), and outer membrane fraction (lane 4) were separated by SDS-PAGE and probed with anti-CmeB (panel I), anti-Cj0089 (panel II), anti-MOMP (panel III), and anti-Cj0091 (panel IV), respectively.
FIG. 5. Generation of deletional and insertional mutations in \textit{cj0089} and \textit{cj0091}. (A) Schematic diagram of the locations of deletional and insertional mutations. The genes are indicated by boxed arrows. The locations of the Kan\textsuperscript{R} insertion are indicated as solid arrows. (B) PCR confirmation of the insertions in Cj89' and Cj91'. Lanes 1, \textit{cj0089} in wild-type; lane 2, \textit{cj0089} in Cj89'; lane 3, \textit{cj0091} in wild-type; lane 4, \textit{cj0091} in Cj91'. (C) Immunoblotting analysis of protein production in various constructs. Wild-type (lane 1), Cj89' (lane 2), and Cj91' (lane 3) were separated by SDS-PAGE and probed with anti-Cj0089 (panel I), anti-Cj0091 (panel II), and anti-MOMP (panel III). MOMP, a 45-kDa outer membrane protein, was used as a loading control.
FIG. 6. Adherence of C. jejuni strain NCTC 11168 and its mutants to INT 407 cells. C. jejuni strain NCTC 11168, Cj89−, and Cj91− were inoculated onto monolayers of INT 407 cells and incubated for 3 h followed by washing. Monolayers were lysed with 1% Triton X-100 in PBS and the Campylobacter cells were enumerated by plate counts. Experiments were conducted in quadruplicate for 3 times. Adherence efficiency was determined using the percentage of bacterial inocula adhered to cells. * P < 0.05
FIG. 7. Colonization of *C. jejuni* strain NCTC 11168 and its mutants in chickens. (a) Approximately $10^7$ CFU of each of NCTC 11168, Cj89', or Cj91' were separately inoculated into three groups of 3-day-old chickens. Cecal samples were collected at 3, 6, and 10 days post inoculation and the numbers of *Campylobacter* were enumerated by plate counts. * P < 0.05 (b) Growth rates of NCTC 11168 and the mutants were determined by measuring OD$_{600}$ of bacterial cultures grown in MH broth.
FIG. 8. Immunoblotting detection of natural anti-Cj0091 antibodies in chickens. Strips of nitrocellulose membranes with rCj0091 were reacted with 4 chicken sera (lanes 2-5) that contained *Campylobacter*-specific maternal antibodies derived from natural infections and one chicken serum (lane 6) that was negative for anti-*Campylobacter* antibodies. Rabbit anti-rCj0091 was applied as a positive control (lane 1).
CHAPTER 3. General Conclusion

In this study, we identified a three-gene operon consisting of cj0089, cj0090, and cj0091, which encode lipoproteins. We showed that this operon is indirectly regulated by CmeR, which functions as a repressor for the multidrug efflux pump, CmeABC. CmeR appears to act as an activator for the lipoprotein-encoding operon because the expression of the lipoproteins was reduced in the cmeR mutant. The findings in this study further confirm the results of our DNA microarrays that CmeR is a pleiotropic regulator and modulates the expression of multiple genes of diverse functions in C. jejuni. Analysis of cellular fractions determined that Cj0089 is located on the inner membrane, while Cj0091 is associated with the outer membrane of Campylobacter cells. At present, it is unknown if Cj0091 faces outward or inward on the outer membrane and the specific location of Cj0091 remains to be determined in future studies. Notably, our work revealed that Cj0091 plays a significant role in C. jejuni adhesion to host cells in vitro and also contributes to the colonization of C. jejuni in the gastrointestinal tracts of chickens. In addition, our results strongly suggested that Cj0091 was produced and immunogenic in chickens that were naturally infected by C. jejuni. Although cj0089 and cj0090 are co-transcribed with cj0091, a definitive function of Cj0089 was not identified and the characterization of Cj0090 was not performed in this work.

Findings from this study established Cj0091 as a newly identified adhesin in C. jejuni and improved our understanding of the pathogenic mechanisms of this enteric pathogen. Cj0091 appears to be highly conserved in different strains of C. jejuni and plays an important role in the interaction between C. jejuni and host cells. In addition, C. jejuni is highly
immunogenic in the animal host. These features of Cj0091 make it a promising candidate for the development of vaccines against *C. jejuni*, which will be pursued in future studies.
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