EXAMINATION OF *CAMPYLOBACTER JEJUNI* PUTATIVE ADHESINS LEADS TO THE IDENTIFICATION OF A NEW PROTEIN, DESIGNATED FLPA, REQUIRED FOR CHICKEN COLONIZATION

By

REBECCA CATHERINE FLANAGAN

A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE IN MICROBIOLOGY

WASHINGTON STATE UNIVERSITY
School of Molecular Biosciences

MAY 2009

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of REBECCA CATHERINE FLANAGAN find it satisfactory and recommend that it be accepted.

____

Michael Konkel, Ph.D., Chair

____

Luying Xun, Ph.D.

____

Nancy Magnuson, Ph.D.

____

Scott Minnich, Ph.D.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my mentor, Dr. Michael Konkel, for his assistance and enthusiasm during my time as a graduate student at WSU. Also, I would like to acknowledge my committee members, Dr. Scott Minnich, Dr. Nancy Magnuson, and Dr. Luying Xun for their assistance and guidance during my graduate research. I thank the past and present members of the Konkel laboratory because without them I could not have finished this research: Dr. Jeffery Christenson, Dr. Kari Sweeney, Dr. Daelynn Buelow, Dr. Tri Duong, Sophia Pacheco, Alison Lane, Charlie Larson, and Jason Neal-McKinney. Additionally, I thank my family for their love and support throughout my time at WSU. I particularly thank Kristen Sukraw for her friendship during my time as a graduate student; my life is richer because of her. Finally, I thank my gracious God for walking with me during these past few years, for guiding me, and showing me how to be more than I ever thought I could be.

EXAMINATION OF *CAMPYLOBACTER JEJUNI* PUTATIVE ADHESINS LEADS TO THE IDENTIFICATION OF A NEW PROTEIN, DESIGNATED FLPA, REQUIRED FOR CHICKEN COLONIZATION

Abstract

by Rebecca Catherine Flanagan, M.S.
Washington State University
May 2009

Chair: Michael E. Konkel

Campylobacter jejuni colonization of chickens is presumably dependent upon multiple surface exposed proteins termed adhesins. Putative *C. jejuni* adhesins include CadF, CapA, JlpA, MOMP, PEB1, Cj1279c, and Cj1349c. We examined the genetic relatedness of ninety-seven *C. jejuni* isolates recovered from human, poultry, bovine, porcine, ovine, and canine sources by multilocus sequence typing (MLST) and examined their profile of putative adhesin-encoding genes by dot blot hybridization. To assess the individual contribution of each protein in bacteria-host cell adherence, the *C. jejuni* genes encoding the putative adhesins were disrupted by insertional mutagenesis. The phenotype of each mutant was judged by performing *in vitro* cell adherence assays with chicken LMH hepatocellular carcinoma epithelial cells and *in vivo* colonization assays with broiler chicks. MLST analysis indicated that the *C. jejuni* isolates utilized in this study were genetically diverse. Dot blot hybridization revealed that the *C. jejuni* genes encoding the putative adhesins, with the exception of *capA,* were conserved amongst isolates. The *C. jejuni* CadF, CapA, Cj1279c, and Cj1349c proteins were found to play a significant role in the bacterium's *in vitro* adherence to chicken epithelial cells, while

CadF, PEB1, and Cj1279c were determined to play a significant role in the bacterium's *in vivo* colonization of broiler chicks. Because Cj1279c promotes the binding of *C. jejuni* to host cells, plays a significant role in *C. jejuni* colonization of chickens, and harbors fibronectin type III domains, we have termed the product encoded by the *Cj1279c* gene FlpA for Fibronectin-like protein A.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS……………………………………………………………iii

ABSTRACT…………………………...………………………………………iv

LIST OF TABLES…………………………………………………………...viii

LIST OF FIGURES………………………………………………………..….ix

CHAPTER

1. INTRODUCTION……………………………………………………...1

    1.1 Background………………………………………………………1

    1.2 Virulence factors…………………………………………………2

    1.3 Putative *C. jejuni* adhesins……………………………………….5

    1.4 References………………………………………………………11

2. EXAMINATION OF *CAMPYLOBACTER JEJUNI* PUTATIVE ADHESINS LEADS TO THE IDENTIFICATION OF A NEW PROTEIN, DESIGNATED FLPA, REQUIRED FOR CHICKEN COLONIZATION

    2.1 Abstract…………………………………………………………20

    2.2 Introduction……………………………………………………..21

    2.3 Materials and Methods………………………………………….24

    2.4 Results…………………………………………………………..32

    2.5 Discussion………………………………………………………36

    2.6 Acknowledgements……………………………………………..42

    2.6 References………………………………………………………43

    2.7 Tables…………………………………………………………...49

    2.8 Figures…………………………………………….……54

3. Future Directions……………………………………………………62

LIST OF TABLES

Table 1. Genes targeted for mutagenesis……………………………………………….49

Table 2. *C. jejuni* genes encoding putative adhesins……………………………………50

Supplemental Table 1. MLST alleles and sequence types of *C. jejuni* isolates……….51

LIST OF FIGURES

Figure 1. MLST analysis of the *C. jejuni* strains used in this study……………………..55

Figure 2. CadF, CapA, Cj1279c, and Cj1349c contribute to *C. jejuni* adherence to LMH cells……………………………………………………………………………………57

Figure 3. CadF, PEB1, and Cj1279c contribute to *C. jejuni* colonization of broiler chickens……………………………………………………………………………….59

Figure 4. Cj1279c is required for efficient cell adherence and chicken colonization…..61

Dedication

In memory of Lynne Winter and every woman

who has ever fought ovarian cancer

Chapter 1

Introduction

1.1 Background

Campylobacter jejuni is a Gram-negative, spiral-shaped, microaerophilic bacterium, which is motile via a bipolar or unipolar flagellum. It is the leading cause of bacterial induced diarrhea in the United States, resulting in an estimated 2.4 million cases annually, and a significant cause of gastroenteritis throughout the world accounting for 5 – 14% of diarrheal illnesses worldwide (2). Experimental *C. jejuni* infections in humans have revealed that as few as 800 bacteria can cause human illness (5). Campylobacteriosis (*C. jejuni*-mediated gastroenteritis) is a self limiting disease, occurring 2 – 5 days upon ingestion of the organism. This disease is characterized by fever, nausea, malaise, abdominal pain, and mild to severe diarrhea, which may contain blood and/or fecal leukocytes (6). Symptoms generally resolve within 10 days. In severe cases antibiotics, such as erythromycin and tetracycline, are used to treat *C. jejuni* infections (1).

In rare cases, *C. jejuni* is associated with serious sequelae such Guillain-Barré syndrome (GBS), an acute autoimmune disease affecting the peripheral nervous system. *C. jejuni* is thought to elicit GBS because *C. jejuni* lipooligosaccharide contains ganglioside-like epitopes which induces an autoimmune response leading to demylination of the peripheral nerves. Guillain-Barré syndrome usually resolves within a few weeks to months, although 15 – 20% of these cases develop into severe neurological paralysis

(54). *C. jejuni* has also been linked to Miller Fisher syndrome, a variant of GBS, as well as inflammatory disorders such as reactive arthritis and myocarditis (39, 50).

C. jejuni infection frequently occurs through the ingestion of *C. jejuni* in undercooked chicken or from the consumption of food products cross-contaminated with raw poultry. Disease may also be contracted via contaminated milk or water. This infection is linked to poultry due to the fact that by 2 to 3 weeks of age most commercial chickens become commensally colonized by as many as 10^8 CFU of *C. jejuni* per gram of cecal contents (47). Consequently, *Campylobacter* organisms are frequently recovered from processed broiler carcasses (51).

1.2 Virulence Factors

C. jejuni virulence is attributed to several properties, including: motility, chemotaxis, adherence, and invasion. After ingestion, *C. jejuni* passes through the stomach to the lumen of the small intestine where it adheres to and colonizes the jejunum and ileum, followed by the colon. As *C. jejuni* enters into the small intestine it is propelled through a layer of viscous mucous towards the intestinal crypts via its flagellum. Upon entering the intestinal crypts, it is proposed that *C. jejuni* translocates from the apical to the basal lateral surface via paracellular or transcellular routes and then attaches to host cell receptors via constitutively expressed proteins referred to as *C. jejuni* adhesins (27).

C. jejuni is characterized by rapid, darting motility via polar flagellum located at either a single or both ends of the organism. The *C. jejuni* flagellum is composed of the basal

body, hook, and filament; the filament is comprised of two highly homologous proteins termed FlaA and FlaB, FlaA being the more highly expressed component (19). In a previous study, Newell *et al.* (40) demonstrated that a non-motile *C. jejuni* isolate failed to colonize the intestinal tract of mice. Also, in a human volunteer study, Black *et al.* was unable to recover non-motile phase variant *C. jejuni* isolates from human stools (5). Secretion of the Cias [*Campylobacter* invasion antigens], which contribute to *C. jejuni* internalization, is dependent on a minimal flagellar apparatus composed of FlaB. As well, the *C. jejuni* flagellum serves in autoagglutination, biofilm formation, and evasion of the immune system (18).

Chemotaxis is defined as the movement of an organism towards or away from a chemical stimulus. *C. jejuni* researchers have demonstrated that *C. jejuni* chemotactic null mutants, *cheY* (53) and *cheA* (8), fail to colonize mice. Hence, chemotaxis appears to play a role in directing *C. jejuni* to host epithelial cells for attachment and colonization.

Extensive studies indicate that the ability of *C. jejuni* to invade the intestinal mucosa plays a significant role in campylobacteriosis. Newell *et al*. (41) found that clinical *C. jejuni* isolates are more invasive than environmental isolates. In corroboration, Everest *et al.* (12) found that *C. jejuni* isolates from individuals with colitis were more invasive than those isolated from individuals with non-inflammatory diarrhea. Numerous studies have attempted to elucidate the role of microfilaments and microtubules in *C. jejuni* invasion, yet the role of each cellular component remains inconclusive. Previous *C. jejuni* studies indicate that invasion is dependent upon microtubules and/or microfilaments (5, 37, 43).

Oelschlaeger *et al.* (42) and Hu and Kopecko (42) demonstrate that *C. jejuni* internalization exploits microfilaments while Monteville *et al.* (36) found that internalization involves both the microtubules and microfilaments. Invasion is also accompanied by alteration of the host cytoskeleton. Recently, activation of the small Rho GTPases Rac1 and Cdc42 was indicated to be required for *C. jejuni* internalization (28).

Bacterial adherence is crucial to host colonization as it prevents host mediated mechanical forces, such as peristalsis, from clearing the bacterium and facilitates infection. Fauchere *et al***.** (13) demonstrated that adherent *C. jejuni* strains recovered from infected individuals were more likely to cause diarrhea and fever in comparison to poorly adherent *C. jejuni* strains that fail to cause campylobacteriosis. Additionally, a previous study demonstrated that a characterized *C. jejuni* outer membrane protein termed, CadF [*Campylobacter* adhesin to Fibronectin (Fn)], is required for colonization of Leghorn chickens (55). Although the mechanism of *C. jejuni* adherence remains to be fully elucidated, researchers have identified several outer membrane *C. jejuni* molecules that bind to cultured epithelial cells. These molecules termed adhesins include: CadF, CapA, JlpA, MOMP, and PEB1. Other *C. jejuni* outer membrane molecules such as flagellum, lipooligosaccharide and P95 are proposed to play a role in *C. jejuni* association to host cells, however additional studies are required to confirm their role in adherence.

1.3 Putative *C. jejuni* Adhesins

CadF

Konkel *et al.* (25) identified the 37 kDa Fn binding protein, CadF. CadF, an extensively studied adhesin, facilitates *C. jejuni* adherence to Fn; Fn is a 250 kDa glycoprotein found in the extracellular matrix and regions of cell-to-cell contact. In corroboration, binding to Fn has been suggested to be a crucial means of host cell adherence for many pathogens such as *Staphylococcus* and *Streptococcus* species (49). *C. jejuni cadF* mutants show a 50% reduction in adherence to human INT 407 intestinal cells relative to a *C. jejuni* wild-type isolate. Additionally, a *C. jejuni cadF* mutant fails to increase the level of tyrosine phosphorylation of INT 407 cells in relation to the wild-type bacteria, indicating that CadF plays a role in alteration of host cell signaling events that possibly lead to cytoskeletal rearrangements (36).

CapA

Ashgar *et al.* (4) identified a putative autotransporter *in silico*, which they termed *Campylobacter* adhesion protein A (CapA). *C. jejuni capA* is a contingency gene, where expression of the functional protein is dependent upon frameshifts within a homopolymeric nucleotide tract located near the 5' end of the *capA* coding region. *C. jejuni capA* was detected in eleven out of twenty *C. jejuni* isolates via real-time PCR and both Southern and Western blotting. Western blotting of *C. jejuni* outer membrane-enriched fractions, as well as immunogold labeling, localized CapA to the bacterial cell surface. Furthermore, a mutant *C. jejuni capA* strain demonstrated decreased

colonization efficiency in chickens as well as a reduction in association to Caco-2 cells (4).

JlpA

Jin *et al.* (20) identified a *C. jejuni* 43.2 kDa protein which they termed JlpA, jejuni lipoprotein A. They localized JlpA to the inner membrane by sarcosyl and Triton X-100 extractions, however JlpA was predominately found in immunoblotted glycine extracts and completely digested by proteinase K, suggesting that JlpA is loosely associated with the *C. jejuni* outer membrane. Disruption of *jlpA* reduces *C. jejuni* adherence to HEp-2 cells by 18 to 19.4% relative to the wild-type strain, but has no effect on *C. jejuni* invasion (20). Further studies demonstrated that JlpA interacts with human heat shock protein (HSP) 90 α on the surface of a HEp-2 cell as *C. jejuni* adherence to HEp-2 cells was reduced four-fold when treated with anti-Hsp90α in comparison to the *C. jejuni jlpA* mutant strain. Finally, the binding of Hsp90α by JlpA induced the pro-inflammatory response in host cells (the activation NF-κB and phosphorylation of IκBα and p38) (21).

MOMP

The *C. jejuni* 43 kDa pore forming protein termed the major outer membrane protein (MOMP), also referred to as *porA,* allows the passage of hydrophilic molecules across the *C. jejuni* outer membrane barrier and provides structural stability to the outer membrane (3, 7). MOMP has also been characterized as an adhesin by Schroeder and Moser (38). Outer membrane sarcosyl extracts and truncated MOMP fragments isolated from proteinase K treated cells localized MOMP to the outer membrane. Sequencing of

the truncated MOMP fragments revealed that 167 N-terminal *porA* residues had been truncated indicating that the C-terminal portion of MOMP may be anchored in the *C. jejuni* outer membrane. MOMP was proposed to be an adhesin because electroeluted MOMP extracts isolated from outer membrane preparations bound INT 407 cells (48). *C. jejuni porA* mutants have yet to be characterized, as mutations in *porA* are lethal to *C. jejuni* due to the critical structural and pore functions MOMP plays.

PEB1

Pei *et al*. (46) purified a 28 kDa *C. jejuni* protein, termed PEB1, and showed that the convalescent sera of *C. jejuni* infected patients recognized this protein. However, immunogold labeling poorly localized PEB1 to the *C. jejuni* outer membrane. Disruption of *peb1A* reduces *C. jejuni* adherence to HeLa cells by 50- to 100-fold and decreases invasion by 15-fold (45). On the other hand, PEB1 protein sequence shows homology to amino acid ABC transport systems in Gram-negative organisms. Signal peptidase I and II cleavage sites were localized to the first 26 amino acid residues of PEB1, suggesting it is exported across the inner and outer membranes (44). Recently, Leon-Kempis *et al.* (31) found that mutation of *Cj0921c*, encoding *peb1A*, impairs L-aspartate and L-glutamate transport and prevents microaerophilic growth. SDS-PAGE and Western blotting of acid-glycine extracts as well as concentrated culture supernatant localized PEB1 to the periplasm and supernatant, suggesting that PEB1 may be secreted across the outer membrane; it was not found to be surface exposed. Consequently, the role of PEB1 as a *C. jejuni* adhesin remains controversial.

Cj1279c and Cj1349c

Cj1279c and Cj1349c are reported to contain Fn Type III domains and to act as a Fn/fibrinogen-binding protein, respectively (http://www.microbesonline.org/, VIMSS ID, 47155 and 47224, respectively). Due to the significant role that the *C. jejuni* fibronectin binding protein CadF plays in host cell association (14), Cj1279c and Cj1349c were included in this study.

P95

C. jejuni P95 was identified by Southern hybridization using degenerate oligonucleotide probes homologous to short amino acid sequences from other Gram-negative bacteria (22). A BLAST search of the P95 amino acid sequence revealed homology with the high-molecular-weight surface exposed adhesins 1 and 2 from *Haemophilus influenzae.* However, the characterization of a *C. jejuni p95* mutant and its localization to the *C. jejuni* outer membrane have yet to be completed. Consequently, additional studies of P95 are required to establish its role in *C. jejuni* adherence.

Flagellin

McSweegan and Walker (33) demonstrated that purified *C. jejuni* flagella bound human INT 407 intestinal cells. As well, Moser *et al.* (37) reported that *C. jejuni* flagella-enriched outer membrane fractions bound INT 407 membrane fractions. On the other hand, Wassenaar *et al.* (52) found that purified flagellin does not competitively inhibit *C. jejuni* binding to INT 407 cells. Hence, further study is required to elucidate the role of flagellin in *C. jejuni*-host cell association.

Lipopolysaccharide

A previous study by McSweegan and Walker (33) suggests that *C. jejuni* lipopolysaccharide (LPS) plays a role in epithelial cell association, as radioactively labeled LPS bound to INT 407 cells and reduced *C. jejuni* adherence to host epithelial cells. However, these data are difficult to interpret because the methods used by McSweegan and Walker likely contaminated the *C. jejuni* LPS with capsular polysaccharide. Consequently, the role of LPS in *C. jejuni* host cell association remains to be determined.

Pilus-Like Appendages

Doig *et al.* (11) observed pilus-like appendages when *C. jejuni* were cultured in medium supplemented with deoxycholate. The appendages were 4-7 nm in diameter and greater than 1 µm in length. Partial sequence analysis of the *C. jejuni* region involving flagellar expression suggested the presence of a pilus encoding gene they termed *pspA* (pilus-synthesis protease). *C. jejuni pspA* mutants were non-piliated relative to the wild-type when grown in the presence of deoxycholate. However, later studies by Gaynor *et al.* (15) showed that these 'pilus-like' appendages were artifacts induced by the culture conditions. These pilus-like appendages were observed, under an electron microscope, in uninoculated growth medium supplemented with deoxycholate as well as medium innoculated with a *C. jejuni pspA* mutant strain. Consequently, while *C. jejuni* may possess pilus-like appendages, such structures remain undocumented.

Purpose of the Study

While *C. jejuni* appears to posses many putative and characterized adhesins, the adhesins have been studied in varying *in vitro* cell lines and *C. jejuni* strains. Consequently it is difficult to interpret the cumulative role that the *C. jejuni* adhesins play in host cell adherence. Additionally, the role of the adhesins *in vivo* has been studied in varying animal models and breeds of chickens, if examined at all. The goal of the following study was to determine the role of the adhesins to adherence *in vitro* using a single *C. jejuni* strain and single epithelial cell line, and to determine their *in vivo* role in host colonization using broiler chicks.

References

1. **Allos, B. M.** 2001. *Campylobacter jejuni* Infections: update on emerging issues and trends. Clin. Infect. Dis. **32:**1201-1206.
2. **Altekruse, S. F., N. J. Stern, P. I. Fields, and D. L. Swerdlow.** 1999. *Campylobacter jejuni*--an emerging foodborne pathogen. Emerg. Infect. Dis. **5:**28-35.
3. **Amako, K., S. N. Wai, A. Umeda, M. Shigematsu, and A. Takade.** 1996. Electron microscopy of the major outer membrane protein of *Campylobacter jejuni*. Microbiol. Immunol. **40:**749-754.
4. **Ashgar, S. S., N. J. Oldfield, K. G. Wooldridge, M. A. Jones, G. J. Irving, D. P. Turner, and D. A. Ala'Aldeen.** 2007. CapA, an autotransporter protein of *Campylobacter jejuni*, mediates association with human epithelial cells and colonization of the chicken gut. J. Bacteriol. **189:**1856-1865.
5. **Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser.** 1988. Experimental *Campylobacter jejuni* infection in humans. J. Infect. Dis. **157:**472-479.
6. **Blaser, M. J., I. D. Berkowitz, F. M. LaForce, J. Cravens, L. B. Reller, and W. L. Wang.** 1979. Campylobacter enteritis: clinical and epidemiologic features. Ann. Intern. Med. **91:**179-185.
7. **Bolla, J. M., E. Loret, M. Zalewski, and J. M. Pages.** 1995. Conformational analysis of the *Campylobacter jejuni* porin. J. Bacteriol. **177:**4266-4271.
8. **Chang, C., and J. F. Miller.** 2006. *Campylobacter jejuni* colonization of mice with limited enteric flora. Infect. Immun. **74:**5261-5271.

9. **Colles, F. M., K. Jones, R. M. Harding, and M. C. Maiden.** 2003. Genetic diversity of *Campylobacter jejuni* isolates from farm animals and the farm environment. Appl. Environ. Microbiol. **69:**7409-7413.

10. **Dingle, K. E., F. M. Colles, D. R. Wareing, R. Ure, A. J. Fox, F. E. Bolton, H. J. Bootsma, R. J. Willems, R. Urwin, and M. C. Maiden.** 2001. Multilocus sequence typing system for *Campylobacter jejuni*. J. Clin. Microbiol. **39:**14-23.

11. **Dolg, P., R. Yao, D. H. Burr, P. Guerry, and T. J. Trust.** 1996. An environmentally regulated pilus-like appendage involved in *Campylobacter* pathogenesis. Mol. Microbiol. **20:**885-894.

12. **Everest, P. H., H. Goossens, J. P. Butzler, D. Lloyd, S. Knutton, J. M. Ketley, and P. H. Williams.** 1992. Differentiated Caco-2 cells as a model for enteric invasion by *Campylobacter jejuni* and *C. coli.* J. Med. Microbiol. **37:**319-325.

13. **Fauchere, J. L., A. Rosenau, M. Veron, E. N. Moyen, S. Richard, and A. Pfister.** 1986. Association with HeLa cells of *Campylobacter jejuni* and Campylobacter coli isolated from human feces. Infect. Immun. **54:**283-287.

14. **Fouts, D. E., E. F. Mongodin, R. E. Mandrell, W. G. Miller, D. A. Rasko, J. Ravel, L. M. Brinkac, R. T. DeBoy, C. T. Parker, S. C. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, S. A. Sullivan, J. U. Shetty, M. A. Ayodeji, A. Shvartsbeyn, M. C. Schatz, J. H. Badger, C. M. Fraser, and K. E. Nelson.** 2005. Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter* species. PLoS Biol. **3:**e15.

15. **Gaynor, E. C., N. Ghori, and S. Falkow.** 2001. Bile-induced 'pili' in *Campylobacter jejuni* are bacteria-independent artifacts of the culture medium. Mol. Microbiol. **39:**1546-1549.

16. **Goulhen, F., E. De, J. M. Pages, and J. M. Bolla.** 2004. Functional refolding of the *Campylobacter jejuni* MOMP (major outer membrane protein) porin by GroEL from the same species. Biochem .J. **378:**851-856.

17. **Guccione, E., R. Leon-Kempis Mdel, B. M. Pearson, E. Hitchin, F. Mulholland, P. M. van Diemen, M. P. Stevens, and D. J. Kelly.** 2008. Amino acid-dependent growth of *Campylobacter jejuni*: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. Mol. Microbiol. **69:**77-93.

18. **Guerry, P.** 2007. *Campylobacter* flagella: not just for motility. Trends Microbiol. **15:**456-461.

19. **Hendrixson, D. R., B. J. Akerley, and V. J. DiRita.** 2001. Transposon mutagenesis of *Campylobacter jejuni* identifies a bipartite energy taxis system required for motility. Mol. Microbiol. **40:**214-224.

20. **Jin, S., A. Joe, J. Lynett, E. K. Hani, P. Sherman, and V. L. Chan.** 2001. JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni,* mediates adherence to host epithelial cells. Mol. Microbiol. **39:**1225-1236.

21. **Jin, S., Y. C. Song, A. Emili, P. M. Sherman, and V. L. Chan.** 2003. JlpA of *Campylobacter jejuni* interacts with surface-exposed heat shock protein 90α and triggers signaling pathways leading to the activation of NF-kB and p38 MAP kinase in epithelial cells. Cell Microbiol. **5:**165-174.

22. **Kelle, K., J. M. Pages, and J. M. Bolla.** 1998. A putative adhesin gene cloned from *Campylobacter jejuni*. Res. Microbiol. **149:**723-733.

23. **Kervella, M., J. M. Pages, Z. Pei, G. Grollier, M. J. Blaser, and J. L. Fauchere.** 1993. Isolation and characterization of two *Campylobacter* glycine-extracted proteins that bind to HeLa cell membranes. Infect. Immun. **61:**3440-3448.

24. **Konkel, M. E., J. E. Christensen, A. M. Keech, M. R. Monteville, J. D. Klena, and S. G. Garvis.** 2005. Identification of a fibronectin-binding domain within the *Campylobacter jejuni* CadF protein. Mol. Microbiol. **57:**1022-1035.

25. **Konkel, M. E., S. G. Garvis, S. L. Tipton, D. E. Anderson, Jr., and W. Cieplak, Jr.** 1997. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. Mol. Microbiol. **24:**953-963.

26. **Konkel, M. E., S. A. Gray, B. J. Kim, S. G. Garvis, and J. Yoon.** 1999. Identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* based on the cadF virulence gene and its product. J. Clin. Microbiol **37:**510-517.

27. **Konkel, M. E., M. R. Monteville, V. Rivera-Amill, and L. A. Joens.** 2001. The pathogenesis of *Campylobacter jejuni*-mediated enteritis. Curr. Issues Intest. Microbiol. **2:**55-71.

28. **Krause-Gruszczynska, M., M. Rohde, R. Hartig, H. Genth, G. Schmidt, T. Keo, W. Konig, W. G. Miller, M. E. Konkel, and S. Backert.** 2007. Role of the

small Rho GTPases Rac1 and Cdc42 in host cell invasion of *Campylobacter jejuni*. Cell Microbiol. **9:**2431-2444.

29. **Kwan, P. S., M. Barrigas, F. J. Bolton, N. P. French, P. Gowland, R. Kemp, H. Leatherbarrow, M. Upton, and A. J. Fox.** 2008. Molecular epidemiology of *Campylobacter jejuni* populations in dairy cattle, wildlife, and the environment in a farmland area. Appl. Environ. Microbiol. **74:**5130-5138.
30. **Labigne-Roussel, A., J. Harel, and L. Tompkins.** 1987. Gene transfer from *Escherichia coli* to *Campylobacter* species: development of shuttle vectors for genetic analysis of *Campylobacter jejuni*. J. Bacteriol. **169:**5320-5323.
31. **Leon-Kempis Mdel, R., E. Guccione, F. Mulholland, M. P. Williamson, and D. J. Kelly.** 2006. The *Campylobacter jejuni* PEB1a adhesin is an aspartate/glutamate-binding protein of an ABC transporter essential for microaerobic growth on dicarboxylic amino acids. Mol. Microbiol. **60:**1262-1275.
32. **Levesque, S., E. Frost, R. D. Arbeit, and S. Michaud.** 2008. Multilocus sequence typing of *Campylobacter jejuni* isolates from humans, chickens, raw milk, and environmental water in Quebec, Canada. J. Clin. Microbiol. **46:**3404-3411.
33. **McSweegan, E., and R. I. Walker.** 1986. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. Infect. Immun. **53:**141-148.
34. **Miller, W. G., S. L. On, G. Wang, S. Fontanoz, A. J. Lastovica, and R. E. Mandrell.** 2005. Extended multilocus sequence typing system for *Campylobacter coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*. J. Clin. Microbiol. **43:**2315-2329.

35. **Monteville, M. R., and M. E. Konkel.** 2002. Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. Infect. Immun. **70:**6665-6671.

36. **Monteville, M. R., J. E. Yoon, and M. E. Konkel.** 2003. Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization. Microbiology **149:**153-165.

37. **Moser, I., W. F. Schroder, and E. Hellmann.** 1992. In vitro binding of *Campylobacter jejuni/coli* outer membrane preparations to INT 407 cell membranes. Med. Microbiol. Immunol. **180:**289-303.

38. **Moser, I., W. Schroeder, and J. Salnikow.** 1997. *Campylobacter jejuni* major outer membrane protein and a 59-kDa protein are involved in binding to fibronectin and INT 407 cell membranes. FEMS Microbiol. Lett. **157:**233-238.

39. **Nachamkin, I.** 2002. Chronic effects of *Campylobacter* infection. Microbes. Infect. **4:**399-403.

40. **Newell, D. G.** 1986. Monoclonal antibodies directed against the flagella of *Campylobacter jejuni*: production, characterization and lack of effect on the colonization of infant mice. J. Hyg. (Lond) **96:**131-141.

41. **Newell, D. G., H. McBride, F. Saunders, Y. Dehele, and A. D. Pearson.** 1985. The virulence of clinical and environmental isolates of *Campylobacter jejuni*. J. Hyg. (Lond) **94:**45-54.

42. **Oelschlaeger, T. A., P. Guerry, and D. J. Kopecko.** 1993. Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. Proc. Natl. Acad. Sci. U S A **90:**6884-6888.

43. **Oyarzabal, O. A., K. S. Macklin, J. M. Barbaree, and R. S. Miller.** 2005. Evaluation of agar plates for direct enumeration of *Campylobacter* spp. from poultry carcass rinses. Appl. Environ. Microbiol. **71:**3351-3354.

44. **Pei, Z., and M. J. Blaser.** 1993. PEB1, the major cell-binding factor of *Campylobacter jejuni*, is a homolog of the binding component in gram-negative nutrient transport systems. J. Biol. Chem. **268:**18717-18725.

45. **Pei, Z., C. Burucoa, B. Grignon, S. Baqar, X. Z. Huang, D. J. Kopecko, A. L. Bourgeois, J. L. Fauchere, and M. J. Blaser.** 1998. Mutation in the *peb1A* locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. Infect. Immun. **66:**938-943.

46. **Pei, Z. H., R. T. Ellison, 3rd, and M. J. Blaser.** 1991. Identification, purification, and characterization of major antigenic proteins of *Campylobacter jejuni.* J. Biol. Chem. **266:**16363-16369.

47. **Sahin, O., T. Y. Morishita, and Q. Zhang.** 2002. *Campylobacter* colonization in poultry: sources of infection and modes of transmission. Anim. Health Res. Rev. **3:**95-105.

48. **Schroder, W., and I. Moser.** 1997. Primary structure analysis and adhesion studies on the major outer membrane protein of *Campylobacter jejuni*. FEMS Microbiol. Lett. **150:**141-147.

49. **Schwarz-Linek, U., M. Hook, and J. R. Potts.** 2004. The molecular basis of fibronectin-mediated bacterial adherence to host cells. Mol. Microbiol. **52:**631-641.

50. **Schwerer, B.** 2002. Antibodies against gangliosides: a link between preceding infection and immunopathogenesis of Guillain-Barre syndrome. Microbes. Infect. **4:**373-384.

51. **Stern, N. J., and S. Pretanik.** 2006. Counts of *Campylobacter* spp. on U.S. broiler carcasses. J. Food Prot. **69:**1034-1039.

52. **Wassenaar, T. M., N. M. Bleumink-Pluym, and B. A. van der Zeijst.** 1991. Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that flaA but not flaB is required for invasion. Embo. J. **10:**2055-2061.

53. **Yao, R., D. H. Burr, and P. Guerry.** 1997. CheY-mediated modulation of *Campylobacter jejuni* virulence. Mol. Microbiol. **23:**1021-1031.

54. **Young, V. B., and L. S. Mansfield.** 2005. *Campylobacter* infection - Clinical context, p. 1-12. *In* J. M. Ketley and M. E. Konkel (ed.), *Campylobacter*: Molecular and Cellular Biology. Horizon Bioscience.

55. **Ziprin, R. L., C. R. Young, L. H. Stanker, M. E. Hume, and M. E. Konkel.** 1999. The absence of cecal colonization of chicks by a mutant of *Campylobacter jejuni* not expressing bacterial fibronectin-binding protein. Avian Dis. **43:**586-589.

Examination of *Campylobacter jejuni* putative adhesins leads to the identification of a new protein, designated FlpA, required for chicken colonization

Rebecca C. Flanagan,[1] Jason M. Neal-McKinney,[1] A. Singh Dhillon,[2] William G. Miller,[3] and Michael E. Konkel[1]

[1]Department of Microbiology, School of Molecular Biosciences, Washington State University, Pullman, WA; [2]Washington State University Avian Health and Food Safety Laboratory, College of Veterinary Medicine, Washington State University, Puyallup, WA; [3]USDA, Western Regional Research Center, Albany, CA.

Running title: *C. jejuni* adhesins

Key words: bacteria-host cell interactions, pathogenesis, virulence determinants, adhesion, cell attachment

*For correspondence. E-mail: konkel@wsu.edu; Tel: (+1) 509 335 5039; Fax: (+1) 509 335 1907.

ABSTRACT

Campylobacter jejuni colonization of chickens is presumably dependent upon multiple surface exposed proteins termed adhesins. Putative *C. jejuni* adhesins include CadF, CapA, JlpA, MOMP, PEB1, Cj1279c, and Cj1349c. We examined the genetic relatedness of 97 *C. jejuni* isolates recovered from human, poultry, bovine, porcine, ovine, and canine sources by multilocus sequence typing (MLST) and examined their profile of putative adhesin-encoding genes by dot blot hybridization. To assess the individual contribution of each protein in bacteria-host cell adherence, the *C. jejuni* genes encoding the putative adhesins were disrupted by insertional mutagenesis. The phenotype of each mutant was judged by performing *in vitro* cell adherence assays with chicken LMH hepatocellular carcinoma epithelial cells and *in vivo* colonization assays with broiler chicks. MLST analysis indicated that the *C. jejuni* isolates utilized in this study were genetically diverse. Dot blot hybridization revealed that the *C. jejuni* genes encoding the putative adhesins, with the exception of *capA,* were conserved amongst the isolates. The *C. jejuni* CadF, CapA, Cj1279c, and Cj1349c proteins were found to play a significant role in the bacterium's *in vitro* adherence to chicken epithelial cells, while CadF, PEB1, and Cj1279c were determined to play a significant role in the bacterium's *in vivo* colonization of broiler chicks. Collectively, the data indicate that Cj1279c is a novel adhesin. Because Cj1279c harbors fibronectin type III domains, we designated the protein FlpA for Fibronectin-like protein A.

INTRODUCTION

Campylobacter jejuni is a Gram-negative, spiral-shaped, microaerophilic bacterium, which is motile via a bipolar or unipolar flagellum. This organism is one of the leading bacterial causes of diarrhea in the United States, and accounts for 5 to 14% of diarrhea worldwide (2). Experimental *C. jejuni* infections in humans have revealed that as few as 800 bacteria can cause human illness (5). Campylobacteriosis (*C. jejuni* mediated gastroenteritis) generally occurs 2 – 5 days after ingestion of the bacterium, and is a self-limiting infection. This disease is characterized by fever, nausea, malaise, abdominal pain, and loose to watery stools, which may contain blood and/or fecal leukocytes (6). *C. jejuni* infections can result in several serious sequelae, including Guillain-Barré syndrome, an acute autoimmune disease affecting the peripheral nervous system (54).

C. jejuni infection frequently occurs through the ingestion of *C. jejuni* in undercooked chicken or from the consumption of food products cross-contaminated with raw poultry. This infection is linked to poultry due to the fact that by 2 to 3 weeks of age most commercial chickens become commensally colonized by as many as 10^8 CFU of *C. jejuni* per gram of cecal contents (47). Not surprisingly, *Campylobacter* organisms are frequently recovered from processed broiler carcasses (51). Bacterial adherence to host epithelial cells appears to be crucial for *C. jejuni* colonization of chickens, as it may prevent host mediated mechanical forces such as peristalsis from clearing the bacterium. Previous work has revealed that one *C. jejuni* adhesin, termed CadF [*Campylobacter* adhesion to fibronectin (Fn)], is required to colonize Leghorn chickens (55).

Researchers have identified a number of *C. jejuni* proteins that bind to cultured epithelial cells. These adhesive proteins include: CadF, CapA, JlpA, MOMP, and PEB1. Konkel *et al.* (25) identified CadF, which is a 37 kDa Fn-binding protein. CadF facilitates *C. jejuni* adherence to Fn, which is a ubiquitous ~ 250 kDa glycoprotein found in the extracellular matrix and regions of cell-to-cell contact. A *C. jejuni cadF* mutant shows a 50% reduction in adhesion to human INT 407 intestinal cells when compared to a *C. jejuni* wild-type isolate (36). Ashgar *et al.* (4) identified a putative autotransporter *in silico*, which was termed CapA, for *Campylobacter* adhesion protein A. *C. jejuni capA* is a contingency gene, where expression of the functional protein is dependent upon frameshifts within a homopolymeric nucleotide tract located near the 5' end of the *capA* coding region. A *C. jejuni capA* mutant demonstrated a reduction in adherence to human Caco-2 colorectal adenocarcinoma epithelial cells and a decrease in colonization efficiency of chickens. Jin *et al*. (20) identified JlpA, jejuni lipoprotein A, which is a 43 kDa protein. Disruption of *jlpA* reduces *C. jejuni* adherence to human HEp-2 epithelial cells by 18 to 19.4% relative to the wild-type strain. Moser *et al*. {Moser, 1997 #19} reported that the major outer membrane protein (MOMP), encoded by *porA*, bound to INT 407 cells. The *C. jejuni* 43 kDa MOMP is also known to allow passage of hydrophilic molecules across the outer membrane and to provide structural stability to the outer membrane (7, 16). *C. jejuni porA* mutants have yet to be characterized, as mutations in *porA* are presumably lethal due to the critical structural and transport functions of the MOMP. Kervella *et al*. (23) characterized PEB1, a *C. jejuni* 28 kDa putative adhesin. Disruption of *peb1A* reduces *C. jejuni* adherence to human HeLa epithelial cells by 50- to 100-fold; such mutants also failed to colonize the intestinal tract

of mice (45). In addition to the proteins listed above, which have been studied previously, Cj1279c and Cj1349c are reported to contain Fn type III domains and to act as a Fn/fibrinogen-binding protein, respectively (http://www.microbesonline.org/, VIMSS ID, 47155 and 47224, respectively). Due to the well-established role of CadF in *C. jejuni* cell adherence and chicken colonization, Cj1279c and Cj1349c were included in this study.

C. jejuni binding to host cells and colonization is a multifactorial process dependent on motility, chemotaxis, and the synthesis of multiple adhesins (27). The goal of this study was to evaluate the contribution of the CadF, CapA, JlpA, PEB1, MOMP, Cj1279c and Cj1349c proteins in *C. jejuni*-host cell interactions. Dot blot assays were used to assess the differences in the content of the putative adhesin encoding genes amongst the *C. jejuni* isolates. To assess the roles of the putative adhesins in promoting host cell binding and host colonization, we generated *C. jejuni* adhesin mutants via insertional mutagenesis and performed *in vitro* adherence and *in vivo* chicken colonization assays. To our knowledge, this is the first time the roles of these proteins have been compared in a single genetic background, and the first time that the functional role of many of these proteins has been examined in chickens (i.e., the natural host). We report that some of the *C. jejuni* proteins play a significant role in chicken colonization, while other proteins are not essential for colonization.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Ninety-seven *C. jejuni* isolates were obtained from human clinical cases, poultry, bovine, porcine (swine), ovine, and canine sources (Supplemental Table 1). All human isolates were obtained from individuals with clinical signs of campylobacteriosis. *C. jejuni* F38011 was isolated from an individual with bloody diarrhea. In total, we used 43 human strains (F38011, 81-176, 81116, M129, H1, H2, H4-7, H9-24, H26-32, and H34-43), 41 poultry strains (RM1221, Turkey, S1, S2B, USDA02-833L, A2a, A5a, A18a, D34a, G11a, Iowa 2, Iowa 4-9, Iowa 11-13, Iowa 15, Iowa 21-26, Iowa 33-36, Iowa 39, Iowa 42, Iowa 44, Iowa 77-81, and Iowa 83), five bovine strains (C913, C973, C1086, C1129, and C1144), five porcine strains (93-55, 93-58, 93-338, 93-343, and 92-1578), two ovine strains (ov48 and ov112), and one canine strain (can1979858). *C. jejuni* isolates were cultured at 37°C under microaerobic conditions (5% O_2, 10% CO_2, 85 % N_2) on Mueller-Hinton agar plates supplemented with 5% citrated bovine blood (MH-blood agar plates). *C. jejuni* strains were subcultured to a fresh plate every 48 h. The *C. jejuni* F38011 *cadF* (kanamycin resistant, Kan^R), *capA* (tetracycline resistant, Tet^R), *jlpA* (Kan^R), *peb1A* (Kan^R), *Cj1278c* (Tet^R), *Cj1279c* (Kan^R), and *Cj1349c* (Kan^R) mutants were generated as outlined below. When appropriate, the growth media were supplemented with antibiotics at the following concentrations: Kan, 50 µg/ml (Sigma, St. Louis, MO) and Tet, 2.0 µg/ml (Sigma).

Motility assay. Motility was determined using MH medium supplemented with 0.4% Select agar (Invitrogen, Carlsbad, CA). Briefly, 10µl of each bacterial suspension in MH broth was added to the surface of the agar and the plates were incubated at 37°C under

microaerobic conditions. Motility was determined by measuring the diameter of the bacterial migration zone after 48 h of incubation.

Multilocus sequence typing. Genomic DNA was isolated from the *C. jejuni* isolates using phenol chloroform extractions. Briefly, bacteria were cultured on MH-blood agar plates and harvested in 5 ml of phosphate-buffered saline (PBS). After incubation for 1 h at 37°C with 500 µl 10% sodium dodecyl sulfate (SDS) and 5 µl proteinase K (20 mg/ml), three phenol and isoamyl chloroform extractions (24 parts chloroform and 1 part isoamyl alcohol) were performed with the aqueous layer retained each time. An equal volume of cold isopropanol and 250 µl of 2.5 M sodium acetate were added to the aqueous layer, prior to incubation at -20°C for 5 min. The DNA was pelleted by centrifugation at 11,600 × *g* for 15 min. The pellet was washed with 70% ethanol, spun at 11,600 × *g* for 15 min, resuspended in sterile water, and RNase treated at 37°C for 1 h. DNA purity, using an OD_{260}/OD_{280} ratio, and concentration was determined using a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE).

C. jejuni housekeeping genes aspartase A (*aspA*), glutamine synthetase (*glnA),* citrate synthase (*gltA*), serine hydroxymethyl transferase (*glyA*), phosphoglucomutase (*pgm*), transketolase (*tkt*), and the ATP synthase alpha subunit (*uncA*) were amplified and sequenced, using the primers described elsewhere (34). PCR was performed using approximately 50 ng of genomic DNA and 1 U *Taq* polymerase (New England Biolabs, Beverly, MA) in a 50 µl reaction volume with 50 pmol of each primer, 1 × MasterAmp PCR buffer (Epicentre, Madison, WI), 1 × MasterAmp PCR enhancer (Epicentre), 2.5

mM $MgCl_2$, and 250 µM (each) dNTPs. Genes were amplified using the following amplification parameters: 94°C for 30 sec, 53°C for 30 sec, and 72°C for 2 min (30 cycles). Amplicons were confirmed by agarose gel electrophoresis and purified on a BioRobot 8000 workstation (Qiagen, Valencia, CA). Cycle sequencing reactions were performed on a Tetrad thermocycler (Bio-Rad, Hercules, CA), using the ABI BigDye terminator cycle sequencing kit (ver. 3.1; Applied Biosystems, Foster City, CA) and standard protocols. Cycle sequencing extension products were purified using DyeEx 96 plates (Qiagen). DNA sequencing was performed on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems), using POP-7 polymer and ABI data collection and sequencing analysis software. Nucleotide sequences were aligned and analyzed using Seqman Pro (ver. 7.2; DNASTAR, Madison, WI). Alleles and sequence types were assigned using MLSTparser3 (Miller *et al*., unpublished); novel alleles and sequence types were submitted to the PubMLST *C. jejuni*/*C. coli* database (http://pubmlst.org/campylobacter/).

Dot blot hybridization. The *C. jejuni* putative adhesin-encoding genes examined in this study were *porA, cadF, capA, jlpA, peb1A, Cj1279c*, and *Cj1349c*. The sequence of each gene from *C. jejuni* NCTC 11168 was obtained from on-line resources (http://www.sanger.ac.uk/Projects/C_jejuni/). Gene-specific probes were generated as outlined below. An internal fragment of each gene was amplified via PCR using the primers listed in Table 1. The amplifications were performed using high fidelity *Taq* DNA polymerase (Invitrogen) with *C. jejuni* NCTC 11168 chromosomal DNA as the template. Genes were amplified using the following parameters: 94°C for 2 min (1

cycle); 94°C for 45 sec, 60°C (-1°C per cycle) for 30 sec, 70°C for 1.5 min (10 cycles); 94°C for 45 sec, 50°C for 30 sec, 70°C for 1.5 min (25 cycles); 70°C for 8 min (1 cycle). The amplified PCR fragments were ligated into the vector pCR2.1 according to the manufacturer's directions (Original TA Cloning Kit, Invitrogen) and electroporated into *Escherichia coli* InvαF'. The purified plasmids were nick-translated using a Nick Translation Kit according to the manufacturer's directions (Roche Applied Science, Indianapolis, IN). One-hundred ng of *C. jejuni* genomic DNA, isolated via phenol chloroform extractions as described above, were vacuum transferred to a genescreen membrane (PerkinElmer, Waltham, MA) using a Schleicher and Schuell Minifold II Slot-Blotter (Jencons, United Kingdom). Depurinating solution (0.25 M HCl) was added to each slot for 4 min, followed by denaturating solution (1.5 M NaOH and 0.5 NaCl) for 3 min, neutralizating solution (1.0 M Tris and 1.5 M NaCl, pH 8.0) for 3 min, and 20 x SSC (3.0 M NaCl and 0.3 M sodium citrate) for 20 min. DNA was cross-linked to the membrane using a Gene Linker UV Chamber, according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

Each membrane was blocked for 15 min at room temperature with 100 μl denatured salmon sperm DNA in hybridization solution [5 ml formamide, 2 ml 5x P buffer (1.0% BSA, 1.0% polyvinyl-pyrrodilone, 1.0% Ficoll, 0.5% sodium pyrophosphate, 5.0% SDS, and 250 mM Tris pH 7.5), 2 ml 50% dextran sulfate, and 0.58 g NaCl] that had been warmed to 50°C. The radioactively-labeled probe was denatured by heating for 15 min at 95°C, chilled on ice for 15 min, and added to the hybridization solution. The membrane was incubated with the hybridization solution at 35°C in a hybridization incubator

(Robbins Scientific, Hudson, NH) overnight. Membranes were washed twice with 2x SSC at 25°C for 10 min, and twice with a 2x SSC and 1% SDS solution at 35°C for 20 min. Autoradiography was performed with Kodak BioMax MR film at -80°C for approximately 2 h.

Generation of *C. jejuni cadF, jlpA, peb1A, Cj1279c,* and *Cj1349c* suicide vectors. The PCR amplicons used as probes for the dot blot hybridizations were removed from the pCR2.1 multiple-cloning site (MCS) and ligated into pBSK-Kan2. The pBSK-Kan2 vector is identical to pBlueScript (Invitrogen), except that the original kanamycin cassette was replaced with one that functions in both *C. jejuni* and *E. coli* (30). The resulting pBSK-Kan2 vectors (pMEK252-pMEK256) were confirmed by DNA sequencing, and were electroporated into *E. coli* InvαF' electrocompetent cells.

Generation of *C. jejuni capA* and *Cj1278c* suicide vectors. DNA regions upstream and downstream of the *C. jejuni capA* and *Cj1278c* genes were amplified by PCR using *Taq* DNA Polymerase (Invitrogen) and the primers listed in Table 1. *C. jejuni* NCTC 11168 chromosomal DNA was used for the amplification of DNA regions flanking *capA,* while *C. jejuni* F38011 chromosomal DNA was used for the regions flanking *Cj1278c*. The reaction conditions were: 94°C for 2 min (1 cycle); 94°C for 45 sec, 63°C (-1°C per cycle) for 30 sec, 70°C for 4 min (8 cycles); 94°C for 45 sec, 50°C for 30 sec, 70°C for 4 min (25 cycles); 70°C for 8 min (1 cycle). The two flanking regions were cloned individually in pCR2.1. Thereafter, one fragment was cloned into the pCR2.1 vector harboring the other fragment, and a tetracycline resistance cassette was inserted between

the two flanking regions. The resulting fragment was then moved into the MCS of pBSK-Kan2. The mutation construct was verified by DNA sequencing.

Generation of *C. jejuni* F38011 mutants. *C. jejuni* F38011 was grown overnight in MH broth with shaking at 37°C under microaerobic conditions to a final OD_{540} of 1.0. Two-hundred ml of culture was centrifuged at 6,000 × *g* for 5 min to pellet the cells. The cells were washed once in sterile water and once in 10% glycerol, and resuspended in 350 µl of 10% glycerol. Approximately 2 µg of a CsCl-concentrated suicide vector was mixed with 50 µl of the electrocompetent *C. jejuni* and pulsed at 2.50 kV. The cells were immediately suspended in 200 µl of MH broth and plated on MH-blood agar plates. After overnight incubation at 37°C in a microaerobic environment, one-half of the growth was streaked onto MH-blood plates containing the appropriate antibiotic (50 µg/ml Kan or 2 µg/ml Tet). After 48 h of incubation, the isolated colonies were screened by PCR using gene-specific primers. Each *C. jejuni* mutant was confirmed using gene-specific primers, and in the case of the *C. jejuni capA* and *Cj1278c* mutants by sequencing the DNA flanking regions. The motility of each *C. jejuni* mutant was assessed prior to use.

Tissue culture. Chicken LMH hepatocellular carcinoma cells (ATCC CRL-2117) were obtained from the American Type Culture Collection (Manassas, VA). Stock cultures of LMH cells were grown in flasks coated with 0.1% gelatin in Waymouth's MB 752/1 medium supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). Cells were maintained at 37°C in a humidified, 5% CO_2 incubator.

***C. jejuni*-LMH binding assay.** LMH cells were seeded to a cell density of 3.0×10^5 cells/ml and incubated for 24 h at 37°C in a humidified, 5% CO_2 incubator. The cells were rinsed once with Minimal Essential Medium (MEM; Invitrogen) supplemented with 1% FBS and inoculated with approximately 3.0×10^7 CFU bacteria. Each plate was then subjected to centrifugation at 600 × *g* for 5 min to promote bacteria-host cell contact and incubated at 37°C for 30 min. To quantitate cell adherence, the *C. jejuni*-inoculated cells were rinsed three times with PBS, and lysed with a solution of 0.1% (v/v) Triton X-100 (Calbiochem, La Jolla, CA) in PBS. Ten-fold serial-dilutions of the samples were made and plated on MH-blood agar plates to determine the number of adherent bacteria. The reported values represent the mean counts ± standard deviation from triplicate wells.

Chicken colonization experiments. All the experiments and procedures described below were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC protocol #3248) at Washington State University. A total of 80 one-week-old chicks were obtained, divided into eight groups, and placed into isolation chambers (Horsfall Bauer isolators) on wire mesh. Water and a commercial chick starter feed were provided *ad libitum*. Each isolator was equipped with two removable metal trays. Fecal matter was collected and autoclaved before disposal. The chicks were inoculated with *C. jejuni* by oral gavage with 0.5 ml of a bacterial suspension ($\sim 10^7$ bacteria); the *C. jejuni* F38011 strain was cultured in Bolton's broth at 42ºC for 16 h under microaerobic conditions prior to inoculation of the birds. One group of 10 chicks was kept as the uninoculated control group. The remaining groups of chicks were inoculated with the *C. jejuni* F38011: 1) wild-type strain; 2) *cadF* mutant; 3) *capA*

mutant; 4) *jlpA* mutant; 5) *peb1A* mutant; 6) *Cj1279c* mutant; and 7) *Cj1349c* mutant. After the chicks were inoculated, the remaining bacterial suspensions were serially diluted and plated on Campy-Cefex agar (43) to confirm the CFU of each inoculum.

The chicks were euthanized and necropsied at 7 days post-inoculation (DPI). The cecum was dissected from each chick, weighed, diluted 1:10 (wt/v) in Bolton's broth media, and thoroughly stomached. For enumeration, serial 10-fold dilutions of the cecal contents were made and plated onto Campy-Cefex agar plates. The plates were incubated in a microaerobic environment at 37ºC and the CFUs were counted after 72 h of incubation. PCR was performed with *C. jejuni cadF* and *capA* specific primers to confirm that the counted colonies were *C. jejuni* (Table 1).

RESULTS

The *C. jejuni* strains used in this study are genetically diverse.

Multilocus sequence typing (MLST) is commonly used for molecular typing of *C. jejuni* isolates (10, 32). We collected a total of 97 isolates from humans, poultry, bovine, porcine, ovine, and canine sources, and assessed their genetic relatedness by MLST (Figure 1). The *C. jejuni* isolates comprised 45 sequence types (Supplemental Table 1). Eighty-four isolates were assigned to one of 18 clonal complexes (CC). The complexes with the greatest number of isolates were CC 21, CC 48, and CC 45 that were comprised of 19, 10, and 10 isolates, respectively. In total, two human isolates and eleven animal isolates did not belong to a CC in the MLST database. We also compared the allelic profiles, or sequence type (ST) of each isolate. The most common ST was ST-21, represented by isolates H2, H10, H12, Iowa 11, Iowa 13, Iowa 15, Iowa 35, and C1129. The second most common ST was ST-50, represented by isolates H6, H34, H36, H37, H40, S1, and 93-58. Several STs were comprised of three to five isolates, whereas 27 STs were represented by a single isolate. Newly identified STs were generated with four human isolates, F38011, H11, H14, and H30, and one poultry isolate USDA02-833L. In total, 105 alleles were identified amongst the seven loci, and a new *pgm* allele (*pgm431*) was reported. Based on the MLST analysis, we concluded that the *C. jejuni* strains used in this study were genetically diverse.

The adhesin-encoding genes, except *capA*, are conserved amongst *C. jejuni* strains.

The presence of genes encoding putative adhesins in the *C. jejuni* strains was determined by dot blot hybridization coupled with gene specific probes. The essential features of

these genes are listed in Table 2. Six of the seven putative adhesin-encoding genes, i.e., *cadF*, *jlpA, peb1A, porA, Cj1279c*, and *Cj1349c*, were detected in every *C. jejuni* strain tested (not shown), indicating that these genes are conserved within *C. jejuni*. One of the seven putative adhesin-encoding genes, *capA*, was not conserved amongst the strains assayed (Figure 1). *C. jejuni capA* was absent in 17 of the 43 (40%) human isolates and from 21 of the 54 (39%) animal isolates. The presence or absence of *capA* often correlated to specific STs. STs 50, 48, and 21, comprising 20 isolates, all possessed *capA* while STs 464, 459, 61, and 45, comprising 15 isolates, lacked *capA*.

CadF, CapA, Cj1279c, and Cj1349c contribute to *C. jejuni* adherence to chicken LMH cells.

To determine the role of the putative adhesins in promoting the binding of *C. jejuni* to cultured chicken epithelial cells, *in vitro* adherence assays were performed with *C. jejuni* mutants and chicken LMH hepatocellular carcinoma epithelial cells (Figure 2). A mutation in the *porA* gene was not attempted, as a mutation of this gene is hypothesized to be lethal due to its critical structural and pore activity {Amako, 1996 #36}. All of the *C. jejuni* mutants (i.e., *cadF*, *capA*, *jlpA, peb1A, Cj1279c*, and *Cj1349c*) generated were motile (not shown). The LMH cell line was chosen for these experiments because it is the only chicken epithelial cell line readily available to researchers. While LMH cells are derived from the liver, previous *C. jejuni* adherence studies indicate similar bacterial-host cell adherence efficiency with LMH and human INT 407 epithelial cells (16, 23). Mutations in *jlpA* and *peb1A* had little effect on the ability of *C. jejuni* to bind to the LMH cells. In contrast, a significant reduction ($P < 0.05$) was observed in the binding of

the *C. jejuni cadF*, *capA*, *Cj1279c*, and *Cj1349c* mutants to LMH cells when compared with the *C. jejuni* wild-type strain. In addition, *C. jejuni* isolates were genetically matched (H11 and H14; Iowa 80 and Iowa 81) based upon MLST and tested for cell adherence; the H11 and Iowa 81 isolates contained *capA* and the H14 and Iowa 80 isolates did not (Figure 1). Strains lacking *capA* showed a significant reduction ($P < 0.05$) in binding to LMH cells relative to strains in possession of the gene (not shown).

CadF, PEB1, and Cj1279c contribute to *C. jejuni* colonization of broiler chickens. To determine the relative importance of each putative adhesin in chicken colonization, we inoculated one-week-old chicks with the defined *C. jejuni* mutants. Eighty chicks were divided into groups, with each group consisting of ten chicks (Figure 3). All chicks were euthanized at 7 days post-inoculation and the number of *C. jejuni* per gram of cecal material was determined. *C. jejuni* was not recovered from any of the uninoculated chicks. Mutations in the *capA*, *jlpA,* and *Cj1349c* genes had little effect on the ability of *C. jejuni* to colonize the chicks, as judged by comparison with the wild-type. In contrast, the *C. jejuni cadF*, *peb1A*, and *Cj1279c* mutants demonstrated a marked impairment in their ability to colonize chicks, as only two of ten chickens inoculated with the *C. jejuni cadF* and *Cj1279c* mutants were colonized. None of the ten chicks inoculated with the *C. jejuni peb1A* mutant were colonized.

Cj1279c is required for efficient cell adherence and chicken colonization.
In silico analysis of *Cj1279c* revealed that this gene is located within a putative operon consisting of 13 genes (http://www.microbesonline.org/). The *Cj1279c* gene is situated

downstream of *Cj1280c* that encodes a putative ribosomal pseudouridine synthase and upstream of eleven *C. jejuni* genes involved in various functions including cellular division and metabolism. To alleviate the concern of a polar effect, the *Cj1278c* gene downstream of *Cj1279c* was mutated. Adherence assays performed with chicken LMH cells demonstrated that the observed phenotype of the *Cj1279c* mutant was not due to a polar effect, as a difference in binding was not observed with the *Cj1278c* mutant relative to the wild-type strain (Figure 4). Although variations were observed from one experiment to another in the number of *C. jejuni* that bound to the chicken LMH cells (Figs. 2 and 4), these results appeared to be due to fluctuations in the MOI. Regardless, the *C. jejuni cadF* and *Cj1279c* mutants consistently showed reductions in cell-binding when compared to the wild-type strain in all experiments performed. Because *Cj1279c* has not been previously characterized, we propose that it is a novel adhesin. As indicated above, the *Cj1279c* mutant demonstrates a reduction in both adherence to chicken LMH cells and in the colonization of chickens. Based on these findings and the fact that Cj1279c contains Fn type III domains, we will refer to the *Cj1279c* gene as *flpA* for Fibronectin-like protein A from this point forward.

DISCUSSION

Bacterial adherence to host epithelial cells is proposed to be critical for chicken colonization, as cell attachment may prevent clearance of the bacteria via host mediated mechanical force. The goal of this study was to assess the conservation of the putative *C. jejuni* adhesin-encoding genes *cadF*, *capA*, *jlpA*, *peb1A*, *porA*, *Cj1279c* (*flpA*), and *Cj1349c* and the contribution of the corresponding proteins in *C. jejuni* host cell interactions. In this study, we found that the *cadF, jlpA, porA, peb1A*, *flpA*, and *Cj1349c* genes were conserved amongst the isolates, whereas the presence of the *capA* gene was variable. We found that the *C. jejuni* CadF, CapA, FlpA, and Cj1349c proteins contribute to the bacterium's *in vitro* adherence to chicken LMH hepatocellular carcinoma epithelial cells, while CadF, PEB1, and FlpA contribute to the bacterium's *in vivo* colonization of broiler chicks. This is the first study to show that FlpA promotes the binding of *C. jejuni* to host cells and plays a role in *C. jejuni* colonization of chickens.

This study was performed with *C. jejuni* isolates collected from humans, poultry, bovine, porcine, ovine, and a canine. These isolates were genetically diverse, as judged by MLST. The isolates were found to comprise 42 unique sequence types, four of which had not been identified previously. The clonal complexes identified amid the *C. jejuni* livestock (i.e., bovine, porcine and ovine) isolates included two complexes, CC42 and CC61, that were determined in previous studies (9, 29) to be associated significantly with bovine and ovine. Furthermore, the eleven clonal complexes identified in the 41 poultry isolates included several poultry-associated complexes [i.e., CC45, CC257 and CC354; {Dingle, 2002 #37}]. Thus, the results from this study are consistent with previous

associations of clonal complex and source. Additionally, the clonal complexes of the human isolates identified in this study were also found within the poultry and livestock isolates, and *vice versa*. Therefore, no predominant food animal source of human infection was identified in this study.

As indicated above, genetic analysis of the adhesin profiles amongst the strains via dot blot assays demonstrated conservation of the *C. jejuni cadF, jlpA, porA, peb1A*, *flpA*, and *Cj1349c* genes. While the dot-blot hybridization assay is stringent enough to detect the presence or absence of the well-conserved adhesin genes, it cannot detect strain-to-strain sequence variations. However, the amino-acid sequences of the putative adhesins CadF, JlpA, PEB1, Cj1279c, and Cj1349 are all greater than 95% identical between *C. jejuni* strains, and CapA is greater than 85% identical between *C. jejuni* strains. The sequence of MOMP is the most variable between strains, however, PorA is required for viability. Fouts *et al.* (14) demonstrated the conservation of the characterized and putative *C. jejuni* adhesins in *C. jejuni* NCTC 11168 and RM1221 strains, and three other *Campylobacter* species. Although *capA* was omitted in the study by Fouts *et al.* (14), Ashgar *et al.* (4) reported that nine of 20 *C. jejuni* human clinical isolates tested in their study lacked the *capA* gene. In agreement with Ashgar *et al.* (4), we found that the *capA* gene was absent from 40% of the *C. jejuni* strains recovered from humans, and was absent from 39% of the *C. jejuni* strains recovered from animals.

While some of the *C. jejuni* proteins examined in this study have been the focus of previous work, this is the first time the functional role of these proteins has been

compared by generating a mutation in these genes within a single genetic background. The *C. jejuni* CadF, CapA, FlpA and Cj1349c proteins were found to play a significant role in the bacterium's *in vitro* adherence to chicken epithelial cells, whereas JlpA and PEB1 did not appear to play a role in cell adherence. Previous work has indicated that the *C. jejuni* lipoprotein JlpA is located predominately in the bacterial inner membrane and can be found loosely associated with the outer membrane and in culture medium during growth. Further, JlpA interacts with the human heat shock protein (HSP) 90α on the surface of human HEp-2 cells and is correlated to induction of the pre-inflammatory response due to the activation of NF-κB and p38 MAP kinase (21). Jin *et al.* (20) reported that disruption of *jlpA* reduces the adherence of *C. jejuni* to human HEp-2 epithelial cells by 18 to 19.4% when compared with a wild-type isolate. We found that insertional mutagenesis of *jlpA* did not result in a reduction in binding to chicken LMH cells. In agreement with the results from the *in vitro* binding assays, the *jlpA* mutant was able to colonize broiler chickens at a level comparable with that of a wild-type isolate.

While we found that a *C. jejuni peb1A* mutant bound to chicken LMH cells at a level comparable to that of a wild-type isolate, the mutant did not colonize broiler chickens. Based on our *in vitro* data and previously published data (12, 24), PEB1 does not appear to act as an adhesin but rather plays a critical role in aspartate and glutamate transport. In support of these findings, Leon-Kempis *et al.* (31) found PEB1 in the periplasm and supernatant, which is typical of an ABC transporter (44), but not in the outer membrane. In addition, the investigators demonstrated that a *peb1A* mutant failed to transport L-glutamate, showed a large reduction in L-aspartate transport, and did not grow in MEM-α

medium supplemented with L-aspartate or L-glutamate. Also relevant, Guccione *et al.* (17) reported that *C. jejuni* growth primarily depends upon four amino acids: aspartate, glutamate, serine, and proline. Interestingly, a *C. jejuni* aspartase mutant, which is unable to utilize aspartate, glutamate, and proline, grows poorly in a complex medium and is impaired in chicken colonization. These findings indicate that amino acid utilization in the chicken intestine is vital to the bacterium's colonization and persistence.

The CapA protein was identified as a putative autotransporter based on *in silico* analysis. Ashgar *et al*. (4) reported that a *capA* knockout reduced the binding of *C. jejuni* to human Caco-2 colorectal adenocarcinoma cells by approximately 30% and failed to colonize and persist in Rhode Island Red chickens. We found that the *capA* gene was not conserved amongst *C. jejuni* isolates. Indeed, the dot blot assay revealed that 15 of the *C. jejuni* poultry isolates utilized in this study lacked the *capA* gene. We also found that the *C. jejuni capA* mutant exhibited a 47% reduction in binding to chicken LMH epithelial cells when compared with the wild-type isolate, yet was able to colonize broiler chickens as efficiently as the wild-type isolate. The reason for the discrepancy in our data and that of Ashgar *et al*. (4) is not known; however, different methods and species of chickens were used in the two studies. Based on our results, we conclude that the CapA protein is an adhesin, but that it is not required for the colonization of broiler chickens.

CadF is a highly conserved 37 kDa outer membrane protein that binds to the extracellular matrix component Fn (24-26, 35). Previous reports have indicated that a *C. jejuni cadF* mutant shows a 60% reduction in binding to immobilized Fn and a 59% reduction in

adherence to INT 407 cells as compared to the wild-type isolate (25, 36). In addition, a *cadF* mutant fails to colonize the intestinal tract of leghorn chickens (55). In this study, the *C. jejuni cadF* mutant demonstrated a 41% reduction in binding to chicken LMH cells and was unable to efficiently colonize broiler chickens. These findings are in agreement with previously published studies (24-26, 35, 36, 55).

Since the Fn-binding protein CadF is critical to *C. jejuni* host cell adherence, we hypothesized that FlpA and Cj1349c may play a role in host cell attachment. Cj1349c has been annotated as a putative Fn/fibrinogen-binding protein. The *Cj1349c* mutant demonstrated a 14% reduction in binding to chicken LMH cells ($P < 0.05$). However, we did not observe reduced colonization of broiler chicks with a *Cj1349c* mutant when compared with the wild-type isolate. Based on the *in vitro* experiments, Cj1349c may act as an adhesin. However, the functional role of Cj1349c *in vivo* is not clear based on the chicken colonization experiments. FlpA contains Fn type III domains. Interestingly, the *flpA* mutant showed a 39% reduction in binding to chicken LMH epithelial cells relative to the wild-type isolate. In addition, the *flpA* mutant failed to efficiently colonize broiler chickens, as only two of ten broiler chicks were colonized. To address the concern that a mutation in *flpA* may have a polar effect, a mutation was generated in *Cj1278c*. The *Cj1278c* mutant did not show a significant reduction in binding to chicken LMH cells relative to the wild-type isolate. These data suggest that FlpA is a novel *C. jejuni* adhesin involved in *C. jejuni*-host cell adherence and chicken colonization.

In summary, this work indicates that the *cadF, jlpA, peb1A, porA, flpA*, and *Cj1349c* genes are conserved amongst *C. jejuni* isolates, whereas the presence of the *capA* gene is variable. We report that the CadF, CapA, FlpA, and Cj1349c proteins facilitate *C. jejuni* adherence to chicken LMH cells. This finding is consistent with the hypothesis that more than one protein contributes to the binding of *C. jejuni* to host epithelial cells. We conclude that both the CadF and FlpA proteins play a significant role in *C. jejuni* colonization of chickens. Based on the *in vivo* assays, it is apparent that the CapA and Cj1349c proteins are not essential for *C. jejuni* to colonize chickens, however, we cannot rule out the possibility that they contribute in the process. We found that the PEB1 protein is required for *C. jejuni* to colonize chickens, but this finding is likely due to that fact that it is involved in amino acid transport required for viability within the host. We were unable to assess the importance of MOMP, primarily due to the fact that mutations in *porA* are likely to be lethal to the bacterium. Based on the results herein, we propose that FlpA (Cj1279c) is a novel *C. jejuni* adhesin. Additional studies are in progress to dissect the function of the FlpA protein.

ACKNOWLEDGMENTS

We thank Travis Ruff for assistance with the dot blots, Dennis Schaberg and Kari Shoaf-Sweeney for assistance with *C. jejuni* inoculation of the chickens and with sample collection, Stuart Perry for animal care, Sylvia K. Weber and Fonda Wier for assistance in preparing chicken tissue samples for plating, and Emma Yee for assistance with MLST. This work made use of the *Campylobacter* MultiLocus Sequence Typing website (http://pubmlst.org/ campylobacter/) developed by Keith Jolley at the University of Oxford. We also thank Charles Larson (School of Molecular Biosciences, Washington State University) for critical review of this manuscript.

This work was supported from funds awarded to MEK from the USDA National Research Initiative's Food Safety 32.0 program (2006-35201-16553 and 2006-35201-17305).

REFERENCES

1. **Altekruse, S. F., N. J. Stern, P. I. Fields, and D. L. Swerdlow.** 1999. *Campylobacter jejuni* - an emerging foodborne pathogen. Emerg. Infect. Dis. **5:**28-35.
2. **Amako, K., S.N. Wai, A. Umeda, M. Sigematsu, and A. Takade.** 1996. Electron microscopy of the major outer membrane protein of *Campylobacter jejuni*. Microbiol. Immunol. **40:**749-754.
3. **Ashgar, S. S., N. J. Oldfield, K. G. Wooldridge, M. A. Jones, G. J. Irving, D. P. Turner, and D. A. Ala'Aldeen.** 2007. CapA, an autotransporter protein of *Campylobacter jejuni*, mediates association with human epithelial cells and colonization of the chicken gut. J. Bacteriol. **189:**1856-1865.
4. **Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser.** 1988. Experimental *Campylobacter jejuni* infection in humans. J. Infect. Dis. **157:**472-479.
5. **Blaser, M. J., I. D. Berkowitz, F. M. LaForce, J. Cravens, L. B. Reller, and W. L. Wang.** 1979. Campylobacter enteritis: clinical and epidemiologic features. Ann. Intern. Med. **91:**179-185.
6. **Bolla, J. M., E. Loret, M. Zalewski, and J. M. Pages.** 1995. Conformational analysis of the *Campylobacter jejuni* porin. J. Bacteriol. **177:**4266-4271.
7. **Colles, F. M., K. Jones, R. M. Harding, and M. C. Maiden.** 2003. Genetic diversity of *Campylobacter jejuni* isolates from farm animals and the farm environment. Appl. Environ. Microbiol. **69:**7409-7413.

8. **Dingle, K. E., F. M. Colles, D. R. Wareing, R. Ure, A. J. Fox, F. E. Bolton, H. J. Bootsma, R. J. Willems, R. Urwin, and M. C. Maiden.** 2001. Multilocus sequence typing system for *Campylobacter jejuni*. J. Clin. Microbiol. **39:**14-23.

9. **Dingle, K. E., F. M. Colles, R. Ure, J. A. Wagenaar, B. Duim, F. J. Bolton, A. J. Fox, D. R. Wareing, and M.C. Maiden.** 2002. Molecular characterization of *Campylobacter jejuni* clones: a basis for epidemiologic investigation. Emerg. Infect. Dis. **8:**949-955.

10. **Fouts, D. E., E. F. Mongodin, R. E. Mandrell, W. G. Miller, D. A. Rasko, J. Ravel, L. M. Brinkac, R. T. DeBoy, C. T. Parker, S. C. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, S. A. Sullivan, J. U. Shetty, M. A. Ayodeji, A. Shvartsbeyn, M. C. Schatz, J. H. Badger, C. M. Fraser, and K. E. Nelson.** 2005. Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter* species. PLoS Biol. **3:**e15.

11. **Goulhen, F., E. De, J. M. Pages, and J. M. Bolla.** 2004. Functional refolding of the *Campylobacter jejuni* MOMP (major outer membrane protein) porin by GroEL from the same species. Biochem. J. **378:**851-856.

12. **Guccione, E., R. Leon-Kempis Mdel, B. M. Pearson, E. Hitchin, F. Mulholland, P. M. van Diemen, M. P. Stevens, and D. J. Kelly.** 2008. Amino acid-dependent growth of *Campylobacter jejuni*: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. Mol. Microbiol. **69:**77-93.

13. **Jin, S., A. Joe, J. Lynett, E. K. Hani, P. Sherman, and V. L. Chan.** 2001. JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. Mol. Microbiol. **39:**1225-1236.

14. **Jin, S., Y. C. Song, A. Emili, P. M. Sherman, and V. L. Chan.** 2003. JlpA of *Campylobacter jejuni* interacts with surface-exposed heat shock protein 90α and triggers signalling pathways leading to the activation of NF-κB and p38 MAP kinase in epithelial cells. Cell. Microbiol. **5:**165-174.

15. **Kervella, M., J. M. Pages, Z. Pei, G. Grollier, M. J. Blaser, and J. L. Fauchere.** 1993. Isolation and characterization of two *Campylobacter* glycine-extracted proteins that bind to HeLa cell membranes. Infect. Immun. **61:**3440-3448.

16. **Konkel, M. E., J. E. Christensen, A. S. Dhillon, A. B. Lane, R. Hare-Sanford, D. M. Schaberg, and C. L. Larson.** 2007. *Campylobacter jejuni* strains compete for colonization in broiler chicks. Appl. Environ. Microbiol. **73:**2297-2305.

17. **Konkel, M. E., J. E. Christensen, A. M. Keech, M. R. Monteville, J. D. Klena, and S. G. Garvis.** 2005. Identification of a fibronectin-binding domain within the *Campylobacter jejuni* CadF protein. Mol. Microbiol. **57:**1022-1035.

18. **Konkel, M. E., S. G. Garvis, S. L. Tipton, D. E. Anderson, Jr., and W. Cieplak, Jr.** 1997. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. Mol. Microbiol. **24:**953-963.

19. **Konkel, M. E., S. A. Gray, B. J. Kim, S. G. Garvis, and J. Yoon.** 1999. Identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter*

coli based on the *cadF* virulence gene and its product. J. Clin. Microbiol. **37:**510-517.

20. **Konkel, M. E., M. R. Monteville, V. Rivera-Amill, and L. A. Joens.** 2001. The pathogenesis of *Campylobacter jejuni*-mediated enteritis. Curr. Issues Intest. Microbiol. **2:**55-71.

21. **Kwan, P. S., M. Barrigas, F. J. Bolton, N. P. French, P. Gowland, R. Kemp, H. Leatherbarrow, M. Upton, and A. J. Fox.** 2008. Molecular epidemiology of *Campylobacter jejuni* populations in dairy cattle, wildlife, and the environment in a farmland area. Appl. Environ. Microbiol. **74:**5130-5138.

22. **Labigne-Roussel, A., J. Harel, and L. Tompkins.** 1987. Gene transfer from *Escherichia coli* to *Campylobacter* species: development of shuttle vectors for genetic analysis of *Campylobacter jejuni*. J. Bacteriol. **169:**5320-5323.

23. **Larson, C. L., D. H. Shah, A. S. Dhillon, D. R. Call, S. Ahn, G. J. Haldorson, C. Davitt, and M. E. Konkel.** 2008. *Campylobacter jejuni* invade chicken LMH cells inefficiently and stimulate differential expression of the chicken CXCLi1 and CXCLi2 cytokines. Microbiology. **154:**3835-3847.

24. **Leon-Kempis Mdel, R., E. Guccione, F. Mulholland, M. P. Williamson, and D. J. Kelly.** 2006. The *Campylobacter jejuni* PEB1a adhesin is an aspartate/glutamate-binding protein of an ABC transporter essential for microaerobic growth on dicarboxylic amino acids. Mol. Microbiol. **60:**1262-1275.

25. **Levesque, S., E. Frost, R. D. Arbeit, and S. Michaud.** 2008. Multilocus sequence typing of *Campylobacter jejuni* isolates from humans, chickens, raw

milk, and environmental water in Quebec, Canada. J. Clin. Microbiol. **46:**3404-3411.

26. **Miller, W. G., S. L. On, G. Wang, S. Fontanoz, A. J. Lastovica, and R. E. Mandrell.** 2005. Extended multilocus sequence typing system for *Campylobacter coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*. J. Clin. Microbiol. **43:**2315-2329.

27. **Monteville, M. R., and M. E. Konkel.** 2002. Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. Infect. Immun. **70:**6665-6671.

28. **Monteville, M. R., J. E. Yoon, and M. E. Konkel.** 2003. Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization. Microbiology. **149:**153-165.

29. **Moser, I., W. Schroeder, and J. Salnikow.** 1997. *Campylobacter jejuni* major outer membrane protein and a 59-kDa protein are involved in binding to fibronectin and INT 407 cell membranes. FEMS Microbiol. Lett. **157:**233-238.

30. **Oyarzabal, O. A., K. S. Macklin, J. M. Barbaree, and R. S. Miller.** 2005. Evaluation of agar plates for direct enumeration of *Campylobacter* spp. from poultry carcass rinses. Appl. Environ. Microbiol. **71:**3351-3354.

31. **Pei, Z., and M. J. Blaser.** 1993. PEB1, the major cell-binding factor of *Campylobacter jejuni*, is a homolog of the binding component in Gram-negative nutrient transport systems. J. Biol. Chem. **268:**18717-18725.

32. **Pei, Z., C. Burucoa, B. Grignon, S. Baqar, X. Z. Huang, D. J. Kopecko, A. L. Bourgeois, J. L. Fauchere, and M. J. Blaser.** 1998. Mutation in the *peb1A* locus

of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. Infect. Immun. **66:**938-943.

33. **Sahin, O., T. Y. Morishita, and Q. Zhang.** 2002. *Campylobacter* colonization in poultry: sources of infection and modes of transmission. Anim. Health Res. Rev. **3:**95-105.

34. **Stern, N. J., and S. Pretanik.** 2006. Counts of *Campylobacter* spp. on U.S. broiler carcasses. J. Food Prot. **69:**1034-1039.

35. **Young, V. B., and L. S. Mansfield.** 2005. *Campylobacter* infection - Clinical context, p. 1-12. *In* J. M. Ketley and M. E. Konkel (ed.), *Campylobacter*: Molecular and Cellular Biology. Horizon Bioscience.

36. **Ziprin, R. L., C. R. Young, L. H. Stanker, M. E. Hume, and M. E. Konkel.** 1999. The absence of cecal colonization of chicks by a mutant of *Campylobacter jejuni* not expressing bacterial fibronectin-binding protein. Avian Dis. **43:**586-589.

Table 1. Genes targeted for mutagenesis

Locus Tag (Gene Designation)[a]	Gene Product (protein)	# of nucleotides /residues	Amplified Fragment(s) in nucleotides	Primers
Cj1478c *(cadF)*	CadF	960/320	620	*cadF*-F[b] TATTTCTATGGTTTAGCAGGTGGAG *cadF*-R[b] GCTCTACCTTCTTTAGTGTCATTGC
Cj0628 / Cj0629 *(capA)*	CapA	3436/1145	1321, 1635	*capA*-F[b] TGAATCGAAGTGGAAAAATAGAAG *capA*-R[b] CCCATTTTTGTATCTTCATAACCT *capA*-SstI-F[c] ATGAGCTCAAAGTTGTTCCTAAGG GTAAAGC *capA*-SstII-R[c] ATACCGCGGAGTTTTATTCATAAAT ATTCCCTTTCC *capA*-SstII-F[c] ATACCGCGGGCTCAGTTTAATTATC TTTGGTAATC *capA*-XhoI-R[c] ATACTCGAGCATTTTACAAGCCCTA TAAGAAGG
Cj0983 *(jlpA)*	JlpA	1119/373	868	*jlpA*-F[b] TCTCAGGACTCTGGAATAAAGATTG *jlpA*-R[b] GTGTGCTATAGTCACTAACAGGGA TG
Cj0921c *(peb1A)*	PEB1	780/260	560	*peb1A*-F[b] TCTAGGTGCTTGTGTTGCATTTAG *peb1A*-R[b] TGTCTACAGAAAACGCATCAACTC
Cj1279c	Cj1279c (FlpA)	1233/411	832	Cj1279c-F[b] TCAGAAGATGGCAAGGTTATAGAA G Cj1279c-R[b] GTTATTGCTATTGATTCAGCTGGAC
Cj1349c	Cj1349c	1308/436	1115	Cj1349c-F[b] TATTTTTGATCTTACTCGTGCAATG Cj1349c-R[b] TTAAGGTATAATCGACCCAATACGA
Cj1278c	Cj1278c	1179/393	1033, 991	Cj1278c-BamHI-F[c] ATATAGGATCCGTATCGTTCTAGTG ATGAAAATCC Cj1278c-SstII-R[c] ATATACCGCGGTTTTAAAATTTGGC ACTACTGAGC Cj1278c-SstII-F[c] ATATACCGCGGGTTTAAAATATAAT TTTTCTTGAA AATTAAGC Cj1278c-BamHI-R[c] ATATAGGATCCTTTTCAGAAACATC ATTTTTCAAACG

[a] The gene number is from the genome sequence from *C. jejuni* NCTC11168.
[b] Indicates the primers used to amplify a DNA fragment for the generation of a suicide vector (gene knockout) and the probe for dot-blot hybridization.
[c] Indicates the primers used to amplify the DNA fragments for construction of the vectors to generate the mutants (i.e., *capA* and *Cj1278c*) via a double-crossover event; the two fragments were cloned into pBSK-Kan2, and disrupted by the insertion of a tetracycline resistance cassette.

Table 2. *C. jejuni* genes encoding putative adhesins

ORF	BLAST	Signal Peptide Cleavage Sites	Genes Within the Putative Operon[a]	Relevant Reference
porA/ Cj1259	Major outer membrane protein, *Campylobacter jejuni*	Residues 22-23, SpI cleavage[b]	None Identified	29
cadF/ Cj1478c	Structural outer membrane porin OprF, *Pseudomonas aeruginosa* 2e-27	Residues 26-27, SpI cleavage[b]	Cj1478c,1477c	18
capA/ Cj0628 and Cj0629	Autotransporter beta-domain, *Campylobacter jejuni*	None Identified	None Identified	1
jlpA/ Cj0983	Surface-exposed lipoprotein, *Campylobacter jejuni*	Residues 17-18, SpII cleavage[c]	None Identified	13
peb1A/ Cj0921c	Amino acid ABC transporter, amino acid-binding protein, *Streptococcus pneumoniae* 1e-52	Residues 26-27, SpI cleavage[b]	Cj0922c 0921c, 0920c, 0919c	15
Cj1279c	Fibronectin Type III domain containing protein lipoprotein, *Sulfurimonas denitrificans* 1e-57	Residues 20-21, SpII cleavage[c]	Cj1280c, 1279c, 1278c, 1277c, 1276c, 1275c, 1274c, 1273c, 1272c, 1271c, 1270c, 1269c, 1268c	ND
Cj1349c	Fibronectin/fibrinogen-binding protein FBP54, *Streptococcus pyogenes* 1.7e-05	None Identified	Cj1350c,1349c , 1348c, 1347c, 1346c, 1345c, 1344c, 1343c, 1342c	ND

[a] Genes within the putative operons were determined using NMPDR.
[b] Putative signal peptide cleavage site.
[c] Putative lipoprotein signal peptide cleavage site.

Supplemental Table 1. MLST alleles and sequence types of *C. jejuni* isolates

Species	Strain	Allele							ST	CC
		aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*		
Human	F38011	7	4	27	68	11	3	6	3644	None
	81-176	1	2	3	27	5	9	3	604	42
	81116	4	7	40	4	42	51	1	267	283
	M129	7	17	5	2	10	3	6	353	353
	H1	1	3	6	4	3	3	3	22	22
	H2	2	1	1	3	2	1	5	21	21
	H4	1	1	2	2	225	3	17	1244	61
	H5	4	7	10	4	1	7	1	45	45
	H6	2	1	12	3	2	1	5	50	21
	H7	2	21	5	2	59	1	5	222	206
	H9	2	4	1	4	19	62	5	475	48
	H10	2	1	1	3	2	1	5	21	21
	H11	9	2	4	62	10	5	17	3645	257
	H12	2	1	1	3	2	1	5	21	21
	H13	2	4	1	2	7	1	5	48	48
	H14	9	2	4	62	10	5	17	3645	257
	H15	2	4	1	2	7	1	5	48	48
	H16	22	146	4	64	74	25	23	1255	1034
	H17	1	1	2	2	225	3	17	1244	61
	H18	1	1	2	2	225	3	17	1244	61
	H19	4	7	10	4	1	7	102	1228	45
	H20	4	7	10	4	1	7	1	45	45
	H21	7	17	5	2	10	3	6	353	353
	H22	24	25	2	10	22	3	6	1477	52
	H23	8	2	5	53	11	3	1	607	607
	H24	24	30	2	2	89	59	6	460	460
	H26	4	7	10	4	1	7	1	45	45
	H27	4	7	10	4	1	7	1	45	45
	H28	2	4	1	2	7	1	5	48	48
	H29	7	17	12	2	10	3	6	452	353
	H30	8	2	1	53	11	3	1	3646	607
	H31	4	7	10	4	1	7	1	45	45
	H32	2	4	1	2	7	1	5	48	48
	H34	2	1	12	3	2	1	5	50	21
	H35	1	3	6	4	3	3	3	22	22
	H36	2	1	12	3	2	1	5	50	21
	H37	2	1	12	3	2	1	5	50	21
	H38	8	10	2	2	11	12	6	354	354
	H39	8	2	2	212	309	253	147	2083	None
	H40	2	1	12	3	2	1	5	50	21
	H41	1	4	2	2	6	3	17	61	61

Supplemental Table 1 (cont.). MLST alleles and sequence types of *C. jejuni* isolates

Species	Strain	Allele							ST	CC
		aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*		
Human	H42	1	2	3	4	5	9	3	42	42
	H43	1	3	6	4	3	3	3	22	22
Poultry	RM1221	8	10	2	2	11	12	6	354	354
	Turkey	2	4	1	2	7	1	5	48	48
	S1	2	1	12	3	2	1	5	50	50
	S2B	8	10	2	2	11	12	6	354	354
	USDA02-833L	9	25	2	10	431	3	6	3647	52
	A2a	24	25	2	2	226	59	221	2827	460
	A5a	33	25	30	82	104	35	17	2828	828
	A18a	8	2	5	53	11	3	105	1212	607
	D34a	1	2	3	3	5	9	3	459	42
	G11a	33	153	2	2	89	43	6	2832	460
	Iowa 2	7	4	5	2	11	1	5	429	48
	Iowa 3	7	4	5	2	11	1	5	429	48
	Iowa 4	7	4	5	2	11	1	5	429	48
	Iowa 5	24	2	2	2	10	3	1	464	None
	Iowa 6	24	2	2	2	10	3	1	464	None
	Iowa 7	24	2	2	2	10	3	1	464	None
	Iowa 8	7	4	5	2	11	1	5	429	48
	Iowa 9	24	2	2	2	10	3	1	464	None
	Iowa 11	2	1	1	3	2	1	5	21	21
	Iowa 12	4	7	10	4	1	1	1	97	45
	Iowa 13	2	1	1	3	2	1	5	21	21
	Iowa 15	2	1	1	3	2	1	5	21	21
	Iowa 21	7	4	5	2	13	3	26	457	353
	Iowa 22	8	10	2	2	378	12	6	2836	354
	Iowa 23	7	4	5	2	13	3	26	457	353
	Iowa 24	8	10	2	2	378	12	6	2836	354
	Iowa 25	8	10	2	2	378	12	6	2836	354
	Iowa 26	8	10	2	2	378	12	6	2836	354
	Iowa 33	7	2	5	302	10	3	6	2837	353
	Iowa 34	7	112	5	2	379	67	6	2838	353
	Iowa 35	2	1	1	3	2	1	5	21	21
	Iowa 36	9	2	4	62	4	5	17	929	257
	Iowa 39	7	84	5	10	119	178	26	1911	None
	Iowa 42	7	84	5	10	119	178	26	1911	None
	Iowa 44	7	265	5	10	119	178	26	2840	None
	Iowa 77	7	84	5	10	119	178	26	1911	None
	Iowa 78	7	84	5	10	119	178	26	1911	None
	Iowa 79	4	7	10	4	42	7	1	137	45
	Iowa 80	4	7	10	4	42	7	1	137	45
	Iowa 81	4	7	10	4	42	7	1	137	45
	Iowa 83	7	84	5	10	119	178	26	1911	None

Supplemental Table 1 (cont.). MLST alleles and sequence types of *C. jejuni* isolates

Species	Strain	Allele							ST	CC
		aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*		
Bovine	C913	1	2	3	3	5	9	3	459	42
	C973	2	1	1	3	2	1	6	8	21
	C1086	2	1	2	3	2	1	5	982	21
	C1129	2	1	1	3	2	1	5	21	21
	C1144	1	2	3	3	5	9	3	459	42
Swine	93-55	2	1	1	3	2	1	6	8	21
	93-58	2	1	12	3	2	1	5	50	21
	93-338	7	1	2	83	2	3	6	441	None
	93-343	2	1	1	3	2	1	6	8	21
	92-1578	2	1	1	3	2	1	6	8	21
Ovine	ov112	1	4	2	2	6	3	17	61	61
	ov48	1	4	2	2	6	3	17	61	61
Canine	1979858	10	27	16	19	10	5	7	403	403

Figure 1. Dendrogram of the *C. jejuni* MLST profiles identified in this study. The MLST sequence types were determined as outlined in 'Materials and Methods'. For each isolate, the allelic sequences were concatenated in the order *aspA*-*uncA*-*glnA*-*gltA*-*glyA*-*pgm*-*tkt* and aligned in ClustalX. The dendrogram was constructed in Mega 4 using the aligned concatenated sequences, the neighbor-joining algorithm and the Kimura two-parameter distance estimation method. Bootstrap values of >75%, generated from 500 replicates, are shown at the nodes. The scale bar represents substitutions per site. The filled circles (●) indicate those *C. jejuni* strains in which the *capA* gene is absent. Labels in parentheses indicate isolate source: H, human; P, poultry; S, porcine (swine); B, bovine; O, ovine; C, canine.

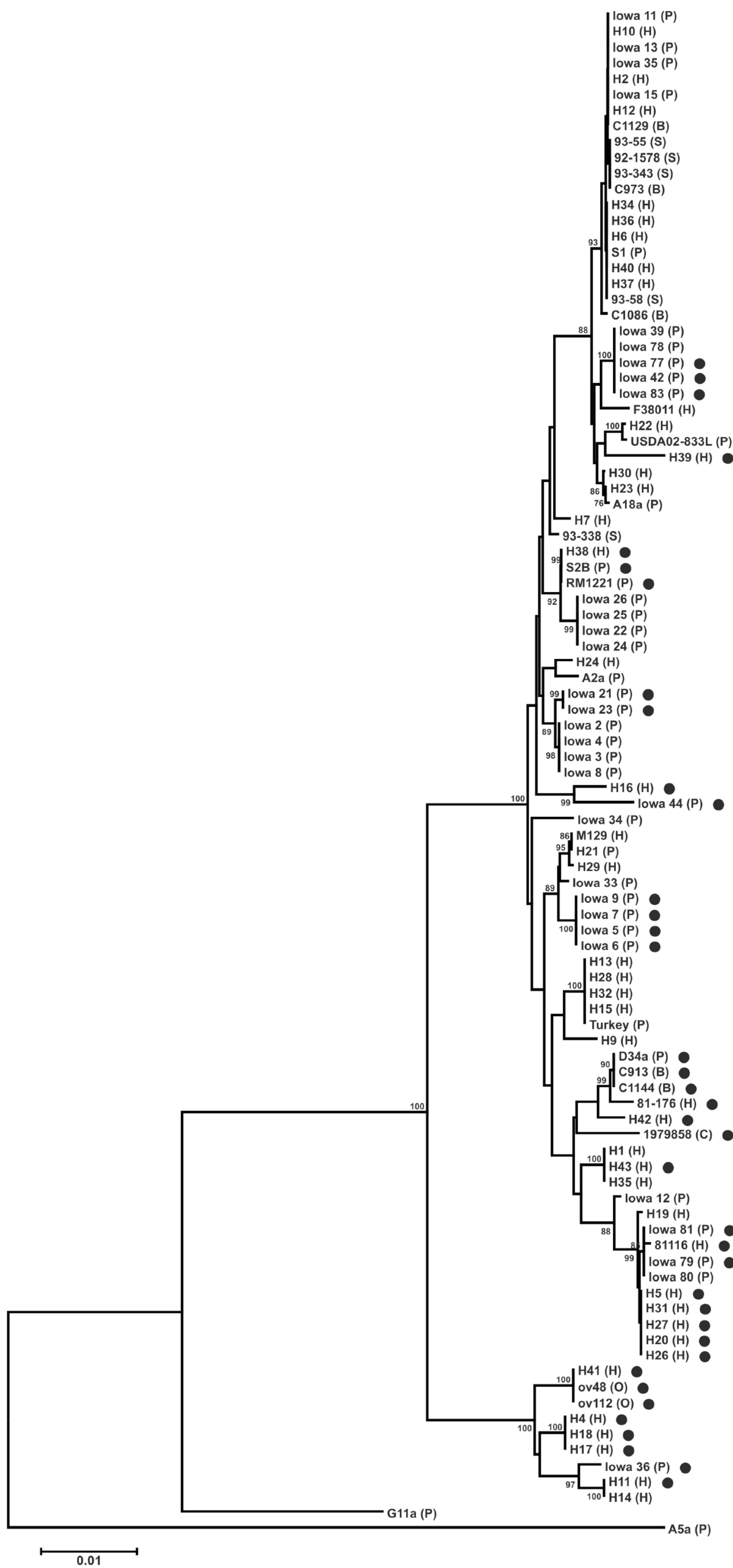

Iowa 11 (P)
H10 (H)
Iowa 13 (P)
Iowa 35 (P)
H2 (H)
Iowa 15 (P)
H12 (H)
C1129 (B)
93-55 (S)
92-1578 (S)
93-343 (S)
C973 (B)
H34 (H)
H36 (H)
H6 (H)
S1 (P)
H40 (H)
H37 (H)
93-58 (S)
C1086 (B)
Iowa 39 (P)
Iowa 78 (P)
Iowa 77 (P)
Iowa 42 (P)
Iowa 83 (P)
F38011 (H)
H22 (H)
USDA02-833L (P)
H39 (H)
H30 (H)
H23 (H)
A18a (P)
H7 (H)
93-338 (S)
H38 (H)
S2B (P)
RM1221 (P)
Iowa 26 (P)
Iowa 25 (P)
Iowa 22 (P)
Iowa 24 (P)
H24 (H)
A2a (P)
Iowa 21 (P)
Iowa 23 (P)
Iowa 2 (P)
Iowa 4 (P)
Iowa 3 (P)
Iowa 8 (P)
H16 (H)
Iowa 44 (P)
Iowa 34 (P)
M129 (H)
H21 (P)
H29 (H)
Iowa 33 (P)
Iowa 9 (P)
Iowa 7 (P)
Iowa 5 (P)
Iowa 6 (P)
H13 (H)
H28 (H)
H32 (H)
H15 (H)
Turkey (P)
H9 (H)
D34a (P)
C913 (B)
C1144 (B)
81-176 (H)
H42 (H)
1979858 (C)
H1 (H)
H43 (H)
H35 (H)
Iowa 12 (P)
H19 (H)
Iowa 81 (P)
81116 (H)
Iowa 79 (P)
Iowa 80 (P)
H5 (H)
H31 (H)
H27 (H)
H20 (H)
H26 (H)
H41 (H)
ov48 (O)
ov112 (O)
H4 (H)
H18 (H)
H17 (H)
Iowa 36 (P)
H11 (H)
H14 (H)
G11a (P)
A5a (P)
0.01

Figure 2. Number of *C. jejuni* bound to chicken LMH hepatocellular carcinoma epithelial cells. Binding assays were performed as outlined in 'Materials and Methods'. Each bar represents the mean ± standard deviation of *C. jejuni* bound to the LMH cells per well of a 24-well plate. The asterisk (*) indicates a statistically significant difference ($P < 0.05$) between the *C. jejuni* F38011 wild-type isolate and an isogenic mutant, as determined using Student's *t*-test.

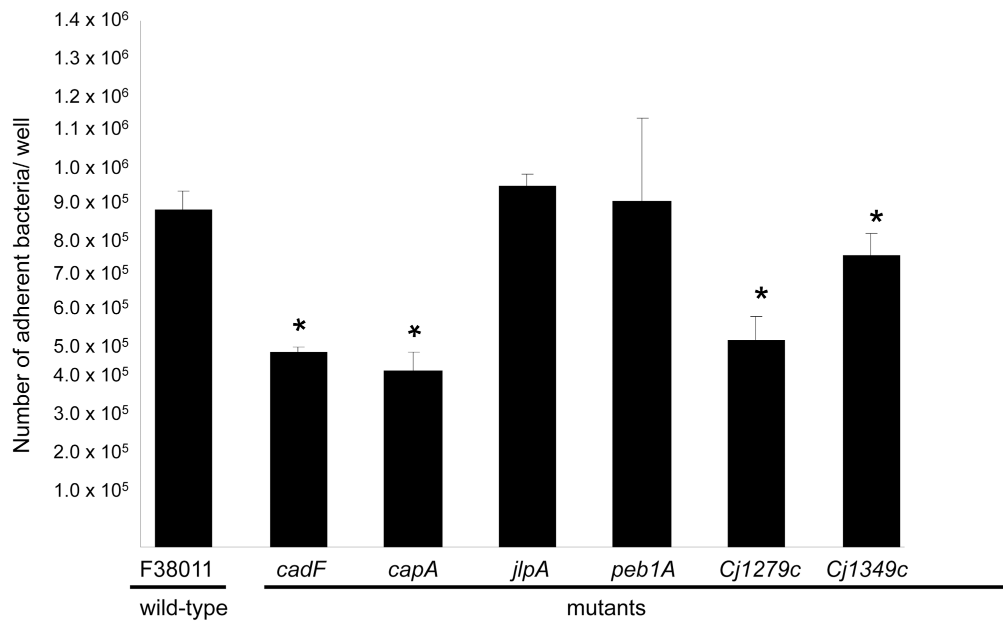

Number of adherent bacteria/ well
1.4 x 10⁶
1.3 x 10⁶
1.2 x 10⁶
1.1 x 10⁶
1.0 x 10⁶
9.0 x 10⁵
8.0 x 10⁵
7.0 x 10⁵
6.0 x 10⁵
5.0 x 10⁵
4.0 x 10⁵
3.0 x 10⁵
2.0 x 10⁵
1.0 x 10⁵
F38011
cadF
capA
jlpA
peb1A
Cj1279c
Cj1349c
wild-type
mutants

Figure 3. CadF, PEB1, and Cj1279c contribute to *C. jejuni* colonization of broiler chickens. Cecal samples were collected at 7 days post inoculation of the broiler chickens. The CFU per gram of cecal content was determined as described in 'Materials and Methods.' The 'N' indicates the number of chickens in the group of 10 that were colonized with *C. jejuni* (limit of detection 10^3 CFU/gram cecal contents). The bar indicates the median CFU for each group, which was determined using all birds within the group. The absence of a bar indicates the number of *C. jejuni* was below the limit of detection.

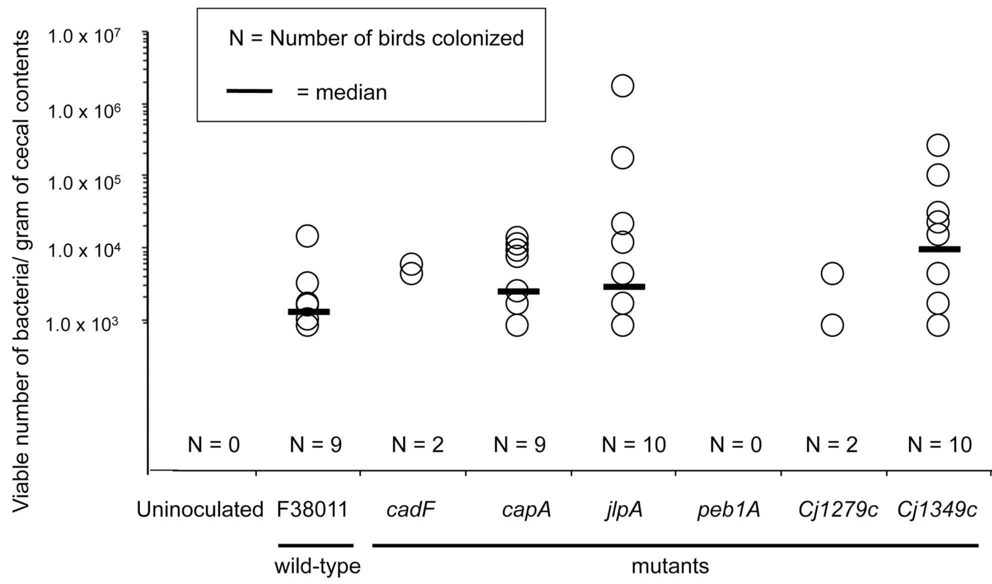
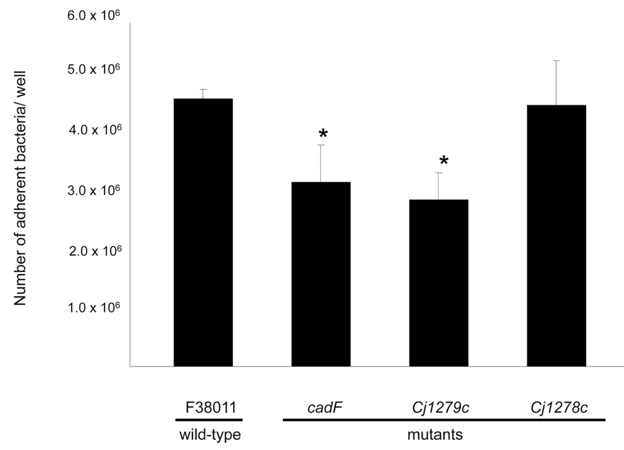

N = Number of birds colonized
= median
Viable number of bacteria/ gram of cecal contents
1.0 x 10^7
1.0 x 10^6
1.0 x 10^5
1.0 x 10^4
1.0 x 10^3
N = 0
N = 9
N = 2
N = 9
N = 10
N = 0
N = 2
N = 10
Uninoculated
F38011
cadF
capA
jlpA
peb1A
Cj1279c
Cj1349c
wild-type
mutants

Figure 4. *Cj1279c* (*flpA*) encodes an adhesin. Binding assays were performed with *C. jejuni* and chicken LMH hepatocellular carcinoma epithelial cells as outlined in 'Materials and Methods'. Each bar represents the mean $\pm$ the standard deviation for the number of *C. jejuni* bound to the LMH cells in each well of a 24-well plate. The asterisk (*) indicates a statistically significance difference ($P < 0.05$) between the *C. jejuni* F38011 wild-type isolate and an isogenic mutant, as determined using Student's *t*-test.


6.0 x 10⁶
5.0 x 10⁶
4.0 x 10⁶
3.0 x 10⁶
2.0 x 10⁶
1.0 x 10⁶
Number of adherent bacteria/ well
*
*
F38011
cadF
Cj1279c
Cj1278c
wild-type
mutants

CHAPTER 5

FUTURE DIRECTIONS

Most often up to 10^8 *Campylobacter jejuni* commensally colonize commercial chickens within 2 to 3 weeks of age. In the present study, using a single genetic screen, we characterized the role that the putative *C. jejuni* adhesins, CadF, CapA, JlpA, MOMP, PEB1, Cj1279c (FlpA), and Cj1349c play in the colonization of chickens and indicated conservation of the *C. jejuni* adhesins, except CapA, amongst a set of diverse *C. jejuni* isolates. With the exception of CadF and CapA, the role of the *C. jejuni* adhesins in the colonization of chickens remained unknown. Herein we show that *C. jejuni* adhesins CadF, PEB1, and Cj1279c play significant roles to the *in vivo* colonization of chickens, and adhesins CadF, CapA, Cj1279c, and Cj1349c play significant roles in the *in vitro* colonization of chicken cells.

Previously, Ashgar *et al.* (4) demonstrated that CapA is required for the efficient colonization of chickens. However, in our study, CapA did not demonstrate a role in the colonization of broiler chickens. On the other hand, our *in vitro* studies of a *capA* mutant suggested that CapA participates in *C. jejuni* host cell adherence. Thus, CapA may play a role to initial bacterial-host cell adherence, such as that observed in an *in vitro* binding assay, but it does not significantly alter persistence in the chicken. This may be due to the fact that a *capA* mutant causes a significant reduction in binding to epithelial cells over the duration of thirty minutes during an adherence assay, yet the mutant is able to colonize broiler chickens over a long period of time as other *C. jejuni* adhesins, such as

CadF, facilitate adherence to the extracellular matrix and thereby permit persistence. As well, we demonstrate that *C. jejuni* environmental isolates possessing the *capA* gene bound in higher numbers to chicken LMH cells and that the genetic presence of *capA* varies amongst the *C. jejuni* strains. Hence, characterization of CapA and its host-cell target warrants further investigation.

In these studies, the novel adhesin FlpA played a significant role to *in vivo* and *in vitro C. jejuni* colonization and adherence. Because the precise role of FlpA remains unclear, further studies are currently under way in our laboratory to characterize FlpA. FlpA will be examined to determine if it is localized to the outer-membrane via sarcosyl extracts and a polyclonal anti-FlpA antibody. Elucidation of the host-cell target should prove interesting due to the fact that FlpA harbors Fn type III domains. Binding of FlpA to Fn will be studied using ELISAs. The cumulative roles of CadF and FlpA to *C. jejuni* adherence also warrants investigation as mutations in both genes within a single strain may indicate additive binding of the two adhesins. Finally, deciphering the host-cell target of FlpA will prove interesting should this adhesin, upon binding a host cell, alter the host cell cytoskeleton.