Examination of *Campylobacter jejuni* Putative Adhesins Leads to the Identification of a New Protein, Designated FlpA, Required for Chicken Colonization

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Received 16 October 2008/Returned for modification 19 January 2009/Accepted 25 March 2009

*Campylobacter jejuni* colonization of chickens is presumably dependent upon multiple surface-exposed proteins termed adhesins. Putative *C. jejuni* adhesins include CadF, CapA, JlpA, major outer membrane protein, 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 bacterium-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 among 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.

*Campylobacter jejuni* is a gram-negative, spiral, microaerophilic bacterium, which is motile via a bipolar or unipolar flagellum. This organism is one of the leading bacterial causes of diarrheal illness in the United States and accounts for 5 to 14% of total human illness (3). Campylobacteriosis is a self-limiting infection. This disease is characterized by fever, nausea, malaise, abdominal pain, and loose-towyatery stools, which may contain blood and/or fecal leukocytes (5). *C. jejuni* infections in humans have revealed that as few as 800 bacteria can cause human illness (4). Campylobacteriosis (*C. jejuni*-mediated gastrointestinalitis) generally occurs 2 to 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-waty stools, which may contain blood and/or fecal leukocytes (5).

*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 g of cecal contents (33). Not surprisingly, *Campylobacter* organisms are frequently recovered from processed broiler carcasses (34). 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 (36).

Researchers have identified a number of *C. jejuni* proteins that bind to cultured epithelial cells. These adhesive proteins include CadF, CapA, JlpA, major outer membrane protein (MOMP), and PEB1. Konkel et al. (18) identified CadF, which is a 37-kDa Fn-binding protein. CadF facilitates *C. jejuni* adherence to Fn, which is a ubiquitous $\sim 250$-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 compared to a *C. jejuni* wild-type isolate (28). Ashgar et al. (3) identified a putative autotransporter in silico, which was termed CapA, for *Campylobacter adhesion protein A*. *C. jejuni* capA is a contingency gene, in which 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. (13) identified JlpA (*jejuni copy protein A*), which is a 43-kDa protein. Disruption of *JlpA* reduces *C. jejuni* adherence to...
jejuni isolates were cultured at 37°C under microaerobic conditions (5% O2, 10% CO2, 85% N2) on Mueller-Hinton (MH) agar plates supplemented with 5% citrated bovine blood (MH-blood agar plates). The radioactively labeled probe was denatured by heating for 15 min at 95°C, mixed with 4 parts formamide and 1 part formamide buffer (50% formamide, 5.0% SDS, 250 mM Tris [pH 7.5]), 2 ml 50% dextran sulfate, and 0.58 g isoamyl chloroform extractions (24 parts chloroform and 1 part isoamyl alcohol) in a 50 ml reaction volume with 50 pmol of each primer, 1× MasterAmp PCR buffer (Epicentre, Madison, WI), 1× MasterAmp PCR enhancer (Epicentre), 2.5 mM MgCl2, and 250 μM (each) dideoxynucleoside triphosphates (dNTPs). Genes were amplified using the following amplification parameters: 94°C for 30 s, 53°C for 30 s, and 72°C for 2 min (30 cycles). Amplicons were confirmed by agarose gel electrophoresis and purified using a BioRobot II 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 (version 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 Sequencher (version 7.2; DNASTAR, Madison, WI). Alleles and sequence types (STs) were assigned using MLSTpars (Miller et al., unpublished observations).

Dot blot hybridization. The C. jejuni putative adhesin-encoding genes examined in this study were porA, cadF, capA, porC, peb1A, MOMP, Cj1279c, and Cj1349c. The sequence of each gene from C. jejuni NCTC 11618 was obtained from online 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 11618 chromosomal DNA as the template. Genes were amplified using the following parameters: 94°C for 2 min (1 cycle); 94°C for 45 s, 60°C (–1°C per cycle) for 30 s, and 70°C for 1 min (10 cycles); 94°C for 45 s, 50°C for 30 s, and 70°C for 1 min (25 cycles); and 70°C for 8 min (1 cycle). The amplified PCR fragments were ligated into the vector pCR2.1 (Invitrogen) and electroporated into Escherichia coli Invotestr (Invitrogen). The purified plasmids were nick translated using a nick translation kit according to the manufacturer’s directions (Roche Applied Science, Indianapolis, IN). One hundred nanograms of C. jejuni genomic DNA, isolated via phenol chloroform extractions as described above, was vacuum transferred to a GeneScreen membrane (Perkin-Elmer, Beverly, MA) overnight. Membranes were washed twice with 2× SSC (3.0 M NaCl plus 0.5 M Na2CO3) at 25°C for 10 min and twice with a solution of 2× SSC and 1% SDS at 35°C 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 that had been warmed to 50°C. The hybridization solution was composed of 5 ml formamide, 2 ml 5X P-buffer (1.0% bovine serum albumin, 1.0% polyvinylpyrrolidone, 1.0% Ficol, 0.5% sodium pyrophosphate, 5.0% SDS, 250 mM Tris [pH 7.5]), 2 ml 50% dextran sulfate, and 0.58 g NaCl. 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 2× SSC at 25°C for 10 min and twice with a solution of 2× SSC and 1% SDS at 35°C for 20 min. Autoradiography was performed with Kodak BioMax MR film at −80°C for approximately 2 h.
Generation of \textit{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 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 E. coli and C. jejuni. The resulting pBSK-Kan2 vectors (pMEK252-pMEK256) were confirmed by DNA sequencing and were electroporated into \textit{C. jejuni}. The PCR amplicons used as probes for the dot blot hybridizations were removed from the pCR2.1 multiple cloning site 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 E. coli and C. jejuni. The resulting pBSK-Kan2 vectors (pMEK252-pMEK256) were confirmed by DNA sequencing and were electroporated into E. coli Inv+ electrocompetent cells.

Generation of \textit{C. jejuni} capA and Cj1278c suicide vectors. DNA regions upstream and downstream of the \textit{C. jejuni} capA and Cj1278c genes were amplified by PCR using \textit{C. jejuni} adhesion assays. All experiments and procedures described below were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC protocol no. 3248) at Washington State University. A total of 80 1-week-old chicks were obtained, divided into eight groups, and placed into isolation chambers (Horsfall Bauer Washington State University). A total of 80 1-week-old chicks were obtained, divided into eight groups, and placed into isolation chambers (Horsfall Bauer Washington State University).
and the CFU were counted after 72 h of incubation. PCR was performed with specific primers to confirm that the counted colonies were C. jejuni (Table 1).

Nucleotide sequence accession number. Novel alleles and STs have been submitted to the PubMLST C. jejuni/C. coli database (http://pubmlst.org/campylobacter/).

RESULTS

The C. jejuni strains used in this study are genetically diverse. MLST is commonly used for molecular typing of C. jejuni isolates (8, 25). We collected a total of 97 isolates from humans, poultry, bovine, porcine (swine), bovine, ovine, and canine sources and assessed their genetic relatedness by MLST (Fig. 1). The C. jejuni isolates comprised 45 STs (see Table S1 in the supplemental material). Eighty-four isolates were assigned to one of 18 clonal complexes (CCs). The complexes with the greatest number of isolates were CC21, CC48, and CC45, which were comprised of 19, 10, and 10 isolates, respectively. In total, 2 human isolates and 11 animal isolates did not belong to a CC in the MLST database. We also compared the allelic profiles, or ST, of each isolate. The most common ST was ST-50, represented by isolates H6, H34, H36, Iowa 11, Iowa 13, Iowa 15, Iowa 35, and C1129. The second most common ST was ST-21, represented by isolates H2, H10, H12, 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 (USDA02-835L). In total, 105 alleles were identified among 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 among 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 among the strains assayed (Fig. 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 (Fig. 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 (2). All of the C. jejuni mutants (i.e., cadF, capA, jlpA, peb1A, Cj1279c, and Cj1349c) generated were motile (not

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<table>
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<tr>
<th>ORF</th>
<th>BLAST identification</th>
<th>Signal peptide cleavage sites</th>
<th>Genes within the putative operon</th>
<th>Relevant reference</th>
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<td>porA/Cj1259</td>
<td>MOMP, C. jejuni</td>
<td>Residues 22–23, SpI cleavage&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Residues 26–27, SpI cleavage&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>capA/Cj0628 and Cj0069</td>
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<td>None identified</td>
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<td>Residues 17–18, SpII cleavage&lt;sup&gt;c&lt;/sup&gt;</td>
<td>None identified</td>
<td>13</td>
</tr>
<tr>
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<td>Residues 26–27, SpI cleavage&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Cj1279c</td>
<td>Fibronectin type III domain containing protein lipoprotein, Sulfurimonas denitrificans 1e-57</td>
<td>Residues 20–21, SpII cleavage&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Cj1349c</td>
<td>Fn/fibrinogen-binding protein FBP54, Streptococcus pyogenes 1.7e-05</td>
<td>None identified</td>
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</table>

<sup>a</sup> Genes within the putative operons were determined using NMPDR.
<sup>b</sup> Putative signal peptide cleavage site.
<sup>c</sup> Putative lipoprotein signal peptide cleavage site.

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FIG. 1. Dendrogram of the C. jejuni MLST profiles identified in this study. The MLST STs were determined as outlined in Materials and Methods. For each isolate, the allelic sequences were concatenated in the order aspA-uncA-glnA-gltA-gluA-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; and C, canine.
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 bacterium-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 compared with the *C. jejuni* wild-type strain. In addition, *C. jejuni* isolates were genetically matched (H11 and H14 to 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 (Fig. 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 1-week-old chicks with the defined *C. jejuni* mutants. Eighty chicks were divided into groups, with each group consisting of 10 chicks (Fig. 3). All chicks were euthanized at 7 dpi 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

![Image](http://iai.asm.org/)
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 2 of 10 chicks inoculated with the C. jejuni cadF and Cj1279c mutants were colonized. None of the 10 chicks inoculated with the C. jejuni peb1A mutant was 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, which encodes a putative ribosomal pseudouridine synthase and upstream of 11 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 (Fig. 4). Although variations were observed from one experiment to another in the number of C. jejuni cells that bound to the chicken LMH cells (Fig. 2 and 4), these results appeared to be due to fluctuations in the multiplicity of infection. Regardless, the C. jejuni cadF and Cj1279c mutants consistently showed reductions in cell binding 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 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 among 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 human, poultry, bovine, porcine, ovine, and canine sources. These isolates were genetically diverse, as judged by MLST. The isolates were found to comprise 42 unique sequence types, 4 of which had not been identified previously. The CCs 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 (7, 21) to be associated significantly with bovine and ovine isolates. Furthermore, the 11 CCs identified in the 41 poultry isolates included several poultry-associated complexes (i.e., CC45, CC257, and CC354) (9). Thus, the results from this study are consistent with previous associations of CC and source. Additionally, the CCs 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 among 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 Cj1349c 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, MOMP is required for viability. Fouts et al. (10) demonstrated the conservation of the characterized and putative C. jejuni adhesins in the C. jejuni NCTC 11168 and RM1221 strains and three other *Campylobacter* species. Although capA was omitted in the study by Fouts et al. (10), Ashgar et al. (3) reported that 9 of 20 C. jejuni human clinical isolates tested in their study lacked the capA gene. In agreement with Ashgar et al. (3), 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 gener-
ating 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 90k on the surface of human HEp-2 cells and is correlated with induction of the proinflammatory response due to the activation of NF-kB and p38 mitogen-activated protein kinase (14). Jin et al. (13) reported that disruption of jlpA reduces the adherence of C. jejuni to human HEp-2 epithelial cells by 18 to 19.4% 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 to 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. (24) found PEB1 in the periplasm and supernatant, which is typical of an ABC transporter (31), but not in the outer membrane. In addition, the investigators demonstrated that a peb1A mutant failed to transport t-glutamate, showed a large reduction in t-aspartate transport, and did not grow in minimal essential medium supplemented with t-aspartate or t-glutamate. Also relevant, Guccione et al. (12) 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. (3) 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 among 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 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 between our data and those of Ashgar et al. (3) 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 (17–19, 27). 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 compared to the wild-type isolate (18, 28). In addition, a cadF mutant fails to colonize the intestinal tract of Leghorn chickens (36). 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 (17–19, 27, 28, 36).

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 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 2 of 10 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 among 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 to 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 (Cj1278c) 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 Multi-Locus Sequence Typing website (http://pubmlst.org/campylobacter/) developed by Keith Jolley at the University of Oxford. We also thank

Locus Sequence Typing website (http://pubmlst.org/campylobacter/).
Charles Larson (School of Molecular Biosciences, Washington State University) for critical review of the manuscript. This work was supported from funds awarded to M.E.K. from the USDA National Research Initiative’s Food Safety 32.0 program (2006-35201-16533 and 2006-35201-17305).

REFERENCES


